Product License Application

For

"A4+"

Sabell Corporation
Suite 200 Manulife House, 603 - 7th Ave SW
Calgary, Alberta
T2P 2T5

Health Canada / Santé Canada

Product Licence
Licence de mise en marché

Product Number/*Numéro de produit*: 80033347

Brand Name/*Marque nominative*: A4+

Issued to/*Émise à:*

Name of licensee/*Nom du titulaire:*

Sabell Corporation
Suite 200 Manulife House, 603-7th Ave. SW
Calgary, Alberta, T2P 2T5
Canada

Authorized for the following/*Autorisé pour ce qui suit:*

Dosage form/*Forme posologique*: Capsule

Recommended route of administration/Voie *d'administration recommandée:*

Oral

Recommended dose/*Dose recommandée:*

Adults : Take two (2) capsules three times per day before meals.

Recommended duration of use/*Durée d'utilisation recommandée:*

Consult a health care practitioner for use beyond 4 weeks.

Recommended use or purpose/*Usage ou les fins recommandés:*

Used in Herbal Medicine as a hepatoprotectant/liver protectant.

Risk Information/*Renseignements sur les risques:*

Cautions and Warnings

Keep out of reach of children.
Consult a health care practitioner prior to use if you are pregnant, breastfeeding, or plan to become pregnant.
Consult a health care practitioner prior to use if you are taking antihypertensive, blood thinning medication, have gallstones, ulcers, liver or bile obstruction.
Consult a health care practitioner if you have excess stomach acid or if symptoms persist or worsen.

Medicinal Ingredients/*Ingrédients médicinaux:*

Proper Name Nom propre	Common Name Nom usuel	Quantity per Dosage Unit *Quantité par unité posologique*	Extract *Extrait*	Potency *Activité*	Source Material *Matière d'origine*
Annona muricata	Soursop	9.7 mg	38.6:1 375 mg	N/A	Leaf
Cordia lutea	Oberal	77.8 mg	7.7:1 600 mg	N/A	Flower(s)
Curcuma longa	Turmeric	9.7 mg	38.6:1 375 mg	N/A	Rhizome

This licence is issued by the Minister of Health under the authority of section 7 of the Natural Health Products Regulations. Sale of the described natural health product, including any changes thereto pursuant to section 11 of the Regulations, is subject to the Food and Drugs Act and to the Natural Health Products Regulations.
Cette licence est émise par la ministre de la Santé en vertu de l'article 7 du Règlement sur les produits de santé naturels. La vente du produit de santé naturel décrit dans la présente, y compris toute modification afférente au sens de l'article 11 du Règlement, est assujettie à la Loi sur les aliments et drogues et au Règlement sur les produits de santé naturels.

Health Canada Santé Canada

Product Number/*Numéro de produit*: 80033347
Brand Name/*Marque nominative*: A4+

Issued/émis le: 2012-07-03	Revised/Amended/Modifié le: N/A

Director General/*Directeur général*
NHPD/*DPSN*

Health Canada Santé Canada

Company Code: 26715
File Number: 178665
Submission Number: 178665

2012-07-03

Mr. Brad Clarke
President & CEO
Sabell Corporation

Suite 200 Manulife House. 603-7th Ave. SW
Calgary
Alberta
T2P 2T5
Canada

Dear Mr. Brad Clarke

Re: Product Licence Issuance - NPN 80033347
Non-Traditional - A4+

The Natural Health Products Directorate (NHPD) has concluded that the product is in compliance pursuant to section 7 of the *Natural Health Products Regulations*. Please find enclosed a copy of the Product Licence hereby authorizing the sale of the product described therein.

Any labels used in the marketing of this product must reflect the information outlined on the product licence and must comply with the labelling requirements as per Part 5 of the *Natural Health Products Regulations*. Please note that you are responsible for ensuring that advertising claims on the label do not contravene Section 9 of the *Food and Drugs Act*. Additional information on acceptable advertising claims can be obtained from the "Consumer Advertising Guidelines for Marketed Health Products (for Nonprescription Drugs including Natural Health Products)" at http://www.hc-sc.gc.ca/dhp-mps/advert-publicit/pol/guide-ldir_consom_consum_e.html#a.1. Section 87 (Labelling and Packaging), specifies the label text information that you are responsible for ensuring is translated into French.

Changes made in respect of a licensed product require the submission of an amendment, notification or a new product licence application as per sections 11, 12 and 13 of the *Natural Health Products Regulations*.

As per Part 6, Section 108 of the *Natural Health Product Regulations*, any Drug Identification Number (DIN) assigned to a product is no longer valid and will be cancelled once an Natural Product Number (NPN) has been issued. For those applications that have not been identified as a Transitional DIN, we recommend the applicant notify the Therapeutic Products Directorate that an NPN has been issued to avoid any administrative fees for DIN products.

As per the *Natural Health Product Regulations*, you are responsible for providing the NHPD with the Canadian site information prior to commencing the importation and/or sale of the natural health product. All information required is outlined in Part 1, Section 22 (1 & 2). For transitional DIN products that are currently on the market, this information is required within 30 days after the day on which the natural health product licence is issued. If this information has not already been provided to NHPD, please submit this information as a notification, as per section 12 (2) (b) of the Natural Health Product Regulations.

If you notice any discrepancies concerning the information on the licence, please submit a notice entitled "Request for Correction to the Product Licence" indicating the corrections to be made, within 60 days after the day on which the product licence is issued, to the Submission Coordinator Vera Kazakova, *tel.*: (613) 948-7155. The File Number (provided at the top right corner of the title page) and Product Number must be quoted on all future correspondence regarding this product.

Health Canada Santé Canada

Yours truly,

Vera Kazakova
Submission Coordinator
Natural Health Products Directorate

encl.:Product Licence
c.c.: Dr. Ravibhushan Singh

PE PharmEng Technology

130-10691 Shellbridge Way
Richmond, BC, V6X 2W8
Tel: (604) 303-0445
Fax: (604) 303-0432

23 Dec 2011

Submission Management Division
Natural Health Products Directorate
2936 Baseline Rd., AL 3300C
Ottawa, ON
K1A 0K9

Dear Submission Management Division,

Re: A4+ Product License Application

In compliance with the Canadian Natural Health Product Regulations, we are submitting a Non-traditional Product License Application for A4+ Capsule, on behalf of Sabell Corporation, Calgary, Alberta, Canada.

The following documents are enclosed as part of this Non-traditional PLA:

1. Cover Letter
2. Product License Application form
3. Designated Party Authorization form
4. Proposed Label Text
5. Evidence and Safety Report
6. Quality Summary Report
7. References

I hope the information provided meets the submission requirements and an NPN can be issued. Should you need additional information, please contact me at 604-303-0445 or ravi.s@pharmeng.com.

Sincerely yours,

Ravibhushan Singh, M.Sc., Ph.D., RAC
PharmEng Technology
A Division of PE Pharma Inc.
Tel: 604-303-0445
Fax: 604-303-0432
ravi.s@pharmeng.com

Health Canada / Santé Canada

PRODUCT LICENCE APPLICATION FORM

Natural Health Products Directorate

Protected when completed

HEALTH CANADA USE ONLY		3. Date/Time of Receipt
1. Submission Number	2. File Number	

Please refer to the Guide for instructions on how to complete this application.

PART 1 – APPLICANT AND CONTACT INFORMATION

A. – APPLICANT OR LICENSEE (This is the product licence holder)

4. Applicant/Company Name*			5. Company Code (if known)
Sabell Corporation			
6. Address: Street/Suite/PO Box*			
Suite 200 Manulife House, 603 - 7th Ave SW			
7. City – Town*	9. Province – State*	8. Country*	10. Postal/ZIP Code*
Calgary	**Alberta**	**Canada**	**T2P 2T5**

B. – SENIOR OFFICIAL (This is the name of the principal contact person for the applicant/company)

11. Name ☒ Mr. ☐ Ms. ☐ Dr.		12. Title	13. Language preferred:
Surname* **Brad**	Given Name* **Clarke**	**President and CEO**	☒ English ☐ French
14. Company Name (* if different from Applicant/Licensee)			15. Address same as "A" ☒
16. Street/Suite/PO Box*			
17. City – Town*	19. Province – State*	18. Country*	20. Postal/Zip Code*

21. Telephone No.*	Ext.	22. Fax No.	23. E-mail
403-261-8888	**224**	**403-264-3310**	**brad@sabell.ca**

C. – CONTACT FOR THIS APPLICATION (This is the contact person for product-specific questions)

24. Contact same as "B" ☐		26. Title	27. Language preferred:
25. Name ☐ Mr. ☐ Ms. ☒ Dr.		Senior Consultant	☒ English ☐ French
Surname* **Singh**	Given Name* **Ravibhushan**		
28. Company Name (*if different from Applicant/Licensee)			29. Address same as "A" ☐
PharmEng Technology			
30. Street/Suite/PO Box*			
130-10691 Shellbridge Way			
31. City – Town*	33. Province – State*	32. Country*	34. Postal/Zip Code*
Richmond	**BC**	**Canada**	**V6X 2W8**

35. Telephone No.*	Ext.	36. Fax No.	37. E-mail
604-303-0445	**250**	**604-303-0432**	**ravi.s@pharmeng.com**

Attach separate sheets (same format) if necessary. Number of pages attached: _____

D. – REPRESENTATIVE IN CANADA (Only required where Address in "A" is not in Canada)

38. Contact same as "C" ☐		40. Title	41. Language preferred:
39. Name ☐ Mr. ☐ Ms. ☐ Dr.			☐ English ☐ French
Surname*	Given Name*		
42. Company Name (* if different from Applicant/Licensee)			43. Address same as "C" ☐
44. Street/Suite/PO Box*			
45. City – Town*	47. Province – State*	46. Country*	48. Postal/Zip Code*

49. Telephone No.*	Ext.	50. Fax No.	51. E-mail

E. – CONTACT TO WHOM THE PRODUCT LICENCE IS TO BE SENT:

52. As Above: B: ☒ C: ☐ D: ☐
Not Applicable: ☐ Name: _____
(check only one box)

* - denotes mandatory ** - if yes, complete Animal Tissue Form

Health Canada / Santé Canada

PART 2 – SUBMISSION TYPE

A. – PRODUCT LICENCE APPLICATION

53. Indicate the type of application (*select one only)

☐ Compendial ☐ Traditional claim ☒ Non-traditional claim ☐ Homeopathic ☐ TPD Category IV/Labelling Standard

☐ Homeopathic DIN (DIN#_____) ☐ Transitional DIN (DIN#_____)

54. Is this formulation hypothetical? ☐ Yes ☒ No

55. NPN/DIN-HM #_____ (* - required for Section B. C, and D. only).

B. – MONOGRAPH REVISIONS AFFECTING AN EXISTING PRODUCT LICENCE

56. ☐ Yes, revisions to the published NHPD Compendial Monograph affect the NPN above.
NHPD Compendial Monograph:_____ Date:_____

C. – PRODUCT LICENCE – AMENDMENT

57. Indicate the affected change to the NPN/DIN-HM above. (select one or more)

☐ Potency
☐ Source material of any of its medicinal ingredients
☐ Addition or substitution of a non-medicinal ingredient not on the NHPD List of Acceptable non-medicinal ingredients
☐ Specification
☐ Deletion or modification of risk information on any labels
☐ Recommended dose
☐ Change to Animal Tissue Form(s)
☐ Recommended use/purpose
☐ Change to or from synthetically manufactured
☐ Recommended duration of use
☐ Change to manufacturing information

D. – PRODUCT LICENCE – NOTIFICATION

58. Indicate the type of change(s) that have been made to the NPN/DIN-HM above. (select one or more)

☐ Addition or substitution of any of its proposed non-medicinal ingredient other than those originally authorized for the product.
☐ Change to the common name of any of its medicinal ingredients
☐ Addition of risk info on any of its labels
☐ Change to the name, address, telephone number, fax number, and/or electronic mail address of the manufacturer, packager, labeller, importer, and distributor.
☐ Sale under a brand name other than the one(s) originally authorized for the product license
☐ Change to the proper name of any of its medicinal ingredients
☐ Change to the name, address, telephone number, fax number, and/or electronic mail address of the applicant or Canadian representative.
☐ Change to a Site Licence number for a Canadian manufacturer, packager, labeller, or importer.
☐ Addition of a site associated with the product.

E. – SUBMISSION CONTENT

59. Type of supporting documents, by volume: check type that is applicable and indicate the volume in which the document is submitted.

			Volume #
Number of Volumes: 1	☐ Animal tissue form(s)	#:_____	_____
☒ Product licence application form	☒ Designated Party Authorization form:		1
☐ Additional pages for Product Information	☒ Label Text	#:_____	1
☐ Additional pages for Site Information	☐ TPD Label Text (Transitional DIN or Homeopathic DIN)	#:_____	_____
☒ Evidence Summary Report:	☒ Quality Summary Report:		1
☒ Safety Summary Report:	☒ Other, Claim Evidence: scientific studies and *aboriginal interviews and monographs*		1

F. – REFERENCE SUBMISSION (if applicable)

60. Other submission that contains the evidence to support the safety, efficacy and/or quality of this particular submission.

Company #:_____ File #:_____ Submission #:_____ NPN/DIN-HM #:_____
Contains information to support: ☐ Safety ☐ Efficacy ☐ Quality Letter of access(es) enclosed: ☐ Yes ☐ Not Applicable

Company #:_____ File #:_____ Submission #:_____ NPN/DIN-HM #:_____
Contains information to support: ☐ Safety ☐ Efficacy ☐ Quality Letter of access(es) enclosed: ☐ Yes ☐ Not Applicable

Company #:_____ File #:_____ Submission #:_____ NPN/DIN-HM #:_____
Contains information to support: ☐ Safety ☐ Efficacy ☐ Quality Letter of access(es) enclosed: ☐ Yes ☐ Not Applicable

G. – NHPD MASTER FILE (if applicable)

61. Master file that contains the evidence to support the safety, efficacy and/or quality of this particular submission.

Master File #:_____ Letter of access enclosed: ☐ Yes ☐ Not Applicable

Contains information to support: ☐ Safety only ☐ Efficacy only ☐ Quality only ☐ Complete submission

Attach separate sheets (same format) if necessary. Number of pages attached:_____

* - denotes mandatory ** - If yes, complete Animal Tissue Form

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PART 3 – SITE INFORMATION

62. Company Name **POS Bio-Sciences** 64. Number, Street – Suite – PO Box **118 Veterinary Road** 65. City **Saskatoon** 66. Province – State: **SK** | 67. Country: **Canada** | 68. Postal Code – Zip Code: **S7N 2R4**	63. ☒ Manufacturer SL# **300354** ☐ Packager SL# ______ ☐ Labeller SL# ______ ☐ Importer SL# ______ ☐ Distributor
62. Company Name **Natural Factors Nutritional Products Ltd.** 64. Number, Street – Suite – PO Box **1550 United Boulevard,** 65. City **Coquitlam** 66. Province – State: **BC** | 67. Country: **Canada** | 68. Postal Code – Zip Code: **V3K 6Y2**	63. ☒ Manufacturer SL# **300059** ☒ Packager SL# **300059** ☒ Labeller SL# **300059** ☐ Importer SL# ______ ☐ Distributor
62. Company Name 64. Number, Street – Suite – PO Box 65. City 66. Province – State | 67. Country | 68. Postal Code – Zip Code	63. ☐ Manufacturer SL# ______ ☐ Packager SL# ______ ☐ Labeller SL# ______ ☐ Importer SL# ______ ☐ Distributor
62. Company Name 64. Number, Street – Suite – PO Box 65. City 66. Province – State | 67. Country | 68. Postal Code – Zip Code	63. ☐ Manufacturer SL# ______ ☐ Packager SL# ______ ☐ Labeller SL# ______ ☐ Importer SL# ______ ☐ Distributor
62. Company Name 64. Number, Street – Suite – PO Box 65. City 66. Province – State | 67. Country | 68. Postal Code – Zip Code	63. ☐ Manufacturer SL# ______ ☐ Packager SL# ______ ☐ Labeller SL# ______ ☐ Importer SL# ______ ☐ Distributor

69. Attach separate sheets (same format) if necessary. Number of pages attached: __0______

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PART 4 – PRODUCT INFORMATION

70. Primary Brand Name* A4+

71. If necessary, attach a separate sheet with other brand names. Number of pages attached: _____

A. – MEDICINAL INGREDIENT(S)

72. Ingredient No.	73. Standard or Grade	74. NHPD Compendial Monograph		75. Proper Name*	76. Common Name	77. Quantity per Dosage Unit*	78. Synthetic*		79. Animal Tissue**	
		Name	Date				Yes	No	Yes	No
1.				*Cordia lutea*	Overo or Overal	77.8 mg		X		x
2.				*Annona muricata*	Graviola or Guanabana	9.7 mg		X		X
3.		Turmeric	2006	*Curcuma longa*	Curcumin or Tumeric	9.7 mg		X		X
4.										
5.										
6.										
7.										
8.										
9.										
10.										
11.										
12.										

80. Ingredient No.	81. Potency (if applicable)		84. Source Information* (If more than one enter on new line)	85. Extract (if applicable)				90. Method of preparation
	82. Amount	83. Constituent		86. Ratio	87. Quantity Crude Equivalent	Original Material 88. Fresh	Original Material 89. Dry	
1.			*Cordia lutea* Flower	3:1	600 mg		X	Hydro-alcoholic extract
2.			*Annona muricata* Leaf	15:1	375 mg		X	Hydro-alcoholic extract
3.			*Curcuma longa* Root	15:1	375 mg		X	Hydro-alcoholic extract
4.								
5.								
6.								
7.								
8.								
9.								
10.								
11.								
12.								

91. Attach separate sheets (same format) if necessary. Number of pages attached: ____0____

* - denotes mandatory

** - if yes, complete Animal Tissue Form

Health Canada / Santé Canada

PART 4 – PRODUCT INFORMATION

B. – NON-MEDICINAL INGREDIENT(S)

92. Ingredient No.	93. Proper Name	94. Common Name*	95. Purpose*	96. Animal Tissue Used**	
				Yes	No
1.		Microcrystalline cellulose	encapsulating agent		X
2.	Cellulose, 2-hydroxypropyl methyl ether	Hypromellose	encapsulating agent		X
3.					
4.					
5.					
6.					
7.					
8,					
9.					
10.					
11.					
12.					

97. Ingredient No.	98. Standard or Grade	99. Quantity	100. Source Information (if more than one enter on new line)
1.			
2.			
3.			
4.			
5.			
6.			
7.			
8,			
9.			
10.			
11.			
12.			

C. – INGREDIENT(S) USED IN PROCESSING

101. "Was animal tissue used in the processing of this product, although not present in the final product?" ** ☐ Yes ☒ No

* - denotes mandatory ** - If yes, complete Animal Tissue Form

Health Canada / Santé Canada

PART 4 – PRODUCT INFORMATION

D. – RECOMMENDED CONDITIONS OF USE

102. Recommended Use or Purpose*
"To alleviate symptoms of liver diseases, promotes healing of the liver and maintains liver health"

103. Dosage Form (one only)*	104. Sterile*	105. Route of Administration*
Capsule	☐Yes ☒ No	**Oral**

106. Duration of Use (if any)
It is recommended that A4+ be taken daily for a minimum of 30 days.

Recommended Dose (repeat for each sub-population group)

107. Sub-population group*	108. Amount to be taken at one time:		111. Frequency	112. Directions of Use
	109. No. of Dosage Units* (e.g. 1, 2, etc.)	110. Dosage Unit* (e.g. capsule, tsp, etc.)		
Adults	**2**	**Capsules**	**3 times daily**	**Take two (2) capsules three times per day before meals**

Risk Information

113. Cautions and Warnings*

Keep out of reach of children

Consult a health care practitioner prior to use if you are pregnant, breastfeeding, or plan to become pregnant.

Consult a health care practitioner prior to use if you are taking antihypertensive, blood thinning medication, have gallstones, ulcers, liver or bile obstruction.

114. Contraindications*

N/A

115. Known Adverse Reactions*

N/A

ATTESTATION

"I attest that the natural health product that is the subject of this product license application will be manufactured, packaged, labelled, distributed and stored:

a) **If the natural health product is imported, in accordance with the 'Good Manufacturing Practices' requirements as set out in Part 3 of the Natural Health Products Regulations or in accordance with requirements that are equivalent to those set out in Part 3, or**
b) **If the natural health product is not imported, in accordance with the 'Good Manufacturing Practices' requirements set out in Part 3 of the Natural Health Products Regulations.**

I, the undersigned, certify that the information and material included in this product licence application is accurate and complete".**

116. Name of Authorized Senior Official[1] (print)*	117. Signature*	118. Date*
RAVIBHUSHAN SINGH	R Singh	2 0 1 1 1 2 2 3

If the signing official is a third party acting on behalf of the Senior Official of the applicant company designated in Part 1 of the application, a designated Party Authorization form must be signed by the Senior Official and filed with the complete application.

* - denotes mandatory ** - if yes, complete Animal Tissue Form

Health Canada / Santé Canada

DESIGNATED PARTY AUTHORIZATION FORM

Natural Health Products Directorate

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FORMULAIRE D'AUTORISATION DE LA PARTIE DÉSIGNÉE

Direction des produits de santé naturels

Protégé une fois rempli

Note: Only submit this document with the application when the party signing the application is a designated party acting on behalf of the applicant or licensee according to paragraph 5(*b*) of the *Natural Health Products Regulations*. A separate authorization is required for each application.

Note : Ne présenter ce document avec la demande que lorsque la partie désignée signe la demande au nom du demandeur ou du titulaire conformément à l'alinéa 5(*b*) du *Règlement sur les produits de santé naturels*. Une autorisation distincte est obligatoire pour chaque demande.

I / Je **Brad Clarke** authorize / donne l'autorisation à **Ravibhushan Singh** of / de

(The Senior Official / Agent principal) (Third party person / Tierce partie)

PharmEng Technology to file a submission with the Natural Health Products Directorate on behalf of / de remplir une présentation à la Direction des produits de santé naturels au nom de **Sabeil Corporation**

(Third party company name / Nom de l'entreprise de la tierce partie) (Applicant/Company name / Nom du demandeur/de l'entreprise)

Signature

Brad Clarke

Print Name / Nom en lettres moulées

President & CEO

Title / Titre

Sabeil Corporation

Applicant/Company name / Nom du demandeur/de l'entreprise

Date: **2011 12 22** (yyyy mm dd / aaaa mm jj)

CONTACT INFORMATION/RENSEIGNEMENTS SUR LE TITULAIRE

Surname / Nom de famille	Given Name / Prénom
☐ Mr. / M. ☐ Ms. / Mme ☒ Dr. **Singh**	**Ravibhushan**
Title / Titre	Language preferred / Langue de préférence
Consultant	☒ English / Anglais ☐ French / Français

Street/Suite/Land Location / Rue/bureau/emplacement: **130-10691 Shellbridge Way**

City - Town / Ville	Province - State / Province/état	Country / Pays	Postal/ZIP Code / Code postal/ZIP
Richmond	**BC**	**Canada**	**V6X 2W8**

Telephone No. / N° de téléphone	Ext. / Poste	Fax No. / N° de télécopieur	E-mail / Adresse électronique
604-303-0445	**250**	**604-303-0432**	**ravi.s@pharmeng.com**

Canada

"A4+"

Inner Label and Outer Label (combined):

Note: Proposed label text is in ***bold italic*** on right hand column of Table below. Otherwise, label text is not applicable ("N/A") or to be determined ("TBA").

Outer Label (principal display panel)	
Brand name consisting of trade name and product name in English	Brand name: ***ISULA*** Product name: ***A4+***
Dosage form	***250 mg Capsules***
Sterile	N/A
Net amount in the immediate container	***84 Capsules***
NPN number	***NPN*** XXXXXXXX
Outer Label (Back/Side Panel)	
Name and postal code of the product licence holder	***Sabell Corporation*** ***Suite 200 Manulife House*** ***603 - 7th Ave SW*** **Calgary, Alberta T2P 2T5**
Name and postal code of Importer (if applicable)	***Sabell Corporation*** ***T2P 2T5***
Medicinal ingredients: Proper name or common name in English, description of source material, quantity, potency	***Each capsule contains:*** ***Cordia lutea.....77.8 mg*** ***Annona muricata.....9.7 mg*** ***Curcuma longa.....9.7 mg***
Recommended use or purpose	***"To alleviate symptoms of liver diseases, promotes healing of the liver and maintains liver health"***
Recommended route of administration, recommended dose, recommended duration of use, if any	***Two (2) capsules to be taken three times per day before meals. It is recommended that A4+ be taken daily for a minimum of 30 days.*** ***Users are encouraged to get a "daily dose" of sunlight on their bodies.*** ***Persons taking this herbal product are recommended to avoid fatty foods, shellfish, pork and red meat.***
Risk information: cautions, warnings, contraindications, known adverse reactions	***"Consult a health care practitioner prior to use if you are pregnant, breastfeeding, or plan to become pregnant."*** ***"Consult a health care practitioner prior to use if you are taking antihypertensive, blood thinning medication, have***

	gallstones, ulcers, liver or bile obstruction." ***"Keep out of reach of children."***
Recommended storage conditions, if any	***Store bottle in a cool dry place***
Expiry date	***TBD***
Lot number	***TBD***
Cautionary statement	***Do not use if seal is broken.***
Non-medicinal ingredients: Common names in English	***Microcrystalline cellulose and Hypromellose***
Quantity of mercury	N/A

Quality Summary Report

For

"A4+"

Sabell Corporation
Suite 200 Manulife House, 603 - 7th Ave SW
Calgary, Alberta
T2P 2T5

Date of Preparation: December 2011

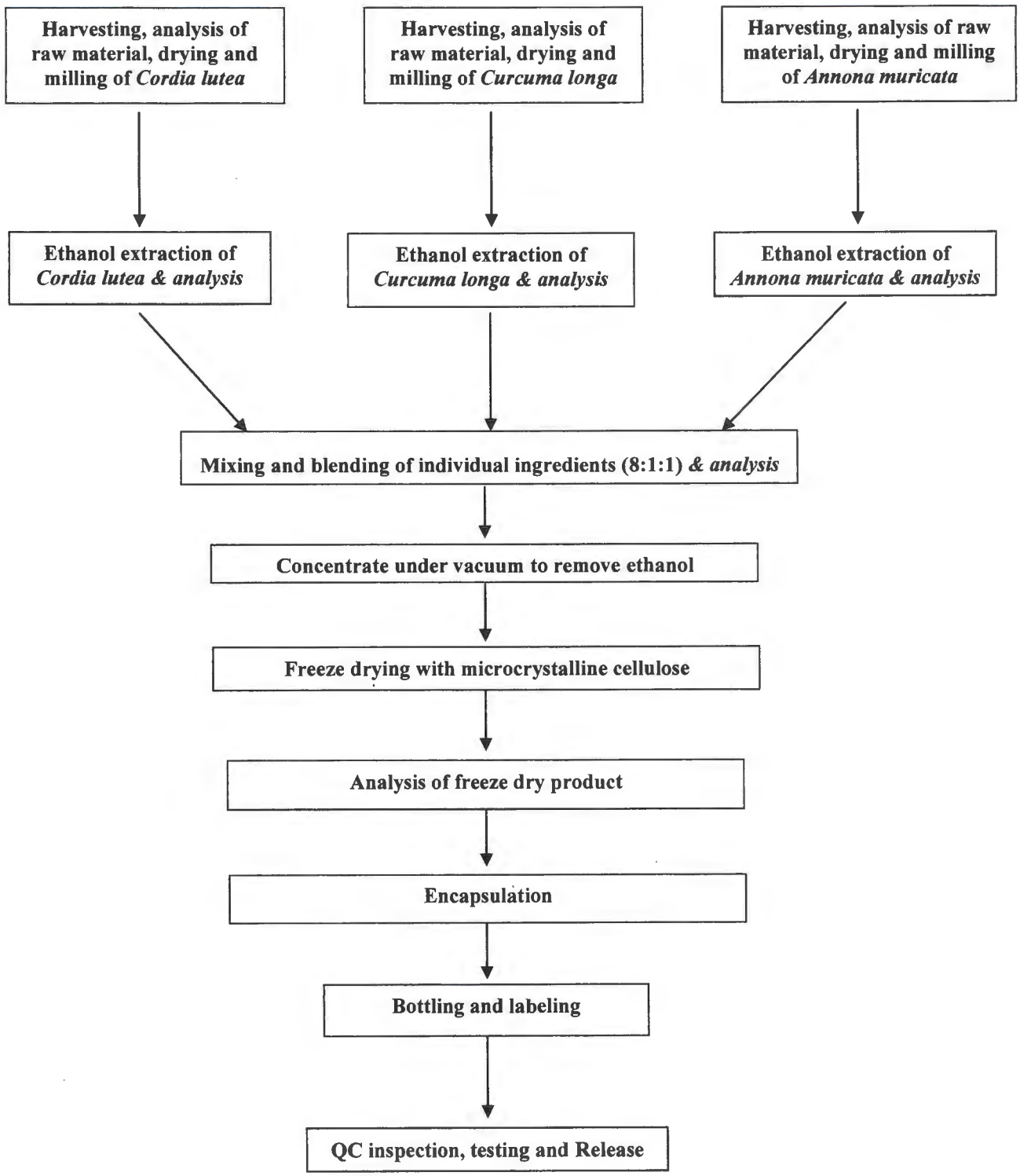
I. Description of Manufacturing Process

A4+ is manufactured under GMP compliance. Sabell Corporation uses their own facilities for the harvesting, drying and milling of the raw materials (*Cordia lutea, Curcuma longa and Annona muricata*). The raw materials are tested for identity and purity prior to use in the manufacturing process. Manufacturing area and equipment used in the manufacturing of A4+ are cleaned/sanitized following in-house procedures before starting the manufacturing activities.

The manufacturing of A4+ involves the following steps:

1) Harvesting, analysis of raw material, drying and milling of the raw materials
2) Ethanol extraction of individual ingredients and analysis
3) Mixing and blending of individual ingredients and analysis
4) Freeze drying of extract with microcrystalline cellulose
5) Analysis of freeze dry product
6) Encapsulation
7) Bottling and labeling
8) QC inspection, testing and Release

A flow diagram illustrating the manufacturing process of A4+;



II. Sterilization and Irradiation Processes

Not applicable.

III. Analytical Procedures

The following tests are performed on Sabell Corporation "A4+".

Test Parameter	Test	Test Method
Physical Identity	Appearance	Visual
	Dissolution	USP
	Weight of capsule	USP
Chemical Identity	*Cordia lutea*	TLC
	Curcuma longa	TLC
	Annona muricata	TLC
Purity	**Microbiological Contamination**	
	Yeast and mould	USP <2021>
	Total aerobic count bacteria	USP <2021>
	E. coli	USP <2022>
	Salmonella spp.	USP <2022>
	Staphylococcus aureus	USP <2022>
	Pseudomonas aeruginosa	USP <2022>
	Heavy metals	
	Arsenic	USP <211>
	Total Mercury	USP <261>
	Lead	USP <251>
	Cadmium	ICP-MS
	Pesticides	USP <561>
Potency/ Quantity	*Cordia lutea*	HPLC
	Curcuma longa	HPLC
	Annona muricata	HPLC

IV. Validation of Analytical Procedures

All compendial test methods are based on validated methods, i.e. USP chapters and hence not applicable.

V. Justification of Specifications

Not applicable.

Specifications used are within the referenced ranges listed in the guidance document "Evidence for Quality of Finished Natural Health Products".

VI. Finished Product Specifications;

Test Parameter	Test	Test Method	Tolerances
Physical Identity	Appearance	Visual	Beige to brown powder Opaque capsule
	Dissolution	USP	USP limits
	Weight of Capsule	USP	250 mg ± 10%
Chemical Identity	**Chemical Identity tests**		
	Cordia lutea	TLC	Positive
	Curcuma longa	TLC	Positive
	Annona muricata	TLC	Positive
Potency/ Quantity	*Cordia lutea*	HPLC	80 – 120%
	Curcuma longa	HPLC	80 – 120%
	Annona muricata	HPLC	80 – 120%
Purity	**Microbiological Contamination**		
	Yeast and Mould	USP <2021>	<10,000 cfu/g
	Total aerobic count	USP <2021>	<100,000 cfu/g
	E. coli	USP <2022>	Absent[1]
	Staphylococcus aureus	USP <2022>	Absent[1]
	Salmonella spp.	USP <2022>	Absent[1]
	Pseudomonas aeruginosa	USP <2022>	Absent[1]
	Heavy Metals		
	Arsenic	USP <211>	< 0.14 µg / kg b.w./day
	Total Mercury	USP <261>	< 0.29 µg / kg b.w./day
	Lead	USP <251>	< 0.29 µg / kg b.w./day
	Cadmium	ICP-MS	< 0.09 µg / kg b.w./day
	Pesticides	USP <561>	USP limits

[1]Absent means $<1x10^1$ cfu/g or $1x10^1$ cfu/ml as per Appendix 4 of the NHPD's June 2007 edition of the "Evidence for Quality of Finished Natural Health Products".

Evidence and Safety Summary Report

For:

"A4+"

Sabell Corporation

Suite 200 Manulife House, 603 - 7th Ave SW

Calgary, Alberta

T2P 2T5

Date of Preparation: December 2011

1.0 Evidence Summary Report

1.1 Recommended use or purpose (health claim):

- **Claim:** "To alleviate symptoms of liver diseases, promotes healing of the liver and maintains liver health"
- Non-Traditional Use claim
- Following published scientific references as well as scientific studies conducted by Sabell corporation that support this claim;
  - Bussmann R.W., Sharon D., Garcia M., "From Chamomile to Aspirin? Medicinal Plant Use Among Clients at Laboratorios Beal in Trujillo", Peru, Ethnobotany Research and Applications, 2009, Vol 7, 399-407.
  - Health Canada, NHPD monograph for Turmeric (*Curcuma longa)* USDA 2008; Date: 2010-02-25.
  - Raintree Nutrition, Tropical Plant Database, Monograph (Database File) for Graviola (*Annona muricata*).
  - Natural Medicines Comprehensive Database, Monograph for Graviola (*Annona muricata*).
  - Samanta A., "Quality of life following the use of A4 plus in a limited number of subjects with chronic hepatitis C: Open-label, non placebo-controlled, preliminary observations", Independent assessment report, 2004.
  - Cabanillas Jose, Nystrom Joseph W, Zambrano Luis, Chicolote Gerardo., "Chronic Hepatitis C Treatment with A4+, Controlled Clinical Test", Lima Peru, May-June 2004.
  - Cabanillas Jose, Joseph Nystrom, Hugo Marquez, S., "Short term effects of A4+ on clinical and biochemical markers in chronic hepatitis C", September 2001.
  - Kosinski, M., "Revised Summary Report on Study Findings of 10 Patients Receiving Nutraceutical Product A4+L for the Treatment for Chronic Hepatitis C Virus (CO-1031)", Quality Metric Inc., July 2004.
  - Coral Jose Gonzalo Cabanillas., "Herbal Compositions and Methods for Treating Hepatic Disorders", Patent Pending (PCT/CA2008/001764), International Filing Date: 03 October 2008.
  - Swain MG., "Evaluation of the Hepatoprotective and Behavioral Effects of the Herbal Compound A4+ in Acute Liver Injury Models", (University of Calgary Report - 2011).

- Cabanillas J., Fong F., "A4+ Interviews with Peruvian Medicine Experts", Peru, January 2009. Translated to English by Mr. Jorge Alvarado, Certified Translator from the Society of Translators and Interpreters of British Columbia, March 2009.
- Tyrrell L., Part 1: "Anti-viral effects of plant extracts on HCV infected cells" and Part 2: **"Effect of Short-term exposure to A4+ plant extract on Natural Killer Cell Activity"** (Li Ka Shing, Institute of Virology, University of Alberta Report - 2011).
- Wallace J., "Evaluation of the Anti-oxidant, Anti-inflammatory and mucosal protective Actions of A4+ and its Constituents" (Penumbra Associates Ltd, 2010).
- Adewole SO, Ojewole JA., "Protective effects of *Annona muricata* Linn. (Annonaceae) leaf aqueous extract on serum lipid profiles and oxidative stress in hepatocytes of streptozotocin-treated diabetic rats". Afr J Tradit Complement Altern Med. 2008; 25; 6(1):30-41.
- Orlando Vieira de Sousa, Glauciemar Del-Vechio Vieira, José de Jesus R. G. de Pinho, Célia Hitomi Yamamoto and Maria Silvana Alves., "Antinociceptive and Anti-Inflammatory Activities of the Ethanol Extract of *Annona muricata* L. Leaves in Animal Models". Int. J. Mol. Sci. 2010, 11, 2067-2078.
- Baskar R, Rajeswari V, Kumar TS., "In vitro antioxidant studies in leaves of *Annona* species". Indian J Exp Biol. 2007; 45(5):480-485.
- A Saravana Kumar, R Gandhimathi, KK Senthil Kumar, Kusuma Praveen Kumar., "Hepatoprotective potential of *Cordia subcordata* Lam. against carbon tetra chloride (CCl4)-induced hepatotoxicity in Wistar albino rats". J Biomed Sci and Res., 2009, Vol 1 (1), 19-26.
- M. Afzala, C. Obuekweb, A. R. Khanc and H. Barakata., "Antioxidant activity of *Cordia myxa* L. and its hepatoprotective potential". EJEAFChe 2007, 6 (6), 2109-2118.
- Thirupathi K., Sathesh Kumar S., Goverdhan P., Ravikumar B., Krishna D. R. and Krishna Mohan G., "Hepatoprotective action of *Cordia dichotoma* against Carbon tetrachloride induced liver injury in rats". Nig. J. Nat. Prod. and Med. 2007, Vol. 11, 37-40.
- D Sobiya Raj, J Jannet Vennila, C Aiyavu, K Panneerselvam., "The hepatoprotective effect of alcoholic extract of *Annona squamosa* leaves on experimentally induced liver injury in swiss albino mice". JIB, 2009, Vol. 5(3), 182-186.
- Mohamed Saleem TS, Christina AJM, Chidambaranathan N, Ravi V, Gauthaman K., "Hepatoprotective activity of *Annona squamosa* Linn. on experimental animal

model". International Journal of Applied Research in Natural Products, 2008, Vol. 1(3), 1-7.

1.2 Critical Overview:

Individually these plants and/or part of these plants (*Cordia lutea, Curcuma longa and Annona muricata*), has been used traditionally for many years. The flower component of *Cordia lutea* has been used as a traditional medicine for centuries, *Curcuma longa* (Turmeric) has a long history of use as a food ingredient as well as an herbal medicine in many cultures and *Annona muricata* has a long, rich history of use in herbal medicine as well as a lengthy recorded indigenous use. In anecdotal reports, this compound has been used for many years to treat individuals with a variety of different liver diseases. A leaf tea form of these three compounds is commonly used by both medical doctors and natural healers in the Peruvian Amazon. Two complete monographs have been published supporting the use of *Curcuma longa* (Turmeric) and *Annona muricata* (Graviola). Both monographs review the effectiveness and long history of use of these ingredients for treating patients with liver disorders.

Sabell Corporation has developed A4+ a Natural Health Product that consists of these three herbal or natural ingredients; *Cordia lutea, Curcuma longa and Annona muricata.* Sabell Corporation has conducted many scientific studies that provide sufficient evidence that A4+ components are pharmacologically active with indications in liver disease.

In-vitro/*in-vivo* studies conducted by Sabell Corporation show the hepatoprotective, anti-oxidant and anti-inflammatory effects of A4+ on liver tissue. The antiviral effect of A4+ also shows that the A4+ component has significant levels of antiviral activity against HCV in cell culture. Toxicology studies conducted in mice and rat shows no overt evidence of toxicity related to the A4+ at any dosage up to the highest administered to female Sprague rats (2000 mg/kg). Toxicity studies indicate no toxic effects, no mortality effects at up to 2,000 mg A4+/kg mice body weight and therefore concluded that, there is no toxicity associated with A4+ consumption.

Two clinical trials have demonstrated that A4+ is well tolerated across a wide range of doses. In these uncontrolled studies, it was found that the addition of daily A4+ either in conjunction with HCV therapy, or alone, showed a significant improvement in the following parameters: depression, health-related QOL as measured by Medical Outcomes Short Form, 36 item survey (SF-36), Hepatitis Quality of Life Quotient (HQLQ); and an improvement in clinical symptoms which included fatigue; dyspepsia; nausea and vomiting; indigestion; right upper quadrant abdominal pain and tenderness; headache; muscle, joint and bone pain. Both studies concluded that A4+ demonstrated a clinical benefit. A4+ is considered a safe product to use with only a minimum of reported mild side effects. The most common side effect of A4+ is mild headache, reported within the first 7 days of taking A4+. This side effect usually abates after the first week.

Cordia lutea: *Cordia* is a genus of flowering plants in the borage family, Boraginaceae. It contains about 300 species of shrubs and trees. Many studies have shown the hepatoprotective potential of different species of *Cordia* and have been used for many years. The flower component of *Cordia lutea* has been used as a traditional medicine for centuries in different cultural paradigms including ancient Peruvian culture.

- Bussmann RW, Sharon D, Garcia M., "From Chamomile to Aspirin? Medicinal Plant Use Among Clients at Laboratorios Beal in Trujillo", Peru, Ethnobotany Research and Applications, 2009, Vol 7, 399-407.
- Saravana Kumar, R Gandhimathi, KK Senthil Kumar, Kusuma Praveen Kumar., "Hepatoprotective potential of *Cordia subcordata* Lam. against carbon tetra chloride (CCl4)-induced hepatotoxicity in Wistar albino rats". J Biomed Sci and Res., 2009, Vol 1 (1), 19-26.
- M Afzala, C Obuekweb, A R Khanc and H Barakata., "Antioxidant activity of *Cordia myxa* L. and its hepatoprotective potential". EJEAFChe 2007, 6 (6), 2109-2118.
- Thirupathi K., Sathesh Kumar S., Goverdhan P., Ravikumar B., Krishna DR and Krishna Mohan G., "Hepatoprotective action of *Cordia dichotoma* against Carbon tetrachloride induced liver injury in rats". Nig. J. Nat. Prod. and Med. 2007, Vol. 11, 37-40.

Curcuma longa (USDA 2008): **Based on Health Canada Monograph, the use or purpose of Turmeric: Oral**

Statement(s) to the effect of:

- Provides antioxidants for the maintenance of good health (ESCOP 2003, Blumenthal et al. 2000, Mills and Bone 2000).
- Used in Herbal Medicine to aid digestion (ESCOP 2003, Williamson 2003, Blumenthal et al. 2000, Mills and Bone 2000).
- (Traditionally) used in Herbal Medicine to help relieve flatulent dyspepsia (carminative) (Mills and Bone 2005, Blumenthal et al. 2000, Wren 1907).
- **Used in Herbal Medicine as a hepatoprotectant/liver protectant** (Boon and Smith 2004, Williamson 2003**).**
- Used in Herbal Medicine to increase bile excretion by the liver (choleretic) and stimulate contraction of the gallbladder (cholagogue) (Mills and Bone 2005, Boon and Smith 2004, Wichtl 2004, Williamson 2002, Blumenthal et al. 2000, Mills and Bone 2000).
- (Traditionally) used in Herbal Medicine as an anti-inflammatory to help relieve joint pain (Winston and Kuhn 2008, Blumenthal et al. 2000).

- Used in Traditional Chinese Medicine (TCM) to eliminate blood stasis, promote the flow of qi and relieve pain of menstruation due to blood stasis (PPRC 2005).
- Traditionally used in Ayurveda to relieve pain and inflammation, and assist healing of minor wounds such as cuts and burns, and minor skin irritations (Paranjpe 2005, Murthy 2004, API 2001 [1990], Kapoor 2001).

Annona Muricata: *Annona* is a genus of flowering plants in the pawpaw/sugar apple family, *Annonaceae*. It is the second largest genus in the family containing approximately 110 species of mostly neotropical and afrotropical trees and shrubs. Many studies have shown the hepatoprotective potential of different species of *Annona* and have been used for many years. *Annona muricata* has a long, rich history of use in herbal medicine as well as a lengthy recorded indigenous use. Raintree Nutrition Monograph for *Annona muricata* (Guanabana/Graviola) also indicates that "*Annona muricata* has a long, rich history of use in herbal medicine as well as a lengthy recorded indigenous use." "In the Brazilian Amazon, a leaf tea is used for liver problems".

- Raintree Nutrition, Tropical Plant Database, Monograph (Database File) for Graviola (*Annona muricata*).
- Adewole SO, Ojewole JA., "Protective effects of *Annona muricata* Linn. (Annonaceae) leaf aqueous extract on serum lipid profiles and oxidative stress in hepatocytes of streptozotocin-treated diabetic rats". Afr J Tradit Complement Altern Med. 2008; 25; 6(1):30-41.
- Orlando Vieira de Sousa, Glauciemar Del-Vechio Vieira, José de Jesus R. G. de Pinho, Célia Hitomi Yamamoto and Maria Silvana Alves., "Antinociceptive and Anti-Inflammatory Activities of the Ethanol Extract of *Annona muricata* L. Leaves in Animal Models". Int J Mol Sci, 2010, 11, 2067-2078.
- Baskar R, Rajeswari V, Kumar TS., "In vitro antioxidant studies in leaves of *Annona* species". Indian J Exp Biol. 2007; 45(5):480-5.
- D Sobiya Raj, J Jannet Vennila, C Aiyavu, K Panneerselvam., "The hepatoprotective effect of alcoholic extract of *Annona squamosa* leaves on experimentally induced liver injury in swiss albino mice". JIB, 2009, Vol. 5(3), 182-186.
- Mohamed Saleem TS, Christina AJM, Chidambaranathan N, Ravi V, Gauthaman K., "Hepatoprotective activity of *Annona squamosa* Linn. on experimental animal model". International Journal of Applied Research in Natural Products, 2008, Vol. 1(3), 1-7.
- Natural Medicines Comprehensive Database, Monograph for Graviola (*Annona muricata*).

In addition to the above references, following studies were conducted by Sabell Corporation that summarizes the efficacy and safety of A4+:

A) Cabanillas Jose, Joseph Nystrom, Hugo Marquez, S., "Short term effects of A4+ on clinical and biochemical markers in chronic hepatitis C", September 2001.

This document summarizes the results of the clinical study entitled "Prospective Study of the Course of Chronic Hepatitis C after the Incorporation to the Daily Diet of A4+ in 6 Patients During 4 Weeks". The primary objective of this study was to evaluate the value of a natural nutraceutical A4+ used in 6 patients with chronic Hepatitis C. Patients with varying severity of Chronic Hepatitis C Genotype 1 were administered 20 grams per day of the product A4+ in the form of a tea infusion steeped in boiling water prior to each of three meals per day for 4 weeks. All patients were provided a balanced diet and exposed to natural sunlight on a daily basis and all patients demonstrated clinical improvement and were uniformly free of adverse reactions to the product.

The majority of patients entering the study had baseline abnormal liver functions. There were no statistical changes in the liver functions in these first 4 weeks of treatment. Likewise, there were no changes in the markers for other organ systems. While being a study of very short time length, we have noted no adverse trend on serum GTT, SGOT, SGPT, bilirubin, alkaline phosphatase, blood counts, and renal function of lipid indices. The study is too small to assign significance to results regarding viral loads or alphafetoprotein.

However, this study did conclude that the natural product A4+ demonstrated a clinical benefit and merits further study as an alternative treatment for those suffering from Chronic Hepatitis C.

B) Samanta A., "Quality of life following the use of A4+ in a limited number of subjects with chronic hepatitis C: Open-label, non placebo-controlled, preliminary observations", Independent assessment report, 2004.

The primary objective of this clinical study was to examine whether A4+ might have a beneficial effect in patients with chronic hepatitis C. The study was an open-label, non-randomized, non-placebo controlled preliminary assessment of the effect of oral ingestion of A4+ on symptoms of chronic hepatitis C. Clinical parameters noted below were evaluated a day prior to the administration of A4+ and then again at 28 days after the use of A4+. The impact of use of A4+ was assessed by comparing the values of these parameters at the start of the study against values at day 28 of the study.

This study included 10 Caucasian volunteer patients. All ten patients were treated with the A4+ formula taken orally 3 times a day for 28 days. Nine out of ten of the patients were long

term sufferers of Hepatitis C Genotype 1. Prior to the start of this study, the tenth patient had successfully treated Genotype 2 Hepatitis C with Interferon, but was diagnosed with severe liver cirrhosis. This patient clearly presented different results compared to the other 9 Chronic Hepatitis C Genotype 1 patients.

The parameters studied for the effect of A4+ on Hepatitis C patients before and after treatment are included below;

Depression: The severity of depression at the start of the study ranged from borderline in 10% of the subjects, mild to moderate depression in 80%, and severe depression in 10%. By the end of study period significant improvement in depression was noted and 90% of the subjects had become free of depression and 10% exhibited mild depression. Noteworthy is the reported change in the Beck Depression Inventory score. Mean score before the use of A4+ was 20.9 (range 11-35) and decreased markedly to 2.7 (range 0-3) 28 days after the use of A4+. Sixty percent of the subjects reported total Beck score of 2 or less, including score of 0-1 in 40% after 28 days of the use of A4+.

Health-Related Quality of life Burden (SF-36 HQLQ): In general, use of A4+ was accompanied by significantly improved health-related quality of life in study subjects by Day 14. By Day 28, the functional status and well-being of nine out of the ten study subjects were restored completely to normal levels. The score improvements observed in this study by Day 28 were on average larger than two standard deviations for nearly all health-related quality of life scales.

Clinical symptoms: Study subjects showed a significant improvement in most of their symptoms. This included improvement in fatigue, right upper quadrant pain and tenderness, dyspepsia, nausea-vomiting, indigestion, headache, muscle and joint/bone pain.

Fatigue: *Before the use of A4+:* Severe fatigue was present in 80%, moderate fatigue in 10% and mild fatigue in 10% of the subjects. *After 28 days of the use of A4+*: Severe fatigue was seen in 20% and 40% each having mild and moderate fatigue.

Dyspepsia: Utilizing the maximum score of 15 for dyspepsia as described above (GSRS), it was rated as mild (score 1-5), Moderate (score of 6-10) and severe (score of 11-15). Before the use of A4+: None of the subjects were free of dyspepsia. Most of the subjects had mild dyspepsia (70%), and 30% had moderate dyspepsia. After 28 days of the use of A4+: Dyspepsia was absent in 90% with 10% had moderate dyspepsia.

Nausea and Vomiting: Was assessed as one of the elements of dyspeptic syndrome domain of GSRS scoring system noted above. Before the use of A4+: Was experienced occasionally by 30%, frequently by 30% and was absent in 40% of the subjects. After 28 days of the use of A4+: Was resolved in 90% of the subjects, and 10 % had only occasional nausea and vomiting.

Indigestion: Utilizing the maximum score of 12 for indigestion as described above (GSRS), indigestion was rated as mild (score 1-5), Moderate (score 6-9) and severe (score 10-12). Before the use of A4+: Most subjects (70%) had mild indigestion, 20% experienced moderate indigestion, and 10% had severe indigestion (Figure 5). After 28 days of the use of A4+: Symptom of indigestion was absent in 70%, while 30% had moderate indigestion.

Nutritional status: In order to ascertain whether the observed changes in the health-related quality of life and symptoms of the study subjects could have been influenced by improved nutritional status, the nutritional status of the study subjects was evaluated both at the beginning and at the end of the study period. Nutritional parameters, including anthropometry and serum transferrin and pre-albumin remained unchanged during the short study period indicating that improvement in symptoms and health-related quality of life were independent of nutritional status.

Routine Liver Chemistry: Serum bilirubin, AST, ALT and albumin did not show any change at the end of the study as compared to pre-study values.

Prothrombin activity and serum cholinesterase: There was a significant increase in Prothrombin activity and serum cholinesterase which suggests possible increased protein synthesis by liver or a decrease in their degradation.

At baseline, prothrombin activity expressed as percent of control was 68.9 + 22.7% and serum cholinesterase was 5194.1 + 1590.1 U/L. After 28 days these increased to 81.2 + 28.0% and 7792.7 + 2218.8 U/L respectively.

TNF-α: Serum TNF-α was increased at the end of 28 days (10.0 + 3.0 pgm/ml) of the use of A4+ as compared to the Day 1 value (7.2 + 2.1 pgm/ml).

Liver size: There was no change in the liver size or echo-texture of the liver during the use of A4+.

Results of this study was summarised by **Kosinski M., "Revised Summary Report on Study Findings of 10 Patients Receiving Nutraceutical Product A4+L for the Treatment for Chronic Hepatitis C Virus (CO-1031)", Quality Metric Inc., July 2004**. Quality Metric Incorporated (QMI) is a health survey provider for the healthcare and life sciences industries. QMI's products and services are designed to measure patient-reported outcomes from clinical studies and provide scientifically valid assessments of physical and mental health.

The conclusions from the HQLQ report from QMI were that on average, treatment with the Nutraceutical Product A4+ was observed to significantly improve the health related quality of life of study patients. By day 14 and by day 28 of treatment, the functional status and well-being of study patients was completely restored to normal levels. The score improvements

observed in this study by day 28 were on average larger than two standard deviations for nearly all health related quality of life scales, which has rarely been observed in the thousands of treatment studies of other chronic diseases involving the SF-36 Health Survey. In addition, the evaluation of individual patient scores over time showed that 9 out of the 10 patients in this study improved by day 28 by a clinically meaningful amount on each of the health related quality of life scales, so the average results were not driven by a couple of outlier patients. The conclusions address the small patient population size of the study (n=10) but also states that despite the lack of statistical power, statistically significant changes were observed across all health related quality of life scale scores.

C) Swain MG., "Evaluation of the Hepatoprotective and Behavioral Effects of the Herbal Compound A4+ in Acute Liver Injury Models" (University of Calgary, Alberta, 2011).

The purpose of this study was to investigate whether A4+ could attenuate the detrimental behavioural (i.e., sickness behaviours) and biochemical effects associated with liver injury in two well-characterized mouse models of liver injury.

The first model was bile duct ligation and resection (BDR). For this the mice were randomly divided into two groups: BDR surgery only (control) and BDR plus A4+ (160 mg/kg/day) administered by oral gavage. Sickness behaviour was evaluated using two well established methods: (1) A social investigation paradigm and (2) open field locomotor activity measurement to assess overall mobility. Result shows that levels of plasma ALT and total bilirubin levels were similar in control and A4+ treated BDR mice at 9 days post surgery, as was time spent in social investigation behaviour. By contrast, BDR animals administered A4+ were significantly more active in overall mobility for both ambulatory movements ($p=0.03$) and enhancement of the number of horizontal movements ($p=0.04$) respectively.

The second model was Concanavalin A (Con A)-induced hepatitis. For this study, one group of mice were pre-treated with vehicle (control) and another group with A4+ (640 mg/kg). As in the first study, biochemical measurements were measured. In addition, livers were dissected and processed for flow cytometry analysis 9FACS) after staining of different cell surface markers to identify immune cell subsets and to identify cytokine production profiles of these cells using intracellular staining. Result shows that plasma ALT levels in the A4+ treated Con A mice were significantly ($p=0.04$) reduced compared to the control Con A mice. Pre-treatment with A4+ had no significant effect on IFN© cell recruitment and activation. In contrast, hepatic recruitment of IFN© expressing NK cells to the liver were significantly ($p<0.05$) increased in the A4+ Con A treated animals compared to Con A controls.

In conclusion, the first study indicated that A4+ has beneficial behavioural modifying effects in a test which examined sickness-related immobility. These improvements occurred in the absence of significant changes in biochemical indices of liver damage.

In the second study A4+ treatment attenuated Con A hepatitis as reflected by a reduction in plasma ALT levels compared to vehicle-treated controls. Pre-treatment with A4+ had no significant effect on IFN© cell recruitment and activation. However, more hepatic NK cells expressed IFN© in mice pre-treated with A4+ which received ConA than in vehicle-treated mice.

Overall, the finding of positive changes in behaviour, liver enzymes and some aspects of immune function in these animal models of hepatic injury indicate a potential clinical relevance for the treatment of patients with hepatic injury.

D) Wallace J., "Evaluation of the Anti-oxidant, Anti-inflammatory and mucosal protective Actions of A4+ and its Constituents" (Penumbra Associates Ltd, 2010).

The purpose of this study was to investigate the anti-oxidant, anti-inflammatory and mucosal protective effects of A4+. The anti-oxidant activity and its constituents were evaluated using an *in vitro* assay in which a stable free radical was allowed to interact with the test substance. The result of this study shows that A4+ exhibited potent anti-oxidant activity. The results suggest that the anti-oxidant effects of A4+ can mainly be attributed to the *Cordia* and *Annona*, but not with *Curcuma*.

Anti-inflammatory action of A4+ was evaluated by one of the most widely used *in vivo* "air pouch" model. This model allows one to determine the effects of a drug on many different aspects of the inflammatory process, thereby allowing for determination of mechanism(s) of action. The results showed that administration of A4+ to the site of inflammation results in reduced levels of inflammatory mediators, or reduced inflammation.

A4+ administration reduced the severity of gastric damage induced by subsequent administration of a potent nonsteroidal anti-inflammatory drug (indomethacin), but only at quite a high dose (≥300 mg/kg) and only when given at least 4 hours prior to the indomethacin. The result suggests that sufficient blood levels of A4+ much be achieved to observe the gastroprotective effect.

These studies demonstrated the anti-oxidant and anti-inflammatory properties of A4+ and provided insight into the mechanisms of action of A4+ for its beneficial effects on hepatitis.

E) Tyrrell L., Part 1: "Anti-viral effects of plant extracts on HCV infected cells" (Li Ka Shing, Institute of Virology, University of Alberta, 2011).

The purpose of this study was to determine the anti-viral effect of A4+ on Hepatitis C virus (HCV) infected cells. The studies were carried out using Huh7.5 cells and a tissue-culture adapted strain of HCV, JFH. Cells were seeded, allowed to adhere and establish, followed by 4hr infection incubation with HCV strain. After infection, cells were washed with fresh media followed by treatment with A4+. Cells were exposed to diluted A4+ powder (0.1, 0.5, 1, 5 and 10 μg/mL in 45% ethanol) for 4 days. This was followed by collection of supernatant and cells for viral titering. The studies were repeated three times and in each, results showed that A4+L component of the A4+ powder indicated significant levels of antiviral activity. Intracellular and extracellular fractions were measured after 4 days of drug treatment, and both fractions showed a significant drop of HCV titres at A4+L concentrations greater than 1 μg/mL. The A4+L showed activity at all concentrations used – but was most active at 1 μg/ml or greater with approximately 90% inhibition of HCV in cell cultures at 10 μg/ml. To prove beyond a doubt that the A4+L drug is antiviral, viral protein levels were visualized by western blot. Cells were plated, infected, and drug treatment was conducted as previously described. After treatment, cells were lysed with RIPA buffer to release cell contents and prevent protein degradation. Protein levels were quantified with a BioRad Protein assay so the same amount of protein could be added to each well. Two HCV antibodies were used to determine viral quantity: NS3 and core. A drop in NS3 is seen in lanes for A4+, A4+L and A4+R, with the greatest being in A4+L. This observation indicates that viral load is decreased as a result of A4+ exposure. In Part 1, it was demonstrated that the A4+ plant extract had significant antiviral activity against HCV in cell culture.

F) Tyrrell L., Part 2: "Effect of Short-term exposure to A4+ plant extract on Natural Killer Cell Activity" (Li Ka Shing, Institute of Virology, University of Alberta, 2011).

The purpose of this study was to determine if the antiviral benefits of the A4+ plant extract attributed to enhancement of Natural Killer (NK) activity. C57/B6 mice were treated with A4+ daily for 14 days. After 14 days, mice were euthanized and their spleens were removed for NK cell preparation. A cell suspension was prepared and NK cells were purified using an Easy Sep Mouse NK Cell Enrichment Kit. Purified NK cells were analyzed by FACS. The result showed that treatment with A4+ didn't enhance NK activity *in vivo*. There may have been a tendency for decreased NK activity with A4+ treatment, but the number of experiments was too low to show statistically significant differences. In this short term experiment the A4 extract were not toxic to the mice. Since the antiviral activity was only shown in lymphocyte free cell culture, it was concluded that the antiviral effect of A4+ on HCV is a direct antiviral effect and is not mediated through a NK immune enhancement.

G) Cabanillas J., Fong F., "A4+ Interviews with Peruvian Medicine Experts", Peru, January 2009. Translated to English by Mr. Jorge Alvarado, Certified Translator from the Society of Translators and Interpreters of British Columbia, March 2009.

The following is a summary of five individual interviews with five different Peruvian Elder Expert Healers in Peru. Each confirms the traditional use of either *Cordia lutea*, *Curcuma longa* (Turmeric), and *Annona muricata* (Graviola) for maintaining liver health. The interviews were performed in Peru in January 2009 by Dr. Jose Cabanillas together with Fenton Fong of PharmEng Technology.

The first three interviews focus on the traditional use of Graviola (aka. *Annona muricata*/Guanabana) with additional references to the use of Turmeric or *Curcuma longa* (aka. Curcuma, Curcumin, "Guisador"). All three experts interviewed indicated that Graviola and Turmeric have been used for centuries to treat conditions of the liver, commonly referred to in the interviews as Hepatitis. All agreed that the use of Graviola and Turmeric is safe and that there are no known side effects. They also confirmed that the preparation and use of alcohol extracts of Graviola and Turmeric is a common practice.

The fourth and fifth interviews focus on the traditional use of *Cordia lutea* (Overal) in traditional Peruvian culture for treating liver illnesses. All agreed that the use of *Cordia lutea* is safe and has no side effects.

Summary, Interview 1: Marcos Pandoro Mesa, age 43. Mr. Mesa indicated that both Guanabana and Curcuma have been used for longer than 50 years; they have been used since 'the time of his grandparents'. He indicated that both ingredients are used to treat the liver as well as blood problems and malaria. Mr. Mesa knew of no side effects, recommended to avoid fatty foods, meat, and acidic foods. The method of preparation he was most familiar with was chopping the roots of the Curcuma plant and boiling in water. He also said that you can chop the root, put it in alcohol, mix it for 3 days then you can drink it. He indicated a use of half a cup every morning before breakfast for 9 days.

Summary, Interview 2: Mr. Ruiz, 56 years old. Mr. Ruiz is employed as a forestry engineer, and said he often works with botanists, shamans and healers in using plants from the Amazon as natural medicines. He said people have been using Guanabana and Curcumin since at least the 1800's, and he knew of no side effects. He indicated that Curcumin is traditionally used for treating the liver, and Guanabana is also used for treating the liver but mostly for cancers, breast and liver cancers and problems of the female reproductive system. He prepares Curcumin by grating the root and soaking in alcohol for 3 days then uses the alcohol in hot water for drinking, and crushes the leaves of the Guanabana and mixes with hot water to prepare a tea-like beverage. He also included that alcohol is commonly used in the Amazon to preserve plant medicines.

Summary, Interview 3: Ramon Aredano Huaman, age 74. Mr. Huaman said that besides treating others, he personally has been using these ingredients for over 60 years, and indicated no side effects. He said Curcuma is always used to treat liver related conditions like yellow fever. He also indicated Curcumin is usually boiled in water with the water later being drunk like a tea, or soaking in alcohol for 3 days and then adding the alcohol to water to drink. For Guanabana he suggested crunching the leaves and mixing them with hot water to be drunk.

The following two interviews focused on *Cordia lutea* (Overal) in traditional Peruvian culture. The two experts interviewed all indicated that the use of Cordia was common in teas for treatment of the liver.

Summary, Interview 4: Hernan Alemen, 62 years old. Mr. Alemen indicated said he uses Cordia lutea himself but also has harvested the flower and sends it to others to be used in teas to treat jaundice and liver problems. He said this plant has been used for well over 100 years, and was used by his grandparents. He knew of no side effects and said pork, and shellfish should be avoided. He recommended taking the tea 3 times per day to treat liver conditions. He recommended preparing from fresh water and perhaps adding honey.

Summary, Interview 5: Rigoberto Leon Carrasco, 75 years old. In this final interview from Mr. Carrasco, the healer said he relies on the teachings of his grandparents and said his grandmother used Cordia when he was a child to treat family members with hepatitis and jaundice. He personally uses the Cordia tea when he feels ill, fatigue, or has inflammation of the liver and claims he has used Cordia to successfully treat his granddaughter who was ill with hepatitis, having not had the hepatitis vaccine. He said there can be different methods of taking this medicine for different conditions but for liver problems it is taken as a tea. He also said this flower can be soaked in alcohol for 8 to 15 days with the alcohol later being used in hot water.

It should be noted that the interview accounts above, show that each of the three constituent plants in A4+ have been used individually in traditional Peruvian medicine for similar, overlapping treatment purposes or healing paradigms (namely: promoting healing of the liver and maintaining liver health) for well over 50 years. Therefore, according to NHPD, the above interview accounts can be considered as acceptable expert opinion report evidence for the efficacy of A4+. The above interview evidence is being used to support each ingredient component of A4+, and A4+ final product because they all have a common therapeutic purpose.

H) Sabell Corporation's Medical Research & Advisory Board

Sabell Corporation is committed to developing a safe and effective treatment for liver diseases using natural/herbal products. Sabell Corporation has recognized the potential of A4+ and chosen to invest in the development and execution of a number of clinical, in vitro, in vivo, toxicology, and product chemistry development studies to prove the safety and efficacy of A4+.

Sabell Corporation has a medical research and advisory board comprised of the following highly distinguished doctors - two of which are recipients of the Order of Canada;

Francis Green, M.D, Ph.D. Department of Pathology & Laboratory Medicine, Faculty of Medicine, University of Calgary Edmonton, Canada	Medical Advisory Committee Chair for Sabell Corporation
Steven K.H. Aung, M.D, College of Integrated Medicine, Faculty of family Medicine, University of Alberta Edmonton, Canada	Recipient of the **Order of Canada** & WHO Consultant. Medical practitioner of Eastern, Western and Integrated Medicine, Advisor on traditional herbal medicine.
John Wallace, *PhD, MBA, FRSC,* Farncombe Family Digestive Health Research Institute, McMaster University, Canada	Evaluation of the Anti-Inflammatory, Anti-Oxidant and Mucosal Protective/Reparative Actions of A4+ and Its Constituents
Lorne Tyrrell,M.D, PhD, FRCP, Professor and CIHR/GSK Chair in Virology, Director, Li Ka Shing Institute of Virology University of Alberta Edmonton, Canada	Recipient of the **Order of Canada**; former Dean , Faculty of Medicine , University of Alberta. Investigations of the Herbal Compound A4+ and its constituents as a Potential Antiviral Therapy for Hepatitis B and Hepatitis C Viruses
Mark G. Swain, MD, MSc, FRCPC, Professor of Medicine, University of Calgary	Hepatologist. Engaging in studies with mice to determine effect of A4+ and Cordia flowers in fibrosis, and the anti-inflammatory effects in liver disease.
Sam Lee, M.D. & Alex Aspinal, M.D, Faculty of Medicine, University of Calgary	Hepatologists. Investigation and preparation for a potential double blind placebo controlled human clinical trial involving

Edmonton, Canada	A4+ in treating the symptoms and quality of life of chronic Hepatitis C Genotype 1 patients
Hugh A. Semple, DVM, PhD, Toxtest, Alberta Innovates – Technology Solutions in Vegreville, Edmonton, Canada	Heading four new GMP A4+ toxicology testing studies at AITF, consulting on product development.
Brian Duff Sloley, PhD, Senior Scientist, Phytovox Inc.	Analytical chemistry research for characterizing marker compounds in the A4+ formulation and individual constituents.
Raimar Loebenberg, PhD, Director, Drug Development and Innovation Centre and Associate Professor, Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta,	A4+ Stability testing and formulation consultation
Dr Sheldon Roth, Professor, Department of Physiology & Pharmacology Faculty of Medicine, Hotchkiss Brain Institute	Advisor on toxicology and A4+ excipient formulation.

The herbal components of A4+ originate from the Amazon rainforest and the Coastal Plains of Peru. The unique formulation for A4+ has been developed by Dr. Jose Cabanillas, a western trained physician who is licensed in Peru and who is also a widely respected and highly regarded expert in natural medicinal plant medicines derived from the Amazon rainforest, the Andes and the Coastal Plains of Peru (http://sabell.ca/jose/bio.htm). Dr Cabanillas provides invaluable cultural expertise and knowledge into the medicinal plant ingredients for a variety of medicinal herbal formulas from Peru. Sabell Corporation has a long standing collaborative relationship with Dr. Cabanillas.

1.3 Dosage and Other Conditions of Use

A4+ is available as (250 mg) capsule in a bottle containing 84 capsules. Each capsule contains 77.8 mg *Cordia lutea*, 9.7 mg *Annona muricata* and 9.7 mg *Curcuma longa*.

Two capsules to be taken three times a day before meals. It is recommended that A4+ be taken daily for a minimum of 30 days. Users are encouraged to get a "daily dose" of sunlight on their bodies. Persons taking this herbal product are recommended to avoid fatty foods, shellfish, pork and red meat.

2.0 Safety Summary Report

2.1 Safety Overview:

Cordia lutea: *Cordia* is a genus of flowering plants in the borage family, Boraginaceae. It contains about 300 species of shrubs and trees. Many studies have shown the hepatoprotective potential of different species of *Cordia* and have been used for many years. The flower component of *Cordia lutea* has been used as a traditional medicine for centuries. It is used traditionally in Peru as a tea infusion or as an alcohol extract to treat jaundice, hepatitis and liver problems. Overall, *Cordia lutea* is a safe substance for human ingestion Peruvian medicine. There are no documented cases of adverse reactions.

Cautionary statement can be placed on the label:

- *Keep out of reach of children."*
- *"Consult a health care practitioner prior to use if you are pregnant, breastfeeding, or plan to become pregnant."*

Curcuma longa **(Turmeric):** Based on Health Canada Monograph, for Turmeric (*Curcuma longa)* USDA 2008; Date: 2010-02-25

- **Caution(s) and Warning(s)**
  - Consult a health care practitioner prior to use if you are pregnant (ESCOP 2003, Brinker 2001, McGuffin et al. 1997).
  - Consult a health care practitioner prior to use if you have gallstones or a bile duct obstruction (ESCOP 2003, Brinker 2001, McGuffin et al. 1997).
  - Consult a health care practitioner prior to use if you have stomach ulcers or excess stomach acid (Brinker 2001, McGuffin et al. 1997).
  - Digestive aid; Relief of flatulent dyspepsia; Hepatoprotectant; Bile excretion; Anti-inflammatory; TCM; Ayurveda:

    Consult a health care practitioner if symptoms persist or worsen

- **Contraindication(s)**

  No statement is required

- **Known Adverse Reaction(s)**

  No statement is required

***Annona muricata* (Graviola):**

Annona is a genus of flowering plants in the pawpaw/sugar apple family, *Annonaceae*. It is the second largest genus in the family containing approximately 110 species of mostly neotropical and afrotropical trees and shrubs. Many studies have shown the hepatoprotective potential of different species of *Annona* and have been used for many years. *Annona muricata* has a long, rich history of use in herbal medicine as well as a lengthy recorded indigenous use. It is used traditionally by many Peruvian and Brazilian residents and elders from both countries to treat jaundice, hepatitis and liver conditions. The Rain Tree information source also has a compiled list of numerous scientific studies that have been conducted to characterize the many potential benefits of *Annona muricata* (Graviola). Overall, *Annona muricata* is a safe substance for human ingestion and there are no documented cases of adverse reactions and has no known food or drug interactions.

- Based on Raintree Nutrition, Tropical Plant Database, Monograph (Database File) for Graviola (*Annona muricata*).

  - **Caution(s) and Warning(s)**

    - It has cardiodepressant, vasodilator, and hypotensive (lowers blood pressure) actions.
    - Large dosages can cause nausea and vomiting. Avoid combining with ATP-enhancers like CoQ10.

  - **Contraindication(s)**

    - Graviola has demonstrated uterine stimulant activity in an animal study (rats) and should therefore not be used during pregnancy.
    - Graviola has demonstrated hypotensive, vasodilator, and cardiodepressant activities in animal studies and is contraindicated for people with low blood pressure. People taking antihypertensive drugs should check with their doctors before taking graviola and monitor their blood pressure accordingly (as medications may need adjusting).
    - Graviola has demonstrated significant *in vitro* antimicrobial properties. Chronic, long-term use of this plant may lead to die-off of friendly bacteria in the digestive tract due to its antimicrobial properties. Supplementing the diet with probiotics and digestive enzymes is advisable if this plant is used for longer than 30 days.
    - Graviola has demonstrated emetic properties in one animal study with pigs. Large single dosages may cause nausea or vomiting. Reduce the usage accordingly if this occurs.
    - Alcohol extracts of graviola leaf showed no toxicity or side effects in mice at 100 mg/kg; however, at a dosage of 300 mg/kg, a reduction in explorative behavior and

mild abdominal constrictions was observed. If sedation or sleepiness occurs, reduce the amount used.

Cautionary statement can be placed on the label:

- *Keep out of reach of children."*
- *"Consult a health care practitioner prior to use if you are pregnant, breastfeeding, or plan to become pregnant."*
- *"Consult a health care practitioner prior to use if you are taking antihypertensive drugs".*

In order to confirm the safety of A4+, the following studies were conducted by Sabell Corporation;

1.0 Semple H., "A 7-Day Repeat Dose (oral) Toxicity Screen on A4+ in Rats", ToxTest Alberta Innovates–Technology Futures, Vegreville, Alberta, 2010.

The purpose of this study was to determine adverse effect resulting from daily, oral administration of A4+, over the course of 7 days in male and female Sprague Dawley rats. A4+ was administered orally for 7 days to rats in aqueous suspensions at dosage of 2000 (high dose), 500 (mid dose), 125 (low dose) and 0 (Controls) mg/kg BW.

Species	:	Sprague Dawley rats
Route / Method	:	Oral / gavage
Number of animals	:	10 (5 male and 5 female) animals/ group
Sex	:	Male and female
Body weight	:	Male (323.3 – 355.7 g) & Female (212.2 – 238.4 g)

Treatment Group	Treatment	No. of Animals/group		Dose Level (mg/kg BW)	Dose Conc. (mg/mL)	Dose Volume (mL/kg BW)
		Male	Female			
1	Control	5	5	0	0	6
2	Low dose A4+	5	5	125	20.81	6
3	Mid dose A4+	5	5	500	83.25	6
4	High dose A4+	5	5	2000	333.0	6

There was no overt evidence of toxicity related to the test article at any dosage administered. There was no apparent effect on general health, including clinical observations, body weight and food consumption. Analysis of clinical pathology and hematology parameters revealed minor statistically significant group differences mostly within the normal range, that were not considered to be biologically relevant test article related effects. The pathologic examination revealed no treatment related macroscopic findings, and one high-dose animal exhibited

myocarditis on histopathological examination. Whether this was treatment related is an open question since only one animal was affected.

In summary, this study did not demonstrate any common or consistent adverse effects at the doses employed and under the conditions of the experimental protocol of this study.

2.0 Semple H., "A 28-day repeat dose oral toxicity study of A4+ in rats", ToxTest Alberta Innovates–Technology Futures, Vegreville, Alberta, 2010.

The purpose of this study was to determine the toxicity of A4+ in rats, when given daily for 28 days. Four groups, Vehicle Control (1), Low dose (2), Mid dose (3) and High dose (4) of 5 female and 5 male Sprague Dawley rats were administered test of reference item by oral gavage daily for 28 days.

Species	:	Sprague Dawley rats
Route / Method	:	Oral / Gavage
Number of animals	:	10 (5 male and 5 female) animals/group
Sex	:	Male and female
Body weight	:	Male (275.3 – 315.6 g) and Female (188.7 – 211.9 g)

Treatment Group	Treatment	No. of Animals/group		Dose Level (mg/kg BW)	Dose Conc. (mg/mL)	Dose Volume (mL/kg BW)
		Male	Female			
1	Control	5	5	0	0	6
2	Low dose A4+	5	5	125	20.81	6
3	Mid dose A4+	5	5	500	83.25	6
4	High dose A4+	5	5	2000	333.0	6

Animals were observed twice daily, weighed weekly and food consumption was measured weekly. During the last week of the study, functional observational batteries were conducted on all Groups 1 to 4 animals. On study day 29, Group 1 to 4 animals were exsanguinated under anesthesis and the collected blood was analyzed for clinical chemistry and hematology including coagulation. The euthanized animals were necropsied and tissues were collected. Selected organs were weighed. Tissues from the high dose and control groups were processed and histopathological examination was conducted.

No significant test item-related effects were observed among clinical observation, body weights, food consumption, functional observational battery, urinalysis, clinical pathology parameters, blood coagulation parameters, mortality or macroscopic and histopathological findings. Small but statistically significant differences in some hematology results and organ weights were deemed to have low biological significance. No animal deaths occurred during the study.

It was concluded that A4+ did not exhibit toxicity, under the conditions of this study and a no adverse effect level (NOAEL) of 2000 mg/kg can be assigned.

3.0 Semple H., "A Mammalian Erythrocyte Micronucleus Study of A4+ in Mice", ToxTest Alberta Innovates–Technology Futures, Vegreville, Alberta, 2010.

The purpose of this study was to evaluate the genotoxic potential of A4+ based upon its ability to induce micronuclei in rodent polychromatophilic erythrocytes (PCE).

Species	:	Balb/c mice
Inbred	:	Balb/C/CNPB
Number of animals	:	60 animals for this study
Sex	:	Male and female
Body weight	:	Male (18.2 – 23.1 g) and female (16.9 – 19.9 g)

Treatment Group	Treatment	No. of Animals/group		Dose Level (µg/g BW)	Dose Conc. (mg/mL)	Dose Volume (µL/g BW)
		Male	Female			
1	Vehicle Control (Reference Item 1)	5	5	0	0	6
2	Low dose A4+	5	5	125	20.81	6
3	Mid dose A4+	5	5	500	83.25	6
4	High dose A4+	5	5	2000	333.0	6
5	Water (Reference Item 3)	5	5	0	0	6
6	Cyclophosphamide (Positive control Reference Item 2)	5	5	40	4.0	10

Under the conditions of the experiment, A4+ was negative for the production of elevated micronucleus counts and did not exhibit bone marrow toxicity. If an acute micronucleus test is negative after 24 hours exposure, another cohort of animals should be examined 48 hours post dosing, or consideration should be given to testing for micronuclei after multiple dosing. In the case of A4+, because the extract is poorly water soluble, oral absorption is likely to be slow. Therefore, the results of an acute micronucleus protocol may not adequately predict the genotoxicity potential of A4+ in human. Therefore, integration of another micronucleus test into a repeated dose oral toxicity study is recommended.

4.0 Semple H., "A Repeated Dose Mammalian Erythrocyte Micronucleus Study of A4+ in Mice", ToxTest Alberta Innovates–Technology Futures, Vegreville, Alberta, 2010.

The purpose of this study was to evaluate the genotoxic potential of A4+ based upon its ability to induce micronuclei in rodent polychromatophilic erythrocytes (PCE).

Species	:	Balb/c mice
Inbred	:	Balb/C/CNPB
Number of animals	:	56 animals for this study
Sex	:	Male and female
Body weight	:	Male (16.2 – 22.2 g) and female 916.1 – 19.1 g)
Dose / Method	:	Treatment group: 1 to 4 (Oral / Gavage) Treatment group: 5 (Intraperitoneal / Injection)

Treatment Group	Treatment	No. of Animals/group		Dose Level (µg/g BW)	Dose Conc. (mg/mL)	Dose Volume (µL/g BW)
		Male	Female			
1	Control	5	5	0	0	6
2	Low dose A4+	5	5	125	20.81	6
3	Mid dose A4+	5	5	500	83.25	6
4	High dose A4+	5	5	2000	333.0	6
5	Cyclophosphamide (Positive control)	8	8	40	4.0	10

No abnormalities were observed during clinical observations. Animals were euthanized 24 hours after the final or in the case of the positive control group, only dosing, both femurs were collected, and the marrows were flushed with fetal bovine serum. From the suspended cells, bone marrow smears were made. The high dose group and both negative and positive control group smears were scored for PCE/200 erythrocytes and for micronucleated PCE 9MNPCE)/2000 PCE.

Statistical analysis of the bone marrow scores revealed that the positive control compound cyclophosphamide reduced PCE scores similarly in both sexes, an indicator of bone marrow toxicity. No changes in PCE scores were associated with high dose A4+ test item. MNPCE scores, the primary indicator of genotoxicity in this test, were significantly higher in the positive control animals of both sexes than in either the high dose A4+ treated animals or the negative controls. The A4+ treated animals of both sexes has similar low MNPCE scores to the control animals with no significant differences between groups.

It was concluded that under the repeated dose conditions of the experiment, A4+ was negative for the production of elevated micronucleus counts and did not exhibit bone marrow toxicity based on the results of this study.

5.0 Cabanillas Jose., "Toxicological Evaluation of the A4+ Formula", University of Iquitos, Lima, Peru, 2009.

Two A4+ toxicity studies conducted; a) The acute toxicity of A4+ was tested using limited dose test in albino rats and b) The acute toxicity of A4+ was tested using limited dose test in albino mice.

a) The acute toxicity of A4+ was tested using limited dose test in albino rats (Holtzmann). The A4+ and a control substance (a saline solution) were administered orally using an intragastric catheter.

Species	:	Albino rat (Rattus novergicus)
Inbred	:	Holtzmann
Number of animals	:	3 animals per experimental group
Sex	:	Male and female
Body weight	:	120 – 160 g
Group 1 (Treatment)	:	These animals were administered a dose of 2,000 mg/kg of A4+
Group II (control)	:	These animals were administered a saline solution (same as A4+ volume)

This experiment was performed using male and female rats, which underwent a week-long quarantine, were divided in two groups composed on three animals of each sex, and were weighed and marked for identification purposes. Before the evaluation, the animals underwent a fasting period of 12 hours; then, the A4+ and the saline were administered to both groups according to the dose. Immediately after the substances were administered, the animals were observed to look for toxic signs at system/organ level: Autonomous, behaviour, sensory, neuromuscular, respiratory, ocular, gastrointestinal, urinary, and others, such as body weight. The body weights of the animals were recorded on the 7^{th} and 14^{th} day after the administration.

After 14 days, the animals were sacrificed following the ethical principals for the animal experimentation; this was followed by a macroscopic study to analyse the size, colour and consistency of the following organs: heart, kidneys, liver, spleen, stomach, lung, brain, ovaries and testicles. The macroscopic analysis of the organs did not find any visible changes where the A4+ was administered at a dose of 2,000 mg/kg.

The results obtained show the innocuousness of the extract at a dose of 2,000 mg/kg A4+, since no mortality and no clinical signs or macroscopic changes were observed, thus finding no evidence of toxicity in the organs.

b) The acute toxicity of A4+ was tested using limited dose test in albino mice (Balb/C/CNPB). The A4+ and a control substance (a saline solution) were administered orally using an intragastric catheter.

Species	:	Albino Mice (Mus musculus)
Inbred	:	Balb/C/CNPB
Number of animals	:	10 animals per experimental group
Sex	:	Male and female
Body weight	:	20 – 25 g
Group 1 (Treatment)	:	These animals were administered a dose of 2,000 mg/kg of A4+

Group II (control) : These animals were administered a saline solution (same as A4+ volume)

The mice underwent a fasting period of 4 hours prior to the experiment; then, the A4+ and saline solution were administered accordingly and the animals were under continuous observation for 4 hours. Upon no occurrence of mortality, the observation period was extended to 14 days after the administered, and then up to 21 days, in order to perform an observation of the recovery of the animals and the reversibility of the effects. The body weight of the animals was recorded at the beginning of the experiment, as well as on the 7^{th} , 14^{th} and 21^{st} day, when possible after the substances were administered, in order to established whether there was a weight loss or gain.

At the end of the experiment, the animals were sacrificed through a cervical dislocation procedure. A necropsy was performed on all the animals that survived until the end of the experiment. The microscopic analysis of the organs did not find any visible changes in the Group I mice where the A4+ was administered at a dose of 2,000 mg/kg. The results obtained show the innocuousness of the A4+ at a dose of 2,000 mg/kg. p.c., since no mortality and no clinical signs or macroscopic changes were observed, and thus there was no evidence of toxicity in the organs.

Drug and Food Interactions:

There are no known interactions for *Curcuma longa* (Turmeric), *Annona muricata* (Graviola) or *Cordia lutea*.

2.2 Risk Information and Risk Mitigation:

According to the information in Section 2.1, the following cautions and warnings have been determined to be included on the product label:

- *"Keep out of reach of children."*
- *"Consult a health care practitioner prior to use if you are pregnant, breastfeeding, or plan to become pregnant."*
- *"Consult a health care practitioner prior to use if you are taking antihypertensive, blood thinning medication, have gallstones, ulcers, liver or bile obstruction."*

3.0 Combination Rationale (if applicable)

In accordance with NHPD guidance (NHPD Safety and Efficacy guidance document, Ver.2, Dec 2006) a combination rationale is not required for the A4+ formulation because "adequate evidence is provided above that support the safety and efficacy of the finished product".

4.0 Non-Medicinal Ingredient Information, if applicable

All non-medicinal ingredients are included in Health Canada's list of acceptable non-medicinal ingredients.

5.0 References

1. Adewole SO, Ojewole JA., Protective effects of *Annona muricata* Linn. (Annonaceae) leaf aqueous extract on serum lipid profiles and oxidative stress in hepatocytes of streptozotocin-treated diabetic rats. Afr J Tradit Complement Altern Med. 2008; 25; 6(1):30-41.
2. Baskar R, Rajeswari V, Kumar TS., In vitro antioxidant studies in leaves of *Annona* species. Indian J Exp Biol. 2007; 45(5):480-485.
3. Bussmann R.W., Sharon D., Garcia M., From Chamomile to Aspirin? Medicinal Plant Use Among Clients at Laboratorios Beal in Trujillo, Peru, Ethnobotany Research and Applications, 2009, Vol 7, 399-407.
4. Cabanillas Jose., "Toxicological Evaluation of the A4+ Formula", University of Iquitos, Lima, Peru, 2009.
5. Cabanillas J., Fong F., A4+ Interviews with Peruvian Medicine Experts, Peru, January 2009. Translated to English by Mr. Jorge Alvarado, Certified Translator from the Society of Translators and Interpreters of British Columbia, March 2009.
6. Cabanillas Jose, Joseph Nystrom, Hugo Marquez, S., "Short term effects of A4+ on clinical and biochemical markers in chronic hepatitis C", September 2001.
7. Cabanillas Jose, Nystrom Joseph W, Zambrano Luis, Chicolote Gerardo., "Chronic Hepatitis C Treatment with A4+, Controlled Clinical Test", Lima Peru, May-June 2004.
8. Coral Jose Gonzalo Cabanillas., "Herbal Compositions and Methods for Treating Hepatic Disorders", International Filing Date: 03 October 2008.
9. D Sobiya Raj, J Jannet Vennila, C Aiyavu, K Panneerselvam., "The hepatoprotective effect of alcoholic extract of *Annona squamosa* leaves on experimentally induced liver injury in swiss albino mice". JIB, 2009, Vol. 5(3), 182-186.
10. Health Canada, NHPD monograph for Turmeric (*Curcuma longa)* USDA 2008; Date: 2010-02-25.
11. Kosinski M., "Revised Summary Report on Study Findings of 10 Patients Receiving Nutraceutical Product A4+L for the Treatment for Chronic Hepatitis C Virus" (CO-1031)", Quality Metric Inc., July 2004.
12. M Afzala, C Obuekweb, AR Khanc and H Barakata., "Antioxidant activity of *Cordia myxa* L. and its hepatoprotective potential". EJEAFChe 2007, 6 (6), 2109-2118.
13. Mohamed Saleem TS, Christina AJM, Chidambaranathan N, Ravi V, Gauthaman K., "Hepatoprotective activity of *Annona squamosa* Linn. on experimental animal model". International Journal of Applied Research in Natural Products, 2008, Vol. 1(3), 1-7.

14. Natural Medicines Comprehensive Database, Monograph for Graviola (*Annona muricata*).

15. Orlando Vieira de Sousa, Glauciemar Del-Vechio Vieira, José de Jesus R. G. de Pinho, Célia Hitomi Yamamoto and Maria Silvana Alves., "Antinociceptive and Anti-Inflammatory Activities of the Ethanol Extract of *Annona muricata* L. Leaves in Animal Models". Int. J. Mol. Sci. 2010, 11, 2067-2078.

16. Raintree Nutrition, Tropical Plant Database, Monograph (Database File) for Graviola (*Annona muricata*).

17. Samanta A., "Quality of life following the use of A4 plus in a limited number of subjects with chronic hepatitis C: Open-label, non placebo-controlled, preliminary observations", Independent assessment report, 2004.

18. Saravana Kumar, R Gandhimathi, KK Senthil Kumar, Kusuma Praveen Kumar., "Hepatoprotective potential of *Cordia subcordata* Lam. against carbon tetra chloride (CCl4)-induced hepatotoxicity in Wistar albino rats". J Biomed Sci and Res., 2009, Vol 1 (1), 19-26.

19. Semple H., "A 7-Day Repeat Dose (oral) Toxicity Screen on A4+" (ToxTest Alberta Innovates–Technology Futures, Vegreville, Alberta, 2010).

20. Semple H., "A 28-day repeat dose oral toxicity study of A4+ in rats" (ToxTest Alberta Innovates–Technology Futures, Vegreville, Alberta, 2010).

21. Semple H., "A Repeated Dose Mammalian Erythrocyte Micronucleus Study of A4+ in Mice" (ToxTest Alberta Innovates–Technology Futures, Vegreville, Alberta, 2010).

22. Semple H., "A Mammalian Erythrocyte Micronucleus Study of A4+ in Mice" (ToxTest Alberta Innovates–Technology Futures, Vegreville, Alberta, 2010).

23. Swain MG., "Evaluation of the Hepatoprotective and Behavioral Effects of the Herbal Compound A4+ in Acute Liver Injury Models", (University of Calgary Report - 2011).

24. Thirupathi K, Sathesh Kumar S, Goverdhan P, Ravikumar B, Krishna DR and Krishna Mohan G., "Hepatoprotective action of Cordia dichotoma against Carbon tetrachloride induced liver injury in rats". Nig. J. Nat. Prod. and Med. 2007, Vol. 11, 37-40.

25. Tyrrell L., Part 1: "Anti-viral effects of plant extracts on HCV infected cells" and Part 2: "Effect of Short-term exposure to A4+ plant extract on Natural Killer Cell Activity" (Li Ka Shing Institute of Virology, University of Alberta, 2011).

26. Wallace J., "Evaluation of the Anti-oxidant, Anti-inflammatory and mucosal protective Actions of A4+ and its Constituents" (Penumbra Associates Ltd, 2010).

Reference # 1

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PROTECTIVE EFFECTS OF *ANNONA MURICATA* LINN. (ANNONACEAE) LEAF AQUEOUS EXTRACT ON SERUM LIPID PROFILES AND OXIDATIVE STRESS IN HEPATOCYTES OF STREPTOZOTOCIN-TREATED DIABETIC RATS

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Abstract

Extracts from various morphological parts of *Annona muricata* Linn. (Annonaceae) are widely used medicinally in many parts of the world for the management, control and/or treatment of a plethora of human ailments, including diabetes mellitus (DM). The present study was undertaken to investigate the possible protective effects of *A. muricata* leaf aqueous extract (AME) in rat experimental paradigms of DM. The animals used were broadly divided into four (A, B, C and D) experimental groups. Group A rats served as 'control' animals and received distilled water in quantities equivalent to the administered volumes of AME and reference drugs' solutions intraperitoneally. Diabetes mellitus was induced in Groups B and C rats by intraperitoneal injections of streptozotocin (STZ, 70 mg kg^{-1}). Group C rats were additionally treated with AME (100 mg kg^{-1} day^{-1}, p.o.) as from day 3 post STZ injection, for four consecutive weeks. Group D rats received AME (100 mg kg^{-1} day^{-1} p.o.) only for four weeks. Post-euthanization, hepatic tissues were excised and processed biochemically for antioxidant enzymes and lipid profiles, such as catalase (CAT), reactive oxygen species (ROS), glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), thiobarbituric acid reactive substances (TBARS), triglycerides (TG), total cholesterol (TC), high density lipoprotein (HDL) and low density lipoprotein (LDL), respectively. Treatment of Groups B and C rats with STZ (70 mg kg^{-1} i. p.) resulted in hyperglycaemia, hypoinsulinaemia, and increased TBARS, ROS, TC, TG and LDL levels. STZ treatment also significantly decreased ($p<0.05$) CAT, GSH, SOD, GSH-Px activities, and HDL levels. AME-treated Groups C and D rats showed significant decrease ($p<0.05$) in elevated blood glucose, ROS, TBARS, TC, TG and LDL. Furthermore, AME treatment significantly increased ($p<0.05$) antioxidant enzymes' activities, as well as serum insulin levels. The findings of this laboratory animal study suggest that *A. muricata* extract has a protective, beneficial effect on hepatic tissues subjected to STZ-induced oxidative stress, possibly by decreasing lipid peroxidation and indirectly enhancing production of insulin and endogenous antioxidants.

Key Words: *Annona muricata* leaf; Aqueous extract; Lipid profiles; Streptozotocin; Oxidative stress; Antioxidants.

Introduction

Diabetes mellitus (DM) is one of the commonest endocrine and metabolic disorders of the 21st century, and a major threat to healthcare worldwide. Numerous experimental and clinical observations have indicated that hyperglycaemia may directly or indirectly contribute to excessive formation of free radicals (Ceriello, 2003). Diabetes is also known to involve oxidative stress and changes in lipid metabolism (Scoppola et al; 2001). The liver

is the main effector organ for maintaining plasma glucose levels within narrow limits. Herrman et al; (1999) reported that streptozotocin (STZ) progressively decreased the volume of hepatocytes and their nuclei, as a result of cytoplasmic changes, and that a basal insulin level is also necessary to maintain the state of aggregation of the endoplasmic reticulum-bound polysomes for secretory protein synthesis. In insulin-deficient animals, loss of rough endoplasmic reticulum reduces amino acid incorporation into protein, and a decrease in rough endoplasmic reticulum-bound ribosomes (Lenk et al; 1992). At the same time, hyperglycaemia can generate a redox imbalance inside the cells, especially in the liver (Gallou et al; 1993). An ideal antidiabetic drug should, therefore, possess both hypoglycaemic and antioxidant properties, without any adverse effect.

Increase in free radical-mediated toxicity is well documented in STZ-treated diabetic rats. Increased formation of free radicals in diabetes mellitus can be a risk factor for the disease, and it occurs as a result of two processes: (i) decreased activity of the body antioxidant systems, and (ii) auto-oxidation of reducing saccharides and formation of adducts with proteins. Antioxidant levels in the blood and tissues are important factors for sensitivity of individual tissues to oxidative stress (Durackova, 1999). Antioxidants have been classified according to their mode of action, and Bonnefont-Rousselot et al., (2000) differentiated them into three main groups, namely: (i) antioxidants that prevent the formation of new reactive oxygen species (ROS) such as caeruloplasmin, metallothioniene, albumin, myoglobin, ferritin and transferrin, (ii) scavenging antioxidants which remove ROS once formed, thus preventing radical chain reactions – these include reduced glutathione (GSH), vitamin E, vitamin C, α-carotene, uric acid and bilirubin, and (iii) enzyme antioxidants that function by catalyzing the oxidation of other molecules. This group includes superoxide dismutase (SOD) that produces hydrogen peroxide from superoxide radicals, glutathione reductase (GSH-R), glutathione peroxidase (GSH-Px) and catalase (CAT) which decompose hydrogen peroxide. Type 2 diabetes mellitus (T2DM) has been associated with an increased risk for developing premature atherosclerosis due to increase in triglycerides and low-density lipoprotein levels, and decrease in high-density lipoprotein levels.

Annona muricata Linn. (Annonaceae) is commonly known as 'Soursop' or 'Graviola'. Because of the 'custard-like texture' of its edible fruit, *Annona muricata* has been grouped with the 'Custard-Apple' plants of the Annonaceae family. It is a deciduous, terrestrial, erect tree of 5–8 metres in height, with an open, roundish canopy. Although a native of America, *Annona muricata* has now naturalized and become established in many tropical countries of the world. The plant has been used medicinally in many tropical African countries for an array of human ailments, especially for parasitic infections and cancer. It has also been used in some African herbal medicine systems for its sedative and antispasmodic properties. In tropical Africa, including Nigeria, the plant is generally used as antiparasitic, antispasmodic, astringent, anticancer, sedative, hypotensive, insecticide, piscicide, vermifuge, and for coughs, fevers, pain and skin diseases (Watt and Breyer-Brandwijk, 1962). The stem-bark and roots of the plant are commonly used as remedies for diarrhoea, dysentery and intestinal worms (Watt and Breyer-Brandwijk, 1962). The fruit pulp of the plant is also used in treating fevers. The unripe fruit of the plant is astringent, and is used in the treatment of intestinal atony and for scurvy (Watt and Breyer-Brandwijk, 1962). In India, the root-bark and leaf of the plant are used as anthelmintic and antiphlogistic agents, while its flowers and fruit pods are used as remedies for catarrh (Watt and Breyer-Brandwijk, 1962).

Several chemical compounds have been isolated from various morphological parts (roots, stem-barks, leaves, fruits, and seeds) of *Annona muricata* Linn. Some of the reported phytochemicals isolated and characterized from various parts of the plant include: annonaceous acetogenins, lactones and isoquinoline alkaloids; tannins, coumarins, procyanidins, flavonoids, pentacyclic terpenoid saponins; p-coumaric acid, stearic acid, myristic acid, stepharine, reticuline, ellagic acid; phytosterols (β-sitosterol, stigmasterol), sugars, alcohols, aldehydes, organic and inorganic acids, metals, inorganic salts, vitamins B and C; stepharine, reticuline, gamma-amino butyric acid (GABA); annonacin, annocatalin, annomonicin, annomuricin, annomuricatin, corossolone, epomuricenin, gigantetrocin, javoricin, muricine, muricinine, muricapentocin, muricoreacin, montanacin, montecristin, muracin, muricatalin, muricin, murisolin, robustocin, solamin, and so on (Watt and Breyer-Brandwijk, 1962; TDRG, 2002).

There are several reasons why medicinal plants should be subjected to scientific scrutiny. First and foremost, many herbal remedies have recognizable therapeutic effects (Bailey and Day 1989); but they may also have toxic side-effect (Keen et al; 1994). Recently, there has been a renewed interest in the use of plant products as antidiabetic agents. The antidiabetic effects of many traditional herbal drugs (phytomedicines) may be ascribed to their flavonoid and other chemical constituents which may also inhibit certain enzymes and possess antioxidant activities. *A. muricata* has a long history of usage in herbal medicine in the tropical areas of South and North America, as well as in West Africa, especially in Western Nigeria. Although all the morphological parts of the plant have been claimed to be useful in traditional medicine, no scientific studies have been carried out to establish the hypolipidaemic and antioxidant effects of the plant. Therefore, the present study was undertaken to investigate the

hypoglycemic, hypolipidemic and antioxidant properties of *A. muricata* leaf aqueous extract in rat experimental paradigm.

Materials and Methods

Ethical consideration

Experimental protocols and procedures used in this study were approved by the Animal Ethics Committee of the University of KwaZulu-Natal, Durban 4000, South Africa; and conform to the *"Guide to the Care and Use of Animals in Research and Teaching"* [Published by the Ethics Committee of the University of Durban-Westville, Durban 4000, South Africa].

Animals

This study was carried out in healthy, male and female Balb C mice (*Mus domesticus*) weighing 20-25 g; and healthy, young adult, Wistar rats (*Rattus norvegicus*) of both sexes weighing 250-300 g. The animals were housed under standard laboratory conditions of light, temperature and humidity. The animals were given free access to food (standard rat pellets) and drinking tap water *ad libitum*. The rats were randomly divided into four experimental groups of 10 rats each: Group A (distilled water-treated 'control'), Group B (STZ-treated), Group C (STZ-+ *A. muricata* leaf extract-treated), and Group D (*A. muricata* leaf extract-treated) rats. All the animals were fasted for 16 hrs, but still allowed free access to drinking tap water, before the commencement of our experiments. The mice were used for acute toxicity testing of the crude plant's extract, while the rats were used for hypoglycaemic and hypolipidaemic evaluations of the plant's extract.

Plant material

Fresh leaves of *Annona muricata* (Linn.) (family: Annonaceae) (locally known as "Soursop" or "Graviola" in English, and "Abo" in Yoruba language of Western Nigeria) were collected in Ile-Ife, Western Nigeria, between April and May, 2006. The leaves were identified by the Taxonomist/Curator of the Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria, as those of *Annona muricata* Linn. (family: Annonaceae). A voucher specimen (S/N. SA003) of the plant has been deposited in the Herbarium of the University's Botany Department.

Preparation of *Annona muricata* leaf aqueous extract

A. muricata fresh leaves were air-dried at room temperature. One kilogram (1 kg) of the air-dried leaves of the plant was milled into fine powder in a Waring commercial blender. The powdered leaf was macerated in distilled water and extracted twice, on each occasion with 2.5 litre of distilled water at room temperature for 48 h (with occasional shaking). The combined aqueous extract solubles were concentrated to dryness under reduced pressure at 60±1°C in a rotary evaporator. The resulting aqueous extract was freeze-dried, finally yielding 36.23 g (i.e., 3.62% yield) of a light green, powdery crude aqueous leaf extract of *A. muricata* (AME). Without any further purification, the crude aqueous extract thus obtained was refrigerated and subsequently used in this study. Aliquot portions of the crude plant extract residue were weighed and dissolved in distilled water for use on each day of our experiments.

Acute toxicity testing

The median lethal dose (LD_{50}) of *A. muricata* leaf aqueous extract (AME) was determined in mice using a modified method of Lorke (Lorke, 1983). Mice fasted for 16 h were randomly divided into groups of eight mice each. Stepwise, graded doses of AME (25, 50, 100, 200, 400, 800, 1600 and 3200 mg kg^{-1}) were separately administered intraperitoneally (i. p.) to the mice in each of the 'test' groups. Each of the mice in the 'control' group was treated with distilled water (3 ml kg^{-1} i.p.) only. The mice in both the 'test' and 'control' groups were then allowed free access to food and drinking tap water, and observed over a period of 48 h for signs of acute toxicity. The number of deaths (caused by the extract in each group) within this period of time was noted and recorded. Log dose-response plots were subsequently constructed for the plant's extract, from which the LD_{50} of the plant's leaf aqueous extract was determined.

Induction of experimental diabetes

Diabetes mellitus was induced (in Groups B and C 'test' rats) by intraperitoneal injections of STZ (70 mg kg^{-1}), freshly dissolved in 0.1 mol L^{-1} citrate buffer (pH 6.3) (Rossini et al., 1978). Diabetic state was confirmed by measuring basal blood glucose concentrations 72 h after STZ injection. Diabetes was allowed to develop and stabilize in these STZ-treated rats over a period of 4-7 days. The 'test' compound [i.e., *Annona muricata* leaf aqueous extract (AME, 100 mg kg^{-1} day^{-1} p.o.)] was administered orally by intragastric intubation to fasted Groups C and D rats. In Group C rats, administration of AME (100 mg kg^{-1}) commenced as from the 3rd day post STZ injection, and continued for the next 4 consecutive weeks.

Biochemical assays
Blood Glucose and serum insulin estimations

Blood samples were obtained by repeated needle puncture of the tail tip veins. Blood samples were obtained 1 day before STZ treatment, and subsequently on each other day after induction of diabetes mellitus. Blood glucose concentrations were determined by means of Bayer Glucometer Elite® and compatible blood glucose test strips. Fasted STZ–treated rats with blood glucose concentrations ≥18 mmol L^{-1} were considered to be diabetic, and used in this study. Serum insulin concentrations were determined by an enzyme-linked immunosorbent assay (ELISA), using a commercial kit (Crystal Chem, Chicago, IL; USA).

Hexokinase (HXK) and glucokinase (GCK) activities

Frozen liver tissue (1 g) was homogenized at 4°C in a 9-ml cold buffer solution (pH 7.4) containing Na-HEPES, 50 mM; KCl, 100 mM; EDTA, 1 mM; $MgCl_2$, 5 mM and dithiothreitol (DTE), 2.5 mM; using a glass-Teflon Potter Homogenizer. The suspension formed was centrifuged at 12000 x g for 1 h at 4°C. The clear supernatant formed was used for the measurement of HXK and GCK activities by the coupled enzyme assay procedure of Davidson and Arion (1987). The incubation mixture contained the following ingredients in a final volume of 1 ml: HEPES, 50 µmol; KCl, 100 µmol; $MgCl_2$, 7.5 µmol; and DTE, 2.5 µmol; fatty acid free bovine serum albumin, 10 mg; NAD^+, 0.5 µmol; G-6-PD, 4 units; liver supernatant, 100 µl for HXK assay or 10 µL for total HXK and GCK assays; and D-glucose, 0.5 µmol for HXK and 10 µmol for total enzyme activities. Both the 'control' and 'test' tubes were pre-incubated at 25±1°C for 5 min. To the 'control' tubes, 0.2 ml of H_2O was added, and to start the reactions in the 'test' tubes, 0.2 ml of a solution containing 0.5 µmol of ATP was added. 'Control' tubes were adjusted to zero absorbance in DU-7 spectrophotometer at 340 nm, and the increase in absorbance in the 'test' tubes at this wavelength was plotted against time period of 15 min. The reaction was found to be linear with time. Total enzyme activities (GCK + HXK) and HXK activities were calculated in terms of mU ml^{-1} of the liver supernatant. One milliunit of the enzyme corresponds to the amount of the enzyme producing 1 nmol of NADH per min under assay conditions at 25±1°C. Hexokinase activities were subtracted from the total HXK + GCK activities to obtain glucokinase activities. Protein content of the liver homogenate was determined by using bicinchoninic acid (BCA) protein assay reagent (Pierce Chemical Company, Rockford, IL, USA).

Catalase activity (CAT)

The activity of catalase (CAT) was measured by using its perioxidatic function according to the method of Johansson and Borg (1988). 50 µL potassium phosphate buffer (250 mM, pH 7.0) was incubated with 50 µL methanol and 10 µL hydrogen peroxide (0.27%). The reaction was initiated by addition of 100 µL of enzyme sample with continuous shaking at room temperature (25±1°C). After 20 minutes, the reaction was terminated by addition of 50 µL of 7.8 M potassium hydroxide. 100 µL of purpald (4-Amino-3-hydrazino-5-mercapto-1,2,4-triazole, 34.2 mM in 480 mM HCl) was immediately added, and the mixture was again incubated for 10 minutes at 25±1°C with continuous shaking. Potassium peroxidate (50 µL of a 65.2 mM solution) was added to obtain a coloured compound. The absorbance was read at 550 nm in a spectrophotometer. Results are expressed as micromoles of formaldehyde produced mg^{-1} protein.

Reactive oxygen species (ROS)

The amount of ROS activity in the liver was measured by using 2′,7′-dichlorofluorescein diacetate (DCF-DA), which gets converted into highly fluorescent DCF by cellular peroxides (including hydrogen peroxide). The

assay was performed as described earlier by Socci et al., (1999). Briefly, the liver tissue (10 mg) was homogenized in 1 ml of ice-cold 40 mM Tris-HCl buffer (pH 7.4), and further diluted to 0.25% with the same buffer and placed on ice. The sample was divided into two equal fractions. In one fraction, 40 µL of 1.25 mM DCF-DA in methanol was added for ROS estimation. The other fraction to which 40 µL of methanol was added, served as a 'control' for tissue auto-fluorescence. All samples were incubated for 15 min in a 37°C water-bath. Fluorescence was determined at 488 nm excitation and 525 nm emission, using a fluorescence plate reader (Tecan Spectra Fluor Plus, Germany). Result are expressed as nmol min^{-1} mg^{-1} protein.

Reduced GSH and oxidized glutathione GSSG levels

Liver GSH and GSSG contents were measured as described by Hissin and Hilf (1973). To measure GSH contents, 4 ml of the liver homogenate was precipitated by adding 1 ml of a 25% metaphosphoric acid and centrifuged at 10,000 x g (Ultracentrifuge, Hitachi, Japan) for 30 min. Supernatant was diluted 20 times with the same buffer, and 100 µL of orthopthaldehyde (OPT) was added. In addition, for GSSG assay, 0.5 ml supernatant was incubated at room temperature with 200 µL of 0.04 mol L^{-1} N-ethylmaleimide solution for 30 min, and to this mixture, 4.3 ml of 0.1 mol L^{-1} NaOH was added. A 100 µL sample of this mixture was taken for the measurement of GSSG, using the procedure described above for GSH assay, except that 0.1 mol L^{-1} NaOH was used as the diluent instead of phosphate buffer. Samples were incubated at room temperature for 15 min and fluorescence was measured using spectrofluorometer (Tecan Spectra Fluor Plus, Germany) at 350 nm (E_x)/420 nm (E_m). The values obtained were ascribed to the amount of glutathione in the liver.

Superoxide dismutase activity (SOD)

Liver SOD activity was assayed by the method of Kakkar et al., (1984). The reaction mixture contained 1.2 ml of sodium pyrophosphate buffer (0.052 mM, pH 7.0), 0.1 ml of phenazine methosulphate (PMS) (186 µM), 0.3 ml of nitro blue tetrazolium (NBT) (300 µM). 0.2 ml of the supernatant obtained after centrifugation (1500 x g, 10 min followed by 10,000 x g, 15 min) of 5% liver homogenate was added to reaction mixture. Enzyme reaction was initiated by adding 0.2 ml of NADH (780 µM), and stopped precisely after 1 min by adding 1 ml of glacial acetic acid. The amount of chromogen formed was measured by recording colour intensity at 560 nm. Results are expressed as units mg^{-1} protein.

Glutathione peroxidase activity (GSH-Px)

Glutathione peroxidase (GSH-Px) activity was measured by NADPH oxidation, using a coupled reaction system consisting of glutathione, glutathione reductase, and cumene hydroperoxide (Tappel, 1978). 100 µL of enzyme sample was incubated for five minutes with 1.55 ml stock solution (prepared in 50 mM Tris buffer, pH 7.6 with 0.1 mM EDTA) containing 0.25 mM GSH, 0.12 mM NADPH and 1 unit glutathione reductase. The reaction was initiated by adding 50 µL of cumene hydroperoxide (1 mg ml^{-1}), and the rate of disappearance of NADPH with time was determined by monitoring absorbance at 340 nm. One unit of enzyme activity is defined as the amount of enzyme that transforms 1 µmol of NADPH to NADP per minute. Results are expressed as units mg^{-1} protein.

Thiobarbituric acid reactive substances (TBARS)

The product of the reaction between malondialdehyde (MDA) and thiobarbituric acid reactive substances (TBARS) was measured by a modified method of Ohkawa et al., (1979). For each sample to be assayed, four tubes were set up containing 100, 150, 200 and 250 µL of tissue homogenate, 100 µL of 8.1% SDS, 750 µL of 20% acetic acid, and 750 µL of 0.8% aqueous solution of TBA. The volume was made up to 4 ml with distilled water, mixed thoroughly and heated at 95°C for 60 minutes. After cooling, 4 ml of n-butanol was added to each tube, the contents mixed thoroughly, and then centrifuged at 3000 rpm for 10 minutes. The absorbance of the clear, upper (n-butanol) layer was measured using a Shimadzu (Japan) UV-1601 spectrophotometer at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of MDA-TBA complex at 1.56 x 10^5 cm^{-1} M^{-1}, and was expressed in µmol TBARS g^{-1} tissue protein.

Determination of serum cholesterol, lipoproteins and triglyceride

Blood samples were collected from tail tip veins of the rats after 16 h of fasting, and transferred to sterilized centrifuge tubes at room temperature. The blood samples were centrifuged for 10 min at 4,000 x g to obtain serum. The serum was stored in a freezer at 0° for later analysis of total cholesterol (TC) and triglyceride (TG), high- and low-density lipoprotein (HDL and LDL)-cholesterols. Aliquots of serum were taken for determination of total cholesterol by enzymatic colorimetric assay method of Allain et al., (1974), and triglycerides determined by enzymatic glycerol phosphate oxidase/peroxidase method of Cheng et al., (1988). Autoanalyzer (Express Plus, Ciba Corning, USA) and Elitech kit were used. Serum high density lipoprotein (HDL)-cholesterol was assayed by precipitation of chylomicrons, while very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) were determined with sodium phosphotungstic acid and magnesium chloride (Rainwater et al; 1995). Centrifugation left only the HDL in the supernatant; their cholesterol content was determined by the method of Virella-Lopes et al., (1977). Estimation of low density lipoprotein (LDL)-cholesterol was done by using empirical formula of Friedewald et al., (1972) for samples with TG levels <4.5 mmol L^{-1}. [LDL-chol] = [Total chol] – [HDL-chol] – ([TG]/2.2); where all concentrations are given in mmol L^{-1}.

Statistical analysis

The data obtained were expressed as means (±SEM), and analyzed by using repeated measures of variance. The differences between the means were analyzed statistically with one-way analysis of variance (ANOVA; 95% confidence interval), and the Bonferroni correction was applied as post hoc test. Values of $p<0.05$ were taken to imply statistical significance.

Results

Acute toxicity testing

Intraperitoneal administrations of stepwise, graded doses of *Annona muricata* leaf aqueous extract (AME, 25–100 mg kg^{-1}) were found to be safe in mice. However, relatively moderate to high doses of the plant's extract (>200 mg kg^{-1} i. p.) were found to be toxic and/or lethal to the animals. The LD_{50} value of the plant's extract was calculated to be 155±20 mg kg^{-1} i. p. in mice. The relatively low LD_{50} value of 155±20 mg kg^{-1} obtained probably suggests that *Annona muricata* leaf aqueous extract is only moderately safe in mice.

Effects of diabetes on body weight and serum insulin

Seventy-two hours after STZ administration, all the rats treated with STZ displayed glucosuria, hyperglycemia, hypoinsulinemia and moderate but insignificant ($p>0.05$) loss of body weight. At the beginning of this study, the baseline weights of all the rats were similar in all groups. At the end of the study period (60 days), however, the diabetic animals presented with significant ($p<0.05$) loss in body weight, as well as insignificant liver weight loss. The initial and final body weights were, however, not significantly different ($p>0.05$) in the 'control' and AME-treated rat groups (Table 1).

Table 1: Changes in body and liver weights of 'control', STZ-treated, STZ- + AME-treated, and AME-treated rat groups just before and after treatment.

Parameters/animal groups	Body weights (g)		Liver weights (g)	
	Initial	Final	Initial	Final
Control	237±13	242±11	9.76±0.2	9.82±0.3
STZ-treated	233±10	217±14[a]	9.27±0.5[a]	8.25±0.6[a]
STZ- + AME-treated	234±12	239±10[b]	9.56±0.4[b]	9.46±0.8[b]
AME-treated	239±20	242±30	9.87±0.9	9.90±0.3

Values are expressed as means (±SEM) of 10 rats. [a] Significant difference ($p<0.05$) between STZ-treated and 'control' groups. [b] Significant difference ($p<0.05$) between AME-treated and STZ-treated groups.

Table 2: Changes in blood glucose concentrations and serum insulin levels of 'control', STZ-treated, STZ- + AME-treated, and AME-treated rat groups during the study period.

Parameters/Days	0	10	20	30	40	50	60
Blood glucose concentrations (mmol L^{-1})							
Control	4.1±0.2	4.2±0.4	4.0±0.6	4.0±0.5	4.1±0.2	4.1±0.3	4.0±0.1
STZ-treated	4.2±0.6	18.8±0.2[a]	20.2±0.4[a]	21.4±0.3[a]	21.2±0.1[a]	22.1±0.3[a]	21.9±0.2[a]
STZ- + AME-treated	4.3±0.2	8.6±0.4[b]	7.6±0.2[b]	6.5±0.3[b]	5.9±0.4[b]	5.3±0.8[b]	5.2±0.6[b]
AME-treated	4.0±0.1	3.9±0.5	3.9±0.3	3.8±0.9	3.9±0.2	3.9±0.1	3.8±0.6
Serum insulin concentrations (μU ml^{-1})							
Control	12.7±1.2	12.9±1.0	12.9±1.3	12.9±1.7	13.01.2	13.1±1.0	12.9±1.8
STZ-treated	12.8±1.5	8.7±1.4[c]	6.3±1.4[c]	5.9±1.3[c]	5.7±2.3[c]	5.5±1.2[c]	5.3±1.2[c]
STZ- + AME-treated	13.2±1.7	11.3±1.2[d]	10.6±1.1[d]	10.9±1.7[d]	11.5±1.2[d]	11.0±0.9[d]	12.2±1.4[d]
AME-treated	12.4±1.3	12.7±1.5	12.9±1.0	13.3±1.6	13.5±1.2	13.5±1.7	13.9±1.5

Values are expressed as means (±SEM) of 10 rats. [a,c] Significant difference ($p<0.05$) between STZ-treated and 'control' groups. [b,d] Significant difference ($p<0.05$) between AME-treated and STZ-treated groups. Values for 'control' group rats are presented as 0 day mean values.

Table 3: Hepatic tissue CAT (μmol mg^{-1} protein), ROS (nmol $min^{-1}mg^{-1}$ protein), GSH (U g^{-1} protein), GSSG (U g^{-1} protein), SOD (U mg^{-1} protein), GSH-Px (U mg^{-1} protein), TBARS (nmol mg^{-1} protein), HXK and GCK (mU mg^{-1} protein) of 'control', STZ-treated, STZ- +AME-treated, and AME-treated rats.

Parameters	Control	STZ-treated	STZ- + AME-treated	ME-treated
Hepatic CAT	0.36±0.4	0.23±0.2[a]	0.32±0.3[b]	0.38±0.2[c]
Hepatic ROS	0.13±0.1	0.29±0.4[a]	0.14±0.5[b]	0.12±0.5[c]
Hepatic GSH	7.22±1.3	3.82±1.2[a]	6.6±1.2[b]	7.80±1.4[c]
Hepatic GSSG	53.1±1.6	71.1±1.3[a]	56.2±1.5[b]	52.7±1.2[c]
Hepatic SOD	24.9±1.4	13.7±1.3[a]	25.7±1.1[b]	25.8±1.6[c]
Hepatic GSH-Px	0.49±0.4	0.28±0.3[a]	0.51±0.2[b]	0.52±0.3[c]
Hepatic TBARS	89±15	148±17[a]	98±14[b]	86±10[c]

Values are expressed as means (±SEM) of 10 rats per group. [a] Significant difference ($p<0.05$) when compared with 'control' group rats. [b,c] Significant difference ($p<0.05$) in the same row when compared with STZ-treated group rats.

Table 4: Serum lipid profiles of 'control' and AME-treated rats.

Experimental days	Control rats				AME-treated rats			
	HDL	LDL (mmol L^{-1})	TC	TRIG (mmol L^{-1})	HDL	LDL (mmol L^{-1})	TC	TRIG (mmol L^{-1})
0	0.86±1.1	0.33±0.3	1.6±1.3	0.9±0.2	0.85±0.6	0.34±0.2	1.6±1.2	0.9±0.1
10	0.85±1.2	0.54±0.2	1.8±1.6	0.9±0.3	0.87±0.5	0.42±0.4	1.7±1.4	0.9±0.2
20	0.86±1.1	0.34±0.5	1.7±1.5	1.1±0.5	0.92±0.7	0.32±0.4	1.6±1.1	0.8±0.5
30	0.87±1.0	0.43±0.4	1.8±1.7	1.0±0.4	0.90±0.6	0.43±0.1	1.7±1.0	0.8±0.2
40	0.87±1.2	0.42±0.2	1.7±1.4	0.9±0.3	0.92±0.4	0.31±0.4	1.6±1.3	0.8±0.2
50	0.88±1.3	0.55±0.3	1.8±1.3	0.8±0.5	0.94±0.2	0.44±0.2	1.7±1.2	0.7±0.5
60	0.89±1.0	0.64±0.4	1.9±1.4	0.8±0.2	0.93±0.4	0.45±0.3	1.7±1.4	0.7±0.6

Values are expressed as means (±SEM) of 10 rats per group. There was no significant difference ($p>0.05$) among the parameters determined for the 'control' and AME-treated rats. Values for T-chol/HDL-chol in both 'control' and AME-treated rats were also not significantly different (2.01±1.2 vs 1.94±1.3, respectively).

Table 5: Serum lipid profiles of 'control', STZ-treated, STZ + AME-treated, and AME-treated rats.

Experimental days	STZ-treated rats				STZ- + AME-treated rats			
	HDL	LDL (mmol L^{-1})	TC	TRIG (mmol L^{-1})	HDL	LDL (mmol L^{-1})	TC	TRIG (mmol L^{-1})
0	0.86±1.4	0.33±0.3	1.6±1.5	0.9±0.2	0.84±0.2	0.40±0.2	1.6±1.3	0.8±0.3
10	0.82±1.3	0.83±0.2	2.2±1.4	1.2±0.3	0.89±0.4	0.90±0.4[a]	2.2±1.9	0.9±0.2
20	0.74±1.4[a]	1.23±0.5[b]	2.7±1.5[c]	1.6±0.5[d]	0.92±0.3	0.77±0.4[a]	2.1±1.5	0.9±0.4
30	0.63±1.3[a]	1.66±0.4[b]	3.2±1.7[c]	2.0±0.4[d]	1.05±0.4	0.58±0.1[c]	2.0±1.3	0.8±0.3
40	0.59±1.1[a]	2.36±0.2[b]	3.9±1.6[c]	2.1±0.3[d]	1.03±0.1	0.51±0.4[c]	1.9±1.6	0.8±0.1
50	0.55±1.2[a]	2.77±0.3[b]	4.5±1.8[c]	2.6±0.5[d]	1.02±0.5	0.46±0.2[c]	1.8±1.5	0.7±0.4
60	0.52±1.3[a]	3.11±0.4[b]	4.9±1.9[c]	2.8±0.2[d]	1.02±0.2	0.46±0.3[c]	1.8±1.7	0.7±0.2

Values are expressed as means (±SEM) of 10 rats per group. [a] Significant decrease ($p<0.05$) when compared with 'control' group rats. [b,c,d] Significant increase ($p<0.05$) in the same column when compared with 'control' group rats. Value for T-chol/HDL-chol in STZ-treated rats was 5.57±1.5 when compared with the 'control' rats' value of 2.14±1.7.

Blood glucose and serum insulin concentrations

The mean blood glucose concentrations and serum insulin levels of the STZ-treated animals are shown in Table 2. In our 'control' set of experiments, pretreatment of the rats with distilled water alone did not significantly modify ($p>0.05$) the animals'serum insulin and blood glucose concentrations. As shown in Table 2, induction of diabetes resulted in a significant increase in the blood glucose levels of the rats. There was a gradual rise in the blood glucose concentrations of the animals as from day 2 following injection of STZ, and the values were significantly higher ($p<0.05$) than those of 'control' animals (Table 2). Furthermore, high levels of blood glucose concentrations of the STZ-treated rats were persistently observed throughout the study period (22.3±0.6 mmol L^{-1}). AME treatment significantly reduced ($p<0.05$-0.001) the blood glucose concentrations of the AME-treated group C diabetic rats. AME treatment also significant increased ($p<0.05$) serum insulin levels of the group C rats.

Biochemical findings

Figure 1 shows the effect of *A. muricata* aqueous leaf extract on hepatic hexokinase and glucokinase activities. In the STZ-treated diabetic rats, both hexokinase and glucokinase activities significantly decreased ($p<0.05$), but the levels returned to almost normal, after AME treatment.

Table 4 shows the effects of *A. muricata* aqueous leaf extract on biochemical variables in STZ-treated animals. There was a clear evidence that STZ-induced hepatic injury was associated with free radical injury and oxidative stress. Oxidative stress was characterized by increased lipid peroxidation and/or altered non-enzymatic and enzymatic antioxidant systems. The effects of STZ and STZ + AME treatments on hepatic tissues' ROS, GSH, SOD, GSH-Px and TBARS are presented in Table 3. The hepatic antioxidant activities of CAT, GSH-Px, SOD and GSH significantly decreased ($p<0.05$), while GSSG, ROS and TBARS significantly increased, in the STZ-treated, diabetic rats. The 'control' group of rats maintained optimal values of the antioxidants studied. AME treatment significantly ($p<0.05$) decreased STZ-induced elevated GSSG, ROS and TBARS, and also significantly increased ($p<0.05$) STZ-induced reduced antioxidant enzyme activities. Furthermore, AME treatment restored the altered activities of antioxidant enzymes like GSH-Px, SOD and GSH, TBARS towards their normal values in the liver.

Serum total cholesterol, triglycerides, HDL and LDL cholesterols, and (T-chol/HDL-chol) in the 'control', STZ-treated, STZ + AME-treated, and AME-treated rats are shown in Tables 4 and 5. Serum total cholesterol, triglycerides, LDL cholesterol and (T-chol/HDL-chol) were significantly elevated ($p<0.05$) in STZ-treated Group B diabetic rats as compared to 'control' Group A rats. Similarly, HDL cholesterol was significantly reduced ($p<0.05$) in STZ-treated group B diabetic rats (Table 5). All the lipid parameters examined were improved towards normal values after AME treatment in Group C rats.

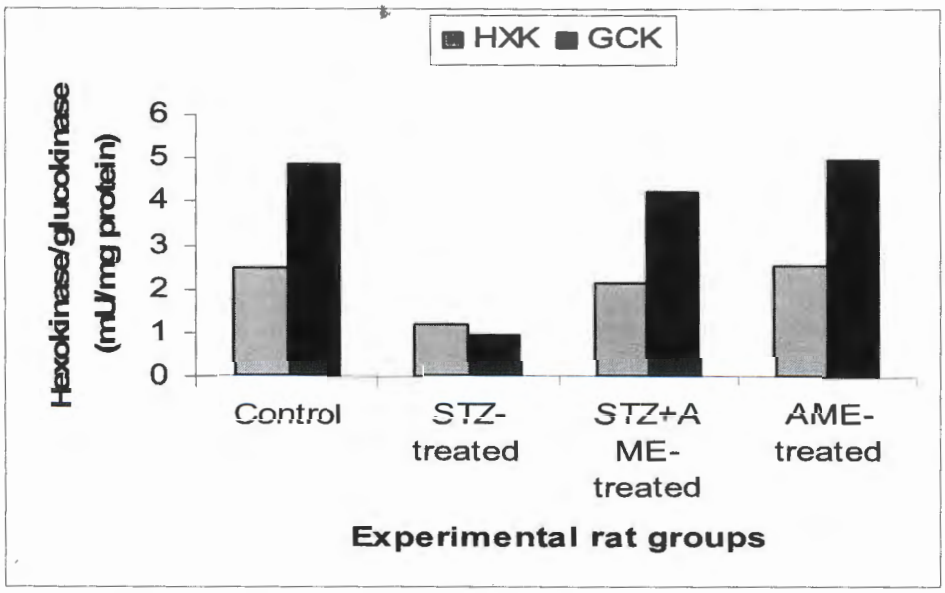

Figure 1. Hepatic hexokinase and glucokinase activities in 'control', STZ-treated, STZ- +AME-treated, and AME-treated groups of rats. The figure shows protective effects of AME on hepatic tissues, and reveals that STZ severely reduced the liver enzymes' activities in diabetic rats.

Discussion

Medicinal plants have been used for centuries in the treatment of diabetes mellitus. Therefore, we have investigated the effects of *A. muricata* leaf aqueous extract on lipid profiles in serum and biomarkers of oxidative stress in hepatocytes of diabetic rats. In diabetes, hypoinsulinaemia increases the activity of fatty acyl coenzyme-A oxidase, which initiates β-oxidation of fatty acids, resulting in lipid peroxidation (Baynes, 1995). Also, protein glycation and glucose auto-oxidation can lead to the formation of free radicals, and this, in turn, can induce lipid peroxidation (Baynes, 1991). Increased lipid peroxidation impairs membrane functions by decreasing membrane fluidity and changing the activity of membrane-bound enzymes and receptors (Baynes, 1995). Oxidative stress in diabetes mellitus could cause disturbances at the level of subcellular organelles, especially in the liver, which is the metabolic 'power-house' of the body. Evidence of mitochondrial alterations in diabetic rats has been noticed for a long time (Gerbitz et al., 1996). Mitochondrial damage can, in turn, generate a further oxidative stress inside the cell; therefore, liver mitochondria from streptozotocin-treated rats are likely to generate increased levels of reactive oxygen species (Kristal et al., 1997). Along with hyperglycaemia and abnormalities in serum lipids, diabetes is usually associated with microvascular and macrovascular complications which are the major causes of morbidity and mortality in diabetic individuals (Virella-Lopes and Virella, 2003). Diabetes can be managed by exercise, diet and drugs. Hypoglycaemic drugs are either too expensive, or possess undesirable side-effects and/or contra-indications. Therefore, the search for more effective and safer hypoglycaemic agents from plants and other natural sources has continued to be an area of interest for many researchers (Krishna et al., 2004).

In the present study, we noticed elevated serum lipids in STZ-treated diabetic rats. Lipids play an important role in the pathogenesis of diabetes mellitus. The level of serum lipids is usually raised in diabetes, and such an elevation represents a risk factor for coronary heart diseases (Mironava et al., 2000). However, in this study, a significant decrease in STZ-induced elevated LDL, TG and TC; and an increase in STZ-induced reduced HDL levels, were observed in AME-treated rats. These alterations could be beneficial in preventing diabetic complications as well as in improving lipid metabolism in diabetics.

The results of the present study also showed an increase in the levels of ROS, GSSG and MDA; and a decrease in GSH, CAT, GSH-Px and SOD contents of hepatic tissues of STZ-treated diabetic rats. Continuous treatment of Group C rats with AME caused significant decreases in the elevated blood glucose and ROS, GSSG and MDA levels of the diabetic rats. A significant elevation of hepatic activities of GSH-Px, CAT, SOD and GSH level were also observed in the AME-treated diabetic rats. It is thought that reactive oxygen free radicals could inactivate and reduce hepatic CAT, SOD, and GSH-Px activities. This speculation is in agreement with the findings of Wohaieb and Godin (Wohaieb and Godin, 1987). Furthermore, the decrease in hepatic GSH and increase in hepatic GSSG, could be due to decreased synthesis, or increased degradation of GSH by oxidative stress in diabetes. The marked decrease in MDA, ROS and GSSG levels in the hepatocytes of AME-treated rats probably suggests that AME exerts antioxidant activity that protects the tissues from the destructive effects of lipid peroxidation (Nicola et al., 1996).

Most of the glucokinase (GCK) in a mammal is found in the liver, and GCK provides approximately 95% of hexokinase activity in hepatocytes. GCK plays an important role in diabetes. It is involved in glucose uptake in the pancreas and liver, which are defective in type 2 diabetes mellitus. Hypoglycaemia or hyperglycaemia may also reduce or alter the functional efficiency of the GCK enzyme molecule, resulting in increasing or decreasing sensitivity of β-cell insulin response to glucose (Zelent et al., 2005). Because insulin is one of, if not the most important, regulators of GCK synthesis, diabetes of all types diminishes GCK synthesis and activity by a variety of mechanisms. Furthermore, it has been shown recently that insulin has a direct stimulatory effect on mitochondrial protein synthesis in isolated rat hepatocytes (Memon et al., 1995). In the present study, GCK activity was lower in STZ-treated diabetic rats as compared with the 'control' rats. The decrease in hepatic GCK could result from hypoinsulinaemia, decreased synthesis, or increased degradation of GCK by oxidative stress in diabetes (Matschinsky and Magnuson, 2004). However, the ability of AME to significantly increase GCK activity of the hepatocytes to optimal level would appear to suggest insulin releasing ability of the plant's extract in AME-treated Group C rats.

The results of the present study also revealed a highly significant decrease in serum insulin levels of STZ-treated diabetic rats. Single daily doses of AME significantly reduced the blood glucose concentrations of diabetic rats, and caused a significant increase in serum insulin levels. The present data, therefore, shows that treatment of diabetic rats with AME caused marked amelioration of hyperglycaemia, with pronounced increase in serum insulin levels. Improvement in insulin action in diabetic rats after AME administration might be attributed to its ability to improve the physical state of plasma membrane through increment of hepatic GSH levels, thereby interfering with the progression of lipid peroxidation.

Although the exact mechanisms of action of AME on the different biochemical variables examined in this study could not be established, a number of earlier investigators have shown that tannins and other polyphenolic compounds (e.g., coumarins), flavonoids, triterpenoid saponins, and a host of other plant secondary metabolites possess hypoglycaemic, hypolipidaemic, hypotensive, anti-inflammatory, and other pharmacological and biochemical properties in various experimental animal models (Ojewole, 2005). *A. muricata* is known to contain ellagic acid, tannis, flavonoids, polyphenolic compounds, triterpenoids, β-sistosterol, and so on (Watt and Breyer-Brandwijk, 1962; TDRG, 2002; Chang, 2001). It is, therefore, not unreasonable to speculate that some of the above chemical constituents of the plant, especially the coumarins, flavonoids and triterpenoids, are probably responsible for the altered biochemical variables in the hepatic tissues, as well as the antidiabetic property of AME, observed with plant's leaf aqueous extract in this study.

Conclusion

Based on our findings, we conclude that STZ treatment is associated with oxidative stress in hepatic tissues, and that *A. muricata* leaf aqueous extract possesses antioxidant activity which is able to inhibit and/or prevent hepatic oxidative damage produced by STZ treatment.

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References

1. Allain, C. C., Poon, L. S., Chon, C. S. G., Richmond, W. and Fu, P. C. (1974). Enzymatic determination of total serum cholesterol. Clin. Chem., **20:** 470-475.
2. Bailey, C. J. and Day, C. (1989). Traditional plant medicines as treatment for diabetes. Diabetes Care, **12:** 553-564.
3. Baynes, J. W. (1991). Role of oxidative stress in development of complications in diabetes.Diabetes, **40:** 405-412.
4. Baynes, J. W. (1995). Reactive oxygen in the aetiology and complications of diabetes. In: Ioannides C, Flatt P.R, (eds.), Drug, diet and disease mechanistic approach to diabetes, Ellis Horwood Limited. Hertfordshire**2:**203-231.
5. Bonnefont-Rousselot, D., Bastard, J. P., Jaudon, M. C. and Delattre, J. (2000). Consequences of diabetic status on the oxidant/antioxidant balance. Diabetes and metabolism (Paris), **26:**163-176.
6. Ceriello, A. (2003). New insights on oxidative stress and diabetic complications may lead to a "causal" antioxidant therapy. Diabetes Care, **26:**1589-1596.
7. Chang, R. F. (2001). Novel cytotoxic annonaceous acetogenins from *Annona muricata*.J. Nat. Prod., **64:** 925-931.
8. Cheng, M. L., Kammerer, C. M. and Lowe, W. F., Dyke, B. and VandeBerg, J.L. (1988). Method for quantitating cholesterolin subfractions of serum lipoproteins separated by gradient gel electrophoresis. Biochem. Genet., **26:** 657-681.
9. Davidson, A. and Arion, W. J. (1987). Factors underlying significant underestimations of glucokinase activity in crude liver extracts: physiological implications of higher cellular activity. Arch. Biochem. Biophys., **253:** 156-167.
10. Durackova, Z. (1999). Oxidative stress. In: Free radicals and antioxidants in Medicine (II). Durackova Z, Bergendi L, Carsky J. (eds.), Slovak Academic Press, Bratislava, pp. 11-38.
11. Friedewald, W. T., Levy, R. I. and Fredickson, D. S. (1972). Estimation of the concentration of low density lipoprotein cholesterol in plasma without the use of preparative ultracentrifuge. Clin. Chem., **18:** 499-502.
12. Gallou, G., Ruelland, A., Legras, B., Maugendre, D., Allanic, H. and Cloarec, L. (1993). Plasma MDA in Types 1 and 2 diabetes. Clin. Chim. Acta, **214:** 227-234.
13. Gerbitz, K. D., Gempel, K. and Brdiczka, D. (1996). Mitochondria and diabetes. Genetic, biochemical and clinical implications of the cellular energy circuit. Diabetes, **45:**113-126.
14. Herrman, C. E., Sanders, R. A., Klaunig, J. E., Schwarz, L. R. and Watkins, J. B. (1999). Decreased apoptosis

as a mechanism for hepatomegaly in streptozotocin-induced diabetic rats. Toxicol. Sci., **50:** 146-151.
15. Hissin, P. J. and Hilf, R. (1973). A fluorometric method for the determination of oxidized and reduced glutathione in tissue. Anal. Biochem., **74:** 214-226.
16. Johansson, L. H. and Borg, L. A. (1988). A spectrophotometric method for determination of catalase activity in small tissue samples. Anal. Biochem., **174:** 331–336.
17. Kakkar, P., Das, B. and Viswanathan, P. N. (1984). A modified spectrophotometric assay of superoxide dismutase, Ind. J. Biochem. Biophys., **21:** 130-132.
18. Keen, R. W., Deacon, A. C. and Delves HT (1994). Indian herbal remedies for diabetes as a cause of lead poisoning. Postgrad. Med. J., **70:** 113-114.
19. Krishna, B., Nammi, S., Kota, M. K. and Krishna Rao, R. V. (2004). Evaluation of hypoglycaemic and antihyperglycaemic effects of *Datura metel* Linn. seeds in normal and alloxan induced diabetic rats. J. Ethnopharmacol., **9:** 95-98.
20. Kristal, B.S., Jackson, C.T., Chung, H.Y., Matsuda, M., Nguyen, H. D. and Yu, B. D., (1997). Deffect at the centre P underlie diabetes-associated mitochondrial dysfunction. Free Rad. Biol. Med., **22:** 823-833.
21. Lenk, S. E., Bhat, D., Blankeney, W. and Dunn, W. A. Jr. (1992). Effects of streptozotocin-induced diabetes on rough endoplasmic reticulum and lysosomes of the rat liver. Am. J. Physiol., **263:** E856-862.
22. Lorke, D. A. (1983). A new approach to practical acute toxicity testing. Arch. Toxicol.,**54:** 275-287.
23. Memon, R. A., Mohan, C. and Bessman, S. P. (1995). Insulin stimulates hepatic mitochondrial protein synthesis. Biochem. Mol. Bio. Int., **37:** 627-634.
24. Matschinsky, F. M. and Magnuson, M. A. (2004). Glucokinase and Glycemic Disease: From Basic to Novel Therapeutics. Karger, Basel.
25. Mironava, M. A., Klein, R. L., Virella, G. T. and Lopes-Virella, M. F. (2000). Anti-modified LDL antibodies, LDL-containing immunune complexes, and susceptibility of LDL to *in vitro* oxidation in patients with type 2 diabetes. Diabetes, **49:** 1033-1049.
26. Nicola, W. G., Ibrahim, K. M., Mikhail, T. H., Girgis, R. B. and Khadr, M. E. (1996). Role of the hypoglycaemic plant extract *Cleome droserifolia* in improving glucose and lipid metabolism and its relation to insulin resistance in fattuy liver. Biol. Chim. Farm., **135:** 507-517.
27. Ohkawa, H., Ohishi, N. and Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem., **95:** 351–358.
28. Ojewole, J. A. O. (2005). Antinociceptive, anti-inflammatory and antidiabetic effects of *Bryophyllum pinnatum* (Crassulaceae) leaf aqueous extract. J. Ethnopharmacol.,**99:**13-19.
29. Rossini, A. A., Williams, R. M., Appel, M. C. and Like, A. A. (1978). Complete protection from low-dose streptozotocin-induced diabetes in mice. Nature, **276:** 182–184.
30. Rainwater, D. L., Ludwig, M. J., Haffner, S. M. and VandeBerg, J. L. (1995). Lipid and Lipoprotein factors associated with variation in Lp(a) density. Arterioscler. Thromb. Vasc. Biol., **15:** 313-319.
31. Scoppola, A., Montecchi, F. R., Mezinger, G. and Lala A. (2001). Urinary mevelonate excresion rate in type 2 diabetes: role of metabolic control. Artherosclerosis, **156:** 357-361.
32. Socci, D. J., Bjugstad, K. B. and Jones, H. C., Pattisapu, J.V. and Arendash, G. W. (1999). Evidence that oxidative stress is associated with the pathophysiology of inherited hydrocephalus in the H-Tx rat model. Exp. Neurol., **155:** 109-117.
33. Tappel, A. L. (1978). Glutathione peroxidase and hydroperoxides. Methods Enzymol.,**52:** 506–513.
34. Technical Data Report on Graviola (TDRG) (*Annona muricata*). Sage Press, Inc. (2002).
35. Virella-Lopes, M. F. L., Stone, P. G. and Colwel, J. A. (1977). Serum High Density Lipoprotein in diabetic patients. Diabetologia, **13:** 285-291.
36. Virella-Lopes, M. F. L. and Virella, G. (2003). The role of immune and inflammatory processes in the development of macrovascular disease in diabetes. Frontiers in Biosc., **8:** 750-768.
37. Watt, J. M. and Breyer-Brandwijk, M. J. (1962). The medicinal and poisonous plants of Southern and Eastern Afica. E. & S. Livingstone Ltd; Edinburgh and London, 2nd edn., pp.58-59.
38. Wohaieb, S. A. and Godin, D. V. (1987). Alterations in free radical tissue defense mechanisms in streptozotocin-induced diabetes in rat: effect of insulin treatment. Diabetes, **36:** 1014-1018.
39. Zelent, D., Najafi, H., Odili, S., Buettger, C., Weik-Collins, H., Li, C., Doliba, N., Grimsby, J. and Matschinsky, F. M. (2005).Glucokinase and glucose homeostasis: proven concepts and new ideas. Biochem. Soc. Trans., **33:** 306-310.

Reference # 2

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In vitro antioxidant studies in leaves of *Annona* species

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Antioxidant potential of leaves of three different species of *Annona* was studied by using different *in vitro* models eg., 1,1-diphenyl-2-picryl hydrazyl (DPPH), 2,2-azinobis-(3-ethylbenzothizoline-6-sulphonate) (ABTS), nitric oxide, superoxide, hydroxy radical and lipid peroxidation. The ethanolic extract of *A.muricata* at 500 µg/ml showed maximum scavenging activity (90.05%) of ABTS radical cation followed by the scavenging of hydroxyl radical (85.88%) and nitric oxide (72.60%) at the same concentration. However, the extract showed only moderate lipid peroxidation inhibition activity. In contrast, the extract of *A.reticulata* showed better activity in quenching DPPH (89.37%) and superoxide radical (80.88%) respectively. *A.squamosa* extract exhibited least inhibition in all *in vitro* antioxidant models excepting hydroxyl radical (79.79%). These findings suggest that the extracts of *A. muricata* possess potent *in vitro* antioxidant activity as compared to leaves of *A. squamosa* and *A.reticulata* suggesting its role as an effective free radical scavenger, augmenting its therapeutic value.

Keywords: *Annona* species, Antioxidant, Free radicals, Lipid peroxidation

Oxidative stress has been implicated in the pathology of many diseases such as inflammatory conditions, cancer, diabetes and aging[1]. Free radicals induced by peroxidation have gained much importance because of their involvement in several pathological conditions such as atherosclerosis, ischemia, liver disorder, neural disorder, metal toxicity and pesticide toxicity[2]. Together with other derivatives of oxygen, they are inevitable byproducts of biological redox reactions[3]. Reactive oxygen species (ROS) such as superoxide anions ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$) and nitric oxide (NO) inactivate enzymes and damage important cellular components causing injury through covalent binding and lipid peroxidation[4]. Antioxidants may offer resistance against the oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation and by other mechanisms and thus prevent disease[5]. Foods rich in antioxidants have been shown to play an essential role in the prevention of cardiovascular diseases, cancer, neurodegenerative diseases, inflammation and problems caused by cell and cutaneous aging[6].

Annona species belonging to Custard Apple family is cultivated all over India for its edible fruit. All parts of *Annona* are used in natural medicine in the tropics. It is considered to be good source of natural antioxidants for various diseases. Therefore, attention in recent times has been focused on the isolation, characterization and utilization of natural antioxidants. Of the species, *Annona squamosa* (AS) is traditionally used for the treatment of epilepsy, dysentery, cardiac problems, fainting, worm infestation, constipation, hemorrhage, dysuria, fever, thirst, malignant tumors, ulcers and also as an abortifacient[7].Fruits of *Annona reticulata* (AR) are astringent, sweet and useful in blood complaints. It is also used as anti-dysentric and anti-helminthic[8]. Its leaves and stem constituents include anonaine, roemerine, corydine, isocorydine and many other aporphine alkaloids[8].

Fruits of *Annona muricata* (AM), also known as Graviola in South America are taken internally for worms and parasites, to cool fevers, to increase mother's milk after child birth and as an astringent for diarrhoea and dysentery. Leaves are considered antitumorous[9], sedative, anti-spasmodoic[10] and hypotensive[11].

Preliminary reports have confirmed the antioxidant potential of AS in different *in vitro* models. This has been attributed to the presence of flavonoids like rutin and hyperoside in leaves[12]. In contrast, the leaves of AM are rich in Annonaceous acetogenins responsible for its antitumorous property[9]. However, no reports

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are available to show the antioxidant property of the plant with relation to its anticancer activity. Hence, the present study has been undertaken to investigate the antioxidant activity of the leaves of *Annona muricata* with reference to the two other species of *Annona* i.e. *A. squamosa* and *A. reticulata* using different *in vitro* models.

Materials and Methods

Materials—All chemicals and solvents were of analytical grade and were obtained from HiMedia Chemicals, Mumbai, India. 2,2-azinobis (3-ethylbenzo-thiozoline-6-sulphonate) (ABTS) was obtained from Sigma Chemicals, USA. The other chemicals used were 1,1-diphenyl, 2-picryl hydrazyl (DPPH), sodium nitroprusside, sulphanilamide, o-phosphoric acid, napthyl ethylene diamine dihydrochloride, ferrous sulphate ($FeSO_4$), thiobarbituric acid (TBA), trichloroacetic acid (TCA), nitroblue tetrazolium (NBT) and ethylene diamine tetra acetic acid (EDTA).

Plant material—The leaves of AS, AR and AM were collected during December 2005 from Coimbatore, Tamil Nadu. The plant material was authenticated by the Agricultural Officer, Horticultural Research Station, Coonoor, Tamil Nadu. Voucher specimens (VR 101, 102, 103) of the samples have been deposited in the herbarium of Dr G R Damodaran College of Science, Coimbatore.

Preparation of the plant extract— About 350 g of the shade dried, powdered leaves of AS, AR and AM were exhaustively extracted with 95% ethanol using Soxhlet apparatus. The residue was filtered and concentrated *in vacuo* to a syrupy consistency. The extract was then stored in a dessicator until further use.

DPPH radical scavenging activity—DPPH scavenging activity was measured by the spectrophotometric method[13]. To a methanolic solution of DPPH (200μM), 0.05ml of the test compounds dissolved in ethanol were added at different concentration (100 – 500 μg /ml). An equal amount of ethanol was added to the control. After 20 min, the decrease in the absorbance of test mixture (due to quenching of DPPH free radicals) was read at 517 nm and the percentage inhibition calculated by using the formula[14]:

$$\text{Inhibition (\%)} = \frac{(\text{control} - \text{test})}{\text{control}} \times 100$$

ABTS radical cation decolorisation assay[15]—In this improved version, ABTS.-, the oxidant is generated by persulfate oxidation of 2,2,-azinobis (3-ethylbenzoline-6- sulfonic acid) – ($ABTS^{2-}$).

ABTS radical cation ($ABTS^{\cdot+}$) was produced by reacting ABTS solution (7mM) with 2.45 mM ammonium persulphate and the mixture were allowed to stand in dark at room temperature for 12 – 16 hr before use. For the study, different concentration (100 – 500 μg/ml) of ethanolic extract (0.5 ml) were added to 0.3 ml of ABTS solution and the final volume was made up with ethanol to make 1 ml. The absorbance was read at 745 nm and the percentage inhibition calculated.

Scavenging of nitric oxide radical[16,17]—Nitric oxide was generated from sodium nitroprusside and measured by Griess' reaction[18,19]. Sodium nitroprusside (5 mM) in standard phosphate buffer solution was incubated with different concentration (100 – 500 μg/ml) of the ethanol extract dissolved in phosphate buffer (0.025 M, pH 7.4) and the tubes were incubated at 25°C for 5 hr. Control experiments without the test compounds, but with equivalent amounts of buffer were conducted in an identical manner. After 5 hr, 0.5 ml of incubation solution was removed and diluted with 0.5 ml of Griess' reagent (1% sulphanilamide, 2% o-phosphoric acid and 0.1% napthylethylene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with napthylethylene diamine was read at 546 nm. The experiment was repeated in triplicate.

Scavenging of hydroxyl radical—Hydroxyl radical scavenging activity was measured according to the method of Kunchandy and Rao[20] by studying the competition between deoxyribose and test extract for hydroxyl radical generated by Fenton's reaction. The reaction mixture contained deoxyribose (2.8 m*M*), $FeCl_3$ (0.1 m*M*), EDTA (0.1 m*M*), H_2O_2 (1 m*M*), ascorbate (0.1 m*M*), KH_2PO_4- KOH buffer (20 m*M*, *p*H 7.4) and various concentrations of the sample extracts in a final volume of 1.0 ml. The reaction mixture was incubated for 1 h at 37°C. Deoxyribose degradation was measured as thiobarbituric acid reacting substances (TBARS) and the percentage inhibition calculated.

Scavenging of superoxide radical—The scavenging activity towards the superoxide radical ($O_2^{\cdot-}$) was measured in terms of inhibition of generation of $O_2^{\cdot}$ (ref. 21). The reaction mixture consisted of phosphate buffer (50 m*M*, *p*H 7.6), riboflavin

(20 µg/0.2 ml), EDTA (12 mM), NBT (0.1 mg/3ml) and sodium cyanide (3 µg/0.2 ml) Test compounds of various concentrations of 100 -500 µg/ml were added to make a total volume of 3 ml. The absorbance was read at 530 nm before and after illumination under UV lamp for 15 min against a control instead of sample. The percentage inhibition was calculated by using the same formula as given above.

In vitro anti-lipid peroxidation assay—Freshly excised goat liver was processed to get 10% homogenate in cold phosphate buffered saline, *p*H 7.4 using glass teflon homogenizer and filtered to get a clear homogenate. The degree of lipid peroxidation was assayed by estimating the TBARS by using the standard method[22] with minor modifications[14]. Different concentrations of the extracts (100 – 500 µg/ml) in water was added to the liver homogenate. Lipid peroxidation was initiated by adding 100 µl of 15 mM ferrous sulphate solution to 3 ml of the tissue homogenate. After 30 min, 100 µl of this reaction mixture was taken in a tube containing 1.5 ml of 10 % TCA. After 10 min, tubes were centrifuged and supernatant was separated and mixed with 1.5 ml of 0.67% TBA in 50 % acetic acid. The mixture was heated for 30 min in a boiling water bath. The intensity of the pink coloured complex formed was measured at 535 nm. The results were expressed as percentage inhibition using the formula as given above.

Statistical analysis—Linear regression analysis was used to calculate the IC_{50} values.

Results

Several concentrations ranging from 100-500µg/ml of the ethanolic extract of AS, AR and AM were tested for their antioxidant activity in different *in vitro* models. It was observed that free radicals were scavenged by the test compounds in a concentration dependent manner in all the models. From the results given in Tables 1-3, it was inferred that, with respect

Table 1— Effect of ethanolic extract of AS on different antioxidant models

[Values are mean of 3 replicates]

Conc. (µg/ml)	Inhibition (%)					
	DPPH	ABTS	Nitric Oxide	Hydroxyl radical	Superoxide radical	Lipid peroxidation
100	75.12	10.45	34.69	37.56	10.29	7.64
200	78.88	33.83	40.27	45.60	26.47	11.70
300	86.23	50.25	45.96	52.46	50.00	26.24
400	87.47	72.14	52.60	69.78	66.91	44.52
500	88.77	88.06	68.03	79.79	77.21	50.83
IC_{50}(µg/ml)	65	300	370	300	300	480

Table 2— Effect of ethanolic extract of AR on different antioxidant models

[Values are mean of 3 replicates]

Conc. (µg/ml)	Inhibition (%)					
	DPPH	ABTS	Nitric Oxide	Hydroxyl radical	Superoxide radical	Lipid peroxidation
100	63.75	3.98	39.47	40.80	13.97	6.31
200	69.38	35.32	47.44	57.51	34.56	9.97
300	75.00	51.74	55.40	65.03	52.92	10.63
400	81.63	73.63	64.39	69.43	66.18	30.23
500	89.37	89.05	71.10	77.72	80.88	35.54
IC_{50} (µg/ml)	80	260	225	215	285	315

Table 3—Effect of ethanolic extract of AM on different antioxidant models

[Values are mean of 3 replicates

Conc. (µg/ml)	Inhibition (%)					
	DPPH	ABTS	Nitric Oxide	Hydroxyl radical	Superoxide radical	Lipid peroxidation
100	72.72	4.48	32.65	44.05	16.18	3.65
200	76.23	27.36	39.02	54.53	33.09	7.30
300	80.74	49.25	43.45	63.73	49.26	12.63
400	83.89	74.00	56.31	69.80	66.18	39.80
500	88.77	90.05	72.60	85.88	78.68	58.47
IC_{50} (µg/ml)	70	305	350	155	300	455

to maximum percentage inhibition in all models viz., ABTS, nitric oxide, hydroxyl radical, lipid peroxidation, the extracts of AM showed 90.05, 72.60, 85.88, 58.47% inhibition respectively at 500µg/ml concentration. In contrast, the extracts of AR showed 89.37 and 80.88 % inhibition in quenching DPPH and superoxide radical respectively at the same concentration. However, the IC_{50} value of AR for ABTS, nitric oxide and hydroxyl radical were shown to be 260, 225 and 215 µg/ml respectively. The extracts of AS showed least inhibition at 500 µg/ml concentration in all the models excepting hydroxyl radical (79.79 %).

Discussion

The peroxidation of membrane lipids initiated by oxygen radicals may lead to cell injury. Initiation of lipid peroxidation by ferrous sulphate takes place through ferryl-perferryl complex[23] or through ·OH radical by Fenton's reaction[24], thereby initiating a cascade of oxidative reactions. The results obtained in the present study may be attributed to several reasons viz., the inhibition of ferryl-perferryl complex formation; scavenging of ·OH or superoxide radical or by changing the ratio of Fe^{3+}/Fe^{2+}; reducing the rate of conversions of ferrous to ferric or by chelation of the iron itself[25].

Free radicals are chemical entities that can exist separately with one or more unpaired electrons. The generation of free radicals can bring about thousands of reactions and thus cause extensive tissue damage. Lipids, proteins and DNA are all susceptible to attack by free radicals[26]. Antioxidants may offer resistance against oxidative stress by scavenging the free radicals.

The 1, 1-diphenyl -2-picryl hydrazyl (DPPH) radical was widely used as the model system to investigate the scavenging activities of several natural compounds such as phenolic and anthocyanins or crude mixtures such as the ethanol extract of plants. DPPH radical is scavenged by antioxidants through the donation of proton forming the reduced DPPH. The color changes from purple to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 517nm. Radical scavenging activity increased with increasing percentage of the free radical inhibition.

DPPH is a relatively stable radical. The assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH which reacts with suitable reducing agent. The electrons become paired off and solution loses color stochiometrically depending on the number of electrons taken up[27]. DPPH was used to determine the proton radical scavenging action of extracts of AS, AR and AM, because it possesses a proton free radical and shows a characteristic absorbance at 517 nm. From the present results, it may be postulated that AR reduces the radical to corresponding hydrazine when it reacts with hydrogen donors in antioxidant principles[21].

The ABTS assay is based on the inhibition of the absorbance of the radical cation $ABTS^{\cdot +}$, which has a characteristic long wavelength absorption spectrum[21]. The results imply that the extracts of AM inhibit or scavenge the $ABTS^{\cdot +}$ radicals since both inhibition and scavenging properties of antioxidant towards $ABTS^{\cdot +}$ radicals have been reported earlier[15, 28].

Nitric oxide is a free radical generated by endothelial cells, macrophages, neurons etc., and involved in the regulation of various physiological process[29]. Excess concentration of NO is associated with several diseases[30,31]. Oxygen reacts with the excess nitric oxide to generate nitrite and peroxy nitrite anions, which act as free radicals[26, 32]. In the present study, the ethanolic extract of AM showed better activity in competing with oxygen to react with nitric oxide and thus the inhibition of generation of anions.

The hydroxy radical scavenging activity is measured as the percentage of inhibition of hydroxy radicals generated in the Fenton's reaction mixture[25] by studying the competition between deoxyribose and the extract for hydrogen radicals generated from Fe^{3+}/ascorbate/EDTA/H_2O_2 systems. The hydroxyl radicals attack deoxyribose which eventually results in TBARS formation. From the present results, it is observed that the extract of AM have better hydroxyl radical scavenging activity as reflected in terms of percentage inhibition.

Superoxides are produced from molecular oxygen due to oxidative enzymes[32] of body as well *via* non-enzymatic reaction such as autooxidation by catecholamines[33]. The scavenging activity towards the superoxide radical ($O_2^{\cdot -}$) is measured in terms of inhibition of generation of $O_2^{\cdot -}$. In the present study, superoxide radical reduces NBT to a blue colored formosan that is measured at 560 nm[34]. The result shows that extract of AR has a potent scavenging activity with increasing percentage inhibition. The

probable mechanism of scavenging the superoxide anions may be due to the inhibitory effect of the extract towards generation of superoxides in the *in vitro* reaction mixture.

Lipid peroxidation is the oxidative degradation of polyunsaturated fatty acids and involves formation of lipid radicals leading to membrane damage. Free radicals induces lipid peroxidation in polyunsaturated lipid rich areas like brain and liver[35]. Initiation of lipid peroxidation by ferrous sulphate takes place through hydroxy radical by Fenton's reaction[25]. The inhibition could be caused by absence of ferryl-perferryl complex or by scavenging the hydroxy radical or the superoxide radicals or by changing the Fe^{3+}/Fe^{2+} or by reducing the rate of conversion of ferrous to ferric or by chelating the iron itself. In this study, *in vitro* lipid peroxidation was induced in goat liver by using $FeSO_4$ and ascorbic acid. The present results show that extract of AM shows better dose-dependent prevention towards generation of lipid peroxides.

From the above results, it can be concluded that ethanolic extracts of leaves of AM showed the most potent *in vitro* antioxidant activity with high percentage inhibition as compared to leaves of AS and AR. This may be attributed to the presence of acetogenins, which probably play a role as an effective free radical scavenger and hence an effective antitumorous agent.

References

1 Marx J L, Oxygen free radicals linked to many diseases. *Science*, 235 (1987) 529.

2 Pandey S, Sharma, Chaturved P & Tripathi B, Protective effect of Rubia cordifolia on lipid peroxide formation in isolated rat mice, *Indian J Exp Biol*, 32 (1994) 180.

3 Ajay Arora, Sairam R K & Srivatsava G C, Oxidative stress and antioxidant system in plants, Curr Sci, 82 (2002) 122.

4 Geesin J G, Gordon J S & Berg R A, Retinoids affect collagen synthesis through inhibition of ascorbate-induced lipid peroxidation in cultured human dermal fibroblasts, *Arch Biochem Biophys*, 278 (1990) 352.

5 Youdim K A & Joseph J A, A possible emerging role of phytochemicals in improving age-related neurological dysfunctions –a multiplicity of effects, *Free Rad Biol Med*, 30 (2001) 583.

6 Pratt D E, Natural antioxidants from plant material, in *Phenolic compounds in foods and their effects on health II: Antioxidants and cancer prevention I* (ACS Symposium Series 507) edited by M Hang, C. Ho & C Lee (American Chemical Society, Washington DC) 1992, 54.

7 Nadkarni A K, in *Indian Materia medica*, Vol I, (Popular Prakasan Ltd., Mumbai, India) 2000, 116.

8 Oliver-Brever, *Medicinal plants in tropical West Africa*. (Cambridge University Press, Cambridge, UK) 1986.

9 Yuan S S, Chang H L, Chen H W, Yeh Y T, Kao Y H, Lin K H, Wu Y C & Su J H, Annonacin, a mono-tetrahydrofuran acetogenin, arrests cancer cells at the G1 phase and causes cytotoxicity in a Bax- and caspase-3-related pathway, *Life Sci*, 72 (2003) 2853.

10 N'gouemo P, Koudogbo B, Pambou Tcivounda H, Akono-Nguema C & Minko M, Effects of ethanol extract of Annona muricata on pentylenetetrazol-induced convulsive seizures in mice, *Phytothe Res*, 11 (1997) 243.

11 Carbajal D, Casaco A, Arruzazzabala L & Gonzalez R, Pharmacological screening of plant decoctions commonly used in Cuban folk medicine, *J Ethnopharmacol*, 33(1991)21.

12 Shirwaikar A, Rajendran K & Dinesh Kumar C, In vitro antioxidant studies of Annona squamosa Linn leaves, *Indian J Exp Biol*, 42 (2004) 803.

13 Sreejayan N & Rao M N A, Free radical scavenging activity by curcuminoids, *Drug Res*, 46(1996) 169.

14 Prasanth Kumar V, Shasidhara S, Kumar M M & Sridhara B Y, Effect of Luffa echinata on lipid peroxidation and free radical scavenging activity, *J Pharm. Pharmacol*, 52 (2000) 891.

15 Re R, Pellegrini N, Protoggenete A, Pannala A, Yang M & Rice-Evans C, Antioxidant activity applying an improved ABTS radical cation assay, *Free Rad Biol Med*, 26 (1999) 1231.

16 Sreejayan N & Rao M N A, Nitric oxide scavenging activity by curcuminoids, *J. Pharm Pharmacol* , 47 (1997) 105.

17 Shirwaikar Annie & Somashekar A P, Anti-inflammatory activity and free radical scavenging studies of Aristoluchia bractoelata Lam., *Indian J Pharm Sci* , 65 (2003) 68.

18 Green L C, Wagner D A, Glogowoski J, Skipper P L, Wishnok J S & Tannenbavm, S R, Analysis of nitrate and 15N in biological fluids, *Anal Biochem*, 126 (1982) 131.

19 Marcocci L, Maguire J J, Droy-Lefaix M T & Packer L, The nitric oxide scavenging property of Ginko biloba extract EGB 761, *Biochim Biophys Res Commun*, 201 (1994) 748.

20 Kunchandy E & Rao M N A, Oxygen radical scavenging activity of curcuminoid, *Int J Pharmacognosy*, 58 (1990) 237.

21 Sanchez-Moreno C, Methods used to evaluate the free radical scavenging activity in foods and biological systems, *Food Sci Tech Int*, 8 (2002) 122.

22 Ohkawa H, Ohishi N & Yagi K, Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, *Anal Biochem*, 95 (1979) 351.

23 Gutteridge J M C, Age, pigments and free radicals: fluoresecent lipid complexes by iron and copper containing proteins, *Biochim Biophys Acta*, 834 (1985) 144.

24 Halliwell B, Superoxide-dependent formation of hydroxyl free radicals in the presence of iron chelates, *FEBS Lett.*, 92 (1978) 321.

25 Braugghler J M, Duncan C A & Chase L R, The involvement of iron in lipid peroxidation. Important of ferrous to ferric iron in initiation, *J Biol Chem*, 261 (1986) 10282.

26 Cotran R S, Kumar V & Collins T, in *Robbin's pathological basis of disease*, 6th ed (Thomson Press (I) Ltd, Noida, India) 1999, 1.

27 Blois M S, Antioxidant determination by the use of stable free radicals, *Nature*, 26 (1958) 1199.

28 Rice-Evans C & Miller N J, Factors influencing the antioxidant activity determined by the ABTS.+ radical cation, *Free Rad Res*, 26 (1997) 195.

29 Lata H & Ahuja G K, Role of free radicals in health and disease, *Indian J Physio Allied Sci*, 57 (2000) 124.

30 Ialenti A, Moncada S & Di Rosa M, Modulation of adjuvant arthritis by endogenous nitric oxide, *Br J Pharmacol*, 110 (1993) 701.

31 Ross R, The pathogenesis of atherosclerosis: a perspective for the 1990's, *Nature*, 362 (1993) 801.

32 Sainani G S, Manika J S & Sainani R G, Oxidative stress: a key factor in pathogenesis of chronic diseases, *Med Uptake*, 1 (1997) 1.

33 Hemmani T & Parihar M S, Reactive oxygen species and oxidative damage, *Indian J Physiol Pharmacol*, 42 (1998) 440.

34 Khanam S, Shivaprasad H N & Kshama D, In vitro antioxidant screening models: a review, *Indian J Pharm Educ*, 38 (2004) 180.

35 Coyle J T & Puttfarcken P, Oxidative stress, glutamate and neurodegenerative diseases, *Science*, 219 (1993) 1184.

Reference # 3

From Chamomile to Aspirin? Medicinal Plant use among clients at Laboratorios Beal in Trujillo, Peru

R.W. Bussmann, D. Sharon and M. Garcia

Research

Abstract

Medicinal plant use in Peru can be tracked back for millennia, and although westernized medicine has become an important factor in the treatment of illnesses, many patients still frequent herbalist shops and retain some herbal knowledge of their own. The present study, undertaken at "Laboratorios Beal," a herbalist practice in Trujillo, Peru, was conducted as a comparison to previous research at Clinica Anticona, a Western style clinic in the same city, to evaluate if patients at a herbal clinic were more likely to use plants for treatment rather than pharmaceuticals, and if their own plant knowledge was more extensive than the knowledge of the patients interviewed at a Western clinic. The results demonstrate that, amongst the patients of the herbal clinic, plants do played only a slightly larger role when compared to the use of pharmaceuticals, indicating that patients at the herbal clinic were as likely to use Western pharmaceuticals as patients at a Western clinic were using herbs, and vise versa. Even at a herbalist shop many patients thought that pharmaceutical medicine to be faster and more effective than herbs, while plants were regarded as safer and free from side-effects. The plant knowledge of individual patients was comparable to the knowledge encountered at a western medicinal facility.

Resumen

El uso de plantas medicinales en el Perú puede ser trazado hacia milenio atrás, y aunque la medicina occidental ha llegado a ser un factor importante en el tratamiento de la enfermedad, todavía muchos pacientes visitan las tiendas de herbolarios y retienen conocimientos propios d las yerbas. El estudio presente, llevado a cabo en "Laboratorios Beal", un consultorio herboristico en Trujillo, Perú, trata de descubrir si las pacientes a tal facilidad usan de preferencia plantas medicinales sobre productos farmacéuticos, y si sus conocimientos de plantas medicinales son más extensivos que los de los clientes de una clínica occidental. Los resultados de nuestro estudio demuestran que, entre los pacientes de la tienda de yerbas, las plantas juegan un papel un poco más grande en comparación con el uso de productos farmacéuticos. Sin embargo, la diferencia es marginal y las preferencias de uso son comparables a esos de una clínica occidental. Por eso aún en la tienda de yerbas muchos pacientes piensan que la medicina farmacéutica es más rápida y efectiva mientras que se consideran a las plantas como más seguras y libre de efecios secundarios. Los conocimientos herboristicos de pacientes individuales son comparable a esos encontrados en una clínica occidental.

Introduction

Traditional Medicine and Complementary Alternative Medicine is used globally and has a rapidly growing economic importance. In developing countries, Traditional Medicine and Complementary Alternative Medicine is often the only accessible and affordable treatment available. In Latin America the WHO Regional Office for the Americas (AMRO/PAHO) reports that 71% of the popula-

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tion in Chile and 40% of the population in Colombia have used Traditional Medicine and Complementary Alternative Medicine (World Health Organization 1999, 2002). In the USA, a national survey reported the use of at least one of 16 alternative therapies increased from 34% in 1990 to 42% in 1997 (Eisenberg *et al.* 1998, UNCTAD 2000).

Traditional and Complementary Alternative Medicine are gaining more and more respect by national governments and health providers (Alves *et al.* 2007). Peru's National Program in Complementary Medicine and the Pan American Health Organization recently compared Complementary Medicine to allopathic medicine in clinics and hospitals operating within the Peruvian Social Security System (EsSalud 2000). A total of 339 patients - 170 being treated with Complementary Alternative Medicine and 169 with allopathic medicine - were followed for one year. Treatments for osteoarthritis; back pain; neuroses; asthma; peptic acid disease; tension and migraine headache; and obesity were analyzed. The results, with 95% significance, showed that the cost of using Complementary Alternative Medicine was less than the cost of Western therapy. In addition, for each of the criteria evaluated - clinical efficacy, user satisfaction, and future risk reduction - Complementary Alternative Medicine's efficacy was higher than that of conventional treatments, including fewer side effects, higher perception of efficacy by both the patients and the clinics, and a 53-63% higher cost efficiency of Complementary Alternative Medicine over that of conventional treatments for the selected conditions.

According to WHO (2002), the most important challenges for Traditional Medicine/Complementary Alternative Medicine for the next years are:

- Research into safe and effective Traditional Medicine and Complementary Alternative Medicine treatments for diseases that represent the greatest burden, particularly among poorer populations.
- Recognition of the role of Traditional Medicine practitioners in providing health care in developing countries.
- Optimized and upgraded skills of Traditional Medicine practitioners in developing countries.
- Protection and preservation of the knowledge of indigenous Traditional Medicine.
- Sustainable cultivation of medicinal plants.
- Reliable information for consumers on the proper use of Traditional Medicine and Complementary Alternative Medicine therapies and products.

Material and Methods

The primary focus of this project has been the ethnobotany of medicinal plants used on the north coast of Peru. Northern Peru represents the "health axis" of the ancient Central Andean cultural area stretching from Ecuador to Bolivia. The traditional use of medicinal plants in this region, which encompasses in particular the Departments of Piura, Lambayeque, La Libertad, Cajamarca and San Martin possibly dates as far back as the first millennium B.C. (north coastal Cupisnique culture) or at least to the Moche period (A.D. 100-800), with healing scenes and healers frequently depicted in ceramics.

Precedents for this study have been established by the late 17th-century plant collections of Bishop Baltasar Jaime Martinez Compañón (Sharon & Bussmann 2005), ethnoarchaeological analysis of the psychedelic San Pedro cactus (Sharon 2000), **curandera** depictions in Moche ceramics (Glass-Coffin *et al.* 2004), research on the medicinal plants of Southern Ecuador and Northern Peru (Bussmann & Sharon 2006a,b, Sharon & Bussmann 2006), and use in field guides on the medicinal plants of the region (Bussmann & Sharon 2007a,b).

In 2006, the authors investigated whether or not the clients of Clinica Anticona in El Porvenir, Trujillo, used Western medicines more than medicinal plants (Bussmann *et al.* 2007). The results demonstrated that - despite parallel use of phytomedicine - pharmaceutical medicines play a slightly larger role in such a setting when compared to the use of medicinal plants. This documented incorporation of Traditional Medicine and Complementary Alternative Medicine into public health has become an important topic in scientific discussion (Mignone *et al.* 2007, Rômulo *et al.* 2007).

In order to investigate if such findings hold true with clients of an herbalist, a comparative study was conducted at an herbal medicine shop called Laboratorios Beal, where Manuel Bejarano Alvarado is a **médico naturista** or herbalist. The results of this study demonstrated that in this setting medicinal plants played a somewhat larger role than pharmaceutical medicines.

Interviews were conducted daily at Laboratorios Beal, a herbal consultation shop in Trujillo, Peru, while clients waited in line to be seen by herbalist Manuel Bejarano Alvarado. The herb store was visited every day for two weeks from 7:30AM to 11:00AM. It was during these hours that the herbalist had the most clients waiting in line. As a requirement of checking their pulses they had to come in with empty stomachs. Clients were approached and informed that the authors were conducting research to investigate whether the population of Trujillo preferred to use medicinal herbs or pharmaceutical medicines. The first step was to explain the purpose of the study and the prior informed consent form (see Bussmann *et al.* 2007), which was signed by those who agreed to participate. The interviews were conducted by using a questionnaire as a guide for conversation, making sure all of the questions were answered. Every day was different in terms of how many people were willing to respond to questionnaires. There were times when there were a lot of people waiting

in line but only a few of them would want to participate. Then there were times when there were very few waiting in line but most of them would want to participate. However, by the end of two weeks, 100 questionnaires had been completed.

In the first few days of the research project the participants were asked to write down the answers on the questionnaires themselves. However, it soon became apparent that the people would be more willing to participate if asked questions with the authors noting the answers. This methodology proved more successful since it was discovered that sometimes the respondents wouldn't answer all the questions in the self-response format or they were confused with regard to the meaning of some of the questions.

The questionnaires were identical to those that were conducted by Bussmann *et al.* (2007) but an additional question ("Do your parents know more, less, or the same as you regarding the use of herbal medicines?") was added in order to ascertain if the knowledge of herbal medicines is increasing, decreasing, or remaining constant amongst that portion of the population of Trujillo represented by the clients of Laboratorios Beal. The informants were also asked to explain the meaning of **susto** (fright), **chucaque** (shame), and **mal daño** (sorcery) in order to investigate if there was consensus amongst the respondents regarding the meaning of these traditional ailments.

Results

Sixty-seven percent of the interviewees were females and 33% were males. More women are represented in this study because there were always more women in the shop than men. Twenty- seven percent were married, 41% were single, 30% were cohabitating with a partner, 1% were divorced, and 1% were widowed. The average age amongst the respondents was about 38 years, 20% were between the ages 12-25, 30% were between the ages of 26-35, 19% were between the ages 36 and 45, 14% were between the ages 46 and 55, 11% were between the ages 57 and 65, and 6% were between the ages 70 and 77. Of the 100 informants, 75% had children and 25% did not. Of those with children, the average number of children was two, but about 42% of the informants had between one and two children. Most single respondents only had one or two children.

In terms of the education level of the respondents, 3% of the informants had no education at any educational institute, 15% had finished elementary school, 15% went to elementary school but didn't finish, 32% finished junior high-school, 12% went to junior high-school but didn't finish, 3% finished high-school, and only one went to high-school but didn't finish. Six percent attended university and graduated, 5% went to a university but didn't graduate, 5% went to a technical institute and graduated, 2% went to a technical institute but didn't graduate, and one person didn't answer the question. Most of the respondents had the education level of a junior high-school graduate.

An interview was also conducted with Manuel Bejarano in order to acquire more information about the nature of his clientele and what were the most common cases amongst those clients. According to Sr. Bejarano, his clients come for a wide range of reasons, but they largely complained of stress or they had problems with their nervous system, inflammation, kidneys and lungs. He also indicated that many of the patients were women and that the average age range was from the age of 30 to the age of 60. Very few of his patients were elderly (**ancianos**) and very few were youths. Sr. Bejarano also stated that most of his patients came from outside of Trujillo, for example, from Lima, Huaraz and central Peru. Many clients indicated that this was their first time at the shop. In fact, they used this reason as their excuse for not wanting to participate or hesitating to participate in the questionnaire. When asked, Sr. Bejarano pointed out that he was recommended to many of his clients by other clients, and that he always had many first-timers as clients.

When asked what **susto** (fright), **chucaque** (shame), and **mal daño** (sorcery) were, it was interesting to find that the results were similar to those that were found by Bussmann *et al.* (2007). In their study, 84% of the respondents knew what **susto** and **chucaque** were while 58% said they knew what **mal daño** was. In the present study 72% said they knew what **susto** was, 82% said they knew what **chucaque** was, and 56% said they knew what **mal daño** was. A second part of the question in the present study asked the participants to explain what each disease was and how it was cured in order to investigate if there was a consensus amongst the population as to the nature of each ailment. Almost every respondent had a basic understanding that **susto** was a sickness where the person has problems with his or her nervous system and therefore loses a lot of weight and, that the sickness was caused by a sudden fright or shock experienced by the person. For example, a dog might have barked at the person unexpectedly. About 16% of those who said they knew what **susto** was said that the sickness was mostly a child's ailment. Only about 2% said that it was simply a sickness, 7% said that it was specifically an effect of being scared by an animal. The part of the answer where the respondents explained how the **susto** was cured was one where the answers varied greatly amongst the participants. Most of the responses basically included that **susto** was cured with herbs, **baños** (baths) and massaging with herbs, beef, eggs or newspaper conducted by a **curandero** or herbalist. The concept of what **chucaque** was, and how the condition was to be treated, very vague with varied answers. This condition often implies that the patients were ashamed (**tiene verguenza**) and included headaches and stomach problems. In addition, a person

who looked at another person in a negative way that could cause the victim to become ill. In most cases **chucaque** was to be cured with lime and salt, by pulling the hairs on the head to remove it, or by using eggs and newspaper, with herbs, and by **reventándolo** (cracking it out by cracking one's back).

Interviewees' understanding of **mal daño** was very basic. It was understood as an ailment that was caused by a person who envied another and wanted to harm that person, as well as a disease caused by sorcery practiced by **brujos** (sorcerers), whether they were hired by others or on their own. It had to be cured by going to the **curandero** or herbalist, and patients did not know how it was cured, other than that it was cured with herbs, baths and massage with herbs.

Another question addressed with what frequency patients used medicinal herbs or pharmaceuticals. Seven percent said they never used herbal medicines. These respondents were often those who mentioned that they were first-timers to the shop. Twenty-three percent of the informants said they used herbal medicines very little. Fifty-four percent said they used them moderately. Sixteen percent said they used them frequently.

In terms of how often interviewees used pharmaceutical medicines, seven percent said they never used them, 48% said they used them very little, 41% said they used them moderately, and 4% said they used them frequently. This indicated that the use of medicinal plants was more prevalent than the use of pharmaceutical medicines.

The participants were also asked what they use more frequently, originally with two choices: herbal medicines or pharmaceutical medicines. In the course of the study two more options ("both equally" and "none") were added. The results show that 48% used herbal medicines more often, 22% used pharmaceutical medicines more frequently, 28% used both equally, and 2% used neither. This is interesting in comparison to Bussmann *et al.* (2007) who found that over 50% of the respondents used pharmaceutical products more, 35% said they used medicinal plants more and 15% said they used both equally.

Patients who answered that they preferred medicinal plants indicated that they used them more because they were better and didn't have side effects. Interestingly, only five of the respondents said they used medicinal plants because they were less expensive. Pharmaceutical medicines were used because patients assumed they acted faster. Other reasons included that the informants had more faith in science and that they did not have enough knowledge about medicinal plants. For those patients using both equally, one of the most popular reasons was that, when medicinal plants did not work, pharmaceutical medicines were employed and *vice versa*. Herbs were used when patients said they only felt "sick" and pharmaceutical medicines when they felt "very sick."

Which medicinal plants were the most popular and how they were used was an important part of the study. Table 1 shows the 10 most commonly used medicinal plants mentioned.

It was obvious that each herb was used for many reasons. Table 2 gives an indication of each respondent's herb usage. Patients at Laboratorios Beal were also asked which pharmaceuticals they used for what reasons. Table 3 shows the ten most important pharmaceuticals used by the respondents. Interestingly pharmaceutical products were mostly used for pain relief and flu.

Another question asked if the respondent's parent knew more, less, or the same about the use of medicinal plants. Seventy-two percent said their parents knew more than

Table 1. Ten most frequently used medicinal plants based on number of respondents using plant in Trujillo, Peru.

Medicinal Plant (number of responses)	Uses
Manzanilla (35)	Stomach aches, tea, stomach problems, cold, asthma, clean eyes, fever, nervous system, pain relief
Cola de Caballo (22)	Inflammation, infection, kidneys, liver, stomach problems
Matico (22)	Flu, stomach aches, pain relief, cough, infection, cold, baths, stomach problems
Eucalipto (20)	Flu, bronchioles, cough, baths, cold, asthma, tea
Chanca Piedra (15)	Kidney, liver, renal problems, gastritis, tea, inflammation
Una de Gato (13)	Inflammation, injuries, kidneys, liver, tea, infection
Anis (12)	Stomachaches, gas, tea, pain relief, cold
Hierba Luisa (12)	Stomach problems, nervous system, tea, blood pressure, diarrhea (infants)
Sabila (11)	Inflammation, stomach problems, urinary tract, injuries, liver, bronchioles, cold, every ailment
Pie de Perro (11)	Inflammation, infection, kidney, ovaries

Table 2. Medicinal plants, uses and indication of relative importance based on number of positive responses reported by people visiting Laboratorios Beal in Trujillo, Peru. Number of positive responses from Bussman *et al.* 2007 from Clinica Anticona in El Porvenir settlement on the outskirts of Trujillo, Peru is provided for comparison. (1. No voucher specimens of plants were collected based upon the interviews so scientific taxa are identified as probable rather than confirmed. 2. Recently introduced plants.)

Patients citing plant	Importance	Importance Bussmann *et al.* 2007	Vernacular plant name (probable scientific taxa)[1]	Use
35	1	1	**Manzanilla** (*Matricaria recutita* L., Asteraceae)[2]	stomach problems, nervous system, pain relief, cold, tea, eye-wash, fever, asthma
22	2	3	**Cola de Caballo** (*Equisetum bogotense* Kunth, Equisetaceae)	infection, inflammation, kidneys, stomach problems
22	2	2	**Matico** (*Piper aduncum* L., Piperaceae)	infection, cough, cold, vaginal infections, stomach problems, flu, kidney infection
22	2	-	**Sauco** (*Sambucus peruviana* Kunth, Caprifoliaceae)	urinary tract problems
20	5	4	**Eucalipto** (*Eucalyptus globulus* Labill., Myrtaceae)[2]	flu, cold, cough, bronchial system, baths, tea, asthma
16	6	18	**Hierba Buena** (*Mentha spicata* L., Lamiaceae)[2]	stomach ache, diarrhea for babies, tea, parasites, allergies, respiratory problems, mucus
14	7	12	**Chanca Piedra** (*Phyllanthus niruri* L., *P. stipulatus* (Raf.) G.L. Webster, *P. urinaria* L., Euphorbiaceae)	kidneys, inflammation, liver
12	8	9	**Anis** (*Pimpinella anisum* L., Apiaceae)[2]	stomach ache, pain relief, cold, nervous system, tea, gas, stomach problems
12	8	13	**Uña de Gato** (*Uncaria tomentosa* (Willd. ex Roem. & Schult.) DC., Rubiaceae)	inflammation, kidney, liver, infections,
11	10	6	**Sabila** (*Aloe vera* (L.) Burm f., Asphodelaceae)	bronchial system, bruises, pain relief, inflammation, liver, cold, urinary tract problems, stomach problems, nervous system, injuries, "everything"
11	10	8	**Hierba Luisa** (*Cymbopogon citratus* (DC.) Stapf., Poaceae)[2]	nervous system, digestion, pain relief, cold, diarrhea of babies, tea, blood pressure, stomach problems
11	10	18	**Pie de Perro** (*Desmodium molliculum* (Kunth) DC., Fabaceae)	infection, inflammation, ovaries, kidneys
8	13	4	**Llanten** (*Plantago linearis* Kunth, Plantaginaceae)	infection, inflammation, pain relief, injuries, stomach ache
7	14	24	**Ajenco** (*Artemisia absinthium* L., Asteraceae)[2]	liver, stomach aches, inflammation, aire, cough
6	15	37	**Oregano** (*Origanum vulgare* L., Lamiaceae)[2]	stomach ache
6	15	37	**Tilo** (*Tilia platyphyllos* Scop., Tiliaceae)[2]	cough, cold, vaginal baths, nervous system
5	17	16	**Toronjil** (*Melissa officinalis* L., Lamiaceae)[2]	nervous system, heart problems, stomach problems, menstruation problems
5	17	9	**Boldo** (*Peumus boldus* Molina, Monimiaceae)	kidney, liver, infection, gastritis
5	17	24	**Molle** (*Schinus molle* L., Anacardiaceae)	Cold, pain relief, susto

Patients citing plant	Importance	Importance Bussmann et al. 2007	Vernacular plant name (probable scientific taxa)[1]	Use
4	21	-	**Cedron** (*Aloysia triphylla* Royle, Verbenaceae)	pain relief, cold, high blood pressure, nervous system, infection
4	21	24	**Apio** (*Apium graveolens* L., Apiaceae)[2]	stomach ache, menstruation problems, gas, digestion
4	21	28	**Coca** (*Erythroxylon coca* Lam., Erythroxylaceae)	liver, kidney, inflammation, flu, tea
4	21	-	**Trinidad** (*Maurla heterophylla* Kunth, Anacardiaceae)	infection
3	25	6	**Linaza** (*Linum sativum* L., Linaceae)[2]	stomach problems, fever, inflammation
3	25	-	**Culen** (*Otholobium glandulosum* (L.) J.W. Grimes, Fabaceae)	tea, cold, stomach problems
3	25	-	**Cerraja** (*Sonchus oleraceus* L., Asteraceae)[2]	stomach aches, nausea, pain relief, nervous system
2	28	37	**Talla, Tara** (*Caesalpinia spinosa* (Molina) Kuntze, Fabaceae)	infection
2	28	-	**Te de Indio** (*Capraria peruviana* Benth., Scrophulariaceae)	stomach problems, gastritis
2	28	24	**Hierba Santa** (*Cestrum auriculatum* L'Hér., Solanaceae)	baths, recogido
2	28	22	**Paico** (*Chenopodium ambrosioides* L., Chenopodiaceae)	diarrhea for babies, stomach aches
2	28	37	**Flor de Overo** (*Cordia lutea* Lam., Boraginaceae)	liver, infection
2	28	-	**Hierba de Toro** (*Cuphea strigulosa* Kunth, Lythraceae)	infection
2	28	-	**Grama Dulce** (*Cynodon dactylon* (L.) Pers., Poaceae)[2]	urinary tract problems, infections
2	28	9	**Cebada** (*Hordeum vulgare* L., Poaceae)[2]	inflammation, stomach problems
2	28	-	**Escorcionera** (*Perezia multiflora* (Bonpl.) Less., Asteraceae)	cough, flu
2	28	-	**Romero** (*Rosmarinus officinalis* L., Lamiaceae)[2]	stomach aches, infection
2	28	-	**Pimpinela** (*Sanguisorba minor* Scop., Rosaceae)	heart problems
2	28	22	**Panisara** (*Satureja pulchella* (Kunth) Briq., Lamiaceae)	tea
2	28	-	**Sen** (*Senna monilifera* H.S. Irwin & Barneby, Fabaceae)	stomach problems
2	28	24	**Flor de Arena** (*Tiquilia paronychioides* (Phil.) A.T. Richardson, Boraginaceae)	kidney, ovaries, gastritis, ulcers
1	42	37	**Culantrillo** (*Adiantum concinnum* Humb. & Bonpl. ex Willd., Adiantaceae)	stomach aches
1	42	37	**Malva Blanca** (*Alcea rosea* L., Malvaeae)	inflammation, baths
1	42	-	**Lancetilla** (*Alternanthera porrigens* (Jacq.) Kuntze, Amaranthaceae)	blood pressure
1	42	-	**Altamisa** (*Ambrosia peruviana* Willd., Asteraceae)	cold
1	42	37	**Guanabana** (*Annona muricata* L., Annonaceae)	inflammation
1	42	-	**Purunrosa** (*Bejaria aestuans* Mutis ex L., Ericaceae)	infection
1	42	-	**Achiote** (*Bixa orellana* L., Bixaceae)	prostate

Patients citing plant	Importance	Importance Bussmann *et al.* 2007	Vernacular plant name (probable scientific taxa)[1]	Use
1	42	-	**Borraja** (*Borago officinalis* L., Boraginaceae)[2]	cold
1	42	13	**Flor Blanca** (*Buddleja utilis* Kraenzl., Loganiaceae)	inflammation
1	42	-	**Manayupa** (*Desmodium molliculum* (Kunth) DC., Fabaceae)	cholesterol
1	42	37	**Papa Semitona** (*Dioscorea tambillensis* R. Knuth, Dioscoreaceae)	infection
1	42	-	**Hinojo** (*Foeniculum vulgare* Mill., Apiaceae)[2]	stomach problems
1	42	-	**Macacha** (*Galvesia fruticosa* Gmel., Scrophulariaceae)	susto
1	42	-	**Hercampuri** (*Gentianella alborosea* (Gilg) Fabris, Gentianaceae)	blood pressure
1	42	-	**Polen** (Insect feces)	stomach aches
1	42	-	**Nogal** (*Juglans neotropica* Diels, Juglandaceae)	cough
1	42	-	**Maca** (*Lepidium meyenii* Walp., Apiaceae)	calcium, strength
1	42	-	Alfalfa (*Medicago sativa* L., Lamiaceae)[2]	head aches
1	42	18	**Poleo** (*Mentha x piperita* L., Lamiaceae)[2]	for eyes
1	42	37	**Mufia** (*Minthostachys mollis* (Kunth) Griseb., Lamiaceae)	digestion
1	42	-	**Albaca** (*Ocimum basilicum* L., Lamiaceae)[2]	stomach aches, cough, diarrhea of babies
1	42	37	**Congona** (*Peperomia inaequalifolia* Ruiz & Pav., Piperaceae)	nervous system
1	42	37	**Pepa Palta** (*Persea americana* Mill., Lauraceae)	diarrhea for babies
1	42	16	**Valeriana** (*Phyllactis rigida* (Ruiz & Pav.) Pers., Valerianaceae)	nervous system
1	42	18	**Chicoria** (*Picrosia longifolia* D. Don, Asteraceae)	dengue fever
1	42	-	**Calahuala** (*Polypodium crassifolium* L., Polypodiaceae)	infection
1	42		**Ruda** (*Ruta graveolens* L., Rutaceae)[2]	susto
1	42	37	**Agua de Papa** (*Solanum tuberosum* L., Solanaceae)	kidney
1	42	-	**Flor de Trebol** (*Trifolium repens* L., Fabaceae)[2]	kidney
1	42	15	**Ortiga Negra** (*Urtica magellanica* Juss. ex Poir., Urticaceae)	blood circulation
1	42	-	**Agua de Chocio** (*Zea mays* L., Poaceae)	kidney

Table 3. The ten pharmaceutical medicines used most frequently in Trujillo, Peru.

Acetaminophen (Panadol ®)(23)	Flu, headache, fever, pain relief, menstruation
Acetaminophen (Paracetamol ®)(23)	Headache, fever, pain relief
Amoxicillin (Amoxicilina)(15)	Infection, pain relief, inflammation, respiratory problems, cough
Altanjina (13)	Headache, fever, blood pressure
Naproxen (Apronax ®)(11)	Headache, inflammation, pain relief,
Ibuprofen (Ibuprofeno)(11)	Pain relief, headache, inflammation
Aspirin (Aspirina) (8)	Heart problems, headache, pain relief, flu, fever
Dolocontrolan ® (6)	Pain relief, infection, gastritis
Aspirin/Acetaminophen (Mejoral ®)(6)	Fever, flu, headache, pain relief
Doloflan ® (6)	Pain relief, inflammation, infection, headache

they did, 7% said they knew less, 11% said they shared the same amount of knowledge, and 10% could not answer the question because they either didn't know their parents, or their parents were dead and they did not remember. Fifty-one percent of the respondents with children said they did teach them about medicinal plants, and 12% said they did not. The question couldn't be applied to 12% of those informants with children because 9% had children that were too young and 3% did not see their children. Of the 12% who answered that they did not teach their children, the reasons given were very diverse. Some stated that they did not have enough knowledge about herbs themselves to teach their children, that the children were not interested in knowing, or that there was no time to teach them. Those who did teach their children about the use of medicinal plants had many reasons as to why they did so. One of the most popular reasons to teach the children was that the respondents wanted their children to have knowledge of how to cure themselves when they were ill. Another popular reason was that they thought herbs were better and healthier.

Discussion and Conclusion

It was no surprise to find that patients at an herbalist's shop used medicinal herbs more frequently than pharmaceutical medicines. People generally assumed that plants are healthier and better to use because they are natural and are thought to not have any side-effects. It is difficult to determine if the knowledge of the use of medicinal plants is growing or decreasing, but the indications are that the last generation knows more than the present. However, most of the present generation does teach their children about the use of medicinal plants. The present study also showed what medicinal plants the respondents used for specific purposes. It would be interesting to evaluate with bioassays the properties of the species used.

It is apparent that the respondents used medicinal herbs more often than pharmaceutical medicines, but only to a small degree. Bussmann *et al.* (2007) showed in their study at a private Western clinic that patients had a preference for pharmaceutical medicines, but also only to a small degree. Similarly, the plant knowledge of patients at both facilities was largely identical, with an essentially overlapping selection of common, mostly introduced, species, and basically the same number of medicinal plants mentioned overall. This indicates that traditional medicinal knowledge is a major part of a people's culture that is being maintained while patients are also embracing the benefits of western medicine.

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Literature Cited

Alves, R.R.N. & R.M.L. Lerecê. 2007. Biodiversity, traditional medicine and public health: Where do they meet? *Journal of Ethnobiology and Ethnomedicine* 3:14.

Bussmann, R.W. & D. Sharon. 2006a. Traditional plant use in Northern Peru: Tracking two thousand years of health culture. *Journal of Ethnobiology and Ethnomedicine* 2:47.

Bussmann, R.W. & D. Sharon. 2006b. Traditional plant use in Southern Ecuador. *Journal of Ethnobiology and Ethnomedicine* 2:44.

Bussmann, R.W. & D. Sharon. 2007a. *Plants of the four winds - The magic and medicinal flora of Peru. Plantas de los cuatro vientos - La flora mágica y medicinal del Perú.* Graficart, Trujillo.

Bussmann, R.W. & D. Sharon. 2007b. *Plants of longevity - The medicinal flora of Vilcabamba. Plantas de longevidad - La flora medicinal de Vilcabamba.* Graficart, Trujillo.

Bussmann, R.W., D. Sharon & A. Lopez. 2007. Blending traditional and western medicine: Medicinal plant use among patients at Clinica Anticona in El Porvenir, Peru. *Ethnobotany Research and Applications* 5:185-199.

Eisenberg, D.M., R.B. Davis, S.L. Ettner, S. Appel, S. Wilkey, M. Van Rompay & R.C. Kessler. 1998. Trends in alternative medicine use in the United States, 1990-1997: Results of a follow-up national survey. *Journal of the American Medical Association* 280:1569-1575.

EsSalud/Organización Panamericana de Salud. 2000. *Estudio Costo-Efectividad: Programa Nacional de Medicina Complementaria. Seguro Social de EsSalud (Study of Cost-Effectiveness: National Program in Complementary Medicine. Social Security of EsSalud).* EsSalud/Organización Panamericana de Salud, Lima.

Glass-Coffin, B., D. Sharon & S. Uceda. 2004. Curanderas a la sombra de la Huaca de la luna. *Bulletin de l'Institut Français d'Études Andines* 33:81-95.

Mignone, J., J. Bartlett, J. O'Neil & T. Orchard. 2007. Best practices in intercultural health: Five case studies in Latin America. *Journal of Ethnobiology and Ethnomedicine* 3:31.

Sharon, D. & R.W. Bussmann. 2005. Plantas medicinales en la Obra del Obispo Don Baltasar Jaime Martínez Compagñon (Siglo XVIII). Pp. 147-165 in *Desde el exterior: El Perú y sus estudios.* Edited by L. Millones & T. Kato. Tercer Congreso Internacional de Peruanistas, Nagoya, 2005. Universidad Nacional Mayor de San Marcos, Lima.

Sharon, D. & R.W. Bussmann. 2006. Avances de la etnobotánica en Trujillo, Peru: El programa MHIRT. *Arnaldoa* 13(2):398-406.

Sharon, D. 2000. *Shamanismo y el Cacto Sagrado - Shamanism and the Sacred Cactus.* San Diego Museum Papers 37.

United Nations Conference on Trade and Development. 2000. *Systems and National Experiences for Protecting Traditional Knowledge, Innovations and Practices. Background Note by the UNCTAD Secretariat.* United Nations Conference on Trade and Development, Geneva. (document reference TD/B/COM.1/EM.13/2).

World Health Organization. 1999. *Consultation Meeting on Traditional Medicine and Modern Medicine: Harmonizing the Two Approaches.* World Health Organization, Geneva. (document reference (WP)TM/ICP/TM/001/RB/98–RS/99/GE/32(CHN)).

World Health Organization. 2002. *WHO Traditional Medicine Strategy 2002–2005.* World Health Organization, Geneva.

Reference # 4

Evaluación Toxicológica de la Fórmula A4+

LABORATORIO DE INVESTIGACIÓN DE PRODUCTOS NATURALES ANTIPARASITARIOS DE LA AMAZONÍA – LIPNAA

UNIVERSIDAD NACIONAL DE AMAZONIA PERUANA – UNAP

SABELL

Proyecto "Evaluación del Potencial Toxicológico del Extracto Liofilizado de la Fórmula A4+, en roedores"

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TOXICOLOGICAL EVALUATION: LIMIT DOSE METHOD

PRESENTATION

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The toxicity of substances can only be identified through two methods (besides theoretical previsions): retrospective studies of intoxication cases and experimental tests using animals and plants.

The use of experimental animals (a method that ancient pharmacologists and toxicologists had already used) was systematised by Trevan (1927) to determine the dose of a substance that could be lethal when administered orally.

Over time, increasing social awareness and pressure from animal protection organisations led to a reduction in the number of experimental animals and changes in the testing techniques, so that animals suffer less, and more information can be obtained through fewer experiments. In addition, experimental animals have started to be replaced by what is called Alternative Methods.

A full toxicological evaluation requires that a substance be tested for toxicity through different methods.

Some methods are performed over a short period and are used to detect the general toxicity using experimental animals. Other methods are long-termed and are aimed to assess the effects of a substance when animals are exposed to it for an extended period of their lives.

INTRODUCTION

A toxic substance usually can enter the body through inhalation, ingestion or dermal contact. It can cause damage to the specific part of the body that was in contact with it, or can be absorbed, transported and spread through the blood to different organs.

The type and intensity of any harmful effects would depend on its concentration, its chemical composition, its physical shape, and how long it remains in an organ.

Possible consequences of the exposure to toxic substances include a wide range of acute, chronic, reversible and irreversible effects.

They can go from the slightest and most common effects such as skin and mucous membrane irritation, to effects in the immunological or endocrine system; hepatic, renal or neurological damage; reproductive changes, congenital malformations, hereditary changes or more harmful diseases such as cancer.

This manual has been designed to carry out experimental procedures using laboratory animals (rodents) to determine whether a substance can be classified as toxic or non-toxic at a specific dose according to a specific test.

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I. TOXICOLOGICAL DEFINITIONS

1.1 DEFINITION AND SCOPE OF TOXICOLOGY

Toxicology can be defined as the science that studies poisons or toxic substances, as well as their detection, effects and antidotes, or, as defined by the World Health Organisation, it is the "discipline that studies the harmful effects of chemical or physical agents (toxic agents) on biological systems, and determines the extent of the damage based on the exposure of live organisms to such agents. It studies the nature and the mechanics of the effects and evaluates the different biological changes caused by the harmful agents". [1]

Given the increasing use of chemicals for multiple purposes and the presence of toxic and chemical pollutants in the air, water, food and other elements in the environment, this discipline has been divided into the following areas: [2]

1.1.2 FORENSIC TOXICOLOGY

This area of specialises in the use of toxicological knowledge to support pathological studies and forensic medicine to determine the causes of death as well as for medical and legal purposes in a situation where a crime is suspected to have occurred.

1.1.3 CLINICAL TOXICOLOGY

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It studies the expected or unusual effects of a therapeutic drug administered to patients to treat their ailments or diseases, while monitoring their condition as well as any improvement that may result from the use of these substances.

1.1.4 OCCUPATIONAL TOXICOLOGY

During the second half of the nineteenth century and throughout the twentieth century, studies on occupational activities in certain industries showed that serious diseases and deaths were caused by exposure to dangerous chemicals and toxic agents in unsafe working conditions. In this field Occupational Toxicology studies the harmful effects that pollutants in the working environment have on the worker's health.

1.1.5 ENVIRONMENTAL TOXICOLOGY

Environmental Toxicology deals with the harmful effects of the chemical substances and toxic agents that are present in the air, water, soil, food and other environmental elements, to which humans, pets, fish, wild life and other elements of the biota are exposed. In other words, it studies the harmful effects that environmental agents have on living organisms.

1.2. TYPES OF TOXICITY

Toxicity is the property of a chemical molecule or compound that enables it to cause damage or a harmful effect on living organisms. These harmful effects can be caused

by the specific circumstances of the exposure. For instance, part of the toxicity may depend on the time during which the living organism was exposed to the substance.[3]

1.2.1 ACUTE EXPOSURE

It is a brief exposure during which the chemical or physical agent is quickly absorbed, either through one or more doses, in a period of 24 hours or less. The effects are immediate.

1.2.2 SUBACUTE EXPOSURE

It results from frequent or repeated exposures over several days or weeks. The effects are relatively lagged.

1.2.3 CHRONICAL EXPOSURE

It results from repeated exposure at low doses over a long period. The effects are caused by the accumulation of a toxic agent in the organs because the amount of the substance that is released is less than the amount that is absorbed, or because the effects that result from repeated exposures are added up. Toxicity can also be based on the specific area where the substance acts.

1.2.3.1 LOCAL EFFECTS

It refers to the specific organ or area of the organism that was in contact with the substance. The area may be the skin, the mucous membrane in the eyes, nose or mouth, or any other part of the respiratory or gastrointestinal system.

1.2.3.2 SYSTEMIC EFFECTS

These are effects on a specific area where the agent acts but is not related to the area of contact, thus showing that the absorption process has been completed. This means that the toxic substance has been absorbed and spread through the blood and is lodged in a target organ, or that is showing its effects in the entire organism.

1.3. ROUTES OF ABSORPTION

The route through which a toxic element gets in contact with the individual is a very important factor in the effects of a substance. The following are the most common routes of absorption: (4)

1.3.1. SKIN

The skin is usually an effective barrier, but many substances can be absorbed through it, especially if it has excoriations or lesions which could be easily penetrated. When a substance is in contact with the epidermis, the skin may work effectively as a barrier, or it may cause irritation or sensitivity in the area of contact and eventually penetrate into the blood stream.

1.3.2. GASTROINTESTINAL

The substance can enter orally (as it is also called) through the ingestion of water, contaminated food, or the toxic substance alone. The absorption of the

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substance inside the gastrointestinal system may be very quick or very slow depending on the substance's intrinsic characteristics.

1.3.3. RESPIRATORY

This is the most common type of exposure in the working environment, especially in factories and manufacturing activities due to the continuous use of volatile substances and/or the presence of corpuscles. One thing that is relevant about this type of absorption is that it can spread locally and damage a specific area, or it can enter directly into the blood stream. However, we must take into account that the factors that determine a reaction to a toxic agent depend on the agent, the recipient and the environment. The factors that should be taken into consideration include the recipient's genetic structure, nutritional state, sex, age and emotional state; the environmental temperature and the oxygen's partial pressure; and the agent's characteristics, such as its structure and chemical composition, the size of its particles, its amount and its concentration level.

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II. TOXICOLOGICAL EVALUATION

2.1. TOXICOLOGICAL EVALUATION USING THE ACUTE TOXIC CLASS TEST (ATC)

SUMMARY

Acute Oral Toxicity was analysed in order to asses the potential acute toxicity of the freeze-dried extract of the A4+ Formula , using the ACUTE TOXIC CLASS (ATC) METHID. This method followed the OECD norms.

To conduct the ATC test, we used Holtzman albino rats *Rattus norvegicus* of both sexes, with an average weight of 120 g. to 160 g. The extract of the A4+ Formula was

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administered orally at a maximum dose of 2000 mg/kg^{-1} of the b.w. using an intragastric cannula; a control group was set up, and was administered a saline solution.

The animals were observed for 14 days after the administration to detect any signs of toxicity; their weights were recorded, and eventually, a necropsy was conducted to perform a macroscopic study of their organs.

The results obtained showed no clinical signs of toxicity or macroscopic effects on the organs studied. Since no mortality was observed at the aforementioned dose, the freeze-dried extract of the A4+ Formula showed a result of ATC 0 and / or NOT CLASSIFICABLE, using the ACUTE TOXIC CLASS METHOD.

Keywords: *A4+ Formula, Acute Toxicity, Acute Toxicity Class*

INTRODUCTION

The Acute Toxic Class method used in this test is a procedure performed in stages, where three animals of the same sex are used in each stage. The procedure takes three

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or four stages to determine the acute toxicity of the substance studied, depending on the mortality and/or the agony symptoms shown by the animals [6].

This procedure can be reproduced, it requires few animals, and it allows us to classify substances in a way that is very similar to other acute toxicity test methods. The Acute Toxicity Class method is based on biometric evaluations [7] [8] [9] [10] with fixed doses, separated in such a way that a substance can be sorted and classified, so that its risk can be evaluated.

It is not necessary to administer doses that are known to cause pain or suffering due to its highly irritating or corrosive effects. Those animals that are dying or show clear signs of pain or deep and continuous suffering are scarified humanly and the interpretation of their results are considered in the same way as those obtained from animals that die during the test.

In principle, this method does not intend to determine a precise LD50, but to provide several levels of exposure in which lethality could be observed, since the death of a certain number of animals is still its main parameter. This method allows us to determine the LD50 only when at least two doses cause a mortality level of more than 0% and less than 100%. The use of doses that have been previously set (regardless of the substance tested) and the explicit relationship between the classification and the number of animals observed in different states improve the consistency in the information provided by different laboratories and the repetitions.

2.1.1 OVERVIEW

Research Centre : Laboratorio de Productos Naturales Antimaláricos de la Amazonía - LIPNAA

Address : AA.HH. Nuevo San Lorenzo "Pasaje Los Paujiles" S/N – San Juan

Area : Toxicology

2.1.2 TESTED SUBSTANCE

CODE : Freeze-dried extract of the A4+ Formula

2.1.3 METHOD USED

Acute Toxic Class (ATC)

2.1.4 EXPERIMENTAL SYSTEM

Species : Rattus Novergicus Rats

Inbred : Holtzman

Experimental Groups : 2 (Treated, Control)

Number of animals : 3 animals per experimental group

Sex : Male and Female

Body weight : 120-160g.

Lodging : Controlled temperature 23±2°C, Light/Dark Cycles: 12-12 hours; layer of sterilised shavings on the base changed every 48 hours.

Diet : Food and water ad-libitum.

2.1.5 EQUIPMENT AND MATERIALS

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- Intragastric Catheter
- Surgical Gloves
- Beaker - 25 ml.
- Graduated Cylinder - 20 ml.
- Stirring Rod or Spatula
- Analytical Scale
- Saline Solution
- Picric Acid
- Clean Cages
- Sterilised Shavings

2.1.6 ROUTE OF ADMINISTRATION

The extract of the A4+ Formula and the saline solution are administered orally using an intragastric catheter.

2.1.7 DOSE

GRUPO I (TREATED): These animals are administered the extract of the medicine plant at a dose of 2,000 mg/kg^{-1} of the body weight (b.w.).

GROUP II (CONTROL): These animals are administered a solvent or saline solution (same volume as the extract's)

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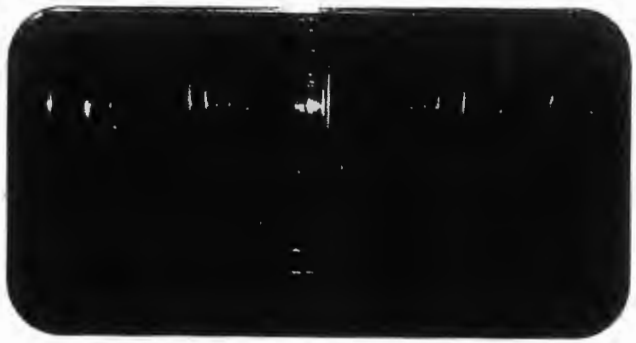
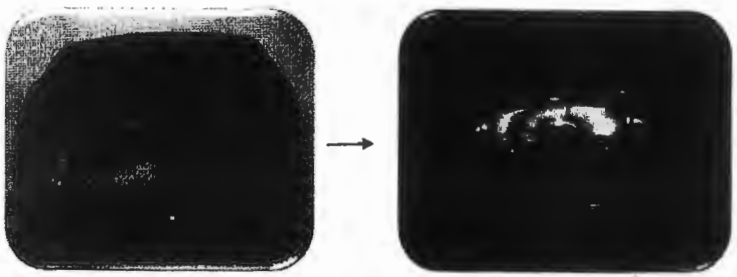
EXPERIMENTAL PROCEDURE

1. This evaluation requires rats of both sexes, which undergo a week-long quarantine.



2. Two study groups are formed by randomly choosing three animals per group/sex, which are weighed and marked for identification purposes.



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3. Before the evaluation, the animals undergo a fasting period of 12 hours; then, the extract of the plant and the control substance are administered to both experimental groups (treated and control respectively) according to the Dose Table. (ANNEX N° 01)

4. The specified doses of the extract of the plant and the control substance are administered orally using an intragastric catheter.

5. The time of administration is immediately recorded and the animals are observed to detect any toxic signs at system or organ level: Autonomous, behaviour, sensory, neuromuscular, respiratory, ocular, gastrointestinal, urinary, and others such as body weight (Annex N°02).

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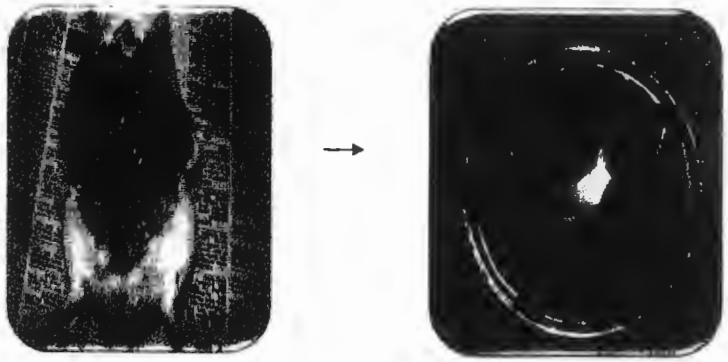
6. The body weight of the animals is recorded on the 7th and 14th day after the substances are administered.

7. At the end of the 14-day period of evaluation, the animals are sacrificed taking into account the ethical principles on experiments with animals.

8. Then, a necropsy and a macroscopic study are performed to make an assessment of the following organs: the heart, kidneys, liver, spleen, stomach, lungs, and brain. The macroscopic study takes into account the relative weights, size, colour, and consistency of the organs.



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2.1.9 RESULTS

a). Clinical Toxic Signs

During the assessment period, no mortality or clinical signs of toxicity were observed among the animals in any of the sexes, neither during the first 24 hours nor over the 14-day period after the administration of the substance. This applies to both groups, the one with the animals that received the freeze-dried extract of the A4+ FORMULA at a dose of 2,000 mg/kg^{-1} of the body weight (b.w.), and the control group that was administered a saline solution.

b). Body Weight

Body weight records of male and female albino rats show that none of them lost weight, and that in fact weight gain was a constant during the study in both groups (the one with the animals that received the freeze-dried extract of the A4+ Formula at a dose of 2,000 mg/kg^{-1} of the b.w., and the control group that was administered a saline solution) (TABLES N° 01 and 02).

c). Macroscopic Evaluation of the Organs

The necropsy showed no evidence of macroscopic effects regarding the colour, consistency and size of the organs studied, such as the brain, heart, lungs, liver, kidneys, spleen, stomach, testicles and ovaries in any of the experimental groups (TABLES N° 03 and 04).

d). Organ Weight

The average weight of the albino rats' organs (both sexes) in both groups (the one treated with the freeze-dried extract of the A4+ Formula and the group that received the saline solution) showed no significant statistical differences (TABLES N° 05 and 06).

RESULT TABLES

TABLE N° 01

Average Body Weight - Male Albino Rats Treated with a Dose of 2,000 mg/kg^{-1} of the A4+ Formula and Those that Were Administered a Saline Solution

Treated ♂	145.6 ± 2.82	158.2 ± 5.43	170.37 ± 6.19
Control ♂	151.3 ± 0.7	165.93 ± 3.82	172.37 ± 6.05

X±SD = Mean and Standard Deviation

TABLE N° 02

Average Body Weight - Female Albino Rats Treated with a Dose of 2,000 mg/kg^{-1} of the A4+ Formula and Those that Were Administered a Saline Solution

Treated ♀	171.63 ± 1.15	172.5 ± 1.26	174.67 ± 3.50
Control ♀	163.2 ± 11.5	164.33 ± 9.18	166.93 ± 9.19

X±SD = Mean and Standard Deviation

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TABLE N° 03

Macroscopic Observation - Organs of Male Albino Rats Treated with the A4+ Formula at a Dose of 2,000 mg/kg^{-1}

ORGANS	NEGATIVE CONTROL	EXTRACT OF THE A4+ FORMULA AT A DOSE OF 2,000mg/Kg
BRAIN	Colour : cream Size : normal Consistency : tender	Colour : cream Size : normal Consistency : tender
HEART	Colour : greyish red Size : normal Consistency : semi-hard and firm texture	Colour : greyish red Size : normal Consistency : semi-hard and firm texture
LUNGS	Colour : creamy pink Size : normal Consistency : gelatinous, tender	Colour : creamy pink Size : normal Consistency : gelatinous, tender
LIVER	Colour : homogeneous vinous red Size : normal Consistency : soft, firm texture, smooth contour	Colour : homogeneous vinous red Size : normal Consistency : soft, firm texture, smooth contour
SPLEEN	Colour : homogeneous vinous red Size : normal Consistency : firm texture, shapely	Colour : homogeneous vinous red Size : normal Consistency : firm texture, shapely
KIDNEYS	Colour : homogeneous greyish red Size : normal Consistency : soft, firm texture	Colour : homogeneous greyish red Size : normal Consistency : soft, firm texture
STOMACH	Colour : cream Size : normal Consistency : soft, elastic texture	Colour : cream Size : normal Consistency : soft, elastic texture
TESTICLES	Colour : homogeneous cream Size : normal Consistency : soft, smooth contour	Colour : homogeneous cream Size : normal Consistency : soft, smooth contour

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TABLE N° 04

Macroscopic Observation - Main Organs of Female Albino Rats Treated with A4+ at a Dose of 2,000 mg/Kg^{-1}

ORGANS	NEGATIVE CONTROL		EXTRACT OF THE FORMULA A4+ DOSE OF 2,000mg/Kg	
BRAIN	Colour	: cream	Colour	: cream
	Size	: normal	Size	: normal
	Consistency	: tender	Consistency	: tender
HEART	Colour	: greyish red	Colour	: greyish red
	Size	: normal	Size	: normal
	Consistency	: semi-hard and firm texture	Consistency	: semi-hard and firm texture
LUNGS	Colour	: creamy pink	Colour	: creamy pink
	Size	: normal	Size	: normal
	Consistency	: gelatinous, tender	Consistency	: gelatinous, tender
LIVER	Colour	: homogeneous vinous red	Colour	: homogeneous vinous red
	Size	: normal	Size	: normal
	Consistency	: soft, firm texture, smooth contour	Consistency	: soft, firm texture, smooth contour
SPLEEN	Colour	: homogeneous vinous red	Colour	: homogeneous vinous red
	Size	: normal	Size	: normal
	Consistency	: firm texture, shapely	Consistency	: firm texture, shapely
KIDNEYS	Colour	: homogeneous greyish red	Colour	: homogeneous greyish red
	Size	: normal	Size	: normal
	Consistency	: soft, firm texture	Consistency	: soft, firm texture
STOMACH	Colour	: cream	Colour	: cream
	Size	: normal	Size	: normal
	Consistency	: soft, elastic texture	Consistency	: soft, elastic texture
OVARIES	Colour	: homogeneous cream	Colour	: homogeneous cream
	Size	: normal	Size	: normal
	Consistency	: soft, corrugated contour	Consistency	: soft, corrugated contour

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TABLE Nº 05

Average Organ Weight - Group of Animals Treated with the A4+ Formula at a Dose of 2,000 mg/Kg^{-1}

Organs	Male (X±SD)	Female (X±SD)
Liver	6.17 ± 0.37	5.81 ± 0.46
Brain	1.29 ± 0.11	1.13 ± 0.08
Heart	0.65 ± 0.05	0.60 ± 0.05
Stomach	0.91 ± 0.04	0.96 ± 0.08
Spleen	0.35 ± 0.03	0.39 ± 0.04
Lung	1.07 ± 0.09	0.93 ± 0.12
Right Kidney	0.54 ± 0.06	0.56 ± 0.08
Left Kidney	0.52 ± 0.05	0.54 ± 0.06
Left Testicle	1.72 ± 0.67	-
Right Testicle	1.25± 0.53	-
Ovaries	-	0.45 ± 0.14

X±SD = Mean and Standard Deviation

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TABLE Nº 06

Average Organ Weight - Control Group of Animals that Were Administered a Saline Solution

Organ	Male X±SD	Female X±SD
Liver	7.80 ± 0.66	6.27 ± 0.56
Brain	1.26 ± 0.20	1.38 ± 0.08
Heart	0.77 ± 0.13	0.7 ± 0.003
Stomach	1.12 ± 0.11	1.19 ± 0.08
Spleen	0.45 ± 0.01	0.54 ± 0.05
Lung	1.52 ± 0.20	1.54 ± 0.07
Right Kidney	0.75 ± 0.05	0.77 ± 0.01
Left Kidney	0.69 ± 0.03	0.68 ± 0.04
Right Testicle	1.99 ± 0.33	-
Left Testicle	1.91 ± 0.41	-
Ovaries	-	1.20 ± 0.63

X±SD = Mean and Standard Deviation

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DISCUSSION

* The toxicological results obtained through these studies showed the non-toxicity of the A4+ at a dose of 2000mg. Kg^{-1} of the b.w., since no clinical signs of toxicity or mortality were found using the ATC test.

* When the consistency and colour of the targeted organs were compared to those of the rats that were administered NaCl 0.9% , no significant differences were found that suggested any toxicity of the substance studied.

* While over the testing period there was an increase in the body weight of the animals in both experimental groups, this increase was not significant, thus suggesting that the substance under study does not produce any deleterious effect, and thus no toxic effect.

CONCLUSIONS

* According to the ACUTE TOXIC CLASS (ATC) Method, the freeze-dried extract of the A4+ Formula did not cause any deaths when administered orally at a dose of 2000 mg/kg^{-1}.

* The freeze-dried extract of the A4+ Formula is classified as ATC. 0 and /or NOT CLASSIFIABLE, through the ACUTE TOXIC CLASS (ATC) Method.

2.2. TOXICOLOGICAL EVALUATION: LIMIT DOSE METHOD

SUMMARY

Acute Oral Toxicity was analysed in order to assess the potential acute toxicity of the freeze-dried extract of the A4+ Formula , using the LIMIT DOSE (LD) METHOD. This method followed the OECD norms.

To conduct the Limit Dose test, we used Balb/C/CNPB albino mice *Mus Musculus* of both sexes, with an average weight of 20 g. to 25 g. The extract of the A4+ Formula was administered orally at a maximum dose of 2000 mg/kg^{-1} of the b.w. using an intragastric cannula; a control group was set up, and was administered a saline solution.

The animals were observed for 14 days after the administration to detect any signs of toxicity; their weights were recorded, and eventually, a necropsy was conducted to perform a macroscopic study of their organs.

The results obtained showed no clinical signs of toxicity or macroscopic effects in the organs studied. Since no mortality was observed at the aforementioned dose, the freeze-dried extract of the A4+ Formula showed a result of NON-TOXIC using the LIMIT DOSE METHOD.

Keywords: *A4+ Formula, Acute Toxicity, Limit Dose*

INTRODUCTION

Acute Toxicity studies are usually designed to show the potential toxicity in terms of LD_{50} or LC_{50} (Median Lethal Dose or Median Lethal Concentration). This value represents the estimated dose that causes death to 50% of a population exposed to a certain substance under testing conditions. [6]

It is important to note that the Acute Toxicity test results are not a synonym of mortality among animals exposed to a substance. More information must be obtained through a properly designed acute toxicity study in order to provide a quick assessment of lethality, and properly identify signs and symptoms in case of lagged toxicity, as well as possible effects on the organs. Male, female, young and adult animals undergo the test to make an assessment taking into account the differences in susceptibility. The Limit Dose Test is used when there is information that suggests that the substance is probably non-toxic or that is toxic only if administered at a dose above a specified Limit Dose.[7,8]

Information about the toxicity of the tested substance can be obtained from similar tested compounds, products, mixes or products, as long as the important components (from the toxicological point of view) and their percentage in the substance are identified.

If there is little or no information about the toxicity of the substance, or if we foresee that it is toxic, the main test is carried out.

A Limit Dose Test can be performed with a single dose of 2,000 mg/kg[1] of the body weight applied to six animals (three per stage). If there is mortality due to the substance, a complementary test at a lower level may be necessary.

2.2.1 OVERVIEW

Research Centre	:	Laboratorio de Productos Naturales Antimaláricos de la Amazonía-LIPNAA
Address	:	AA.HH. Nuevo San Lorenzo "Pasaje Los Paujiles" S/N – San Juan
Area	:	Toxicology

2.2.2 TESTED SUBSTANCE

CODE : Freeze-dried extract of the A4+ Formula

2.2.3 METHOD USED

Limit Dose Method

2.2.4 EXPERIMENTAL SYSTEM

Species : Albino mice *Mus musculus*
Balb/C/CNPB inbred
Albino Rats *Rattus norvegicus*
Holtzmann inbred

Number of Animals :10 animals per sex and per dose group
The size of the groups guarantees that all possible effects can be observed over the evaluation period.

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Sex	: Male and Female.
Body Weight	: Mice - From 20 g to 25 g.
	Rats - From 150 g to 200 g.
Lodging	: Cage with a layer of shavings on the base
Diet	: Pelleted feed for mice

Environment:

Room Temperature	:	22 °C + 3 °C
Relative Humidity	:	30 % to 70%
Light/Dark Cycle	:	12 H and 12 H
Exposure to light	:	Position of the cages was changed every 3 days, so that all cages are equally exposed to light.

2.2.5 QUARANTINE

A 7-day period, in which animals are subject to an adaptation process, and to perform a control in aspects such as:

- Parasitological
- Hematological, hemoglobin, hematocrit, leukocyte formula and count.

2.2.6 ROUTE OF ADMINISTRATION

The substance is administered orally as long as it is technically possible to use that route when administered to humans; otherwise, other routes may be considered, such as intraperitoneal, endovenous or dermal, among others.

Maximum volume to be administered:

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Mouse: 1 ml / 20 g of the body weight

Rat: 3 ml/100 g of the body weight

2.2.7 DOSIFICATION

The products to be evaluated in this Protocol will have a maximum dose of 2,000 mg/kg (According to the Dose Table).

Inoculation Volume Factor: 0.02 ml/ of the animal's body mass

Concentration: 10 %.

2.2.8 EXPERIMENTAL PROCEDURE

1. This evaluation requires rats of both sexes, which undergo a week-long quarantine.

2. Two study groups are formed by randomly choosing three animals per group/sex, which are weighed and marked for identification purposes.

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3. The specified dose of the extract of the plant and the saline solution are administered orally through a solution or suspension using an intragastric catheter. The mice undergo a fasting period of 4 hours previous to the experiment. After the administration of the substance, they are put under continuous observation for toxicity symptoms for 4 hours.

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If animals die within 24 hours after been administered the extract, a lower dosage level is then considered.

If there is no mortality, the observation period is extended to 14 days and then to 21 days, in order to observe the reversibility and recovery of the animals during the test period.

In order to determine if there was a gain or loss of weight, the weights are recorded at the beginning of the observation period as well as on the 7th, 14th and if possible, the 21st day of the observation period.

4. At the end of the experiment, the animals are sacrificed using the cervical dislocation method, which consists on separating the cranium from the cervical spine by applying pressure to the base of the cranium and the cervical spine. By doing this, there is no sensibility to pain, since the spinal is separated from the brain.

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5. A necropsy is performed on all the animals that survived until the end of the experiment and those that died during the experiment. The colour, size, and weight of their organs were evaluated; these organs were kept in 4% formol whenever a histopathological procedure is performed within 48 hours later, or 10% formol if the histopathological procedure is performed more than 48 hours later.

2.2.9 RESULTS

a) Clinical Toxic Signs

During the assessment period, no mortality or clinical signs of toxicity were observed among the animals (both sexes), neither during the first 24 hours nor over the 14-day period after the administration of the substance. This applies to both groups, the one with the animals that received the freeze-dried extract of the A4+ Formula at a dose of 2,000 mg/kg^{1} of the b.w., and the control group.

b) Body Weight

Body weight records of male and female albino mice show that none of them lost weight, but that weight gain was a constant during the study in both groups (the

one with the animals that received the freeze-dried extract of the A4+, at a dose of 2,000 mg/kg^{-1} of the b.w., and the control group (TABLES N° 01 and 02).

c) Macroscopic Evaluation of the Organs

The necropsy showed no evidence of macroscopic effects with regards to the colour, consistency and size of the organs studied, such as the brain, heart, lungs, liver, kidneys, spleen, stomach, testicles and ovaries in any of the experimental groups (TABLES N° 03 and 04).

d) Evaluation of the Organs' Weights

The relative and average weight of the albino mice's organs (both sexes) in both groups (the one treated with the freeze-dried extract of the A4+ Formula and the control group), showed no significant statistical differences (TABLES N° 05 and 06).

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RESULT TABLES

TABLE N° 01

Average Body Weight - Male Albino Mice Treated with the A4+ Formula at a Dose of 2,000 mg/Kg^{-1} and Those that Were Administered a Saline Solution

Experimental Group	Day 7 (g)	Day 14 (g)
Treated♂	23.35 ± 0.78	22.12± 0.69
Control ♂	21.64± 0.69	22± 1.15

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X±SD = Mean and Standard Deviation

TABLE N° 02

Average Body Weight - Female Albino Mice Treated with the A4+ Formula at a Dose of 2,000 mg/Kg^{-1} and Those that Were Administered a Saline Solution

Treated ♀	25.28 ± 1.32	25.9 ± 1.64
Control ♀	22.5 ± 1.08	21.76 ± 2.28

X±SD = Mean and Standard Deviation

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TABLE N° 03

Macroscopic Observation - Organs of Male Albino Mice Treated with the A4+ Formula at a Dose of 2,000 mg/kg^{-1}

OR	CONTROL	DOSE OF 2000 mg/kg
BRAIN	Colour : cream Size : normal Consistency : tender	Colour : cream Size : normal Consistency : tender
HEART	Colour : greyish red Size : normal Consistency : semi-hard and firm texture	Colour : greyish red Size : normal Consistency : semi-hard and firm texture
LUNGS	Colour : creamy pink Size : normal Consistency : gelatinous, tender	Colour : creamy pink Size : normal Consistency : gelatinous, tender
LIVER	Colour : homogeneous vinous red Size : normal Consistency : soft, firm texture, smooth contour	Colour : homogeneous vinous red Size : normal Consistency : soft, firm texture, smooth contour
SPLEEN	Colour : homogeneous vinous red Size : normal Consistency : firm texture, shapely	Colour : homogeneous vinous red Size : normal Consistency : firm texture, shapely
KIDNEYS	Colour : homogeneous greyish red Size : normal Consistency : soft, firm texture	Colour : homogeneous greyish red Size : normal Consistency : soft, firm texture
STOMACH	Colour : cream Size : normal Consistency : soft, elastic texture	Colour : cream Size : normal Consistency : soft, elastic texture
TESTICLES	Colour : homogeneous cream Size : normal Consistency : soft, smooth contour	Colour : homogeneous cream Size : normal Consistency : soft, smooth contour

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TABLE N° 04

Macroscopic Observation - Main Organs of Female Albino Mice Treated with the A4+ Formula at a Dose of 2,000 mg/Kg^{-1}

ORGANS	NEGATIVE CONTROL	EXTRACT OF THE A4+ FORMULA - LIMIT DOSE OF 2,000mg/Kg^{-1} of the b.w.
BRAIN	Colour : cream Size : normal Consistency : tender	Colour : cream Size : normal Consistency : tender
HEART	Colour : greyish red Size : normal Consistency : semi-hard and firm texture	Colour : greyish red Size : normal Consistency : semi-hard and firm texture
LUNGS	Colour : creamy pink Size : normal Consistency : gelatinous, tender	Colour : creamy pink Size : normal Consistency : gelatinous, tender
LIVER	Colour : homogeneous vinous red Size : normal Consistency : soft, firm texture, smooth contour	Colour : homogeneous vinous red Size : normal Consistency : soft, firm texture, smooth contour
SPLEEN	Colour : homogeneous vinous red Size : normal Consistency : firm texture, shapely	Colour : homogeneous vinous red Size : normal Consistency : firm texture, shapely
KIDNEYS	Colour : homogeneous greyish red Size : normal Consistency : soft, firm texture	Colour : homogeneous greyish red Size : normal Consistency : soft, firm texture
STOMACH	Colour : cream Size : normal Consistency : soft, elastic texture	Colour : cream Size : normal Consistency : soft, elastic texture
OVARIES	Colour : homogeneous cream Size : normal Consistency : soft, corrugated contour	Colour : homogeneous cream Size : normal Consistency : soft, corrugated contour

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TABLE N° 05

Average Organ Weight - Group of Animals Treated with the A4+ Formula at a Dose of 2,000 mg/Kg^{-1}

Organs	Males X ± SD	Females X ± SD
Liver	1.6 ± 0.20	1.42 ± 0.29
Brain	0.37 ± 0.07	0.38 ± 0.08
Heart	0.14 ± 0.01	0.16 ± 0.02
Stomach	0.19 ± 0.01	0.16± 0.02
Spleen	0.14 ± 0.06	0.19 ± 0.07
Lung	0.35 ± 0.08	0.41 ± 0.06
Right Kidney	0.26 ± 0.04	0.24 ± 0.02
Left Kidney	0.24 ± 0.04	0.22 ± 0.04
Right Testicle	0.17 ± 0.04	-
Left Testicle	0.16 ± 0.03	-
Ovaries	-	0.28 ± 0.04

X±SD = Mean and Standard Deviation

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TABLE N° 06

Average Organ Weight - Control Group of Animals that Were Administered a Saline Solution

Organs	Male X±SD	Female X±SD
Liver	1.42 ± 0.3	1.41 ± 0.27
Brain	0.34 ± 0.05	0.32± 0.04
Heart	0.12 ± 0.02	0.13± 0.02
Stomach	0.15 ± 0.03	0.22± 0.04
Spleen	0.2 ± 0.07	0.13± 0.06
Lung	0.32 ± 0.04	0.37 ± 0.05
Right Kidney	0.24± 0.03	0.24± 0.04
Left Kidney	0.21± 0.03	0.21± 0.03
Right Testicle	0.13 ± 0.04	-
Left Testicle	0.13± 0.04	-
Ovaries	-	0.26± 0.1

X±SD = Mean and Standard Deviation

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DISCUSSION

* The toxicological results obtained through these studies showed the innocuousness of the freeze-dried extract of the A4+ Formula at a dose of 2000 mg. Kg^{-1} of the b.w., since no clinic showing toxicity or mortality were found using the Limit Dose test.

* When the consistency and colour of the targeted organs were compared to those of the mice in the group that was administered NaCl 0.9%, no significant differences were found that suggested any toxicity of the substance studied.

CONCLUSIONS

* According to the Limit Dose (LM) Method, the freeze-dried extract of the A4+ Formula did not cause any deaths when administered orally at a dose of 2000 mg/kg^{-1}.

* The A4+ Formula freeze-dried aqueous extract is thus classified as NON-TOXIC, based on the Limit Dose Method.

BIBLIOGRAPHY

1. Corey, G. 1988. Vigilancia en epidemiología ambiental. OPS/OMS 193 pp.
2. Durham, W.B. 1974. Toxicology of enviromental pollutants in Industrial Pollution [N.I. Sax] Van Nostrand Reinhold Company. Chapter 1: 1-9.
3. SSA 1993.Manual de toxicología. Dirección de Salud Ambiental, Subsecretaría de Regulación y Fomento Sanitario, Secretaría de Salud. 183 pp.
4. Gutiérrez Ruiz, M.C. and T. Fortuol van der Goes. 1997. Conceptos básicos de toxicología ambiental En Introducción a la toxicología ambiental. [L.A. Albert] OPS/Mexico State Government. Chap. 5: 53-79.
5. British Toxicology Society Working Party on Toxicity (1984). Special report: a new approach to the classification of substances and preparations on the basis of their acute toxicity. Human Toxicol., 3, 85-92.
6. Van den Heuvel, M.J., Dayan, A.D. and Shillaker, R.O. (1987). Evaluation of the BTS approach to the testing of substances and preparations for their acute toxicity. Human Toxicol. 6, 279-291.
7. Van den Heuvel, M.J., Clark, D.G., Fielder, R.J., Koundakjian, P.P., Oliver, G.J.A., Pelling, D., Tomlinson, N.J. and Walker, A.P. (1990). The international validation of a fixed-dose procedure as an alternative to the classical LD50 test. Fd. Chem. Toxicol. 28, 469-482 (3).
8. Whitehead, A. and Curnow, R.N. (1992). Statistical evaluation of the fixed-dose procedure. Fd. Chem. Toxicol., 30, 313-324.
9. Stallard, N. and Whitehead, A. (1995). Reducing numbers in the fixed-dose procedure. Human Exptl.Toxicol., 14, 315-323.
10. Stallard, N., Whitehead, A. and Ridgeway, P. (2002). Statistical evaluation of the revised fixed dose procedure. Hum. Exp. Toxicol., 21, 183 -196.

SABELL

11. Heddle, J.A. (1973). A Rapid *In Vivo* Test for Chromosomal Damage, Mutation Res., 18, 187-190.

12. Schmid, W. (1975). The Micronucleus Test, Mutation Res., 31, 9-15.

13. Heddle, J.A., Salamone, M.F., Hite, M., Kirkhart, B., Mavournin, K., MacGregor, J.C. y Newell, G.W.(1983). The Induction of Micronuclei as a Measure of Genotoxicity. Mutation Res. 123: 61-118.

14. Mavournin, K.H., Blakey, D.H., Cimino, M.C., Salamone, M.F. y Heddle, J.A. (1990). The *In Vivo* Micronucleus Assay in Mammalian Bone Marrow and Peripheral Blood. A report of the U.S. Environmental Protection Agency Gene-Tox Program, Mutation Res., 239, 29-80.

15. MacGregor, J.T., Schlegel, R. Choy, W.N. and Wehr, C.M. (1983). Micronuclei in Circulating Erythrocytes: A Rapid Screen for Chromosomal Damage During Routine Toxicity Testing in Mice. En: "Developments in Science and Practice of Toxicology", ed. A.W. Hayes, R.C. Schnell y T.S. Miya, Elsevier, Amsterdam, 555-558.

16. Guidance Document on Acute Oral Toxicity Testing. OECD (2001). Environmental Health and Safety Monograph Series on Testing and Assessment N. 24. Paris.

17. OECD (2,000). Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation. Environmental Health and Safety Monograph Series on Testing and Assessment N. 19.

18. AVAILABLE: http://ec.europa.eu/environment/dansub/pdfs/annex5b_es.pdf.

19. Thielmann, K. Principios de metodología en Bioquímica Clínica. RDA: Editorial Leipzig ;1973.

20. Wolford ST, Schroer RA, Gohs FX, Gallo PP, Brodeck M, Falk HB, e al. Reference range data base for serum chemistry and hematology values in laboratory animals. J Toxicol Environm Health. 1986; 18:161-88. Received: August 20, 2006. Passed: August 30, 2006, MSc. *Yana Gonzalez Torres*. Centro Nacional para la producción de animales de laboratorio Finca Tirabeque, Carretera El Cacahual

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21. AVAILABLE: http://www.respyn.uanl.mx/i/2/ensayos/toxicologia.htm.

22. AVAILABLE: http://www.proyectosfindecarrera.com/toxicologia.htm.

SABELL

23. Gonzáles T. Y., Scull C. I., Bada B. A., Gonzáles N. B., Fuentes M. D., Arteaga P. et al. (2003). Ensayo de toxicidad a dosis repetidas del extracto acuoso de *Morinda royoc* L. en ratas Cenp:SPRD. La Habana, Cuba. Disponible en: URL: http://www.bvs.sld.cu/revistas/pla/vol8_2_03/pla05203.htm.

24. SODI KOFF, CH. (1996) Pruebas diagnósticas y de laboratorio en las enfermedades de pequeños animales. 2da Edición. Editorial MOSBY – ESPAÑA.

25. Hardy R. M. (1992). Enfermedades del hígado y su tratamiento. En: Ettinger, S. Tratado de Medicina Interna Veterinaria: enfermedades del perro y el gato. Vol. 2, 3ª ed., Inter-Médica. Buenos Aires, Argentina.

26. Espinoza O., Bustos-Obregón E., Suja J. (2002) Efecto del parathion sobre el índice de apoptosis en hepatocitos de ratones CF1. Rev. chil. anat. v.20 n.1. Chile. Disponible en: URL: http://www.scielo.cl/scielo.php

27. KATHLEEN M. (1999). Laboratorio Clínico y Pruebas de Diagnóstico. Editorial Manual Moderno. México. 49 – 62.

28. BRAUN JP, AKTAS M, LEFEBVRE H, RICO AG, TOUTAIN PL. (1993). Clinical enzymology for the assessment of organ damage: Interspecific differences. Comp Haematol Int. 3: 27 – 32.

29. KOHN DF, CLIFFORD CB. (2002). Biology and diseases of rats. In: Fox JG, Anderson LC, Loew FM, Quimby FW, editors. Laboratory animal medicine. Amsterdam: Academic Press; p. 121 – 65. [Chapter].

30. ANGEL, G. (1996). Interpretación Clínicas del laboratorio. 5ª Edición. Editorial Médica Panamericana.

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ANNEX

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1. CRITERIA USED IN THE DESIGN OF TRADITIONAL TOXICOLOGY PROTOCOLS

a) Animal Species to be Used

In general, and due to economic reasons, small animals are used, such as mice, hamsters, rats, guinea pigs, rabbits and dogs. The animals must be kept in a good vivarium to ensure that the animals used are healthy, with a good inbred, appropriately fed and kept with scrupulous hygiene.

b) Total Number of Animals

In any group of subjects, some subjects are sensitive to a toxic substance while others are more resistant. There is now a trend to reduce the number of animals as much as possible by improving the mathematical techniques.

c) Routes of Administration that Must Be Used

The route of administration depends on the type of product and the possible route through which the substance would be absorbed by humans. Therefore, an oral, cutaneous or parenteral medication would require the same route of administration. Similarly, an air pollutant would be studied by administering through inhalation and cutaneous contact.[22]

d) Study Period

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Toxicity studies can be carried out through a single administration (Acute Toxicity), through short treatments (Subchronic Toxicity or Repeated Dose), or medium or long-termed treatments (Chronic Toxicity). [22]

3. GLOSSARY

- **Drug:** When we talk about drugs, the purpose of their ingestion is to feel pleasure or ease. [22]

- **Dose:** It is the amount of the substance that is absorbed by the organism in a certain period, and it usually depends on the weight of the individual. [22]

- **Useless Dose:** It does not produce any noticeable or known effect that could be linked to it. A dose is considered to be useless when no effect can be noticed in the short term.

- **Therapeutic Dose:** It is the amount required to alleviate a problem that exists in our organism.

- **Toxic Dose:** It is the amount that causes a damaging effect on our organism.

- **Lethal Dose (LD):** It is the amount that causes death to an individual. A Lethal Dose is not the same for all the individuals in the population of subjects in this study.

- **Lethal Dose 50 (LD50):** It is the dose that would cause death to 50% of the population.

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- **Minimum Lethal Dose (LDm):** It is the lowest dose that has caused the death of an individual.

- **Lethal Time (LT):** It is the time that elapses between the ingestion of the dose and the death of the individual.
- **Lethal Time 50 (LT50):** It is the time that elapses until 50% of the individuals in the population die.
- **Acute Oral Toxicity:** Harmful effects that appear after a single dose of the substance or multiple doses administered within a 24-hour period. [22]
- **Delayed Death:** It means that the animal does not die or does not seem to be dying within a period of 48 hours, but it dies later, during the 14-day observation period. [22]
- **Limit Dose:** Maximum limit dose to be use in the test (2,000 mg/kg).
- **Acute Oral Toxicity:** Harmful effects that appear after a single dose of the substance or multiple doses administered within a 24-hour period.
- **Delayed Death:** It means that the animal does not die or does not seem to be dying within a period of 48 hours, but it dies later, during the 14-day observation period.

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- **Visible Toxicity:** It is a general term that describes clear signs of toxicity after a substance has been administered. Thus, if a fixed dose that is just above is administered, most animals can be expected to experience strong pain and continuous signs of suffering and agony.

- **Micronucleus:** It is a small nucleus, which is additional to the separate, main nucleus, and created after the telophase of the mitosis or the meiosis by of lagged chromosome fragments or full chromosomes.

- **Normochromic Erythrocyte:** A mature erythrocyte that does not have ribosomes and can be differentiated from the Polychromic Erythrocyte (immature) through the selective dying of the ribosomes.

- **Polychromic Erythrocyte:** Immature erythrocyte that is in an intermediate transforming stage which still has ribosomes and can be differentiated from the Polychromic Erythrocyte (mature) through the selective dying of the ribosomes.

- **OECD:** *Organization for Economic Co-operation and Development*

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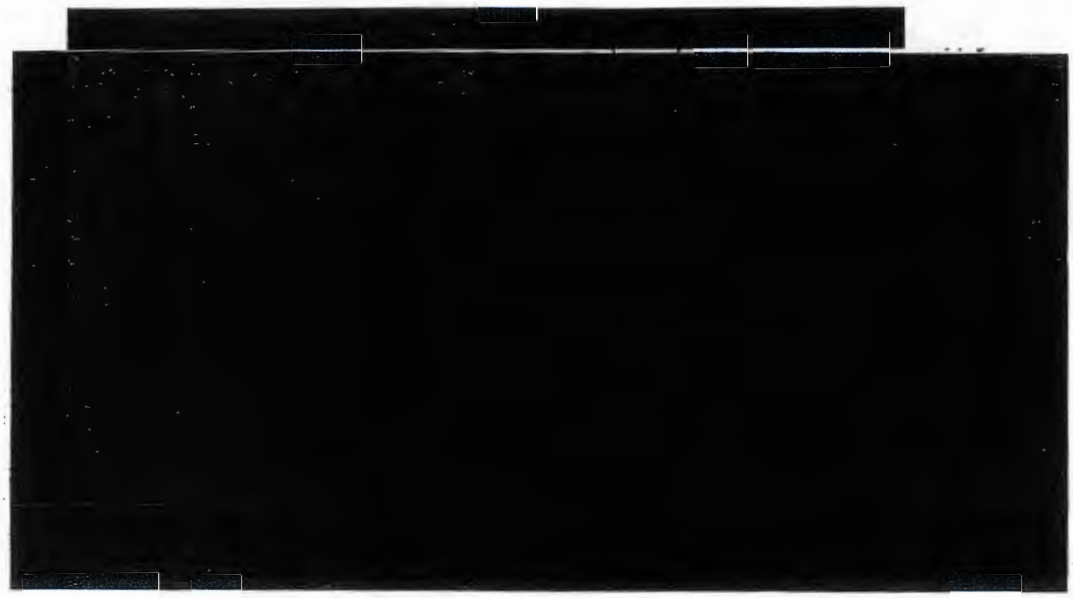
4. DOSE TABLE



V.I. = Dose x W / [Concentration] Volume of the Inoculum = V.I. (ml) Animal's Weight = W (grams)

[redacted] = mg/kg. b.w. [redacted] = ml/g.b.m [redacted] = g/ml

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5. ACUTE TOXICITY OBSERVATIONS TO IDENTIFY THE ORGANS LINKED TO THE AGENT'S ACTIVITY

ORGAN/SYSTEM	TOXIC SIGNS
AUTONOMOUS	Salivation, nasal discharges, diarrhea, urination, piloerection, exophthalmos, relaxed nictitating membranes, rhinorrhea, perspiration.
BEHAVIOUR	Sedation, head bowed, seating with the head in upright position, severe depression. Restlessness, excessive self-grooming, irritability, aggressive behaviour, defensive hostility, fierceness, bizarre activity, confusion.
SENSORY	Right reflex, sensitivity to pain, corneal reflex, reflex in the rear limbs, sensitivity to sound and touch.
NEUROMUSCULAR	Decreased or increased activity, fasciculations, fears, weakness, absent or reduced reflexes in the rear limbs, muscular tone, ataxia, convulsions, severe numbing, weak rear limbs.
CARDIOVASCULAR	Variation (increase or decrease) in the cardiac frequency, vasoconstriction, vasodilatation, hemorrhage.
RESPIRATORY	Gasping, dyspnea, apnea, hypopnea.
OCULAR	Lacrimation, ptosis, nystagmus, mydriasis, miosis, cycloplegia, pupillary light reflex.
GASTROINTESTINAL	Salivation, nausea, diarrhea, defecation (with or without blood), constipation.
CUTANEOUS	Piloerection, alopecia, shaking (wet dog), erythema, edema, swelling, necrosis.

Taken from: McNamara, B.P. New concepts in safety evaluation Mehlman, M.A. Shapiro, R.E. and Blumenthal, H. Eds. Hemisphere Publishing, New York (1976). Chap. 4 According to Ecobichon, D.J. (1992)

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Reference # 5

Disc: A4+ - Interviews With Peruvian Medicine Experts – Jan 2009

Notes by the translator are indicated between brackets "[ ]".
Key Excerpts from Interviewer highlighted in [illegible]
Key Excerpts from Interviewee highlighted in Yellow

Isula Interview Expert # 1

Interpreter:	Your... your name, and your... and what is it what you do, eh? Like... what is your job? I mean, do you see patients? What do you do? Your name first.
Interviewee:	[Incomprehensible]
Interpreter:	Let's see if you can talk... talk as loud as possible, closer, come... your name, eh, Marco...
Interviewee:	[illegible]
Interpreter:	What is it what you do? What do you do for a living? Where do you work at? What do you work with?
Interviewee:	[illegible]
Interpreter:	How old are you?
Interviewee:	43 years old.
Interpreter:	Eh, have you been using, eh, the *guisador* for these past years?
Interviewee:	Yes, the *guisador*.
Interpreter:	The *guanabana*, have you ever used, maybe the, this, these plants?
Interviewee:	Yes, a lot, a lot, yes.
Interpreter:	Have you, eh, say if you have used the, the *guanabana* and the *guisador* for the liver...
Interviewee:	Yes, for that yes...
Interpreter:	Say the name of the two, let's see...
Interviewee:	Eh, *guanabana*... the *guisador*...
Interpreter:	[illegible]
Interviewee:	[illegible]
Interpreter:	What do you use the *guisador*, those plants for?
Interviewee:	[illegible]
Interpreter:	So the two plants, the two ingredients are for the liver...
Interviewee:	For the liver, yes [incomprehensible].
Interpreter:	[illegible]
Interviewee:	[illegible]

[1] "Guisador" is a Spanish term for Curcuma or Tumeric.

Interpreter: [redacted]

Interviewee: [redacted]

Interpreter: What else?

Interviewee: [redacted]

Interpreter: Ah, have you ever used it in powder? No.

Interviewee: No, no.

Interpreter: Have you used it in powder? No.

Interviewee: Yes, in powder yes, to, to eat, also prepared to be eaten with food, it gives a lot of flavour, excellent flavour...

Interpreter: [redacted]

Interviewee: [redacted]

Interpreter: Before or after breakfast?

Interviewee: [redacted]

Interpreter: For how long?

Interviewee: [redacted]

Interpreter: [redacted]

Interviewee: [redacted]

Interpreter: Is there any contraindication? For example, can you eat no matter what or can you do no matter what? Can you take alcohol and take that? Yes or not...

Interviewee: During the treatment...

Interpreter: I mean, what is... How do you take it? With what you cannot take it?

Interviewee: Eh, during, during the treatment, what we do... you don't eat, eh, very fatty foods, you're not allowed to take, eat fatty foods... eh, acids, during that time, during the nine-day period, you don't eat acids, fatty foods...

Interpreter: And what else you don't eat?

Interviewee: Well, eh, hard things, meat, broths are fine, not meat, not meat, pork. All of that...

Interpreter: ... We're done...

Interviewee: Ok...

Interpreter: You're very kind, eh...

Isula Interview Expert # 2

Interpreter: Your name and your profession and your age...

Interviewee: [Speaking in English]: [Incomprehensible] [redacted] [End of what he spoke in English]

Interpreter: And eh, what do you do? Are you, eh, have you used medicines? Are you, do you consider yourself a, a person that uses jungle, jungle medicines, I mean, like a healer, right?

Interviewee: [redacted]

Interpreter: [redacted]

Interviewee: [redacted]

Interpreter: How... how old are you?

Interviewee: Fifty-six... [smiles, laughs]...

Interpreter: You, are you a, are you a person, eh, are you an authority in traditional... historic medicine?

Interviewee: No, no, no, I, I know how medicine is used because in the botanical projects, eh, the first people I usually ask for information are the *chamanes* [healers], or...

Interpreter: [redacted]

Interviewee: [redacted]

Interpreter: [redacted]

Interviewee: [redacted]

Interpreter: And what are the benefits of...?

Interviewee: [redacted]

Interpreter: [redacted]

Interviewee: [redacted]

Interpreter: [redacted]

Interviewee: [redacted]

Interpreter: But also in alcohol, right?

Interviewee: Usually when it's very, very, very strong, they stop taking alcohol, I mean, they take pure water, with water...

Interpreter: [redacted]

Interviewee: [redacted]

Interpreter: But it is also prepared with alcohol...

Interviewee: Sometimes, but not usually...

Interpreter: [redacted]

Interviewee: [redacted]

Interpreter: [redacted]

Interviewee: [redacted]

Interpreter: [Talking to somebody else]: Tito, bring number 12. [Talking again to the interviewee]: [redacted]

Interviewee: [redacted]

Interpreter: How?

Interviewee: [redacted]

Interpreter: For example, for the hepatitis, how would it be prepared if it is with alcohol, how would it be?

Interviewee: [redacted]

Interpreter: And how much do you take per day and for how long for a, for the hepatitis?

Interviewee: [redacted]

Interpreter: [redacted]

Interviewee: [redacted]

Interpreter: Thank you.

Isula Interview Expert #3

Interpreter: Mr. Ramoncito, stand over here please, I'm going to ask you a couple of questions, how... closer, closer, get closer... ok, eh... first tell me your name.

Interviewee: Ramon [incomprehensible]

Interpreter: What?

Interviewee: [redacted]

Interpreter: [redacted]

Interviewee: [redacted]

Interpreter: How many years more or less?

Interviewee: Eh, [incomprehensible] years

Interpreter: [redacted]

Interviewee: [redacted]

Interpreter: What is your age?... What is your age?

Interviewee: Seven-four

Interpreter: [redacted]

Interviewee: [redacted]

Interpreter: [redacted]

Interviewee: [redacted]

Interpreter: What else?

Interviewee: [redacted]

Interpreter: [redacted]

Interviewee: [redacted]

Interpreter: How many times a day?

Interviewee: [redacted]

Interpreter: How many per day more or less?
Interviewee: [redacted]
Interpreter: [redacted]
Interviewee: [redacted]
Interpreter: [redacted]
Interviewee: [redacted]
Interpreter: [redacted]
Interviewee: [redacted]
Interpreter: [redacted]
Interviewee: [redacted]
Interpreter: [redacted]
Interviewee: [redacted]
Interpreter: Ok Mr. Ramon, ok thank you, eh...

Tumbes Interview Expert #1

Interpreter: Ok, what's your name? What's your name, eh...? [Talking to somebody else]: Give me some flowers.
Interviewee: [redacted]
Interpreter: [Talking again to the Interviewee]: And are you from here? Where are you from? From Tumbes?
Interviewee: Tumbes, I belong to... I belong to a district called Casitas, around here...
Interpreter: I mean, in Tumbes, what do you do? Do you see, eh, who... what is your job? Do you see patients? What is...?
Interviewee: No, I work at a butchery.
Interpreter: [redacted]
Interviewee: [redacted]
Interpreter: [redacted]
Interviewee: [redacted]
Interpreter: [redacted]
Interviewee: [redacted]
Interpreter: Is there any other way to prepare it? How else is it prepared...? With what can it be prepared?
Interviewee: [redacted]
Interpreter: [redacted]
Interviewee: [redacted]

[redacted]

Interpreter: [redacted]
Interviewee: [redacted]
Interpreter: [redacted]
Interviewee: [redacted]
Interpreter: But how much do you give to him? Like this, a handful...?
Interviewee: Yes, more or less like this, as much as this [The interviewee shows the flowers in his hand].
Interpreter: And how is it used? Do you wash yourself with it? Do you take it? What do you do?
Interviewee: [redacted]
Interpreter: That's, eh.... what? Before the meal? After the meal? How do you take it?
Interviewee: [redacted]
Interpreter: [redacted]
Interviewee: [redacted]
Interpreter: [redacted]
Interviewee: [redacted]
Interpreter: You mean...
Interviewee: [redacted]
Interpreter: [redacted]
Interviewee: [redacted]
Interpreter: Can you eat pork, or whatever?
Interviewee: [redacted]
Interpreter: Ok
Interviewee: Not pork.
Interpreter: Right, not with this, what else should not...?
Interviewee: Certain things, of course, certain foods should not be eaten, of course... foods have to be avoided too, they can be harmful...
Interpreter: What kind of foods should be avoided more or less?
Interviewee: [redacted]

Tumbes Interview Expert #2

Interpreter: Ready, eh, how are you my friend? What's your name? What's your name?
Interviewee: [redacted]
Interpreter: [redacted]
Interviewee: [redacted]
Interpreter: [redacted]

Interviewee: [redacted]

Interpreter: [redacted]

Interviewee: [redacted]

Interpreter: [redacted]

Interviewee: [Nods] [redacted]

Interpreter: So, what is, what about your patients...? What happened? Let's see, tell me about your experience with the patients to whom you have given the *Overal*.

Interviewee: [redacted]

Interpreter: Eh... and you basically work curing people? Is that your, your job?

Interviewee: Every now and then, to people, to neighbours and many others, thanks to the experience that I got from my grandparents, we are using it for that.

Interpreter: [redacted]

Interviewee: [redacted]

Interpreter: [redacted]

Interviewee: [redacted]

Interpreter: What do you take it for?

Interviewee: [redacted]

Interpreter: [redacted]

Interviewee: [redacted]

Interpreter: For how long?

Interviewee: [redacted]

Interviewee: [redacted]

Interpreter: [redacted]

Interviewee: [redacted]

Reference # 6

SHORT TERM EFFECTS OF A4+ ON CLINICAL AND BIOCHEMICAL MARKERS IN CHRONIC HEPATITIS C – SEPTEMBER 2001

Jose Cabanillas C., M.D., Joseph Nystrom, M.D., Hugo Marquez, S., M.D.

SUMMARY

To evaluate the value of a natural nutraceutical named A4+ used in patients with chronic hepatitis C, 6 HIV negative volunteer patients with varying severity and past treatments were administered A4+ with each meal for 4 weeks. All were provided a balanced diet and exposed to natural sunlight. The product was without side effects. All patients demonstrated profound clinical improvement, even with reversal of anasarca in one patient. While being a study of very short time length, we have noted no adverse trend on serum GTT, SGOT, SGPT, bilirubin, alkaline phosphatase, blood counts, and renal function of lipid indices. The study is too small to assign significance to results regarding viral loads or alphafetoprotein. We conclude that the natural product A4+ demonstrated profound clinical benefit and merits further study as an alternative for those suffering from this disease.

DATA

R.R.47YRMALE.....R.W.66YRFEM.....B.B.44YRMALE.....P.P.45YRMALE.....R.D.47YRFEM.....J. M.47YRFEM

Clinical Changes after 4 weeks of A+

Anorexia	improved	improved	improved	improved	improved
Fatigue	improved	improved	improved	improved	improved
Headaches	improved	improved	improved	improved	improved
Nausea	improved	improved	improved	improved	improved
Tremors	n/a	n/a	improved	improved	n/a
Anasarca	n/a	n/a	improved	n/a	n/a
Depression	n/a	improved	imrpoved	improved	n/a
Weight Kg	+3.5	-2.1	-4.2	-0.5	-0.6 -1.2

Laboratory Changes after 4 weeks of A+

SerumGGT [normal 8-37]	94 > 79	52 > 53	36 > 37	21 > 37	53 > 61 49 > 48
SGOT [normal0-40]	61> 73	35 > 46	95 > 70	114 > 221	63 > 112 49 > 51
SGPT [normal 0-38]	137 > 153	46 > 53	34 > 24	180 > 206	98 > 178 59 > 54
Tot. Billirubin [normal 0-1]	0.8 > 0.4	0.7 > 0.6	1.6 > 2.7	0.5 > 0.8	0.6 > 0.7 0.5 > 0.6

AlkalinePhos	110 > 101	132 > 128	232 > 237	115 > 128
167 > 171	79 > 103			
[normal 90-270]				
WBC	8.2 > 7.2	10.8 > 13.8	4.1 > 2.7	7.2 > 6.6
9.4 > 7.1	5.0 > 4.7			
[normal 4.1-10.9k]				
Hct	49 > 49	44 > 44	40 > 41	44 > 43
47 > 45	44 > 43			
[normal 38-48]				
Platelets	243 > 324	167 >175	84 > 80	144 > 174
229 > 227	90 > 172			
[normal 140-440K]				
HDL	30 >31	36 >44	28 > 35	27 > 28
45 > 41	35 > 50			
[normal 40-70]				
Serum Creatinine	0.9 > 0.8	0.7 > 0.9	1.1 > 0.8	0.9 > 0.9
0.5 > 0.8	0.6 > 0.9			
[normal 0.7-1.4]				
AFP	? > 5.4	4.2 > 6.2	7.3 > 12	? > 2.3
? > 7.0	5 > 6.4			
[normal 0.8-8.5]				
HepC RNAxPCR	414 > 710k	over 850 > 836k	180 > 113k	528 > 817k
398 > 792k	221 > 840k			

Discussion

Accepted the fact that Hepatitis C is a growing epidemic which threatens individuals during their productive years on a worldwide level and for which effective therapy is not only extremely costly but widely ineffective and poorly tolerated it is understandable that searches for alternatives to conventional treatments can and should be conducted. Scant study into the use of natural products in the course of hepatitis C are found in western literature. The principal investigator of our study has extensive experience in the use of natural products in people afflicted with a wide range of pathologies. Prior to this study the natural product A4+ had been used with great success in other cases of Hepatitis C. This is the first time we attempt to document the use of A4+ in a prospective study of chronic hepatitis C. While studies for conventional therapies in the treatment of hepatitis C customarily last 1 or more years, so far we have data including only an initial 4 week period.

All patients demonstrated clinical improvement and were uniformly free of adverse reactions to the product. The majority of patients entering the study had baseline abnormal liver functions. There were no statistical changes in the liver functions in these first 4 weeks of treatment. Likewise, there were no changes in the markers for other organ systems. The viral load increased in 4 patients and decreased in 2 patients. The alpha fetoprotein was above normal limits in only one patient. All patients will continue to be followed.

Conclusion

This study of the effects of a natural product termed A4+ as a nutraceutical in the alternative treatment of chronic hepatitis C included 6 patients with varying severity. While the study was of short duration as compared to those utilizing conventional therapies, all patients tolerated the product very well. No consistent or statistical significant change in viral load has yet been detected. There is, so far, no significant change from baseline liver enzyme levels. Most importantly, all patients reported dramatic improvement in symptomology. We expect to do further prospective studies and to follow up with all patients to demonstrate continued improvement in all parameters.

Reference # 7

Chronic Hepatitis C Treatment with $A4^{+}$

Controlled Clinical Test

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Lima – Peru
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INTRODUCTION

According to the **World Health Organization,** the prevalence of hepatitis C (HCV) infection worldwide is approximately 170,000,000 people. **The Centers for Viral Disease Control and Prevention in North America** (CDC) estimate that at least4,000,000 people in the U.S. (1.8% of the population) have been infected by the HCV, according to positive results in VHC antibody tests, and 100,000 new cases are reported each year in this country[1-2].

In most people with HCV, the immune system is unable to eradicate the virus. According to the CDC's most recently collected data, 70% of people infected with HCV will develop chronic hepatitis, which is defined as the presence of detectable HCV RNA for six months or longer[3]. Many of this people will develop liver conditions as a result of the disease; the course of progression and the manifestation of symptoms may occur only decades later. Between 20 % and 30% of chronically infected patients are at risk of developing **hepatic cirrhosis** or even **hepatocellular carcinoma** [4-5], since it has proven to be oncogenic in humans[6]. Chronic hepatitis may result in end-stage liver failure, which is the most frequent cause for liver transplant in most of the countries where it is performed.[7].

The course of chronic hepatitis C progression varies among individuals and, therefore, it is not possible to accurately predict which HCV-infected patients will go on to develop cirrhosis, liver cancer or end-stage liver disease. However, several cofactors appear to affect the severity of the disease. In some, but not in all studies, HCV subtypes **1a** y **1b del VHC** have been associated with more rapid progression, more extensive liver damage, and reduced response to treatment.[8].

Alcohol consumption is likely to be the most important external factor. Excessive alcohol use alone can damage the liver, in conjunction with HCV; and the additive effects of the virus can lead to a more severe disease. The person's **age** can also affect disease progression; people over the age of 50 tend to have more rapid progression and develop more severe symptoms. Research indicates that children's immune systems can fight better and eradicate the virus more frequently that those of the adults. **Gender** also seems to have an effect on progression; men tend to develop more severe liver disease. Finally, individuals who are **immunologically compromised**, due to HIV, to the use of drugs that suppress the immune system or to any other cause, tend to experience rapid HCV disease progression[9].

Over time, while liver cells are infected and destroyed, individuals may develop progressive liver damage. This process begins with persistent inflammation, and proceeds to fibrosis or fibrinogenesis, steatosis, and cirrhosis of the liver. When normal liver cells are replaced with fibrous tissue, fat and scar tissue, they are unable to carry out their functions of metabolism.

According to these data, HCV infection is a **worldwide health problem** and there is a need for effective therapy. Hepatitis C treatments are not perfect and there is still a lot of research to be conducted. Recent progress has improved the chances of maintaining the highest viral suppression.

It is known that Interferon (IFN) is the only drug that has shown some efficacy in the inhibition of viral replication and, therefore, in the clinical and histological progression of chronic HCV infection. Many therapeutic schemes were tested since the first original work by Hoofnagle in 1986 [10] until the introduction in May of 1999 of **Pegylated Interferon alpha-PEG** [11]. Standard doses of 3,000,000 units 3 times a week, as well as high doses have been tested both by induction and by escalation schemes.[12-13]. Combined therapy with **Ribavirin** was approved in the year 2,000 by the FDA, for treatment-naive patients as well as for non-responders and relapsers Clinical trials for this kind of patients had already been conducted before. [14-15] Compared with Interferon, the combined therapy reduced the risk of not having a sustained virological negativization, in 28% of the patients with a previous treatment; 33% in relapsers and 11% in those that had not responded before. Regardless of previous treatment, the combined therapy reduced the risk of not obtaining a sustained normalization of biochemical tests or an histological improvement; but it significantly increased the risk of discontinuing treatment due to various adverse side effects.[16].

The observations of **Dr. José Cabanillas**, on positive and encouraging results about the clinical manifestations of HCV (chronic hepatitis C) patients treated with "**active organic ingredients** (**A4^{+}**), motivated us to conduct a 30-day controlled clinical test that would allow us to asses the clinical, hematological, biochemical, ultrasound and anatomic manifestations in a group of 10 voluntary patients who carry chronic hepatitis C.

Methods

We conducted a controlled clinical test in 10 Caucasian, adult patients with a confirmed diagnosis of chronic hepatitis C. Some of them had been treated before without satisfactory results. They were patients with chronic hepatopaty symptomatology who voluntarily underwent the **therapy with A4^{+}.**

Out of the10 patients included in the study, 5 were men and 5, women, and their ages ranged from 37 to 58 years. The estimated period, by anamnesis, of **time of virus inoculation** was 30 years for those with the longest time and 4 years for those with the shortest period. The duration **of the disease** observed in the patients was established by the means of the appearance of symptoms and serologic diagnosis from 12 to 1 year. **Table 1**

Table 1

Chronic Hepatitis C (HCV)

Possible Inoculation Period and Disease Duration

CASES	1	2	3	4	5	6	7	8	9	10
PATIENTS	S.R	S.T.	O.P.	L.R.H.	Ch.D.	W.S.	F.S.	D.S.	H.G.	S.L.
Sex	M	M	M	M	M	F	F	F	F	F
Age (years)	51	37	46	52	46	58	46	47	56	53
Inoculation Period (y)	34	18	21	6	28	32	4	15	26	33
Disease Duration (y)	7	7	5	3	12	5	1	5	10	12

Source: Dr. José Cabanillas & Colleagues
Lima – Peru

To assess the current state of the disease, clinical, serological, biochemical, ultrasound and histo-pathological tests were conducted.

A second generation ELISA system was used to confirm the presence of seric **anti–VHC**. Moreover, we conducted an investigation of **Hepatitis B**, by the means of investigating surface **antigen (HBs Ag) and core antigen (HBc Ag.).** Results are shown in **Table 2.**

Table 2
Chronic Hepatitis C (HCV)
Serologic Evaluation

CASES	1	2	3	4	5	6	7	8	9	10
PATIENTS	S.R	S.T.	O.P.	L.R.H.	Ch.D.	W.S.	F.S.	D.S.	H.G.	S.L.
Anti-HCV	+	+	+	+	+	+	+	+	+	+
Antigen HBs Ag	-	-	-	-	-	-	-	-	-	-
Antigen HBc Ag	-	-	-	+	-	+	+	-	-	-

Source: Dr. José Cabanillas & Colleagues
Lima – Peru

HCV FIBROSURE (550123) was used to determine the state of **histo-pathological lesions** in patients. The HCV FIBROSURE is a noninvasive blood test for assessing liver state in hepatitis C patients. Developed by hepatologists at the **PITIE–SALPETRIERE HOSPITAL and BIO PREDICTIVE** in Paris (France), it is only available in the United States through LAB–CORP.

The HCV FIBROSURE provides an easily accessible alternative to liver biopsy, which is used to assess **liver fibrosis and necroinflammatory activity** in HCV patients, while liver biopsies have been traditionally used in hepatitis C patients to provide important information on disease prognosis, as well as potential lateral response, they are considered an invasive procedure that can cause complications and frequently accompanied by transitory pain.

The HCV FIBROSURE combines the quantitative results of six serum biochemical markers**: Alpha2-Macroglobulin, Haptoglobulin, Apolipoprotein A1, Bilirubin, Gamma Glutamyl Transpeptidase (GGT) and Glutamic Piruvic Transaminase (GPT)** with age and gender, in a patented artificial algorithm to determine the degree of liver fibrosis and the level of ongoing necroinflammatory activity.

Results obtained with HCV FIBROSURE constitute a reliable quantitative assessment of fibrogenetic and inflammatory liver activity in HCV patients.

The results of the HCV FIBROSURE are shown in **Table 3** and **Table 4**

Table 3
Chronic Hepatitis C (HCV)
HCV FIBROSURE-Biochemical Indicators

Biochemical Indicator Normal Value	Alpha2 Macroglobulin 110-276 mg/dl.	Hapto Globulin 34-200 mg/dl.	Apo-lipo Proteein A1 110-205mg/dl.	Total bilirubin 0.1-1.2mg/dl	Gama glutamyl Transpeptidase 0.0-60 IU/L.	Piruvic Transaminase 0.0-40 IU/L.
PATIENTS						
Stad, Raymond	437	16	125	1.20.	191	330
Smith, Troy	293	102	120	1.20	91	61
O'Connor, Patrick	424	107	99	0.40	53	71
Langen, Ron Harry	337	60	116	0.60	71	54
Charron, Daniel	312	124	110	0.50	32	34
White, Susan	291	<15	83	1.70	34	21
Fetterroll, Susan	149	180	116	0.30	57	49
Doyle, Susan	236	138	165	0.30	27	37
Hutchines, Geraldine	185	138	152	0.50	26	40
Stockert, Linda	437	67	106	0.90	36	181

Source: Dr. José Cabanillas & Colleagues
Lima – Peru

Table 4
Chronic Hepatitis C (HCV)
HCV FIBROSURE-Histopatologic Evaluation

	Fibrosis Score	Necrosis Score	Fibrosis Score Diagnosis	Inflammatory Necrosis Score Diagnosis
Normal Value	**0.0-0.21**	**0.0-0.17**		
PATIENTS				
Stad, Raymond	0.96	0.96	**F4** cirrhosis	**A3** Severe Activity
Smith, Troy	0.67	0.50	**F3** fibrous bridges w/ numerous septa	**A1-2** Minimal Activity
O'Connor, Patrick	0.70	0.58	**F3** fibrous bridges w/ numerous septa	**A2** Moderate Activity
Langen, Ron Harry	0.50	0.22	**F2** fibrous bridges w/ numerous septa	**A0-1** No Activity
Charron, Daniel	0.76	0.48	**F4** cirrhosis	**A1-2** Minimal Activity
White, Susan	0.90	0.20	**F4** cirrhosis	**A0-1** No Activity
Fetterroll, Susan	0.10	0.23	**F0** no fibrosis	**A0-1** No Activity
Doyle, Susan	0.12	0.16	**F0** no fibrosis	**A0** No Activity
Hutchines, Geraldine	0.16	0.18	**F0** no fibrosis	**A0-1** No Activity
Stockert, Linda	0.81	0.89	**F4** cirrhosis	**A3** Severe Activity

Source: Dr. José Cabanillas & Colleagues
Lima – Peru

We conducted a study using THREE-DIMENSIONAL ULTRASOUND to determine liver volumetry, the characteristics of its borders and the ultrasound alterations of the liver parenchyma. The assessment of characteristics of the portal vein, the spleen and the presence of ascites are included in the study. **Table 5**

Table 5
Chronic Hepatitis C (HCV)
Three-dimensional ultrasound

	Stad, Raymond	Smith, Troy	O'Connor, Patrick	Langen, Ron Harry	Charron, Daniel
Liver	Hepatomegaly	Hepatomegaly	normal	Hepatomegaly	Hepatomegaly
Size of right lobe	165 mm.	153 – 156 mm.	138 mm.	168 mm.	151-153 mm.
Size of left lobe	114 – 116 mm.	103 – 116 mm.	92 mm.	121 mm.	113-122 mm.
Borders	Regular	Regular	Regular	Regular	Regular
Diffuse echogenicity	Slight Increase	Moderate Increase	Moderate Increase	Moderate Increase	Moderate Increase
Types of echos	Low-amplitude	High-amplitude	Medium-amplitude	Medium-amplitude	Medium-amplitude
Focal Injuries	no signs observed.	no signs observed.	no signs observed.	no signs observed.	no signs observed.
Portal Vein	Normal Appearance	Brightness in portal wall	Normal	Portal hypertension	Normal
Measurements	12mm.	11 – 13 mm.	12-13 mm.	16 mm.	13 mm.
Spleen	Splenomegaly	Splenomegaly	Normal	Splenomegaly	Normal
Measurements	132 x 66 mm.	157 x 81 mm.	92 x 41mm.	135 x 45 mm.	91 x 45 mm.
Splenic vein	8 mm.	8 mm.		4 mm.	
Ascites	Not present	Not present	Not present	Not present	Not present

Source: Dr. José Cabanillas & Colleagues
Lima – Peru

Table 5 (Cont.)
Chronic Hepatitis C (HCV)
Three-dimensional ultrasound

	White, Susan	Fetterroll, Susan	Doyle, Susan	Hutchines, Geraldine	Stockert, Linda
Liver	Decreased	Hepatomegaly	Normal	Normal	Hepatomegaly
Size of right lobe	108 mm.	186mm.	133 mm.	130 mm.	146-149 mm.
Size of left lobe	83 mm.	142mm.	104 mm.	104 mm.	105-124 mm.
Borders	Regular	Regular	Regular	Regular	Regular
Diffuse echogenicity	High Increase	Slight Increase	Slight Increase	Slight Increase	Moderate Increase
Types of echos	High-amplit = cirrhosis	Low-amplitude	Low-amplitude	Low-amplitude	Medium and High
Focal Injuries	no signs observed.	no signs observed.	no signs observed.	no signs observed.	no signs observed.
Portal vein	Hypertension Signs	Hypertension Signs	Normal	Normal	Brightness in portal wall
Measurements	13mm.	11 – 14 mm.	11 mm.	10 mm.	15 mm.
Spleen	Splenomegaly	normal	Normal	Normal	Splenomegaly
Measurements	121 x 51 mm.	106 x 62 mm.	87 x 45mm.	97 x 51 mm.	112 x 58 mm.
Splenic vein					
Ascites	present	Not present	Not present	Not present	Not present

Source: Dr. José Cabanillas & Colleagues
Lima – Peru

The biochemical study of the alterations produced by chronic hepatitis C (HCV) is based on 4 criteria. First: tests to measure liver synthesis capacity. Second: tests to measure alterations due to architectural disorders caused by fibrosis that lead to intrahepatic obstruction. Third: tests to measure necroinflammatory activity in the hepatocytes where it was present, a test to assess focal alterations due to possible hepatocarcinoma. And fourth: tests to measure liver purifying function.

Tests to measure liver synthesis capacity are considered as the study of liver function reserve, once **CHOLINESTERASE** has been determined, since there is evidence[20] that it is produced in the liver and that it decreases with chronic hepatitis, having an average life of 28 days; the assessment of **PREALBUMIN** that indicates the nutritional state of chronic hepatitis patients; and **PROTHROMBIN TIME**, considering that they did not present a deficit of vitamin K and that we did not find, in any case, obstruction of extrahepatic biliary tracts that could inhibit absorption, resulting in a synthesis function of the hepatocyte. See **Table 6**

Table 6
Chronic Hepatitis C (HCV)
Hepatic Synthesis Activity

	Cholinesterase	Prealbumin	Prothrombin time	Concentr. of Prothrombin
Normal Values	**<5,500 U/L**	**<20 mgr/dl**	**11 sec.**	**100%**
PATIENTS				
Stad, Raymond	3,742	16.0	15.0 sec.	65.0
Smith, Troy	8,018	13.0	15.0 sec.	65.0
O'Connor, Patrick	6,241	20.0	13.0 sec.	80.0
Langen, R. Harry	4,631	15.0	14.0 sec.	75.0
Charron, Daniel	4,390	28.0	14.0 sec.	75.0
White, Susan	2,321	7.0	18.0 sec.	51.0
Fetterroll, Susan	5,408	27.0	13.0 sec.	80.0
Doyle, Susan	5,408	25.0	13.0 sec.	80.0
Hutchines, Geraldine	6,696	20.0	14.0 sec.	75.0
Stockert, Linda	5,086	14.6	13.0 sec.	80.0

Source: Dr. José Cabanillas & Colleagues
Lima - Peru

In order to assess liver structural alterations caused by fibrosis and hepatocyte degeneration, both the determination of bilirubin and alkaline phosphatase dosage were taken into consideration. It had already been determined by the means of ultrasound that there was no obstruction of the extrahepatic biliary ducts and, therefore, any increase could be interpreted as intrahepatic alterations in the structure. See **Table 7**.

Table 7
Chronic Hepatitis C (HCV)
Evaluation of intrahepatic structure

	Total bilirubin	Direct bilirubin	Indirect bilirubin	Alkaline phosphatase
Normal Values	**0.3 - 1.0 mgl./ dl**	**0.0 – 0.3 mgl/dl**	**0.3 – 1.0 mgl/dl**	**40 – 129 U/L**
PATIENTS				
Stad, Raymond	1.90	0.60	1.30	97
Smith, Troy	7.2.2.	0.60	1.60	116
O'Connor, Patrick	0.70	0.30	0.40	70
Langen, R. Harry	1.00	0.40	0.60	99
Charron, Daniel	1.00	0.20	0.80	78
White, Susan	3.00	1.20	1.80	196
Fetterroll, Susan	0.60	0.20	0.40	72
Doyle, Susan	0.70	0.20	0.50	58
Hutchines, Geraldine	0.80	0.20	0.60	77
Stockert, Linda	1.30	0.40	0.90	76

Source: Dr. José Cabanillas & Colleagues
Lima - Peru

Tests that measure alterations in the hepatocyte due to viral damage in its membrane are considered as the study of hepatocyte injury and the necroinflammatory activity it can lead to, once the **GLUTAMIC PIRUVIC TRANSAMINASES (GPT) and the GLUTAMIC OXALOACETIC TRANSAMINASES (GOT)** have been determined., as well as the determination of **TRANSFERRIN,** knowing that an increase is due to the release of iron stored in liver cells when they suffer necrosis. The determination of **GAMMA GLUTAMYL TRANSPEPTIDASE,** whose activity in serum increases moderately when there is diffuse liver damage, but it is a guiding enzyme in hepatic toxic lesions due to alcohol 21. And finally, a tumor maker was determined, the **ALPHA-FETOPROTEIN** (AFP), since it increases with chronic and acute liver disease such as cirrhosis and hepatitis, although it rarely exceeds 50-75 ng/ml. Most authors agree that values over 100ng/ml. correlate with primitive hepatocellular cancer[22, 23]. See **Table 8**

TABLE 8
Chronic Hepatitis C (HCV)
Necro-Inflammatory Activity Evaluation

	GPT	GOT	GGT	Transferrin	AFP
Normal Values	**0.0 – 38.0 UI**	**0.0 – 40.0 UI**	**9.0 – 35.0 U/L**	**300 – 360 ug/dl**	**0.8 – 8.5 ng/ml**
PATIENTS					
Stad, Raymond	546	340	186	295	231.0
Smith, Troy	97	62	108	330	7.7
O'Connor, Patrick	93	65	60	331	4.3
Langen, R. Harry	113	96	70	404	6.0
Charron, Daniel	22	21	27	310	1.4
White, Susan	28	48	36	243	3.1
Fetterroll, Susan	71	55	72	410	2.5
Doyle, Susan	50	41	27	377	5.4
Hutchines, Geraldine	48	29	24	306	4.4
Stockert, Linda	306	214	33	347	3.0

Source: Dr. José Cabanillas & Colleagues
Lima - Peru

Finally, we conducted a study of the liver purifying function, by the means of determining AMMONIA IN BLOOD SERUM.

The TNF-ALPHA test was added (tumor necrosis factor), with the intention of measuring hepatic necrosis.

We also included a PLATELET COUNT since an increase in of portal circulation pression and splenomegaly (checked via ultrasound) could be leading to platelet deficiency. See **Table N° 9**

Table 9
Chronic Hepatitis C (HCV)
Evaluation of Hepatic Alterations

	Ammonia	TNF-alpha	Platelet count
Normal values	**25 – 80 ug/dl**	**0.0 – 8.1 pg/ml**	**140,000 – 440,000 x mm**
PATIENTS			
Stad, Raymond	36	10.0	85,000
Smith, Troy	56	7.5	216,000
O'Connor, Patrick	25	7.1	257,000
Langen, R. Harry	27	8.0	130,000
Charron, Daniel	79	4.5	243,000
White, Susan	122	7.5	125,00
Fetterroll, Susan	39	6.4	381,000
Doyle, Susan	47	4.9	319,000
Hutchines, Geraldine	44	5.2	200,000
Stockert, Linda	33	11.1	196,000

Source: Dr. José Cabanillas & Colleagues
Lima - Peru

To complement the study, **chest x-rays** were performed in all patients to rule out lung pathology; they were negative in all cases; we calculated the value of **blood sugar** with the purpose of investigating **Diabetes Mellitus**; a study of **renal function** by the means of serum dosage of urea and creatinine; an evaluation of the **nutritional state** through dosage of **total and fractioned proteins** and finally we determined **thyroid-stimulating hormone** (TSH) to rule out the presence of **thyroid** pathology. The results are shown in **Table 10** and **11**.

Table 10
Chronic Hepatitis C (HCV)
Hematologic Evaluation

	Count	Count	Formula						Constant Values			Hemoglobin	Hematocrit
	Erythrocytes	Leukocytes	E	B	A	S	M	L	Corpusculars				
			%	%	%	%	%	%	VCM	HbCM	CHbCM	gr/dl	%
PATIENTS													
Stad, Raymond'	4,610,000	4,140	3	1	0	62	5	29	102.0	34.6	33.6	15.8	47.1
Smith, Troy	5,420,000	9,150	3	0	1	64	7	25	93.0	31.6	33.9	17.1	50.4
O'Connor, Patrick	4,780,000	14,800	0	0	2	69	3	26	93.7	32.1	34.3	15.4	44.8
Langen, R. Harry	4'330,000	4,100	4	0	4	50	5	37	91.6	30.4	33.2	13.2	39.6
Charron, Daniel	5,220,000	11,600	4	1	1	67	5	22	90.7	30.5	33.6	15.9	47.3
White, Susan	3,980,000	6,420	0	0	0	64	6	30	95.4	31.9	33.4	12.7	37.9
Fetterroll, Susan	4,200,000	8,110	0	0	0	8	5	27	84.8	28.5	33.6	12.0	35.6
Doyle, Susan	3,900,000	5,480	2	0	0	54	6	38	89.8	31.0	34.6	12.1	35.0
Cchines, Geraldine	4,530,000	7,620	0	0	0	71	6	23	94.9	31.2	32.8	14.1	43.0
Stockert, Linda	4,910,000	4,340	1	0	2	44	4	49	90.3	29.6	32.8	14.5	44.3

Source: Dr. José Cabanillas & Colleagues
Lima – Peru

Table 11
Chronic Hepatitis C (HCV)
General Biochemical Evaluation

	Basal glucose	Total proteins	Sero albumin	Sero globulin	Serum urea	Serum creatinine	Thyroid- stimulating hormone
Normal values	**75-110 mg/dl**	**6.0-8.0 gr/dl**	**3.5-5.0 gr/dl**	**2.0-3.0 gr/dl**	**15.0-40.0 mg/dl**	**0.7-1.4 mg/dl**	**0.2-6.8 m UI/ml**
PATIENTS							
Stad, Raymond	93.0	7.60	4.10	3.50	35.0	1.1	5.0
Smith, Troy	210	8.20	3,4,5.	3.70	38.0	1.0	1.3
O'Connor, Patrick	84.0	7.50	4.40	3.10	28.0	1.0	0.8
Langen, R. Harry	88.0	7.10	4.40	2.70	27.0	1.0	1.5
Charron, Daniel	90.0	7.60	4.70	2.90	37.0	0.9	1.2
White, Susan	77.0	6.10	3.0	3.10	14.0	0.8	1.6
Fetterroll, Susan	119.0	7.40	4.50	2.90	15.0	0.8	5.0
Doyle, Susan	82.0	7.90	4.60	3.30	16.0	0.9	6.4
Hutchines, Geraldine	77.0	6.10	3.0	3.10	14.0	0.8	1.5
Stockert, Linda	78.0	9.0	4.90	4.10	20.0	0.90	2.3

Source: Dr. José Cabanillas & Colleagues
Lima – Peru

CLINIC HISTORIES

CASE PRESENTATION

1. STAD , RAYMOND
2. SMITH, TROY
3. O'CONNOR, PATRICK
4. LANGEN, R. HARRY
5. CHARRON, DANIEL
6. WHITE, SUSAN
7. FETTERROLL, SUSAN
8. DOYLE, SUSAN
9. HUTCHINES, GERALDINE
10. STOCKERT, LINDA

Clinic History 1

Date: 16-05-2004

.- Personal Information

Name	**Raymond Stad**
Date of birth	February 12, 1953
Age	51
Place of birth	Vancouver – Canada
Address	2913 Panorama Drive North Vancover B.C. V7G 2A4
Telephone number	604 -290 -7373
Marital Status	common-law
Occupation	worker in shipping terminal

II- Medical Record:

1.- Family Medical Record:

Mother with congestive heart failure.
Others unknown. He denies any family history of hepatitis.

2.- Personal Pathological Record:

Pneumonia in adolescence.
Renal lithiasis at age 44.
He reports diagnosis of portal hypertension and lack of platelets.
He denies any blood transfusion.
He denies surgical history.

3.- Hepatitis C Record:

Estimated date of contact	1970
Reason of exposure	possibly due to the use of drugs

4.- Allergy Record:

Drugs	sulfas
Food	he doesn't report any
Other	he doesn't report any

5.- Harmful Habits Record:

Tobacco	smoked until 1974
Marihuana	from 1974 until 2003
Alcohol	he doesn't consume
Coffee	until 6 years ago
Drugs	he doesn't report any

6.- Treatment Record:

He used Interferon and Ribavirin for 2 months in 2002.
Other: cat's claw, timosin, vitamin B, garlic, vera Reishi.

III.- Present Disease:

Duration of disease	7 years
Onset	insidious
Course	progressive

Symptoms and Signs:

Symptoms: feeling of general discomfort, exhaustion, severe fatigue, extreme weakness, depression. General muscular aching and arthralgia, difficulty sleeping, increased urinary frequency at night. He doesn't report digestive problems: abdominal swelling, constipation, diarrhea, nausea, vomiting, blood in stools or abdominal pain.

He doesn't report headaches, dizziness, blurred vision, shortness of breath or any other pain.

Signs: mild jaundice.

Physical Examination:

Height	1.80 m. (5'8´´)
Weight	97 Kg. (215 pounds)
Body mass index	33
Blood pressure	148/90 mm. Hg.
Heart rate	78 beats per minute
Respiratory rate	18 breaths per minute
Temperature	36.5° C

The patient is a well developed and well nourished Caucasian male, with no feeling of fatigue, that walks without apparent difficulty.

Physical Examination- Preferential.

Patient with mild conjunctival icterus.

Soft, tender, non-sensitive abdomen. No presence of collateral circulation or hernia. Deep palpation revealed a small increase of liver size. Normal intestinal sounds. No presence of ascites. The spleen can't be palpated.

Further Clinical Examination.

Warm, dry, non-turgid skin. No rashes or skin lesions are observed.
Pearl ears, nose and throat. Pinkish and moist oral mucus. Normal oropharynx.

Flexible neck. No adenopathies in neck, axilla and inguinal regions.

RESPIRATORY.- clear lungs. No abnormal sounds.

CARDIOVASCULAR.- regular rate and rhythm, without abnormal sounds or murmurs.

MUSCULOSKELETAL.- no weakness or atrophy, no limitation to articular movement. He walks normally.

EXTREMITIES.- no deformities, cyanosis or edema.

NEUROPSYCHIATRIC.- awake, alert and well oriented.

Normal state of mind. Symmetric reflexes.

IV.- Other Data:

4/10/04 LIVER BIOPSY.- fibrosis in phase 4/4 according to LUDWING's criteria. (Cirrhosis) focal iron stains classified as 1/4 inside Kupffer cells.

7/6/02 ABDOMINAL ULTRASOUND.- 19 cm. increase in liver size, fat infiltration. Mild hepatosplenomegaly.

10/1/00 viral load 2,106,111 copies/ml.

12/2/02	GPT (ALT)	545 U/L
	GOT (AST)	321 U/L
	GGT	164 U/L
	Ferritin	1783 ug/

Clinic History 2

Date: 16-05-2004

I.- Personal Information:

Name	**Troy D. Smith**
Date of birth	January 27, 1967
Age	37
Place of birth	Penticton – Canada
Address	1202 Penticton Av. Penticton, B.C.V2A 2N4
Telephone number	250-490 -8049
Marital Status	married
Occupation	truck driver

II- Medical Record:

1.- Family Medical Record:

Father with arterial hypertension.
Mother with coronary disease and psoriasis.

2.- Personal Pathological Record:

History of hypertension.
Peptic ulcer.
Pneumonia.
Renal calculus.
Fractures due to accident.

3.- Hepatitis C Record:

In 1986, truck accident, multiple reconstructive surgeries are performed on him (face and skull, as well as fractured leg). He received blood transfusion.
Reason of exposure: possibly due to blood transfusion.

4.- Allergy Record:

Food	peanuts
Drugs	he doesn't report any
Other	he doesn't report any

5.- Harmful Habits Record:

Tobacco	he doesn't smoke
Alcohol	he doesn't consume
Coffee	occasionally
Drugs	he doesn't consume

6.- Treatment Record:

ADVIL, calcium, magnesium and tylenol.

III.- Present Disease:

Duration of disease	8 years
Onset	insidious
Course	progressive

Symptoms and Signs:

Symptoms: feeling of fatigue, "I always have to fight to stay awake", light-headed, he frequently forgets things. He reports acidity, chronic abdominal pain, nausea. Perennial articular pain, depression and mood changes.

Signs: no signs have been observed.

Physical Examination:

Height	1.80 m. (5'8'')
Weight	97 Kg. (215 pounds)
Body mass index	32
Blood pressure	140/100 mm. Hg.
Heart rate	72 beats per minute
Respiratory rate	18 breaths per minute
Temperature	36.5° C

The patient is a well developed and well nourished Caucasian male.

Physical Examination- Preferential

Tenderness in abdomen, with mild sensitivity in right superior quandrant. No organomegaly, masses or liquids upon deep palpation. Normal intestinal sounds.

Further Clinical Examination.

Warm, dry skin Normal turgency with some scarring.

Ears, nose and throat: facial surgery scarring.

Equal pupils, reactive to light, with mild conjunctival icterus. Pinkish and moist oral mucus. No erythema.

Flexible neck.. No adenopathies in neck, axilla and inguinal regions.

RESPIRATORY.- clear lungs upon auscultation. No abnormal noises, rhonchi or wheezing. CARDIOVASCULAR.- regular rate and rhythm. No abnormal acceleration, sounds or murmurs. Tracheotomy scarring.

MUSCULOSKELETAL.- no weakness or atrophy, intact articular movement. He walks normally.

EXTREMITIES.- scarring in right thigh and back of the hip. No deformities, cyanosis or edema.

NEUROPSYCHIATRIC.- awake, alert and oriented 3. Normal state of mind. Deep symmetric reflexes in tendons. No trembling. Craneal nerves from II to XII are intact.

IV.- Other Data:

12/2/96 LIVER BIOPSY.- he presents mild fibrosis in portal triad. Mild fat infiltration. Moderate iron stains.

10/8/96 GPT (ALT) = 297UI/L
Iron 36 Umd/L

4/18/00 GOT (AST) = 97 UI/L
GPT (ALT) = 163 UI/L

Clinic History 3

Date: 16-05-2004

.- Personal Information

Name_____ **Patrick O'connor**
Birth Date_____ November 19, 1957
Age_____ 47
Place of birth_____Vancouver – Canada
Address_____Apt. 103 2407 Wall Street Vancouver British.
Telephone_____604 – 562 - 0570
Marital Status_____single
Occupation_____carpenter

II- Medical Record:

1.- Family Medical Record:

Father with arteriosclerotic disease
Mother with con arthritis
Brother with hepatitis C
Sister with hepatitis B and asthma

2.- Personal Pathological Record:

Appendectomy in 1970
Aneurysm repair in 1991
He denies any blood transfusion

3.- Hepatitis C Record:

Estimated date of contact_____1983
Reason of exposure_____possibly due to the use of drugs

4.- Allergy Record:

Medicaments_____he doesn't report any
Food_____he doesn't report any
Other_____he doesn't report any

5.- Harmful Habits Record:

Tobacco	consumes up to date
Marihuana	since 1974 to date
Alcohol	quit in 1999
Coffee	occasionally
Drugs	valium from 1979 to 1987
Cocaine	from 1979 until 1987
Heroine	in 1983

6.- Treatment Record:

He used Interferon for 6 months in 2000
He used Dialanton 1992

III.- Present Disease:

Duration of disease	5 years
Onset	insidious
Course	progressive

Symptoms and Signs:

Symptoms: chronic fatigue, mild headache, back pain and mild shoulder pain. He presents pain around the thoracic box, chronic pain in articulations, myalgia, nauseas and heartburn on occasion. He doesn't report constipation or diarrhea. He does not present fever, stinging or lack of oxygen.

Signs: no signs have been observed.

Physical Examination:

Height	1.80 m.
Weight	72.3 Kg. (159 libras)
Body mass index	26
Blood pressure	144/80 mm. Hg.
Heart rate	56 beats per minute
Respiratory rate	18 breaths per minute
Temperature	36.7° C

The patient is a well developed and well nourished Caucasian male, without acute exhaustion.

Physical Examination- Preferential.

Soft, tender, non-sensitive abdomen. No presence of collateral circulation or hernia upon deep palpation. No hepatosplenomegaly. Normal intestinal sounds. No presence of ascites.

Further Clinical Examination.

Warm, dry, non-turgid skin. No rashes or skin lesions are observed. He presents craniotomy scar.

Pearl ears, nose and throat. Pinkish and moist oral mucus. Normal oropharynx. Equal pupils, reactive to light, clear sclerotic, no jaundice, mild facial angioma.

Flexible neck. No adenopathies in neck, axilla and inguinal regions.

RESPIRATORY.- clear lungs. No abnormal sounds.

CARDIOVASCULAR.- regular rate and rhythm, without abnormal sounds or murmurs.

MUSCULOSKELETAL.- no weakness or atrophy, no limitation to articular movement. He walks normally.

EXTREMITIES.- no deformities, cyanosis or edema. Scar on right knee.

NEUROPSYCHIATRIC.- awake, alert and oriented 3.

Normal state of mind. Deep symmetric reflexes in tendons.

IV.- Other Data:

11/4/99 LIVER BIOPSY.- mild chronic hepatitic with slight fibrosis, with portal inflammation in phase 2 according to LUDWING's criteria, globular inflammation in phase 1, fibrosis in phase 1-2.

4/22/04	GPT (ALT)	105 U/L
	GOT (AST)	321 U/L

Clinic History 4

Date: 16-05-2004

I.- Personal Information:

Name	**Ron Harry Lagen**
Date of birth	August 06,, 1953
Age	51
Place of birth	Casteglar – Canada
Address	# B, 105 – 7th Av. Casteglar
Telephone number	250-354-9683
Marital Status	single
Occupation	writer, journalist

II Medical Record:

1.- Family Medical Record:

Father with chronic alcoholism
Mother with arterial hypertension

2.- Personal Pathological Record:

Pneumonia in 1992
Non- specified sexual transmitted disease
Bone fracture in 1980
Chronic alcoholism
Hepatitis B at 25 years old
Bronchial asthma for the past 10 years
Foot cyst extirpation in 1974

3.- Hepatitis C Record:

Estimated date of contact	1998
Reason of exposure	possibly due to the use of drugs

4.- Allergy Record:

Medicaments	aspirin
Food	he doesn't report any
Other	cats

5.- Harmful Habits Record:

Tobacco	consumes with moderation
Marihuana	occasionally
Alcohol	frequently until 2 years ago
Coffee	little
Drugs	Ativan

6.- Treatment Record:

He used Pegasys – Interferón until April 2004
Ventolin and other bronchodilator up to date
Vitamins C and D

III.- Present Disease:

Duration of disease	3 years
Onset	insidious
Course	progressive

Signs and Symptoms

Symptoms: fatigue, weight loss, stinging (pruritus), congestion, abdominal pain, heartburn, diarrhea, general pain in articulations, myalgia, weakness, depression and anxiety.

Signs: no signs have been observed.

Physical Examination:

Height	1.76 m. (5'9´´)
Weight	88 Kg. (166 pounds)
Body mass index	25
Blood pressure	110/60 mm. Hg.
Heart rate	88 beats per minute
Respiratory rate	18 breaths per minute
Temperature	36.5° C

The patient is a well developed and well nourished Caucasian male, without acute exhaustion.

Physical Examination- Preferential.

Tender abdomen, pain on epigastry upon palpation and painful sensitivity in right superior quandrant. Liver left lobe palpable at 7 cm under of costal rim.

Further Clinical Examination.

Warm, dry, non-turgid skin. No rashes or skin lesions are observed.

Pearl ears, nose and throat. Equal pupils, reactive to light, clear sclerotic. Some facial angiomas are observed. Clear tympanic membranes, pinkish and moist oral mucus with mild sublingual varicose veins, normal oropharynx.

Flexible neck, no jugular ingurgitation.

No adenopathies in cervix, axilla and inguinal regions. He shows mild gynecomastia in chest.

RESPIRATORY.- clear lungs upon auscultation. No abnormal noises, rhonchi or wheezing.

CARDIOVASCULAR.- regular rate and rhythm, without abnormal cardiac sounds, systolic ejection sound grade1/6 at right sternal border, without irradiation.

EXTREMITIES.- no deformities, cyanosis or edema, no bruises.

NEUROPSYCHIATRIC.- no motor sensory deficiency.
Craneal nerves from II to XII are intact.
The patient is alert and well oriented.

IV.- Other Data:

Negative chest X ray.

4/10/04	GOT (AST)	145 U/L
	GPT (ALT)	181 U/L
	Viral load	1 Million
	Platelets	127,000/mm^3

Clinic History 5

Date: 16-05-2004

I.- Personal Information:

Name	**Daniel Charron**
Date of birth	March 16, 1958
Age	46
Place of birth	Quebec – Canada
Address	3022 – CH du pont, vals des ronte San
Telephone number	819-457-1737
Marital Status	single
Occupation	social worker

II Medical Record:

1.- Family Medical Record:

Father died of heart disease at 53
Mother died of multiple sclerosis at 53
Brother died of lung cancer at 44

2.- Personal Pathological Record:

Fracture of tibia in 1976

3.- Hepatitis C Record:

Estimated date of contact	1998
Reason of exposure	possibly due to tatoo

4.- Allergy Record:

Medicaments	he doesn't report any
Food	he doesn't report any
Other	he doesn't report any

5.- Harmful Habits Record:

Tobacco	consumes regularly up to date
Marihuana	yes
Alcohol	little
Coffee	with moderation
Drugs	he doesn't report any

6.- Treatment Record:

He used Rebetron - Interferon from July 2001 until June 2002

III.- Present Disease:

Duration of disease	14 years
Onset	insidious
Course	progressive

Signs and Symptoms

Symptoms: general discomfort, fatigue, lack of concentration, muscular pain and arthralgia, difficulty to sleep, dizziness, alterations in digestion, nausea, semi-liquid stools, bloating after eating, depression.

Signs: no signs have been observed.

Physical Examination:

Height	1.72 m. (5'6'')
Weight	76 Kg. (170 pounds)
Body mass index	27
Blood pressure	120/70 mm. Hg.
Heart rate	76 beats per minute
Respiratory rate	18 breaths per minute
Temperature	36.5° C

The patient is a well developed and well nourished Caucasian male, without acute exhaustion.

Physical Examination- Preferential.

Tender abdomen, slight pain upon superficial and deep contact in right superior quadrant. The spleen and liver can't be palpated.

Further Clinical Examination.

Tattoos on right arm and chest, with mild erythema on chest possibly due to sun exposure.

Equal pupils, reactive to light, with mild conjunctival icterus. Pinkish and moist oral mucus.
Normal tympanic membranes. No erythema.

Flexible neck, no jugular ingurgitation.
No adenopathies in cervix, axilla and inguinal regions. He shows mild gynecomastia in chest.

RESPIRATORY.- clear lungs upon auscultation. No abnormal noises, rhonchi or wheezing.

CARDIOVASCULAR.- regular rate and rhythm, regular sounds, no acceleration.

EXTREMITIES.- no deformities, cyanosis or edema, no bruises.

NEUROPSYCHIATRIC.- no motor sensory deficiency.
Craneal nerves from II to XII are intact.
The patient is alert and well oriented.

IV.- Other Data:

Negative chest X rays.

6/13/01 LIVER BIOPSY.- periportal inflammation with necrosis grade 4, moderate intralobular degeneration grade 3, significant portal inflammation grade 4, compatible with moderate fibrosis.

3/7/01	Viral Count	545,000 c/UI
12/4/01	Viral Count	<600,000
4/5/03	GOT (AST)	19U/L
	GPT(ALT)	27U/L

Clinic History 6

Date: 16-05-2004

I.- Personal Information:

Name	**Susan White**
Date of birth	November 10, 1945
Age	58
Place of birth	Martella – Canada
Address	1373 – 8 Martella Rd. Ladysmith B.C. V 9 G
Telephone number	250-245-7654
Marital Status	divorced
Occupation	nursing assistant

II Medical Record:

1.- Family Medical Record:

Father with congestive heart disease and Diabetes Mellitus.
Mother with non-specified liver disease.
Brother with non-specified cancer.

2.- Personal Pathological Record:

Gastric ulcers
Non-specified fractures
Thyroid disorders (hipotiroidismo)
Rheumathoid arthritis
Pneumonia in 2004
Hepatorenal syndrome
Appendectomy in 1964
Hysterectomy in 1972

3.- Hepatitis C Record:

Estimated date of contact	1962
Reason of exposure	blood transfusion

4.- Allergy Record:

Medicaments	she doesn't report any
Food	she doesn't report any
Other	she doesn't report any

5.- Harmful Habits Record:

Tobacco_____quit 2 years ago
Marihuana_____no
Alcohol _____no
Coffee_____no
Drugs_____antidepressants daily

6.- Treatment Record:

She used Pegasys - Interferon from July 2002 until January 2003
She used Rivavirin in combination with treatment above
Folic acid 5mg. daily
Raberazole 10 mg.
Wellbutrin 150 mg. daily
Espironolactone 40 mg daily
Furosemine 40 mg daily
Conjugated estrogens 80 mg daily
Atrovent 4 puff per day
QVAR 2 puff per day
Salbutamol 2 puff per day
Tylenol for pain

III.- Present Disease:

Duration of disease_____ 30 years
Onset_____ insidious
Course_____ progressive

Signs and Symptoms

Symptoms: feeling of general discomfort, exhaustion, severe fatigue, extreme weakness, depression. Muscular pain, arthralgia, chronic back pain, difficulty to sleep, digestive problems such as chronic abdominal discomfort, heartburn and nausea.

Signs: Mild conjuctival jaundice
Edema in lower extremities
Ecchymosis and petechies in body

Physical Examination:

Height	1.72 m. (5'3´´)
Weight	80 Kg. (177 pounds)
Body mass index	30.5
Blood pressure	114/80 mm. Hg.
Heart rate	104 beats per minute
Respiratory rate	18 breaths per minute
Temperature	36.5° C

Patient with low energy, extreme fatigue, no fever or shivering.

Physical Examination- Preferential.

Tenderness in abdomen, with mild sensitivity in right superior quandrant. Normal intestinal sounds. Spleen and liver cannot be palpated.

Further Clinical Examination.

Normal ears, nose and throat. Equal pupils, reactive to light, with mild conjunctival icterus. Pinkish and moist oral mucus. Pinkish and moist oral mucus with sublingual varicose veins. Facial angioma.

Flexible neck, no jugular ingurgitation. No adenopathies in cervix, axilla and inguinal regions. She shows mild gynecomastia in chest.

RESPIRATORY.- clear lungs upon auscultation. No abnormal noises, rhonchi or wheezing.

CARDIOVASCULAR.- regular rate and rhythm, regular sounds, no acceleration.

EXTREMITIES.- marked edema found within 10 cm under tibia. Presence of circular bruise with a 2.8 cm de diameter in calf. Good irrigation, intact pulses.

NEUROPSYCHIATRIC.- no motor sensory deficiency.
Craneal nerves from II to XII are intact.
Difficulty to concentrate.

IV.- Other Data:

8/3/99 LIVER BIOPSY.- fibrous bands, with moderate chronic inflammatory infiltrations, normal iron deposits, ludwing's criteria, portal inflammation 3, lobular inflammation 2, fibrosis 4, cirrhosis stadium.

3/31/04	Platelet	134,000 mm^3
	Total bilirubin	65 mol/ L
	GOT (AST)	321 U/L
	GPT (ALT)	49 U/L

4/21/04 Bilirubin 37 Umol/L
GALLBLADDER X RAY.- Gallstone.

Clinic History 7

Date: 16-05-2004

I.- Personal Information:

Name	**Susan Fetterroll**
Date of birth	August 22, 1957
Age	46
Place of birth	New Jersey – USA
Address	2692 Hayes Ropad. Shuy Lerville, NJ 12871
Telephone number	518-695-5659
Marital Status	single
Occupation	gardener and elder caregiver

II Medical Record:

1.- Family Medical Record:

Mother died at 69 of unknown causes
Ignores information about fahter

2.- Personal Pathological Record:

Vocal cord surgery in 1992
Tubal ligation in 1988
Extirpation of benign breast tumor in 1990
She reports history of phlebitis and eczema

3.- Hepatitis C Record:

Estimated date of contact_____she doesn't report
Reason of exposure_____possibly due to the use of drugs

4.- Allergy Record:

Medicaments_____she doesn't report any
Food_____she doesn't report any
Other_____she doesn't report any

5.- Harmful Habits Record:

Tobacco	no
Drugs	all kinds
Alcohol	yes
Coffee	no
Medicine	Xanax 0.25 mg Lexapro 10 mg

6.- Treatment Record:

She doesn't report treatment for hepatitis.

III.- Present Disease:

Duration of disease	1 year
Onset	insidious
Course	progressive

Signs and Symptoms

Symptoms: fatigue, nasal congestion, general pain, depression, sleep alteration, heartburn, articular pain, general myalgia and anxiety.

Signs: no signs have been observed.

Physical Examination:

Height	1.72 m. (5'4'')
Weight	88 Kg. (193 pounds)
Body mass index	43
Blood pressure	134/84 mm. Hg.
Heart rate	88 beats per minute
Respiratory rate	16 breaths per minute
Temperature	36.5° C

The patient is a well developed and well nourished Caucasian female that does not present exhaustion.

Physical Examination- Preferential.

Tenderness in abdomen, with mild sensitivity in right superior quandrant. Normal intestinal sounds. No hernia. Normal liver and spleen size.

Further Clinical Examination.

Warm, dry skin with mild angioma. No rashes or lesions.

Normal ears, nose and throat. Equal pupils, reactive to light, clear, white sclerotic. Normal tympanic membranes. Pinkish and moist oral mucus.

Flexible neck, no jugular ingurgitation. No adenopathies in cervix, axilla and inguinal regions. She shows mild gynecomastia in chest.

RESPIRATORY.- clear lungs upon auscultation, no rhonchi or wheezing.

CARDIOVASCULAR.- regular rate and rhythm, regular sounds, no acceleration or abnormal noises.

EXTREMITIES.- no deformities, cyanosis or edema, no bruises.

NEUROPSYCHIATRIC.- no motor sensory deficiency.
Craneal nerves from II to XII are intact.
The patient is alert and well oriented.

IV.- Other Data:

Negative chest X rays.

1/8/04	Viral load	33, 600,000 UI/ml
	GOT (AST)	321 U/L
	GPT (ASP)	79 U/L
	Total bilirubin	1 mg/dl
	AFP	3, 4 ng/ml
	Creatinine	0.7 mg/dl

Clinic History 8

Date: 16-05-2004

I.- Personal Information:

Name_____ **Susan Doyle**
Date of birth _____ September 26, 1956
Age_____ 47
Place of birth_____ Belmont - Canada
Address_____ 2712 Belmont Av. Victoria B.C.
Telephone number_____ 250-595-9944
Marital Status_____single
Occupation_____rehabilitation therapist

II Medical Record:

1.- Family Medical Record:

Doesn't know info about father
Mother with arthritis and thyroid disease

2.-Personal Pathological Record:

Rheumatic fever at 11
Vertebral fracture in 1996
Hypothyroidism
Extirpation of ovarian cyst
Gallbladder surgery in 1979

3.- Hepatitis C Record:

Estimated date of contact_____1979
Reason of exposure_____surgery

4.- Allergy Record:

Medicaments_____sulfas
Food_____she doesn't report any
Other_____she doesn't report any

5.- Harmful Habits Record:

Tobacco	quit 6 years ago
Marihuana	yes
Alcohol	twice a week
Coffee	yes
Drugs	she doesn't report any

6.- Treatment Record:

She doesn't report treatment for hepatitis.

III.- Present Disease:

Duration of disease	5 years
Onset	insidious
Course	progressive

Signs and Symptoms

Symptoms: general discomfort, exhaustion, lack of concentration, difficulty to sleep, irritability and depression. Digestive alterations, nausea in the morning, semi-liquid stools, muscular pain and arthralgia, headaches.

Signs: no signs have been observed.

Physical Examination:

Height	1.60 m (5'1'')
Weight	50 Kg. (110 pounds)
Body mass index	21
Blood pressure	114/74 mm. Hg.
Heart rate	72 beats per minute
Respiratory rate	16 breaths per minute
Temperature	36.5° C

The patient is a well developed and well nourished Caucasian female that does not present exhaustion.

Physical Examination- Preferential.

Sensitivity in right superior and left inferior quandrans. No visceromegaly.

Further Clinical Examination.

Warm, dry, non-turgid skin.

Normal ears, nose and throat. Equal pupils, reactive to light, clear sclerotic. Pinkish and moist oral mucus with mild sublingual varicose veins. Normal oropharynx.

Flexible neck, no jugular ingurgitation. No adenopathies in cervix, axilla and inguinal regions. She shows mild gynecomastia in chest.

RESPIRATORY.- clear lungs upon auscultation, no rhonchi or wheezing.

CARDIOVASCULAR.- regular rate and rhythm, regular sounds, no acceleration or abnormal noises.

EXTREMITIES.- no deformities, cyanosis or edema, no bruises.

NEUROPSYCHIATRIC.- no motor sensory deficiency.
Craneal nerves from II to XII are intact.
The patient is alert and well oriented.

IV.- Other Data:

Negative chest X rays.

8/6/99 LIVER BIOPSY.- chronic hepatitis, with activity grade 1 to 2, fibrosis grade 1 to 2. No coloration due to iron.

4/1/04	Negative serologic hepatitis A and B	
	GOT (AST)	321 U/L
	GPT (ASL)	57U/L
	Total bilirubin	5 Mol/L

Clinic History 9

Date: 16-05-2004

I.- Personal Information:

Name	**Geraldine Hutchings**
Date of birth	June 19, 1947
Age	56
Place of birth	Vancouver - Canada
Address	1313-1030 Burnaby St. Vancouver B.C. VGE
Telephone number	604-632-9699
Marital Status	divorced
Occupation	transportation worker

II Medical Record:

1.- Family Medical Record:

Mother with hypertension
Brother with liver disease

2.- Personal Pathological Record:

Scarlet fever
Repetitive urinary infections
Traffic accident 30 years ago

3.- Hepatitis C Record:

Estimated date of contact	30 years
Reason of exposure	use of needles

4.- Allergy Record:

Medicaments	she doesn't report any
Food	she doesn't report any
Other	she doesn't report any

5.- Harmful Habits Record:

Tobacco	no
Marihuana	yes
Alcohol	yes
Coffee	no
Drugs	she doesn't report any

6.- Treatment Record:

She reports Interferon for 1 month and Pegasys.
Progestagens, antidepressants

III.- Present Disease:

Duration of disease	5 years
Onset	insidious
Course	progresivo

Signs and Symptoms

Symptoms: articular general pain, moderate fatigue, chronic liquid stools, loss of shor-term memory, back pain, weakness and depression.

Signs: no signs have been observed.

Physical Examination:

Height	1.80 m (6'0'')
Weight	65 Kg. (142 pounds)
Body mass index	24
Blood pressure	136/80 mm. Hg.
Heart rate	78 beats per minute
Respiratory rate	14 breaths per minute
Temperature	36.5° C

The patient is a well developed and well nourished Caucasian female that does not present exhaustion.

Physical Examination- Preferential.

Tender abdomen suave, so sensitivity.
No visceromegaly. Positive intestinal sounds.

Further Clinical Examination.

Warm, dry, turgid skin.

Normal ears, nose and throat. Equal pupils, reactive to light, clear sclerotic, no jaundice, slightly injected. Clear tympanic membranes. Pinkish and moist oral mucus. Normal oropharynx.

Flexible neck, no jugular ingurgitation. No adenopathies in cervix, axilla and inguinal regions. She shows mild gynecomastia in chest.

RESPIRATORY.- clear lungs upon auscultation, no rhonchi or wheezing or other noises.

CARDIOVASCULAR.- regular rate and rhythm, regular sounds, no acceleration.

EXTREMITIES.- no deformities, cyanosis or edema, no bruises.

NEUROPSYCHIATRIC.- no motor sensory deficiency.
Craneal nerves from II to XII are intact.
The patient is alert and well oriented.

IV.- Other Data:

20/03/ 2004

GOT (AST)	321 U/L
GPT (ALT)	46 U/L

1/20/04

Viral load	444,023 copies/ml

19 /07/2001 LIVER BIOPSY.- macrovesicular steatosis with mild non-specific portal and lobular inflammation. No fibrosis, cirrhosis or negative iron staining.

Clinic History 10

Date: 16-05-2004

I.- Personal Information:

Name	**Linda Stockert**
Date of birth	December 28, 1950
Age	53
Place of birth	Vancouver - Canada
Address	115-2200 Highbury Street Vancouver. B.C.
Telephone number	604-876-7487
Marital Status	single
Occupation	sales representative

II Medical Record:

1.- Family Medical Record:

Father with hypertension and Gillian Barre Syndrome
Mother with arterial hypertension.

2.- Personal Pathological Record:

Sexually transmitted disease
Curettage in 1988
Uterine neck biopsy in 1974
Extirpation of benign urethral polyp in 1980
Breast surgery in 1981and 1999
Total abdominal hysterectomy in 2000

3.- Hepatitis C Record:

Estimated date of contact	1971
Reason of exposure	surgery

4.- Allergy Record:

Medicaments	sulfas and andanesthetics
Food	dairy products and eggs
Other	dust, dog hair

5.- Harmful Habits Record:

Tobacco	no
Marihuana	no
Alcohol	no
Coffee	no
Drugs	she doesn't report any

6.- Treatment Record:

Korean herbs in 2000

III.- Present Disease:

Duration of disease	12 years
Onset	insidious
Course	progressive

Signs and Symptoms

Symptoms: fatigue, abdominal pain, heartburn, bloating after eating with gas production and painful discomfort in right superior quadrant of abdomen. Back, neck and articular pain. General pain, rigidity, headaches, weakness and difficulty to think clearly.

Signs: no signs have been observed.

Physical Examination:

Height	1.72 m (5'9´´)
Weight	62 Kg. (140 pounds)
Body mass index	21
Blood pressure	118/80 mm. Hg.
Heart rate	84 beats per minute
Respiratory rate	16 breaths per minute
Temperature	36.5° C

The patient is a well developed and well nourished Caucasian female that does not present acute exhaustion.

Physical Examination- Preferential.

Tenderness in abdomen, with mild epigastric sensitivity in right superior quandrant. No visceromegaly. Normal intestinal sounds.

Further Clinical Examination.

Warm, dry, turgid skin.

Normal ears, nose and throat. . Equal pupils, reactive to light, clear sclerotic, no jaundice, slightly injected. Clear tympanic membranes. Pinkish and moist oral mucus. Normal oropharynx.

Flexible neck, no jugular ingurgitation. No adenopathies in cervix, axilla and inguinal regions. She shows mild gynecomastia in chest.

RESPIRATORY.- clear lungs upon auscultation, no rhonchi or wheezing or other noises.

CARDIOVASCULAR.- regular rate and rhythm, regular sounds.

EXTREMITIES.- no deformities, cyanosis or edema, no bruises.

NEUROPSYCHIATRIC.- no motor sensory deficiency.
Craneal nerves from II to XII are intact.
The patient is alert and well oriented.

IV.- Other Data:

Negative chest X rays.

12/4/00 LIVER BIOPSY.- slightly active chronic hepatitis C, no cirrhosis. No iron staining.

4/1/04	Negative serologic hepatitis A and B	
3/26/04	GOT (AST)	107 U/L
	GPT (ALT)	35 U/L
	Viral load	63,700 copies/ml

EVALUATION OF CLINIC HISTORIES

1. DEFINITION OF TERMS

2. RESULTS

Definition of symptoms

To evaluate and tabulate the discomforts presented by patients, expressed in different manners, we made two lists of symptoms : general and gastrointestinal. We also made a group of signs related to chronic hepatitis found in the clinical examinations of patients. They are cited below:

General symptoms:

1.- **General discomfort.-** expressed as upset feeling, feeling uncomfortable or feeling of being sick.

2.- **Severe fatigue.-** expressed as feeling of exhaustion, difficulty to walk or climb stairs, feeling of weakness.

3.- **Lack of concentration.-** expressed as difficulty to carry out work, feeling light headed, feeling of decreased abilities.

4.- **Sleep disorders.-** expressed as difficulty to fall asleep, awaken in the middle of the night, interrupted sleep, having to sleep some hours during the afternoon.

5.- **Depression.-** expressed as feeling diminished, discouraged, depressed almost all the time.

6.- **Articular pain.-** expressed as pain in articulations, with or without movement, erratic and of varied intensity.

7.- **Muscular pain.-** expressed as general pain in different muscular masses along the body.

8.- **Headaches.-** expressed as cephalalgia and dizziness.

Gastrointestinal symptoms:

1.- **Indigestion.-** expressed as abdominal discomfort and heartburn.

2.- **Dyspepsia**.- expressed as feeling full, abdominal bloating, slow digestion or failure to digest food.

3.- **Nausea.-**expressed as feeling nauseous.

4.- **Abdominal pain.-** in relation to food or not.

5.- **Intestinal dysfunction.-** expressed as presence of semi-liquid stools, occasional diarrhea or constipation.

A summary of the 10 clinic histories allows us to establish a table of symptoms and signs that were present in patients in relation to Chronic hepatitis, and that we classified as follows:

General symptoms: general discomfort, severe fatigue, lack of concentration, sleep disorders, depression, articular pain, muscular pain and headache.

Gastrointestinal: indigestion with heartburn, dyspepsia with abdominal bloating, nausea, abdominal pain and intestinal dysfunction.

Signs: jaundice, ascites, collateral circulation, pain upon palpation, palpable spleen, presence of ecchymosis and edema in lower extremities.

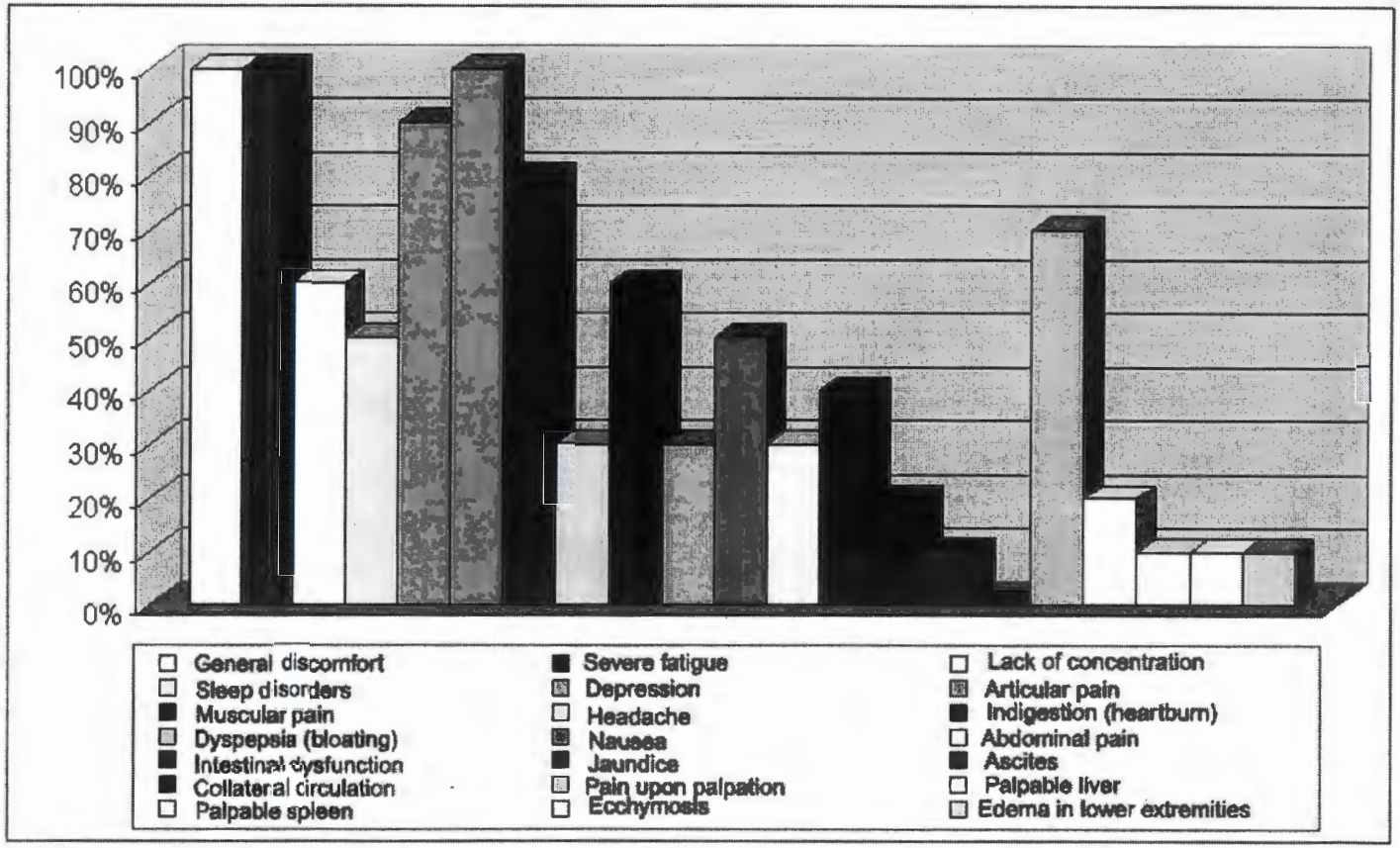
The results are shown in **Table 12 and Figure 1.**

Table 12
Chronic Hepatitis C (HCV)
Symptoms and signs

Síntomas	Stad, R.	Troy, S.	O'Connor	Langen	Charron	White	Fetterroll	Doyle	Hutchines	Stockert	
I) General											
1.- General discomfort	+	+	+	+	+	+	+	+	+	+	**100%**
2.- Severe fatigue	+	+	+	+	+	+	+	+	+	+	**100%**
3.- Lack of concentration	-	+	-	-	+	+	-	+	+	+	**60%**
4.- Sleep disorder	+	-	-	-	+	+	+	+	-	-	**50%**
5.- Depression	+	+	+	+	+	+	+	+	+	-	**90%**
6.- Articular pain	+	+	+	+	+	+	+	+	+	+	**100%**
7.- Muscular pain	+	-	+	+	+	+	+	+	-	+	**80%**
8.- Headache	-	-	+	-	-	-	-	+	-	+	**30%**
II) Gastrointestinal											
1.- Indigestion (heartburn)	-	+	+	+	+	+	+	-	-	-	**60%**
2.- Dyspepsia (bloating)	-	-	-	-	+	+	-	-	-	+	**30%**
3.- Nausea	-	+	+	-	+	+	-	+	-	-	**50%**
4.- Abdominal pain	-	+	-	+	+	-	-	-	-	+	**30%**
5.- Intestinal dysfunction	-	-	-	+	+	+	-	+	+	-	**40%**
Signs											
1.- Jaundice	+	-	-	-	-	+	-	-	-	-	**20%**
2.- Ascites	-	-	-	-	-	+	-	-	-	-	**10%**
3.- Collateral circulation	-	-	-	-	-	-	-	-	-	-	**0%**
4.- Pain upon palpation	-	+	-	+	+	+	+	+	-	+	**70%**
5.- Palpable liver	+	-	-	+	-	-	-	-	-	-	**20%**
6.- Palpable spleen	-	-	-	+	-	-	-	-	-	-	**10%**
7.- Ecchymosis	-	-	-	-	-	+	-	-	-	-	**10%**
8.- Edema in lower extremities	-	-	-	-	-	+	-	-	-	-	**10%**

Source: Dr. José Cabanillas & Colleagues
Lima - Peru

Figure 01
Chronic Hepatitis C (HCV)
Symptoms and Signs:

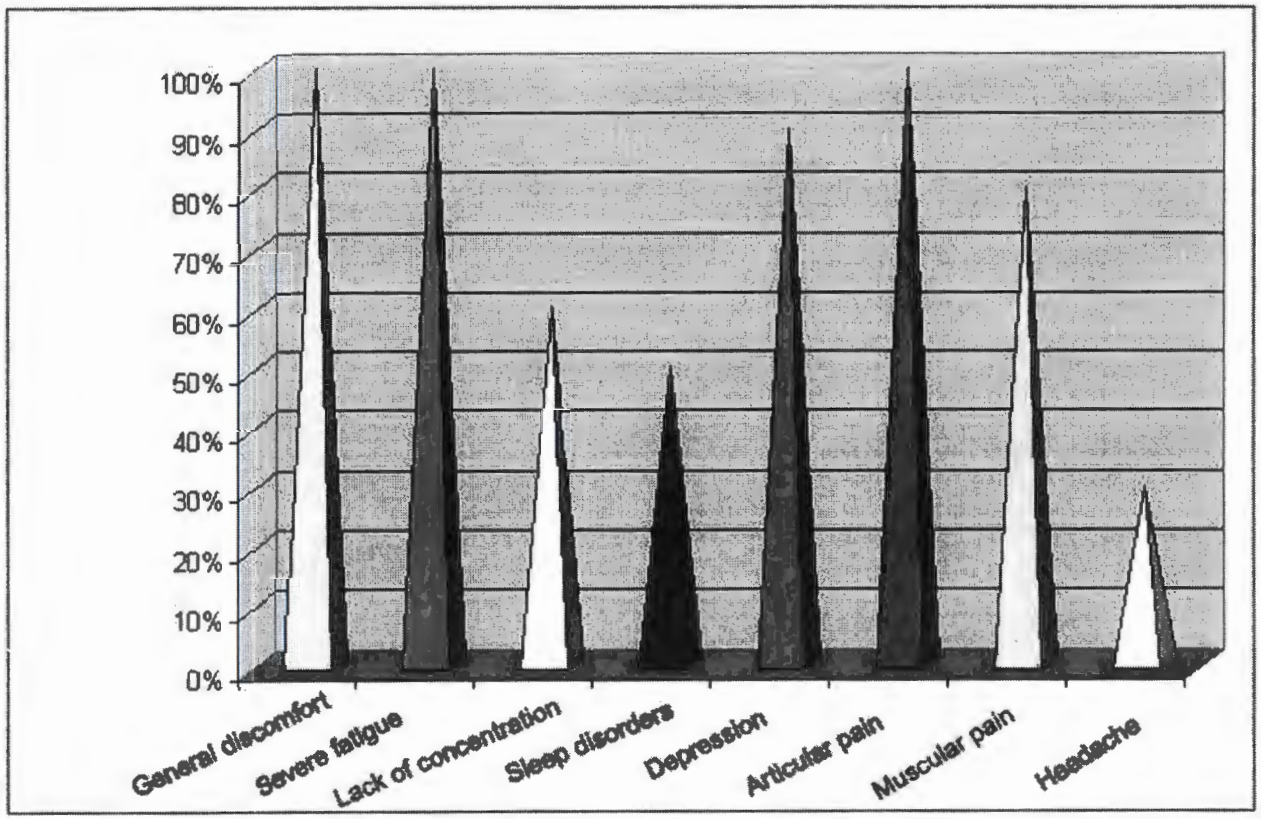


Source: Dr. José Cabanillas & Colleagues
Lima – Peru

The study of clinical histories allows us to establish that in the **general symptoms** group; a feeling of general discomfort, of fatigue, and osteoarticular pain was present in all ten patients, what constitutes 100%; depression was present in 90%; muscular pain attained a frequency of 80%; while lack of concentration and sleep problems were present in 60 and 50% respectively; and headache attained 30%.

See Table 12 and Figure 02

Figure 02
Chronic Hepatitis C (HCV)
General symptoms:

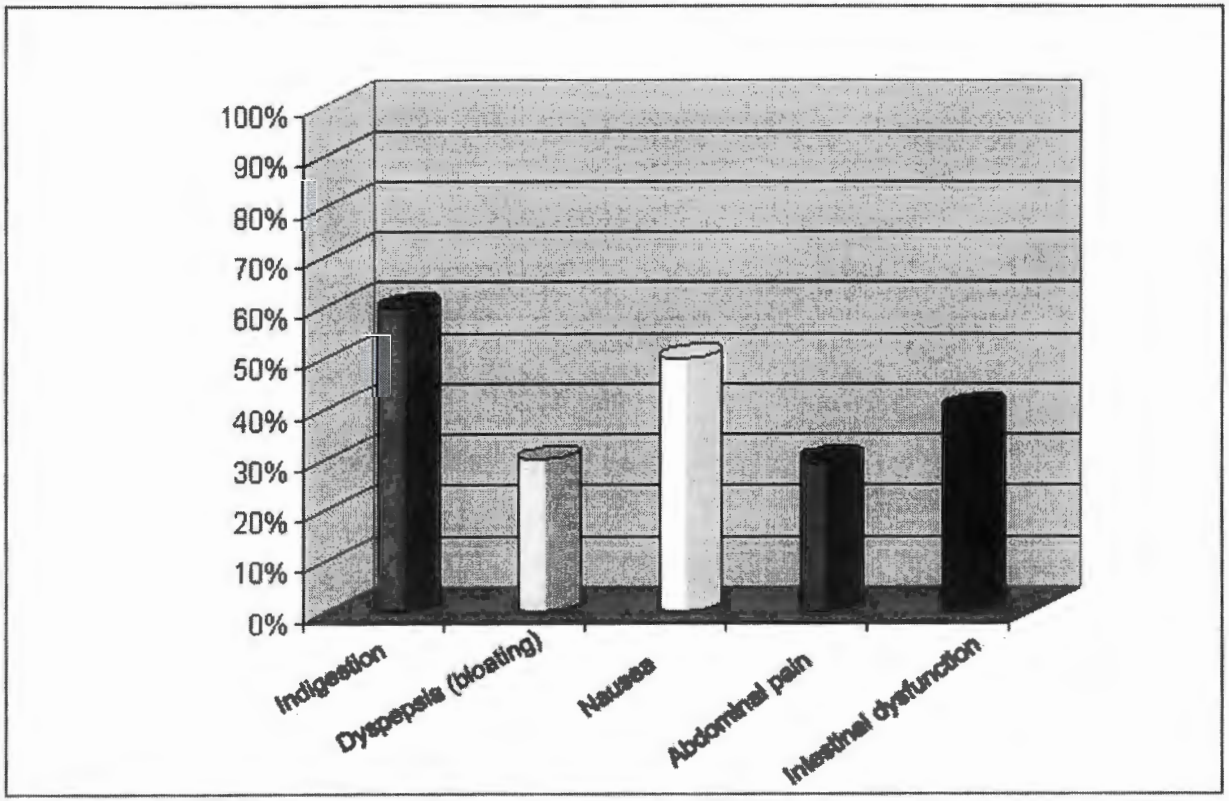


Source: Dr. José Cabanillas & Colleagues
Lima – Peru

In the group of **gastro-intestinal symptoms**: indigestion, referred to as abdominal discomfort and/or heartburn was present in 60% of the cases; nausea was present in 50%, intestinal dysfunction attained 40%; while dyspepsia, stomach bloating and abdominal pain were present in 30% of the cases.

See Table 12 and Figure 3

Figure 3
Chronic Hepatitis C (HCV)
Gastrointestinal symptoms:

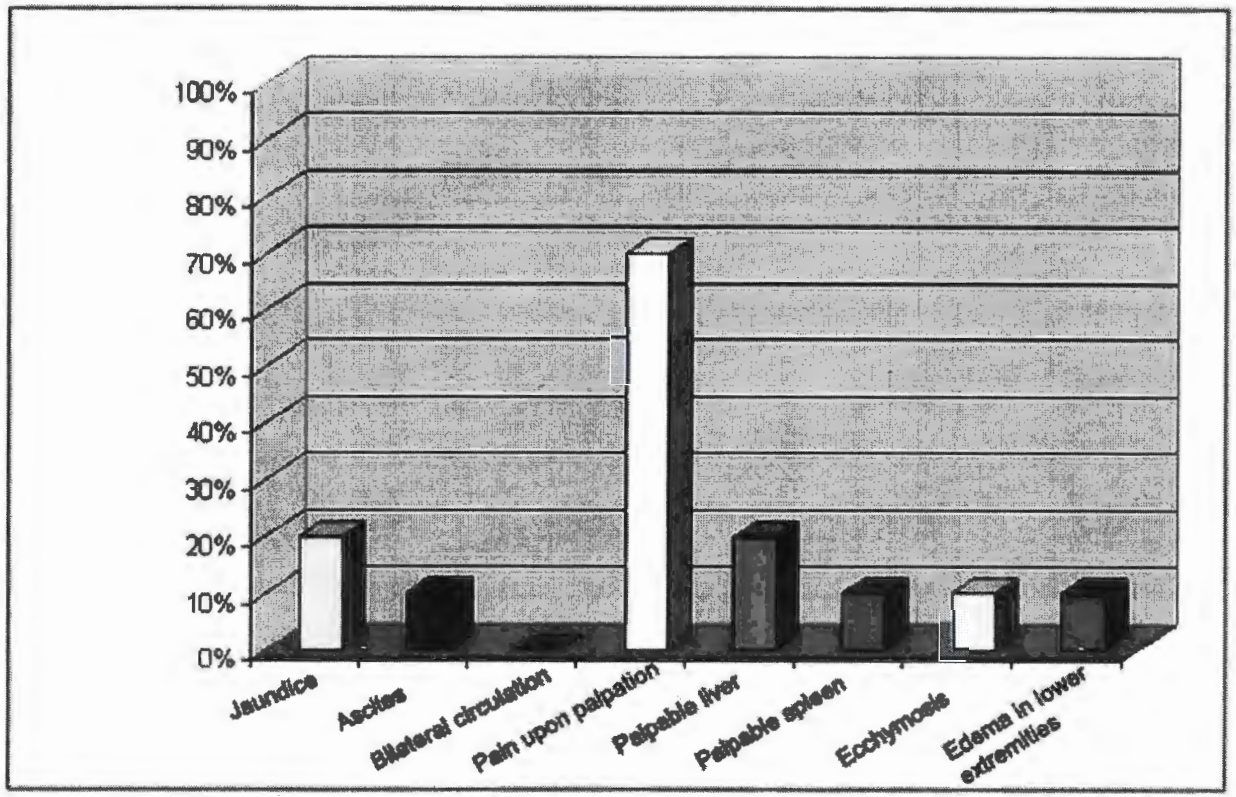


Source: Dr. José Cabanillas & Colleagues
Lima – Peru

In the clinical findings collected through clinical examination, the most frequent **sign** were abdominal pain, palpitation primarily at the level of the right hypochondrio and epigastrium, which was present in 70% of the cases; jaundice and palpable liver were found in 20%; while the presence of ascites, palpable spleen, ecchymosis, and edema in lower extremities were present in only one case representing 10%; no patient showed collateral circulation, 0%.

See Table 12 and Figure 4

Figure 04
Chronic Hepatitis C (HCV)
Signs



Source: Dr. José Cabanillas & Colleagues
Lima – Peru

Diagnosis

The assessment of the clinical records, as well as the information obtained with the HCV Fibrosure, the ultrasounds and the laboratory analysis, allowed us to establish the diagnosis and stadium of each patient.

Stad, Raymond

Chronic Hepatitis C (HCV)
Stadium: cirrhosis
Arterial Hypertension
Low platelet count

Smith, Troy

Chronic Hepatitis C (HCV)
Stadium: fibrosis 3
Diabetes mellitus

O'Connor, Patrick

Chronic Hepatitis C (HCV)
Stadium: fibrosis 3

Langen, R. Harry

Chronic Hepatitis C (HCV)
Chronic Hepatitis B
Stadium: fibrosis 2
Low platelet count

Charron, Daniel

Chronic Hepatitis C (HCV)
Stadium: cirrhosis

White, Susan

Chronic Hepatitis C
Chronic Hepatitis B
Stadium: cirrhosis
Low platelet count
Hepatorenal synd.

Fetterroll, Susan

Chronic Hepatitis C (HCV)
Chronic Hepatitis B
F0 no fibrosis

Doyle, Susan

Chronic Hepatitis C (HCV)
F0 no fibrosis

Hutchines, Geraldine

Chronic Hepatitis C (HCV)
F0 no fibrosis

Stockert, Linda

Chronic Hepatitis C (HCV)
Stadium: cirrhosis

Treatment

All patients underwent treatment with **Active Organic Ingredients: A4+**; 40 ml. orally, three times a day for 28 days, that was complemented with phototherapy by exposure of chest, arms and legs to sun rays during some hours a day.

There was no controlled diet; fat intake was low but there was no quantity restriction; different fruits were administered. Alcohol intake was not permitted.

Carbohydrate or salt intake was restricted for those patients that also suffer from other pathologies (diabetes mellitus, arterial hypertension) who also received regular treatment for their pathologies.

Patients that were taking other drugs, such as sedatives or tranquilizers, did not discontinue their previous treatment.

Evolution

After **14 days**, the clinical symptomatology of patients was controlled and they underwent biochemical analysis; a new evaluation was performed after **28 days**. It included the clinical examination, evolution of symptomatology, ultrasound control and laboratory tests, to be compared to those performed at the beginning of the study.

We will now present the results obtained in the control evaluation 28 after the **ULTRASOUND**, which are: in **six cases** a **favorable** evolution shown by a lower increase of diffuse Echogenicity in comparison with the first evaluation or a decrease in liver and spleen size; in **three cases** the ultrasound showed **stable** sings when compared to the first evaluation and in **one case**, the evolution was **unfavorable** with an increase of **Echogenicity** and an increase in volume of liquid of ascites. **Table 13**

Table 13
Chronic Hepatitis C (HCV)
Three-dimensional ultrasound (control after 28 days)

	Stad, Raymond	Smith, Troy	O'Connor, Patrick	Langen, Ron Harry	Charron, Daniel
	Favorable Evol.	**Stable Evol.**	**Stable Evol.**	**Stable Evol.**	**Stable Evol.**
Decrease	Echogenicity	Remained the same	Remained the same	Remained the same	Echogenicity
Liver	Hepatomegaly	Hepatomegaly	normal	Hepatomegaly	Hepatomegaly
Left lobe size	165 mm.	153 – 156 mm.	138 mm.	168 mm.	151-153 mm.
Right lobe size	114 – 116 mm.	103 – 116 mm.	92 mm.	121 mm.	113-122 mm.
Borders	Regular	Regular	Regular	Regular	Regular
Diffuse echogenicity	Slight Increase	Moderate Increase	Moderate Increase	Moderate Increase	Moderate Increase
Types of echos	Low-amplitude	High-amplitude	Medium-amplitude	Medium-amplitude	Medium-amplitude
Focal Injuries	no signs observed.	no signs observed.	no signs observed.	no signs observed.	no signs observed.
Portal vein	Normal Appearance	Brillo en pared portal	Normal	Hipertensión portal	Normal
Measurements	12mm.	11 – 13 mm.	12-13 mm.	16 mm.	13 mm.
Spleen	Splenomegaly	Splenomegaly	Normal	Splenomegaly	Normal
Measurements	132 x 66 mm.	157 x 81 mm.	92 x 41mm.	135 x 45 mm.	91 x 45 mm.
Splenic vein	8 mm.	8 mm.		4 mm.	
Ascites	Not present	Not present	Not present	Not present	Not present

Source: Dr. José Cabanillas & Colleagues
Lima – Peru

Favorable evolution was pointed as a **decrease of the increase of echogenicity**, that the liver presented in the first control or a **decrease in hepatic volume**.

Table 13 (Cont.)
Chronic Hepatitis C (HCV)
Three-dimensional ultrasound (control after 28 days)

	White, Susan Evoluc. Desfavorable	Fetterroll, Susan Favorable Evol.	Doyle, Susan Favorable Evol.	Hutchines, Geraldine Favorable Evol.	Stockert, Linda Favorable Evol.
Decrease	Increase ascites	Echogenicity	Liver volume	Echogenicity	Echogenicity
Liver	Decreased	Hepatomegaly	Normal	Normal	Hepatomegaly
Right lobe size	108 mm.	186mm.	133 mm.	130 mm.	146-149 mm.
Left lobe size	83 mm.	142mm.	104 mm.	104 mm.	105-124 mm.
Borders	Regular	Regular	Regular	Regular	Regular
Diffuse echogenicity	High Increase	Slight Increase	Slight Increase	Slight Increase	Moderate Increase
Types of echos	high = cirrhosis	Low-amplitude	Low-amplitude	Low-amplitude	Medium and High
Focal Injuries	no signs observed.	no signs observed.	no signs observed.	no signs observed.	no signs observed.
Portal vein	Hypertension Signs	Hypertension Signs	Normal	Normal	Brillo en pared portal
Measurements	13mm.	11 – 14 mm.	11 mm.	10 mm.	15 mm.
Spleen	Splenomegaly	normal	Normal	Normal	Splenomegaly
Measurements	121 x 51 mm.	106 x 62 mm.	87 x 45mm.	97 x 51 mm.	112 x 58 mm.
Splenic vein					
Ascites	present	Not present	Not present	Not present	Not present

Source: Dr. José Cabanillas & Colleagues
Lima – Peru

Favorable evolution was pointed as a **decrease of the increase of echogenicity**, that the liver presented in the first control or a **decrease in hepatic volume**.

As part of the control of tests to measure **liver synthesis capacity**, determination of **CHOLINESTERASE** was performed after 14 and 28 days; while determination of **PREALBUMIN** and **PROTHROMBIN TIME** was only performed after 28 days of treatment.

Since cholinesterase is a product of the synthesis of hepatic metabolism, an increase of its values can be interpreted as an improvement of the function of the hepatocyte.

When analyzing the values found in the determination of **CHOLINESTERASE** between the **initial and the final controls**, we could observe that in **three cases** there was a **high increase** (between 71.1 and 83.1%), in **two cases** there was a **moderate increase** (between 62.1 and 54.9%), in **four cases** there was a **regular increase** (between 34.0 and 40.0%) and in **one case** the increase was **minimum** (15.7%).

See Table 14 and Figure 5

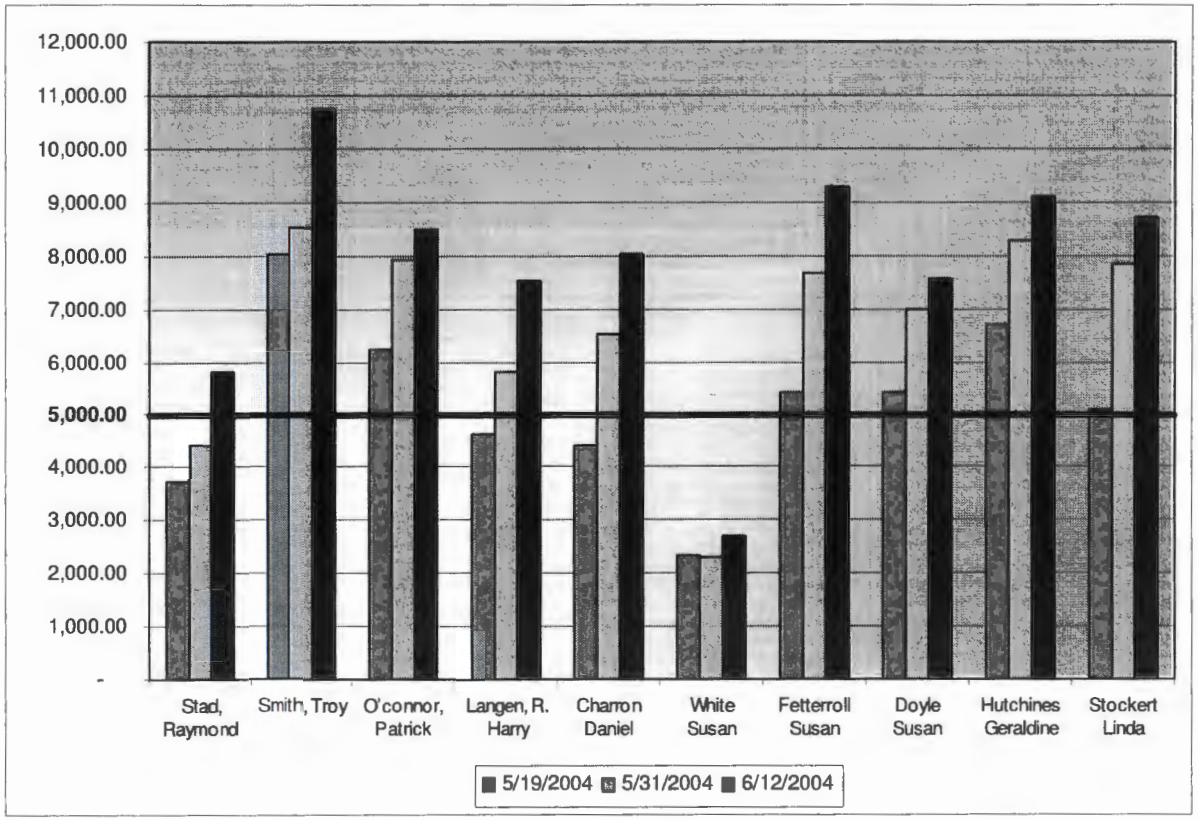
Table 14
Chronic Hepatitis C (HCV)
Evolution of Cholinesterase

	Cholinesterase	Cholinesterase	Cholinesterase	Modification
Date of control	**5/19/04**	**5/31/04**	**6/12/04**	
PATIENTS				
Stad, Raymond	3,742 U/L	4,397 U/L	5,797 U/L	Increase 54.9%
Smith, Troy	8,018 U/L	8,540 U/L	10,749 U/L	Increase 34.0%
O'Connor, Patrick	6,241 U/L	7,905 U/L	8,505 U/L	Increase 36.2%
Langen, R. Harry	4,631 U/L	5,818 U/L	7,507 U/L	Increase 62.1%
Charron, Daniel	4,390 U/L	6,507 U/L	8,039 U/L	Increase 83.1%
White, Susan	GOT (AST) 321 U/L	2,299 U/L	2,687 U/L	Increase 15.7%
Fetterroll, Susan	5,408 U/L	7,673 U/L	9,280 U/L	Increase 71.5%
Doyle, Susan	5,408 U/L	6,973 U/L	7,573 U/L	Increase 40.0%
Hutchines, Geraldine	6,696 U/L	8,284 U/L	9,106 U/L	Increase 35.9%
Stockert, Linda	5,086 U/L	7,828 U/L	8,706 U/L	Increase 71.1%

Source: Dr. José Cabanillas & Colleagues
Lima – Peru

Since **cholinesterase** is a product of the synthesis of hepatic metabolism, an **increase** of its values is interpreted as an improvement of liver function.

Figure 05
Chronic Hepatitis C (HCV)
Evolution of Cholinesterase



Source: Dr. José Cabanillas & Colleagues
Lima – Peru

Since **cholinesterase** is a product of the synthesis of hepatic metabolism, an **increase** of its values is interpreted as an improvement of liver function.

When analyzing the values found in the determination of **PREALBUMIN** between the **initial and the final controls**, we could observe that in **three cases** there was a **significant increase** (between 19.5 and 26.9%), in **four cases** there was a **significant decrease** (between 18.0 and 28.2%) and in **three cases** there were **no significant variations** (between 0.0 and 5.0%).

See Table 15 and Figure 6

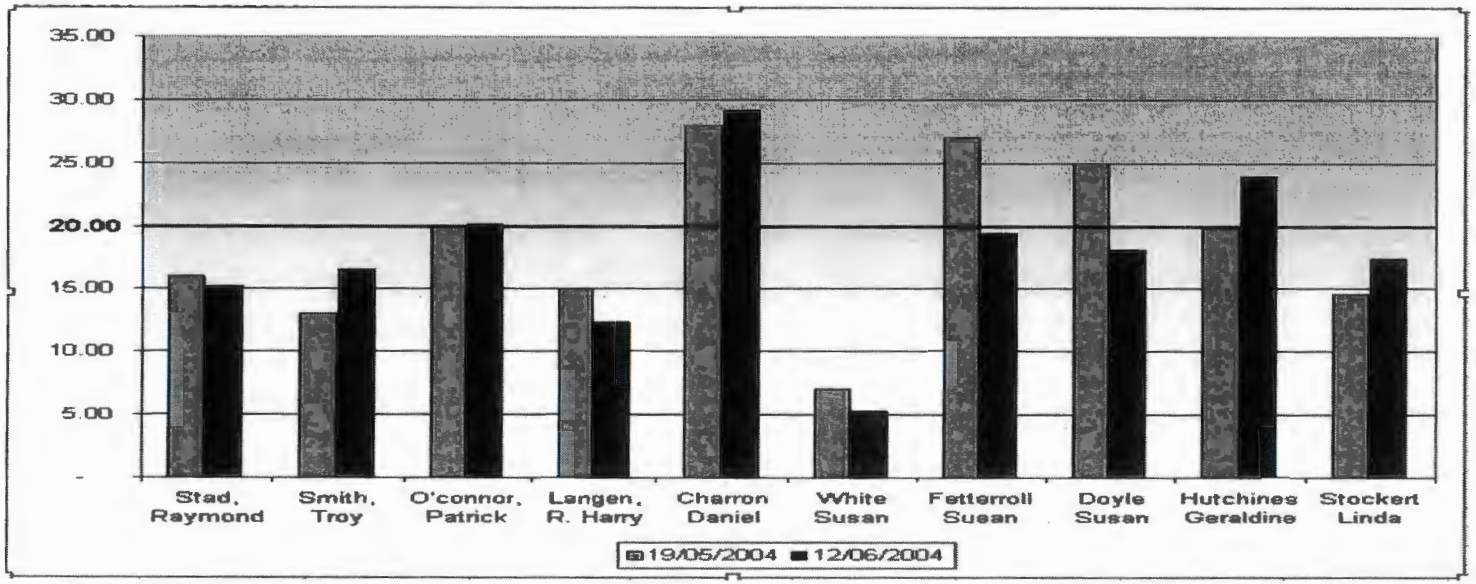
Table 15
Chronic Hepatitis C (HCV)
Evolution of Prealbumin

	Prealbumin	Prealbumin	Modification	
Dates of control	**5/19/04**	**6/2/04**		
PATIENTS				
Stad, Raymond	16.0 mgr/dl	15.2 mgr/dl	Decreased	5.0%
Smith, Troy	13.0 mgr/dl	16.5 mgr/dl	Increased	26.9%
O'Connor, Patrick	20.0 mgr/dl	20.1 mgr/dl	No variation	0.0%
Langen, R. Harry	15.0 mgr/dl	12.3 mgr/dl	Decreased	18.0%
Charron, Daniel	28.0 mgr/dl	29.2 mgr/dl	Increased	0.4%
White, Susan	7.0 mgr/dl	5.3 mgr/dl	Decreased	24.7%
Fetterroll, Susan	27.0 mgr/dl	19.4 mgr/dl	Decreased	28.2%
Doyle, Susan	25.0 mgr/dl	18.1 mgr/dl	Decreased	27.6%
Hutchines, Geraldine	20.0 mgr/dl	23.9 mgr/dl	Increased	19.5%
Stockert, Linda	14.6 mgr/dl	17.4 mgr/dl	Increased	24.2%

Source: Dr. José Cabanillas & Colleagues
Lima – Peru

Since **prealbumin** is a product of the synthesis of hepatic metabolism, an **increase** of its values is interpreted as an **improvement of liver function**.

Figure 06
Chronic Hepatitis C (HCV)
Evolution of Prealbumin



Source: Dr. José Cabanillas & Colleagues
Lima - Peru

Since **prealbumin** is a product of the synthesis of hepatic metabolism, an **increase** of its values is interpreted as an **improvement of liver function**.

When analyzing the values found in the determination of **PROTHROMBIN TIME** between the **initial and the final controls**, established through comparison of **PROTHROMBIN CONCENTRATION** percentages, we could observe that in **four cases** there was an increase in a **high percentage** (between 24.8% and 33.3%); in **four cases** there was a **moderate increase** (between 19.2% and 8.8%); in **one case** there was a **decrease** of 9.0%, while in the remaining case there was **no significant variation**, with a decrease of 0.5%. See **Table 16 and Figure 7**

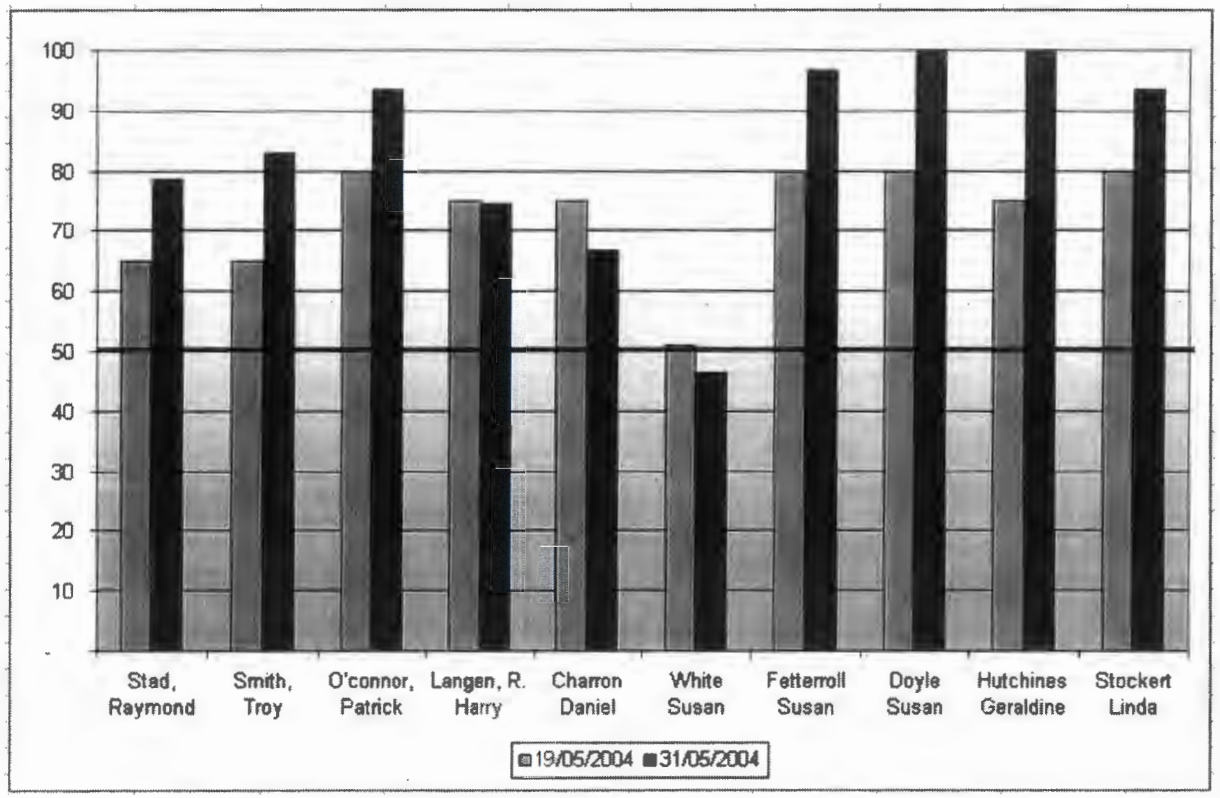
Table 16
Chronic Hepatitis C (HCV)
Evolution of Prothrombin time

	Prothrombin time	Concentr. of Prothrombin	Prothrombin time	Concentr. of Prothrombin	Modification
Dates of control	**5/19/04**	**5/19/04**	**6/12/04**	**6/12/04**	
PATIENTS					
Stad, Raymond	15.0 seg.	65.0%	13.2 seg.	78.6%	Increased 24.8%
Smith, Troy	15.0 seg.	65.0%	12.8 seg.	83.0%	Increased 27.7%
'Connor, Patrick	13.0 seg.	80.0%	12.0 seg.	93.6%	Increased 17.0%
angen, R. Harry	14.0 seg.	75.0%	13.6 seg.	74.6%	Decreased 0.5%
Charron, Daniel	14.0 seg.	75.0%	12.5 seg.	66.7%	Increased 16.0%
White, Susan	18.0 seg.	51.0%	18.4 seg.	46.3%	Decreased 9.0%
Fetterroll, Susan	13.0 seg.	80.0%	11.8 seg.	96.6%	Increased 19.2%
Doyle, Susan	13.0 seg.	80.0%	11.5 seg.	100%	Increased 25.0%
tchines, Geraldine	14.0 seg.	75.0%	11.5 seg.	100%	Increased 33.3%
Stockert, Linda	13.0 seg.	80.0%	12.0 seg.	93.6%	Increased 8.8%

Source: Dr. José Cabanillas & Colleagues
Lima – Peru

Since **prothrombin** is a product of the synthesis of hepatic metabolism, an **increase** of its values is interpreted as an **improvement of liver function**.

Figure 07
Chronic Hepatitis C (HCV)
Evolution of Prothombin Concentration



Normal Values

Source: Dr. José Cabanillas & Colleagues
Lima – Peru

Since **prothrombin** is a product of the synthesis of hepatic metabolism, an **increase** of its values is interpreted as an **improvement of liver function**.

As part of the tests conducted to assess **liver structural alterations** caused by fibrosis and hepatocyte degeneration, determination of **BILIRUBIN** and **ALKALINE PHOSPHATASE** was perfomed.

The comparative analysis of bilirubin control between the **beginning and the end** of treatment showed that out of the **four cases** with values above normal, three attained a **decrease** of initial values in 4.6%, 36.9% and 15.4%, while in the remaining case there was an increase of 23.3%. In the other **six cases** values, that were within normal limits, stayed **the same**. See Table 17 and Figure 8

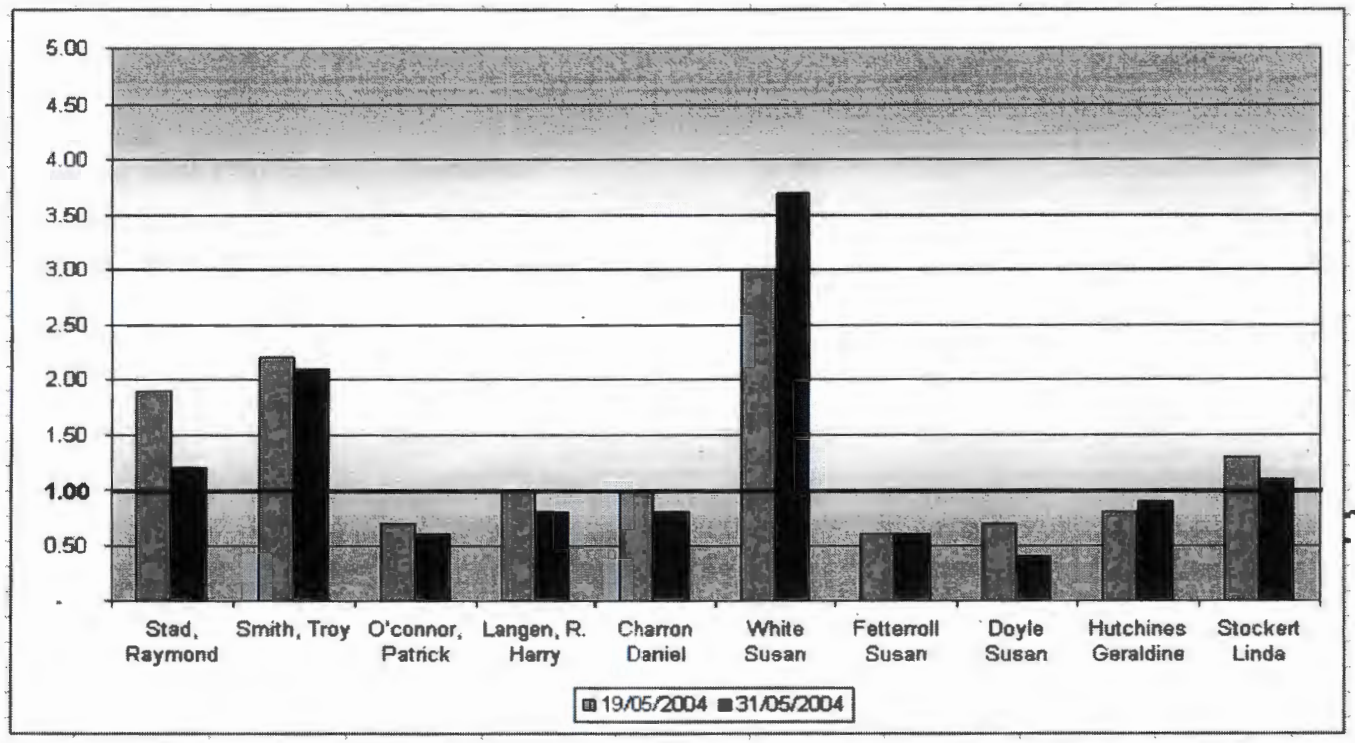
Table 17
Chronic Hepatitis C (HCV)
Evolution of Bilirubin

	Bilirubin						
	Total	Direct	Indirect	Total	Direct	Indirect	Modifications
Dates of control	5/19/04	5/19/04	5/19/04	12/06/2004	6/12/04	6/12/04	
PATIENTS							
Stad, Raymond	1.90 mg/dl	0.60 mg/dl	1.30 mg/dl	1.20 mg/dl	0.50 mg/dl	0.70 mg/dl	Decreased 36.9%
Smith, Troy	2.20 mg/dl	0.60 mg/dl	1.60 mg/dl	2.10 mg/dl	0.60 mg/dl	1.50 mg/dl	Decreased 4.6%
O'Connor, Patrick	0.70 mg/dl	0.30 mg/dl	0.40 mg/dl	0.60 mg/dl	0.20 mg/dl	0.40 mg/dl	Normal
Langen, R. Harry	1.00 mg/dl	0.40 mg/dl	0.60 mg/dl	0.80 mg/dl	0.30 mg/dl	0.50 mg/dl	Normal
Charron, Daniel	1.00 mg/dl	0.20 mg/dl	0.80 mg/dl	0.80 mg/dl	0.20 mg/dl	0.60 mg/dl	Normal
White, Susan	3.00 mg/dl	1.20 mg/dl	1.80 mg/dl	3.70 mg/dl	1.40 mg/dl	2.30 mg/dl	Increased 23.3%
Fetterroll, Susan	0.60 mg/dl	0.20 mg/dl	0.40 mg/dl	0.60 mg/dl	0.10 mg/dl	0.50 mg/dl	Normal
Doyle, Susan	0.70 mg/dl	0.20 mg/dl	0.50 mg/dl	0.40 mg/dl	0.10 mg/dl	0.30 mg/dl	Normal
Hutchines, Geraldine	0.80 mg/dl	0.20 mg/dl	0.60 mg/dl	0.90 mg/dl	0.20 mg/dl	0.70 mg/dl	Normal
Stockert, Linda	1.30 mg/dl	0.40 mg/dl	0.90 mg/dl	1.10 mg/dl	0.30 mg/dl	0.80 mg/dl	Decreased 15.4%

Source: Dr. José Cabanillas & Colleagues
Lima – Peru

Since **bilirubin** increases when liver structure is distorted, its **decrease** is interpreted as an **improvement.**

Figure 08
Chronic Hepatitis C (HCV)
Evolution of Bilirubin



Normal Values

Source: Dr. José Cabanillas & Colleagues
Lima – Peru

Since **bilirubin** increases when liver structure is distorted, its **decrease** is interpreted as an **improvement.**

The comparative analysis of **ALKALINE PHOSPHATASE** controls between the **beginning and the end** of treatment showed that in the case that presented high values at the beginning there was an **increase** of 23.9%, while in the **remaining nine cases values were maintained within normal limits**. See **Table 18 and Figure 9.**

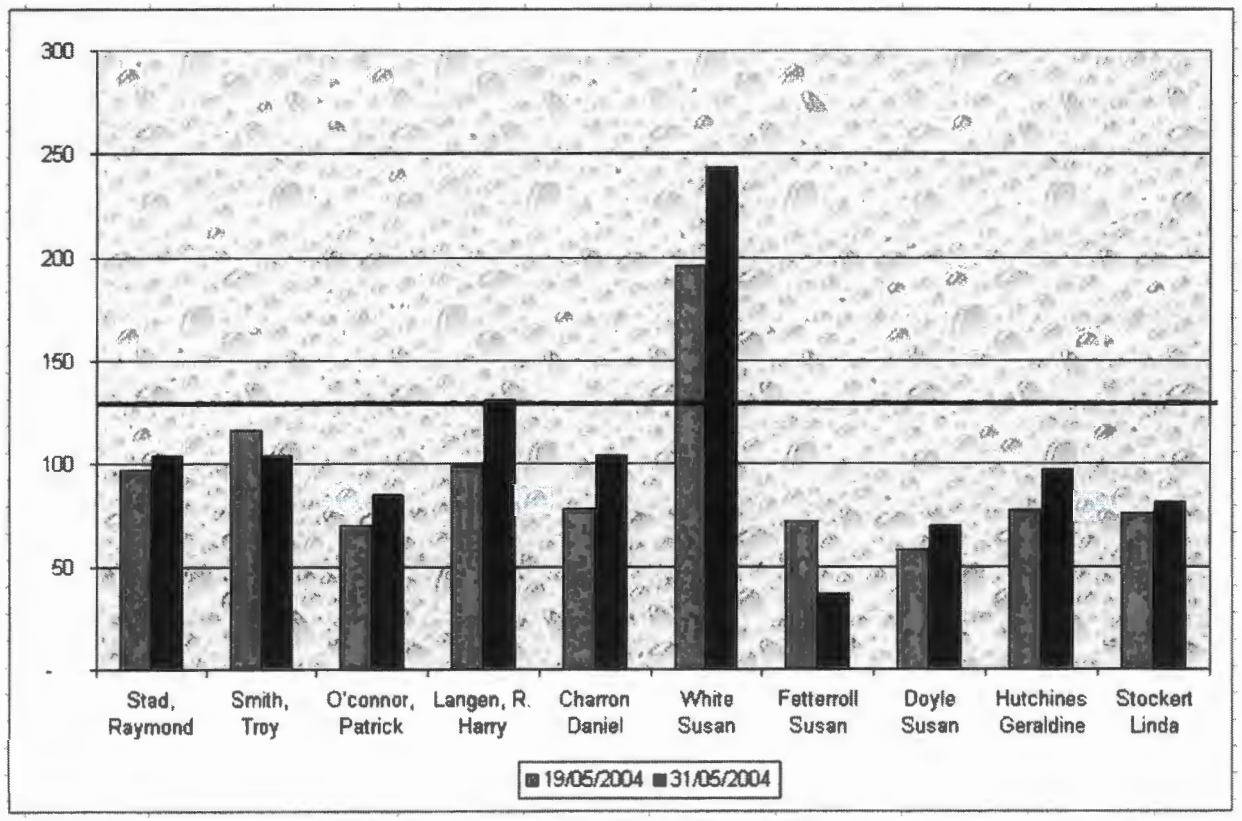
Table 18
Chronic Hepatitis C (HCV)
Evolution of Alkaline Phosphatase

	Alkaline phosphatase	Alkaline phosphatase	Modifications
Dates of control	**5/19/04**	**6/12/04**	
PATIENTS			
Stad, Raymond	97.0 U/L	104.0 U/L	Normal
Smith, Troy	116.0 U/L	104.0 U/L	Normal
O'Connor, Patrick	70.0 U/L	85.0 U/L	Normal
Langen, R. Harry	99.0 U/L	131.0 U/L	Normal
Charron, Daniel	78.0 U/L	104.0 U/L	Normal
White, Susan	196.0 U/L	243.0 U/L	Increased 23.9%
Fetterroll, Susan	72.0 U/L	37.0 U/L	Normal
Doyle, Susan	58.0 U/L	70.0 U/L	Normal
Hutchines, Geraldine	77.0 U/L	92.0 U/L	Normal
Stockert, Linda	76.0 U/L	81.0 U/L	Normal

Source: Dr. José Cabanillas & Colleagues
Lima – Peru

Since **alkaline phosphatase** increases when liver structure is distorted, its **decrease** is interpreted as an **improvement.**

Figure 09
Chronic Hepatitis C (HCV)
Evolution of Alkaline Phosphatase



Normal Values

Source: Dr. José Cabanillas & Colleagues
Lima – Perú

Since **alkaline phosphatase** increases when liver structure is distorted, its **decrease** is interpreted as an **improvement.**

The comparative analysis of **Glutamic Piruvic Transaminase** controls between the **beginning** and the **end** of treatment showed that there was a **decrease** of values in **six cases**, percentages ranged from 6.2 to 84.7 %, in **two cases** there was an **increase** between 16.1 and 57.5 %, while in the remaining **two cases** values remained within **normal numbers**.

A decrease in values of Transaminase is interpreted as a decrease of inflammatory activity on the hepatocyte, specially when it is due to the toxic effect of alcohol.

See Table 19 and Figure 10

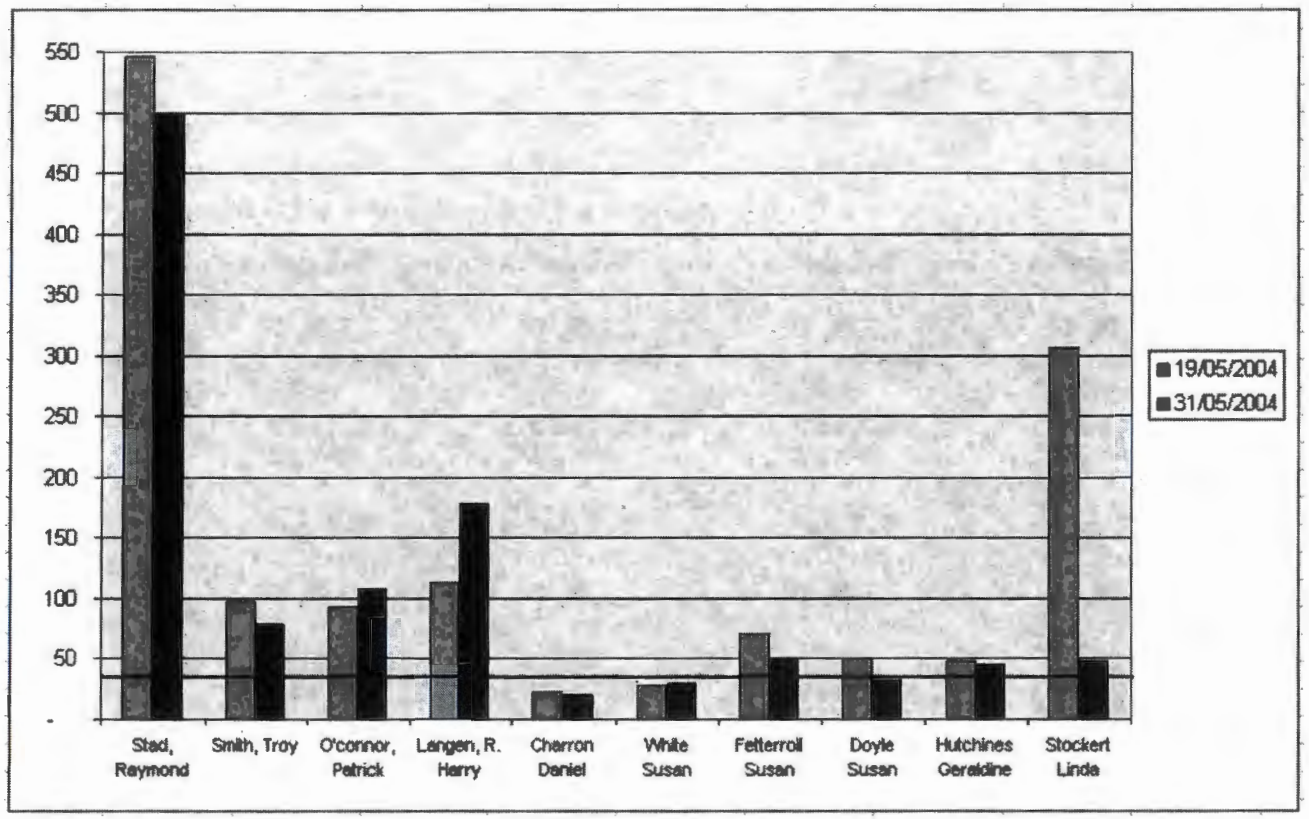
Chronic Hepatitis C (HCV)
Evolution of GPT

	GPT	GPT	Modifications
Dates of control	5/19/04	6/12/04	
PATIENTS			
Stad, Raymond	546 UI	500 UI	Decreased 8.4%
Smith, Troy	97 UI	79 UI	Decreased 18.6%
O'Connor, Patrick	93 UI	108 UI	Increased 16.1%
Langen, R. Harry	113 UI	178 UI	Increased 57.5%
Charron, Daniel	22 UI	20 UI	Normal
White, Susan	28 UI	29 UI	Normal
Fetterroll, Susan	71 UI	51 UI	Decreased 28.2%
Doyle, Susan	50 UI	32 UI	Decreased 36.0%
Hutchines, Geraldine	48 UI	45 UI	Decreased 6.2%
Stockert, Linda	306 UI	48 UI	Decreased 84.7%

Source: Dr. José Cabanillas & Colleagues
Lima – Peru

A **decrease** of **GPT (ALT)** is interpreted as a **decrease** of **inflammatory activity** on the hepatocyte .

Figure 10
Chronic Hepatitis C (HCV)
Evolution of GPT



Source: Dr. José Cabanillas & Colleagues
Lima – Perú

A **decrease** of **GPT (ALT)** is interpreted as a **decrease** of **inflammatory activity** on the hepatocyte

The comparative analysis of **Glutamic Oxaloacetic Transaminase** controls between the **beginning** and the **end** of treatment showed that in **five cases** values **decreased** between 3.8 and 74.3 %; while in **two cases** values **increased** in 30.2 and 31.2 %; in the other **three cases** values remained within **normal limits**.

A decrease in values of Transaminase is interpreted as a decrease of inflammatory activity on the hepatocyte, specially when it is due to the toxic effect of alcohol.

See Table 20 and Figure 11

Chronic Hepatitis C (HCV)
Evolution of GOT

	GOT	GOT	Modifications
Dates of control	**5/19/04**	**6/12/04**	
PATIENTS			
Stad, Raymond	340 UI	327UI	Decreased 3.8%
Smith, Troy	62 UI	52 UI	Decreased 16.1%
O'Connor, Patrick	65 UI	71 UI	Decreased 9.2%
Langen, R. Harry	96 UI	125 UI	Increased 30.2%
Charron, Daniel	21 UI	19 UI	Normal
White, Susan	48 UI	63 UI	Increased 31.2%
Fetterroll, Susan	55 UI	41UI	Decreased 25.5%
Doyle, Susan	41 UI	31UI	Normal
Hutchines, Geraldine	29 UI	32 UI	Normal
Stockert, Linda	214 UI	55 UI	Decreased 74.3%

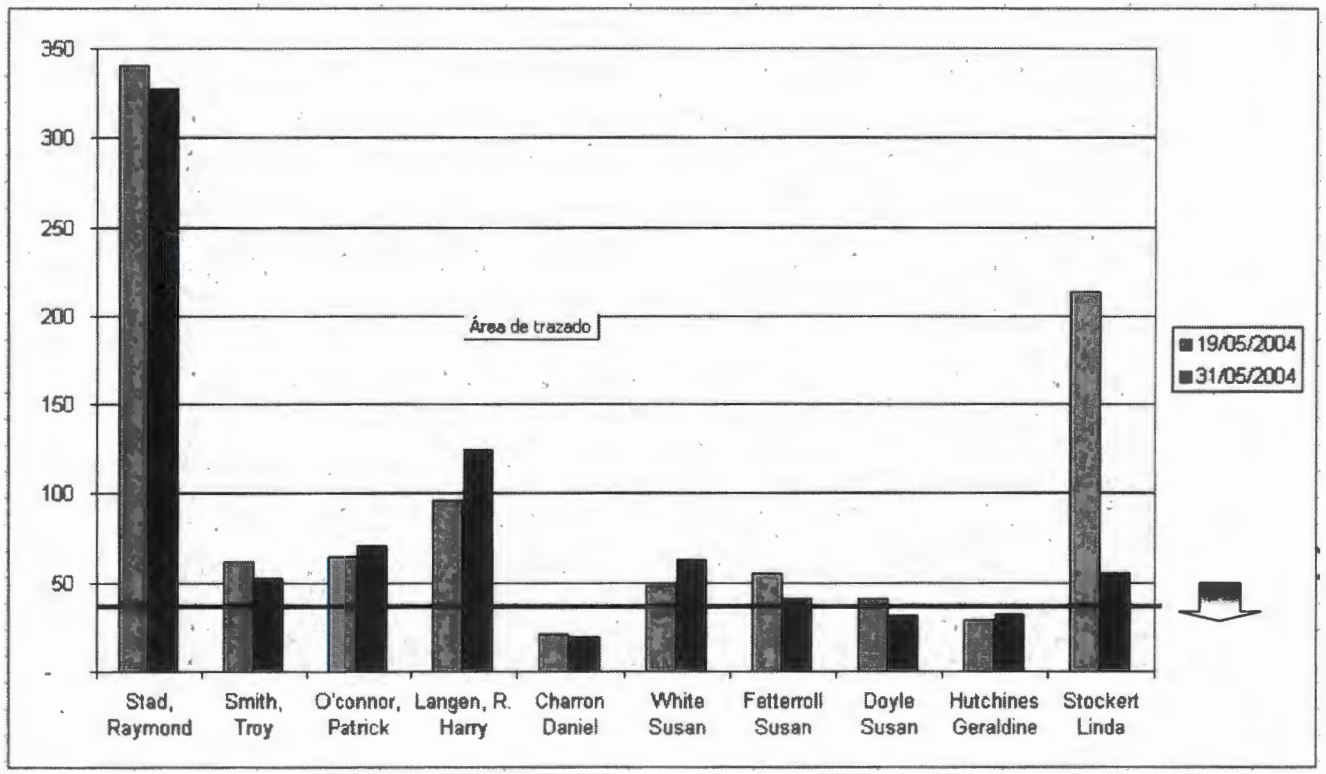
Source: Dr. José Cabanillas & Colleagues
Lima – Peru

A **decrease** of **GOT (AST)** is interpreted as a **decrease** of **inflammatory activity** on the hepatocyte.

Figure 11

Chronic Hepatitis C (HCV)

Evolution of GOT



Normal Values

Source: Dr. José Cabanillas & Colleagues
Lima – Peru

A **decrease** of **GOT (AST)** is interpreted as a **decrease** of **inflammatory activity** on the hepatocyte.

The comparative analysis of determination of **Gamma Glutamyl Transpeptidase** enzyme between the beginning and the end of the assay showed a **decrease** between 11.7 and 48.6% in **three cases**, while there was an increase of 5.7 and 2.1 % in **two of the cases**; in the other **five cases** values remained within **normal limits**.

A decrease in values of Gamma Glutamyl Transpeptidase is interpreted as a decrease of inflammatory activity on the hepatocyte, specially when it is due to the toxic effect of alcohol.

See Table 21 and Figure 12

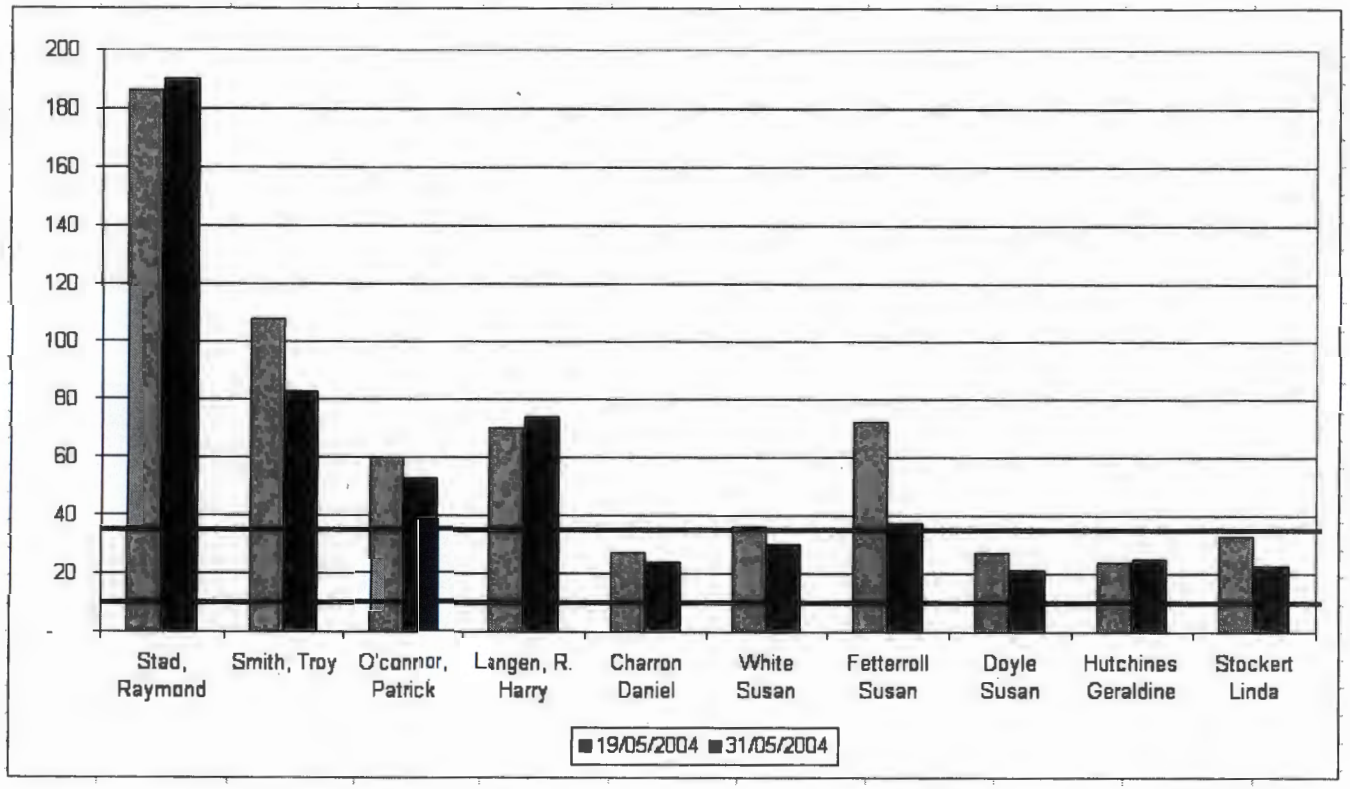
TABLE 21
Chronic Hepatitis C (HCV)
Evolution of GGT

	GGT	GGT	Modifications
Dates of control	**5/19/04**	**6/12/04**	
PATIENTS			
Stad, Raymond	186 U/L	190 U/L	Increased 2.1%
Smith, Troy	108 U/L	83 U/L	Decreased 23.1%
O'Connor, Patrick	60 U/L	53 U/L	Decreased 11.7%
Langen, R. Harry	70 U/L	74 U/L	Increased 5.7%
Charron, Daniel	27 U/L	24 U/L	Normal
White, Susan	36 U/L	30 U/L	Normal
Fetterroll, Susan	72 U/L	37 U/L	Decreased 48.6%
Doyle, Susan	27 U/L	21 U/L	Normal
Hutchines, Geraldine	24 U/L	25 U/L	Normal
Stockert, Linda	33 U/L	23 U/L	Normal

Source: Dr. José Cabanillas & Colleagues
Lima – Peru

A **decrease** of **GGT** is interpreted as a **decrease** of **inflammatory activity**, specially when it is due to the toxic effect of alcohol.

Figure 12
Chronic Hepatitis C (HCV)
Evolution of GGT



Source: Dr. José Cabanillas & Colleagues
Lima – Peru

A **decrease** in values of **GGT** is interpreted as a **decrease** of **inflammatory activity** on the hepatocyte, specially when it is due to the toxic effect of alcohol.

In both tests, the determination of **TRANSFERRIN** showed values within normal limits in **seven** out of the ten patients that took part in the study; out of the **three** patients that had values above normal in the first test, **one** went **down to normal**, whereas in the other **two cases**, they maintained their above normal values.

See Table 22 and Figure 13

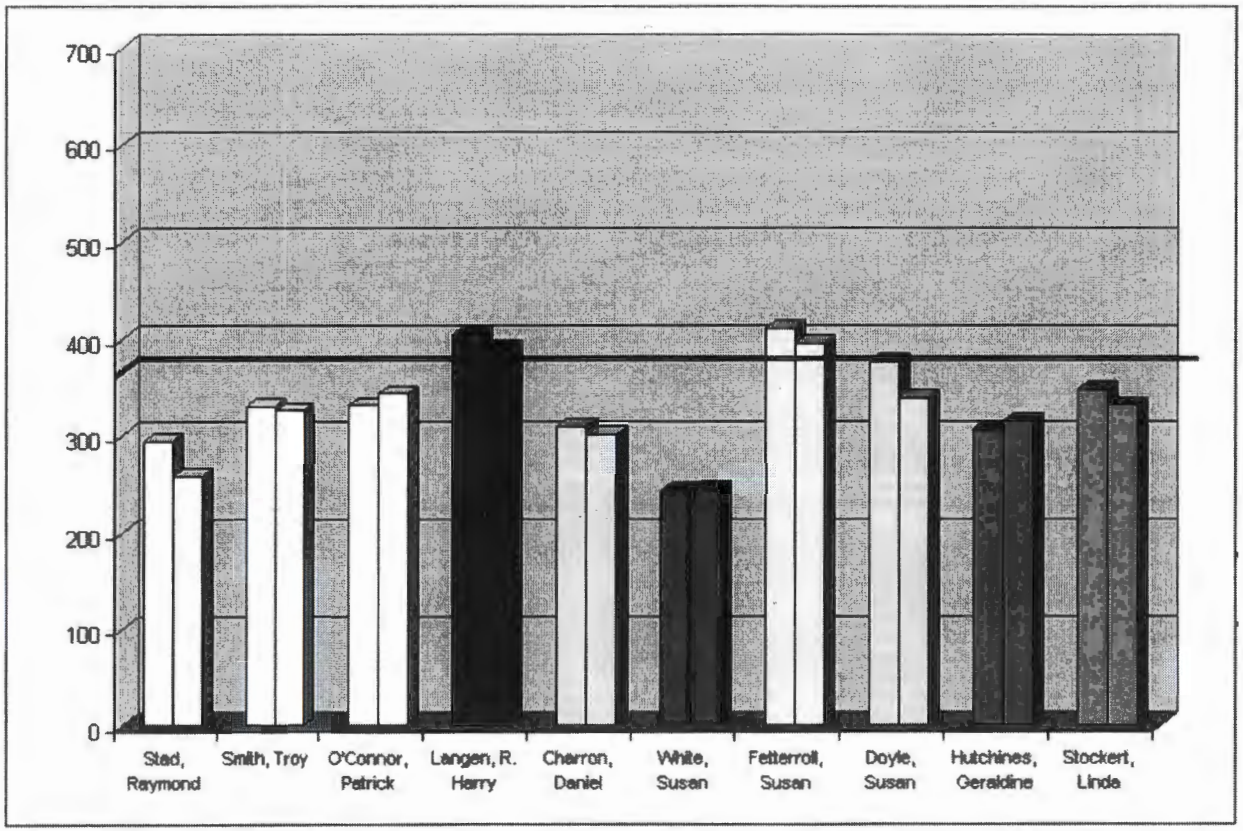
TABLE 22
Chronic Hepatitis C (HCV)
Evolution of Transferrin

	Transferrin		Modifications
Dates of control	**5/19/04**	**6/12/04**	
PATIENTS			
Stad, Raymond	295	259	Normal
Smith, Troy	330	326	Normal
O'Connor, Patrick	331	343	Normal
Langen, R. Harry	404	390	Decreased 3.5%
Charron, Daniel	310	301	Normal
White, Susan	243	246	Normal
Fetterroll, Susan	410	395	Decreased 3.7%
Doyle, Susan	377	339	Decreased 10.1%
Hutchines, Geraldine	306	315	Normal
Stockert, Linda	347	331	Normal

Source: Dr. José Cabanillas & Colleagues
Lima – Peru

An **increase of transferrin** produces accumulation in the liver, which leads to **anatomical damage**.

Figure 13
Chronic Hepatitis C (HCV)
Evolution of Transferrin



Source: Dr. José Cabanillas & Colleagues
Lima – Peru

An **increase of transferrin** produces accumulation in the liver, which leads to **anatomical damage**.

The **initial** determination of **ALPHA FETO-PROTEINS** showed that in **nine** of the cases studied **values were within normal limits**; at the end of the treatment, **eight** maintained **values within normal range**, while **one** of them experienced a **mild increase** going beyond normal limits. The **other** case started with quite high values that even **increased** by the time the **second control** was conducted.

See Table 23 and Figure 14

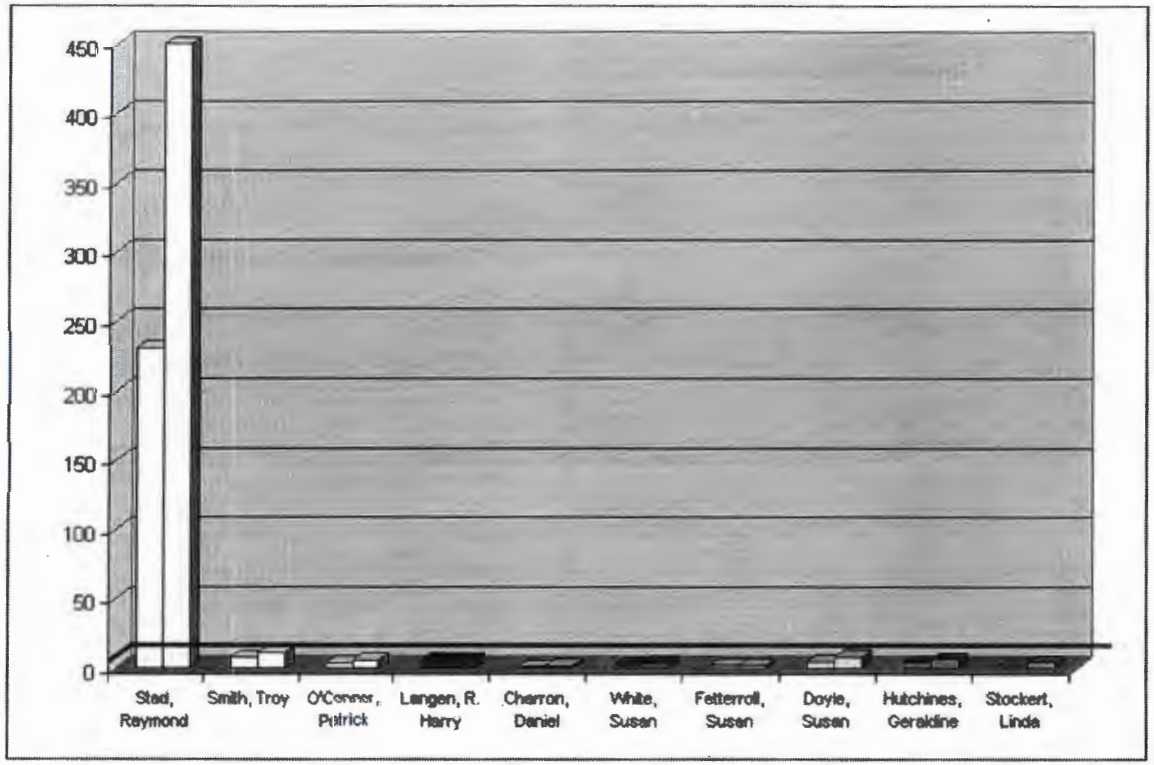
Table 23
Chronic Hepatitis C (HCV)
Evolution of Alpha Feto-Protein

	AFP		Modificaciones
Dates of control	**5/19/04**	**6/12/04**	
PATIENTS			
Stad, Raymond	231.0	454.0	Increased 96.54%
Smith, Troy	7.7	11.0	Increased 42.85%
O'Connor, Patrick	4.3	6.4	Normal
Langen, R. Harry	6.0	6.9	Normal
Charron, Daniel	1.4	1.6	Normal
White, Susan	3.1	3.8	Normal
Fetterroll, Susan	2.5	2.5	Normal
Doyle, Susan	5.4	8.2	Normal
Hutchines, Geraldine	4.4	6.9	Normal
Stockert, Linda	3.0	4.5	Normal

Source: Dr. José Cabanillas & Colleagues
Lima – Peru

AFP is **a tumor marker** used as an indicative of **gastrointestinal cancer.**

Figure 14
Chronic Hepatitis C (HCV)
Evolution of Alpha Feto-Protein



Source: Dr. José Cabanillas & Colleagues
Lima-Peru

AFP is **a tumor marker** used as an indicative of **gastrointestinal cancer.**

Values in the determination of **AMMONIA** were, in the **first control, within normal limits** in **nine** of the cases; such values remained **equally normal by the end of the treatment**. The **remaining case** started with **values above normal** that were also maintained.

See Table 24 and Figure 15

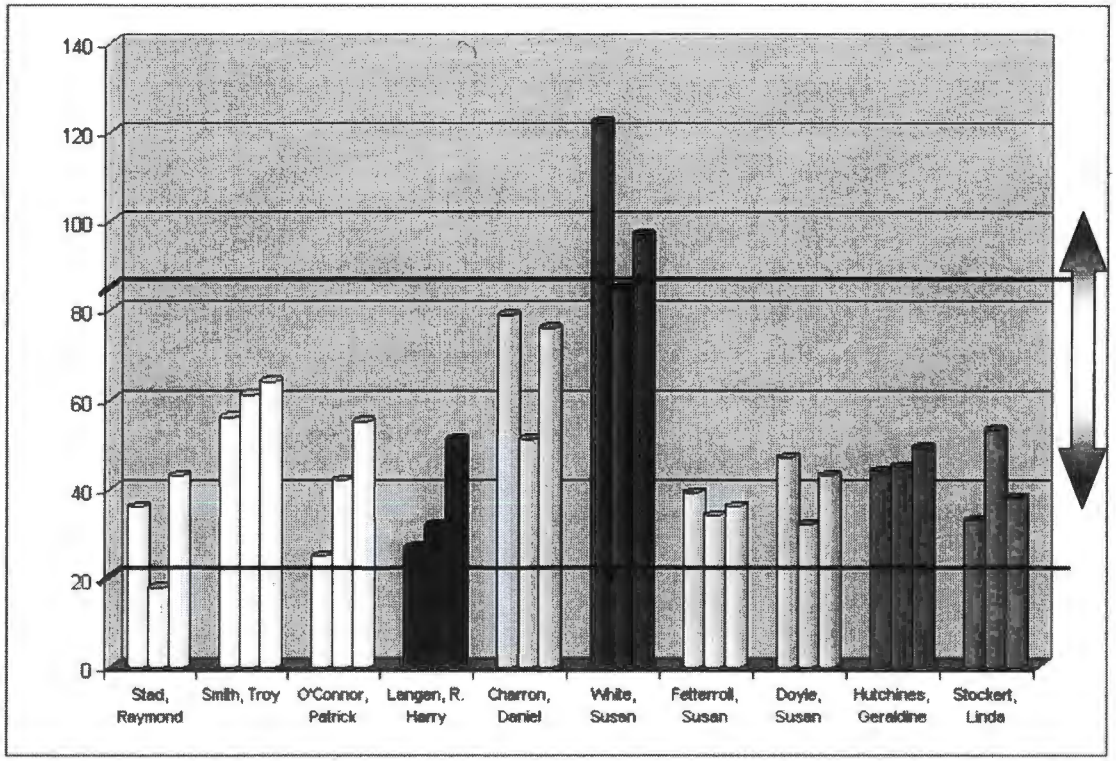
Table 24
Chronic Hepatitis C (HCV)
Evolution of Ammonia

	Ammonia			Modifications
Dates of control	**5/19/04**	**5/31/04**	**6/12/04**	
PATIENTS				
Stad, Raymond	36	18	43	Normal
Smith, Troy	56	61	64	Normal
O'Connor, Patrick	25	42	55	Normal
Langen, R. Harry	27	32	51	Normal
Charron, Daniel	79	51	76	Normal
White, Susan	122	85	97	Decreased 28.49%
Fetterroll, Susan	39	34	36	Normal
Doyle, Susan	47	32	43	Normal
Hutchines, Geraldine	44	45	49	Normal
Stockert, Linda	33	53	38	Normal

Source: Dr. José Cabanillas & Colleagues
Lima – Peru

Ammonia is an internal toxic product. Its **increase** indicates **liver failure**.

Figure 15
Chronic Hepatitis C (HCV)
Evolution of Ammonia



Source: Dr. José Cabanillas & Colleagues
Lima – Peru

Ammonia is an internal toxic product. Its **increase** indicates **liver failure**.

The control of **PLATELET COUNT** at the **beginning** of the assay showed that seven patients had numbers within **normal limits**, which remained equal by the **end** of the treatment. The other **three** cases presented, at the beginning, values **below the minimum established as normal**, 140,000/mm3 , that were also **maintained** at the same range by the end of the treatment.

See Table 25 and Figure 16

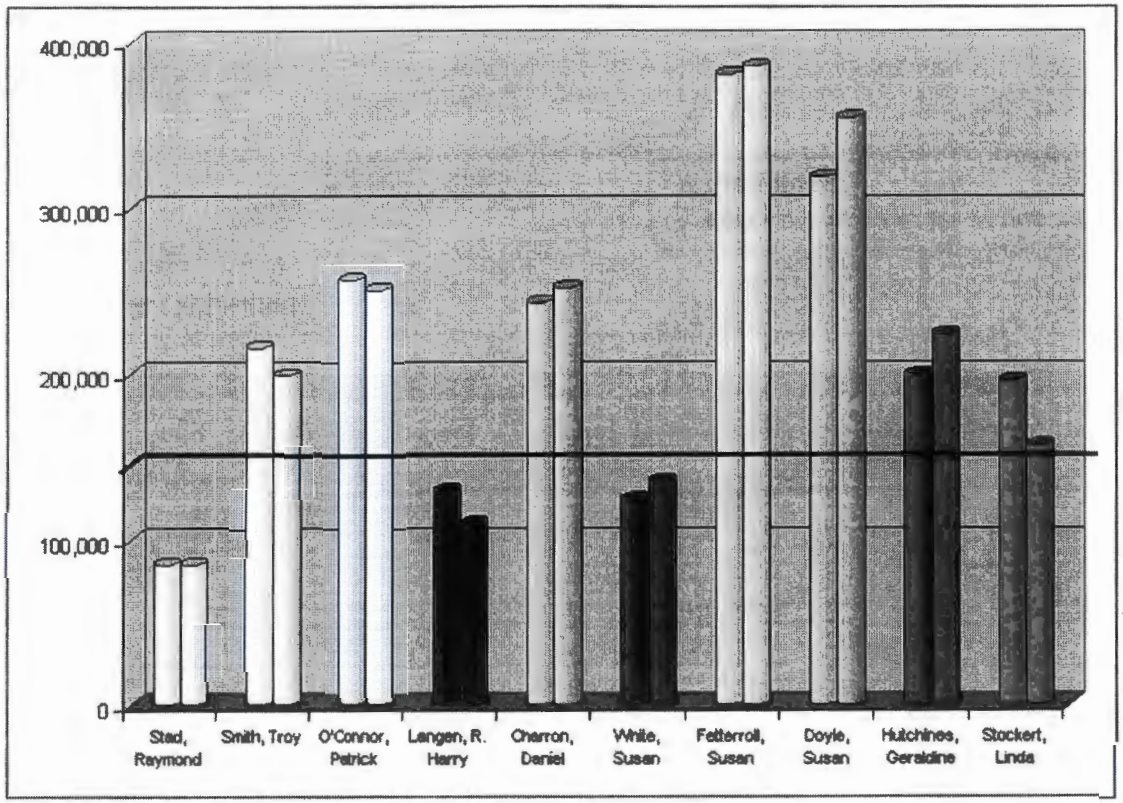
Table 25
Chronic Hepatitis C (HCV)
Evolution of Platelet Count

	Evolution of Platelet Count		Modifications
Dates of control	**5/19/04**	**6/12/04**	
PATIENTS			
Stad, Raymond	85,000	85,200	Values below normal
Smith, Troy	216,000	199,000	Normal
O'Connor, Patrick	257,000	251,000	Normal
Langen, R. Harry	130,000	110,000	Values below normal
Charron, Daniel	243,000	252,000	Normal
White, Susan	125,000	136,000	Values below normal
Fetterroll, Susan	381,000	386,000	Normal
Doyle, Susan	319,000	355,000	Normal
Hutchines, Geraldine	200,000	224,000	Normal
Stockert, Linda	196,000	157,000	Normal

Source: Dr. José Cabanillas & Colleagues
Lima – Peru

A **decrease in platelet count** indicates **enlargement of the spleen.**

Figure 16
Chronic Hepatitis C (HCV)
Evolution of Platelet Count



Source: Dr. José Cabanillas & Colleagues
Lima – Peru

A decrease in platelet count indicates **enlargement of the spleen.**

For the **three controls** that we conducted, values in the determination of **TUMOR NECROSIS FACTOR** (TNF-Alpha) **fluctuated** sometimes within the normal range and sometimes above it in an **alternate and irregular** manner. Thus not allowing us to make any comments on it as proof of evolution in the present study.

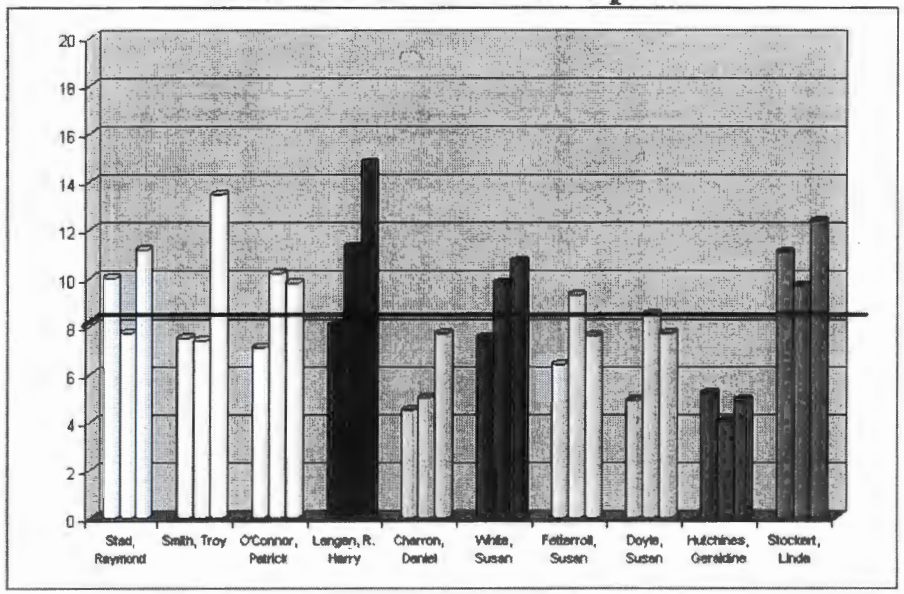
See Table 26 and Figure 17

Table 26
Chronic Hepatitis C (HCV)
Evaluation of TNF-Alpha

	TNF-alpha		
	5/19/04	5/31/04	6/12/04
PATIENTS			
Stad, Raymond	10.0	7.7	11.2
Smith, Troy	7.5	7.4	13.5
O'Connor, Patrick	7.1	10.2	9.8
Langen, R. Harry	8.0	11.3	14.8
Charron, Daniel	4.5	5.0	7.7
White, Susan	7.5	9.8	10.7
Fetterroll, Susan	6.4	9.3	7.6
Doyle, Susan	4.9	8.5	7.7
Hutchines, Geraldine	5.2	4.0	4.9
Stockert, Linda	11.1	9.7	12.4

Source: Dr. José Cabanillas & Colleagues
Lima – Peru

Figure 17
Chronic Hepatitis C (HCV)
Evaluation of TNF-Alpha



Source: Dr. José Cabanillas & Colleagues
Lima – Peru

Evaluation of Clinical Aspects

Evolution of symptoms and signs

1. General symptoms:

The clinical control at the end of the assay showed a decrease in general symptomatology. **Severe fatigue, lack of concentration and osteoarticular pain** disappeared in some patients and their presence was reduced by 40% in the cases studied; **general discomfort and depression** as well as **muscular pain** were reduced and only present in 30% of patients; whereas **sleep disorders** and the **presence of headache** only reached 20% and 10% respectively.

Moreover, we must mention that in some of the cases in which **general symptoms** persisted, patients experienced varied decreases in the intensity of such symptoms.

2. Gastrointestinal symptoms:

The evolution of **gastrointestinal symptoms** also showed a decrease at the end of the process. **Indigestion and intestinal dysfunction** was present in 30% of cases; **dyspepsia and nausea** reached 20 and 10% of patients whereas **abdominal pain** was no longer present in any of the cases 0%.

We must also mention that those patients in which gastrointestinal discomfort did not disappear, experienced a decrease in intensity; except for one patient that reported an increase of nausea.

3. Signs:

During the treatment, the sign of **pain upon palpation of right hypochondrium** was reduced to 20%; whereas other signs such as **jaundice and palpable liver** remained present in 20% of cases; **palpable spleen** was reduced to 10% whereas other signs such as **ascites, ecchymosis and edema in lower extremities** remained present in one patient, that is, 10% of cases; no patient presented **collateral circulation**.

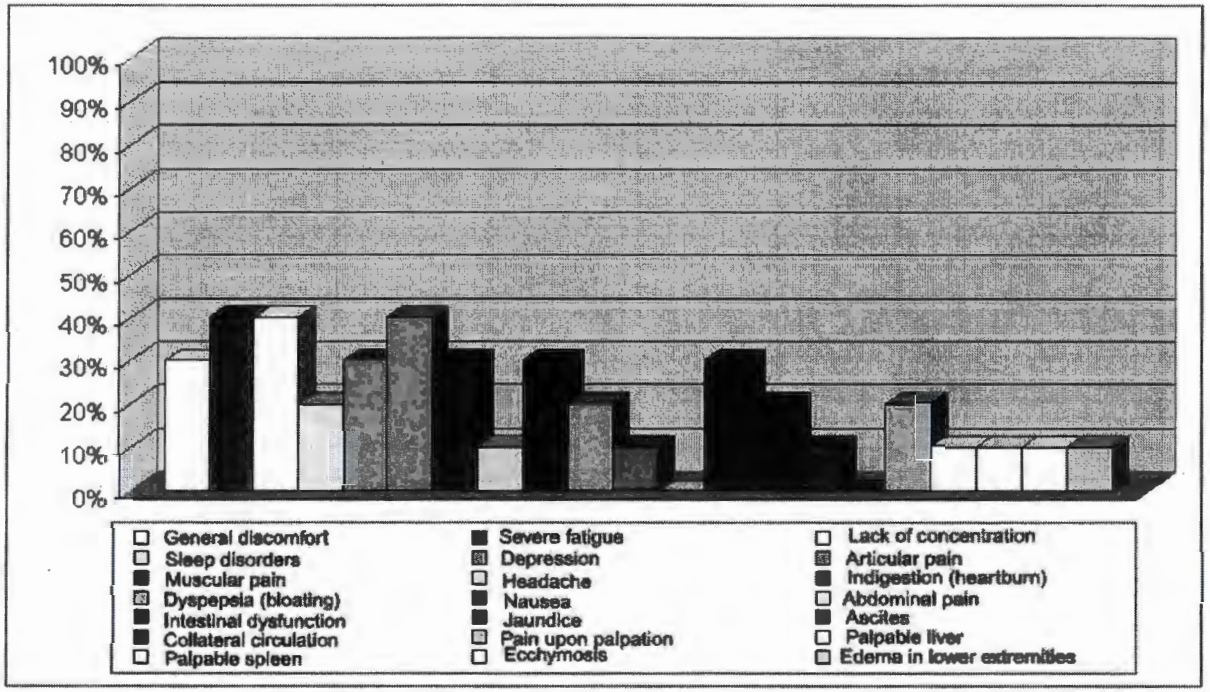
See Table 27 and Figure 18

Table 27
Chronic Hepatitis C (HCV)
Evolution of Symptoms and Signs

Symptoms	Stad, R.	Troy, S.	O'Connor	Langen, H.	Charron	White	Fetterroll	Doyle	Hutchines	Stockert	
I) General											
1.- General discomfort	-	-	-	-	-	+	-	+ decre.	-	+ decre.	30%
2.- Severe fatigue	-	-	-	-	-	+decre.	-	+	+ decre.	+ decre.	40%
3.- Lack of concentration	-	+ decre.	-	-	-	+	-	+	+ decre.	+ decre.	40%
4.- Sleep disorders	-	-	-	-	-	+	-	+ decre.	-	-	20%
5.- Depression	-	-	+ decre.	-	-	+	-	+	-	-	30%
6.- Articular pain	-	-	-	+ decre.	-	+	-	+ decre.	+decre.	+decre.	40%
7.- Muscular pain	-	-	-	+ decre.	-	+	-	-	-	+decre.	30%
8.- Headache	-	-	-	-	-	-	-	-	-	+ decre.	10%
II) Gastrointestinal											
1.- Indigestion (heartburn)	-	+ decre.	-	+ decre.	-	+	-	-	-	-	30%
2.- Dyspepsia (bloating)	-	-	-	-	-	+	-	-	-	+ decre.	20%
3.- Nausea	-	-	-	-	-	+incre.	-	-	-	-	10%
4.- Abdominal pain	-	-	-	-	-	-	-	-	-	-	0%
5.- Instestinal dysfunction	-	-	-	+ decre.	-	+	-	-	+ decre.	-	30%
Signs											
1.- Jaundice	+	-	-	-	-	+	-	-	-	-	20%
2.- Ascites	-	-	-	-	-	+incre.	-	-	-	-	10%
3.- Collateral circulation	-	-	-	-	-	-	-	-	-	-	0%
4.- Pain upon palpation	-	-	-	+ decre.	-	+	-	-	-	-	20%
5.- Palpable liver	-	-	-	+	-	-	-	-	-	-	10%
6.- Palpable spleen	-	-	-	+	-	-	-	-	-	-	10%
7.- Ecchymosis	-	-	-	-	-	+	-	-	-	-	10%
8.- Edema in lower extremities	-	-	-	-	-	+incre.	-	-	-	-	10%

Source: Dr. José Cabanillas & Colleagues
Lima – Peru

Figure 18
Chronic Hepatitis C (HCV)
Evolution of Symptoms and Signs
After Treatment



Source: Dr. José Cabanillas & Colleagues
Lima - Peru

Comparative study of symptoms and signs before and after treatment

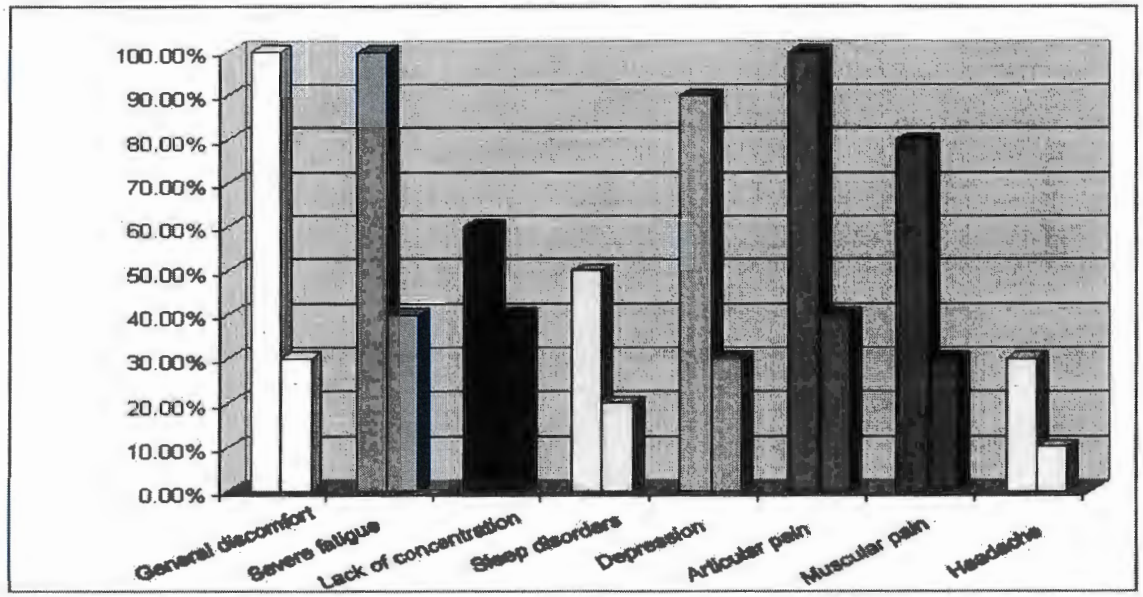
The following graphs allow us to observe, in a comparative study, the frequency of presence of symptoms and signs in the cases studied before and after treatment:

1. General symptoms: **Figure 19**

2. Gastrointestinal symptoms: **Figure 20**

3. Clinical signology: **Figure 21**

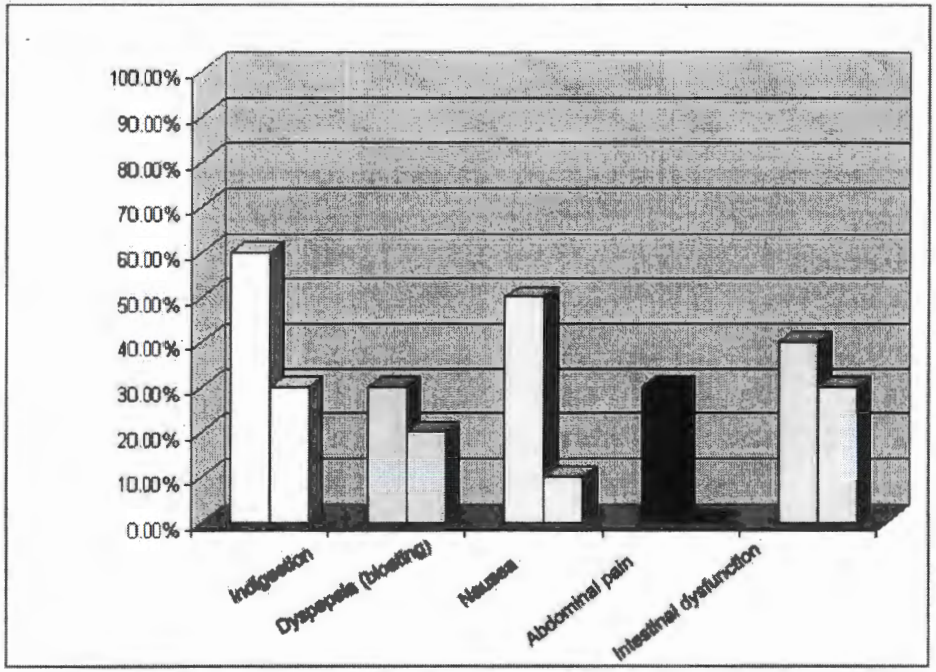
Figure 19
Chronic Hepatitis C (HCV)
Comparative study of symptoms and signs before and after treatment



Source: Dr. José Cabanillas & Colleagues
Lima – Peru

Such **decrease** in the intensity of **symptoms** indicates **patient improvement** after the first 28 days of treatment.

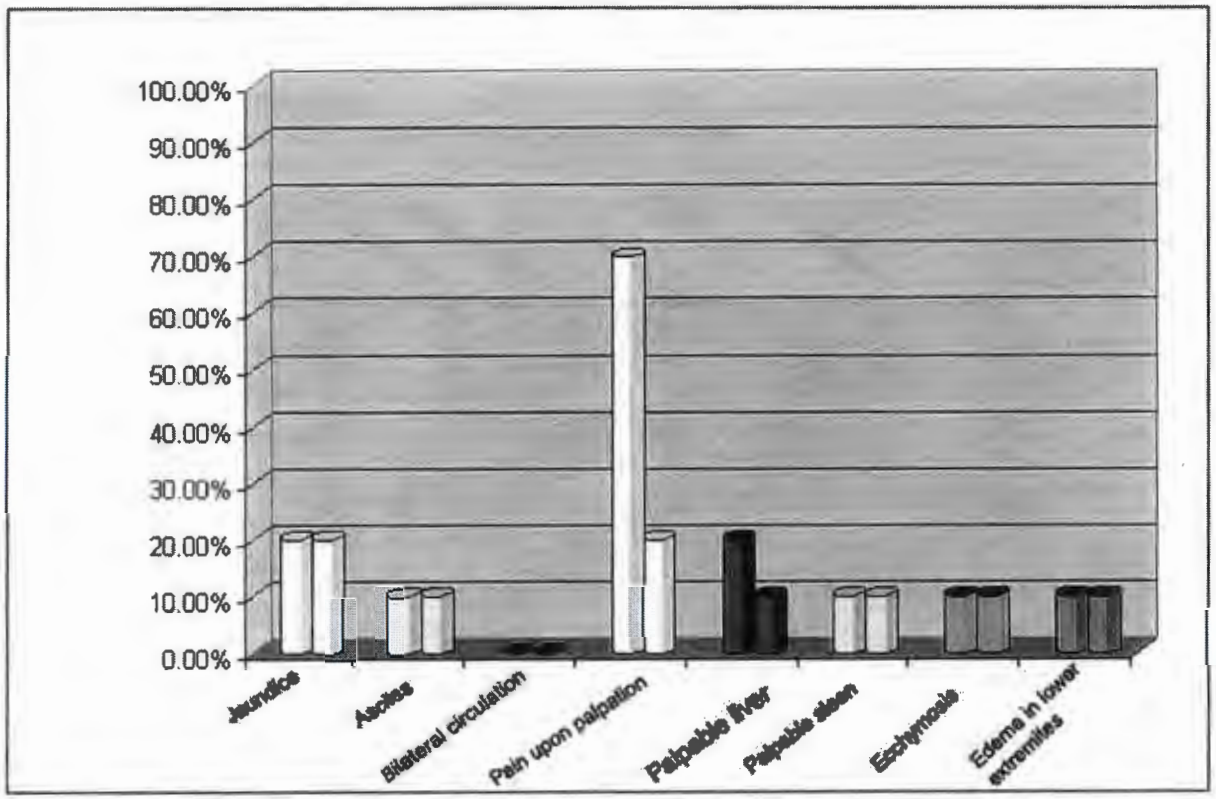
Figure 20
Chronic Hepatitis C (HCV)
Comparative study of symptoms and signs before and after treatment



Source: Dr. José Cabanillas & Colleagues
Lima – Peru

Such **decrease** in the intensity of **symptoms** indicates **patient improvement** after the first 28 days of treatment.

Figure 21
Chronic Hepatitis C (HCV)
Comparative study of symptoms and signs before and after treatment



Source: Dr. José Cabanillas & Colleagues
Lima – Peru

Such **decrease** in the intensity of **symptoms** indicates **patient improvement** after the first 28 days of treatment.

Final Comments

At the beginning of this study, all of the patients manifested the general symptomology associated with chronic Hepatitis C, as well as varying degrees of severity of digestive problems. Although study participants ranged from three to thirty years in managing their disease, in each case, these symptoms created limitations in their work, as well as in their family and social relationships. In addition, the patients reported varying degrees of depression and anxiety related to their futures.

Clinical symptoms:
Study subjects showed a dramatic improvement in the majority of their symptoms. These included improvement in fatigue, right upper quadrant (liver area) pain and tenderness, dyspepsia, nausea-vomiting, indigestion, headaches, muscle and joint/bone pains.

Prothrombine activity and serum cholinesterase:
There was a significant increase in Prothrombine activity and serum cholinesterase which suggests possible increased protein synthesis by the liver, or at very least, a decrease in their degradation. This improvement in these specific vital liver functions demonstrates one of the most promising indications that A4+L is initiating the liver's recovery.

Health-Related Quality of Life:
The efficacy of A4+ to improve the quality of life was tested by administering the 'SF - 36 Quality of Life Survey' and by Day 14, a markedly improved quality of life was reported by 90% of the study subjects. By Day 28, the patients' well-being and capacity to function were restored to normal levels in 90% of the subjects observed. The score improvements observed in this study by Day 28 were, on average, greater than two standard deviations for nearly all health-related quality of life scales. This degree of improvement has rarely been observed in the thousands of treatment studies of other chronic diseases involving the SF-36 Health Survey.25. The SF - 36 Health Survey is the most commonly used, accepted and generally standardized scale for measurement of patient 'Quality of Life'.

Depression:
The severity of depression at the start of the study ranged from borderline in 10% of the subjects, mild to moderate depression in 80%, and severe depression in 10%. By the end of the observation period statistically significant improvement in depression was noted and 90% of the subjects reported being free of depression. By the 28th day, the majority of the patients showed marked progress in their degree of relief from depression as well as their changed attitude toward their own futures. Each patient expressed their desire to return to work, and to their family and social relationships. Most important to their mental health was their positive attitude to recuperating their capacity to be 'normal '.

In summary, by day 28 of the study period, the patients had already improved in every aspect of Hepatitis C symptomology. This was demonstrated by the bio -chemical liver testing (in relation to hepatic synthesis) and liver inflammation (shown in ultrasound testing). These tests, in combination with the marked improvement demonstrated by the results on 'Quality of Life SF-36' scale, and the Beck Depression Inventory, provide evidence, of

marked decrease of liver inflammation, near elimination of Hepatitis C symptoms, and an overall sense of hope for leading a healthy, 'normal' life. With such highly positive significant physical and mental indicators of marked improvement, in such a high percentage of the patients, continued research of A4+L is clearly warranted.

The results obtained in the ultrasound and different biochemical tests that indicate an improvement in the functional and/or anatomic state of the liver must be evaluated in the new control stages.

There was no case in which the patients reported unpleasant side effects from the therapy performed on them; however, hematological and renal function control tests must be performed periodically.

This work constitutes a very optimistic alternative treatment for patients with chronic Hepatitis C.

The Authors

Bibliography

1. WHO. Hepatitis C: global prevalence. Wkly Epidemiol Rec 1997; 72: 341-4

2. Heintger, T; Wands J R.: Hepatitis C virus: epidemiology and transmission Hepatology 1997; 26; 521-6

3. Yano M; Kumada H, Kage M; The long term pathological evolution of chronic hepatitis C. Hepatology 1996; 23: 1334-40

4. Tremolada F, Casarin C, Alberti: Long tern follow-up of no-A non-B (type C) post-transfusion hepatitis. J Hepatol 1992; 16:273-81

5. Working Group on the evaluation of carcinogenic risk to humans, International agency for research on cancer. Word Health Organization. Monographs on the evaluation of carcinogenic risk to humans 1994; 59: 165-221

6. Rao KV, Anderson RC: Long tern results and complications in renal transplant recipients. Observation en the second decade. Transplantation 1998; 45: 45-52

7. Grotz WH, Peters TH, Schalayer HJ, et al.: Immunosuppressive therapy and hepatitis C virus infection: the clinical course of liver desease. J Mol Med 1996; 74: 407-12

8. Desmet VJ, Gerber M, Hoofnagle JH, Manns Sheuer PJ. : Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology 1994; 19: 1513-20

9. Knodell RG, Ishak KG, Black WC, Chen TS, Craig R, Kaplowitz N, et al. Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. Hepatology 1981; 1: 431-5

10. Hoofnagle JH, Mullen KD, Jones DB, et al.: Treatment of chronic non-A, non-B hepatitis with recombinant human alpha interferon. A preliminary report. N Engl J Med 1986; 315: 1575-8

11. Lin R, Roach E, Zimmerman M, Strasser S, Farrel GC. : Interferon alpha 2b for chronic hepatitis C: effects of dose increment and duration of treatment and response rate. Results of the first multicentre. Australian trial. J Hepatol 1995; 23: 487-96

12. Lino S, Hino K, Kuroki T, Suzuki H, Yamamoto S.: Treatment of chronic hepatitis C with study high-dose interferon alpha 2b. A multicentre study. Dig Dis Sci 1993; 38: 612-8

13. Hakosako Y, Shirahama T, Katou M, Nakagawa K, Oba K, Mitamura K.: A controlled study to determine the optimal dose regimen of interferon alpha 2b in chronic hepatitis C. Am J Gastroenterol 1995; 90: 1246-9

14. Mc Hutchison JG, Poynard T.: Combination therapy with interferon plus Ribavirin for the initial treatment of chronic hepatitis C. Semin Liver Dis 1999; 19 (suppl 1) : 57-65

15. Davil GL.: Combination therapy with interferon alpha and Ribavirin as treatment of interferon relapse in chronic hepatitis C. Semin Liver Dis 1999; 19 (suppl 1): 49-55

16. Arus Soler E; Rivera L; Fernández A y otros: Tratamiento de la hepatitis crónica C con interferón alfa 2 recombinante. Controlled Clinical Test Rev. Med. Cuba 2000; 39(1): 12-20

17. Gabo KA, Herlong HF, Torbenson MS, et al.: Role of liver biopsy in magament of chronic hepatitis C: A systematic review. Hepatology 2002; 36: 161-72

18. Albanis E, Friedman SL.: Non-invasive markers of hepatic fibrosis. Clin Pers Gastroenterol 2002; 5: 182-87

19. Poynard T, Imbert-Bismut F, Retziu V et al.: An overvien of biochemicol markeds (fibro-test-actitest) diagnostic volucin chronic liver desease: A non-invasive alternative to liver biopsy. Boston Mass; American Association for the Study of Liver Deseases (AASLD) Nov. 2003. Abstract 03.

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(54) Title: HERBAL COMPOSITIONS AND METHODS FOR TREATING HEPATIC DISORDERS

(57) Abstract: Herbal compositions and their use in the prevention and/or treatment of hepatitis are provided The herbal compositions comprise an extract of flowers, leaves, and roots from the plant genera *Cordia*, *Annona*, and *Curcuma*, respectively, wherein the specific species are *Cordia lutea*, *Annona muricata* and *Curcuma longa*

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Composition for Treating Hepatitis Containing an Extract of *Cordia lutea* Flowers, *Annona muricata* Leaves, and *Curcuma longa* Roots

The present application claims priority to U.S. Provisional Patent Application No. 60/977,256 filed October 3, 2007.

FIELD OF THE INVENTION

The present application relates to novel herbal compositions and their use in the prevention and/or treatment of hepatic disorders. More particularly, the herbal compositions of the present application comprise at least one species of the plant genera *Cordia*, *Annona* or *Curcuma*, or extracts thereof, or combinations thereof.

BACKGROUND OF THE INVENTION

The formation of oxygen radical species (ORS) is involved in the pathogenesis of many acute and chronic diseases, ranging from inflammatory-immunologic diseases to myocardial infarction and cancer. Some of the deleterious effects from the excessive formation of ORS include lipid peroxidation of the membrane lipids, oxidative damage to nucleic acids and carbohydrates, as well as oxidation of sulfhydryl proteins and other sensitive groups. The defence provided by antioxidant systems is essential for survival. Detoxification of the ORS in a cell is carried on by enzymatic and non-enzymatic systems which constitute the antioxidant defence system (Middleton Jr. E., Chithan K., and Heoharides T.C. The Effects of Plant Flavonoids on Mammalian Cells: Implications for Inflammation, Heart Disease, and Cancer. *Pharmacol Rev* 52:673-751, 2000).

Lipid peroxidation can be biologically important in the exacerbation of a tissue lesion due to the potential cytotoxicity of the final products resulting from peroxidation. For example, products of lipid peroxidation of the cells may be carcinogenic. Recently, emphasis has been put on the role that lipid peroxidation plays in the development of arteriosclerosis, strokes, myocardial infarction, damage to the brain and spinal cord after suffering ischemia, cancer, inflammation, iron toxicity, and hepatotoxicity induced by chemical and biological agents (Middleton Jr. E., Chithan K., and Heoharides T.C. The

Effects of Plant Flavonoids on Mammalian Cells: implications for Inflammation, Heart Disease, and Cancer. *Pharmacol Rev* 52:673-751, 2000).

Chronic hepatic diseases cause thousands of deaths in the world every year and are the tenth leading cause of death in the United States. Currently, hepatic disorders, particularly those caused by viral infections, are a serious health issue, and their successful treatment constitutes a big challenge. There is no effective treatment for a majority of the hepatopathies. Currently some patients with viral hepatitis are treated with Interferon (IFN); however, IFN therapy has been successful only in about 25% of the cases.

IFN is not available to all the patients, as the six-month therapy required is expensive. In addition, this treatment has several secondary effects like severe flu-like symptoms, lethargy, hair loss, and bad taste in the mouth. IFN attacks the virus via the immune system, but it does not reverse the damage caused by the infection, like hepatic cirrhosis or diminished functionality of the spleen.

Other treatments, such as Ribavirin therapy, improve the results in medical and histological exams, especially in combination with IFN. However, costs of treatment are also high and there is a significant risk of suspending the treatment due to adverse effects (Mc Hutchison, J.G. and Poynard T. Combination therapy with interferon plus Ribavirin for the initial treatment of chronic Hepatitis C. *Semin Liver Dis* 1999; **19** (suppl 1): 57-65; Davil G.L. Combination therapy with interferon alpha and Ribavirin as treatment of interferon relapse in chronic hepatitis C. *Semin Liver Dis* 1999; **19** (suppl 1): 49-55).

The World Health Organisation (WHO) estimates that 3% of the world's population has been infected with Hepatitis C, and that there are around 170 million chronic carriers who are at risk for developing cirrhosis and/or liver cancer. The WHO cannot afford to treat 170 million people in the world with medications like Rebetron, which consists of Ribavirin and Interferon alpha 2B, whose treatment costs USD 2,000 per month, for 6-12 months; in addition, these treatments require extensive medical support to manage the adverse effects caused by the medications.

Since there is no therapy or synthetic medication effective and safe enough to treat hepatopathies, many patients have turned to alternative medicine based on natural elements. Despite significant progress in modern medicine, medicinal plants remain as a necessary element when it comes to developing accurate, safe and effective treatments for hepatic disorders. In recent years, there has been a shift towards the therapeutic evaluation of herbal products to treat liver diseases, some of which are proving safe and moderately effective.

Several scientific publications point out the fact that many groups of metabolites from vegetal origin show antioxidant and hepatoprotective activity. This is observed particularly amongst phenols, especially those belonging to the benzenoid group, where tournefolal, tournefolic acids A and B, and the ethylester from the tournefolic acid, isolated from the aerial parts, that is, the stem, leaves, flowers and fruit, of the *Tournefortia sarmentosa*, show antioxidant activity and inhibit the peroxidation of low-density lipoproteins (Lin Y.L., Chang Y.Y., Kuo Y.H., Shiao M.S. Anti-lipid-peroxidative principles from *Tounefortia samentosa*. *J Nat Prod.* 2002 May; **65(5)**:745-7). Curcumin, also a benzenoid, shows a captivating activity on superoxide anions (Kunchandy E., Rao M.N.A. *Int. J. Pharmaceut.*, **57**: 173-176 (1989)) and nitric acid (Sreejayan N., Rao M.N.A. *J. Pharm. Pharmacol.*, **49**: 105-107 (1997)) in experimental models showing the inhibition of lipid peroxidation in rat liver (Reddy A.C., Lokesh B.R. *Food Chem. Toxicol.*, **32**: 279-283 (1994)).

Other phenolic elements, such as tannins, show antioxidant (Satoh, K., Sakagami, H., 1996. Ascorbyl radical scavenging activity of polyphenols. *Anticancer Res.* **16**: 2885-2890) and hepatoprotective (Miyamoto, K.I., Nomura, M., Murayama, T., Furukawa, T., Hatano, T., Yashida, T., Koshiura, R., Okuda, T., 1993. Antitumor activities of ellagitannins against sarcoma -180 in mice. *Biol. Pharm. Bull.* **16**: 379-387) activity, inhibiting lipid peroxidation in hepatic microsomes and mitochondria (Okuda T., Kimuar Y., Yoshida T., Hatano T., Okunda H., Arichi S. Studies on the activities of tannins and related compounds from medicinal plants and drugs. I. Inhibitory effects on lipid peroxidation in mitochondria and microsomes of liver. *Chem. Pharm. Bull.* **31**: 1625-1631 (1983)). Tannic acid reduced the incidence of hepatic neoplasia in mice

(Hirose M., Ozaki K., Takaba K., Fukushima S., Shirai T., Ito, N. Modifying effects of the naturally occurring antioxidants gamma – oryzanol, phytic acid, tannic acid and n-tritriacontane-16, 18-dione in a rat wide-spectrum organ carcinogenesis model. *Carcinogenesis* 12: 1971-1921 (1991)). Results from extensive clinical research showed the effectiveness and safety of the polyphenols when it comes to treating hepatobiliary dysfunctions and digestive problems, such as a sensation of fullness, loss of appetite, nausea and abdominal pain. In addition, these elements have been found to have preventive and hepatoprotective effects against gastropathy induced by non-steroidal anti-inflammatories (Ruiyc. Advances in pharmacological studies of silymarin. *Mem Inst Oswaldo Cruz* 1991; 86:79-85; Scevola D, Barbacini G, Grosso A, Bona S, Perissoud D. Flavonoids and hepatic cyclic monophosphates in liver injury. *Boll Ins Steroter Milan* 1984; 63:77-82).

Coumarins are another kind of polyphenolic compounds that can be found in abundance in the vegetable kingdom. Many of them show interesting biological activity, for instance, the 4-methoxycoumarins have cholerectic properties (Takeda, S.; Aburada, M.J. *Pharmacobio-Dyn.* 4: 724 (1981)). The 7,8 dihydroxy-4-methylcoumarin and the 7,8-diacetoxy-4-methylcoumarin have antioxidant properties, and thus are considered effective scavengers of oxygen radicals (Raj, H.G.; Parmar, V.S.; Jain, S.C.; Priyadarsini, K.I.; Mittal, J.P.; Goel, S.; Poonam; Himanshu; Malhotra, S.; Singh, A.; Olsen, C.E.; Wngel, *J. Bioorg. Med. Chem.* 6: 833 (1998)). In addition, this group of molecules show protective effects against toxicity induced by a known oxidant (t-butylhydroperoxide) in HepG2 cells and primary human hepatocyte cultures (Bernard Refouvelet, Catherine Guyon, Yves Jacquot, Corinne Girard, Herve Fein, Francoise Bevalotb, Jean-Francois Robert a, Bruno Heyd, Georges Mantion, Lysiane Richert, Alain Xicluna, Synthesis of 4-hydroxycoumarin and 2, 4-quinolinediol derivatives and evaluation of their effects on the viability of HepG2 cells and human hepatocytes culture. *European Journal of Medicinal Chemistry* 39: 931-937 (2004)).

The terpenoids are other group of metabolites derived from plants that also show antioxidant (Zhu M, Chang Q, Wong LK, Chong FS, Li RC. Triterpene antioxidants from Ganoderma lucidum. *Phytotherapy Research* 13: 529-31 (1999)) and

hepatoprotective (James, L.P., Mayeux, P.R., Honson, J.A. Acetaminophen-induced hepatotoxicity. *Drug Metabolism Disposition* 31: 499-506 (2003)) activity. Triterpene celastrol shows a powerful inhibitory effect against lipid peroxidation in the hepatic mitochondria. *In vitro* and *in vivo* experiments, as well as other clinical tests, have shown the effects of gastroprotective (Zhu M, Chang Q, Wong LK, Chong FS, Li RC. Triterpene antioxidants from Ganoderma lucidum. *Phytotherapy Research* 13: 529-31 (1999)) and hepatoprotective activity of several terpenoids, such as the oleanic acid, ursolic acid, alpha-hederine, glycyrrhizin and lupeol (Liu, J., Liu, Y., Mao, Q. The effects of 10 triterpenoid compounds on experimental liver injury in mice. *Fundamental and Applied Toxicology* 22: 34-40 (1994)) (Sunitha S., Nagaraj M., Varalakshmi P. Hepatoprotective effect of lupeol and lupeol linoleate on tissue antioxidant defence system in cadmium-induced hepatotoxicity in rats. *Fitoterapia* 72: 516-523 (2001)).

There is a need for a safe and effective treatment for viral and non-viral hepatic disorders that address some of the disadvantages of current treatment methods.

SUMMARY OF THE INVENTION

According to a broad aspect of the invention, herbal compositions are provided comprising at least one species of the plant genera *Cordia*, *Annona* or *Curcuma*, or combinations thereof. In one embodiment, the at least one species is a species of the plant genus *Cordia*, for example, *Cordia lutea*. In another embodiment, the herbal composition comprises a species of each of the plant genera *Cordia*, *Annona* and *Curcuma*.

In another embodiment, an herbal composition is provided comprising *Cordia* spp flowers, *Annona* spp leaves, or *Curcuma* spp roots, or combinations thereof. In a further embodiment, an herbal composition is provided comprising *Cordia lutea* flowers, *Annona muricata* leaves and *Curcuma longa* roots. The herbal compositions of the present invention are useful for the prevention and/or treatment of hepatic disorders.

In another broad aspect, the invention is related to herbal compositions comprising extracts of at least one of the plant genera *Cordia*, *Annona* and *Curcuma*, or

At least one of the advantages of the herbal compositions of the present invention is their safety and effectiveness. For example, the herbal compositions comprising extracts of the plant genera of interest were found to be non-toxic at cell and tissue level, non-toxic in acute toxicity studies in mice and rats, and to be safe for human use.

The herbal compositions of the present invention could also act prophylactically through prevention of viral infection or other agents that cause hepatic disorders. Therefore, they can be used to treat hepatic disorders caused by viral infection, autoimmune reactions, consumption of xenobiotics and all those disorders that could compromise hepatic function. Treatment can be therapeutic and/or prophylactic.

The herbal compositions of the present invention can be administered orally or parenterally (topical, rectal, intravenous, intramuscular or hypodermic). Treatment can be administered orally in a liquid or solid form (*e.g.*, tablet), in one dose, multiple doses or through a slow-discharge or deposit method. In the alternative, herbal compositions can be in the form of a tea-like substance where hot water can be added to form a hot or cold drink.

In another broad aspect of the present invention, a method is provided to obtain hydroalcoholic extracts from a selected plant organ for use in the preparation of an herbal composition of the present invention, comprising:

- drying the selected plant organ and grinding the dried plant organ to obtain a powder having a particle size in range of about 0.35 mm to about 0.1 mm;
- macerating the powder in a hydroalcoholic solution for about 6 to about 8 days at around room temperature to obtain an extract of the plant organ;
- concentrating the extract by evaporation in a rotoevaporator; and
- freeze drying and sterilising the extract.

It is understood that other concentrating methods known in the art can also be used.

The herbal compositions of the present invention possess antioxidant activity in isolated hepatocytes in rats, *e.g.*, a decrease in the malonyldialdehyde (MDA) levels when hepatocytes are exposed to an inducer of lipid peroxidation, and a recovery in the glutathione (GSH) levels upon damaging induction. Thus, the hepatoprotective properties of the herbal compositions of the present invention may in part be as a result of their antioxidant properties and the inhibition of the spread of free radicals.

The herbal compositions of the present invention also possess regenerative or proliferative properties. Thus, in one aspect of the present invention, the herbal compositions of the present invention are useful for the regeneration of liver cells in patients.

The presence of chemical groups of secondary metabolites, such as phenols, tannins, terpenoids, lactones and coumarins, in the selected plant organs of the genera and species of interest may explain, at least in part, some of the biological actions observed.

In another broad aspect, the invention is related to a method for the treatment or prevention of hepatic disorders in a patient comprising administering to a patient a therapeutically effective amount of an herbal composition of the present invention. The hepatic disorder may be caused by a viral infection, such as hepatitis B and/or C, or a non-viral hepatic disorder, such as fibrosis, cirrhosis and non-viral hepatitis.

For purposes of summarizing the invention and the advantages achieved over the prior art, certain objects and advantages of the invention have been described above. Of course, it is to be understood that not necessarily all such objects or advantages may be achieved in accordance with any particular embodiment of the invention. Thus, for example, those skilled in the art will recognize that the invention may be embodied or carried out in a manner that achieves or optimizes one advantage or group of advantages as taught herein without necessarily achieving other objects or advantages as may be taught or suggested herein.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed

description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

For the purposes of the present invention, the term "extract" means a concentrate of water-soluble and/or alcohol-soluble and/or other appropriate solvent-soluble, such as hexane-soluble and chloroform-soluble, plant components from the portion of the plant extracted and can be in liquid or solid (*e.g.*, powder) form.

The invention will now be described in terms of following examples.

Example 1

Hydroalcohol extract of *Cordia* spp: 250 g of *Cordia lutea* flowers were dried by dehydration and then macerated with 1-1.5 litres of a hydroalcoholic solution (ethanol-water in a ratio of about 65:35 to about 75:25) for 6 to 8 days at room temperature. The macerated flowers in ethanol were then concentrated at low pressure using a standard rotoevaporator. The residual formed was then freeze-dried and sterilized. A 14-22g mass was obtained in the raw extract, yielding 6-9% of the sample mass. A 5 mg/ml stock solution was prepared for testing by dissolving 5mg in 0.7% ethanol/distilled water.

Example 2

Hydroalcohol extract of *Annona* spp: 250 g of *Annona muricata* leaves were dried using an oven at a temperature of about 45 to 55 degrees Celsius. The dried leaves were then subjected to a grinding process using a standard blade grinder. The powder obtained was sifted until particle size fraction measured between about 0.35 to about 0.10 millimetres. The powder was then macerated with 1-1.5 litres of a hydro-alcoholic solution (ethanol-water in a ratio of about 65:35 to about 75:25) for 6 to 8 days at room temperature. The macerated powder in ethanol was then concentrated at low pressure using a standard rotoevaporator. The residual was then freeze-dried and sterilised. A 14-22g mass was

obtained in the raw extract, yielding 5-8% of the sample mass. A 5 mg/ml stock solution was prepared for testing by dissolving 5mg in 0.7% ethanol/distilled water.

Example 3

Hydroalcohol extract of *Curcuma* spp: 250 g of *Curcuma longa* roots were dried using an oven at a temperature of 45 to 55 degrees Celsius. The dried roots were then subjected to a grinding process using a standard blade grinder. The powder obtained was sifted until particle size fraction measures between 0.35 and 0.10 millimetres. Then it is macerated with 1-1.5 litres of a hydro-alcoholic solution (ethanol-water 65:35 to 75:25) for 6 to 8 days at room temperature. This is then concentrated at low pressure using a standard rotoevaporator. The residual is then freeze-dried, sterilised. A 10-16g mass is obtained in the raw extract, yielding 4-7% of the sample mass. A 5 mg/ml stock solution was prepared for testing by dissolving 5mg in 0.7% ethanol/distilled water.

Example 4

An herbal composition comprising extracts of *Cordia lutea* flowers, *Annona muricata* leaves, and *Curcuma longa* roots was prepared as follows. The freeze-dried raw extracts obtained in Examples 1, 2 and 3 were mixed in that order in the following proportions: 8 mg:1 mg:1 mg to give a weight ratio of 8:1:1 (1:0.125:0.125), respectively, and a 5 mg/ml stock solution was prepared for testing by dissolving 5mg of the freeze-dried mixture in 0.7% ethanol/distilled water.

Example 5

Preparation of Hepatocytes

Extracts of herbal compositions of the present invention were tested using primary rat hepatocyte cultures to determine their antioxidant effects *in vitro*. Typically, tests were performed using male rats (Sprague Dawley) provided by CRIFFA (Santa Perpetua de La Mogoda, Barcelona), which were held at the Fe de Valencia Hospital Research Centre until the isolation of the hepatocytes was performed. The handling and sacrifice of the animals was carried out in accordance to national regulations, the

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The hepatoprotective effect of alcoholic extract of *Annona squamosa* leaves on experimentally induced liver injury in swiss albino mice

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Abstract

Annona squamosa is a multipurpose tree with edible fruits and is a source of medicinal and industrial products. The alkaloids present in *Annona squamosa*, a medicinal plant has proved to have antioxidant activity. The present study has been designed to evaluate the hepatoprotective effect of custard apple *(Annona squamosa)* in Diethylnitrosamine (DEN) induced swiss albino mice. Analysis of selected biochemical parameters (Total protein, Glutamyl Oxaloacetate Transaminase (GOT), Glutamyl Pyruvate Transaminase (GPT), Alkaline Phosphatase (ALP), Acid Phosphatase (ACP), Alpha Fetoprotein (AFP), Total and Direct bilirubin) in serum and tissue and also histopathological studies in liver are carried out in control and experimental mice. The levels of GOT, GPT, ALP, Total and Direct Bilirubin (both in serum and tissue), ACP, AFP (only in serum) are increased, and it is decreased in DEN induced along with *Annona squamosa* extract groups. But total proteins are found to decrease in DEN induced mice and increase in DEN induced along with *Annona squamosa* extract groups. Histopathology also confirms the hepato protective effect of *Annona squamosa*. So, our study has been recommended the herbal treatment of hepatoprotective effect using this plant for effective remedial. Further characterization and purification of the individual component in this plant is also suggested in formulating the strategy of treatment.

Keywords: *Annona squamosa*, Diethylnitrosamine, Biochemical analysis and Herbal therapy.

INTRODUCTION

Drugs are an important cause on liver injury. Drug-induced liver injury is a major health problem that challenges not only health care professionals but also the pharmaceutical industry and drug regulatory agencies (Michael *et al.*, 2005). The rate of hepatotoxicity has been reported to be much higher in developing countries like India (8% - 30%) compaired to that in advanced countries (2% - 3%) with a similar dose schedule (Sharma 2004). The use of medicinal plants to treat human diseases has been performed for millenniums. Nowadays, it is known that 80% of the world population have already taken medicinal plants and 30% are prescribed by physicians (De Silva OC *et al.*, 2003).

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The curative properties of drugs are due to the presence of complex chemical substances of varied composition in one or more parts of these plants. These plant metabolites in one, according to their composition, are grouped as alkaloids, glycosides, corticosteroids, essential oils, etc. Presence of alkaloids (Mukhlesur RM *et al.*, 2005 and Farrell PHJ *et al.*, 1975), terpinoids (Farrell PHJ *et al.*, 1975), glycosides (Farrell PHJ *et al.*, 1975), flavanoids (Kotkar HM *et al.*, 2002), corticosteroids, essential oils has been reported in the plant *Annona squamosa*. The plant is described as anticytotoxic (Mukhlesur RM *et al.*, 2005 and Farrell PHJ *et al.*, 1975), antimicrobial (Kotkar HM *et al.*, 2002), antioxidant (Shirwaikar A *et al.*, 2004 and Kumar CD *et al.*, 2004), antipesticidal (Parthasarathi BVV *et al.*, 2005), antiheadlice (Tiangda CH *et al.*, 2000), anti HIV effects (Wu YC *et al.*, 1996) and vaso relaxant effects (Morita H *et al.*, 2006).

This plant *Annona squamosa* is commonly called custard apple in English and Sharifa in Hindi and sitaphalam in Telugu in India. ***Annona squamosa*** is a multipurpose tree with edible fruits and is a source of medicinal and industrial products and it possesses

potent bioactive principles in all it parts. Various phytochemical, pharmacological, antibacterial and antiovulatory studies have already been carried out with seed extraction. Ayurvedic practitioners use an extract from the stem and leaves as an indigenous uterotomic drug (Gupta RK *et al.*, 2005). The plant is traditionally used for the treatment of epilepsy, dysentery, cardiac problems, ruinting, worm infestation, constipation, hemorrhage, dysuria, fever, thirst, malignant tumors and ulcers and also as an abortifacient (Shirwaikar A *et al.*, 2004). The alkaloids present in *Annona squamosa,* a medicinal plant has proved to have antioxidant activity (Shirwaikar A *et al.*, 2004 and Kumar CD *et al.*, 2004). Aporphine alkaloids, terpine derivatives, glycoside and a novel diazepine, squamolone are isolated from this plant. An ethanolic extract of the leaves and stem is reported to have anti cancer activity (Farrell PHJ *et al.*, 1975). Annotemoyin-1, Annotemoyin-2, Squamocin and Cholesteryl glucopyranoside are isolated from the seeds of *Annona squamosa.* These compounds and plant extracts has been showed remarkable antimicrobial and cytotoxic activities (Mukhlesur RM *et al.*, 2005). Flavonoid isolated from aqueous extract of *Annona squamosa* has been showed antimicrobial activity (Kotkar HM *et al.*, 2002). Bullatacin is one such compound that possessed antitumoral and pesticidal activity *invivo* (Ahmmadsahib KI *et al.*, 1993). *Annona squamosa* is said to show varied medicinal effects, including insecticidal, anti-ovulatory and abortifacient (Damaseeno DC *et al.*, 2002). So far there are no reports of clear biochemical and histopathological results indicating the hepatoprotective effect of *Annona squamosa* inducing DEN. So the present study has been designed to evaluate the hepatoprotective effect of custard apple *(Annona squamosa)* in Diethylnitrosamine (DEN) induced swiss albino mice. This would help to plan the strategy of treatment of hepatic problems using this plant.

MATERIALS AND METHODS

Preparation of leaf extract

Annona squamosa cultivation is most extensive in India and the fruit is exceedingly popular and abundant in markets. The plant material is collected, shade dried and powdered. 50 g of the dried powder has taken and mixed with 300 ml of anhydrate ethanol and kept at room temperature for 36 hours and the final extract has been taken using the soxlet apparatus (Kumar CD *et al.*, 2004).

Experimental induction of Liver injury in swiss albino mice

Selection of animals

Swiss albino mice weighing 30 to 40 g are purchased from Tamilnadu Veterinary and Animal Sciences University, Hyderabad. These mice are reared in animal house which has a well ventilated and lighted environment and also has all the facilities like racks, cages, washing and sterilization. 24 swiss albino mice weighing about 30 to 40 g are used as experimental animals. The animals are acclimatized in laboratory condition and randomly selected and also divided into 4 groups (Group I, II, III and IV), thus each group contains 6 animals. These mice are fed with normal rodent pellet diet for one month.

Treatment with Diethylnitrosamine (DEN)

A single 200 mg/Kg dose of Diethylnitrosamine (DEN) induces both extensive ethylation of liver DNA and cell necrosis, resulting in the generation of initiated hepatocytes eventually leading to hepatocellular carcinoma. The carcinogen has been administered at a standard dose of 200mg/Kg body weight, as an intra peritonial injection (Solt D *et al.*, 1976).

Group I

These groups of mice are served as control and these animals are fed with normal diet without any treatment.

Group II

The second groups are pre-treated with ethanolic extract of *Annona squamosa* at a concentration of about 5 g/Kg of body weight for 30 days.

Group III

The third group animals are treated as test animals. These animals are induced with DEN at a concentration of about 100 mg/Kg of body weight (dosage-two times, via Intra peritonial) to induce Liver injury and animals are pretreated with ethanolic extract of *Annona squamosa* about 5 g/Kg of body weight for 30 days.

Group IV

The fourth group animals are used as experimental animals. These animals are induced with liver damage by administrating DEN at a concentration of about 100 mg/Kg of body weight for two dosages has been given to animals via Intra peritonial (IP).

The grouping of animals is shown in Table 1 *[Supplementary data]*.

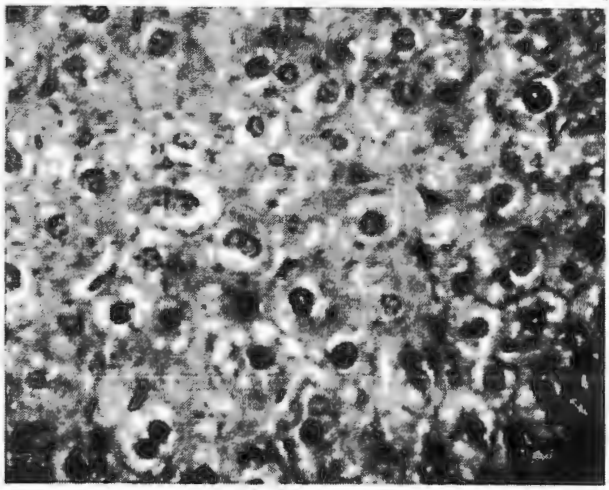


Figure 1: Normal diet animals: It shows a normal hepatic cell with well preserved cytoplasm, prominent nucleus and central vein.

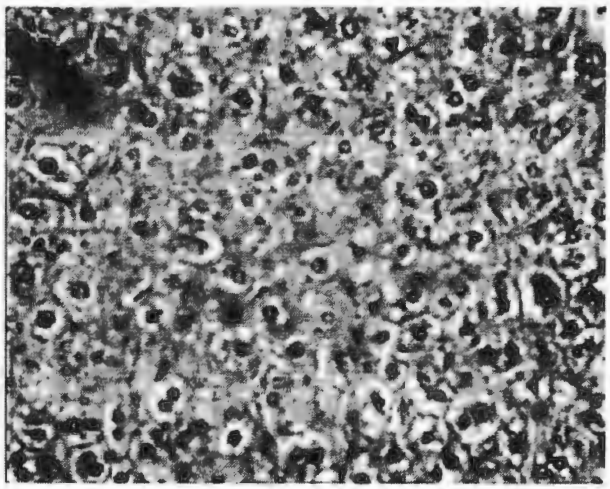


Figure 2: *Annona squamosa* treated mice's: It shows a normal lobular pattern with minimum pooling of blood in the sinusoidal spaces.

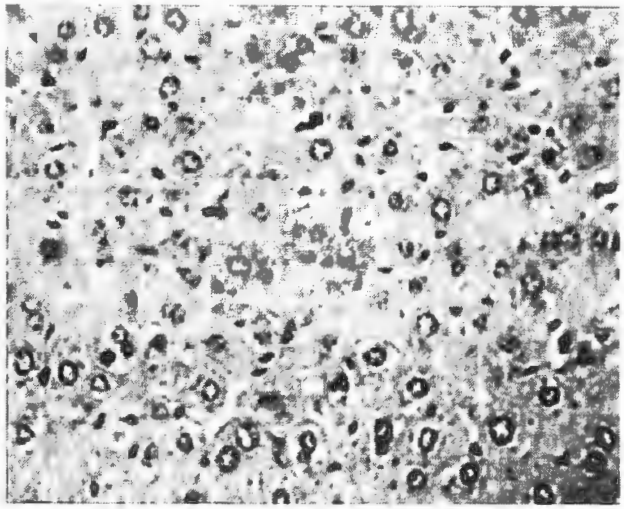


Figure 3: *Annona squamosa* treated along with the induction of DEN: It shows a recovered pattern of necrosis and blood pooling of sinusoidal space.

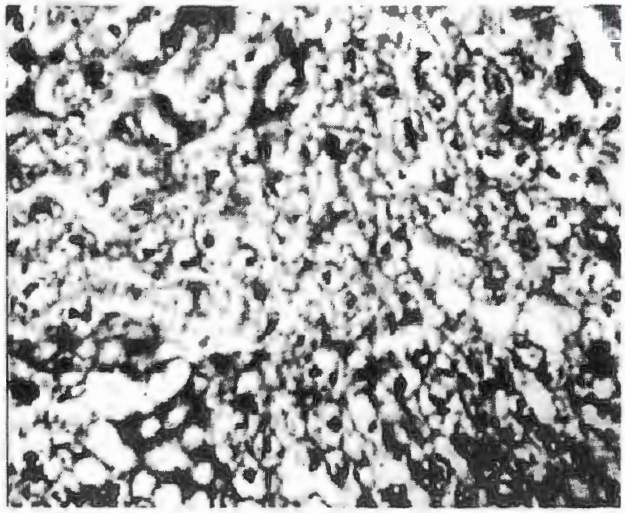


Figure 4: Induction of DEN: It reveals centrilobular necrosis, dilated sinusoidal spaces and necrosis with blood pooling in sinusoidal spaces and central venual.

Biochemical studies

After the treatment of the animals for 30 days duration, the animals are fasted overnight and weighed. Mice are sacrificed under chloroform anesthesia. They are bled by cardiac puncture and blood has been collected using insulin syringes. The obtained blood is allowed to coagulate and the serum is separated. Liver is quickly excised off, washed in saline, blotted dry and stored at 4°C. A known weight of liver tissue is homogenized using 0.1M phosphate buffer, pH 7.4; it is then centrifuged and used for various biochemical studies. Both the serum and liver tissue samples are subjected to further biochemical and tissue analysis.

Analysis of selected biochemical parameters

Following biochemical analysis parameters are analyzed in serum liver tissue.

Total protein level in both serum and liver tissue

Many plasma proteins, including albumin, fibrinogen and most globulins are formed in the liver. The plasma proteins level may be decreased; it causes some liver diseases and its dysfunction. The total proteins are estimated by Lowry's method (Lowry OH *et al.*, 1951 and Varley H *et al.*, 1991).

Serum and tissue Glutamyl Oxalo acetate Transaminase (GOT)

An enzyme that is normally present in liver and heart cells. Serum GOT (SGOT) is released into blood when the liver or heart is damaged. The blood SGOT levels are thus elevated with liver damage. Some medications

can also raise SGOT levels. SGOT is also called Aspartate aminotransferase (AST) (Walker PG *et al.*, 1972 and Varley H *et al.*, 1991).

Serum and tissue Glutamyl Pyruvate Transaminase (GPT)

Serum GPT (SGPT) is an enzyme that is normally present in liver and heart cells. It is released into blood when the liver or heart is damaged. The blood SGPT levels are thus elevated with liver damage. It is also called Alanine aminotransferase (ALT) (Walker PG *et al.*, 1972 and Varley H *et al.*, 1991).

Serum and tissue Alkaline Phosphatase (ALP)

It is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids. The alkaline phosphatase of normal serum in adults appears to be mainly derived from the liver. Increase in alkaline phosphatase activity occurs mainly in disease of liver and biliary tract (Walker K *et al.*, 1974 and Varley H *et al.*, 1991).

Serum Acid Phosphatase (ACP)

The determination of phosphatases can be helpful in monitoring any therapeutic response (Walker K *et al.*, 1974 and Varley H *et al.*, 1991).

Alpha Fetoprotein (AFP)

AFP can be particularly useful in early identification of liver injury and an aggressive tumors associated with hepatocellular carcinoma (HCC). Increased serum levels in adults are also seen in acute hepatitis, colitis and ataxia telangiectasia. The AFP level was estimated using ELISA kit method.

Bilirubin level in both serum and liver tissue

When the liver's function is impaired or biliary drainage blocked some of the conjugated bilirubin leaks out of the hepatocytes and appears in the urine. The presence of this conjugated bilirubin in the urine can be tested for clinically, and is reported as an increase in urine bilirubin (Varley H *et al.*, 1991).

Histopathological studies in liver

Animals are sacrificed to remove the liver. The liver has fixed in Bouin's solution for 12 hours, and then embedded in paraffin using conventional methods (Galighor AE *et al.*, 1976), cut into 5micrometre thick sections and stained using haematoxylin-eosin dye. The sections are then observed under microscope for degeneration, fatty changes, necrotic changes and evidence of hepatotoxicity if any. Results of the histopathological studies are shown in the Figures (Fig. 1 - Fig. 4).

Statistical analysis

Data are expressed as Mean, Median and standard Deviation. The significance of the difference between the means of the tests and control studies are established by applying the student's 't' test for independent samples.

RESULTS AND DISCUSSION

The results of the biochemical analysis are shown in Table 2 *[Supplementary data]*. The levels of GOT,GPT, ALP, Total and Direct Bilirubin (Both in serum and tissue), ACP, AFP (Only in serum) are increased in DEN induced mice and it is decreased in DEN induced along with *Annona squamosa* extract groups, but it still remains higher when compared to group I. Its level is decreased in group II when compared to group I. But only the amount of serum and tissue protein are decreased in DEN induced mice and it is increased in DEN induced along with *Annona squamosa* extract groups, but it still remains lower when compared to group I. Its level in group II is slightly increased when compared to group I.

Histopathological studies in liver of swiss albino mice

Group I: The liver sections of control animal are showed the normal hepatic cells with well-preserved cytoplasm, prominent nucleus and central vein (Fig. 1). Group II: The histological pattern of the liver of mice has pretreated with *Annona squamosa* extract shows a normal lobular pattern with minimum pooling of blood in the sinusoidal spaces (Fig. 2). Group III: The histopathological pattern of the liver of mice has pretreated with *Annona squamosa* extract along with DEN shows a minimal inflammation with moderate portal triaditis and their lobular architecture is normal (Fig. 3). Group IV: The liver section of DEN induced mice has revealed the centrilobular necrosis, dilated sinusoidal spaces and necrosis with blood pooling in sinusoidal spaces and central venual (Fig. 4).

Thus the results confirm the hepato protective effect of *Annona squamosa*. Based on the above findings from biochemical and histological studies, the plant, *Annona squamosa* is strongly recommended in the herbal treatment of hepatic problems for effective remedy. Further characterization and purification of the individual component in this plant is suggested in formulating the strategy of treatment.

References

Michael P, *et al.* (2006) Mechanisms of Drug-Induced Liver Injury. *The AAPS Journal*. 8: 48-54.

Sharma SK. (2004) Antituberculosis drugs and hepatotoxicity. *Infect Genet Evol.* 4:167-170.

De Silva OC, *et al.* (2003) Proliferative effect of medicinal plants and laser on liver regeneration. A considerable experimental model: from an experimental model to clinical applications. *Acta CirBras.* 18: 10-22.

Mukhlesur RM, *et al.* (2005) Anti microbial and Cycotoxic constituents from the seeds of *Annona Squamosa. Filtoterapia.* 76: 484-9.

Farrell PHJ, *et al.* (1975) High resolution two-dimensional electrophoresis of proteins. *Biol. Chem.* 250: 4007-12.

Kotkar HM, *et al.* (2002) Antimicrobial and Pesticidal activity of partially purified flavonoids of *Annona Squamosa. Pest Mang. Sci.* 58: 33-7.

Shirwaikar A, *et al.* (2004) *Invitro* antioxidant studies of *Annona Squamosa Linn.* Leaves. *J. Ethnopharmacol.* 91: 171-5.

Kumar CD, *et al.* (2004) *Invitro* antioxidant studies of *Annona squamosa Linn.* Leaves. *Indian J. Exp. Biol.* 42: 803-7.

Parthasarathi BVV, *et al.* (2005) Differential cytotoxic effects of *Annona squamosa* seed extract on human tumor cell lines: Role of reactive oxygen species and glutathione. *J. Biosci.* 30: 237-44.

Tiangda CH, *et al.* (2000) Anti-headlice activity of a preparation of *Annona squamosa* seed extract. *Southeast Asian J. Trop. Med. Public Health.* 31: 174-7.

Wu YC, *et al.* (1996) Identification of ent-16 beta, 17-dihydroxykauran-19-oic acid as an anti-HIV principle and isolation of the new diterpenoids annosquamosins A and B from *Annona squamosa. J. Nat. Prod.* 59: 635-7.

Morita H, *et al.* (2006) Vasorelaxant activity of cyclic peptide, cyclosquamosin B from *Annona squamosa. Bioorg. Med. Chem. Lett.* 16: 4609-11.

Gupta RK, *et al.* (2005) Nutrition and Hypoglycemic Effect of fruit pulp of *Annona squamosa* in normal healthy and alloxan-Induced Diabetic Rabbits. *Ann-Nuts Metab.* 49: 407-13.

Ahmmadsahib KI, *et al.* (1993) Mode of action of bullatacin; A potent antitumor and pesticidal Annonaceous acetogenins. *Life sci.* 53: 1113-8.

Damaseeno DC, *et al.* (2002) Effects of *Annona Squamosa* extract on early pregnancy in rats. *Phytomedicine.* 9: 667-72.

Solt D, *et al.* (1976) New principles for the analysis of chemical carcinogenesis. *Nature.* 263: 701-3.

Lowry OH, *et al.* (1951) Protein measurement with Folin-phenol Reagent. *J. Biol. chem.* 193: 265-75.

Walker PG, *et al.* (1972) Standardization of clinical enzyme assays: a reference method for aspartate and alanine transaminases. *J..Clin..Pathol.* 25: 940-4.

Walker K, *et al.* (1974) Acid & Alkaline phosphatase in serum, Methods in Enzymatic Analysis, (Academic Press, London).

Varley H, *et al.* (1991) Practical clinical biochemistry, (CBS Publishers and distributors: New Delhi). ISSN: 9780433338031, 535-45,891-918,735-45,1013-35.

Galighor AE and Kozloff EN, *et al.* (1976) Essentials of practical micro technique, 2nd edition, (lea and finger, New York). ISSN: 9780812103564, pp: 210-6.

Reference # 10

Health Canada Santé Canada

TURMERIC

Date: March 3, 2008

Proper name(s): *Curcuma longa* L. (Zingiberaceae) (USDA 1994)

Common name(s): Turmeric, common turmeric, yellow ginger, Indian saffron (McGuffin et al. 2000), curcuma (Mills et al. 2006; Blumenthal et al. 2000)

Source material(s): Rhizome (ESCOP 2003; Blumenthal et al. 2000)

Route(s) of administration: Oral

Dosage form(s): Those suited to the allowable route(s) of administration
This monograph is not intended to include food-like dosage forms such as bars, chewing gums or beverages.

Use(s) or Purpose(s): Statement(s) to the effect of:

- Traditionally used in Herbal Medicine to help relieve flatulent dyspepsia (carminative) (Mills and Bone 2005; Blumenthal et al. 2000; Wren 1907).
- Used in Herbal Medicine to aid digestion (ESCOP 2003; Williamson 2003; Blumenthal et al. 2000; Mills and Bone 2000).

Dose(s): Preparations equivalent to 1-14 g dried rhizome, per day (Mills and Bone 2005; ESCOP 2003; Williamson 2003; Blumenthal et al. 2000; Mills and Bone 2000)

See Appendix 1 for examples of appropriate dosage preparations, frequencies of use and directions for use, according to cited references. The purpose of Appendix 1 is to provide guidance to industry.

Canada

Duration of use: No statement required.

Risk information: Statement(s) to the effect of:

Caution(s) and warning(s):

- Consult a health care practitioner if symptoms persist or worsen.
- Consult a health care practitioner prior to use if you are taking antiplatelet medication or blood thinners (Mills and Bone 2005; Brinker 2001).
- Consult a health care practitioner prior to use if you have gallstones (ESCOP 2003; Brinker 2001; McGuffin et al. 1997).
- Consult a health care practitioner prior to use if you have stomach ulcers or excess stomach acid (Brinker 2001; McGuffin et al. 1997).

Contraindication(s):

- Do not use if you have a bile duct obstruction (ESCOP 2003; Brinker 2001; McGuffin et al. 1997).
- Do not use if you are pregnant (ESCOP 2003; Brinker 2001; McGuffin et al. 1997).

Known adverse reaction(s): No statement required.

Non-medicinal ingredients: Must be chosen from the current NHPD *List of Acceptable Non-medicinal Ingredients* and must meet the limitations outlined in the list.

Specifications: Must comply with the minimum specifications outlined in the current NHPD *Compendium of Monographs*.

References cited:

Blumenthal M, Goldberg A, Brinkmann J, editors. Herbal Medicine: Expanded Commission E Monographs. Boston (MA): Integrative Medicine Communications; 2000.

Brinker F. Herb Contraindications and Drug Interactions, 3rd edition. Sandy (OR): Eclectic Medical Publications; 2001.

ESCOP 2003: ESCOP Monographs: The Scientific Foundation for Herbal Medicinal Products, 2nd edition. Exeter (UK): European Scientific Cooperative on Phytotherapy and Thieme; 2003.

McGuffin M, Hobbs C, Upton R, Goldberg A, editors. American Herbal Products Association's Botanical Safety Handbook. Boca Raton (FL): CRC Press; 1997.

McGuffin M, Kartesz JT, Leung AY, Tucker AO, editors. Herbs of Commerce, 2nd edition. Silver Spring (MD): American Herbal Products Association; 2000.

Mills E, Dugoua J, Perri D, Koren G. Herbal Medicines in Pregnancy and Lactation: An Evidence-Based Approach. London (UK): Taylor and Francis Medical; 2006.

Mills S, Bone K. Principles and Practice of Phytotherapy. Toronto (ON): Churchill Livingstone; 2000.

Mills S, Bone K. The Essential Guide to Herbal Safety. St. Louis (MO): Elsevier Churchill Livingstone; 2005.

USDA 1994: United States Department of Agriculture, Agricultural Research Service, National Genetic Resources Program. Germplasm Resources Information Network (GRIN). *Curcuma longa* L. National Germplasm Resources Laboratory, Beltsville (MD). [Accessed 2008-01-24]. Available from: http://www.ars-grin.gov/cgi-bin/npgs/html/tax_search.pl

Williamson EM. Potter's Herbal Cyclopaedia: The Authoritative Reference work on Plants with a Known Medical Use. Saffron Walden (UK): The C.W. Daniel Company Limited; 2003.

Wren RC. Potter's Cyclopedia of Botanical Drugs and Preparations. London (UK): Potter and Clark; 1907.

References reviewed:

Aggarwal BB, Goel A, Kunnumakkara AB. Curcumin as "Curecumin": From kitchen to clinic. Biochemical Pharmacology 2008; (75):787-809.

Felter HW. The Eclectic Materia Medica, Pharmacology and Therapeutics. Sandy (OR): Eclectic Medical Publications; 1983 [Reprint of 1922 original].

Felter HW, Lloyd JU. King's American Dispensatory, Volume 2, 18th edition. Sandy (OR): Eclectic Medical Publications; 1983 [Reprint of 1898 original].

Gerard J. The Herbal or General History of Plants. The Complete 1633 Edition as Revised and Enlarged by Thomas Johnson. NY (NY): Dover Publications; 1975.

Grieve M. A Modern Herbal, Volume 2. New York (NY): Dover Publications; 1971 [Reprint of 1931 Harcourt, Brace & Company publication].

Hoffmann D. Medical Herbalism. Rochester (VT): Healing Arts Press; 2003.

Mills S. The Dictionary of Modern Herbalism. Wellingborough (UK): Thorsons Publishers Ltd; 1985.

Moerman DE. Native American Ethnobotany. Portland (OR): Timber Press; 1998.

Williamson EM, Evans FJ, Wren RC. Potter's New Cyclopaedia of Botanical Drugs and Preparations. Saffron Walden (UK): C.W. Daniel Company Limited; 1988.

Appendix 1: Examples of appropriate dosage preparations, frequencies of use and directions for use

Dried rhizome:

- 1-4 g, per day (Mills and Bone 2005)
- 1.5-3 g, per day (ESCOP 2003; Blumenthal et al. 2000)
- 1-4 g, per day (Williamson 2003; Blumenthal et al. 2000)
- 1-3 g, per day (Blumenthal et al. 2000)
- 0.5-1 g, several times per day (Blumenthal et al. 2000)

Infusion:

- 3-9 g dried rhizome, per day (Mills and Bone 2005)
- 1.3 g dried rhizome, 2 times per day (Blumenthal et al. 2000)

Directions for use: Pour 150 ml of boiling water on dried rhizome and steep for 10 to 15 minutes (Blumenthal et al. 2000).

Fluidextract:

- 5-14 g dried equivalent, per day
  (1:1, 5-14 ml) (Mills and Bone 2005)
- 1.5-3 g dried equivalent, per day
  (1:1, 1.5-3 ml) (Blumenthal et al. 2000)

Directions for use: Take 4-5 equal doses throughout the day (Mills and Bone 2000).

Tincture: 2 g dried equivalent, per day
(1:5, 10 ml) (Blumenthal et al. 2000)

Reference # 11

QUALITYMETRIC
INCORPORATED

July 27, 2004

Mr. L.B. (Brad) Clarke
President & CEO
Organetix, Inc. (related company to Sabell Corporation)
603 7th Ave. SW
Calgary, Alberta T2P 2T5, Canada

Subject: **Revised Summary Report on Study Findings of 10 Patients Receiving Nutraceutical Product A4+L for the Treatment for Chronic Hepatitis C Virus (CO-1031)**

Dear Mr. Clarke,

This letter responds to your request, on behalf of Organetix, Inc., (Organetix) to QualityMetric Incorporated (QualityMetric) for a summary report on the evaluation of the health-related quality of life burden associated with Chronic Hepatitis C and the impact of treatment with nutraceutical product A4+L. The summary report documents the methods used to analyze the study data and provides detailed information on the interpretation of the health-related quality of life burden associated with chronic hepatitis C and the meaningfulness of treatment response.

Please feel free to contact me if you have any questions about the data presented in this report.

Best regards,

Mark Kosinski, MA,
Senior Scientist

Attachment: as noted

cc: Jakob B. Bjorner, MD, PhD, Deputy Chief Science Officer, QualityMetric Incorporated
Paul G. Dion, CPA, Controller, QualityMetric Incorporated,
John E. Ware, Jr., PhD, CEO, Chief Science Officer, and Chairman of the Board, QualityMetric Incorporated
Martha S. Bayliss, MSc, Senior Scientist and Director, Business Development
David P. Luster, Account Manager
Dr. Arun Samanta

Introduction

Historically, the effects of treatment interventions have been defined in physiologic and biologic terms, using conventional clinical indices to measure treatment success. Conventional clinical indices provide valuable information about the course and severity of a disease, but they tell us little about the functional impairments (physical, emotional, social and role) associated with a disease that are important to patients in their every day lives. Certainly, patients with more severe disease tend to have worse functional status, but there is a growing body of evidence showing that correlations between clinical measures and how patients feel and how they are able to function in daily activities are only weak to moderate. Therefore, to obtain a complete picture of a patient's health status, measures of functioning and well-being, or more commonly referred to as health-related quality of life, must be measured in conjunction with conventional clinical indices to fully understand the burden associated with a disease and assess the benefits of treatment to the patient. In this study a well-validated health-related quality of life instrument, the Hepatitis Quality of Life Questionnaire (HQLQ), was used to assess the functional impairment associated with the chronic hepatitis C virus and the impact of treatment with the Nutraceutical Product A4+L.

Methods

Study Design and Subjects

The sample consisted of 10 patients with chronic hepatitis C virus. Patients received treatment with the Nutraceutical Product A4+L for a period of 28 days. Health-related quality of life was assessed at day 1 prior to the administration of treatment and at days 14 and 28 post-treatment.

Health-Related Quality of Life Measure

The Hepatitis Quality of Life Questionnaire (HQLQ) is a validated health-related quality of life instrument for studying patients with chronic hepatitis C.[1] The HQLQ is based on a core set of generic health measures from the SF-36 Health Survey[2] and is supplemented with additional generic and disease-specific measures thought to characterize the experience of living with the chronic hepatitis C virus.

The SF-36 Health Survey consists of eight multi-item scales that measure physical functioning (PF), role limitations due to physical health (RP), bodily pain (BP), general health perceptions (GH), vitality (VT), social functioning (SF), role limitations due to emotional problems (RE), and mental health (MH). These eight multi-item scales are further aggregated and scored as summary measures of physical (PCS) and mental (MCS) health status. Scores on the SF-36 scales and summary measures are norm-based, where the mean is 50 and the standard deviation is 10 in the general U.S. population and higher scores indicate better health status. The main advantage of the norm-based scoring of SF-36 scales and summary measures is easier interpretation. The general U.S. population norm is built into the scoring algorithm. Accordingly, all scores above of below 50 can be interpreted as above and below the norm of the general population. And because the standard deviations for each scale are standardized at 10, it is easier to see exactly how far above or below the norm a score is in standard deviation or effect size units.[3]

Additional health measures included in the HQLQ consist of two generic and two disease-specific scales of health.[1] These scales were added to the HQLQ to capture effects of chronic hepatitis C virus not captured by the SF-36. The two additional generic scales measure health distress and positive well-being. The two additional disease-specific scales measure limitations in every day activities because of hepatitis and health distress due to hepatitis. These scales are scored on a 0-100 scale, where a higher score indicates more favorable health status.

Health-Related Quality of Life Burden

General population norms were used to estimate the health-related quality of life burden associated with having the chronic hepatitis C virus. Norms for the SF-36 Health Survey come from the 1998 National Survey of Functional Health Status.[3] The meaningfulness of the health-related quality of life burden associated with the chronic hepatitis C virus was determined in three ways. First, we compared SF-36 scale and summary measure scores of the 10 study patients with baseline SF-36 scores of 8

published clinical studies involving chronic hepatitis C patients.[4,5,6,7,8,9,10,11] Second, we compared SF-36 scale and summary measure scores of the 10 study patients with clinical norms for the following chronic conditions: hypertension, congestive heart failure (CHF), myocardial infarction (MI), diabetes, and clinical depression. SF-36 clinical norms come from the Medical Outcomes Study.[12] Third, to interpret the meaning of differences in SF-36 scale scores between the 10 study patients and the general population norms, the content of several items was examined. For example, we compared the percentage of the 10 study patients that reported limitations in climbing stairs (an item in the SF-36 physical functioning scale) to the percentage of the general population that reported limitations in climbing stairs. Lastly, using the clinical cutpoint score (42 and below) for depression on the mental summary scale,[13] we computed the percentage of the 10 study patients screening positive for depression at the baseline visit.

Impact of Treatment on Health-Related Quality of Life

The impact of treatment with the Nutraceutical Product A4+L on health-related quality of life was evaluated in 2 ways. First, mean SF-36 scale scores at day 14 and day 28 of the 10 study patients were compared to day 1 scores and against normative scores from the general population. The aim of these comparisons was to determine how close treatment restored patient's functional status back to normal. Second, we tested the statistical significance of the changes in SF-36 and HQLQ scale scores from baseline to day 14 and day 28 using Student's t-test. These tests determine whether the change in scores were significantly different from zero. The changes in scale scores were translated into treatment effect sizes by dividing the mean changes in scale scores of the 10 study patients by the standard deviation of the general population scale scores.[14] Lastly, to interpret the meaning of the changes in SF-36 and HQLQ scale scores the content of several items was examined. For example, we compared the percentage of the 10 study patients that reported limitations in climbing stairs (an item in the SF-36 physical functioning scale) at baseline with the percentage reporting limitations in climbing stairs at day 14 and day 28.

Results

Health-Related Quality of Life Burden

Figure 1 presents the mean day 1 SF-36 scale and summary measure scores of the 10 study patients in comparison to the general population norms (scores of 50 on all scales and summary measures) and in comparison to patients with CHF and clinical depression. Mean SF-36 scale and summary measure scores of the 10 study patients were 1.5 to 2.5 standard deviations below general population norms, indicative of significant health-related quality of life burden. Comparison of scores with CHF and clinical depression norms suggest that the health-related quality of life burden associated with chronic hepatitis C should be considered clinically significant. With few exceptions, SF-36 scale and summary measure scores of the 10 study patients were below the norms for CHF and clinical depression. In addition, SF-36 scale and summary measure scores of the 10 study patients were also below the norms for hypertension, MI, and diabetes (see Table 2).

In comparison to other patients with chronic hepatitis C the 10 patients in this study showed greater average HRQoL burden. Table 1 presents the mean day 1 SF-36 scale and summary measure scores of the 10 study patients against baseline SF-36 scores observed in 8 published studies involving patients with chronic hepatitis C patients. Overall, the 10 patients in this study scored lower on all SF-36 scales and summary measures compared with baseline scores observed across the 8 published studies.[4,5,6,7,8,9,10,11] In two of the published studies (Study 5 and Study 8), the average HRQoL burden observed of chronic hepatitis patients approached the level observed with the 10 patients in this study, however the HRQoL burden observed in these two studies were compounded by other clinical factors. For example, in Study 5 the chronic hepatitis C patients suffered from significant emotional problems, which had a significant impact on those SF-36 scales measuring emotional well-being (Social Functioning, Role Emotional, Mental Health, and Mental Summary scales). In study 8, the 15 chronic hepatitis patients had hepatocellular carcinoma, which appeared to have a significant impact across all SF-36 scales. In a separate attachment, copies of each of

these 8 studies have been provided to assist in determining the equivalence in clinical characteristics between the 10 patients enrolled in this study and the patients participating in the 8 published studies.

To further our interpretation of the health-related quality of life burden associated with chronic hepatitis C, Table 4 shows that at day 1: 1) 100% of study patients reported limitations in walking more than one mile compared to only 21.9% in the general population; 2) 50% of study patients reported limitations in climbing 1 flight of stairs compared to only 7.1% in the general population; 3) 55.5% of study patients reported limitations in walking 100 yards compared to 14.1% in the general population; 4) 100% of study patients reported having difficulty performing at work compared to only 45% in the general population; 5) 30% of study patients reported that pain interfered with their ability to work compared to 9.5% in the general population; 6) 90% of study patients reported "fair" or "poor" health compared to 13.1% in the general population; 7) 0% of study patients reported "excellent" health compared to 15.6% in the general population; 8) 90% of study patients reported feeling tired all or most of the time compared to 13.4% in the general population; 9) 70% of study patients reported that their health interfered with usual social activities compared to 7.3% in the general population; 10) 100% of study patients reported that they accomplished less at work due to emotional problems compared to 37.4% in the general population; 11) 30% of study patients reported being downhearted and depressed all or most of the time compared to 3.1% in the general population; 12) 70% of study patients reported that hepatitis limited their ability to perform daily work all or most of the time; and 13) 60% of study patients reported that hepatitis limited their ability to engage in usual social activities. Lastly, as shown in Figure 2, 90% of study patients screened positive for depression at day 1.

Impact of Treatment with the Nutraceutical Product A4+L *on Health-Related Quality of Life*

Figure 3 plots the mean SF-36 scale and summary measure scores at day 1, day 14 and day 28 for the 10 study patients against general population norms (scores of 50). As shown, at day 14 there was substantial improvement with all SF-36 scale and

summary measure scores in comparison to day 1 scores, and by day 28, the mean scores of the 10 study patients were at or above general population norms for all SF-36 scales and summary measures.

Table 5 provides significance testing for the changes in SF-36 and HQLQ scale scores from baseline to day 14 and baseline to day 28. As shown, the changes in scores from baseline to day 14 and day 28 were statistically significant across all SF-36 and HQLQ scales. In treatment effect size terms, the changes in SF-36 and HQLQ scores from baseline to day 14 ranged from 0.77 to 1.46, which are considered large treatment effect sizes.[15] By day 28, for the majority of the SF-36 and HQLQ scales (10 of 14 scales) the treatment effect size exceeded 2.0, meaning that on average the score changes on these 10 scales were greater than 2 standard deviations. Inspection of the frequency distributions of the change scores at day 28 show that 9 of the 10 patients improved by a clinically meaningful amount on all SF-36 and HQLQ scales.

Table 6 and Figures 6-15 provide a patient-by-patient account of HRQoL scores at days 1, 14 and 28. These data show that the HRQoL of 6 patients (patients 1, 2, 3, 6, 7, and 8) was restored to normal or above normal levels (for example all SF-36 scores were at or above 50 at day 28). For 3 of the patients (patients 4, 5, and 9) their HRQoL was restored to near normal levels (for example several SF-36 scale scores were at or above 50 and some were slightly below 50 by 2-4 points). It should be pointed out that although not all aspects of HRQoL were restored to normal for these 3 patients, their day 28 scores were significantly better than day 1 scores. Only one patient (patient 10) showed no HRQoL improvement from day 1 to days 14 and 28.

The data in Table 4 provides a means for interpreting the meaningfulness of the changes in SF-36 and HQLQ scale scores. As shown in Table 4 there were significant reductions in the percentage of study patients who reported physical, social, role, and emotional limitations from day 1 to days 14 and 28. For example, at day 1, 100% of the study patients reported limitations in walking more than one mile. After 14 days of treatment the percentage of the study patients that reported limitations in walking more

than one mile was reduced to 70% and by day 28, none of the study patients reported limitations in walking more than one mile. The data in Table 4 also show that: 1) the percentage patients that reported limitations in climbing one flight of stairs dropped from 50% at day 1, to 20% at day 14 and to 10% at day 28; 2) the percentage of patients that reported limitations in walking 100 yards dropped from 55% at day 1, to 20% at day 14 and to 10% at day 28; 3) the percentage of patients that reported difficulty performing at work dropped from 100% at day 1, to 90% at day 14 and to 50% at day 28; 4) the percentage of patients that reported pain interfered with work dropped from 30% at day 1, to 10% at days 14 and 28; 5) the percentage of patients that reported "fair" or "poor" health dropped from 90% at day 1, to 33% at day 14 and to 10% at day 28; 6) the percentage of patients that reported excellent health increased from 0% at day 1, to 20% at day 28; 7) the percentage of patients that reported feeling tired all or most of the time dropped from 90% at day 1, to 30% at day 14 and to 11% at day 28; 8) the percentage of patients that reported their health limited them from engaging in social activities all or most of the time dropped from 70% at day 1 to 10% at day 14, and 0% at day 28; 9) the percentage of patients that reported accomplishing less due to emotional problems all or most of the time dropped from 100% at day 1 to 90% at day 14, and 10% at day 28; 10) the percentage of patients that reported feeling downhearted and depressed all or most of the time dropped from 30% at day 1 to 0% at days 14 and 28; 11) the percentage of patients that reported that hepatitis limited them in performing at work all or most of the time dropped from 70% at day 1 to 20% at day 14, and 10% at day 28; and 12) the percentage of patients that reported that hepatitis limited them in engaging in normal social activities all or most of the time dropped from 60% at day 1 to 10% at days 14 and 28. Note that by day 28 the percentage of study patients that reported physical, social, role, and emotional limitations was nearly equal to or lower than the percentage reporting limitations in the general population.

The clinical significance of the changes in the SF-36 mental health scales can be seen in Figure 2. Figure 2 shows significant reductions in the percentage of study patients that screened positive for depression. At day 1, 9 out of 10 patients screened positive for depression. By day 14 the percentage of patients who screened positive for

depression dropped to 40%, or 4 in 10 patients, and by day 28 none of the patients screened positive for depression.

Conclusion

Overall, the health-related quality of life of the 10 chronic hepatitis C patients that participated in this study was substantially compromised. On average, the patients participating in this study scored one and one-half to two and one-half standard deviations below general population norms, which was larger than the average health-related quality of life burden observed in other studies of chronic hepatitis C.[4,5,6,7,8,9,10,11,12,16] Although, one should carefully considered the differences in the clinical characteristic between patients in this study and chronic hepatitis C patients participating in other studies before drawing any conclusions. Regardless, comparisons of health-related quality of life scores with other chronic conditions (hypertension, CHF, MI, diabetes, and clinical depression) suggest that the burden observed in this study should be considered clinically significant.

On average, treatment with the Nutraceutical Product A4+L was observed to significantly improve the health related quality of life of study patients by day 14 and by day 28 the functional status and well-being of study patients was completely restored to normal levels. The score improvements observed in this study by day 28 were on average larger than two standard deviations for nearly all health related quality of life scales, which has rarely been observed in the thousands of treatment studies of other chronic diseases involving the SF-36 Health Survey.[17] In addition, the evaluation of individual patient scores over time showed that 9 out of the 10 patients in this study improved by day 28 by a clinically meaningful amount on each of the health related quality of life scales, so the average results were not driven by a couple of outlier patients.

A limitation of this study concerns the sample size. Obviously with 10 patients the generalizability of the results of this study is limited. With such a small sample the patients participating in this study are not likely to be representative of the average

chronic hepatitis C patient. The health-related quality of life burden observed in this study in comparison to other studies would seem to support this impression. The small sample size in this study also posed another concern with respect to statistical power. With 10 patients the power to detect statistically significant changes in health-related quality of life scale scores was seriously compromised, meaning that it would take extremely large changes in scores to be considered statistically significant. Despite the lack of statistical power, we observed statistically significant changes across all health related quality of life scale scores.

References

1. Bayliss MS, Gandek B, Bungay KM, et al. A questionnaire to assess the generic and disease-specific health outcomes of patients with chronic hepatitis C. Quality of Life Research 1998;7:39-55.

2. Ware JE, Sherbourne CD. The MOS 36-item short-form health survey (SF-36). I. Conceptual framework and item selection. Medical Care 1992;30(6):473-483.

3. Ware JE, Kosinski M, Dewey JE. How to Score Version 2 of the SF-36 Health Survey. Lincoln, RI: QualityMetric Incorporated, 2000.

4. Bonkovsky HL, Woolley JM, And The Consensus Interferon Study group. Reduction of health-Related Quality of Life in Chronic Hepatitis C and improvement with Interferon Therapy. *Hepatology* 1999.

5. Chong CA, Gulamhussein A, Heathcote EJ, Lilly L, Sherman M, Naglie G et al. Health-state utilities and quality of life in hepatitis C patients. *American Journal of Gastroenterology* 2004.

6. Fontana RJ, Moyer CA, Sonnad S, Lok ASF, Sneed-Pee N, Walsh J et al. Comorbidities and quality of life in patients with interferon-refractory chronic hepatitis C. *Am J Gastroenterol* 2001; 96(1):170-178.

7. Fontana RJ, Hussain KB, Schwartz SM, Moyer CA, Su GL, Lok AS. Emotional distress in chronic hepatitis C patients not receiving antiviral therapy. *J Hepatol* 2002; 36(3):401-407.

8. Hussain KB, Fontana RJ, Moyer CA, Su GL, Sneed-Pee N, Lok AS. Comorbid illness is an important determinant of health-related quality of life in patients with chronic hepatitis C. *American Journal of Gastroenterology* 2004.

9. Kleinman L, Zodet MW, Hakim Z, Aledort J, Barker C, Chan K et al. Psychometric evaluation of the fatigue severity scale for use in chronic hepatitis C. *Qual Life Res* 2000; 9(5):499-508.

10. Kramer L, Bauer E, Funk G, Hofer H, Jessner W, Steindl-Munda P et al. Subclinical impairment of brain function in chronic hepatitis C infection. *Journal of Hepatology* 2004.

11. Moyer CA, Fontana RJ, Hussain K, Lok ASF, Schwartz S. The Role of Optimism/Pessimism in HRQOL in Chronic Hepatitis C Patients. *Journal Of Clinical Psychology in Medical Settings* 2003.

12. Tarlov AR, Ware JE, Greenfield S, et al. The Medical Outcomes Study: An application of methods for monitoring the results of medical care. Journal of the American Medical Association 1989;262:925-930.

13. Ware JE and Kosinski M. SF-36 Physical and Mental Health Summary Scales: A manual for users of Version 1, second edition, Lincoln, RI: QualityMetric Incorporated, 2001.

14. Kazis LE, Anderson JJ, Meehan RF. Effect sizes for interpreting changes in health status. Medical Care 1989;37(3):S178-S189.

15. Cohen J. Statistical *Power Analysis for the Behavioral Sciences* – Second Edition. Hillsdale, NJ: Lawrence, Erlbaum Associates, 1988.

16. Ware JE, Bayliss MS, Mannocchia M, et al. Health-related quality of life in chronic hepatitis C: Impact of disease and treatment response. Hepatology 1999;30:550-555.

17. Turner-Bower D, Bartley PJ, Ware JE. SF-36 Health Survey Bibliography: Third Edition (1988-2000). Lincoln, RI: QualityMetric Incorporated, 2002.

Tables

Table 1. HRQoL Burden of Hepatitis C Patients Participating in a 28 Day Study of Organetix Compound: Comparison of Mean Baseline SF-36 Scale Scores Across 8 HRQoL Study Involving Chronic Hepatitis C Patients

	Organetix (N=10)	Study 1 (N=220)	Study 2 (N=100)	Study 3 (N=642)	Study 4 (N=107)	Study 5 (N=78)	Study 6 (N=1,215)	Study 7 (N=123)	Study 8 (N=15)*	Mean[1]
PF	36.2	45.4	47.9	48.9	50.4	39.9	49.6	44.9	35.8	45.4
RP	27.2	45.5	45.8	47.0	48.0	38.9	47.5	43.5	32.9	43.6
BP	37.3	46.0	51.6	50.1	51.2	40.5	52.0	45.6	42.5	47.4
GH	26.8	41.1	43.8	45.9	49.0	34.9	45.7	40.1	34.0	41.8
VT	35.2	46.2	46.2	47.8	50.9	40.5	48.6	45.3	38.8	45.5
SF	26.9	44.9	44.5	47.9	50.6	37.2	47.5	44.1	36.5	44.2
RE	25.6	45.2	46.8	48.0	50.9	36.7	47.7	43.0	38.7	44.6
MH	34.5	41.4	41.3	48.8	51.6	36.2	47.6	41.3	45.2	44.2
PCS	34.2	45.7	49.1	48.1	49.1	40.5	49.5	44.9	34.6	45.2
MCS	29.8	43.6	43.0	48.1	51.6	36.7	47.1	42.6	42.8	44.4

1 Mean = Average of scale scores across the 8 published studies.

Study 1 = Hussain et al., 2001
Study 2 = Kramer et al., 2002;
Study 3 = Bonkovsky et al., 1999;
Study 4 = Fontana et al., 2001;
Study 5 = Fontana et al., 2002;
Study 6 = Kleinman et al., 2000;
Study 7 = Moyer et al., 2003;
Study 8 = Chong et al., 2003

*Note that the 15 patients in study 8 had hepatocellular carcinoma.

Table 2. HRQoL Burden of Hepatitis C Patients Participating in a 28 Day Study of Organetix Compound: Comparison of SF-36 Scale and Summary Measure Scores to Patients with Chronic Conditions from the Medical Outcomes Study

	Study Data: 10 Hepatitis C Patients						Chronic Conditions from Medical Outcomes Study									
	Day 1 N=10		Day 2 N=10		Day 3 N=10		Hypertension N=1,937		CHF N=192		MI N=102		Diabetes N=499		Depression N=476	
SF-36 Scales	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
PF	36.2	8.3	44.2	9.7	50.3	9.4	45.3	11.4	35.4	12.0	43.7	11.1	42.6	12.3	45.8	11.3
RP	27.2	7.0	38.0	12.0	48.8	9.4	44.8	11.3	37.7	10.8	42.2	11.5	42.8	11.7	40.6	11.3
BP	37.3	8.4	45.3	10.5	50.7	11.0	49.9	10.7	47.7	11.5	49.4	10.9	48.4	11.4	45.2	11.4
GH	26.8	6.3	38.8	11.5	48.4	12.9	46.0	9.6	38.8	10.0	43.9	9.2	42.6	10.1	41.6	10.5
VT	35.2	5.9	47.7	11.0	57.7	12.4	49.8	10.2	44.3	10.3	49.6	9.5	48.3	10.4	41.9	9.9
SF	26.9	9.0	39.9	8.3	48.7	8.2	50.8	9.4	46.2	12.4	49.4	10.1	48.7	10.9	39.1	11.7
RE	25.6	10.2	36.4	14.9	52.0	6.6	48.0	11.3	45.3	13.1	46.4	12.2	47.2	11.8	36.0	12.6
MH	34.5	10.0	49.2	7.0	55.1	5.3	51.1	10.2	50.9	10.5	50.3	9.8	50.5	10.8	33.8	11.5
PCS	34.2	6.1	41.8	9.9	48.2	11.5	45.5	10.9	36.5	10.9	43.5	10.1	42.6	11.1	47.6	11.6
MCS	29.8	8.2	43.9	8.8	54.8	5.1	51.7	9.8	51.4	10.2	50.9	9.7	51.2	10.2	34.0	12.3

Table 3. HQLQ Hepatitis-specific Scale Scores for Hepatitis C Patients Participating in a 28 Day Study of the Nutraceutical Product A4+L.

	Study Data					
	Day 1		Day 14		Day 28	
	(N=10)		(N=10)		(N=10)	
	Mean	SD	Mean	SD	Mean	SD
HQLQ Hepatitis-specific scales						
Health distress (generic)	28.50	25.93	61.00	21.71	84.00	18.97
Positive well-being	45.00	14.53	66.00	16.80	80.50	15.71
Hepatitis-specific limitations	35.33	25.73	60.00	28.28	81.33	30.11
Hepatitis-specific health distress	33.17	29.03	59.00	25.69	84.50	14.42

HQLQ = Hepatitis Quality of Life Questionnaire.

Table 4. HRQOL Burden of Hepatitis C Patients Participating in a 28 Day Study of the Nutraceutical Product A4+L

	Day 1	Day 14	Day 28	General U.S. Population (N=1982)
Physical Functioning				
Limitations in walking more than a mile				
"any limitations"	100.0%	70.0%	30.0%	21.9%
Limitations climbing 1 flight of stairs				
"any limitations"	50.0%	20.0%	10.0%	7.1%
Limitations walking 100 yards				
"any limitations"	55.5%	20.0%	10.0%	14.1%
Role Physical				
Difficulty performing at work				
"any limitations"	100.0%	90.0%	50.0%	45.0%
Bodily Pain				
Pain interfered at work				
"quite a lot/extremely"	30.0%	10.0%	10.0%	9.5%
General Health				
Fair or Poor Health Evaluation	90.0%	33.3%	10.0%	13.1%
Excellent Health Evaluation	0.0%	0.0%	20.0%	15.6%
Vitality				
Feeling Tired				
"all or most of the time"	90.0%	30.0%	11.1%	13.4%
Social Functioning				
Health limited social activities				
"all or most of the time"	70.0%	10.0%	0.0%	7.3%
Role Emotional				
Accomplish less at work				
"any limitations"	100.0%	90.0%	20.0%	37.4%
Mental Health				
Downhearted and depressed				
"all or most of the time"	30.0%	0.0%	0.0%	3.1%
HQLQ Hepatitis-Specific Limitations				
Hepatitis limited daily work				
"all or most of the time"	70.0%	20.0%	10.0%	n/a
Hepatitis limited normal social activities				
"all or most of the time"	60.0%	10.0%	10.0%	n/a

n/a - general U.S. population norm not available.
HQLQ = Hepatitis Quality of Life Questionnaire.

Table 5. Health-Related Quality of Life Outcomes among 10 Chronic Hepatitis C Patients

	Baseline to Day 14			Baseline to Day 28		
	Mean Δ (SE)	t-statistic	ES	Mean Δ (SE)	t-statistic	ES
SF-36 Scales						
Physical Functioning	8.04 (2.6)	3.13**	0.80	14.13 (2.1)	5.43***	1.41
Role Physical	10.77 (2.2)	4.84***	1.08	21.55 (2.8)	7.83***	2.16
Bodily Pain	8.03 (1.7)	4.63***	0.80	13.36 (2.3)	5.80***	1.34
General Health	11.98 (2.5)	4.76***	1.19	21.55 (3.7)	5.76***	2.16
Vitality	12.49 (2.3)	5.48***	1.25	22.48 (3.5)	6.41***	2.25
Social Functioning	13.09 (1.8)	7.06***	1.31	21.82 (3.0)	7.17***	2.18
Role Emotional	10.89 (2.7)	4.02**	1.09	26.43 (2.5)	10.52***	2.64
Mental Health	14.64 (3.3)	4.47**	1.46	20.56 (2.8)	7.21***	2.06
Physical Summary	7.67 (1.8)	4.15**	0.77	14.05 (2.8)	4.92***	1.41
Mental Summary	14.15 (1.9)	7.49***	1.42	25.07 (2.3)	11.03***	2.51
Hepatitis Distress (generic)	32.50 (5.2)	6.28***	1.41	55.50 (8.8)	6.31***	2.39
Positive Well-Being	21.00 (6.2)	3.37**	0.98	35.50 (7.1)	4.98***	1.65
Hepatitis Specific Limitations	24.67 (8.2)	3.01**	1.09	46.00 (8.7)	5.26***	2.06
Hepatitis Specific Distress	25.83 (6.6)	3.94***	1.08	51.33 (7.8)	6.57***	2.15

Mean Δ = mean change in score from baseline
SE = Standard error of change score
ES = Effect Size as determined by dividing the mean change score by the standard deviation of the scale score observed in the general population.

Table 6. Baseline, Day 14, and Day 28 SF-36 and HQLQ Scale Scores for 10 Hepatitis C Patients

	Patient 1			Patient 2			Patient 3		
Scales	Day 1	Day 14	Day 28	Day 1	Day 14	Day 28	Day 1	Day 14	Day 28
PF	31.8	52.8	57.0	38.1	48.6	52.8	25.5	44.4	50.7
RP	27.5	47.1	56.9	32.4	44.6	47.1	17.7	17.7	56.9
BP	33.0	51.1	62.1	37.2	46.1	50.3	46.1	55.4	62.1
GH	25.8	43.4	63.9	23.4	31.5	41.0	23.4	48.2	55.3
VT	33.4	55.2	70.8	33.4	45.9	52.1	45.9	58.3	58.3
SF	24.1	45.9	56.9	24.1	40.5	45.9	13.2	29.6	51.4
RE	24.8	44.2	55.9	28.7	44.2	55.9	17.0	9.2	55.9
MH	35.9	55.6	64.1	33.1	52.8	58.5	16.2	50.0	52.8
PCS	30.6	48.9	59.0	35.6	42.1	45.1	34.3	45.7	56.4
MCS	30.4	50.1	62.3	28.2	47.0	55.9	18.8	33.6	54.0
HD	50.0	75.0	100.0	20.0	65.0	95.0	0.0	40.0	95.0
PWB	35.0	85.0	100.0	35.0	60.0	70.0	35.0	60.0	80.0
HLIM	20.0	86.7	100.0	40.0	66.7	80.0	33.3	40.0	100.0
HHD	55.0	85.0	100.0	30.0	75.0	85.0	0.0	20.0	80.0

	Patient 4			Patient 5			Patient 6		
Scales	Day 1	Day 14	Day 28	Day 1	Day 14	Day 28	Day 1	Day 14	Day 28
PF	33.9	40.2	48.6	33.9	29.7	48.6	33.9	48.6	54.9
RP	27.5	37.3	37.3	25.0	39.7	44.6	37.3	44.6	56.9
BP	37.2	46.1	51.1	29.2	33.4	37.2	46.1	46.9	62.1
GH	25.8	25.8	31.5	21.0	25.8	41.0	25.8	33.9	63.9
VT	30.2	36.5	52.1	36.5	39.6	58.3	33.4	49.0	70.8
SF	29.6	35.0	45.9	24.1	35.0	45.9	29.6	40.5	56.9
RE	20.9	32.6	44.2	24.8	48.1	52.0	32.6	44.2	55.9
MH	30.3	38.7	50.0	27.5	44.4	47.2	44.4	44.4	52.8
PCS	34.7	39.6	41.9	30.2	27.9	41.7	35.7	44.9	60.6
MCS	25.8	34.3	49.0	27.3	47.9	52.7	36.3	43.5	57.3
HD	65.0	80.0	85.0	0.0	35.0	70.0	30.0	65.0	100.0
PWB	45.0	50.0	75.0	40.0	70.0	85.0	50.0	80.0	100.0
HLIM	40.0	66.7	80.0	20.0	33.3	80.0	60.0	80.0	100.0
HHD	80.0	80.0	85.0	0.0	25.0	80.0	15.0	75.0	95.0

	Patient 7			Patient 8			Patient 9		
Scales	Day 1	Day 14	Day 28	Day 1	Day 14	Day 28	Day 1	Day 14	Day 28
PF	50.7	54.9	57.0	44.4	48.6	57.0	44.4	48.6	50.7
RP	37.3	56.9	56.9	27.5	32.4	56.9	22.6	39.7	42.2
BP	46.1	62.1	51.1	37.2	41.4	55.4	41.4	46.1	46.1
GH	43.4	60.1	60.1	28.2	43.0	52.9	28.2	48.2	45.8
VT	45.9	70.8	70.8	33.4	39.6	61.5	30.2	45.9	52.1
SF	45.9	56.9	51.4	35.0	40.5	56.9	24.1	45.9	45.9
RE	48.1	55.9	55.9	24.8	36.4	55.9	24.8	36.4	52.0
MH	47.2	64.1	58.5	44.4	47.2	61.3	24.7	47.2	52.8
PCS	43.8	57.2	55.9	35.9	41.8	54.5	39.9	47.2	44.3
MCS	47.6	62.8	59.2	34.0	41.2	59.5	21.0	42.7	52.9
HD	70.0	100.0	90.0	25.0	55.0	95.0	0.0	65.0	70.0
PWB	60.0	95.0	85.0	50.0	40.0	95.0	25.0	55.0	60.0
HLIM	86.7	93.3	100.0	46.7	60.0	93.3	0.0	73.3	80.0
HHD	75.0	80.0	100.0	40.0	40.0	90.0	26.7	75.0	80.0

Table 6. Baseline, Day 14, and Day 28 SF-36 and HQLQ Scale Scores for 10 Hepatitis C Patients

	Patient 10		
Scales	Day 1	Day 14	Day 28
PF	25.1	25.5	25.5
RP	17.7	20.1	32.4
BP	19.9	24.9	29.2
GH	23.4	28.2	28.2
VT	30.2	36.5	30.2
SF	18.7	29.6	29.6
RE	9.2	13.1	36.4
MH	41.6	47.2	52.8
PCS	21.2	23.2	22.9
MCS	28.2	36.1	45.5
HD	25.0	30.0	40.0
PWB	75.0	65.0	55.0
HLIM	6.7	0.0	0.0
HHD	10.0	35.0	50.0

PF = SF-36 Physical Functioning
RP = SF-36 Role Physical
BP = SF-36 Bodily Pain
GH = SF-36 General Health
VT = SF-36 Vitality
SF = SF-36 Social Functioning
RE = SF-36 Role Emotional
MH = SF-36 Mental Health
HD = HQLQ Health Distress
PWB = HQLQ Positive Well-Being
HLIM = HQLQ Hepatitis Specific Limitations
HHD = HQLQ Hepatitis Specific Distress

Figures

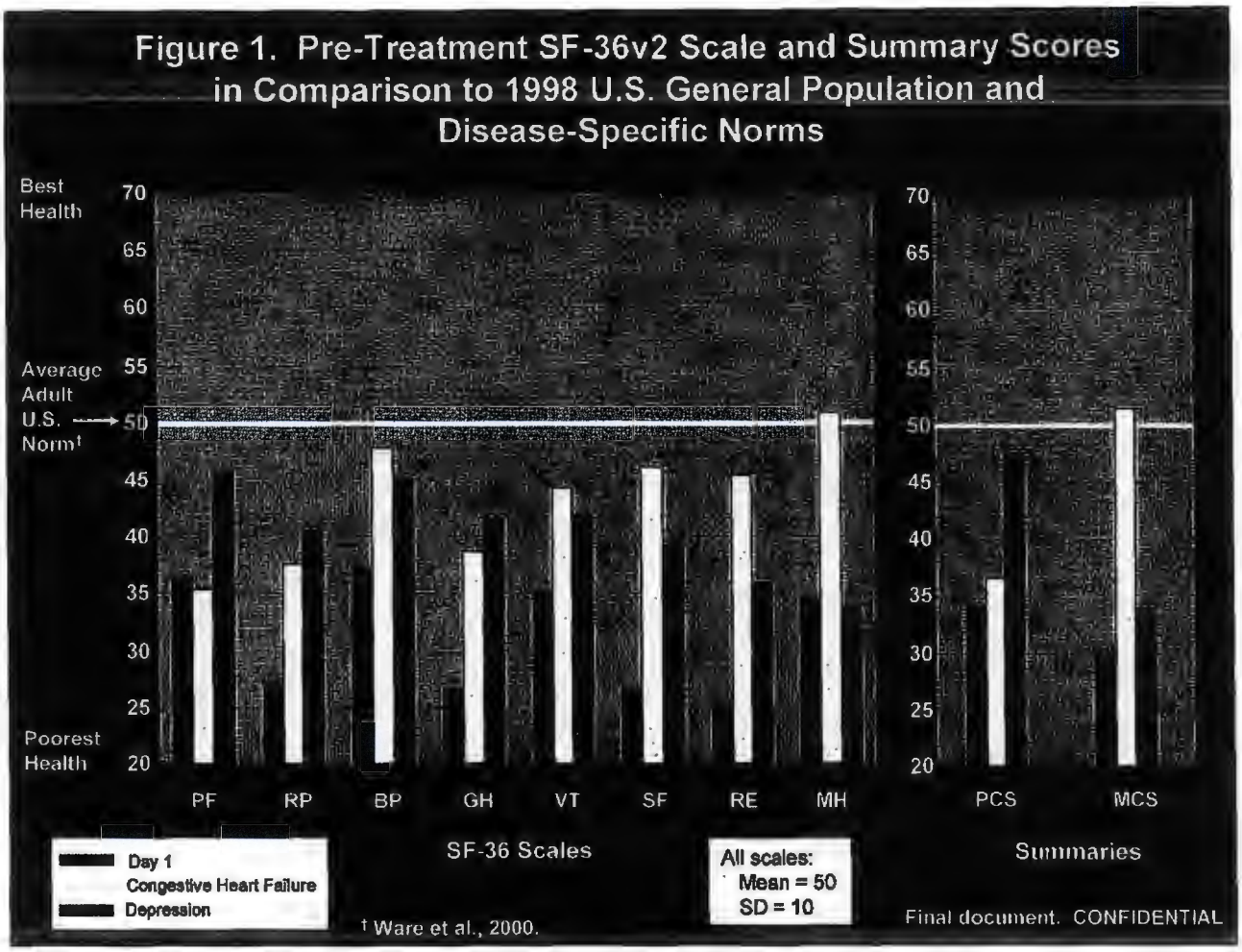


Figure 1. Pre-Treatment SF-36v2 Scale and Summary Scores in Comparison to 1998 U.S. General Population and Disease-Specific Norms
Best Health
Average Adult U.S. Norm†
Poorest Health
70
65
60
55
50
45
40
35
30
25
20
PF
RP
BP
GH
VT
SF
RE
MH
PCS
MCS
SF-36 Scales
Summaries
Day 1
Congestive Heart Failure
Depression
All scales:
Mean = 50
SD = 10
† Ware et al., 2000.
Final document. CONFIDENTIAL

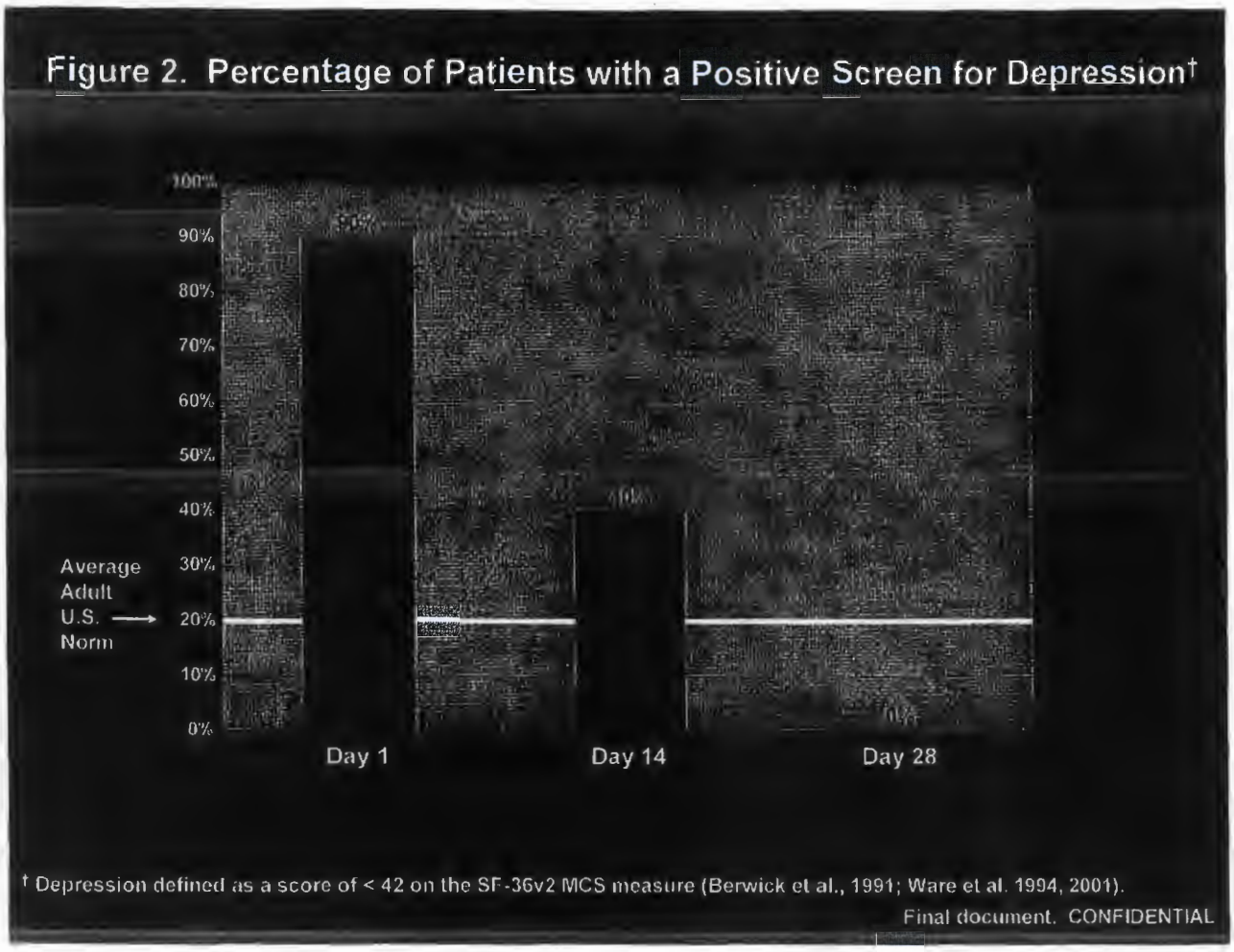


Figure 2. Percentage of Patients with a Positive Screen for Depression†
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Average Adult U.S. Norm
Day 1
Day 14
Day 28
† Depression defined as a score of < 42 on the SF-36v2 MCS measure (Berwick et al., 1991; Ware et al. 1994, 2001).
Final document. CONFIDENTIAL

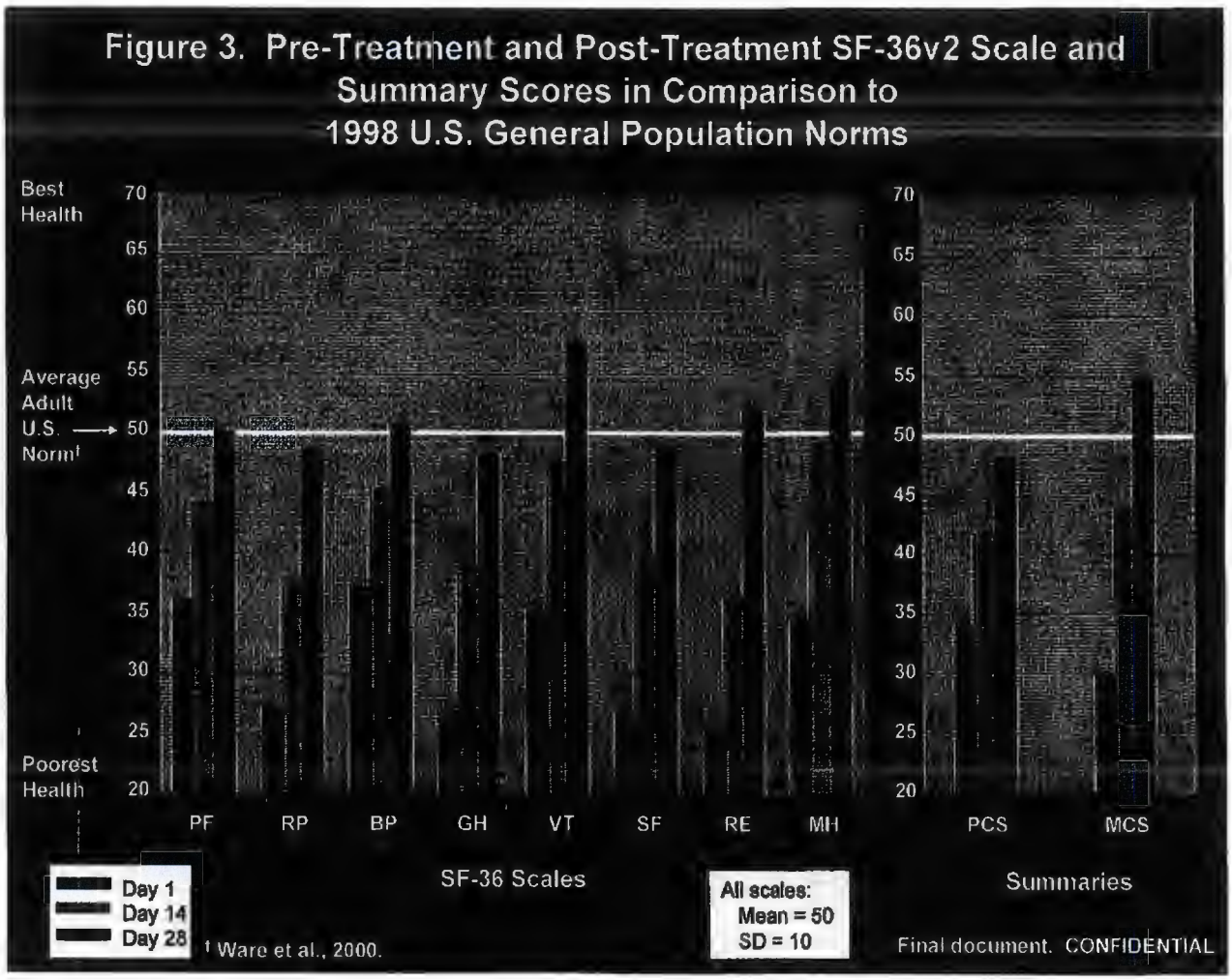
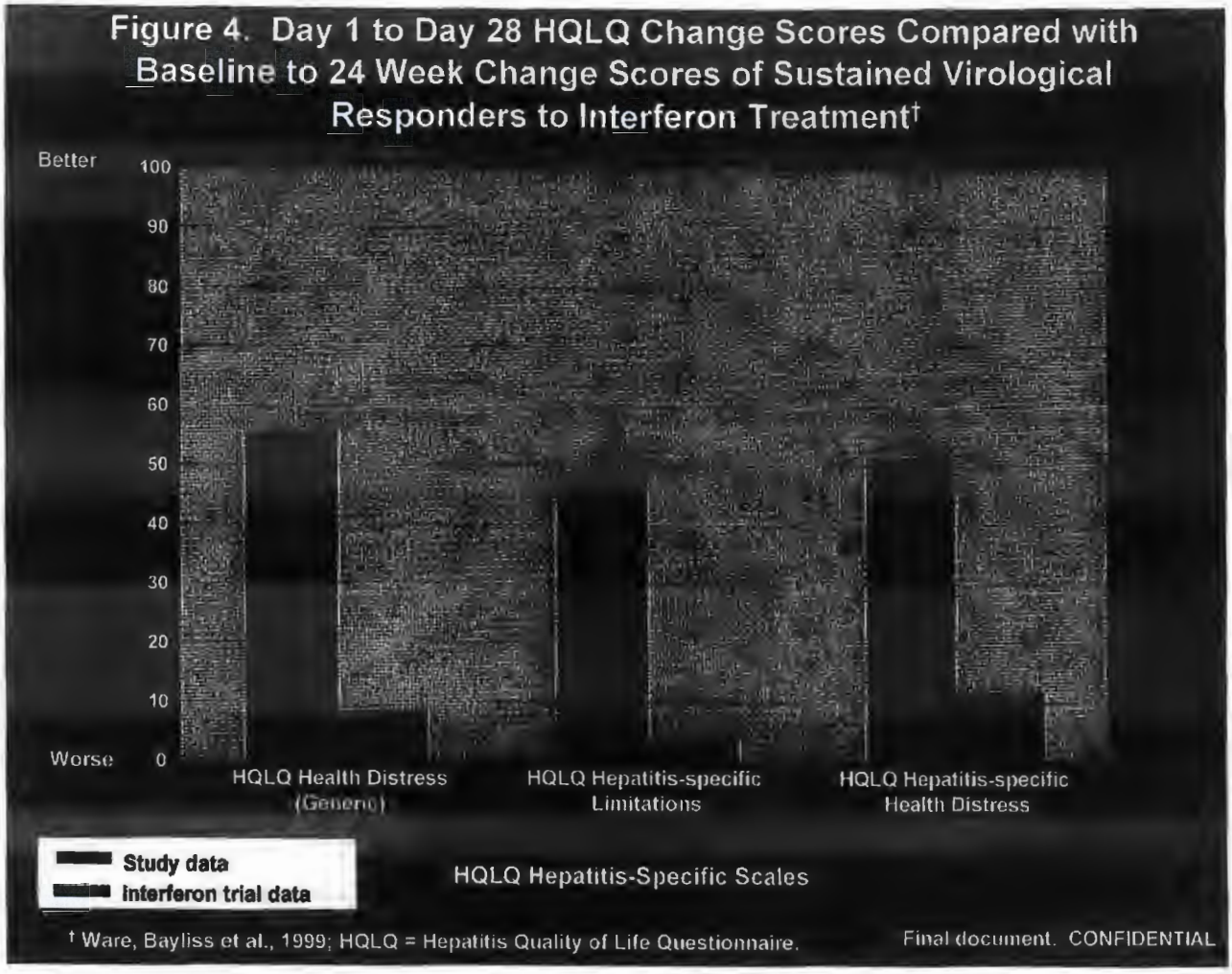


Figure 3. Pre-Treatment and Post-Treatment SF-36v2 Scale and Summary Scores in Comparison to 1998 U.S. General Population Norms
Best Health
Average Adult U.S. Norm[1]
Poorest Health
70
65
60
55
50
45
40
35
30
25
20
PF
RP
BP
GH
VT
SF
RE
MH
PCS
MCS
SF-36 Scales
Summaries
Day 1
Day 14
Day 28
All scales:
Mean = 50
SD = 10
[1] Ware et al., 2000.
Final document. CONFIDENTIAL



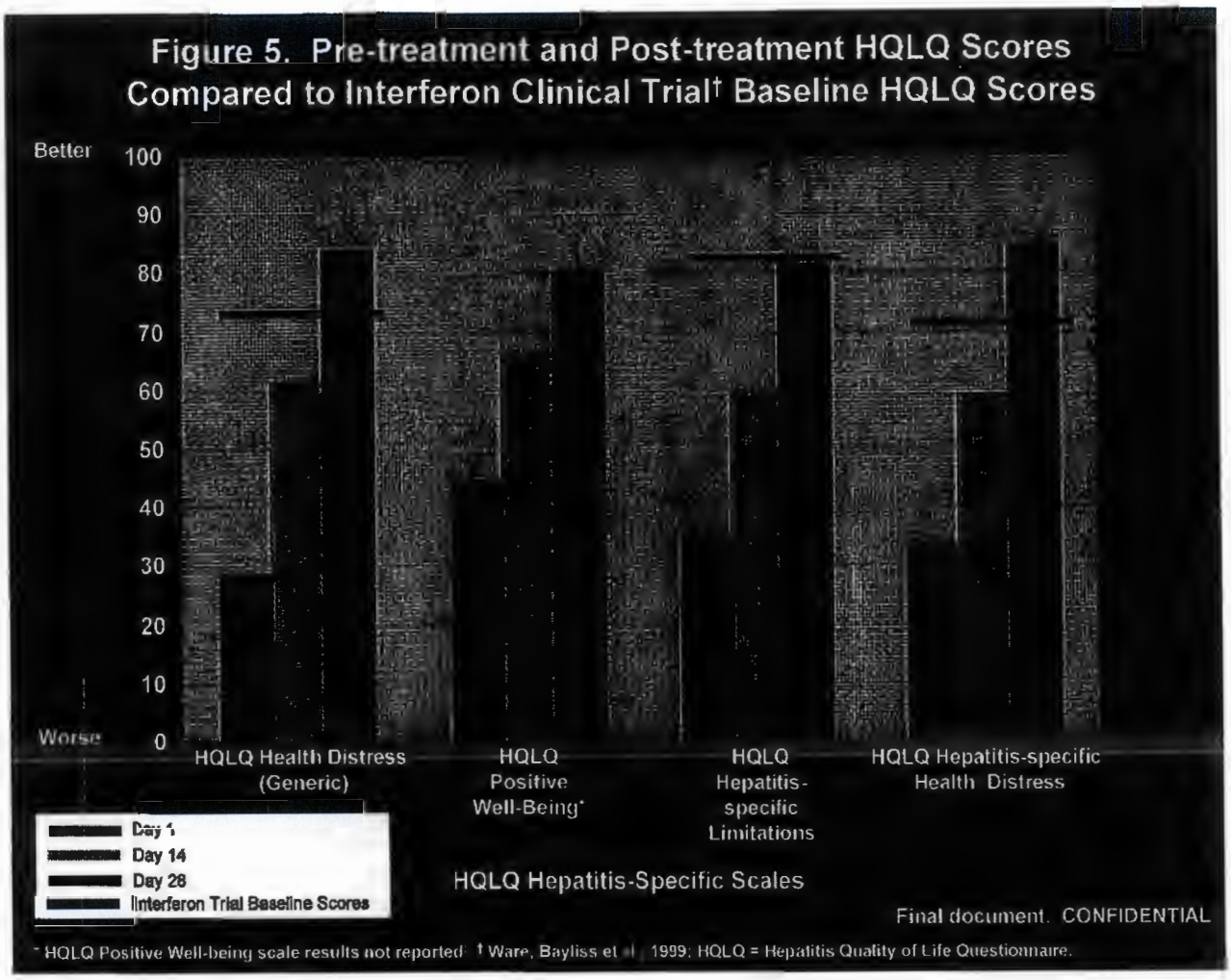


Figure 5. Pre-treatment and Post-treatment HQLQ Scores Compared to Interferon Clinical Trial† Baseline HQLQ Scores
Better
100
90
80
70
60
50
40
30
20
10
Worse
0
HQLQ Health Distress (Generic)
HQLQ Positive Well-Being*
HQLQ Hepatitis-specific Limitations
HQLQ Hepatitis-specific Health Distress
Day 14
Day 28
Interferon Trial Baseline Scores
HQLQ Hepatitis-Specific Scales
Final document. CONFIDENTIAL
* HQLQ Positive Well-being scale results not reported † Ware, Bayliss et al 1999; HQLQ = Hepatitis Quality of Life Questionnaire.

Figure 6. Profile of SF-36 Scale Scores Before (Day 1) and After (Days 14 and 28) Treatment of Chronic Hepatitis C: Patient 1

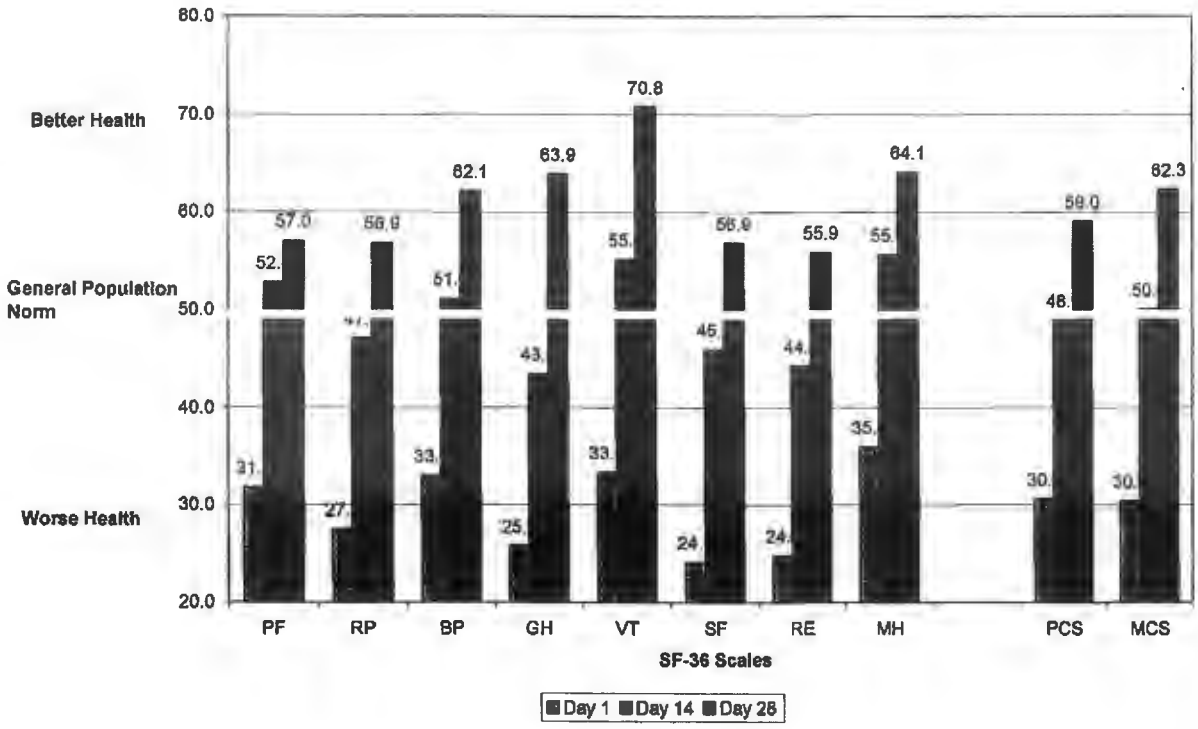


Figure 7. Profile of SF-36 Scale Scores Before (Day 1) and After (Days 14 and 28) Treatment of Chronic Hepatitis C: Patient 2

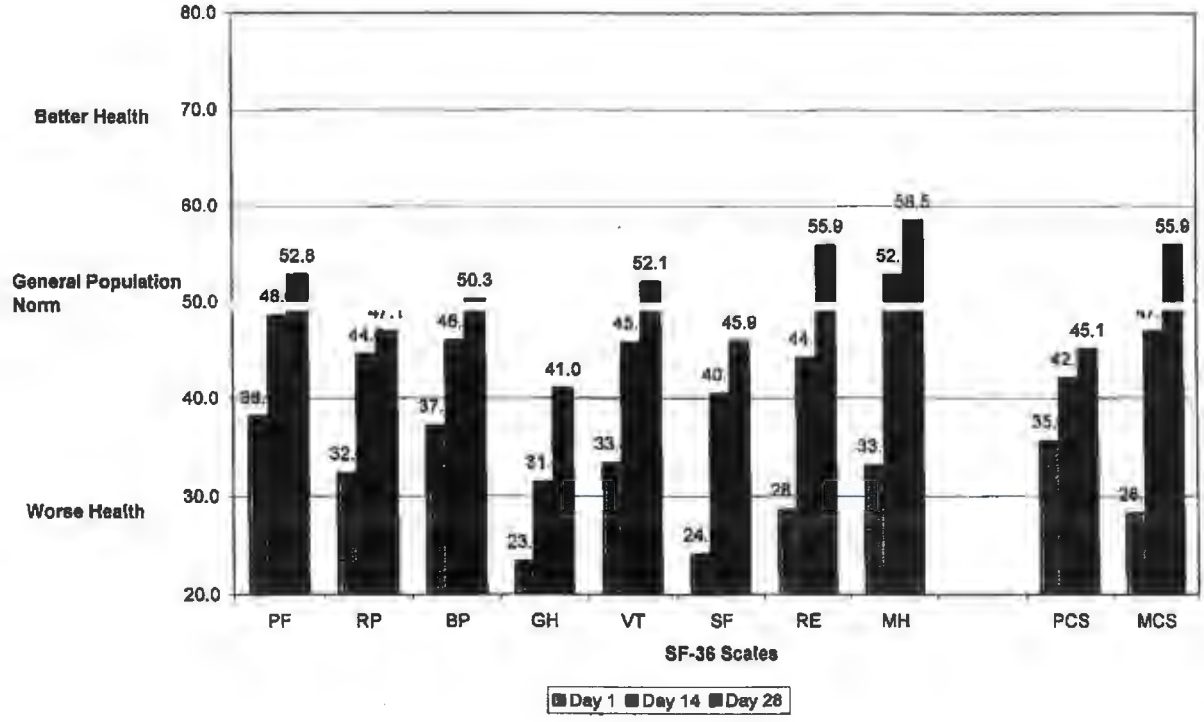


Figure 8. Profile of SF-36 Scale Scores Before (Day 1) and After (Days 14 and 28) Treatment of Chronic Hepatitis C: Patient 3

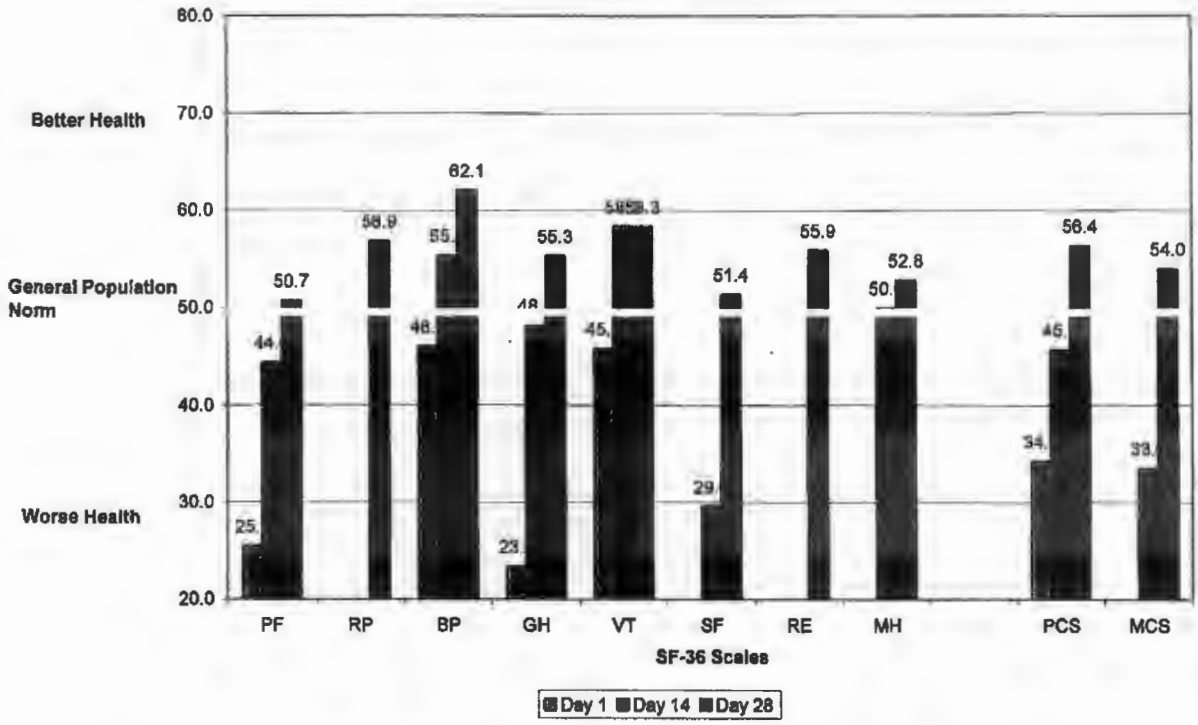


Figure 9. Profile of SF-36 Scale Scores Before (Day 1) and After (Days 14 and 28) Treatment of Chronic Hepatitis C: Patient 4

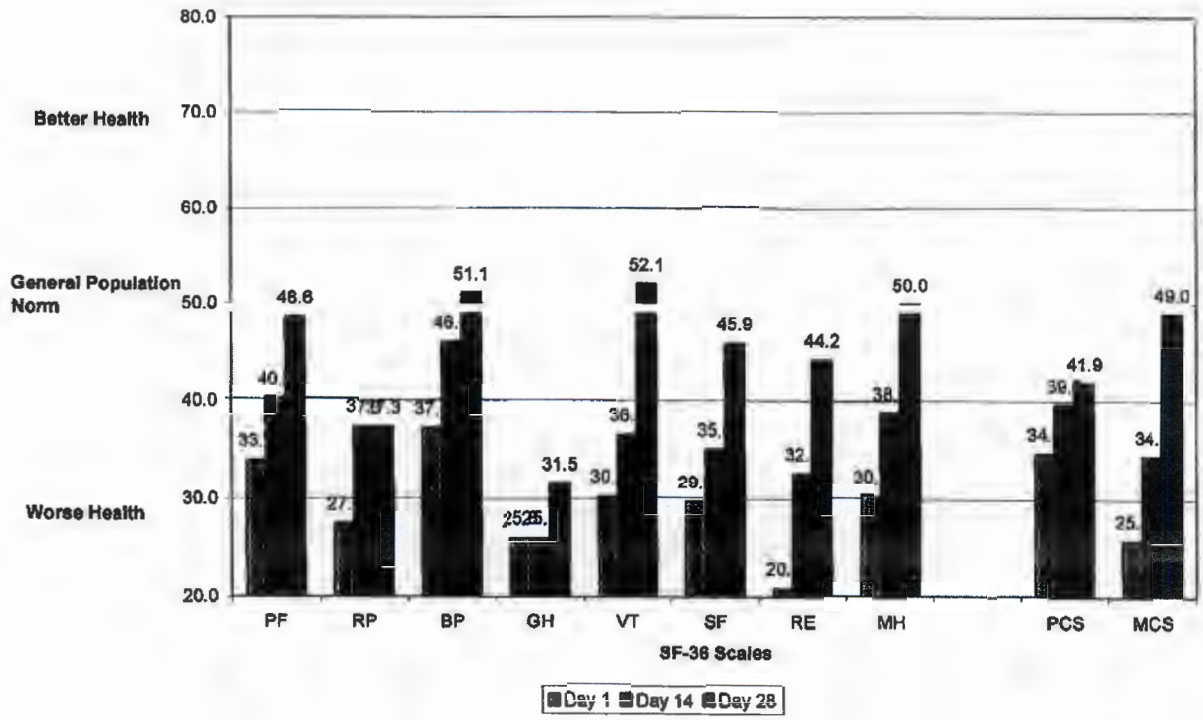


Figure 10. Profile of SF-36 Scale Scores Before (Day 1) and After (Days 14 and 28) Treatment of Chronic Hepatitis C: Patient 5

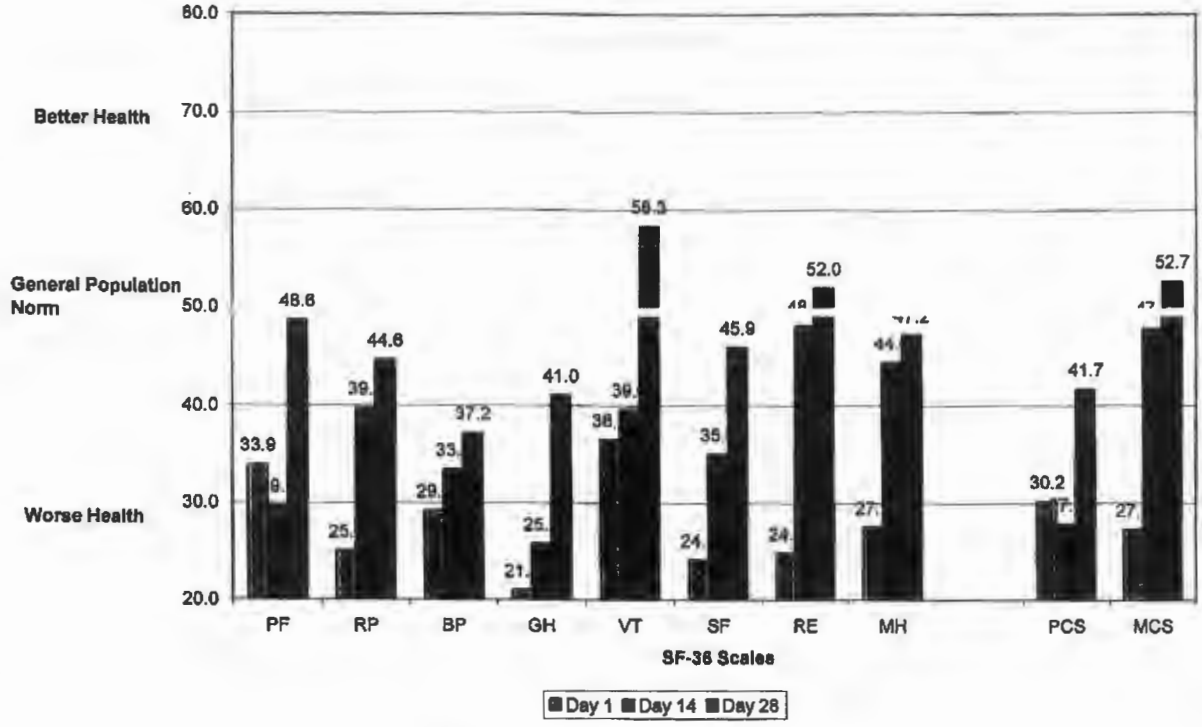


Figure 11. Profile of SF-36 Scale Scores Before (Day 1) and After (Days 14 and 28) Treatment of Chronic Hepatitis C: Patient 6

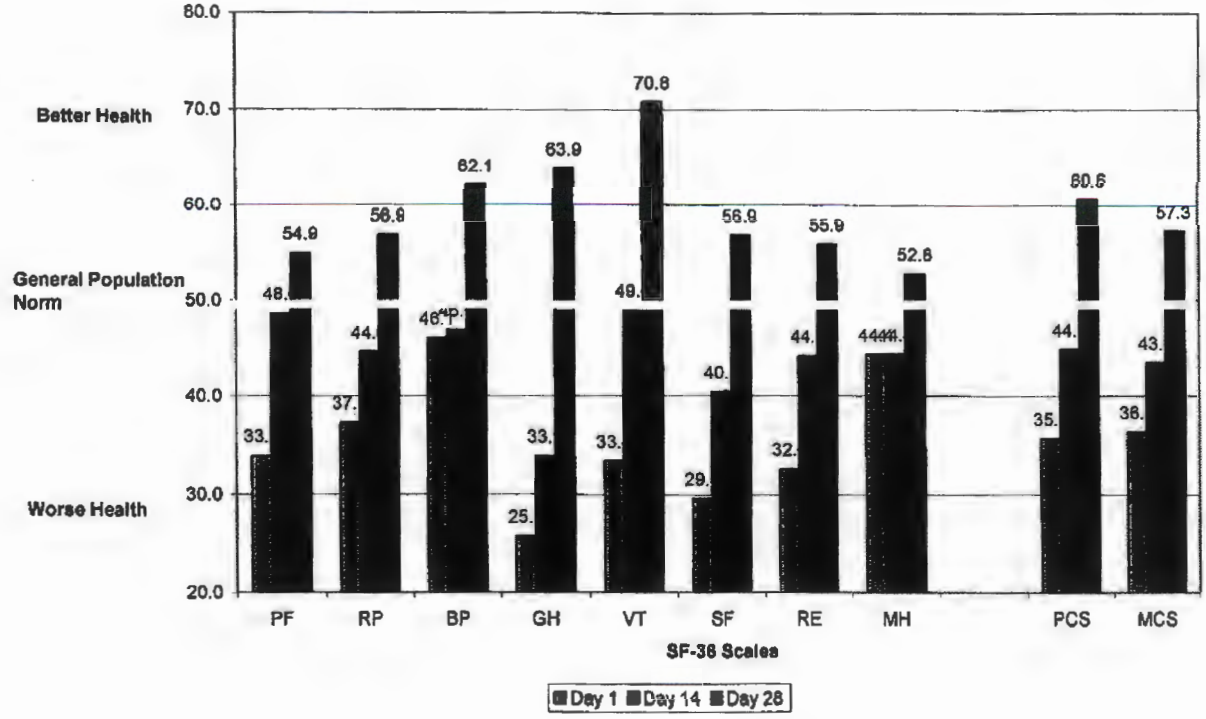


Figure 12. Profile of SF-36 Scale Scores Before (Day 1) and After (Days 14 and 28) Treatment of Chronic Hepatitis C: Patient 7

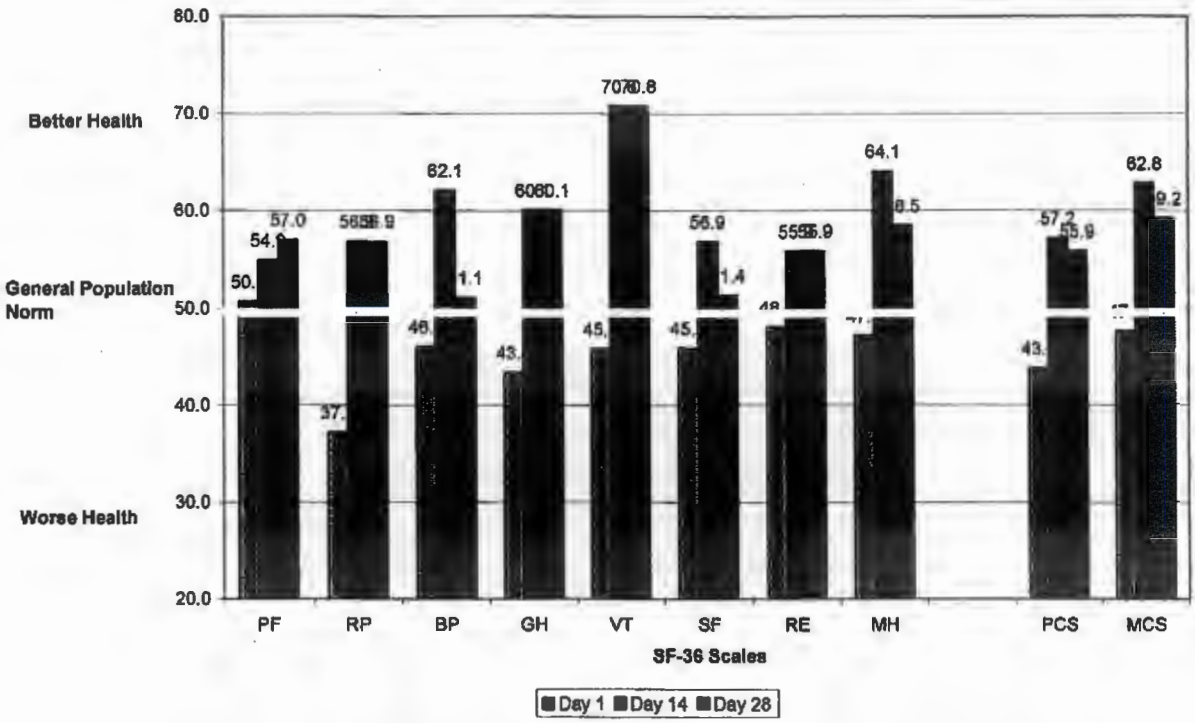


Figure 13. Profile of SF-36 Scale Scores Before (Day 1) and After (Days 14 and 28) Treatment of Chronic Hepatitis C: Patient 8

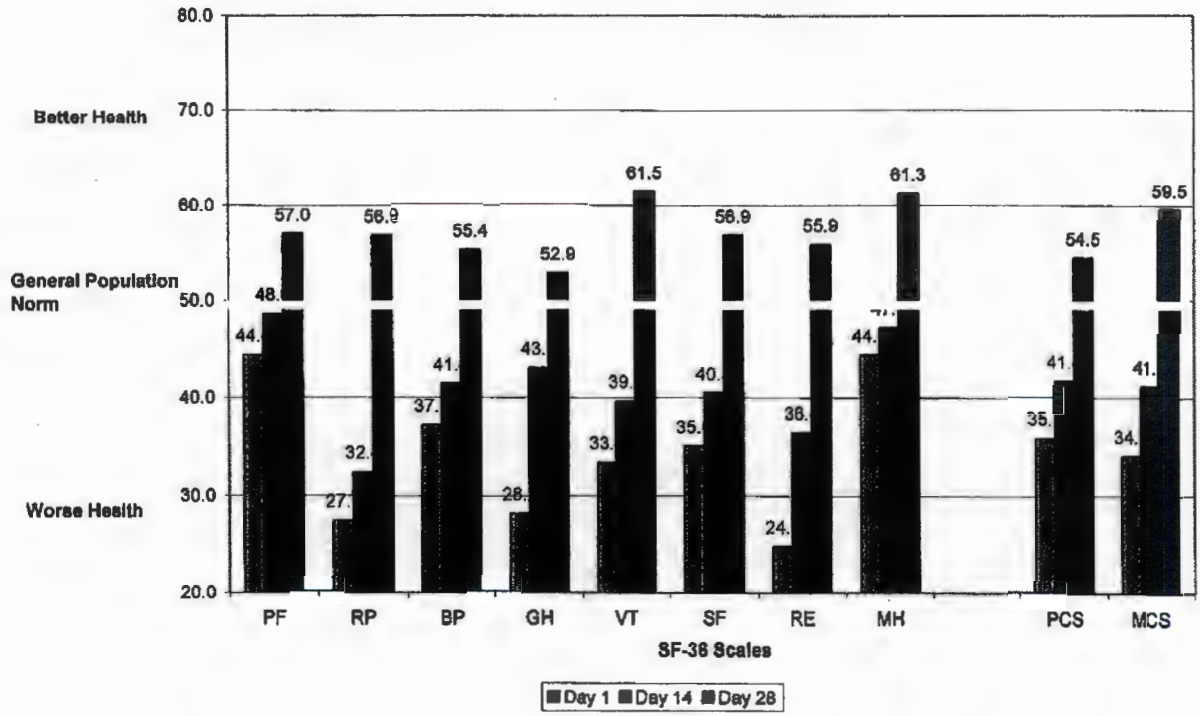


Figure 14. Profile of SF-36 Scale Scores Before (Day 1) and After (Days 14 and 28) Treatment of Chronic Hepatitis C: Patient 9

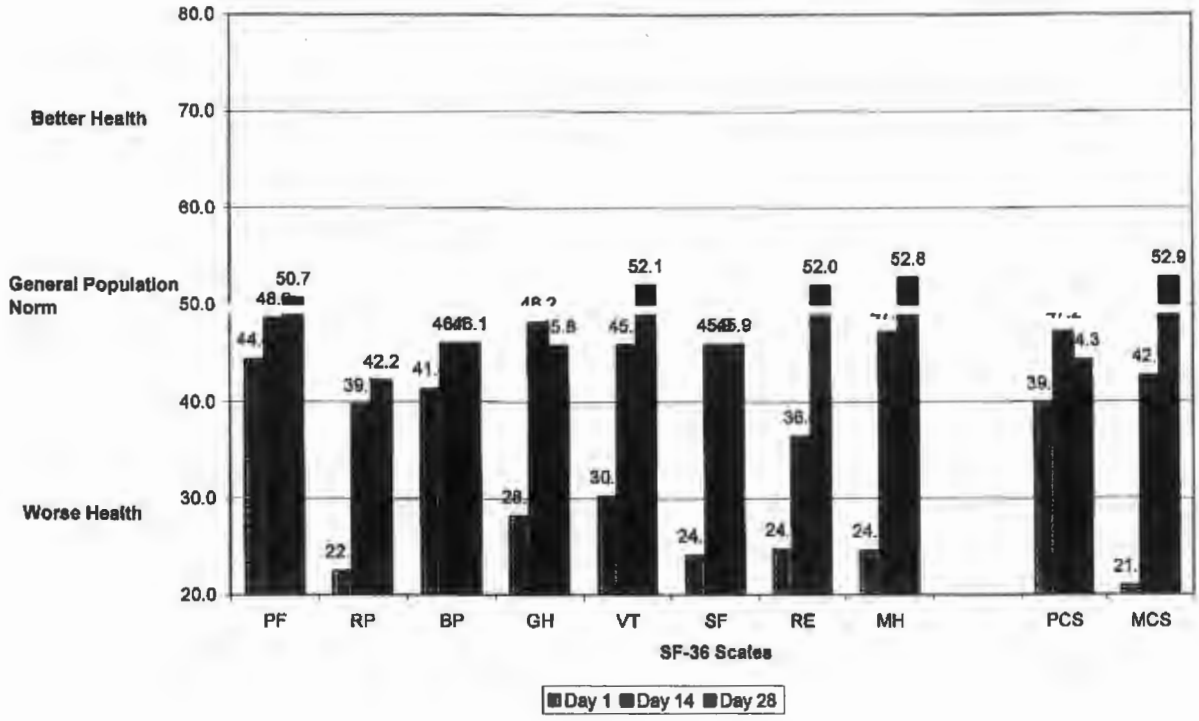
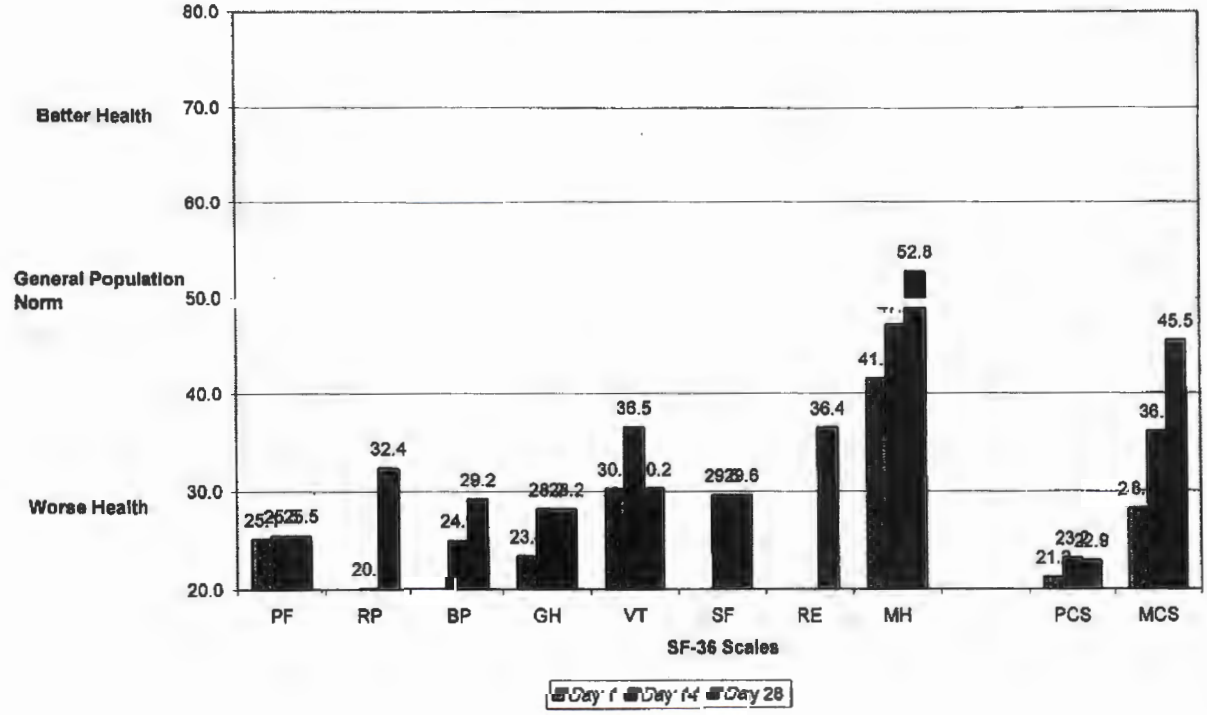


Figure 15. Profile of SF-36 Scale Scores Before (Day 1) and After (Days 14 and 28) Treatment of Chronic Hepatitis C: Patient 10



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ANTIOXIDANT ACTIVITY OF *CORDIA MYXA* L. AND ITS HEPATOPROTECTIVE POTENTIAL

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ABSTRACT

Protective role of *Cordia myxa L.* (CM) against liver fibrosis induced by carbon tetrachloride (CCl_4) or thioacetamide (TA) was investigated. Plant was extracted in different solvents and the extracts were evaluated for their phenolic content and antioxidant activity. Phenolic content was measured using Folin-Ciocalteu reagent and was calculated as gallic acid equivalents. Antiradical activity of *C. myxa* extracts was measured by α,α-diphenyl-β-picrylhydrazyl (DPPH) assay and was compared to ascorbic acid. One milligram of the crude extract was found to be equivalent to 15μg of ascorbic acid. Protective role of *C. myxa* against carbon tetrachloride or thioacetamide induced fibrosis was assessed in serum aspartate transaminase (AST), glutamate transaminase (ALT) and alkaline phosphatase (ALP). Level of these enzymes significantly improved in rats after administration of (CCl_4) + CM, or (TA) + CM as compared to rats that were treated alone with CCl_4 or TA. It was found that the fresh *C. myxa* extract offered better protection against liver fibrosis induced by these chemicals.

KEYWORDS

Cordia myxa L, Bumber, antioxidant, hepatoprotective, DPPH ((α,α-diphenyl-β-picrylhydrazyl)), ascorbate, antiradical, flavonoids, polyphenol, thioacetamide, carbontetrachloride

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INTRODUCTION

Cordia myxa L. locally known as "Bumber" is popularly used for its efficacy in chest and urinary infections (Alami and Macksad, 1974). It is also used for its anthelmintic, diuretic, demulcent, antidiarrheal, antigastritic, antiworm properties and also as a liver tonic. Several preparations of Cordia species have been used in traditional medicine for osteoarticular diseases. Analgesic, antiinflammatory and antiarthritic activities of *C. francisci, C. martinicensis, C. myxa, C. serratifolia* and *C. ulmifolia* have been studied in rats (Ficarra et al., 1995). Petroleum ether extract, especially of *C. francisci, C. myxa* and *C. serratifolia* leaves has been reported to carry significant analgesic, anti-inflammatory and anti-arthritic activities (Ficarra et al., 1995; Al-Awadi et al., 2001).

Sugars, flavonoids and alkaloid content of five Cordia species have been analyzed by TLC and reverse phase HPLC chromatographic techniques (Al-Awadi, 1987; Ifzal and Qureshi, 1976). These species have yielded four flavone glycosides, robinin, rutin, datiscoside and hesperidin, one flavone aglycone, dihydrorobinetin and two phenolic derivatives, chlorgenic and caffeic acids. C. myxa seed oil (Tiwari et al., 1980) and its photosynthetic pigments have been analyzed (Afzal et al., 2004).

Steatohepatitis has recently been increasing as a cofactor influencing the progression of fibrosis, cirrhosis, adenoma and carcinoma in liver. In the present study, protective role of *C. myxa* in chemically induced fibrosis by (CCl_4) or (TA) is being reported. Liver damage induced by carbon tetrachloride has been studied extensively for more than 50 years. Carbon tetrachloride is metabolized through cytochrome P450 enzyme in liver to give tricholoromethyl radicals that are involved in free radical mediated oxidation reactions. Thioacetamide is known for attenuation of glutathione peroxidase leading to oxidative stress. We planed to compare the efficacy of *C. myxa,* in liver fibrosis induced by both of these chemicals, to study the therapeutic strategies and for evaluating chemoprevention strategies for steatohepatitis for which no specific drugs are available.

MATERIALS AND METHODS

C. Myxa fruit and leaves were collected from local private yards during summer 2003. Green fruit mixed with semi-ripe (greenish pink) fruit of 3-4 cm in diameter was collected. Plant leaves, green on the outside and purple on the inside, were also collected. Leaves were washed under running water, dried between folds of filter papers and were stored at -20° C in zipped plastic bags until used. The fruit, washed under running water, was wiped dry with paper towel, and stored in plastic zipped bags at –20° C until used. Plant material was also completely dried under shade and powdered for later use. Seven extracts from frozen and dried plant material were prepared in different solvents and a representative extraction procedure is presented.

All chemicals used in this study were purchased from Sigma Co (St. Louis, MO, USA). DPPH (α,α-diphenyl-β-picrylhydrazyl) and ascorbate were purchased from Fluka, Switzerland.

Preparation of plant extract

Frozen fruit material (50.0 g) was thawed and the outer thin skin was removed manually. Initially, the pulp was crushed at low temperature (4° C), transferred into 100 ml cold phosphate buffer (50mM, pH 6.75) and stirred for 12 hrs. The brown extract was passed through a cheese cloth, centrifuged at 3000 rpm for 30 min and the clear supernatant was freeze-dried to an amorphous powder. This was labeled as fruit extract # 1. After the above extraction, the remaining gel like material on seeds was hand macerated and re-extracted into 100 ml phosphate buffer (50mM, pH 6.75), followed by centrifugation and lyophilization to give extract # 2. In the case of an organic extraction, solvent was removed on a rotavapor under reduced pressure and at low temperature. All extracts given in Table 1, were prepared as follows:

1. Fresh fruit extraction into phosphate buffer
2. Fruit flesh (gel) extracted into phosphate buffer: It was prepared as extract # 1.
3. Crushed fruit was stirred in methanol for 12 hr and filtered. The solvent was removed on a rotavapor.
4. Dried fruit extraction into phosphate buffer: Powdered dry fruit was extracted into phosphate buffer and processed as for extract # 1
5. Dry fruit methanol extract: It was prepared as extract # 2
6. Leaf extraction: It was prepared as extract # 1
7. Leaf extraction into 50% methanol: It was prepared as extract # 2.

Table 1. Plant extract % yields and their phenol content

#	Plant Extract	Yield$^{\varphi}$	Phenols$^{¥}$
1	Fresh fruit extraction into phosphate buffer	28.3	11.8
2	Fruit (gel) extracted into phosphate buffer	31.4	11.1
3	Fresh fruit methanol extract	22.5	6.4
4	Dry fruit extraction into phosphate buffer	43.7	7.8
5	Dry fruit methanol extract	29.1	9.3
6	Green leaf extraction into phosphate buffer	17.3	9.5
7	Green leaf 50% methanol extract	21.6	9.6

φ gm kg^{-1}
¥ mg/g of extract

Animals and their treatment

54 Male adult Sprague-Dawley rats weighing 250±10g were used in this study. Rats were fed with standard chow diet composed of 0.25% sodium, 0.22% magnesium, 11ppm copper,

0.5% phosphorus, 0.71 calcium, 0.25% methionine, 5.9% raw ash, 5.3% cellulose, 14.7% protein, and 2.6% fat. All animals were maintained under controlled conditions at 22-24° C, 50-60% humidity, 12 h light-dark cycle and over corn cob bedding. Animals were randomly divided into nine groups, six rats in each group, and were treated for two weeks. Results were statistically analyzed by paired student t-test and one-way Anova using SPSS software. All animals were fed by gavage according to the protocol as given under. In order to examine dose response, two concentrations of plant extract 50 mg/ml and 500 mg/ml in normal saline were prepared. A 0.2% carbon tetrachloride solution was prepared by stirring 2 ml of the organic solvent in one liter of normal saline solution for 12 hrs.

Group 1 Control, received 1 ml of normal saline daily for two weeks.

Group 2. Received 1ml of CCl_4 aqueous solution twice daily, each at 10AM and 3PM for one week, followed by a normal diet for another week.

Group 3. Received 1ml of thioacetamide aqueous solution (40 mg/ml) twice daily, each at 10 AM & 3 PM for one week, followed by a normal diet for the second week.

Group 4. Received 1ml of plant extract # 2 (50 mg/ml) twice daily each at 10 AM & 3 PM for two weeks.

Group 5. Received 1ml of plant extract # 2 (500 mg/ml) twice daily, each at 10 AM & 3 PM for two weeks.

Group 6. Received 1ml of CCl_4 solution twice daily each at 10 AM and 3 PM for the first week followed by 1ml solution of (50 mg/ml) plant extract # 2, twice daily, each at 10 AM & 3 PM, for the second week.

Group 7. Received 1ml of CCl_4 solution twice daily each at 10 AM and 3 PM for the first week followed by 1ml of (500 mg/kg) plant extract # 2 twice daily, each at 10 AM & 3 PM, for the second week.

Group 8. Received 1ml of thioacetamide solution (40 mg/ml) twice daily each at 10 AM and 3 PM for the first week followed by 1ml of (50mg/ml) plant extract # 2, twice daily each at 10 AM & 3 PM, for the second week.

Group 9. Received 1ml of thioacetamide solution (40mg/ml) twice daily each at 10 AM and 3 PM, for the first week followed by 1ml of (500mg/ml) plant extract # 2, twice daily each at 10 AM & 3 PM, for the second week.

Serum Preparation

Animals were anesthetized with an IM injection of 500 µl of Ketamine solution (1 mg/ml). Blood was collected through cardiac puncture and spun at 3000 rpm for 20 min. and clear serum was taken. Duplicate aliquots (1 ml) were frozen till further use.

Enzyme assay

AST, ALT and ALP were assayed in fresh serum by kits supplied by Randox (UK) according to the standard procedures described by the manufacturers.

Total phenol content and antiradical assay

Total phenols were measured using Folin-Ciocalteu reagent and antiradical assay was carried out using DPPH according to the established procedure (Schwarz et al.,2001).

RESULTS AND DISCUSSION

Extracts used in this study and their yields are given in Table 1. Extraction results showed that the fresh fruit extract # 2 yielded maximum amount of the material on fresh fruit weight bases justifying its use in different assays. Shade drying of fruit radically changed its color from greenish pink to blackish brown that may indicate aerial oxidation/polymerization of the plant components.

Polyphenols are known bioactive molecules that are ubiquitously distributed in plant species, influencing their morphology, growth, reproduction as well as their resistance to parasites and environmental stress (Bravo, 1988). Flavonoid molecules are of particular interest since these demonstrate a whole spectrum of biological activities (Di Carlo et al., 1999; Hollman and Arts, 2001; Jayaprakasam, 2005; Aruoma, 2002). Many of the polyphenols have been identified as powerful antioxidants (Luximon-Ramma et al., 2002; Bahorun et al., 2003; Kaur and Kapoor, 2002; Proteggente et al., 2002; Luximon-Ramma et al., 2003; Martinez-Valverde et al., 2002; Szeto et al., 2002; Everett et al., 2002; Chu et al., 2002; Aruoma, 2003; Halvorsen et al., 2002), cardioprotective (Bagchi et al. 2003), anticancer (Lambert and Yang, 2003), antithrombotic and antihypertensive (Cheng et al., 1993) agents. For their quantitative measurement in tissues, these compounds have been assayed by many methods (Hertog et al., 2000; Pascual-Teresa et al., 2000) and the positive influence of these natural products is attributed to their antioxidant activities.

The phenolic content in seven extracts of *C. myxa* are shown in Table 1, and are indicated as μg/g gallic acid equivalent. Extracts # 1 and # 2 had similar amount of phenolic content while extracts 5-7 showed diminished but similar amount of phenolic content. The dry fruit extract # 4 showed maximum amount of extractable material but lesser amount of phenolic content, Table 1.

Table 2. Antiradical activity of extracts

	% INHIBITION						
Plant ext. Conc.(μg/ml)	Ext #1	Ext # 2	Ext # 3	Ext # 4	Ext # 5	Ext # 6	Ext # 7
1000	5.77	16.34	6.33	4.24	14.03	5.88	7.73
800	4.58	11.6	10.7	3.42	11.08	2.28	5.00
500	2.04	13.1	3.73	1.95	4.57	1.80	2.10
400	2.90	5.72	1.30	*5.87*	4.73	1.80	1.77
200	3.30	8.82	*2.92*	*2.12*	5.05	0.50	0.81
100	4.40	0.16	*5.68*	2.28	3.15	1.31	6.92
50	6.62	0.82	*1.13*	0.16	2.05	1.14	2.57

1. Fresh fruit, buffer extract. 2. Gel-Like material, buffer extract. 3. Fresh Fruit, methanol extract. 4. Dry fruit, buffer extract. 5. Dry fruit, methanol extract. 6. Green leaf, buffer extract. 7. Green leaf, 50% methanol extract.

Antiradical activity as assayed by DPPH model system

Antiradical activity is based on a reduction of 2,2'-diphenyl-1-picryl-hydrazyl (DPPH) which is a stable free radical. Antiradical activity of *C. myxa* was determined using DPPH assay as described in the literature (Schewarz et al., 2001; Gorinstein et al., 2004). This assay was carried out on all seven extracts and the results are shown in Table 2. The extracts # 2 and # 5 showed better antiradical activity as compared to other extracts Table 2. Extract # 2 showed the maximum antiradical activity at all 4 different concentrations of the extract. In order to assess the antiradical efficacy of the extracts 2 and 5, these were compared with ascorbate as a standard antioxidant agent. Results (Table 3) showed that extract # 2 was two fold more active at all three extract concentrations suggesting that dried plant material had lost some of its antiradical activity. This was further confirmed from an assessment of antiradical activity of the dried fruit extract # 5 and its comparison with other polyphenols like gallic acid and pyrogallol, Table 4. The extract # 5 exhibited a reduced percentage inhibition as compared to standard polyphenols. These results perceptibly showed that extract # 2 had stronger antiradical activity as compared to dry fruit extract # 5. Moreover extract # 2 was richer in vitamin C equivalence (Table 3, Fig. 1) and consequently stronger antioxidant, suggesting that the fresh fruit could be more effectual to combat liver cirrhosis as compared to dried fruit.

Table 3. Antiradical activity of *C. myxa* fruit extract # 2 & 5 and their equivalence to ascorbic acid

Plant Extract	Conc. µg/ml	DPPH % Inhibition	Ascorbate Equivalence µg/ml
Extract # 2	1000	25.1	15.0
	800	21.1	12.7
	500	12.8	7.8
Extract # 5	1000	10.5	6.3
	800	9.9	6.0
	500	8.1	4.9

Hepatic enzyme measurements

54 Male rats were randomly distributed into nine groups with six animals in each group. The animals were treated with carbon tetrachloride or thioacetamide and plant extract # 2 as given in materials and methods. Serum alanine transaminase, aspartate transaminase and alkaline phosphatase were measured using standard enzyme kits and the results are summarized in Table 5. Animals in groups G_2 and G_3 showed statistically higher levels (p 0.005) of hepatic enzymes in response to carbontetrachloride (Ramkumar and Anuradha, 2005; Yim et al., 2006; Liu et al., 2006; Kanter et al., 2005; Fan et al., 2006) and thioacetamide (Amali et al., 2006; Sehrawat et al., 2006; Mendez-Lopez et al., 2005; Galisteo et al., 2006; Gan et al., 2005) indicating cirrhotic liver induced by these chemicals. Carbon tetrachloride and thioacetamide are known hepatic toxins that induce liver cirrhosis through oxidative stress (Ramkumar and Anuradha, 2005; Yim et al., 2006; Liu et al., 2006; Kanter et al., 2005; Fan et al., 2006; Amali et al., 2006; Sehrawat et al., 2006; Mendez-Lopez et al., 2005). While level of these enzymes in animals in G_4 and G_5 were at normal level as compared to G_1 indicating that fruit extract had no effect on normal liver of animals. Level of hepatic enzymes in G_6, G_7 and G_8, G_9 animals significantly declined (p 0.05) as compared to group G_2, G_3 animals but were still higher as compared to control group of rats

in G_1. These results indicated that the effect of fruit extract was dose independent and cirrhotic liver could not be fully recovered with one week treatment of these animals. The values obtained showed that the fruit extract had a dose-independent protective effect on liver damage induced by carbon tetrachloride or thioacetamide.

Table 4. Antiradical activity of *C myxa* fruit extract # 5 and its comparison with pyrogallol and gallic acid antioxidant activity

Plant extract Conc. mg/ml	DPPH % Inhibition Plant extract	Conc. µg/ml	DPPH % Inhibition Pyrogallol	DPPH % Inhibition Gallic acid
50	38.9	100	51.6	38.3
25	20.6	50	43.5	39.1
12.5	13.8	25	29.5	23.1
6.25	9.4	12.5	16.0	12.4
3.12	6.40	6.25	10.7	6.90

Table 5. Enzyme level in carbon tetrachloride and thioacetamide-induced hepatic damage and treatment with *C. myxa* extract

	Enzyme levels (µ/l) in carbon tetrachloride-induced liver damage					
	G1	G2	G4	G5	G6	G7
ALT	36.4±4.8	53.6±3.3	36.5±4.7	32.7±4.3	47.6±3.4	42.5±3.6
AST	32.6±3.4	55.7±4.6	35.7±4.5	32.8±4.4	45.4±3.5	42.8±4.2
ALP	68.4±3.8	95.2±3.5	71.6±3.3	70.7±3.5	75.4±3.7	71.6±5.1
	Enzyme levels (µ/l) in thioacetamide-induced liver damage					
	G1	G3	G4	G5	G8	G9
ALT	36.4±4.8	48.5±3.3	36.5±4.7	32.7±4.3	42.5±4.3	41.4±4.2
AST	32.6±3.4	47.4±3.6	35.7±4.5	32.8±4.4	41.3±4.4	40.9±4.8
ALP	68.4±3.8	84.3±3.9	71.6±3.3	70.7±3.5	76.3±4.7	72.5±4.1

G = animal group as defined in treatment protocol

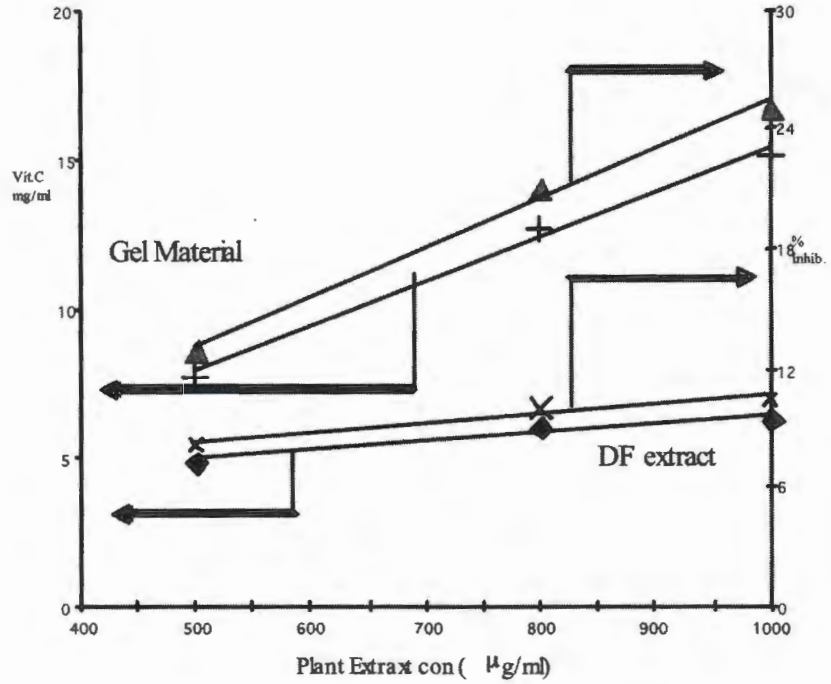

Fig.1 Dry fruit (#5) and gel material extract (#2) antiradical activity and equivalence to ascorbic acid

CONCLUSION

Cordia myxa L. has strong antioxidant activity that may be responsible for its antifibrotic activity against carbontetrachloride or acetamide induced fibroisis.

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REFERENCES

1. Afzal, M., Obuekwe, C., Shuaib, N. and Barakat, H. (2004). Photosynthetic pigment profile of *Cordia myxa* L. and its potential in folklore medicinal application. Journal of Food Agriculture and Environment, 2(20), 114-120.
2. Alami, R. and Macksad, A. (Ed.) (1974). Medicinal plants in Kuwait, Al-Assriya Press, Kuwait. p6.
3. Al-Awadi, F.M. Srikumar, T.S., Anum, J.T. and Khan, I. (2001). Antiinflammatory effects of Cordia myxa fruit on experimentally induced colitis in rats. Nutrition, 17(5), 391-396.

4. Amali, A.A., Rekha, R.D., Lin, C.J., Wang, W.L., Gong, H.Y., Her, G.M. and Wu, J.L. (2006). Thioacetamide induced liver damage in zebrafish embryo as a disease model for steatohepatitis. *Journal of Biomedical Science* 3, 1-8.
5. Aruoma, O.I. (2002). Neuroprotection by dietary antioxidants: new age of research. *Nahr Food,* 46, 381-382.
6. Aruoma, O.I. (2003). Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. *Mutation Research,* 523/524, 9-20.
7. Bagchi, D., Sen, C.K., Ray, S.D., Das, D.K., Bagchi, M., Preuss, H.G. and Vinson, J.A. (2003). Molecular mechanisms of cardioprotection by a novel grape seed proanthocyanidin extract. *Mutation Research,* 523/524, 87-97.
8. Bahorun, T., Aumjaud, E., Ramphul, H., Rycha, M., Luximon-Ramma, A., Trotin, F. and Aruoma, O.I. (2003). Phenolic constituents and antioxidant capacities of Crataegus monogyna (hawthorn) callus extracts. *Nahr Food,* 47, 191-198.
9. Bravo, L. (1988). Polyphenols: chemistry, dietary sources, metabolism and nutritional significance, *Physiology Reviews,* 56, 317-333.
10. Cheng, T.J. Hsu, L.F. and Chen, F.H. (1993). Antihypersensitive principles from the leaves of *Melastome candidum. Planta Medica,* 59, 405-407.
11. Chu,Y.F., Sun, J., Wu, X. and Liu, R.H. (2002). Antioxidant and antiproliferative activities of common vegetables. *Journal Agriculture and Food Chemistry,* 50, 6910-6916.
12. Di Carlo, G., Mascolo, N., Ice, A.A. and Capasso, F. (1999). Old and new aspects of a class of natural therapeutic drugs. *Life Science,* 65, 337-353.
13. Everett, S.M., Drake, I.M., White, K.L.M., Maostone, N.P., Chalmers, D.M., Schorah, C.J. and Axon, T.R. (2002). Antioxidant vitamin supplements do not reduce reactive oxygen species activity in *Helicobacter pylori* gastritis in the short term. *British Journal Nutrition,* 87, 3-11.
14. Fan, J.G., Tian, L.Y., Cai, X.B., Qian, Y., Yang, Z.R. and Xu, Z.J. (2006). Effect of hyperglycemia on the progress of rat liver fibrosis induced by carbon tetrachloride. *Zhon Gan Zang Bing Za Zhi* 14(1), 58-60.
15. Ficarra, R. Ficarra, P. Tommasini, S. Calabro, M.L. Ragusa, S. Barbera, R. and Rapisarda, A. (1995). Leaf extract of some Cordia species ; analgesic and anti-inflammatory activities as well as their chromatographic analysis. *Farmacolgia*, 50(4), 245-256.
16. Gan, B.H., Ng, G.L., Bay, B.H. and Chang, C.F. (2005). Altered CD38 expression in thioacetamide-induced rat model of liver cirrhosis. *Liver International*, 25(6), 1233-1242.
17. Galisteo, M., Duarez, A., Montilla, M.P., Fernandez, M.I., Gil, A. and Navarro, M.C. (2006). Protective effects of Rosmarinus tomentosus ethanol extract on thioacetamide-induced liver cirrhosis in rats. *Phytomedicine*, 13(1-2), 101-108.
18. Gorinstein, S., Haruenkit, R., Park, Y.S., Jung, S.T., Zachweija, Z., Jastrzebski, Z., Katrich, E., Trakhtenberg, S. and Belloso, O.M. (2004). Bioactive compounds and antioxidant potential in fresh and dried Jaffa sweeties, a new kind of citrus fruit. *Journal of Science Food* and *Agriculture,* 84, 1459-1463.
19. Halvorsen, B.L., Holte, K., Myhrstad, M.C.W., Barikmo, I., Hvattum, E., Renberg, S.F., Wold, A.B., Haffnre, K., Baugerod, H., Andersen, L.F., Moskaug, J.O., Jacobs, D.R. and Blomhoff, R. (2002). A systematic screening of total antioxidants in dietary plants. *Journal of Nutrition,* 132, 461-471.
20. Hertog, M.G.L., Hollman, P.C.H. and Katan, M.B. (2000). Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands. *Journal of Agriculture and Food Chemistry,* 40, 2379-2383.
21. Hollman, P.C.H. and Arts, I.C.W. (2001). Flavonoids, flavones and flavonols – nature, occurrence and dietary burden. *Journal of Science Food and Agriculture,* 80, 1081-1093.
22. Ifzal, S.M. and Qureshi, A. (1976). Studies on *Cordia myxa.* Part I. The monosaccharide and polysaccharide components of fruits of *Cordia myxa. Pakistan Journal Scientific and Industrial Research,* 19(2), 64-65.
23. Jayaprakasam, B., Vareed, S.K., Olson, L.K. and Nair, M.G. (2005). Insulin secretion by bioactive anthocyanins and anthocyanidins present in fruits. *Journal of Agriculture and Food Chemistry,* 53, 28-31.
24. Kanter, M., Coskun, O. and Budancamanak, M. (2005). Hepatoprotective effects of Nigella sativa L and Urtica dioica L on lipid peroxidation, antioxidant enzyme systems and liver enzymes in carbon tetrachloride-treated rats. World Journal of Gastroentrology, 11(42), 6684-6688.

25. Kaur, C. and Kapoor, H.C. (2002). Anti-oxidant activity and total phenolic content of some Asian vegetables. *International Journal of Food Sciience and Technology,* 37, 153-161.
26. Lambert, J.D. and Yang, C.S. (2003). Cancer chemopreventive activity and bioavailability of tea and tea polyphenols: a review. *Mutation Research,* 523/524, 210-208.
27. Liu, S.Q., Yu, J.P., Chen, H.L., Luo, H.S., Chen, S.M. and Yu, H.G. (2006). Therapeutic effects and molecular mechanisms of ginkgo biloba extract on liver fibrosis in rats. American Journal Clinical Medicine, 34(1), 99-114.
28. Luximon-Ramma, A., Bahorun, T., Soobratte, M.A. and Aruoma, O.I. (2002). Antioxidant activities of phenolic, proanthocyanidin and flavonoid components in extracts of Cassia fistula. *Journal of Agriculture and Food Chemistry,* 50, 5042-5047.
29. Luximon-Ramma, R. Bahorun, T. and Crozier, A. (2003). Antioxidant actions and phenolic and vitamin C contents of common Mauritius exotic fruits. *Journal of Science Food and Agriculture,* 83, 496-502.
30. Martinez-Valverde, I., Preiago, M.J., Provan, G. and Chesson, A. (2003). Phenolic compounds, lycopene and antioxidant activities in commercial varieties of tomato (*Lycopersicum esculentum*). *Journal of Science Food and Agriculture,* 82, 323-330.
31. Mendez-Lopez, M., Mendez, M., Lopez, L., Aller, M.A., Gonzalez-Pardo, H., Nava, M.P., Sanchez-Patan, F., Arias, J. and Arias, J.L. (2005). Increased cytochrome oxidase activity in adrenal glands of thioacetamide-cirrhotic rats. *Phytomedicine,* 28, 26-27.
32. Pascual-Teresa, S.D., Santos-Buelga, C. and Rivas-Gonzalo, J.C. (2000). Quantitative analysis of flavan-3-ols in Spanish foodstuffs and beverages. *Journal of Agriculture and Food Chemistry,* 48, 5331-5337.
33. Proteggente, A.R., Pannala, A.S., Paganga, G., Van Buren, L., Wagner, E., Wiseman, S., Van de Put, F., Dacombe, C. and Rice-Evans, C. (2002). The antioxidant activity of regularly consumed fruit and vegetables reflects their phenolic and vitamin C composition. *Free Radical Research,* 36, 217-233.
34. Ramkumar, K.M. and Anuradha, C.V. (2005). Short-term dietary restriction modulates liver lipid peroxidation in carbon tetrachloride-intoxicated rats. Journal of Basic Clinical Physiology and Pharmacology, 16(4), 245-256.
35. Schewarz, K., Bertelsen, G., Nissen, L.R., Gardener, P.T., Heinonen, M.I., Hopia, A., Huyah-Ba, T., Lambelet, P., McPhail, D., Skibsted, L.H. and Tijburg, L. (2001). Investigation of plant extracts for the protection of processed foods against lipid oxidation. Comparison of antioxidant assays based on radical scavenging, lipid oxidation and analysis of the principal antioxidant compounds. *European Food Research Technology,* 212, 319-329.
36. Sehrawat, A., Khan, T.H., Prasad, L. and Sultana, S. (2005). Butea monosperma and chemomodulation: Protective role against thioacetamide-mediated hepatic alterations in Wistar rats. Phytomedicine, 3(3), 157-163.
37. Szeto, Y.T., Tomlinson, B. and Benzie, I.F.F. (2002). Total antioxidant and ascorbic acid content of fresh fruits and vegetables: implications for dietary planning and food preservation. *British Journal of Nutrition,* 87, 55-59.
38. Tiwari, R.D., Srivastava, K.C., Shukla, S. and Bajpai, R.K. (1980). Chemical examination of the fixed oil of the seeds of *Cardia myxa. Planta Medica,* 15(3), 240-244.
39. Yim, H.K., Jung, Y.S. and Kim, Y.C. (2006). Contrasting Changes in Phase I and Phase II Metabolism of Acetaminophen in Male Mice Pretreated with Carbon Tetrachloride. Basic Clinical Pharmacology and Toxicology, 98(2), 225-230.
40. Wassel, G., El-Menshawi, B., Saeed, A., Mahran, G. and Reich, J. (1987). New sources of pyrrolizidine alkaloids: Genus Cordia (Ehretiaceae) and Schismus (Gramineae). *Journal of Science and Pharmacy,* 55(3), 163-166.

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Original Article

Hepatoprotective activity of *Annona squamosa* Linn. on experimental animal model

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Summary: Natural remedies from medicinal plants are considered to be effective and safe alternative treatment for liver toxicity. Our aim was to demonstrate the hepatoprotective effect of alcoholic and water extract of *Annona squamosa* (custard apple) hepatotoxic animals with a view to explore its use for the treatment of hepatotoxicity in human. These extracts were used to study the Hepatoprotective effect in isoniazid + rifampicin induced hepatotoxic model. There was a significant decrease in total bilirubin accompanied by significant increase in the level of total protein and also significant decrease in ALP, AST, ALT and γ-GT in treatment group as compared to the hepatotoxic group. In the histopathological study the hepatotoxic group showed hepatocytic necrosis and inflammation in the centrilobular region with portal triaditis. The treatment group showed minimal inflammation with moderate portal triaditis and their lobular architecture was normal. It should be concluded that the extracts of *Annona squamosa* were not able to revert completely hepatic injury induced by isoniazid + rifampicin, but it could limit the effect of these drugs in liver. The effect of extracts compared with standard drug silymarin.

Industrial relevance: A clear definition of herbal product is required at this stage, so as to provide a proper focus and strategy for the development of the industry. The development of herbal products only as medicinal inputs would clearly identify the potential beneficiaries and enable the medical practitioners to recognize the products as such. This would inevitably lead to quicker development in the field and pave the way for providing a scientific and technological explanation and justification for the use of the products in the medicinal sector. Today a substantial number of drugs are developed from plants. The majority of these involve the isolation of active ingredient found in a particular medicinal plant and its subsequent modification. A semi-synthetic analogue of such a compound could typically be a useful pharmaceutical product. Most of the synthetic drugs available in the market have more side effects specifically inducing hepatotoxicity. Drugs discovered from herbs will give good therapeutic medicine with fewer side effects and lower cost. The present study will help the industry to develop herbal medicine in the treatment of hepatotoxicity with fewer side effects. In future the development of formulation by these plant constituents will give good hepatoprotective medicine at lower cost.

Keywords: Annona squamosa, Isoniazid, Hepatoprotective activity.

Introduction

Drug-induced liver injury is a major health problem that challenges not only health care professionals but also the pharmaceutical industry and drug regulatory agencies. According to the United States Acute Liver Failure Study Group, drug-induced liver injury accounts for more than 50% of acute liver failure, including hepatotoxicity caused by overdose of acetaminophen (39%) and idiosyncratic liver injury triggered by other drugs (13%) (Michael and Cynthia 2005). Drugs are an important cause on liver injury. Approximately 75% of the idiosyncratic drug reaction results in liver transplantation (or) death (Zimmerman 1978). Drug-induced liver toxicity is a common cause of liver injury. It accounts for approximately one-half of the cases of acute liver failure and mimics all forms of acute and chronic liver disease (Kaplowitz 2001). Different types of drugs such as acetaminophen, chloroquine and isoniazid are inducing hepatotoxicity in world. Isoniazid and rifampicin, the first line drugs used for tuberculosis therapy are associated with hepatotoxicity (Tasduq et al., 2005). The rate of hepatotoxicity has been reported to be much higher in developing countries like India (8% - 30%) compaired to that in advanced countries (2% - 3%) with a similar dose schedule (Sharma 2004). WHO estimated that in 2004 alone 8.9 million new cases of tuberculosis arose and 1.7 million deaths due to tuberculosis that year. Developing countries and population with HIV infection suffer disproportionately (WHO report 2006). The reported incidence of anti-tuberculosis drugs induced hepatotoxicity indicated that the developing countries having difficulties in systematic steps for prevention and management of anti-tuberculosis drugs induced hepatotoxicity. These include patient and regimen selection to optimize benefits over risks, effective staff and patient education, ready access to care for patients, good communication among providers, and judicious use of clinical and biochemical monitoring.

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There is a growing interest in herbal remedies because of their effectiveness, minimal side effects in clinical experience and relatively low cost. Herbal drugs or their extracts are prescribed widely, even when their biological active compounds are unknown (Gupta et al., 2005). Therefore, studies with plant extracts are useful to know their efficacy and mechanism of action and safety. Natural remedies from medicinal plants are considered to be effective and safe alternative treatment for hepatotoxicity. The plant *Annona squamosa* (annonaceae) is commonly called as custard apple in English sharifa in Hindi (Morton 1987). This plant is reputed to possess varied medicinal properties (Watt 1972). It is used as an insecticidal agent has been investigated by several workers (Cheema et al., 1985). Free radical scavenging activity of *Annona squamosa* (Shirwaikar et al., 2004) was reported in the leaf extracts. Hypoglycemic and antidiabetic effect of *Annona squamosa* (Kaleem et al., 2006, Gupta et al., 2005) was reported in the leaf extract. From the bark of *Annona squamosa*, a bioactive acetogenin with anticancer activity (Hopp et al., 1998, Li et al., 1990, Hopp et al., 1997) have been isolated. Flavonoids from leaves (Seetharaman 1986), Aporphine alkaloids (Bhakuni 1972, Bhaumik 1979), glycoside (Forgacs 1980) and squamoline (Yang and Chi-ming 1972) were isolated from this plant.

In the ayurvedhic system of medicine, herbal extracts but not purified compounds have been used from centuries, because many constituents with more than one mechanism of action are considered to be beneficial. Here we report detailed studies on the hepatoprotective activity of ethanolic and aqueous extracts from leaves of *Annona squamosa*, with a view to provide scientific evidence on modern lines.

Materials and Methods

Chemicals: Isoniazid and rifampicin as a pure were purchased from Micro labs, India. Bilirubin, Total Protein, Alkaline phosphatase (ALP), Alanine transaminases (ALT), Aspartate transaminases (AST), and Gamma glutamate transpeptase (γ-GT) were assayed by using kits from Ranbaxy diagnostic, New Delhi.

Animals: Wistar strain of rats, weighing about 150 – 200 g were obtained from institute animal center, KMCP, Madurai and used in the experiments. The protocol was approved by the Institute's Animal Ethical Committee (IAEC No. KMCP/07/60/IAEC/0017). Animals were kept in animal house at an ambient temperature of 25°C and 45 – 55% relative humidity, with 12 h each of dark and light cycles. Animals were fed pellet diet and water ad-libitum. CPCSEA guidelines for laboratory animal facility (IJP 2003; 35: 257-274) was followed.

Preparation of Plant Extract: Leaves of *Annona squamosa* were collected from the medicinal garden of institute in November and identified by Dr. D. Stephen, Department of botany, American college, Madurai. The shade dried leaves were powdered to get a course granule. About 750 g of dried powder was extracted with ethanol and water by continuous hot percolation, using soxhlet apparatus. The resulted dark – brown extract was concentrated up to 100 ml on Rota vapour under reduced pressure. The concentrated crude extracts were lyophilized in to powder (5 & 15 g respectively) and used for the study.

Induction of experimental hepatotoxicity: Isoniazid and rifampicin (100 mg/kg bw) solution were prepared separately in sterile distilled water. Rats were treated with isoniazid, co-administered with rifampicin for 21 days by ip route (Jiang et al., 2004).

In order to study the effect of ethanolic and aqueous extract of *Annona squamosa* in rat 350 mg/kg bw (Gupta et al., 2005) and 300 mg/kg bw (Kaleem et al., 2006) were used respectively. Silymarin (2.5 mg/kg bw) was used as a standard drug in this study (Parthasarathy et al., 2007). Rats were divided into five groups as following protocol.

Treatment Protocol:

GROUP I	:	Normal control (n=6, the animals were given normal saline only)
GROUP II	:	Hepatotoxic control (n=6, the animals were given INH+RIF for 21 Days)
GROUP III	:	Treatment group (n=6, the animals were given INH+RIF+EEAS for 21 days)
GROUP IV	:	Treatment group (n=6, the animals were given INH+RIF+AEAS for 21 days)
GROUP V	:	Standard group (n=6, the animals were given INH+RIF+Silymarin for 21 days)

Rats were treated as per the treatment protocol. Body weights of these rats were monitored sequentially in control and experimental animals for a period of 21 days.

Biochemical estimation: Rats were sacrificed 1 hour after administration on day 21. The blood was collected by retro orbital artery bleeding. Blood samples were centrifuged for 10 minutes at 3000 rpm to separate the serum. ALP, ALT, AST, γ-GT, Total protein and Bilirubin levels were estimated from the serum by using standard kits (Rajesh et al., 2005). Results of biochemical analysis are given in the Table 1.

Histopathological studies: The livers were excised quickly and fixed in 10% formalin and stained with haemotoxylin and eosin and then observed under microscope for degeneration, fatty changes, necrotic changes and evidence of hepatotoxicity if any. Results of the histopathological studies are shown in the Figures (1-5).

Statistics: The results were expressed as Mean ± SEM. Statistical analysis was carried out by using ONEWAY ANOVA followed by Newman-Keul's multiple tests.

Results

Biochemical parameters

Control Group (G1) (Table 1): The basal levels of liver enzymes (ALP, AST, ALT and γ-GT) in control were 117.39 ± 2.1 IU/dl, 118.39 ± 5.9 IU/dl, 30.99 ± 0.38 IU/dl and 90.31 ± 1.73 IU/dl respectively. Total Bilirubin and Total protein levels were 0.55 ± 0.01 mg/dl and 0.47 ± 0.01 mg/dl respectively.

Toxic Control (G2) (Table 1): There was significant increase in total Bilirubin (1.76 ± 0.04 mg/dl), accompanied by significant decrease in level of Total protein (0.35 ± 0.04 mg/dl) and also significant increase in ALP (343.44 ± 7.55

IU/dl), AST (568.72 ± 5.25 IU/dl), ALT (174.41 ± 12.64 IU/dl) and γ-GT (170.24 ± 5.99 IU/dl) as compared to the control.

Treatment Groups (Table 1):

EEAS Groups (G3): There was significant decrease in total Bilirubin (1.24 ± 0.04 mg/dl), accompanied by significant increase in level of Total protein (8.1 ± 0.11 mg/dl) and also significant decrease in ALP (250.55 ± 11.49 IU/dl), AST (364.84 ± 4.66 IU/dl), ALT (76.02 ± 3.75 IU/dl) and γ-GT (156.68 ± 1.75 IU/dl) as compared to the toxic control.

AEAS Groups (G4): There was significant decrease in total Bilirubin (1.62 ± 0.04 mg/dl), accompanied by significant increase in level of Total protein (8.8 ± 0.07 mg/dl) and also significant decrease in ALP (251.99 ± 9.39 IU/dl), AST (366.96 ± 7.39 IU/dl), ALT (89.46 ± 5.55 IU/dl) and γ-GT (146.70 ± 3.67 IU/dl) as compared to the toxic control.

SIL Groups (G5): There was significant decrease in total Bilirubin (1.42 ± 0.03 mg/dl), accompanied by significant increase in level of Total protein (7.85 ± 0.01 mg/dl) and also significant decrease in ALP (211.29 ± 11.23 IU/dl), AST (347.88 ± 7.82 IU/dl), ALT (55.52 ± 3.48 IU/dl) and γ-GT (100.29 ± 1.04 IU/dl) as compared to the toxic control.

Histopathological studies

Control Groups (G1) (Fig No: 1): Hepatocytes of the normal control group showed a normal lobular architecture of the liver.

Toxic Control (G2) (Fig No: 2): In the INH + RIF treated group the liver showed hepatocytic necrosis and inflammation also observed in the centrilobular region with portal triaditis.

EEAS Groups (G3) (Fig No: 3): EEAS pretreated group showed minimal inflammation with moderate portal triaditis and their lobular architecture was normal.

AEAS Groups (G4) (Fig No: 4): AEAS pretreated group showed minimal inflammation with moderate portal triaditis and their lobular architecture was normal.

SIL Groups (G5) (Fig No: 5): Silymarin pretreated group showed normal hepatocytes and their lobular architecture was normal.

Discussion

Although *Annona squamosa* is reported to possess varied medicinal properties such as insecticidal, antiovulatory and antitumor activities (Nonfon et al., 1990), there is no previous report about the hepatoprotective activity of this plant. The present investigation reports the hepatoprotective effects of EEAS and AEAS extracts of leaves of this plant.

In the present study, hepatotoxicity model in Wistar rats was successfully produced by administering INH and RIF (100 mg/kg per day each) intraperitonially. Three-fold rise above the normal upper limits in the measured serum transaminases in INH+RIF group on day 21 of the experiment was a biochemical indication of liver injury.

During the metabolism of INH, hydrazine is produced directly (from INH) or indirectly (from acetyl hydrazine). From earlier study it is evident that hydrazine play a role in INH-induced liver damage in rats, which is consistent with the report by Sarich et al (Garner et al., 2004). The combination of INH and RIF was reported to result in higher rate of inhibition of biliary secretion and an increase in liver cell lipid peroxidation, and cytochrome P450 was thought to be involved the synergistic effect of RIF on INH (Ramaiah et al., 2001). However, its role in INH-induced hepatotoxicity is unclarified, as INH itself is an inducer of CYP2E1 (Shakun and Shman'ko 1985).

A small retrospective analysis of patients who developed hepatic dysfunction whilst on antituberculosis drugs, hospitalized in a unit between 1st January 1991 and 31st December 1992, was recently undertaken. Out of 1,181 patients who received RIF, INH with or without pyrazinamide and other drugs, 142 developed clinically symptomatic dysfunction. An assumption that the vast majority of hepatic dysfunction episodes should have occurred with in 2 months of commencement of antituberculosis chemotherapy was made, as generally reported (Girling 1982). In previous report also says that there did not seem to be clear evidence that Isoniazid proves much more injuries than Rifampicin and, in this connection, they consider that it is the combination of these two drugs that confer the additive, or even synergistic, potential of liver toxicity than either agent alone, as conjectured (Yasuda et al., 1990, Wu et al., 1990 and Steele et al., 1991).

INH is metabolized in the liver primarily by acetylation and hydrolysis, and it is these acetylated metabolites that are thought to be hepatotoxins (Wu et al., 1990). Previous report in rats suggest that the hydrazine metabolite of INH and is subsequent effect on CYP2E1 induction is involved in the development of INH-induced hepatotoxicity (Jiang et al 2004) and also oxidative stress as one of the mechanism for INH + RIF induced hepatic injury (Perettie et al., 1987).

Extracts of *Annona squamosa* improved liver function by decreasing the serum ALT, AST and alkaline phosphate levels in hepatotoxic rats. Although there will be an increase of AST and ALT in heart and liver diseases, the increase of AST is more in heart disease and ALT in higher in liver disease. Total bilirubin a byproduct of the breakdown of red blood cells in the liver, bilirubin is a good indicator of liver function. High levels will cause icterus (jaundice) and are indicative of damage to the liver and bile duct (Rajesh et al., 2005). Extracts of *Annona squamosa* reduced the ALP as well as the total bilirubin levels, indicating its protective effect over liver and improvement in its functional efficiency.

Hepatocellular disintegrate and the inflammation in the liver was observed in the centrilobular region by histopathological examination in isoniazid-rifampicin treated groups. Previous study also showed the same result in isoniazid treated groups (Jiang et al., 2004, Ravinder et al., 2006). Simultaneously administered ethanolic and aqueous extracts of *Annona squamosa* prevented the induction of histopathological injuries in INH+RIF co-treated animals.

In this study the results suggest that the statistically significant different in biochemical parameters in toxic control group G2, indicate that hepatic damage has been induced by INH + RIF. Following treatment with EEAS, AEAS and Silymarin all the parameters were reduced and total protein restored to normal value.

Table 1. Effect of ethanolic and aqueous extracts of *Annona squamosa* in different biochemical parameters in INH + RIF induced-hepatotoxic rats.

GROUPS	TOTAL BILIRUBUN (mg/dl)	TOTAL PROTEIN (mg/dl)	ALP (IU/dl)	AST (IU/dl)	ALT (IU/dl)	γ-GT (IU/dl)
NORMAL CONTROL (G1)	0.55 ± 0.01	0.47 ± 0.01	117.39 ± 2.1	118.39 ± 5.99	30.99 ± 0.38	90.31 ± 1.73
TOXIC CONTROL (G2)	1.76 ± 0.04♦	0.35 ± 0.04♦	343.44 ± 7.55♦	568.72 ± 5.25♦	174.41 ± 12.64♦	170.24 ± 5.99♦
EEAS GROUP (G3)	1.24 ± 0.04***	8.1 ± 0.11***	250.55 ± 11.49***	364.84 ± 4.66***	76.02 ± 3.75***	156.68 ± 1.75**
AEAS GROUP (G4)	1.62 ± 0.04*	8.8 ± 0.11***	251.99 ± 9.39***	366.96 ± 9.39***	89.46 ± 5.55***	146.70 ± 3.67***
SIL GROUP (G5)	1.42 ± 0.03***	7.85 ± 0.01***	211.29 ± 11.23***	347.88 ± 7.82***	55.52 ± 3.48***	100.29 ± 1.04***

Values are expressed as Mean ± SEM

♦ p<0.001 vs Control (G1) *P<0.05 vs Toxic Control (G2) **p<0.01 vs Toxic Control (G2) ***p<0.001 vs Toxic Control (G2)

Figure 1. Hepatocytes of the normal control group showed a normal lobular architecture of the liver.

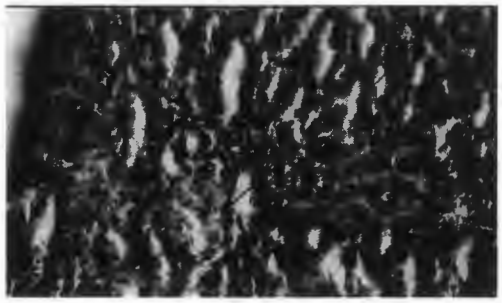

Figure 2. Hepatocytes of the INH + RIF treated group showed liver cell necrosis and inflammation also observed in the centrilobular region with portal triaditis.

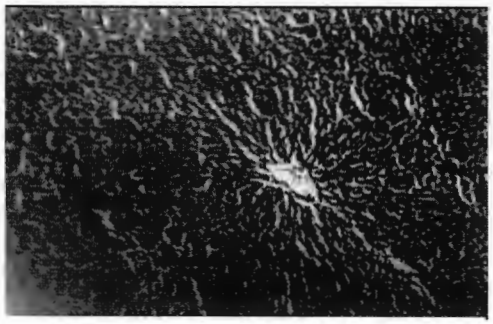

Figure 3. Hepatocytes of the EEAS pretreated group showed minimal inflammation with moderate portal triaditis and their lobular architecture was normal.

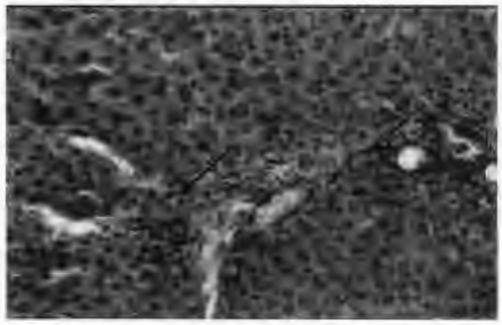

Figure 4. Hepatocytes of the AEAS pretreated group showed minimal inflammation with moderate portal triaditis and their lobular architecture was normal.

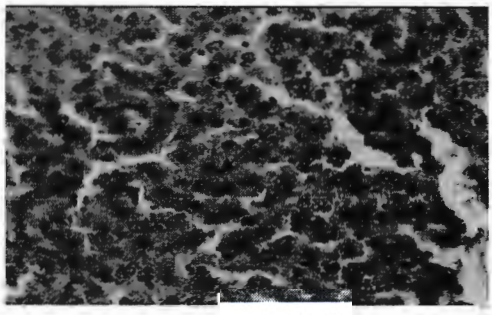

Figure 5. Hepatocytes of the Silymarin pretreated group showed normal hepatocytes and their lobular architecture was normal.

The estimation of γ-GT (gamma glutamyl transpeptidase) levels is a valuable screening test with high negative predictive value for liver disease (Nemesanszky 1996). Drugs and chemicals are known to increase γ-GT activity by the induction of hepatic microsomal enzymes (Kim et al., 1977). Comparatively in EEAS and AEAS treated groups significant changes occurred in total Bilirubin and γ-GT levels. This suggests that the extracts have hepatoprotective effect. However, treatment with these extracts could not completely reverse the hepatic injury, but had resulted in early hepatic damage.

The reason for hepatoprotective effect of the extracts may be that *Annona squamosa* Linn contain flavonoids which might have scavenged the free radical offering hepato protection. Previous report also suggests that the protective role of *Annona squamosa* leaf extracts could be due to the antioxidative effect of flavonoids present in the leaf (Kaleem et al., 2006).

The above are preliminary indications and further detailed studies are necessary to find out whether the action of the extracts is due to one or more of the above–mentioned possible mechanism or not. Thus the extracts of *Annona squamosa* seems to be useful in controlling hepatic injury in drug induced hepatotoxicity. Purification of extracts and identification of the active principle may yield a good hepatoprotective drug.

Conclusion

The results obtained from the analysis of biochemical parameters and histopathological studies, enabled me to conclude that the extracts of *Annona squamosa* were not able to revert completely hepatic injury induced by INH + RIF, but it could limit the effect of INH + RIF to the extent of necrosis. As the two extracts EEAS and AEAS produced same effect it is concluded that there is no difference in the extract treatment. These results indicate that it is worth undertaking further studies on possible usefulness of the extracts of the leaves of *Annona squamosa* in hepatotoxicity.

References

Bhakuni DS, Tewari S, Dhar MM. 1972. Aporphine alkaloids of *Annona squamosa*. Phytochemistry 11:1819–1822.

Bhaumik PK, Mukhergee B, Juneau JP, Bhacca NS, Mukherjee. 1979. Alkaloids from leaves of *Annona squamosa*. Phytochemistry 18:1584–1586.

Cheema PS, Dixit RS, Koshi T, Perti SL. 1985. Insecticidal properties of the seed oil of *Annona squamosa* Linn. J Sci Ind Res 17:132.

Forgacs P, Desconclois JF, Provost R, Tiberghien et Touche A. 1980. Un Nouvel Heteroside Nitre Extrait D' *Annona squamosa*. Phytochemistry 19:1251–1252.

Garner P, Holmes A, Ziganahina L. 2004. Tuberculosis. Clin Evid 11:1081-1093.

Girling DJ. 1982. Adverse effects of antituberculosis drugs. Drugsb 23:56-61.

Gupta RK, Kesari AN, Murthy PS, Chandra R, Tandon V, Watal G. 2005. Hypoglycemic and antidiabetic effect of ethanolic extract of leaves of *Annona squamosa* L. in experimental animals. J Ethnopharmacol 99(1):75-81.

Hopp DC, Zeng L, Gu ZM, Kozlowski JF, McLaughlin JL.1997. Novel mono-tetrahydrofuran ring acetogenins, from the bark of *Annona squamosa*, showing cytotoxic selectivities for the human pancreatic carcinoma cell line, PACA-2. J Nat Prod 60:581-6.

Hopp DC, Alali FQ, Gu ZM, McLaughlin JL. 1998. Mono-THF ring annonaceous acetogenins from *Annona squamosa*. Phytochemistry 47:803-9.

Jiang YUE, Ren-xiu PENG, Jing YANG, Rui KONG, Juan LIU. 2004. CYP2E1 mediated isoniazid-induced hepatotoxicity in rats. Acta Pharmacol Sin 25(5):699-704.

Kaleem M, Asif M, Ahmed Q.U, Bano B. 2006. Antidiabetic and antioxidant activity of *Annona squamosa* extract in streptozotocin-induced diabetic rats. Singapore Med J 47(8):670-675.

Kaplowitz N. 2001. Drug-induced liver disorders: implications for drug development and regulation. Drug Saf 24:483–90.

Kim NK, Vasmineh WG, Frejar EF, Goldman AL, Theologides A. 1977. Value of alkaline phosphatase, 5'-neucleotidase gamma glutamyl transferase and glutamate dehydrogenase activity measurements (Single and combine) in serum in diagnosis of metastases to the liver. Clin Chem 23:2034-38.

Li XH, Hui YH, Rupprecht JK, Liu YM, Wood KV, Smith DL, Chang CJ, McLaughlin JL. 1990. Bullatacin, bullatacinone, and squamone, a new bioactive acetogenin, from the bark of *Annona squamosa*. J Nat Prod 53:81-6.

Michael P. Holt, Cynthia Ju. 2006. Mechanisms of Drug-Induced Liver Injury. The AAPS Journal 8 (1):E48-E54.

Morton J. 1987. Sugar apple. Fruits Warm Climate 69-72.

Nemesanszky E. 1996. Enzyme test in hepatobiliary disease. In: Donald W Moss and Sidney B Rosarki, editors. Enzyme test in diagnosis. New York: Oxford University press. p 25-29.

Nonfon M, Lieb F, Moeschler H, Wendish D. 1990. Four anonyms from *Annona squamosa*. Phytochemistry 29:1951-1954.

Parthasarathy R, Nivethetha M, Brindha P. 2007. Hepatoprotective activity of *Caesalpinia bonducella* seeds on paracetamol induced hepatotoxicity in male albino rats. Indian Drugs 44(5):401-404.

Peretti E, Karlaganis G, Lauterburg BH. 1987. Acetylating of Acetylhydrazine, the toxic metabolite of isoniazid, in humans: inhibition by concomitant administration of isoniazid. J Pharmacol Exp Ther 243:686-689.

Rajesh KG, Achyut NK, Geeta W, Murthy PS, Ramesh C, Vibha T. 2005. Nutritional and Hypoglycemic Effect of Fruit Pulp of *Annona squamosa* in Normal Healthy and Alloxan-Induced Diabetic Rabbits. Ann Nutr Metab 49:407–413

Ramaiah SK, Apte U, Mehendale HIM. 2001. Cytochrome P4502E1 induction increases thioacetamide liver injury in diet-restricted rats. Drug Metab Dispos 29:1088-95.

Ravinder P, Kim V, Arbab S, Kartar S, Satya VR. 2006. Effect of garlic on isoniazid and rifampicin-induced hepatic injury in rats. World J Gastroenterol 12(4):636-639.

Seetharaman TR. 1986. Flavonoids from the leaves of *Annona squamosa* and Polyalthia longifolia. Fitoterapia 57:189-198.

Skakun NP, Shman'ko VV. 1985. Synergistic effect of rifampicin on hepatotoxicity of isoniazid. Antibiot Med Biotekhnol 30:185-9.

Sharma SK. 2004. Antituberculosis drugs and hepatotoxicity. Infect Genet Evol 4:167-170.

Shirwaikar A, Rajendran K, Kumar CD. 2004. In vitro antioxidant studies of *Annona squamosa* Linn. leaves. Indian J Exp Biol 42: 803-7.

Steele MA, Burk RF, Des Prez RM. 1991. Toxic hepatitis with isoniazid and rifampicin: a meta-analysis. Chest 99:465-471.

Tasduq SA, Peerzada K, Koul S, Bhat R, Johri RK. 2005. Biochemical manifestation of anti-tuberculosis drugs induced hepatotoxicity and the effect of Silymarin. Hepatol Res 31:132-135.

Watt G. 1972. Periodical Experts: A Dictionary of the Economic Products of India. Vol 1:260 p.

WHO report. 2006. World Health Organization. Global tuberculosis control: WHO/HTM/TB/2006.362. Geneva.

Wu J-W, Leev S-D, Yeh P-F. 1990. Isoniazid-Rifampicin induced hepatitis in hepatitis B carriers. Gastroentrology 98:502-504.

Yang TH, Chi-Ming C. 1972. Structure of squamolone, a novel diazepine from *Annona squamosa* L. J Chin Chem Soc (Taipei) 19:149–151.

Yasuda K, Sato A, Chida K. 1990. Pulmonary tuberculosis with chemotherapy related liver dysfunction. Kekkadu 65:407-413.

Zimmerman HJ. 1978. Drug-induced liver disease. Drugs 16 (1):25-45.

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Article

Antinociceptive and Anti-Inflammatory Activities of the Ethanol Extract of *Annona muricata* L. Leaves in Animal Models

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Abstract: Antinociceptive and anti-inflammatory activities of the ethanol extract from *Annona muricata* L. leaves were investigated in animal models. The extract delivered per oral route (p.o.) reduced the number of abdominal contortions by 14.42% (at a dose of 200 mg/kg) and 41.41% (400 mg/kg). Doses of 200 and 400 mg/kg (p.o) inhibited both phases of the time paw licking: first phase (23.67% and 45.02%) and the second phase (30.09% and 50.02%), respectively. The extract (p.o.) increased the reaction time on a hot plate at doses of 200 (30.77% and 37.04%) and 400 mg/kg (82.61% and 96.30%) after 60 and 90 minutes of treatment, respectively. The paw edema was reduced by the ethanol extract (p.o.) at doses of 200 (23.16% and 29.33%) and 400 mg/kg (29.50% and 37.33%) after 3 to 4 h of application of carrageenan, respectively. Doses of 200 and 400 mg/kg (p.o.), administered 4 h before the carrageenan injection, reduced the exudate volume (29.25 and 45.74%) and leukocyte migration (18.19 and 27.95%) significantly. These results suggest that *A. muricata* can be an active source of substances with antinociceptive and anti-inflammatory activities.

Keywords: *Annona muricata*; Annonaceae; antinociceptive activity; anti-inflammatory activity

1. Introduction

Annona muricata L. (Annonaceae), commonly known as soursop, is found from Central America to South America, including the North, Northeast and Southeast regions of Brazil [1,2]. Traditionally, the leaves are used for headaches, insomnia, cystitis, liver problems, diabetes, hypertension and as an anti-inflammatory, antispasmodic and antidysenteric [1,2]. The decoction of the leaves have parasiticide, antirheumatic and antineuralgic effects when used internally, while the cooked leaves, applied topically, fight rheumatism and abscesses [1-3].

Among the chemical constituents found in *A. muricata,* the alkaloids (reticulin, coreximine, coclarine and anomurine) [4,5] and essential oils (β-caryophyllene, δ-cadinene, epi-α-cadinol and α-cadinol) [6,7] stand out. However, species of the Annonaceae family, including *A. muricata,* have also been targeted for investigation due to appurtenant substances in the acetogenins class [8] that have been isolated from different parts of the plant [9]. For example, annomuricins A and B, gigantetrocin A, annonacin-10-one, muricatetrocins A and B, annonacin, goniothalamicin [10], muricatocins A and B, annonacin A, (2,4-*trans*)-isoannonacin, (2,4-*cis*)-isoannonacin [11], annomuricin C, muricatocin C, gigantetronenin [12], annomutacin, (2,4-*trans*)-10R-annonacin-A-one, (2,4-*cis*)-10R-annonacin-A-one [13], annopentocins A, B and C, *cis*- and *trans*-annomuricin-D-ones [14], annomuricine, muricapentocin [15], muricoreacin and murihexocin C [16] and annocatacin A and B [17] were identified in the leaves. These acetogenins have cytotoxic properties against tumor cell lines [10-17] and molluscicidal activity [18]. In addition, *A. muricata* leaf extracts have antioxidant [19] and molluscicidal properties [20].

A. muricata ethnomedicinal use, especially for inflammation, rheumatism and neuralgy, still lacks scientifically supported pharmacological and clinical validation. In this sense, the aim of the present study was to investigate the antinociceptive and anti-inflammatory properties of the ethanol extract from *A. muricata* leaves using experimental animal models.

2. Results and Discussion

2.1. Acute Toxicity

At the doses administered per oral route (p.o.), the ethanol extract from *A. muricata* leaves was toxic to animals with LD_{50} of 1.67 g/kg (95% confidence intervals 1.24-2.26 g/kg). This result served as a parameter for dosage definition in the experiments of antinociceptive and anti-inflammatory activities.

2.2. Writhing Response Induced by Acetic Acid in Mice

Doses (p.o.) of 200 and 400 mg/kg of *A. muricata* extract significantly reduced ($p < 0.01$ and $p < 0.001$, respectively) the abdominal contortions induced by acetic acid to 57.87 ± 1.55 s and 39.62 ± 1.97 s compared to the respective control (67.62 ± 2.03 s) (Table 1).

Table 1. Effects of the ethanol extract from *A. muricata* leaves on acetic acid-induced writhing in mice.

Group	Dose (mg/kg)	Number of writhes	Inhibition (%)
Control	Saline	67.62 ± 2.03	-
	100	67.50 ± 1.74	-
Ethanol Extract	200	57.87 ± 1.55**	14.42
	400	39.62 ± 1.97***	41.41
Indomethacin	10	18.25 ± 0.80***	73.01

Data are mean ± s.e.m. of eight mice. **$P < 0.01$, ***$P < 0.001$ *vs.* control group.

2.3. Effects on Formalin-Induced Nociception in Mice

The intraplantar injection of formalin promoted a biphasic characteristic response (Table 2). The time spent licking in the first phase (0-5 min) was 86.62 ± 3.18 s and in the second phase (15-30 min) was 93.87 ± 2.73 s for the control group. After 60 min of treatment, doses (p.o.) of 200 and 400 mg/kg of extract significantly inhibited ($p < 0.001$) the first phase at 23.67 and 45.02% and the second phase at 30.09 and 50.20%, respectively, when compared to the control.

Table 2. Effects of the ethanol extract from *A. muricata* leaves on formalin-induced nociception in mice.

Group	Dose (mg/kg)	Duration of paw licking (s)			
		First phase	Inhibition (%)	Second phase	Inhibition (%)
Control	Saline	86.62 ± 3.18	-	93.87 ± 2.73	-
	100	85.87 ± 2.88	-	91.25 ± 3.07	-
Ethanol Extract	200	66.12 ± 1.54***	23.67	65.62 ± 1.72***	30.09
	400	47.62 ± 2.13***	45.02	46.75 ± 1.68***	50.20
Morphine	1	16.25 ± 1.44***	81.24	19.37 ± 0.94***	79.36

First phase = 0–5 min after formalin injection; second phase = 15–30 min.
Data are mean ± s.e.m. of eight mice. ***$P < 0.001$ *vs.* control group.

2.4. Effects on Hot-Plate Latency Assay in Mice

The *A. muricata* ethanol extract increased the latency time of mice exposed to the hot plate (Table 3). After 60 and 90 min of treatment, doses (p.o.) of 200 (30.77 and 37.04%) and 400 mg/kg (82.61 and 96.30%) increased significantly ($p < 0.05$ and $p < 0.001$, respectively) the latency time in the respective control group. Morphine proved to be a potent analgesic, increasing the latency time

within the evaluation periods. Naloxone, an opioid antagonist, blocked the morphine action but did not completely alter the antinociceptive effect of the tested extracts.

Table 3. Effects of the ethanol extract from *A. muricata* leaves on the reaction time (s) of mice exposed to the hot-plate test.

Group	Dose (mg/kg)	Time after drug administration (s)			
		0 min	30 min	60 min	90 min
Control	Saline	5.50 ± 0.80	6.12 ± 0.44	6.50 ± 0.50	6.75 ± 0.79
Ethanol Extract	100	5.37 ± 0.80	6.25 ± 0.62	7.12 ± 0.29	7.25 ± 0.45
	200	5.50 ± 0.78	6.75 ± 0.45	8.50 ± 0.50*	9.25 ± 0.67*
	400	5.75 ± 0.72	7.37 ± 0.80	11.87 ± 0.64***	13.25 ± 0.84***
Morphine	1	5.75 ± 0.65	9.62 ± 0.82**	13.75 ± 1.10***	16.87 ± 0.93***
Naloxone + Morphine	1 + 1	6.00 ± 0.68	7.87 ± 0.69	8.00 ± 0.46*	7.87 ± 0.55
Naloxone + Extract	1 + 400	5.62 ± 0.68	7.25 ± 0.75	8.75 ± 0,45**	10.87 ± 0.83**

Data are mean ± s.e.m. of eight mice. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *vs.* control group.

2.5. Effects on Carrageenan-induced Edema in Rats

The *A. muricata* ethanol extract anti-inflammatory effect evaluated by the paw edema method induced by carrageenan is shown in Table 4. Edema inhibition was observed 3 h after carrageenan application of doses (p.o.) of 200 (0.73 ± 0.06; 23.16 %; $p < 0.05$) and 400 mg/kg (0.67 ± 0.04; 29.47 %; $p < 0.01$). 4 h after carrageenan injections, the doses of 200 (0.53 ± 0.03; $p < 0.01$) and 400 mg/kg (0.47 ± 0.02; $p < 0.001$) reduced the respective paw edema (29.33 and 37.33%). In this time, indomethacin also reduced the paw edema (42.67%).

Table 4. Effects of the ethanol extract from *A. muricata* leaves on carrageenan-induced paw edema in rats.

Group	Dose (mg/kg)	Volume of hind paw (mL)			
		1 h	2 h	3 h	4 h
Control	Saline	0.53 ± 0.06	0.72 ± 0.05	0.95 ± 0.06	0.75 ± 0.06
Ethanol Extract	100	0.52 ± 0.09	0.68 ± 0.06	0.80 ± 0.06	0.63 ± 0.04
	200	0.50 ± 0.10	0.65 ± 0.09	0.73 ± 0.06*	0.53 ± 0.03**
	400	0.48 ± 0.07	0.60 ± 0.04	0.67 ± 0.04**	0.47 ± 0.02***
Indomethacin	10	0.47 ± 0.10	0.58 ± 0.05	0.62 ± 0.06**	0.43 ± 0.02***

Data are mean ± s.e.m. of six rats. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *vs.* control group.

2.6. Effects on Carrageenan-Induced Pleurisy in Rats

The pleurisy effects demonstrated that doses (p.o.) of 200 ($p < 0.01$) and 400 mg/kg ($p < 0.001$) of the extracts significantly reduced the exudate volume (Figure 1) and the number of total leukocytes (Figure 2). The exudate volume was decreased by 29.25 and 45.74% at doses (p.o.) of 200 and 400 mg/kg compared to the respective control. Leukocyte migration inhibition also occurred from doses

(p.o.) of 200 (12.91 ± 0.32 × 10^3 cells/mm^3; $p < 0.001$) and 400 mg/kg (11.37 ± 0.44 × 10^3 cells/mm^3; $p < 0.001$). Indomethacin reduced the exudate volume and the leukocyte migration.

Figure 1. Effects of the ethanol extract from *A. muricata* leaves on pleural exudation induced by carrageenan in rats. Data are mean ± s.e.m. of six rats. $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* control group.

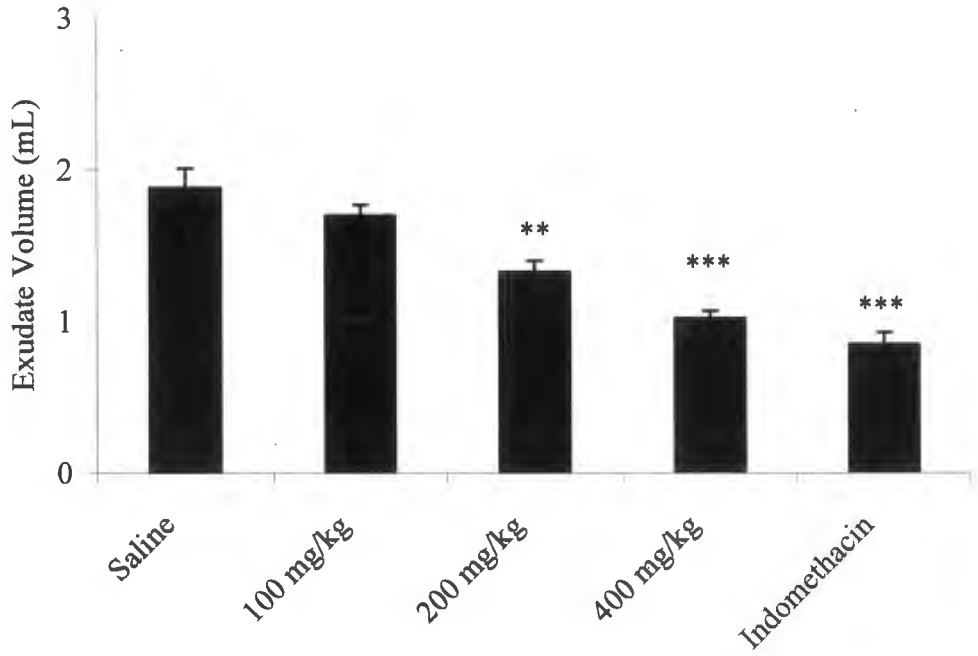
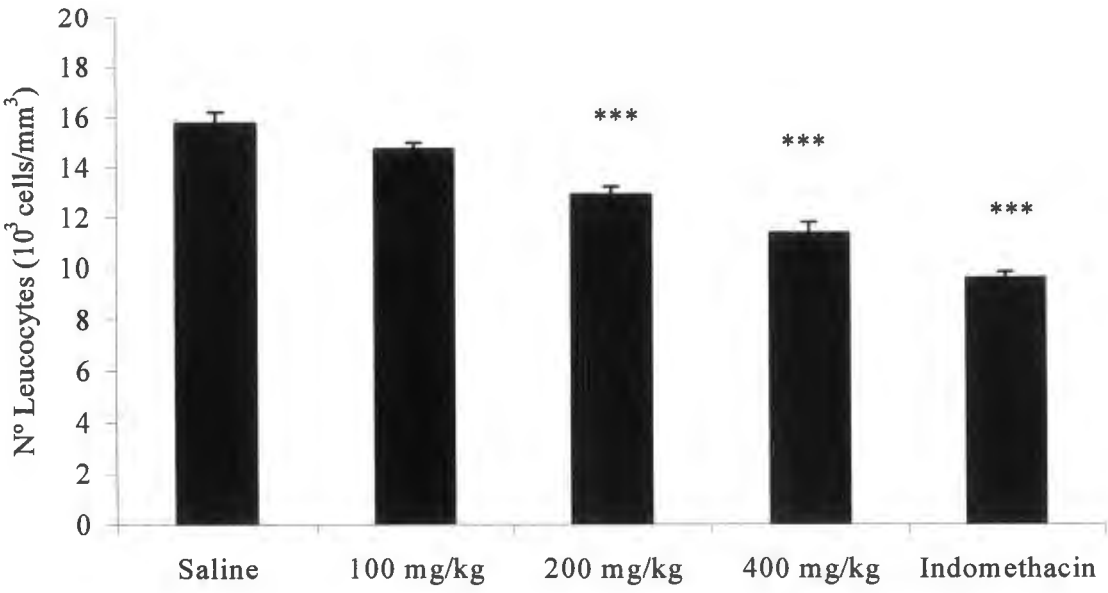


Figure 2. Effects of the ethanol extract from *A. muricata* leaves on number of leucocytes in carrageenan-induced pleurisy in rats. Data are mean ± s.e.m. of six rats. $^{***}P < 0.001$ *vs.* control group.



The acute toxicity test showed that the *A. muricata* leaves ethanol extract doses tested were toxic to mice. However, the largest dose administered (400 mg/kg) is less than the lowest dose applied for determination of the LD_{50} (0.5 g/kg or 500 mg/kg). Studies have demonstrated that isolated acetogenins from the *A. muricata* leaves are toxic to tumor cells [11-17] and molluscicides [18]. It is possible that the toxic effect of the ethanol extract could be due to the presence of these substances. However, the pharmacological doses definition of the ethanol extract was not described in the literature. In the present study, the LD_{50} was used to define the doses that were administered to the animals.

Based on the pharmacological tests results, the *A. muricata* ethanol extract has antinociceptive and anti-inflammatory activities, being firstly reported in the literature. Intraperitoneal administration of acetic acid releases prostaglandins and sympathomimetic system mediators like PGE_2 and $PGF_{2\alpha}$ and their levels were increased in the peritoneal fluid of the acetic acid induced mice [21]. Thus, the antinociceptive effect of the ethanol extract could be mediated by peripherical effects, including the prostaglandin synthesis inhibition. The antinociceptive effect was also demonstrated by the biphasic response time of paw licking induced by formalin [22]. The first phase (0 to 5 min) corresponds to the neurogenic stage as an intensely painful process for the activation of nociceptive pathways, while inflammation mediators are produced after 15 minutes of formalin application (second phase) [22,23]. Substance P and bradykinin act as mediators in the first phase, while histamine, serotonin, prostaglandin and bradykinin are involved in the nociceptive response of the second stage [23]. The central action was confirmed in the hot plate test (200 and 400 mg/kg), showing that the maximum effect is reached after 90 minutes. This test is considered to be sensitive to drugs acting at the supraspinal modulation level of the pain response [24], suggesting at least a modulatory effect of the extract. In this study, antinociceptive action did not depend entirely on the opioid system, because naloxone treatment did not completely reverse the produced effect [25,26]. The formalin induced algesia test also indicated a possible anti-inflammatory activity (the second phase was reduced from 200 mg/kg).

The anti-inflammatory activity was confirmed by the paw edema induced by carrageenan in rats, a model widely used to study anti-inflammatory substances. Carrageenan induces paw edema resulting in the release of mediators such as histamine, serotonin, bradykinin, substance P and a platelet activating factor and prostaglandins [27-33]. In this study, oral treatment with the *A. muricata* extract significantly inhibited the paw edema. This evidence suggests that the anti-inflammatory actions of the ethanol extract are related to inhibition of one or more signaling intracellular pathways involved with these mediators effects.

Pleurisy produced by intrapleural injection of carrageenan leads to the formation of exudate in the pleural cavity [34,35] and leukocyte migration [35,36]. It is a method that assesses the inflammatory infiltrate and confirms the obtained paw edema results. Non-steroidal anti-inflammatory drugs, such as indomethacin, inhibit the accumulation of exudates and mobilization of leukocytes between 3 and 6 h after application of carrageenan [35,37]. By reducing the volume of exudate and the leukocyte migration, the *A. muricata* ethanol extract confirmed the results of the paw edema (Table 4 and Figures 1 and 2).

Plants belonging to the Annonaceae family have been investigated for its antinociceptive and anti-inflammatory properties [25,26,38]. However, considering the compounds isolated from *A. muricata*,

these properties are not been reported for the alkaloids [4,5] and acetogenins [8-18]. Antinociceptive and anti-inflammatory activities have been attributed to essential oil of *Dennettia tripetala* (Annonaceae) [38], but such activities are not described for the major components [6,7] identified in *A. muricata*. Additional studies are necessary to establish the possible correlation between activities and chemical composition of this plant.

3. Experimental Section

3.1. Plant Material and Extraction

The plant material used in this study was collected in Juiz de Fora, State of Minas Gerais, Brazil, in February 2008. The species was identified by Dr Fátima Regina Gonçalves Salimena and a voucher specimen (CESJ number 48236) was deposited in the Herbarium of the Universidade Federal de Juiz de Fora, Brazil. Dried and powdered leaves (600 g) were exhaustively extracted in 95% ethanol (2.5 L) by static maceration for 3 weeks at room temperature with renewal of solvent every 2 days. The ethanol extract was filtered and evaporated under a rotary evaporator at controlled temperature (50-60 °C). This material was placed in a desiccator with silica to yield 36.40 g. The dried extract was dissolved using 1% DMSO in normal saline for pharmacological studies.

3.2. Chemicals

Drugs and reagents used in this study (and their sources) were as follows: acetic acid (Vetec Química Farm Ltda, Rio de Janeiro, RJ, Brazil), formaldehyde (Reagen Quimibrás Ind. Química S.A., Rio de Janeiro, RJ, Brazil), morphine hydrochloride (Merck Inc., Whitehouse Station, NJ, USA), naloxone and indomethacin (Sigma Chemical Co, St Louis, MI, USA).

3.3. Animals

Male Wistar rats (90-110 days) weighing 200-240 g and male Swiss albino mice (50-70 days) weighing 25-30 g were used in the experiments. The animals were provided by the Central Biotery of the Universidade Federal de Juiz de Fora. The animals were divided into groups and kept in plastic cages (47 × 34 × 18 cm) under a 12 h light/12 h dark cycle at room temperature (22 ± 2 °C), with free access to Purina rations and water. Animal care and the experimental protocol followed the principles and guidelines suggested by the Brazilian College of Animal Experimentation (COBEA) and were approved by the local ethical committee.

3.4. Acute Toxicity

Groups of ten mice received oral doses of 0.5, 1, 1.5, 2 and 3 g/kg of ethanol extract from *A. muricata*, while the control group received the vehicle (saline). The groups were observed for 48 h and mortality at end of this period was recorded for each group [39]. The LD_{50} (50% lethal dose) was determined by probit test using a log plot of percentage death *versus* dose [40]. The determination of LD_{50} served to define the doses used in experiments of pharmacological activities.

3.5. Acetic Acid-Induced Writhing Response in Mice

Antinociceptive activity was evaluated using the test of abdominal writhing induced by acetic acid in mice [41]. Animals were divided into groups of eight mice. Control mice received an i.p. injection of acetic acid 0.6% (0.25 mL) and 10 min later the writhes were counted over a period of 20 min. One group of mice received indomethacin (10 mg/kg) by the per oral route (p.o.) as a reference compound, and the other three groups received the extract at doses (p.o.) of 100, 200 and 400 mg/kg, 1 h before the acetic acid injection.

3.6. Formalin-Induced Nociception in Mice

Mice received subplantar injections of 20 μL 2.5% formalin (in 0.9% saline) and the time of paw licking (in seconds) was determined over 0-5 min (first phase - neurogenic) and 15-30 min (second phase - inflammatory) after formalin injection [22]. Animals (n = 8) were pretreated p.o. with extract (100, 200 or 400 mg/kg; 0.1 mL per 10 g body weight) or the reference compound, subcutaneous morphine (1 mg/kg), 1 h before administration of formalin. Control animals were treated with sterile saline (10 mL/kg).

3.7. Hot-Plate Latency Assay in Mice

Animals were placed on a hot-plate (Model LE 7406, Letica Scientific Instruments, Barcelona, Spain) heated at 55 ± 1 °C [42]. Three groups of mice (n = 8) were treated p.o. with ethanol extract (100, 200 or 400 mg/kg; 0.1 mL per 10 g body weight); the control group received sterile saline (10 mL/kg). Measurements were performed at time 0, 30, 60 and 90 min after drug administration, with a cut-off time of 40 s to avoid lesions to the animals' paws. The effect of pretreatment with naloxone (1 mg/kg, subcutaneously) on the analgesia produced by the ethanol extract (400 mg/kg) was determined in a separate group of animals. Morphine (1 mg/kg, subcutaneously), in the absence and presence of naloxone treatment, was used as a reference.

3.8. Carrageenan-Induced Edema in Rats

Anti-inflammatory activity was assessed on the basis of inhibition of paw edema induced by the injection of 0.1 mL of 2% carrageenan (an edematogenic agent) into the subplantar region of the right hind paw of the rat [43]. Male Wistar rats were divided into groups of six animals which received p.o. doses of extract (100, 200 and 400 mg/kg; 0.1 mL per 10 g body weight), saline or indomethacin (10 mg/kg) 1 h before the injection of carrageenan. In the left paw, used as a control, 0.1 mL of sterile saline was injected. 1, 2, 3 and 4 h after injection of carrageenan, the measure of edema was made by the difference between the volume displaced by the right paw and the left paw using a plethysmometer (model LE 7500, Letica Scientific Instruments, Barcelona, Spain).

3.9. Carrageenan-Induced Pleurisy in Rats

Pleurisy was induced in male Wistar rats by intrapleural administration of 0.5 mL 2% carrageenan suspension in saline solution between the third and fifth ribs on the right side of the mediastinum [37].

Extract (100, 200 and 400 mg/kg), saline or indomethacin (10 mg/kg) p.o. were given 60 min before injection of the irritant. Animals were killed 4 h after carrageenan injection, and the skin and pectoral muscles were retracted. A longitudinal incision was made between the third and fifth ribs on each side of the mediastinum. The exudate was collected and transferred to a 15 mL conical centrifuge tube and the total volume determined. A 50 μL aliquot of the exudate was used to determine the total leucocyte count in Neubauer chambers.

3.10. Calculations and Statistical Analysis

Data are expressed as mean ± s.e.m. Statistical significance was determined by one-way analysis of variance followed by the Student–Newman–Keuls test. *P* values below 0.05 were considered significant. The percentage of inhibition was calculated by using

$$100 - T \times 100/C(\%) \text{ or } T \times 100/C - 100(\%)$$

where C and T indicate non-treated (vehicle) and drug-treated, respectively.

4. Conclusions

The results obtained in this study confirm the ethnomedicinal use of the ethanol extract from *A. muricata* leaves. The data analysis supported the antinociceptive and anti-inflammatory activities, suggesting a potential for therapeutic purposes. However, further studies should be conducted to ensure its safe usage.

Acknowledgements

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References and Notes

1. Di Stasi, L.C.; Hiruma-Lima, C.A. *Plantas Medicinais na Amazônia e na Mata Atlântica*, 2nd ed.; Editora UNESP: São Paulo, Brazil, 2002; pp. 87-112.
2. Sousa, M.P.; Matos, M.E.O.; Matos, F.J.A.; Machados, M.I.L.; Craveiro, A.A. *Constituintes Químicos Ativos e Propriedades Biológicas de Plantas Medicinais Brasileiras*, 2nd ed.; Editora UFC: Fortaleza, Brazil, 2004; pp. 281-283.
3. Lorenzi, H.; Matos, F.J.A. *Plantas Medicinais No Brasil: Nativas e Exóticas*, 2nd ed.; Instituto Plantarum: Nova Odessa, Brazil, 2008; pp. 62-63.
4. Leboeuf, M.; Legueut, C.; Cavé, A.; Desconclois, J.F.; Forgacs, P.; Jacquemin, H. Alkaloids of Annonaceae. XXIX. Alkaloids of *Annona muricata*. *Planta Med.* **1981**, *42*, 37-44.
5. Leboeuf, M.; Cavé, A.; Bhaumik, P.K.; Mukherjee, B.; Mukherjee, R. The phytochemistry of the annonaceae. *Phytochemistry***1982**, *21*, 2783-2813.
6. Pélissler, Y.; Marion, C.; Kone, D.; Lamaty, G.; Menut, C.; Besslere, J.M. Volatile components of *Annona muricata* L. *J. Essent. Oil Res.* **1994**, *6*, 411-414.
7. Kossouoh, C.; Moudachirou, M.; Adjakidje, V.; Chalchat, J.C.; Figuérédo, G. Essential oil chemical composition of *Annona muricata* L. leaves from Benin. *J. Essent. Oil Res.* **2007**, *19*, 307-309.

8. Rupprecht, J.K.; Hui, Y.H.; McLaughlin, J.L. Annonaceous acetogenins: a review. *J. Nat. Prod.* **1990**, *53*, 237-278.
9. Cavé, A.; Figadère, B.; Laurens, A.; Cortes, D. Acetogenins from Annonaceae. *Fortschr. Chem. Org. Naturst.* **1997**, *70*, 281-288.
10. Wu, F.E.; Gu, Z.M.; Zeng, L.; Zhao, G.X.; Zhang, Y.; McLaughlin, J.L.; Sastrodihardjo, S. Two new cytotoxic monotetrahydrofuran Annonaceous acetogenins, annomuricins A and B, from the leaves of *Annona muricata*. *J. Nat. Prod.* **1995**, *58*, 830-836.
11. Wu, F.E.; Zeng, L.; Gu, Z.M.; Zhao, G.X.; Zhang, Y.; Schwedler, J.T.; McLaughlin, J.L.; Sastrodihardjo, S. Muricatocins A and B, two new bioactive monotetrahydrofuran Annonaceous acetogenins from the leaves of *Annona muricata*. *J. Nat. Prod.* **1995**, *58*, 902-908.
12. Wu, F.E.; Zeng, L.; Gu, Z.M.; Zhao, G.X.; Zhang, Y.; Schwedler, J.T.; McLaughlin, J.L.; Sastrodihardjo, S. New bioactive monotetrahydrofuran Annonaceous acetogenins, annomuricin C and muricatocin C, from the leaves of *Annona muricata*. *J. Nat. Prod.* **1995**, *58*, 909-915.
13. Wu, F.E.; Zhao, G.X.; Zeng, L.; Zhang, Y.; Schwedler, J.T.; McLaughlin; J.L.; Sastrodihardjo, S. Additional bioactive acetogenins, annomutacin and (2,4-trans and cis)-10R-annonacin-A-ones, from the leaves of *Annona muricata*. *J. Nat. Prod.* **1995**, *58*, 1430-1437.
14. Zeng, L.; Wu, F.E.; Oberlies, N.H.; McLaughlin, J.L.; Sastrodihadjo, S. Five new monotetrahydrofuran ring acetogenins from the leaves of *Annona muricata*. *J. Nat. Prod.* **1996**, *59*, 1035-1042.
15. Kim, G.S.; Zeng, L.; Alali, F.; Rogers, L.L.; Wu, F.E.; McLaughlin, J.L.; Sastrodihardjo, S. Two new mono-tetrahydrofuran ring acetogenins, annomuricin E and muricapentocin, from the leaves of *Annona muricata*. *J. Nat. Prod.* **1998**, *61*, 432-436.
16. Kim, G.S.; Zeng, L.; Alali, F.; Rogers, L.L.; Wu, F.E.; Sastrodihardjo, S.; McLaughlin, J.L. Muricoreacin and murihexocin C, mono-tetrahydrofuran acetogenins, from the leaves of *Annona muricata*. *Phytochem.* **1998**, *49*, 565-571.
17. Chang, F.R.; Liaw, C.C.; Lin, C.Y.; Chou, C.J.; Chiu, H.F.; Wu, Y.C. New adjacent Bis-tetrahydrofuran Annonaceous acetogenins from *Annona muricata*. *Planta Med.* **2003**, *69*, 241-246.
18. Luna, J.S.; Carvalho, J.M.; Lima, M.R.; Bieber, L.W.; Bento, E.S.; Franck, X.; Sant'ana, A.X. Acetogenins in *Annona muricata* L. (Annonaceae) leaves are potent molluscicides. *Nat. Prod. Res.* **2006**, *20*, 253-257.
19. Baskar, R.; Rajeswari, V.; Kumar, T.S. *In vitro* antioxidant studies in leaves of *Annona* species. *Indian J. Exp. Biol.* **2007**, *45*, 480-485.
20. Santos, A.F.; Sant'Ana, A.E.G. Molluscicidal properties of some species of *Annona*. *Phytomedicine* **2001**, *8*, 115-120.
21. Deraedt, R.; Jouquey, S.; Delevallée, F.; Flahaut, M. Release of prostaglandins E and F in an algogenic reaction and its inhibition. *Eur. J. Pharmacol.* **1980**, *51*, 17-24.
22. Hunskaar, S.; Hole, K. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. *Pain* **1987**, *30*, 103-114.
23. Shibata, M.; Ohkubo, T.; Takahashi, H.; Inoki, R. Modified formalin test; characteristic biphasic pain response. *Pain* **1989**, *38*, 347-352.

24. Yaksh, T.L.; Rudy, T.A. Studies on direct spinal action of narcotics in production of analgesia in rat. *J. Pharmacol. Exp. Ther*. **1977**, *202*, 411-428.
25. Sousa, O.V.; Del-Vechio-Vieira, G.; Amaral, M.P.H.; Pinho, J.J.R.G.; Yamamoto, C.H.; Alves, M.S. Efeitos antinociceptivo e antiinflamatório do extrato etanólico das folhas de *Duguetia lanceolata* St. Hil. (Annonaceae). *Lat. Am. J. Pharm.* **2008**, *27*, 398-402.
26. Sousa, O.V.; Del-Vechio-Vieira, G.; Kaplan, M.A.C. Propriedades analgésica e antiinflamatória do extrato metanólico de folhas de *Annona coriacea* Mart. (Annonaceae). *Lat. Am. J. Pharm.* **2007**, *26*, 872-877.
27. Di Rosa, M.; Giroud, J.P.; Willoughby, D.A. Studies on the mediators of the acute inflammatory response induced in rats in different sites by carrageenan and turpentine. *J. Pathol.* **1971**, *104*, 15–29.
28. Seibert, K., Zhang, Y.; Leahy, K.; Hauser, S.; Masferrer, J.; Perkins, W.; Lee, L.; Isakson, P. Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 12013-12017.
29. Nantel, F.; Denis, D.; Gordon, R.; Northey, A.; Cirino, M.; Metters, K.M.; Chan, C.C. Distribution and regulation of cyclooxygenase-2 in carrageenan-induced inflammation. *Br. J. Pharmacol.* **1999**, *128*, 853–859.
30. Stochla, K.; Maślinśki, S. Carrageenan-induced oedema in the rat paw-histamine participation. *Agents Actions* **1982**, *12*, 201–202.
31. Hwang, S.B.; Lam, M.H.; Li, C.L.; Shen, T.Y. Release of platelet activation factor and its involvement in the first phase of carrageenin-induced rat foot edema. *Eur. J. Pharmacol.* **1986**, *120*, 33–41.
32. De Campos, R.O.; Alves, R.V.; Kyle, D.J.; Chakravarty, S.; Mavunkel, B.J.; Calixto, J.B. Antioedematogenic and antinociceptive actions of NPC 18521, a novel bradykinin B_2 receptor antagonist. *Eur. J. Pharmacol.* **1996**, *316*, 277-286.
33. Gilligan, J.P.; Lovato, S.J.; Erion, M.D.; Jeng, A.Y. Modulation of carrageenan-induced hind paw edema by substance P. *Inflammation* **1994**, *18*, 285–292.
34. Ammendola, G.; Di Rosa, M.; Sorrentino, L. Leucocyte migration and lysosomal enzymes release in rat carrageenin pleurisy. *Agents Actions* **1975**, *5*, 250-255.
35. Almeida, A.P.; Bayer, B.M.; Horakova, Z.; Beaven, M.A. Influence of indomethacin and other anti-inflammatory drugs on mobilization and production of neutrophils: studies with carrageenan-induced inflammation in rats. *J. Pharmacol. Exp. Therap*. **1980**, *214*, 74-79.
36. Capasso, F.; Dunn, C.J.; Yamamoto, S.; Willoughby, D.A.; Giroud, J.P. Further studies on carrageenan-induced pleurisy in rats. *J. Pathol.* **1975**, *116*, 117-124.
37. Vinegar, R.; Truax, J.F.; Selph, J.L. Some quantitative temporal characteristics of carrageenin-induced pleurisy in the rat. *Proc. Soc. Exp. Biol. Med.* **1973**, *143*, 711-714.
38. Oyemitan, I.A.; Iwalewa, E.O.; Akanmu, M.A.; Olugbade, T.A. Antinociceptive and antiinflammatory effects of essential oil of *Dennettia tripetala* G. Baker (Annonaceae) in rodents. *Afr. J. Tradit. Complement. Altern. Med.* **2008**, *5*, 355-362.
39. Dietrich, L. A new approach to practical acute toxicity testing. *Arch. Toxicol.* **1983**, *54*, 275-287.
40. Litchfield, J.T.; Wilcoxon, F. A simplified method of evaluating dose-effect experiments. *J. Pharmacol. Exp. Therap.* **1949**, *96*, 99-113.

41. Collier, H.D.J.; Dinnin, L.C.; Johnson, C.A.; Schneider, C. The abdominal response and its suppression by analgesic drugs in the mouse. *Br. J. Pharmacol. Chemother.* **1968**, *32*, 295-310.
42. Eddy, N.B.; Leimbach, D. Synthetic analgesics. II. Dithienylbutenyl and dithienylbutilamines. *J. Pharmacol. Exp. Ther.* **1953**, *107*, 385-393.
43. Winter, C.A.; Risley, E.A.; Nuss, G.W. Carrageenin-induced edema in hind paw of the rat as an assay for anti-inflammatory drugs. *Proc. Soc. Exp. Biol. Med.* **1962**, *111*, 544-547.

Reference # 16

RAINTREE NUTRITION

TROPICAL PLANT DATABASE

Database File for:

GRAVIOLA

(Annona muricata)

PLANT IMAGES

Photos

Family: Annonaceae
Genus: *Annona*
Species: *muricata*
Synonyms: *Annona macrocarpa, A. bonplandiana, A. cearensis, Guanabanus muricatus*
Common names: Graviola, soursop, Brazilian paw paw, guanábana, guanábano, guanavana, guanaba, corossol épineux, huanaba, togebanreisi, durian benggala, nangka blanda, cachiman épineux
Part Used: Leaves, fruit, seeds, bark, roots

From *The Healing Power of Rainforest Herbs*:

GRAVIOLA

HERBAL PROPERTIES AND ACTIONS

Main Actions	Other Actions	Standard Dosage
• kills cancer cells	• relieves depression	Leaves
• slows tumor growth	• reduces spasms	**Infusion:** 1 cup 3 times daily
• kills bacteria	• kills viruses	**Tincture:** 2-4 ml 3 times daily
• kills parasites	• reduces fever	**Capsules:** 2 g 3 times daily
• reduces blood pressure	• expels worms	
• lowers heart rate	• stimulates digestion	
• dilates blood vessels	• stops convulsions	
• sedates		

TECHNICAL PLANT DATA GRAVIOLA

WEB RESOURCES GRAVIOLA

graviola
Product Search

- Website Search

Graviola is a small, upright evergreen tree, 5–6 m high, with large, glossy, dark green leaves. It produces a large, heart-shaped, edible fruit that is 15–20 cm in diameter, is yellow-green in color, and has white flesh inside. Graviola is indigenous to most of the warmest tropical areas in South and North America, including the Amazon. The fruit is sold in local markets in the tropics, where it is called *guanábana* in Spanish-speaking countries and *graviola* in Brazil. The fruit pulp is excellent for making drinks and sherbets and, though slightly sour-acid, can be eaten out of hand.

Tribal & Herbal Medicine Uses

All parts of the graviola tree are used in natural medicine in the tropics, including the bark, leaves, roots, fruit, and fruit seeds. Different

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properties and uses are attributed to the different parts of the tree. Generally, the fruit and fruit juice are taken for worms and parasites, to cool fevers, to increase mother's milk after childbirth, and as an astringent for diarrhea and dysentery. The crushed seeds are used against internal and external parasites, head lice, and worms. The bark, leaves, and roots are considered sedative, antispasmodic, hypotensive, and nervine, and a tea is made for various disorders toward those effects.

Graviola has a long, rich history of use in herbal medicine as well as a lengthy recorded indigenous use. In the Peruvian Andes, a leaf tea is used for catarrh (inflammation of mucous membranes) and the crushed seed is used to kill parasites. In the Peruvian Amazon the bark, roots, and leaves are used for diabetes and as a sedative and antispasmodic. Indigenous tribes in Guyana use a leaf and/or bark tea as a sedative and heart tonic. In the Brazilian Amazon a leaf tea is used for liver problems, and the oil of the leaves and unripe fruit is mixed with olive oil and used externally for neuralgia, rheumatism, and arthritis pain. In Jamaica, Haiti, and the West Indies the fruit and/or fruit juice is used for fevers, parasites and diarrhea; the bark or leaf is used as an antispasmodic, sedative, and nervine for heart conditions, coughs, flu, difficult childbirth, asthma, hypertension, and parasites.

Plant Chemicals

Many active compounds and chemicals have been found in graviola, as scientists have been studying its properties since the 1940s. Most of the research on graviola focuses on a novel set of chemicals called *Annonaceous acetogenins*. Graviola produces these natural compounds in its leaf and stem, bark, and fruit seeds. Three separate research groups have confirmed that these chemicals have significant antitumorous properties and selective toxicity against various types of cancer cells (without harming healthy cells) publishing eight clinical studies on their findings. Many of the acetogenins have demonstrated selective toxicity to tumor cells at very low dosages—as little as 1 part per million. Four studies were published in 1998 which further specify the chemicals and acetogenins in graviola which are demonstrating the strongest anticancerous, antitumorous, and antiviral properties. In a 1997 clinical study, novel alkaloids found in graviola fruit exhibited antidepressive effects in animals.

Annonaceous acetogenins are only found in the Annonaceae family (to which graviola belongs). These chemicals in general have been documented with antitumorous, antiparasitic, insecticidal, and antimicrobial activities. Mode of action studies in three separate laboratories have recently determined that these acetogenins are superb inhibitors of enzyme processes that are only found in the membranes of cancerous tumor cells. This is why they are toxic to cancer cells but have no toxicity to healthy cells. Purdue University, in West Lafayette, Indiana, has conducted a great deal of the research on the acetogenins, much of which, has been funded by The National Cancer Institute and/or the National Institute of Health (NIH). Thus far, Purdue University and/or its staff have filed at least nine U.S. and/or

international patents on their work around the antitumorous and insecticidal properties and uses of these acetogenins.

In 1997, Purdue University published information with promising news that several of the Annonaceous acetogenins were " . . . not only are effective in killing tumors that have proven resistant to anti-cancer agents, but also seem to have a special affinity for such resistant cells." In several interviews after this information was publicized, the head pharmacologist in Purdue's research explained how this worked. As he explains it, cancer cells that survive chemotherapy can develop resistance to the agent originally used as well as to other, even unrelated, drugs. This phenomenon is called *multi-drug resistance* (MDR). One of the main ways that cancer cells develop resistance to chemotherapy drugs is by creating an intercellular pump which is capable of pushing anticancer agents out of the cell before they can kill it. On average, only about two percent of the cancer cells in any given person might develop this pump—but they are the two percent that can eventually grow and expand to create multi-drug-resistant tumors. Some of the latest research on acetogenins reported that they were capable of shutting down these intercellular pumps, thereby killing multi-drug-resistant tumors. Purdue researchers reported that the acetogenins preferentially killed multi-drug-resistant cancer cells by blocking the transfer of ATP—the chief source of cellular energy—into them. A tumor cell needs energy to grow and reproduce, and a great deal more to run its pump and expel attacking agents. By inhibiting energy to the cell , it can no longer run its pump. When acetogenins block ATP to the tumor cell over time, the cell no longer has enough energy to operate sustaining processes—and it dies. Normal cells seldom develop such a pump; therefore, they don't require large amounts of energy to run a pump and, generally, are not adversely affected by ATP inhibitors. Purdue researchers reported that 14 different acetogenins tested thus far demonstrate potent ATP-blocking properties (including several found only in graviola). They also reported that 13 of these 14 acetogenins tested were more potent against MDR breast cancer cells than all three of the standard drugs (adriamycin, vincristine, and vinblastine) they used as controls.

The Annonaceous acetogenins discovered in graviola thus far include: annocatalin, annohexocin, annomonicin, annomontacin, annomuricatin A & B, annomuricin A thru E, annomutacin, annonacin, annonacinone, annopentocin A thru C, cis-annonacin, cis-corossolone, coribin A thru D, corepoxylone, coronin, corossolin, corossolone, donhexocin, epomuricenin A & B, gigantetrocin, gigantetrocin A & B, gigantetrocinone, gigantetronenin, goniothalamicin, iso-annonacin, javoricin, montanacin, montecristin, muracin A thru G, muricapentocin, muricatalicin, muricatalin, muri-catenol, muricatetrocin A & B muricatin D, muricatocin A thru C muricin H, muricin I, muricoreacin, murihexocin 3, murihexocin A thru C, murihexol, murisolin, robustocin, rolliniastatin 1 & 2, saba-delin, solamin, uvariamicin I & IV, xylomaticin

Biological Activites and Clinical Research

In an 1976 plant screening program by the National Cancer Institute,

graviola leaves and stem showed active toxicity against cancer cells and researchers have been following up on these findings since. Thus far, specific acetogenins in graviola and/or extracts of graviola have been reported to be selectively toxic *in vitro* to these types of tumor cells: lung carcinoma cell lines; human breast solid tumor lines; prostate adenocarcinoma; pancreatic carcinoma cell lines; colon adenocarcinoma cell lines; liver cancer cell lines; human lymphoma cell lines; and multi-drug resistant human breast adenocarcinoma. Researchers in Taiwan reported in 2003 that the main graviola acetogenin, *annonacin*, was highly toxic to ovarian, cervical, breast, bladder and skin cancer cell lines at very low dosages saying; ". . . annonacin is a promising anti-cancer agent and worthy of further animal studies and, we would hope, clinical trials."

An interesting *in vivo* study was published in March of 2002 by researchers in Japan, who were studying various acetogenins found in several species of plants. They inoculated mice with lung cancer cells. One third received nothing (the control group), one third received the chemotherapy drug adriamycin, and one third received the main graviola acetogenin, annonacin (at a dosage of 10 mg/kg). At the end of two weeks, five of the six in the untreated control group were still alive and lung tumor sizes were then measured. The adriamycin group showed a 54.6% reduction of tumor mass over the control group—but 50% of the animals had died from toxicity (three of six). The mice receiving annonacin were all still alive, and the tumors were inhibited by 57.9%—slightly better than adriamycin—and without toxicity. This led the researchers to summarize; "This suggested that annonacin was less toxic in mice. On considering the antitumor activity and toxicity, annonacin might be used as a lead to develop a potential anticancer agent."

Current Practical Uses

Cancer research is ongoing on these important *Annona* plants and plant chemicals, as several pharmaceutical companies and universities continue to research, test, patent, and attempt to synthesize these chemicals into new chemotherapeutic drugs. In fact, graviola seems to be following the same path as another well known cancer drug – Taxol. From the time researchers first discovered an antitumorous effect in the bark of the pacific yew tree and a novel chemical called taxol was discovered in its bark - it took thirty years of research by numerous pharmaceutical companies, universities, and government agencies before the first FDA-approved Taxol drug was sold to a cancer patient (which was based on the natural taxol chemical they found in the tree bark). With graviola, it has taken researchers almost 10 years to successfully synthesize (chemically reproduce) the main antitumorous chemical, annonacin. These acetogenin chemicals have a unique waxy center and other unique molecular energy properties which thwarted earlier attempts, and at least one major pharmaceutical company gave up in the process (despite knowing how active the natural chemical was against tumors). Now that scientists have the ability to recreate this chemical and several other active acetogenins in the laboratory, the next step is to

change the chemical just enough (without losing any of the antitumorous actions in the process) to become a novel chemical which can be patented and turned into a new patented cancer drug. (Naturally-occurring plant chemicals cannot be patented.) Thus far, scientists seem to be thwarted again—every time they change the chemical enough to be patentable, they lose much of the antitumorous actions. Like the development of taxol, it may well take government agenies like the National Cancer Institute and the National Institute of Health to step forward and launch full-scale human cancer research on the synthesized unpatentable natural plant chemical (which will allow any pharmaceutical company to develop a cancer drug utilizing the research as happened with taxol) to be able to make this promising therapy available to cancer patients in a timely fashion.

In the meantime, many cancer patients and health practitioners are not waiting... they are adding the natural leaf and stem of graviola (with over 40 documented naturally-occurring acetogenins including annonacin) as a complementary therapy to their cancer protocols. After all, graviola has a long history of safe use as a herbal remedy for other conditions for many years, and research indicates that the antitumorous acetogenins are selectively toxic to just cancer cells and not healthy cells—and in miniscule amounts. While research confirms that these antitumorous acetogenins also occur in high amounts in the fruit seeds and roots of graviola, different alkaloid chemicals in the seeds and roots have shown some preliminary *in vitro* neurotoxic effects. Researchers have suggested that these alkaloids might be linked to atypical Parkinson's disease in countries where the seeds are employed as a common herbal parasite remedy. Therefore, using the seeds and root of graviola is not recommended at this time.

The therapuetic dosage of graviola leaf, (which offers just as high of an amount of acetogenins as the root and almost as much as the seed) is reported to be 2-3 grams taken 3 or 4 times daily. Graviola products (capsules and tinctures) are becoming more widely available in the U.S. market, and now offered under several different manufacturer's labels in health food stores. As one of graviola's mechanisms of action is to deplete ATP energy to cancer cells, combining it with other supplements and natural products which increase or enhance cellular ATP may reduce the effect of graviola. The main supplement which increases ATP is a common antioxidant called Coenzyme Q10 and for this reason, it should be avoided when taking graviola.

Graviola is certainly a promising natural remedy and one that again emphasizes the importance of preserving our remaining rainforest ecosystems. Perhaps—if enough people believe that the possible cure for cancer truly is locked away in a rainforest plant—we will take the steps needed to protect our remaining rainforests from destruction. One researcher studying graviola summarized this idea eloquently: "At the time of preparation of this current review, over 350 Annonaceous acetogenins have been isolated from 37 species. Our preliminary efforts show that about 50%, of over 80 Annonaceous species screened, are significantly bioactive and are worthy of fractionation;

thus, this class of compounds can be expected to continue to grow at an exponential rate in the future, provided that financial support for such research efforts can be found. With the demise of the world's tropical rain forests, such work is compelling before the great chemical diversity, contained within these endangered species, is lost '

GRAVIOLA PLANT SUMMARY

Main Actions (in order):
anticancerous, antitumorous, antimicrobial, antiparasitic, hypotensive (lowers blood pressure)

Main Uses:

1. for cancer (all types)
2. as a broad-spectrum internal and external antimicrobial to treat bacterial and fungal infections
3. for internal parasites and worms
4. for high blood pressure
5. for depression, stress, and nervous disorders

Properties/Actions Documented by Research:
antibacterial, anticancerous, anticonvulsant, antidepressant, antifungal, antimalarial, antimutagenic (cellular protector), antiparasitic, antispasmodic, antitumorous, cardiodepressant, emetic (causes vomiting), hypotensive (lowers blood pressure), insecticidal, sedative, uterine stimulant, vasodilator

Other Properties/Actions Documented by Traditional Use:
antiviral, cardiotonic (tones, balances, strengthens the heart), decongestant, digestive stimulant, febrifuge (reduces fever), nervine (balances/calms nerves), pediculicide (kills lice), vermifuge (expels worms)

Cautions: It has cardiodepressant, vasodilator, and hypotensive (lowers blood pressure) actions. Large dosages can cause nausea and vomiting. Avoid combining with ATP-enhancers like CoQ10.

Traditional Preparation: The therapeutic dosage is reported to be 2 g three times daily in capsules or tablets. A standard infusion (one cup 3 times daily) or a 4:1 standard tincture (2–4 ml three times daily) can be substituted if desired. See Traditional Herbal Remedies Preparation Methods page if necessary for definitions.

Contraindications:

- Graviola has demonstrated uterine stimulant activity in an animal study (rats) and should therefore not be used during pregnancy.
- Graviola has demonstrated hypotensive, vasodilator, and cardiodepressant activities in animal studies and is contraindicated for people with low blood pressure. People taking antihypertensive drugs should check with their doctors before taking graviola and monitor their blood pressure accordingly (as medications may need adjusting).
- Graviola has demonstrated significant *in vitro* antimicrobial

properties. Chronic, long-term use of this plant may lead to die-off of friendly bacteria in the digestive tract due to its antimicrobial properties. Supplementing the diet with probiotics and digestive enzymes is advisable if this plant is used for longer than 30 days.

- Graviola has demonstrated emetic properties in one animal study with pigs. Large single dosages may cause nausea or vomiting. Reduce the usage accordingly if this occurs.
- One study with rats given a stem-bark extract intragastrically (at 100 mg/kg) reported an increase in dopamine, norepinephrine, and monomine oxidase activity, as well as a inhibition of serotonin release in stress-induced rats.
- Alcohol extracts of graviola leaf showed no toxicity or side effects in mice at 100 mg/kg; however, at a dosage of 300 mg/kg, a reduction in explorative behavior and mild abdominal constrictions was observed. If sedation or sleepiness occurs, reduce the amount used.

Drug Interactions: None have been reported; however, graviola may potentiate antihypertensive and cardiac depressant drugs. It may potentiate antidepressant drugs and interfere with MAO-inhibitor drugs. See contraindications above.

WORLDWIDE ETHNOMEDICAL USES	
Brazil	for abscesses, bronchitis, chest problems, cough, diabetes, diarrhea, dysentery, edema, fever, intestinal colic, intestinal parasites, liver problems, neuralgia, nervousness, pain, parasites, rheumatism, spasms, worms
Caribbean	for chills, fever, flu, indigestion, nervousness, palpitations, rash, spasms, skin disease, and as a sedative
Curaçao	for childbirth, gallbladder problems, nervousness, and as a sedative and tranquilizer
Haiti	for digestive sluggishness, coughs, diarrhea, fever, flu, heart conditions, lactation aid, lice, nerves, parasites, pain, pellagra, sores, spasms, weakness, wounds, and as a sedative
Jamaica	for asthma, fevers, heart conditions, hypertension, lactation aid, nervousness, parasites, spasms, water retention weakness, worms, and as a sedative
Malaysia	for boils, coughs, diarrhea, dermatosis, hypertension, rheumatism, and to reduce bleeding
Mexico	for diarrhea, dysentery, fever, chest colds, ringworm, scurvy, and to reduce bleeding
Panama	for diarrhea, dyspepsia, kidney, stomach ulcers, worms
Peru	for diabetes, diarrhea, dysentery, fever, hypertension indigestion, inflammation, lice, liver disorders, parasites, spasms, tumors, ulcers (internal), and as a sedative
	for blood cleansing, fainting, flu, high blood pressure

Trinidad	insomnia, lactation aid, palpitations, ringworms
U.S.A.	for cancer, depression, fungal infections, hypertension, intestinal parasites, tumors
West Indies	for asthma, childbirth, diarrhea, hypertension, lactation aid, parasites, worms
Elsewhere	for arthritis, asthma, bile insufficiency, childbirth, cancer, diarrhea, dysentery, fever, heart problems, kidney problems, lactation aid, lice, liver disorders, malaria, pain, ringworm, scurvy, stomach problems, and as a sedative

† The statements contained herein have not been evaluated by the Food and Drug Administration. The information contained in this plant database file is intended for education, entertainment and information purposes only. This information is not intended to be used to diagnose, prescribe or replace proper medical care. The plant described herein is not intended to treat, cure, diagnose, mitigate or prevent any disease. Please refer to our Conditions of Use for using this plant database file and web site.

Third-Party Published Research on Graviola

All available third-party documentation and research on graviola be found at **PubMed**. A partial listing of the third-party published research on graviola is shown below:

Anticancerous & Antitumor Actions:

Kojima, N. "Systematic synthesis of antitumor Annonaceous acetogenins." *Yakugaku Zasshi.* 2004; 124(10): 673-81.
Tormo, J. R., et al. "*In vitro* antitumor structure-activity relationships of threo/trans/threo mono-tetrahydro-furanic acetogenins: Correlations with their inhibition of mitochondrial complex I." *Oncol. Res.* 2003; 14(3): 147-54.
Yuan, S. S., et al. "Annonacin, a mono-tetrahydrofuran acetogenin, arrests cancer cells at the G1 phase and causes cytotoxicity in a Bax- and caspase-3-related pathway." *Life Sci.* 2003 May: 72(25): 2853-61.
Liaw, C. C., et al. "New cytotoxic monotetrahydrofuran Annonaceous acetogenins from *Annona muricata*." *J. Nat. Prod.* 2002; 65(4): 470-75
Gonzalez-Coloma, A., et al. "Selective action of acetogenin mitochondrial complex I inhibitors." *Z. Naturforsch.* 2002; 57(11-12): 1028-34.
Chang, F. R., et al. "Novel cytotoxic Annonaceous acetogenins from *Annona muricata*." *J. Nat. Prod.* 2001; 64(7): 925-31.
Jaramillo, M. C., et al. "Cytotoxicity and antileishmanial activity of *Annona muricata* pericarp." *Fitoterapia.* 2000; 71 (2): 183-6.
Betancur-Galvis, L., et al. "Antitumor and antiviral activity of Colombian medicinal plant extracts." *Mem. Inst. Oswaldo Cruz.* 1999; 94(4): 531-35.
Kim, G. S., et al. "Muricoreacin and murihexocin C, mono-tetrahydrofuran

acetogenins, from the leaves of *Annona muricata*." *Phytochemistry*. 1998; 49(2): 565-71.
Kim, G. S., et al. "Two new mono-tetrahydrofuran ring acetogenins, annomuricin E and muricapentocin, from the leaves of *Annona muricata*." *J. Nat. Prod.* 1998; 61(4): 432-36.
Nicolas, H., et al. "Structure-activity relationships of diverse Annonaceous acetogenins against multidrug-resistant human mammary adenocarcinoma (MCF-7/Adr) cells." *J. Med. Chem.* 1997; 40(13): 2102-6.
Zeng, L., et al. "Five new monotetrahydrofuran ring acetogenins from the leaves of *Annona muricata*." *J. Nat. Prod.* 1996; 59(11): 1035-42.
Wu, F. E., et al. "Two new cytotoxic monotetrahydrofuran Annonaceous acetogenins, annomuricins A and B, from the leaves of *Annona muricata*." *J. Nat. Prod.* 1995; 58(6): 830-36.
Oberlies, N. H., et al. "Tumor cell growth inhibition by several Annonaceous acetogenins in an *in vitro* disk diffusion assay." *Cancer Lett.* 1995; 96(1): 55-62.
Wu, F. E., et al. "Additional bioactive acetogenins, annomutacin and (2,4-trans and cis)-10R-annonacin-A-ones, from the leaves of *Annona muricata*." *J. Nat. Prod.* 1995; 58(9): 1430-37.
Wu, F. E., et al. "New bioactive monotetrahydrofuran Annonaceous acetogenins, annomuricin C and muricatocin C, from the leaves of *Annona muricata*." *J. Nat. Prod.* 1995; 58(6): 909-5.
Wu, F. E., et al. "Muricatocins A and B, two new bioactive monotetrahydrofuran Annonaceous acetogenins from the leaves of *Annona muricata*." *J. Nat. Prod.* 1995; 58(6): 902-8.
Sundarrao, K., et al. "Preliminary screening of antibacterial and antitumor activities of Papua New Guinean native medicinal plants." *Int. J. Pharmacog.* 1993; 31(1): 3-6.

Antimicrobial Actions:

Takahashi, J.A., et al. "Antibacterial activity of eight Brazilian Annonaceae plants." *Nat. Prod. Res.* 2006; 20(1): 21-6.
Betancur-Galvis, L., et al. "Antitumor and antiviral activity of Colombian medicinal plant extracts." *Mem. Inst. Oswaldo Cruz* 1999; 94(4): 531-35.
Antoun, M. D., et al. "Evaluation of the flora of Puerto Rico for *in vitro* cytotoxic and anti-HIV activities." *Pharmaceutical Biol.* 1999; 37(4): 277-280.
Padma, P., et al. "Effect of the extract of *Annona muricata* and *Petunia nyctaginiflora* on Herpes simplex virus." *J. Ethnopharmacol.* 1998; 61(1): 81–3.
Sundarrao, K., et al. "Preliminary screening of antibacterial and antitumor activities of Papua New Guinean native medicinal plants." *Int. J. Pharmacog. 1993* 31(1): 3–6.
Misas, C. A. J., et al. "Contribution to the biological evaluation of Cuban plants. IV." *Rev. Cubana Med. Trop.* 1979; 31(1): 29–35.

Antidepressant & Antistress Actions:

Padma, P., et al. "Effect of *Annona muricata* and *Polyalthia cerasoides* on brain neurotransmitters and enzyme monoamine oxidase following cold immobilization stress." *J. Natural Remedies* 2001; 1(2): 144–46.
Hasrat, J. A., et al. "Screening of medicinal plants from Suriname for 5-HT 1A ligands: Bioactive isoquinoline alkaloids from the fruit of Annona muricata." *Phytomedicine*. 1997; 4(20: 133-140.
Padma, P., et al. "Effect of alcohol extract of *Annona muricata on* cold immobilization stress induced tissue lipid peroxidation." *Phytother. Res.* 1997; 11(4): 320-327.
Hasrat, J. A., et al. "Isoquinoline derivatives isolated from the fruit of *Annona muricata* as 5-HTergic 5-HT1A receptor agonists in rats: unexploited antidepressive (lead) products." *J. Pharm. Pharmacol.* 1997; 49(11): 1145–49.

Antiparasitic, Antimalarial, & Insecticidal Actions:

Luna, J. S., et al. "Acetogenins in *Annona muricata* L. (Annonaceae) leaves are potent molluscicides." *Nat. Prod. Res.* 2006; 20(3): 253-7.
Jaramillo, M. C., et al. "Cytotoxicity and antileishmanial activity of *Annona muricata* pericarp." *Fitoterapia*. 2000; 71(2): 183–6.
Alali, F. Q., et al. "Annonaceous acetogenins as natural pesticides; potent toxicity against insecticide-susceptible and resistant German cockroaches (*Dictyoptera: Blattellidae*)." *J. Econ. Entomol.* 1998; 91(3): 641-9.

Antoun, M. D., et al. "Screening of the flora of Puerto Rico for potential antimalarial bioactives." *Int. J. Pharmacog.* 1993; 31(4): 255–58.
Heinrich, M., et al. "Parasitological and microbiological evaluation of Mixe Indian medicinal plants (Mexico)." *J. Ethnopharmacol.* 1992; 36(1): 81–5.
Bories, C., et al. "Antiparasitic activity of *Annona muricata* and *Annona cherimolia* seeds." *Planta Med.* 1991; 57(5): 434–36.
Gbeassor, M., et al. "*In vitro* antimalarial activity of six medicinal plants." *Phytother. Res.* 1990; 4(3): 115–17.
Tattersfield, F., et al. "The insecticidal properties of certain species of *Annona* and an Indian strain of *Mundulea sericea* (SuplI)." *Ann. Appl. Biol.* 1940; 27: 262–73.

Anticonvulsant, Antispasmodic, & Smooth Muscle Relaxant Actions:
N'gouemo, P., et al. "Effects of ethanol extract of *Annona muricata* on pentylenetetrazol-induced convulsive seizures in mice." *Phytother. Res.* 997; 11(3): 243–45.
Feng, P. C., et al. "Pharmacological screening of some West Indian medicinal plants." *J. Pharm. Pharmacol.* 1962; 14: 556–61.

Hypotensive & Cardiodepressant Actions
Carbajal, D., et al. "Pharmacological screening of plant decoctions commonly used in Cuban folk medicine." *J. Ethnopharmacol.* 1991; 33(1/2): 21–4.
Feng, P. C., et al. "Pharmacological screening of some West Indian medicinal plants." *J. Pharm. Pharmacol.* 1962; 14: 556–61.
Meyer, T. M. "The alkaloids of *Annona muricata*." *Ing. Ned. Indie.* 1941; 8 (6): 64.

Reference # 17

Quality of life following the use of A4 plus in a limited number of subjects with chronic hepatitis C: Open-label, nonplacebo-controlled, preliminary observations

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I. INTRODUCTION:

The following is an analysis of data provided by Organetix Inc on the use of A4+ (herbal product) in patients with chronic hepatitis C.

In symptomatic patients with chronic hepatitis C, fatigue[1, 2] , depression[2, 3, 4] and impairment of memory ("brain fog") and cognitive function[5] are common. Other symptoms include right upper abdominal discomfort and tenderness, dyspepsia, nausea, indigestion, and muscle/joint pain. There is growing evidence that patients with hepatitis C have a significant impairment of health-related quality of life.[6,7,8] The mechanism of impaired health-related quality of life (HRQL) in these patients is not known and studies have failed to establish a correlation between HRQL and parameters of liver disease and its severity.[6,7, 9,10]

The present study assessed the effect of A4+ on the symptoms of hepatitis C and on the health-related quality of life.

II. OBJECTIVE:

The primary objective of this pilot study was to examine whether A4+ might have a beneficial effect in patients with chronic hepatitis C. At this preliminary stage of testing, finding conclusive evidence to definitively show that A4+ caused the observed changes was not within the scope of the study design and was not the stated goal.

III. STUDY DESIGN and METHODS:

Study Design: The study was an open-label, non-randomized, non-placebo controlled preliminary assessment of the effects of oral ingestion of A4+ on symptoms of chronic hepatitis C. Clinical parameters noted below were evaluated a day prior to administration and at 28 days after the use of A4+. The impact of use of A4+ was assessed by comparing the values of these parameters at the start of the study against their values at day 28 of the study.

Number of study subjects: Ten subjects with chronic hepatitis C who were currently not on anti viral treatment participated in the study.

Study material: The study material is an herbal product called A4+ whose chemical composition is unidentified as of now. It is an alcohol extract of a mixture of herbs that has been previously evaluated by Organetix Inc. for safety and toxicity and has been found to be well tolerated.

The parameters studied:

1. Depression.
2. Health-related quality of life.
3. Clinical Symptoms: fatigue; dyspepsia; nausea and vomiting; indigestion; right upper abdominal pain and tenderness; headache; muscle and joint/bone pain.
4. Nutritional parameters: To eliminate the effect of nutritional status as a possible cause of the changes in the symptoms and quality of life following the use of A4+ nutritional parameters were monitored before and after the use of A4+. The nutritional parameters studied included anthropometric measurements (skin fold measurements) and measurements of serum transferrin and pre-albumin.
5. Ancillary observations: routine liver chemistry: Serum albumin, ALT, AST, Alkaline Phosphatase, Prothrombin activity, Cholinesterase, TNF- alfa, Liver necro-inflammatory activity and liver fibrosis index score using Fibrosure test (calculated utilizing serum Haptoglobin, apolipo-protein-A-1, alfa2 macroglobin, bilirubin, GGTP and ALT) and liver size was measured by ultrasound.

Depression (Beck Depression Scale[11]), health-related quality of life[9] (Hepatitis C specific SF-36 HQLQ-v2), fatigue (Fatigue Severity Scale[12, 13]), dyspepsia, nausea and vomiting and indigestion, (modified Gastrointestinal Symptom Rating Scale-GSRS[14]) were assessed using standard questionnaires.

Standard well established anthropometric parameters, functional testing of grip strength using hand dynamometer and seum transferrin and prealbumin were measured to assess the nutritional status [15, 16] before and after 28 days of the use of A4+.

Right upper abdominal pain, headache, muscle and joint/bone pain were assessed using a ten point scale ranging from 0 (none) to 10 (severe) in escalating grades. Using this scale, patients were categorized as having mild (1-4), moderate (5-8) and severe (9-10) pain.

IV. RESULTS:

1. DEPRESSION:

Beck Depression Inventory (BDI) is a well established, validated and reproducible tool for measuring depression.[11] It is a 21-item questionnaire that measures the severity of various manifestations of depression. Each of the 21 items is rated on a scale of 0-3, giving an additive total score of 0-63. Higher scores are indicative of greater degree of severity of depression. A score of less than 9 is considered normal, 10-16 is borderline depression, 17-20 is mild depression, 21 -30 is moderate depression and above 30 is severe depression. Scores in the range of 0-3 are very unusual in population norms.

Before the use of A4+: Borderline depression was seen in 10%, mild depression in 60%, moderate depression in 20% and severe depression in 10% of the subjects. The mean BDI score was 21 and indicates moderate depression (Figure 1).

After 28 days of the use of A4+: Most of the subjects (90%) became free of depression and one (10%) exhibited mild depression (Figure 1). It is important to note that at the end of the study period 60% of the study subjects had a total Beck Inventory score of 2 or less including 0-1 in 40%.

Assessment of depression using SF-36 mental health scale revealed similar response at the end of the study. Ninety percent of the study subjects screened positive for depression at the start of the study and by 28 days none of the subjects screened positive for depression.

To further explore the effect of A4+ on the somatic and functional elements of the Beck Depression Inventory, following were analyzed (Figure 2):

Loss of energy: *At the start of the study*, thirty percent of the study subjects reported that they "have to push [themselves] very hard to do something," (moderate loss of energy), while 70% stated that it "takes an extra effort to get started at doing something," (mild loss of energy). *After use of A4+*, 90% reported that they could "work as well as before" the illness (No loss of energy); only 10% stated they had to push themselves very hard (Moderate loss of energy).

Irritability: *At the start of the study*, 10% reported they "feel irritated all the time," (moderate irritability) and 90% stated they get "annoyed or irritated more easily than

before" (mild irritability). *After use of A4+*, 90% stated were "no more irritated than ever before" (no irritability), while 10% stated they got "irritated more easily than before" (mild irritability).

Indecisiveness: *At the start of the study*, 60% stated they "put off making decisions more than" before the illness (mild indecisiveness), 20% reported they "have greater difficulty in making decisions" than before (moderate), and 20% reported they made decisions "about as well as ever" (No indecisiveness). *After use of A4+*, 90% stated they "made decisions as well as ever" (no indecisiveness), and 10% stated they "put off making decisions more than they did before" the illness (mild indecisiveness).

Worry about health status: *At the start of the study*, 40% reported they were "very worried about physical problems and [that it was] hard to think of much else" (moderate health worry), 20% stated they were "so worried about ... physical problems that [they could] not think about anything else" (severe health worry), and 30% stated they were "worried about physical problems such as aches and pains; or upset stomach; or constipation" (Mild health worry). Ten percent reported being "no more worried about [their] health than usual."(no health worry). *After use of A4+*, 90% stated they were no more concerned about their health status than before the illness (no health worry), and only 10% indicated they were so worried that they could not think of much else (moderate health worry).

2. Health-Related Quality of Life Measure:

The data analysis of this parameter was done by QualityMetric, Inc., and the following are excerpts from their report, which is attached (Attachment 1). Figures and tables cited in this section are located in the report.

Before the use of A4+ the health-related quality of life burden:

Figure 1 in the QualityMetric report presents the mean day 1 SF-36 scale and summary measure scores of the 10 study patients in comparison to the general population norms (scores of 50 on all scales and summary measures) and in comparison to patients with CHF and clinical depression.

Mean SF-36 scale and summary measure scores of the 10 study patients were 1.5 to 2.5 standard deviations below general population norms, indicative of significant health-related quality of life burden.

Comparison of scores with CHF and clinical depression norms suggest that the health-related quality of life burden associated with chronic hepatitis C should be considered clinically significant. *With few exceptions, SF-36 scale and summary measure scores of the 10 study patients were below the norms for CHF and clinical depression. In addition, SF-36 scale and summary measure scores of the 10 study patients were also below the norms for hypertension, MI, and diabetes* (see Table 2).

In comparison to other patients with chronic hepatitis C, the 10 patients in this study showed greater than average HRQoL burden. Table 1 presents the mean day 1 SF-36 scale and summary measure scores of the 10 study patients against baseline SF-36 scores observed in 8 published studies involving patients with chronic hepatitis C patients. *Overall, the 10 patients in this study scored lower on all SF-36 scales and summary measures compared with baseline scores observed across the 8 published studies.* [4,5,6,7,8,9,10,11] In two of the published studies (Study 5 and Study 8), the average HRQoL burden observed of chronic hepatitis patients approached the level observed with the 10 patients in this study. *However, the HRQoL burden observed in these two studies were compounded by other clinical factors.* For example, in Study 5 the *chronic hepatitis C patients suffered from significant emotional problems*, which had a significant impact on those SF-36 scales measuring emotional well-being (Social Functioning, Role Emotional, Mental Health, and Mental Summary scales). *In study 8, the 15 chronic hepatitis patients had hepatocellular carcinoma*, which appeared to have a significant impact across all SF-36 scales. In a separate attachment, copies of each of these 8 studies have been provided to assist in determining the equivalence in clinical characteristics between the 10 patients enrolled in this study and the patients participating in the 8 published studies.

Table 4 of the QualityMetric report shows that at day 1: 1) 100% of study patients reported limitations in walking more than one mile compared to only 21.9% in the general population; 2) 50% of study patients reported limitations in climbing 1 flight of stairs compared to only 7.1% in the general population; 3) 55.5% of study patients reported limitations in walking 100 yards compared to 14.1% in the general population; 4) 100% of study patients reported

having difficulty performing at work compared to only 45% in the general population; 5) 30% of study patients reported that pain interfered with their ability to work compared to 9.5% in the general population; 6) 90% of study patients reported "fair" or "poor" health compared to 13.1% in the general population; 7) 0% of study patients reported "excellent" health compared to 15.6% in the general population; 8) 90% of study patients reported feeling tired all or most of the time compared to 13.4% in the general population; 9) 70% of study patients reported that their health interfered with usual social activities compared to 7.3% in the general population; 10) 100% of study patients reported that they accomplished less at work due to emotional problems compared to 37.4% in the general population; 11) 30% of study patients reported being downhearted and depressed all or most of the time compared to 3.1% in the general population; 12) 70% of study patients reported that hepatitis limited their ability to perform daily work all or most of the time; and 13) 60% of study patients reported that hepatitis limited their ability to engage in usual social activities. Lastly, as shown in Figure 2, 90% of study subjects screened positive for depression at day 1.

Impact of Treatment after 28 Days of Use of A4+ on Health-Related Quality of Life

Figure 3 plots the mean SF-36 scale and summary measure scores at day 1, day 14 and day 28 for the 10 study patients against general population norms (scores of 50). As shown, at day 14 there was substantial improvement with all SF-36 scale and summary measure scores in comparison to day 1 scores, and by day 28, the mean scores of the 10 study patients were at or above general population norms for all SF-36 scales and summary measures.

Table 5 provides the changes in SF-36 and HQLQ scale scores from baseline to day 14 and baseline to day 28. As shown, the changes in scores from baseline to day 14 and day 28 were statistically significant across all SF-36 and HQLQ scales. In treatment effect size terms, the changes in SF-36 and HQLQ scores from baseline to day 14 ranged from 0.77 to 1.46, which are considered large treatment effect sizes.[15] By day 28, for the majority of the SF-36 and HQLQ scales (10 of 14 scales) the treatment effect size exceeded 2.0, meaning that on average the score changes on these 10 scales were greater than 2 standard deviations. Inspection of the frequency distributions of the change in the scores at day 28 show that 9 of the 10 patients improved by a clinically meaningful amount on all SF-36 and HQLQ scales.

Table 6 provides a patient-by-patient account of HRQoL scores at days 1, 14 and 28. These data show that the HRQoL of 6 patients (patients 1, 2, 3, 6, 7, and 8) was *restored to normal*

or above normal levels (for example all SF-36 scores were at or above 50 at day 28). For 3 of the patients (patients 4, 5, and 9) their HRQoL was restored to *near normal levels* (for example several SF-36 scale scores were at or above 50 and some were slightly below 50 by 2-4 points). It should be pointed out that although not all aspects of HRQoL were restored to normal for these 3 patients, their day 28 scores were significantly better than day 1 scores. Only one patient (patient 10) showed no HRQoL improvement from day 1 to days 14 and 28.

As shown in Table 4, there were significant reductions in the percentage of study patients who reported physical, social, role, and emotional limitations from day 1 to days 14 and 28. For example, at day 1, 100% of the study patients reported limitations in walking more than one mile. After 14 days of treatment the percentage of the study patients that reported limitations in walking more than one mile was reduced to 70% and by day 28, none of the study patients reported limitations in walking more than one mile. The data in Table 4 also show that: 1) the percentage of patients that reported limitations in climbing one flight of stairs dropped from 50% at day 1, to 20% at day 14 and to 10% at day 28; 2) the percentage of patients that reported limitations in walking 100 yards dropped from 55% at day 1, to 20% at day 14 and to 10% at day 28; 3) the percentage of patients that reported difficulty performing at work dropped from 100% at day 1, to 90% at day 14 and to 50% at day 28; 4) the percentage of patients that reported pain interfered with work dropped from 30% at day 1, to 10% at days 14 and 28; 5) the percentage of patients that reported "fair" or "poor" health dropped from 90% at day 1, to 33% at day 14 and to 10% at day 28; 6) the percentage of patients that reported excellent health increased from 0% at day 1, to 20% at day 28; 7) the percentage of patients that reported feeling tired all or most of the time dropped from 90% at day 1, to 30% at day 14 and to 11% at day 28; 8) the percentage of patients that reported their health limited them from engaging in social activities all or most of the time dropped from 70% at day 1 to 10% at day 14, and 0% at day 28; 9) the percentage of patients that reported accomplishing less due to emotional problems all or most of the time dropped from 100% at day 1 to 90% at day 14, and 10% at day 28; 10) the percentage of patients that reported feeling downhearted and depressed all or most of the time dropped from 30% at day 1 to 0% at days 14 and 28; 11) the percentage of patients that reported that hepatitis limited them in performing at work all or most of the time dropped from 70% at day 1 to 20% at day 14, and 10% at day 28; and 12) the percentage of patients that reported that hepatitis limited them in engaging in normal social activities all or most of the time dropped from 60% at day 1 to 10% at days 14 and 28. Note that by day 28 the percentage of study patients that reported

physical, social, role, and emotional limitations was nearly equal to or lower than the percentage reporting limitations in the general population.

Figure 2 shows significant reductions in the percentage of study patients that screened positive for depression. At day 1, 9 out of 10 patients screened positive for depression. By day 14 the percentage of patients who screened positive for depression dropped to 40%, or 4 in 10 patients, and by day 28 none of the patients screened positive for depression.

Overall, the health-related quality of life of the 10 chronic hepatitis C patients that participated in this study was substantially compromised. On average, the patients participating in this study scored one and one-half to two and one-half standard deviations below general population norms, which was larger than the average health-related quality of life burden observed in other studies of chronic hepatitis C. [4,5,6,7,8,9,10,11,12,]

On average, treatment was observed to significantly improve the health related quality of life of study patients by day 14 and by day 28 the functional status and well-being of study patients was completely restored to normal levels. *The score improvements observed in this study by day 28 were on average larger than two standard deviations for nearly all health related quality of life scales, which has rarely been observed in the thousands of treatment studies of other chronic diseases involving the SF-36 Health Survey.*

3. **Clinical Symptoms:**

A. Fatigue:

The Fatigue Severity Scale (FSS) was used to measure the presence and severity of fatigue[12,13] and the impact of oral ingestion of A4+ on the same. Use of FSS in the assessment of fatigue severity in chronic hepatitis C has been validated [13]. The scale consists of nine items each of which is rated on a seven-point scale ranging from 1 (strongly disagree) to 7 (strongly agree) in escalating grades. This gives a total score range of 0 to 63, with a higher score indicating increased fatigue severity. Using this scale, patients were categorized as having mild (1-20), moderate (21-40) and severe (41-63) fatigue (Figure 3).

Before the use of A4+: Severe fatigue was present in 80%, moderate fatigue in 10% and mild fatigue in 10% of the subjects (figure 3).

After 28 days of the use of A4+: Severe fatigue was seen in 20%, and 40% each having mild and moderate fatigue. (Figure 3).

B. Gastrointestinal Symptoms: The gastrointestinal symptoms of dyspepsia, nausea & vomiting, and indigestion were assessed before and after the use of A4+ using the standard Gastrointestinal Symptom Rating Scale (GSRS). GSRS is a clinical rating scale that has been validated in the assessment of gastrointestinal symptoms in patients with dyspeptic symptoms.[14] It consists of three domains of dyspeptic syndrome (five items) indigestion syndrome (four items), and bowel dysfunction syndrome (six items) and each item is rated on a scale of 0 (absence of symptoms) to 3 (maximum severity of symptom). As only the dyspepsia and indigestion were being examined in the present study, only these domains of the GSRS were used. The total 5 items for dyspepsia and 4 for indigestion with a rating scores of 0 to 3 gives a maximum score of 15 for dyspepsia and 12 for indigestion.

Dyspepsia: Utilizing the maximum score of 15 for dyspepsia as described above (GSRS), it was rated as mild (score 1-5), Moderate (score of 6-10) and severe (score of 11-15).

Before the use of A4+: None of the subjects were free of dyspepsia. Most of the subjects had mild dyspepsia (70%), and 30% had moderate dyspepsia (Figure 4).

After 28 days of the use of A4+: Dyspepsia was absent in 90% with 10% had moderate dyspepsia (Figure 4).

Nausea and Vomiting: Was assessed as one of the elements of dyspeptic syndrome domain of GSRS scoring system noted above.

Before the use of A4+: Was experienced occasionally by 30%, frequently by 30% and was absent in 40% of the subjects.

After 28 days of the use of A4+: Was resolved in 90% of the subjects, and 10 % had only occasional nausea and vomiting.

Indigestion: Utilizing the maximum score of 12 for indigestion as described above (GSRS), indigestion was rated as mild (score 1-5), Moderate (score 6-9) and severe (score 10-12).

Before the use of A4+: Most subjects (70%) had mild indigestion, 20% experienced moderate indigestion, and 10% had severe indigestion (Figure 5).

After 28 days of the use of A4+: Symptom of indigestion was absent in 70%, while 30% had moderate indigestion (Figure 5)

Right upper abdominal pain, headache, muscle and joint/bone pain were assessed using a ten point scale ranging from 0 (none) to 10 (severe) in escalating grades. Using this scale, patients were categorized as having mild (1-4), moderate (5-8) and severe (9-10) pain.

C. Right Upper Abdominal Pain:

Before the use of A4+: Mild pain was present in 40%, moderate pain in 40%, severe pain in 10%, and 10% had no pain in the right upper abdomen (Figure 6).

After 28 days of the use of A4+: Besides 10% of the subjects who did not have pain, 60% additional subjects became free of pain. Pain was mild in 20%, severe pain persisted in 10% (Figure 6).

D. Right Upper Abdominal Tenderness:

This was graded arbitrarily on a four-point scale ranging from 0 (none) to 4 (severe) in escalating grades. Using this scale tenderness was categorized as mild (1-2), moderate (3) and severe (4).

Before the use of A4+: Mild tenderness in the right upper abdomen was present in 60%, moderate in 20%, and 20% had no tenderness (Figure 6).

After 28 days of the use of A4+: Tenderness in the right upper quadrant was absent in 80% and the remaining 10% each had mild and moderate tenderness (Figure 6).

E. Muscle and Joint/Bone Pain:

Before the use of A4+: Mild pain was present in 20%, moderate pain in 60%, Severe pain in 20%.

After 28 days of the use of A4+: Pain was absent in 40%, was mild in 50%, and was severe in 10%.

F. Headache:

Before the use of A4+: Mild headache was present in 50%, moderate in 30%, and 20% had no headache,

After 28 days of the use of A4+: Headache was absent in 60%, while the remaining 40% had only mild headache.

4. **Nutritional parameters (including anthropometric measurements and serum transferrin and pre-albumin):**

Nutritional status is known to influence functional ability, quality of life including daily physical and emotional performance both in disease free conditions and in various illnesses including liver disease. It is important that quality of life and other functional status assessment be interpreted with reference to this important variable.

Table 1 provides the mean and standard deviation of all the above indicated nutritional parameters at baseline and after 28 days. As shown in this table there was no significant change in any of the nutritional parameters during the study period suggesting that the observed changes in the quality of life after the use of A4+ were not related to alteration in nutritional status of the subjects.

A. Anthropometry:

The standard parameters: measured were Triceps skin fold (TSF), Subscapular skin fold (SSF), Midarm muscle circumference (MAMC), Arm muscle area (AMA) which measures fat and muscle body composition.[15, 16] Changes in these parameters at baseline would indicate pre-existing nutritional status that could contribute to a compromised functional status, performance level or decreased quality of life.

The baseline TSF (mean 18.4 cm, range 10.3-34.0 cm), SSF (mean 20.5 cm, range 11.7-36.0 cm), MAMC (mean 27.1 cm, range 19.7-32.1 cm) and AMA (mean 110.2 percent of normal, range 57-151) remained unchanged at 28 days (Table 1).

B. Testing of Grip Strength: This is a test of muscle function which can improve earlier than other anthropometric parameters and is of value to monitor when change in nutritional status could be a variable that can influence treatment outcome.[15, 16]

The hand Grip Strength remained unchanged at 28 days (mean 45.3 percent of normal, range 31-61) as compared to baseline (mean 44.3 percent of normal, range 25-67).

C..Serum transferrin and prealbumin:

These are serum proteins that can capture change in nutritional status over a short period of time.[15, 16] They are therefore indicators of change in nutritional status over a short period of time particularly when a treatment response is assessed.

Baseline transferrin (mean 335.3 mcg/dl, range 243-410 mcg/dl) and prealbumin (mean18.1mg/dl, range 7-28.6 mg/dl) did not change after 28 days of the use of A4+ (Transferrin: mean 324.4 mcg/dl, range 246-395 mcg/dl; Prealbumin: mean 17.7 mg/dl, range 5.3-29.2 mg/dl, Table 1).

5. Ancillary Observations:

The primary and only objective of the study was to examine whether the use of A4+ had any effect on the symptoms and quality of life of the ten subjects included in the study. However, ancillary data were obtained to screen for any effect of A4+ on the following parameters: routine liver chemistry, prothrombin activity, serum cholinesterase and chemical markers of continued liver necrosis and inflammation (TNF alfa). These data were collected at baseline and again after 28 days of administration of A4+. Table 2 depicts these values and also shows if there was change of any significance using statistical calculations.

A. Routine Liver Chemistry: These tests are the standard biochemical markers used commonly and routinely to screen for liver injury. They are the measurements of serum bilirubin, AST, and ALT. Favorable treatment response is accompanied by a decrease in the measured values.

As shown in Ttable 2 the mean values of these tests did not show any statistically significant difference at the end of 28 days (Serum bilirubin 1.22 $\pm$ 0.9 mg/dl, Alb 4.12 $\pm$ 0.5 gm%, AST 81.6 $\pm$ 91.2 IU, ALT 104.7 $\pm$ 145.9 IU) compared to the baseline (Serum bilirubin 1.32 $\pm$ 0.8 mg/dl, Alb 4.3 $\pm$ 0.5 gm%, AST 95.1 $\pm$ 102.7 IU, ALT 140.9 $\pm$ 163.2 IU).

B. Prothrombin activity and serum cholinesterase: These two tests are used to assess stabilization of liver injury.[17,18] An improvement in liver status is accompanied by an increase in their measured values.

As shown in Table 2 and Figure 7, there was a significant increase in the serum levels of both, prothrombin and cholinesterase after 28 days of the use of A4+. At baseline, prothrombin activity expressed as percent of control was 68.9 ± 22.7% and serum cholinesterase was 5194.1 ± 1590.1 U/L. After 28 days these increased to 81.2 ± 28.0% and 7792.7 ± 2218.8 U/L respectively.

*C. **TNF alfa:*** At the tissue level, hepatitis is characterized by liver cell injury, inflammation, cell death (necrosis) and scarring (fibrosis). Liver biopsy is the 'gold standard' to assess and monitor these changes. Non-invasive blood tests or other imaging modalities (ultrasound, CT scan, MRI) lack the sensitivity to assess this key element of disease severity and progression. Blood tests like TNF alfa is still a research and investigational tool.

Serum TNF alfa is increased in the presence of liver tissue injury and inflammation. As shown in Table 2 and Figure 7, the serum level of TNF alfa was higher at the end of 28 days (10.0 ± 3.0 pgm/ml) of the use of A4+ than that noted at the baseline (7.2 ± 2.1 pgm/ml).

*D. **Fibrosure test:*** At the present time only one set of data is available on this parameter. My understanding is that, this test will be repeated periodically at selected intervals.

*E. **Liver size:*** Liver size was measured by ultrasound at the start and after 28 days of the use of A4+. It did not change in size after 28 days (Right lobe 147.0 ± 23 cm, Left lobe 110.4 ± 14.2 cm) of the use of A4+ from that at the start of the study (Right lobe 148.3 ± 22.2 cm, Left lobe 109.6 ± 16.2 cm).

V. Summary of Findings:

Before discussing conclusions of this study the constraints and scope of the study must be recognized.

The *primary objective* of this preliminary pilot project was to examine the possible impact of A4+ on pre-selected parameters of depression, health-related quality of life and symptoms of chronic

hepatitis C. At this preliminary stage of testing, finding conclusive evidence to definitively show that A4+ caused the observed changes was not within the scope of the study design and was not the stated goal.

It was the operative decision that, to undertake a study designed to seek conclusive and definitive answers about the effects of A4+ was logistically and financially not possible and operationally inconceivable at this time. Concerns about interpreting the outcome of a study that used an extremely small number of subjects and that lacked a placebo control were raised initially, but the study design and methods were not altered due to operational and other restrictions. Similarly, the idea of keeping the subjects in their own environment during the study period and utilizing a centralized laboratory was deemed not executable for such a preliminary pilot project. Additionally, the unknown composition of A4+, and lack of knowledge about its bio-availability were highlighted but were judged to be issues for future projects. Given these limitations, the study lacks the statistical power to arrive at definitive conclusions or to make general statement of universal applicability of A4+ in hepatitis C. The results must be considered in light of the narrow confines of the stated objective of the project and the above mentioned limitations.

*1. **Demographics of study population:***

The study population consisted of 5 females and 5 males with a mean age of 43.8 years (37-56 years). Concurrent medical conditions (hypothyroidism, chronic bronchial asthma, chronic arthritis, and panic attack) were present in 60% of the subjects. Twenty percent of the population had a history of intravenous substance abuse. No identifiable risk factor for hepatitis C was found in 40% of the subjects. The socio-economic mix of the study subjects was homogeneous. The mean estimated duration of hepatitis C was 23 $\pm$ 9.5 years and 70% of the subjects were infected with Genotype 1 of the hepatitis C virus.

*2. **Features at Start of the Study:***

*A. **Depression:***

Using the Beck Depression Inventory, borderline depression was detected in 10% of the subjects, mild to moderate depression in 80%, and severe depression in 10%. In comparison, the reported incidence of depression in other studies prior to commencement of treatment in patients with hepatitis C typically varies from 10% to 44%.[2,3,4]

B. Health-Related Quality of Life Burden:

The mean SF-36 scale and summary measure score in the ten study subjects, when compared with those of the general population (Figure 1 of QualityMetric report), and patients with other chronic diseases (e.g., congestive heart failure, clinical depression, hypertension, myocardial infarction and diabetes mellitus) were markedly low. Specifically these scores were 1.5 to 2.5 standard deviations below the general population norms, indicating a significant health-related quality of life burden among these study participants.

In comparison to other patients with chronic hepatitis C, the 10 participants in this study showed greater than average HRQoL burden than that reported in the published literature involving patients with chronic hepatitis C. Overall, the 10 patients in this study scored lower on all SF-36 scales and summary measures compared with baseline scores observed across the 8 published studies. In two of the published studies of patients with chronic hepatitis C, where the average HRQoL burden even came close to approaching the level observed in this study, there were other important *compounding factors*. In one study the *chronic hepatitis C patients suffered from significant emotional problems* and the other study *the 15 chronic hepatitis patients had hepatocellular cancer. Both these clinical compounding factors appeared to have a significant impact across all SF-36 scales in the two studies.*

Further analysis comparing the frequency and severity of specific elements of health-related quality of life burden (such as limitations in waking more than one mile, limitations in climbing one flight of stairs, limitations in walking 100 yards, difficulty performing at work, pain interfering with ability to work, perceived health status as "fair" or "poor", feeling tired all or most of the time, etc.) of the study subjects with the control population showed severe limitations in the study population (Table 4 of QualityMetric report).

C. ***Clinical symptoms:*** At the start if the study, fatigue was present in 100% of the subjects and was severe in 80%; right upper quadrant pain was present in 90% and right upper quadrant tenderness in 80%; muscle and/or joint pain of some degree was present in 100% of the subjects; mild to moderate headache in 80%; dyspepsia of mild to moderate nature in 100%; nausea/vomiting either occasionally or frequently in 70%; and indigestion to some degree in all of the subjects. Published reports indicate that in symptomatic patients with chronic hepatitis C, fatigue[1,

[2] and depression[2, 3, 4] are common. The prevalence of fatigue is estimated to be between 40 and 60%.[1, 2]

3. ***Impact of A4+:***

A. Depression: The severity of depression at the start of the study ranged from borderline in 10% of the subjects, mild to moderate depression in 80%, and severe depression in 10%. By the end of study period significant improvement in depression was noted and 90% of the subjects had become free of depression.

Noteworthy is the reported change in the Beck Depression Inventory score. *Mean score before the use of A4+ was 20.9 (range 11-35) and decreased markedly to 2.7 (range 0-3) 28 days after the use of A4+. Sixty percent of the subjects reported total Beck score of 2 or less,* including score of 0-1 in 40%. at 28 days of the use of A4+. Such a score is unusual in population norms.

B. Health-Related Quality of life Burden:

In general, use of A4+ was accompanied by significantly improved health-related quality of life in study subjects by Day 14. By Day 28, the functional status and well-being of study *subjects were restored completely to normal levels.* The score improvements observed in this study by Day 28 were on average larger than two standard deviations for nearly all health-related quality of life scales, *which has rarely been observed in the thousands of treatment* studies *of other chronic diseases* involving the SF-36 Health Survey.[25]

C. Clinical symptoms: Study subjects showed a significant improvement in most of their symptoms. This included improvement in fatigue, right upper quadrant pain and tenderness, dyspepsia, nausea-vomiting, indigestion, headache, muscle and joint/bone pain.

D. Nutritional status: In order to ascertain whether the observed changes in the health-related quality of life and symptoms of the study subjects could have been influenced by improved nutritional status, the nutritional status of the study subjects was evaluated both at the beginning and at the end of the study period. Nutritional parameters, including anthropometry and serum transferrin and pre-albumin remained unchanged during the short

study period indicating that improvement in symptoms and health-related quality of life were independent of nutritional status.

E. Routine Liver Chemistry: Serum bilirubin, AST, ALT and albumin did not show any change at the end of the study as compared to pre-study values.

F. Prothrombine activity and serum cholinesterase: There was a significant increase in Prothrombin activity and serum cholinesterase which suggests possible increased protein synthesis by liver or a decrease in their degradation.

G. TNF alfa: Serum TNF alfa was increased at the end of 28 days (10.0 ± 3.0 pgm/ml) of the use of A4+ as compared to the Day 1 value (7.2 ± 2.1 pgm/ml). In patients with hepatitis C this is indicative of continued liver inflammation and injury.

H. Liver size: There was no change in the liver size or echo-texture of the liver during the use of A4+.

VI. DISCUSSION AND CONCLUSION:

1. The finding of an increased health-related quality of life burden in the study subjects as compared to the general population and those with other chronic diseases like hypertension, and type II diabetes is not unusual and confirms similar reports of increased health-related quality of life burden in patients with chronic hepatitis C as compared to the general population and patients[6-10, 19] with chronic diseases.[9, 10, 21]

2. The scores of all eight domains of SF-36 were markedly decreased in the study population. Finding a decrease in scores of all eight domains of SF-36 is not unusual in patients with chronic hepatitis C and has been reported by other observers.[7,8, 10, 21] In addition, the domains that most consistently scored the lowest in this study namely role physical (RP), general health (GH), Role emotional (RE) and social function (SF) are among the domains that received low scores in other studies.[10, 19, 21] This suggests that the qualitative perception of HRQL had a similar spectrum even though the magnitude differed profoundly both at the beginning and end of the study.

The mechanism of decreased health-related quality of life in patients with hepatitis C is not understood. Studies have failed to establish a correlation between HRQL and parameters of liver disease and its severity.[6,7,9,10] However, recent studies suggest that "brain fog" exhibited by hepatitis C-viremic patients may be the result of a regional imbalance in brain metabolites and/or chemicals.[5]

3. The magnitude of the decrease in the health-related quality of life in the present study is strikingly different from the typical decrease reported by other investigators in patients with chronic hepatitis C.[6, 7, 19, 22] This difference could be related to a number of factors: The small size of the current study population leaves open the possibility that the sample is not reflective of general spectrum of patients with chronic hepatitis C. In addition, the present study could have an element of patient selection bias towards recruiting patients with greater health-related quality of life burden. The fact that these subjects were seeking to participate in the study might suggest that their motivation to participate in the study was driven by the severity of their symptoms and that those with less compromised health-related quality of life did not opt to participate. On the other hand, historical cohorts reported from various treatment protocols with comparatively lower health-related quality of life burden could reflect yet another type of selection bias in favor of less symptomatic patients. Historical cohorts of patients from various interferon based treatment protocols (e.g. the consensus interferon study group[15]) had well defined inclusion and exclusion criteria that excluded patients with depression and may have similarly excluded other patients with significantly compromised health-related quality of life burden. Similar factors could have influenced the reported lower health-related quality of life burden in other historical cohorts of hepatitis C. It is also possible that in the present study the long duration of illness altered the subjective perception of the disease and general well-being by the study participants. Finally most of the subjects in the small population sample of the present study were not treatment naïve: they were either prior treatment failures or intolerant to interferon. It is not unlikely that the fear of perceived progression of disease related to prior treatment failure and the lack of means to eradicate the viral infection adversely influenced their subjective response to questions about health-related quality of life burden. Diagnostic work-up and "labeling" of the disease condition in patients with hepatitis C[22] and other chronic disease[23] is known to decrease the subjective self-assessment of quality of life indicating increased disease burden.

4. After taking A4+, there was a significant improvement in health-related quality of life at Days 14 and 28, and in clinical symptoms at the end of study period. Although improvement in health-related quality of life has been seen in patients who fail to eradicate the hepatitis C virus, the reported change is small.[19, 22] Significant improvement in quality of life in patients with hepatitis C has been reported only after viral clearance.[19, 23] In the current study, the magnitude of HRQL improvement far exceeds that reported in any published reports involving patients with hepatitis C.[19, 23] In fact, the degree of score improvements observed in this study by Day 28 *has rarely been observed in the thousands of treatment studies of several other chronic diseases* involving the SF-36 Health Survey.[20] The physiology of the response in the present study is unclear. Since the subjects stayed together, the possibility remains that environmental factors, group-suggestion and/or peer 'support-group' phenomena may have somewhat influenced the participants' subjective perception of the degree of change in the HRQL burden.

5. There was an increase in the serum prothrombin activity and serum cholinesterase. These changes could suggest either an increase in liver protein synthesis or a decrease in the breakdown of these proteins. However, the concomitant increase in TNF alfa indicates continued liver inflammation, making it unlikely that the increase in prothrombin activity and serum cholinesterase reflected stabilization of liver injury. Alternately, it is unclear whether such an increase simply reflected a fluctuation seen in liver chemistry in patients with hepatitis C. The significance of the increase of these markers must be determined through a better designed, double-blind, placebo-controlled, large population study.

In conclusion, the study subjects reported an improvement in Health-Related Quality of Life and other symptoms while taking A4+. However, on careful analysis of the study data, one finds that after the use of A4+, the scores of most of the observed parameters are clustered at the extreme end of well-being. "*By day 28, for the majority of the SF-36 and HQLQ scales (10 of 14 scales) the treatment effect size exceeded 2.0, meaning that on average the score changes on these 10 scales were greater than 2 standard deviations.*" This was seen in 9 of the 10 study subjects. (Qualitymetrix report.) This clustering is also reflected in the analysis of SF-36 which shows that after 28 days of having used of A4+, "*the functional status and well-being of study subjects were*

restored completely to normal levels" and "*the HRQoL of 6 patients (60%) was restored to normal or above normal levels.*" Notably, these results were seen in the study subjects whose stated decrease in HRQL at the start of the study was of a magnitude never before reported in patients with hepatitis C. As discussed earlier, when the results of the present study are compared with those from other published reports on the treatment response of historical patient cohorts with hepatitis C and other chronic illnesses, the outcomes in the present study appear to be statistical outliers.

The nature of the study participants' response highlighted above and the limitations of the study itself raise concerns about making broad claims about the efficacy of A4+. As indicated previously, the significance of the observations made in this study about A4+'s impact on the liver needs to be verified through a double-blind study involving a larger sample size with a placebo control.

Bibliography

1. Poynard T, Cacoub P, Ratziu V. et al. Fatigue in patients with chronic hepatitis C. Jour Viral Hepat. 9:295-303, 2002

2. Dwight, MM, Kowdley, KV, Russo, JE, et al. Depression, fatigue and functional disability in patients with chronic hepatitis c. Jour. Psychosomatic Res. 49:311-317, 2000

3. Lehman,CL, Cheung, RC. Depression, anxiety, post-traumatic stress and alcohol-related problems among veterans with chronic hepatitis c. Amer. Jour. Gastro 97:2640-2646, 2002

4. Rowan PJ, Tabasi, S, Abdul-Latif, M, Kunik, ME, Lel-Serag,H. Psychosocial factors are the most common contraindications for antiviral therapy at initial evaluation in veterans with chronic hepatitis C. Jour. Clinical Gastro 38:530-534, 2004

5. Fotton DM, Thoms HC, Murphy CA et al. Hepatitis C and cognitive impairment in a cohorrt of patients with mild liver disease. Hepatology 35:433-439, 1998

6. Fontana RJ, Moyer CA, Sonnad S, Lok ASF, Sneed-Pee N, Walsh J et al. Comorbidities and quality of life in patients with interferon-refractory chronic hepatitis C. *Am J Gastroenterol* 96:170-178, 2001

7. Foster GR, Goldin RD, Thomas HC. Chronic hepatitis C virus infection causes a significant reduction in quality of life in absence of cirrhosis. Hepatoloy 27: 209-212,1998

8. Chong CA, Gulamhussein A, Heathcote EJ, Lilly L, Sherman M, Naglie G et al. Health-state utilities and quality of life in hepatitis C patients. *American Journal of Gastroenterology* 2004

9. Bayliss MS, Gandek B, Burgay KM etal. A questionnaire to assess the generic and disease specific quality of life. Qual Life Res 7:39-55, 1998

10. Carithers RL, Sugano D, Bayliss M. Health assessment for chronic hepatitis C infection. Dig Dis Scien 41 (suppl): 75-80, 1996

11. Beck, AT, Steer, RA. Manual for the Beck Depression Inventory. San Antonio, Tx; Psychological Corporation

12. Krupp, LB, LaRocca, NG, Muir-Nash, J. The Fatigue Severity Scale. Application to patients with multiple sclerosis and systemic lupus erythematosus. Arch Neurol 46:1121-1123, 1989

13. Klienman L, Zodel MW, Hakim Z. et al Psychometric evaluation of fatigue severity scale for use in chronic hepatitis C. Qual Life Res 9:499-508,2000

14. Svedlund J, Ingemar S, Dotevall G. GSRS a clinical rating scale for gastrointestinal symptoms in patients with irritable bowel syndrome and peptic ulcer disease. Dig Dis Sci 33:129-134,1988

15. Hensrud DD. Nutritional screening and assessment. Med Clin North Amer 83:1525-1546, 1999

16. Mendenhall CL, Moritz TE, Morgan TR et al. Protein energy malnutrition in severe alcoholic hepatitis: Diagnosis and response to treatment. Jour Parent Enter nutrition 19:258-265, 1995

17. Popovic L, Batinica S, Mestrovic T, et al. The value of cholinesterase activity after Kasai operation. Pediatr Srg Internat 19:605-607, 2003

18. Qing L, Zhong-Ping D, Chun H, Chun-Hui Z. Evaluation of effect of hybrid bioartificial liver using end-stage liver disease model. World Jour Gastro 10: 1379-1381, 2004

19. Bonkovsky HJ, Michael Wooley, and consensus interferon study group. Reduction of health-related quality of life in chronic hepatitis C and improvement with interferon. Hepatology 29:264-270, 1999

20. Turner-Bower D, Bartley PJ, Ware JE, SF-36 Health Survey Bibliography. Third edition (1988-2000). Lincoln, RI, QualityMetric Inc. 2002

21. Rodger AJ, Jolley D, Thompson SC. Et al. The impact of diagnosis of hepatitis c virus on quality of life. Hepatology30:1299-1301, 1999

22. Ware JE, Bayliss MS, Mannocchia M. et alo. Health-related quality of life in chroni hepatitis C: Impact of disease and treatment response. Hepatology 30:550-555,1999

23. McHutchison JG, Ware JE, Bayliss MS, Pianko S. et al. The effect of interferon alpha-2bin combination with ribavirin on health-related quality of lifre and work productivity. Jour Hepatolo 34:140-147, 2001.

EFFECT ON DEPRESSION

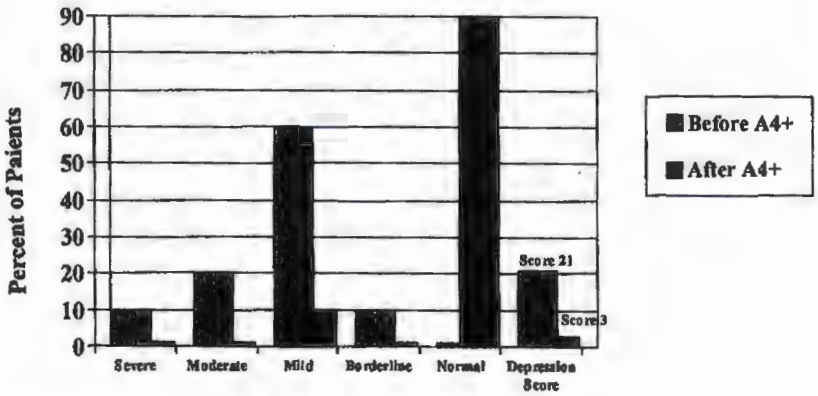

Figure 1.

EFFECT ON SOMATIC AND FUNCTIONAL ELEMENTS OF BECK INVENTORY

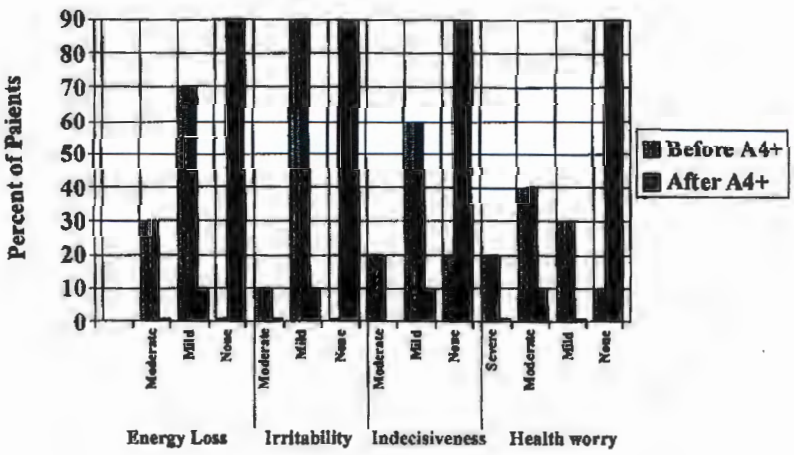

Figure 2.

EFFECT ON FATIGUE

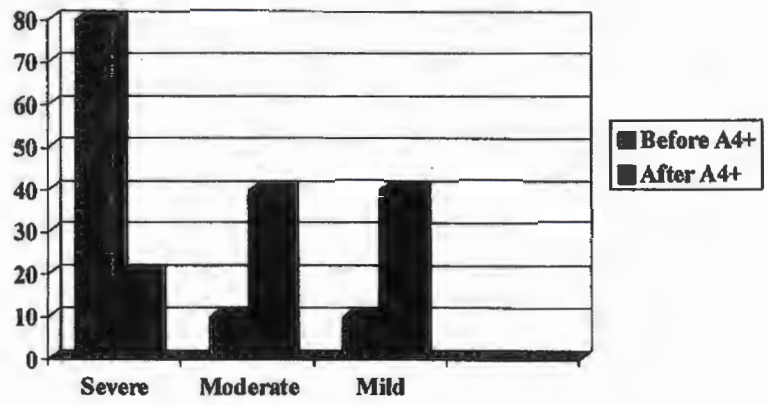

Figure 3

EFFECT ON DYSPEPSIA

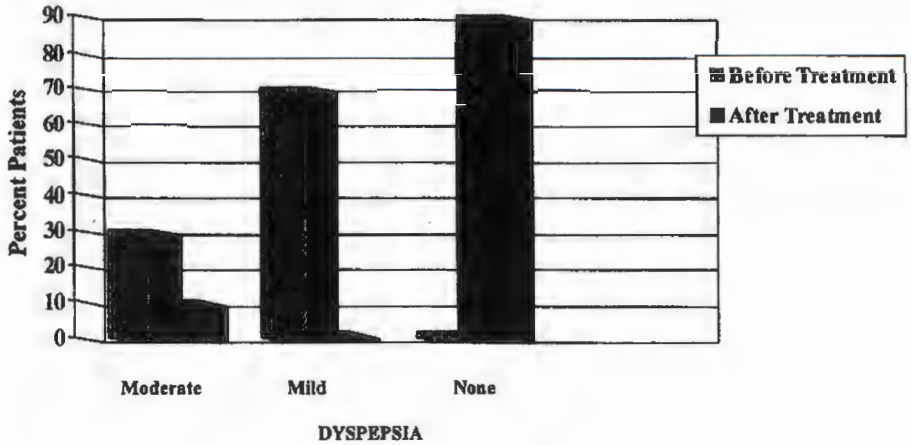

Figure 4

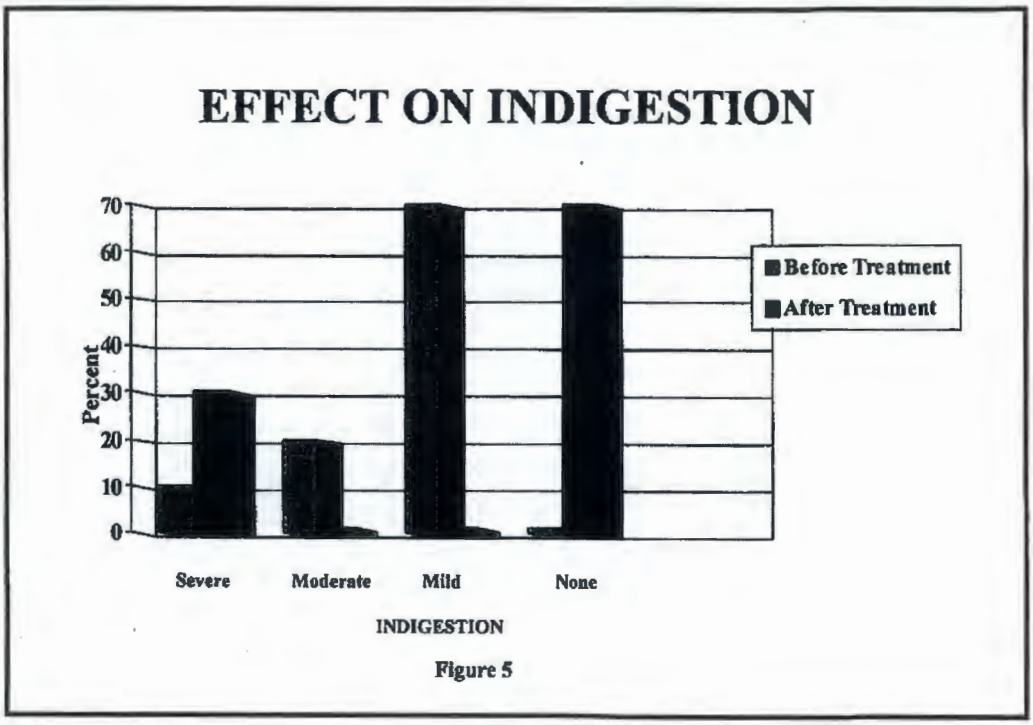

Figure 5

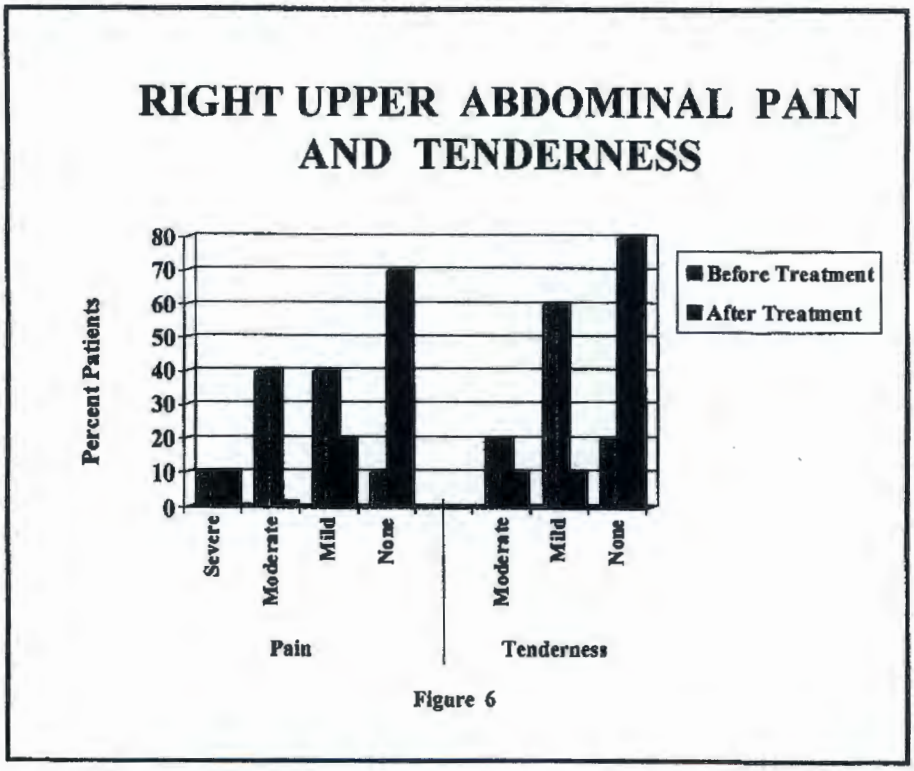

Figure 6

EFFECT ON SERUM PROTHROMBIN, CHOLINESTERASE AND TNF -ALFA

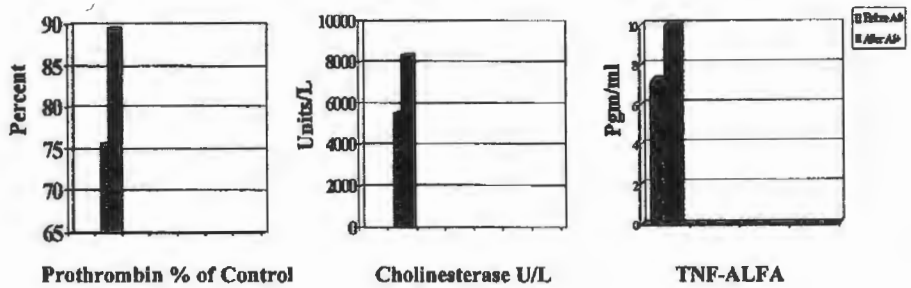

Figure 7

TABLE 1

	Before A4+	After 28 days of use of A4+
TSF (cm)	18.4 ± 6.4	17.5 ±4.3
SSF (cm)	20.5 ± 8.5	19.4 ± 8.9
MAMC (cm)	27.1 ± 4.0	26.9 ± 3.8
AMA (% of normal)	110.2 ± 31.0	109.5 ± 29.2
Hand Grip (% of normal)	44.3 ± 11.7	45.34 ± 14.8
Transferrin (mcg/dl)	335.3 ± 51.5	324.4 ± 48.4
Prealbumin (mg/dl)	18.1 ± 6.2	17.7 ± 6.4

TABLE 1: Nutritional parmeters in the study subjects before, and 28 days after the Use of A4 plus

Attachment 1: QualityMetrix report; analysis of the quality of life data

Reference # 18

Hepatoprotective potential of *Cordia subcordata* Lam. against carbon tetra chloride (CCl_4)-induced hepatotoxicity in Wistar albino rats.

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ABSTRACT

Aim: To investigate the phytoconstituents, acute oral toxicity and hepatoprotective activity of ethanol (90%) extract of *Cordia subcordata* Lam. (EECS) using CCl_4 induced hepatotoxicity in male Wistar albino rats.

Methods: The EECS at doses of 100, 200 and 400mg/kg, p.o and the standard drug Liv.52 (40mg/kg, p.o) were administered for 7 days in CCl_4 intoxicated rats. The hepatoprotective activity was assessed by using various biochemical parameters like SGOT, SGPT, alkaline phosphatase (ALP) and acid phosphatase (ACP), also total bilirubin and urea along with histopathological studies of liver tissue. The biochemical changes and histopathological studies were observed on 4^{th} and 8^{th} day.

Results: EECS at tested doses significantly decrease ($P<0.001$) the elevated levels of the hepatic enzymes, total bilirubin and urea in a dose dependent manner after 3days whereas it's subsequent return towards near normal after 7days indicating the recovery of hepatic cells. In the liver sections of the rats treated with EECS extracts for 7 days, the normal cellular architecture was retained as compared to Liv.52, thereby furtherly confirming the potent hepatoprotective effect of EECS.

Conclusion: The EECS afforded significant protection against CCl_4 induced hepatocellular injury.

KEY WORDS: Hepatotoxicity, CCl_4, Hepatic Enzymes, *Cordia subcordata* Lam. Hepatoprotective, EECS

INRODUCTION

The liver regulates many important metabolic functions, detoxification, and secretory functions in the body. Hepatic injury is associated with distortion of these metabolic functions **[1].** Thus, liver diseases remain one of the serious health problems and its disorders are numerous with no effective remedies. Despite, considerable progress in the treatment of liver diseases by oral hepatoprotective agents, search for newer drugs continues because the existing synthetic drugs have several limitations **[2-4].** So, the search for new medicines is still ongoing. Because liver performs many vital functions in the human body and damage of liver causes unbearable problems. **[5, 6].** Keeping this fact in view, the present study was undertaken to investigate the hepatoprotective activity of *Cordia subcordata* Lam. leaves against carbon tetrachloride-induced hepatic damage in albino rats.

***Cordia subcordata* Lam.** Family: Boraginaceae) is a medium-sized spreading tree to 12m tall with grayish grooved flaking bark. Leaves alternate, petiolate, the petiole about half as long as blade, broadly ovate and entire, often wavy-margined, the apex obtuse to short-pointed, base rounded, the blade up to 20 cm long. Flowers showy, orange, trumpet -shaped, unscented and borne in small axilary or terminal clusters. Fruit a globose drupe up to 3 cm long, surrounded by the enlarged calyx. Flowers and fruit usually available throughout the year. The seeds float and are highly resistant to salt water, thus the species is common in

coastal areas. In Tahiti, the leaves are used in remedies for bronchitis and asthma where the leaves probably act as a purgative. The plant is also used in the treatment of hepatic infections, cirrhosis of the liver and inflammation of the lymph nodes. It is also used to treat albumin present in the urine. Cook Islanders use the leaves in remedies for abdominal swellings and urinary tract infections **[7-9]**.

However, there are no ethnomedicinal information and scientific findings for the above said traditional claim for hepatic disorders. Therefore, to justify the traditional claims the present study was undertaken to find out if ethanol extract of *Cordia subcordata* Lam. leaves demonstrates the hepatoprotective activity against CCl_4-induced liver damage in rats. Hence, the present study was designed to verify the claims of the native practitioners.

MATERIALS AND METHODS

Plant collection

The Plant material of *Cordia subcordata* Lam. leaves was collected from Tirunelveli District, in the Month of August 2008. The plant was authenticated by Dr.V.Chelladurai, Research Officer Botany. C.C.R.A.S., Govt. of India. The voucher specimen (CHE-SA-CS-08) of the plant was deposited at the college for further reference.

Preparation of plant extract

The leaves of *Cordia subcordata* Lam. were dried in shade, separated and made to dry powder. It was then passed through the 40 mesh sieve. A weighed quantity (80gm) of the powder was subjected to continuous hot extraction in Soxhlet Apparatus. The extract was evaporated under reduced pressure using rotary evaporator until all the solvent has been removed to give an extract sample. Percentage yield of ethanolic extract of *Cordia subcordata* was found to be 16.5 % w/w.

Preliminary phytochemical screening

The phytochemical examination of ethanolic (90%) extract of *Cordia subcordata* Lam. leaves was performed by the standard methods **[10].**

Animals used

Wistar albino rats (150-220g) of either sex were obtained from the animal house in C.L. Baid Metha College of Pharmacy, Chennai. The animals were maintained in a well-ventilated room with 12:12 hour light/dark cycle in polypropylene cages. The animals were fed with standard pellet feed (Hindustan Lever Limited., Bangalore) and water was given *ad libitum*. Ethical committee clearance was obtained from IAEC (Institutional Animal Ethics Committee) of CPCSEA (Ref No. IAEC / XIII / 01 / CLBMCP / 2008 - 2009).

Acute Toxicity Study

The acute toxicity of 90% ehanolic extract of *Cordia subcordata* was determined as per the OECD guideline no. 423 (Acute Toxic Class Method). It was observed that the test extract was not mortal even at 2000mg/kg dose. Hence, $1/20^{th}$ (100mg/kg), $1/10^{th}$ (200mg/kg) and $1/5^{th}$ (400mg/kg) of this dose were selected for further study **[11].**

Carbon tetrachloride-induced hepatotoxicity in rats

The liver protective effect was evaluated using the carbon tetrachloride (CCl_4) model described by Visweswaram et al. **[12].** Wistar albino rats (150-220gm) were divided into six groups of six rats each and were subjected to the following treatments: Group-I served as normal control received distilled water (1 ml/kg, p.o) for 7days. Group II -VI received

0.75 ml/kg CCl_4 administered orally as single dose. After 36 hours, Groups III-VI received EECS with doses of 100, 200 and 400mg/kg, p.o and the standard drug Liv.52 with dose of 40mg/kg, p.o, respectively once daily for 7days. The blood was collected by puncturing the retro-orbital sinus of three rats from each group on 4^{th} day of treatment and 8^{th} day after the treatment respectively. From the collected blood samples, serum was separated to assess various biochemical parameters.

Biochemical estimation

The separated serum was subjected to estimate SGOT and SGPT by Reitman and Frankel method, alkaline phosphatase (ALP) and acid phosphatase (ACP) by Kind and King method, bilirubin by Malloy and Evelyn method and urea by Bousquet method **[13-16].** The rats were then sacrificed by bleeding and the liver was carefully dissected, cleaned of extraneous tissue, and part of the liver tissue was immediately processed for histopathological investigation.

Histopathological studies

The tissues of liver were fixed in 10% formalin and embedded in paraffin wax. Sections of 4-5 microns thickness were made using rotary microtome and stained with haematoxylin-eosin and histological observations were made under light microscope **[17, 18].**

Statistical analysis

The data were expressed as mean ± standard error mean (S.E.M).The Significance of differences among the group was assessed using one way and multiple way analysis of variance (ANOVA). The test followed by Dunnett's test p values less than 0.05 were considered as significance.

RESULTS

The results of preliminary phytochemical screening of the ethanoloic extract of *Cordia subcordata Lam.* revealed that presence of alkaloids, flavonoids, carbohydrates, glycosides, tannins, terpeniods and absence of saponins and steroids.

Acute toxicity study

Acute toxicity study in which the animals treated with the EECS at a higher dose of 2000 mg/kg did not manifest any significant abnormal signs, behavioral changes, body weight changes, or macroscopic findings at any time of observation. There was no mortality in the above-mentioned dose at the end of the 14 days of observation.

Effect of EECS on CCl_4 – induced hepatotoxicity

The results of EECS on carbon tetrachloride-induced hepatotoxicity were represented in **Table 1 and Table-2.** The CCl_4 only treated animals exhibited a significant increase (P<0.001) the levels of SGOT, SGPT, alkaline phosphatase (ALP) and acid phosphatase (ACP) and also total bilirubin and urea when compared to the normal control group on both 4^{th} and 8^{th} day, indicating hepatocellular damage.

The EECS at tested doses (group III-V) produced a significant reduction (P<0.001) in the CCl_4-induced elevated levels of SGOT, SGPT, alkaline phosphatase (ALP) and acid phosphatase (ACP), also total bilirubin and urea when compared to the CCl_4 only treated animals (group-II) after 3days of treatment and reduced furthermore to the normalcy on 8th day although the lowest dose (100 mg/kg) tested could produced significant reduction even after 3days of treatment **(Table 1).** Overall, EECS at tested doses significantly reduced the levels of hepatic enzymes, total bilirubin and urea in a dose dependent manner.

Table-1: EECS on CCl_4-induced alteration of hepatic enzymes, serum bilirubin and urea in rat liver after 3 days

Design of Treatment	Biochemical parameters					
	SGOT(U/ml)	SGPT(U/ml)	ALP (KA Units)	ACP(KA Units)	Bilirubin(mg/dl)	Urea(mg/dl)
Group-I: Normal control (DW-1 ml/kg; p.o)	49.31 ± 1.16	60.61 ± 0.06	17.60 ± 0.92	4.02 ± 0.17	0.82 ± 0.03	36.41 ± 1.53
Group-II: CCl_4 (0.75 ml/kg; p.o)	186.20 ± 1.35*ᵃ	154.20 ± 1.35*ᵃ	46.00 ± 1.02*ᵃ	5.93± 0.03*ᵃ	3.06 ± 0.04*ᵃ	96.82 ± 1.26*ᵃ
Group-III:EECS (100 mg/kg; p.o)	124.46 ± 1.46*	132.46 ± 0.46*	38.42 ± 1.72*	5.23 ± 0.01*	1.61 ± 0.03*	78.24 ± 1.15*
Group-IV:EECS (200 mg/kg; p.o)	74.03 ± 1.41*	97.03 ± 1.41*	29.80 ± 1.85*	4.58 ± 0.16*	1.24 ± 0.04*	57.12 ± 1.13*
Group-V:EECS (400mg/kg; p.o)	66.40 ± 1.72*	71.40 ± 1.02*	27.02 ± 0.05*	4.26 ± 0.03*	1.12 ± 0.02*	48.60 ± 0.92*
Group-VI: Liv.52 (40 mg/kg; p.o)	59.74 ± 1.32	65.74 ± 0.92*	25.82 ± 1.85*	4.11 ± 0.02*	1.01 ± 0.03*	42.06 ± 0.94*

Values are Mean ± SEM of 6 animals each in a group.
*ᵃ *P*<0.001 , when compared group I *Vs* group-II,
**P*<0.001, when compared group II *Vs* group III, IV, V and VI
EECS = ethanol (90%) extract of *Cordia subcordata*, CCl_4 = Carbon tetrachloride
DW=distilled water

Table-2: EECS on CCl_4-induced alteration of hepatic enzymes, serum bilirubin and urea in rat liver after7 days

Design of Treatment	Biochemical parameters					
	SGOT(U/ml)	SGPT(U/ml)	ALP (KA Units)	ACP(KA Units)	Bilirubin(mg/dl)	Urea(mg/dl)
Group-I: Normal control (DW-1 ml/kg; p.o)	49.31 ± 1.16	60.61 ± 0.06	17.60 ± 0.92	4.02 ± 0.17	0.82 ± 0.03	36.41 ± 1.53
Group-II: CCl_4 (0.75 ml/kg; p.o)	159.20 ± 1.03*ᵃ	114.14 ± 1.41*ᵃ	46.12 ± 0.91*ᵃ	4.02 ± 0.13*ᵃ	2.12 ± 0.04*ᵃ	89.17 ± 0.54*ᵃ
Group-III: EECS (100 mg/kg; p.o)	108.46 ± 1.15*	95.13 ± 0.25*	28.13 ± 0.04*	3.61 ± 0.04*	1.40 ± 0.05*	67.24 ± 1.15*
Group-IV: EECS (200 mg/kg; p.o)	65.20 ± 0.91*	73.07 ± 0.62*	21.01± 0.64*	3.26 ± 0.14*	1.18 ± 0.02*	48.12 ± 1.13*
Group-V: EECS (400 mg/kg; p.o)	57.17 ± 1.24*	64.02 ± 0.04*	17.04 ± 0.02*	3.15 ± 0.02*	1.00 ± 0.01*	39.60 ± 1.43*
Group-VI: Liv.52 (40 mg/kg; p.o)	52.13 ± 0.81*	61.12 ± 0.43*	15.34 ± 0.92*	3.09 ± 0.03*	0.90 ± 0.02*	34.46 ± 0.62*

Values are Mean ± SEM of 6 animals each in a group. *ᵃ *P*<0.001 , when compared group I *Vs* group-II,
**P*<0.001, when compared group II Vs group I, III, IV, V and VI
EECS= ethanol (90%) extract of *Cordia subcordata*, CCl_4 = Carbon tetrachloride.DW=distilled water

After 7 days, the hepatic enzymes levels were almost restored to the normal after treating with EECS at the dose of 400mg/kg, p.o.

A standard drug, Liv.52 at a dose of 40 mg/kg (group-VI) administered orally produced a significant reduction ($p<0.001$) compared to CCl_4 only treated animals (group-II) on both 4th and 8th day and these protective effects almost close to EECS 400mg/kg, p.o.

Effect of EECS on histolopathological change:

Histopathological examination of liver sections of control group showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces and central vein on both 4th and 8th day **(Fig.1a** and **1b).**

Disarrangement of normal hepatic cells with centrilobular necrosis, vacuolization of cytoplasm and fatty degeneration were observed (on both 4th and 8th day) in CCl_4 intoxicated rats **(Fig.2a and 2b).**

The liver sections (on both 4th and 8th day) of the group-V rats treated with EECS (400mg/kg, p.o) showed a sign of protection as it was evident by the moderate accumulation of fatty lobules, absence of necrosis and vacuoles **(Fig. 3a and 3b).** Almost similar sign of protection was shown in the liver sections of Liv.52 at a dose of 40 mg/kg treated rats **(Fig. 4a and 4b).**

Fig. 1 (a): Normal control treated group on 4th day (100x)

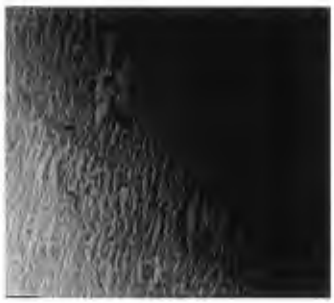

Fig. 1(b): normal control treated group on 8th day (100x)

Fig. 2(a): CCl_4 treated group on 4th day (100x)

Fig. 2(b):CCl_4 treated group on 8th day (100x)

Fig. 3 (a): CCl_4 supplemented with EECS 400 treated group on 4th day (100x)

Fig. 3 (b): CCl_4 supplemented with EECS 400 treated group on 8th day (100x)

Fig. 4(a): CCl_4 with Liv.52 (40mg/kg, p.o) treated group on 4th day (100x)

Fig. 4(b): CCl_4 with Liv.52 (40mg/kg, p.o) treated group on 8th day (100x)

DISCUSSION AND CONCLUSION

The present studies were performed to assess the hepatoprotective activity of ethanol (90%) extract of *Cordia subcordata* leaves in rats against carbon tetrachloride as hepatotoxin to prove its claims in folklore practice against liver disorders.

It is well documented that carbon tetrachloride-induced hepatic injury is commonly used as an experimental method for the study of hepatoprotective effects of drugs or medicinal plants' extracts, by in vivo and in vitro techniques **[19-21].** Carbon tetrachloride (CCl_4) is a potent hepatotoxin producing centrilobular hepatic necrosis. It is accumulated in hepatic parenchyma cells and metabolized to CCl_3 by liver cytochrome P450-dependent monooxygenases **[22].**

Usually, the extent of hepatic damage is assessed by histopathological evaluation and the level of hepatic enzymes ALT, AST and ALP release in circulation **[23].** The administration of CCl_4 resulted in a significant increase in the serum SGOT, SGPT, alkaline phosphatase (ALP) and acid phosphatase (ACP) and also total bilirubin and urea within 36 hours **[24, 25].** The rise in serum levels of AST, ALT, ALP and ACP has been attributed to the damaged structural integrity of the liver, because they are cytoplasmic in location and released into circulation after cellular damages **[26].**

In our study, the biochemical changes were observed after each 3 and 7 days. Thereby, it was found that, the administration of EECS at doses of 100, 200 and 400mg/kg, p.o for 3 days resulted in significantly decreases the CCl_4-induced elevated levels of the hepatic enzymes SGOT, SGPT, alkaline phosphatase (ALP) and acid phosphatase (ACP) in a dose dependent manner. These results indicating the production of structural integrity of hepatocytic cell membrane or regeneration of damaged liver cells by the extracts. Whereas, the EECS extracts at tested doses decreases the CCl_4-induced elevated level of hepatic enzymes in rats, and its subsequent return towards near normalcy after 7days. Reduction in the levels of SGOT and SGPT towards the normal value is an indication of regeneration process. Reduction of ALP levels with concurrent depletion of raised bilirubin level suggests the stability of the biliary function during injury with CCl_4.

Bilirubin is the conventional indicator of liver diseases **[27].** The rise in the levels of serum bilirubin is the most sensitive and confirms the intensity of jaundice **[28].** These biochemical restorations may be due to the inhibitory effects on cytochrome P450 or/and promotion of its glucuronidation **[29].** The marked elevation of bilirubin and urea level in the serum of group II CCl_4 intoxicated rats were significantly decreased in the groups III-V EECS treated animals after 3days. Whereas, after 7 days of treatment, bilirubin and urea level in the serum CCl_4 intoxicated rats subsequently return towards near normalcy in the groups III-V EECS treated animals. These results further substantiate *Cordia subcordata* as a potent hepatoprotective agent.

It has been reported that Liv.52 protects liver from the hepatotoxicity of carbon tetrachloride **[30, 31].** An appreciable protective effect was observed even after 3 days compared with 7 days treatment using marketed product (Liv.52). The extent of production by extracts appeared to depend on the duration of treatment. Overall, these results suggest that the EECS could protect the liver against damage induced by CCl_4 when comparable with Liv.52.

The attributivity of the observed alterations of SGOT, SGPT, alkaline phosphatase (ALP) and acid phosphatase

(ACP), serum ALT were confirmed by histopathological studies of liver sections which reveal that the normal liver architecture was disturbed by hepatotoxin (CCl_4) intoxication. In the liver sections of the rats treated with EECS extract for 7 days, the normal cellular architecture was retained as compared to Liv.52, thereby further confirming the potent hepatoprotective effect of *Cordia subcordata* leaves.

Further research is needed to isolate and purify the active principle involved in hepatoprotection of this plant as well as to confirm the mechanisms responsible for hepatoprotective activity. The present finding provides scientific evidence to the ethnomedicinal use of *Cordia subcordata* in treating hepatic disorders.

Acknowledgement

Authors are sincerely thankful to Dr. S. Venkatraman, M.Sc., M.D., Ph.D., Director and Mr.P.Muralidharan, M.Pharm. (Ph.D), Department of Pharmacology, C.L. Baid Metha Foundation for Pharmaceutical Education and Research, for their contribution and facilities provided regarding our Research work.

REFERENCES

[1] Wolf, P.L., *Indian Journal of Clinical Biochemistry* 1999, 14, 59–90.

[2] Handa, S.S., Sharma, A., and Chakraborti, K.K., *Fitoterapia* 1986, 57, 307-45.

[3] Venkateswaran, S., Pari, L., Viswanathan, P., Menon, V.P., *J Ethnopharmacol,* 1997, 57, 161–167.

[4] Latha, U., Rajesh, M.G,, Latha, M.S., *Ind Drugs*, 1999, 36, 470–473.

[5] Lupper, S., *Altern Med Rev*, 1999, 4, 179–189.

[6] Mitra, S.K., Venkataranganna, M.V., Sundaram, R., and Gopumadhavan, S.,

[7] *J. Ethnopharmacol,* 1998, 63, 181-186.

[8] Weiner, M.A., Secrets of Fijian Medicine. Govt. Printer, Suva, Fiji, 1984, 70.

[9] Whistler, W.A., Polynesian Herbal Medicine. Everbest, Hong Kong, 1992, 138-139.

[10] Whistler, W.A., J. Ethnopharmacol., 1985, 13 (3), 239-280.

[11] Harbone, J.P., Phytochemical Methods, A Guide to modern technique of plant analysis, (Chapmann and Hall, London), 1973, pp. 1-271.

[12] OECD, 2002. Acute oral toxicity. Acute oral toxic class method guideline 423 adopted 23.03.1996. In: Eleventh Addendum to the, OECD, guidelines for the testing of chemicals organisation for economical co-operation and development, Paris, June, 2000.

[13] Visweswaram, D., Rajeswara Rao, P., Satyanarayana, S., *Indian J Pharmacol.,* 1994, 26, 301 – 303.

[14] Reitman, S., Frankel, S., *Am J Clin Path.,* 1957, 28, 56-62.

[15] Kind, P.R.N., King, E.J., *J. Clin. Path.,* 1954, 7, 132-136.

[16] Malloy, H.T., Evelyn, K.A., *J Biol Chem,* 1937, 119, 481-485.

[17] Bousquet, B.F., Julien, R., Bon, R., Dreux, C., *Ann Biol Clin.,* 1971, 29, 415.

[18] Luna, L.G., Methods of Armed Forces Institute of Pathology, London, 1966, pp1–31.

[19] Galigher, A.E., and Kozloff, E.N., Essential Practical Microtechnique, 2nd edn, Lea and Febiger, Philadelphia 1971, 77–210.

[20] Kiso, Y., Tohkin, M., Hikino, H., *Planta Med.*, 1983, 49, 222–225.

[21] Allis, J.W., Ward, T.R., Seely, J.C., Simmons, J.E., *Fundamental Appl Toxicol.*, 1990, 15, 558–570.

[22] Cornelius, C.E., Animal Models in Liver Research. San Diego: Academic Press; 1993, 37, 341.

[23] Recknagel, R.O., Glende, E.A., Jr, Dolak, J.A., Waller, R.L., *Pharmacol Ther.,* 1989, 43, 139–154.

[24] Plaa, G., Charbonneau, M., In: Hayes, A.W. (Ed.), Principles and Methods of Toxicology. Raven Press, New York, 1994, pp 841–846.

[25] Wachstein, M., *Gastroenterol.,* 1959, 37, 525- 37.

[26] Max Wachstein., Elizabeth Meisel., and Carmen Falcon., *Am J Pathol.,* 1962, 40(2), 219–241.

[27] Sallie, R., Tredger, J.M., William, R., *Biopharm Drug Disp.,* 1991, 12, 251-259.

[28] Girish, S., Achliya, Sudhir., Wadodkar, G., Avinash, Dorle K., *J Ethnopharmacol.,* 2004, 90, 229-232.

[29] Cavin, C., Mace, K., Offord, E.A., Schilter, B., *Food Chem Toxicol.,* 2001, 39, 549–556.

[30] Drotman, R.B., Lawhorn, G.T., *Drug Chem Toxicol.,* 1978, 1, 163-171.

[31] Karandikar, S.M., Joglekar, G.V., Chitale, G.K., and Balwani, J.H., *Acta Pharmacol Toxicol.,* 1963, 20, 274-280.

[32] Meena, Kataria., and Singh, L.N., *Indian J Experimental Biol.*, 1997, 35, 655-657.

Reference # 19

ToxTest®

FINAL REPORT

A 7-Day Repeat Dose (Oral) Toxicity Screen on A4+

TEH-146
Sabell Corporation

FINAL REPORT

STUDY TITLE: **A 7-DAY REPEAT DOSE (ORAL) TOXICITY SCREEN ON A4+**

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Sponsor: Sabell Corporation

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Study Initiation Date: January 20, 2010

Study Completion Date: June 24, 2010

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SUMMARY

The intent of this study was to determine adverse effects resulting from daily, oral administration of Sabell A4+ dried extract, over the course of 7 days in male and female Sprague Dawley rats. Sabell A4+ dried extract was administered orally for 7 days to rats in aqueous suspensions at dosages of 2000 (high dose), 500 (mid dose), 125 (low dose) and 0 (controls) mg/kg BW. There was no overt evidence of toxicity related to the test article at any dosage administered. There was no apparent effect on general health, including clinical observations, body weight and food consumption. Analysis of clinical pathology and hematology parameters revealed minor statistically significant group differences mostly within the normal range, that were not considered to be biologically relevant test article related effects. These should be flagged as parameters to observe closely in future studies. The pathologic examination revealed no treatment related macroscopic findings, and one high-dose animal exhibited myocarditis on histopathological examination. Whether this was treatment related is an open question since only one animal was affected. The proposed 28 day repeated dose oral toxicity study is expected to reveal any treatment related pathologies and changes to clinical chemistry and hematology parameters in a more consistent way.

In summary, this study did not demonstrate any common or consistent adverse effects at the doses employed and under the conditions of the experimental protocol of this study. It was recommended that the 28-day repeated dose oral toxicity study on A4+ be conducted at the same doses.

COMPLIANCE STATEMENT

A 7-DAY REPEAT DOSE (ORAL) TOXICITY SCREEN ON A4+

This non-GLP study was conducted in accordance with ToxTest® Standard Operating Procedures.

The undersigned have reviewed and approved the report for final issuance.

Study Director:

_____ _____

Hugh Semple, PhD, DVM Date

Test Facility Management:

_____ _____

Donna M. Day Date

A 7-DAY REPEAT DOSE (ORAL) TOXICITY SCREEN ON A4+

1.0 GENERAL POINTS

1.1 Test Article

A4+

1.2 Nature and Purpose

The purpose of this study was to determine the toxicity of Sabell A4+ when given daily for 7 days in order to support an NHP product license and human clinical trials applications.

1.3 Study Plan No.

TEH-146 (Appendix A)

1.4 Supervisory Personnel

Head, Animal Care: Shawna Bast
Veterinary Care:. Dr. Hugh Semple
Statistician: Michelle Hiltz
Head, Pathology/Data Management/Pharmacy: Marja Rynsewyn

1.5 Test Method/Guideline

The design was a range-finding study in support of a 28-day repeated dose oral toxicity study to be conducted under GLP according to OECD 407.

1.6 Schedule of the Study

Experimental starting date: January 27, 2010
First day of acclimation: January 22, 2010
First day of pre-test period: January 25, 2010
Dosing date: January 29, 2010
Necropsy day: February 5, 2010
Experimental completion date: February 5, 2010

2.0 TEST ARTICLE INFORMATION

2.1 Test Article

- Name or Code: A4+
- Supplier: Sabell Corp.
- Appearance: Tan powder
- Purity: To be supplied by the sponsor. The sponsor assumes responsibility for characterization including identity, purity and stability of the bulk test article under the test conditions used in this study.
- Batch Number: 1-2009

- Storage: Room temperature
- Retest Date: March 31, 2010
  Fate of Test Item: Held for other studies

2.2 Vehicle

- Name or Code: Nanopure water with 0. 2% v/v of simethicone USP
- Supplier: In-house water, PCCA for simethicone (Lot #C128706 PCCA, exp. Sep. 16, 2011, Store @ room temperature)
- Batch Number: TR-365/1
- Storage: Room temperature
- Expiry Date: Feb. 23, 2010

2.3 Reference Article

- Name or Code: Vehicle: Nanopure water with 0. 2% v/v of simethicone USP
- Supplier: In-house, PCCA for simethicone (Lot #C128706 PCCA, exp. Sep. 16, 2011, Store @ room temperature)
- Batch Number: TR-365/1
- Storage: Room temperature
- Expiry Date: Feb. 23, 2010

2.4 Formulation of the Test Article

- Preparation: The dose materials, unless otherwise requested by the sponsor were calculated and prepared assuming the test article is 100% pure. An appropriate amount of A4+ dried extract was finely ground with a mortar and pestle. Appropriate amounts were weighed into Falcon tubes, 0.002 mL of simethicone USP/mL dosing solution, and an appropriate volume of water was added to make sufficient quantities of test article suspension for administering for each day's dosing. The mixtures were vortexed or mixed by a magnetic stirrer and stir bar to make a suspension. The suspension was again vortexed or mixed by a magnetic stirrer and stir bar immediately before taking up each dose for administration.
- Storage conditions after formulation: room temperature
- Special handling requirements after formulation: The suspension was vortexed to make it homogeneous before administration or mixed with magnetic stirrer and stir bar.
- Fate of remaining dosing solutions: Held until end of study, then discarded.
- Frequency of Preparations: Weighed out weekly for daily preparation.

2.5 Fate of Test Article

Unused test article will be held for use in future studies upon completion of the study. When all scheduled studies have been finished, the test article will be returned to the sponsor.

2.6 Test Article Concentration Analysis

Determination of concentration of the test article in the dose material was conducted. A 2 ml aliquot of the dose material was stored at below -15°C and shipped on ice to Dr. Duff Sloley, Phytovox, Inc. for analysis. The method is described in Appendix E.

3.0 EXPERIMENT PROCEDURE

3.1 Test System and Environment

3.1.1 *Species, Strain, Supplier and Specifications*

- Species/Strain: Rat, Sprague-Dawley
- Supplier: Charles River Canada Inc.
- Number of Animals in the Study: 40
- Age at Initiation of Treatment: 8-10 weeks
- Sex: Male and female
- Weight Range at Initiation of Treatment (grams): Male: 323.3-355.7 g
  Female: 212.2-238.4 g

3.1.2 *Environment and Husbandry*

The animal room environment was controlled with targeted conditions:

- Housing: individual
- Temperature: 18 - 26°C
- Relative Humidity: 30 - 70%
- Air Changes: 10 - 15 per hour
- Light Cycle: ~12 hours light
- Caging: Shoebox cages with environmental enrichment
- Bedding: Anderson's Bed-O-Cobs, Lot # 091808

3.1.3 *Diet and Water*

- Diet: Certified Rodent Chow – Irradiated (Certified Pico Rodent Diet Lot AUG 17 09 3A from LabDiet)
- Water: *ad libitum* reverse osmosis UV sanitized water

The study director has reviewed the feed, and bedding for contaminants and found none present.

3.2 Acclimation Procedures

- Animal Health Procedure: Rats were observed daily and body weights taken on the first day of the pre-test period. A health status report was generated prior to animals being released to the study (randomization).
- Acclimation Period: Animals were acclimated to rack and position for 7 days.
- Allocation to Treatment Group: Rats were randomized to group with a computer generated randomization procedure using a SAS PROC PLAN procedure to minimize differences in body weight between treatment groups.
- Identification of the Animals: temporary identification with permanent marker
- Identification Numbers: Specified in raw data
- Identification of the Cage: Cage card

3.3 Treatment

3.3.1 *Experimental Design*

This study involved administration of three dose levels of A4+ plus vehicle control once daily to male and female rats for 7 days as per Table 1, followed by euthanasia and necropsy.

Table 1. Study Design with Dosing Details

Treatment Group	Treatment	No. of Animals per Group		Dosage Level (mg/kg)	Dosage Concentration (mg/ml)	Dosage Volume (ml/kg)
		Male	Female			
1	Vehicle Control	5	5	0	0	6
2	Low dose A4+	5	5	125	20.81	6
3	Mid dose A4+	5	5	500	83.25	6
4	High dose A4+	5	5	2000	333	6

3.3.2 *Rationale for Dose Selection*
These doses were calculated as large multiples of the human dose.

3.3.3 *Route and Method of Administration*
- Route: oral
- Method: gavage
- Rate of Dosing: bolus
- Volume Administered: 6 mL/ kg
- Dosing was completed before 12:00 PM.

3.3.4 *Frequency and Duration of Treatment*
- Frequency: Once daily
- Number of administrations: 7

3.4 Examinations Performed During the Test Period

3.4.1 *Observations*
Once daily regular observations were done during the pre-test period.

Detailed clinical examinations were performed once daily prior to dosing on study days 1-7 as well as on Study Day 8, prior to euthanasia, Regular observations were made each day in the afternoon. Additional observations were done as deemed necessary for animal welfare.

3.4.2 *Body Weight*
Body weights were taken on all animals on the day of randomization, prior to randomization, Study Day 1 prior to dosing, and on Study Day 8 prior to euthanasia.

3.4.3 *Food Consumption*
Animals were fasted overnight beginning the afternoon of Study Day 7. Animals did not have food returned prior to necropsy on Study Day 8. Food consumption was measured on Study Day -4, on Study Day 1 and on Study Day 7 after food was taken away for overnight fast prior to Necropsy.

3.4.4 Blood Collection
Blood was collected on Study Day 8 prior to euthanasia via venipuncture of the abdominal vena cava and assayed for the following parameters:

Clinical Chemistry:

- Alanine Aminotransferase
- Albumin
- Albumin/Globulin ratio (calculated)
- Alkaline Phosphatase
- Aspartate Aminotransferase
- Calcium
- Chloride
- Cholesterol
- Creatine Kinase
- Creatinine
- Globulin (calculated)
- Glucose
- Sorbitol dehydrogenase
- Phosphorus
- Potassium
- Sodium
- Total Protein
- Total Bilirubin
- Triglycerides
- Urea Nitrogen

Hematology:

- Red Blood Cell count and morphology
- White Blood Cell count
- Differential White Blood Cell count from the instrument (agranulocytes and granulocytes.
- Hematocrit
- Hemoglobin
- Mean Cell Hemoglobin
- Mean Cell Volume
- Mean Cell Hemoglobin Concentration
- Platelet count

3.4.5 *Euthanasia*

Animals were euthanized via exsanguination under isoflurane anesthesia.

3.4.6 *Necropsy*

All rats underwent a necropsy under the supervision of a Board Certified Veterinary Pathologist. Any animals found dead or euthanized prior to the end of the study underwent a necropsy. Samples of the following tissues were collected for histopathological examination: liver, kidneys, adrenals, spleen, heart, lung, thymus, brain, testes or ovaries. In addition, any grossly abnormal tissues were examined and collected into 10% neutral buffered formalin at the discretion of the study director and pathologist. Histopathology was performed on all tissues from high dose and control animals only. Tissues from the mid and low dose animals were retained for future examination if deemed necessary by the study director and sponsor.

3.4.6.1 Organ Weights

The following tissues were weighed: liver, kidneys, adrenals, spleen, heart, lung, thymus, brain, testes or ovaries.

3.4.7 *Histology*

Histology was done on tissues and organs with gross lesions and others at the discretion of the pathologist, veterinarian or study director.

4.0 STATISTICAL ANALYSES

The Statistical report can be found in Appendix C.

- All statistical analyses were performed using SAS Release 9.2 for Windows XP.
- Statistical procedures were selected based on the distribution of the data and the validity of the assumptions. Statistical significance was declared when $p \leq 0.05$.
- All statistical analyses were done separately for each sex.
- Body weights from study day 8 were compared between treatment groups (1-4) using an analysis of variance with the body weights from study day 1 included as a covariate. In addition, body weights on study day 1 (pre-dose) were compared between treatment groups (1-4) using an analysis of variance.
- Average daily food consumption from study day 1 to 7 were compared between treatment groups (1-4) using an analysis of variance with the average daily food consumption from study day -4 to 1 (pre-dose) included as a covariate. In addition, average daily food consumption from study day -4 to 1 was compared between treatment groups (1-4) using an analysis of variance. Average daily food consumption (g/day) for each individual during each time period was calculated by dividing the total individual food consumption (in grams) by the number of days within the time period.
- Clinical chemistry and hematology parameters were compared between groups (1-4) using a one-way analysis of variance with the exception of TBIL, which was compared between groups using Cochran-Mantel-Haenszel's row mean score statistic.
- Organ weights were analyzed as absolute weights and as a proportion of total body weight using a one-way analysis of variance model to compare treatment groups (1-4).
- For statistical analysis of body weight, food consumption, organ weights, and clinical chemistry and hematology parameters, if the model revealed statistical significance ($p \leq 0.05$), Tukey-Kramer adjusted comparisons were used to determine if pairwise differences existed between treatment groups 1 to 4.

5.0 ARCHIVES

The following materials will be maintained in the archives of the test facility:

- Study plan and (if applicable) amendment(s).
- Raw data and final report.
- Organs collected and stored in 10% Neutral Buffered Formalin.
- Blocks.
- Slides.
- Analytical results.

6.0 RESULTS

6.1 Clinical Observations

Detailed clinical observations can be found in Appendix B, Table B1.

One control female had mild forelimb alopecia on Study Days 6 and 7. Two males, one from the mid dose group and one from the high dose group exhibited increased lung sounds from Study Day 5 onwards, but temperatures remained normal.

6.1.1 *Interpretation*

The alopecia is not considered to be treatment related. The animals exhibiting increased lung sounds may have aspirated a small amount of test item on Study Day 5; however, because the temperatures remained normal, it is unlikely that the observations indicate a significant health problem.

6.2 Body Weights

Detailed individual animal body weights can be found in Appendix B, Table B2. Summary statistics for body weight can be found in Appendix C, Table 1.

There were no differences among groups of either males or females at the beginning and the end of the study. The animals grew over the course of the study.

6.2.1 *Interpretation*

The lack of group differences at the beginning of the study indicates that the randomization process was conducted correctly. None of the treatments caused changes in body weight.

6.3 Food Consumption

Detailed food consumption data can be found in Appendix B, Table B3. Summary statistics for food consumption are found in Appendix C, Table 2.

There were no differences in food consumption among groups of either males or females at the beginning and end of the study. Food consumption was greater from study Day 1 to 7 than from Study Day -4 to Study Day 1.

6.3.1 *Interpretation*

During the study, there were no treatment related effects on food consumption. Food consumption increased as the animals grew.

6.4 Clinical Pathology

Detailed clinical pathology data can be found in Appendix B, Table B4. Summary statistics for clinical chemistry parameters are found in Appendix C, Table 3. Results of the statistical analysis of clinical chemistry parameters can be found in Appendix C, Table 4.

The high-dose males had significantly lower AST than controls. For ALT, the analysis of variance showed a significant group affect however, there were no pairwise differences observed. The mid and high dose females had significantly higher serum phosphate than the controls, and

the mid dose males had higher phosphate than the low dose males. The low dose males had higher cholesterol levels than the control males (+31%). In the males, the low and mid dose groups had significantly higher sodium levels than controls. The low dose females had higher potassium levels than the mid dose females or controls.

6.4.1 *Interpretation*

Minor statistically significant differences among groups such as those observed for AST and ALT in this study cannot be considered to be biologically significant.

Although statistically significant group differences were seen in phosphorus levels in both sexes, all group means were within normal range, and thus the differences cannot be considered to be biologically significant.

Cholesterol levels in the males differed between the low dose and control groups. The control level of cholesterol was the lowest among all the groups, and the mean was slightly below the normal range. Therefore, the difference was not considered to be biologically significant with respect to treatment effect.

Groupwise differences were observed in both sodium and potassium levels, however, the standard deviations were small, and for both electrolytes, all of the means were well within the normal range.

In summary, none of the groupwise statistical differences were deemed due to treatment effects.

6.5 Hematology

Hematology values can be found in Appendix B, Table B5a and blood cell morphology observations can be found in Appendix B, Table B5b. Summary statistics for hematology parameters can be found in Appendix C, Table 5. Results of statistical analyses of hematology parameters can be found in Appendix C, Table 6.

Hemoglobin values for high dose females were significantly greater than controls. Mid dose males had significantly higher hematocrit than control males. Low dose females had a lower mean MCV value than controls. In both females and males, MCHC was lower in the low dose group than in the controls.

6.5.1 *Interpretation*

All groupwise differences were minor variations within the normal range for each parameter and were not deemed to be treatment related.

6.6 Organ Weights

Detailed organ weight data can be found in Appendix B, Table B6. The summary statistics for organ weights are presented in Appendix C, Table 7. The results of statistical analysis or organ weights are presented in Appendix C, Table 8.

No differences among groups were observed for organ weights.

6.6.1 *Interpretation*

The A4+ treatments did not affect organ weight.

6.7 Macroscopic Examination

The Pathology report can be found in Appendix D. One female from the mid dose group had an ovarian cyst, which was interpreted as an incidental finding.

6.7.1 *Interpretation*
No treatment related effects of A4+ were found on macroscopic examination.

6.8 Histopathologic Examination

Results of the histopathologic examination can be found in the pathology report, Appendix D. Tissues from the low and mid dose group were not examined for histopathology.

One female in the high dose group was found to have an interstitial myocarditis.

6.8.1 *Interpretation*
While the myocarditis-affected female was a single animal, and no other animals in the high-dose group had any unexpected lesions, it will be necessary to evaluate the significance of this finding with respect to the 28 day study prior to making any conclusions about whether it is test article related. No other potentially treatment related effects of A4+ were found on histopathologic examination. It was therefore concluded that the test article did not have any common or consistent anatomic-pathologic effects at the doses employed and under the conditions of the experimental protocol of this study.

7.0 DEVIATIONS

7.1 Study Event

SDH Level 1control values recovering low. SDH Level 2 recovering low at the end of sampling.

7.2 Study Plan

Body weights were not taken on the first day of pretest period (on the day of arrival), but were taken four days later.

Neither deviation was deemed to have a negative impact upon the study.

8.0 CONCLUSIONS

No significant treatment related effects were encountered in the study. For the few findings that raised questions over whether they were treatment related, the planned 28 day repeated dose oral toxicity study will provide a more sensitive examination of the highlighted organ systems. It is recommended that the same test item doses as used in this study be used for the 28 day repeated dose oral toxicity study.

Reference # 20

ToxTest®

FINAL REPORT

A 28-DAY REPEAT DOSE ORAL TOXICITY STUDY OF A4+ IN RATS

TEH-148
Sabell Corporation

FINAL REPORT

STUDY TITLE: **A 28-DAY REPEAT DOSE ORAL TOXICITY STUDY OF A4+ IN RATS**

STUDY PLAN NO.: TEH-148

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Study Initiation Date: February 8, 2010

Study Completion Date: March 29, 2011

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APPENDICIES

SUMMARY

The purpose of this study was to determine the toxicity of Sabell A4+ when given daily for 28 days in order to support an NHP product license and human clinical trials applications. The design was a repeat dose oral toxicity study integrating a micronucleus genotoxicity test conducted under GLP according to OECD 407and 474 guidelines. Four groups (Control (1); Low dose (2), 125 mg/kg A4+; Mid dose (3), 500 mg/kg A4+; High Dose (4), 2000mg/kg A4+) of 5 female and 5 male Sprague Dawley rats were administered test or reference item by oral gavage daily for 28 days. A fifth satellite group of rats served as the positive control group (5) for micronucleus arm of the study conducted at the end. Group 5 rats received 20 mg/kg of cyclophosphamide via intraperitoneal injection once one day prior to the study termination on day 29.

Animals were observed twice daily, weighed weekly and food consumption was measured weekly. During the last week of the study, functional observational batteries were conducted on all Groups 1 to 4 animals. On study day 29, Groups 1 to 4 animals were exsanguinated under anesthesia and the collected blood was analyzed for clinical chemistry and hematology including coagulation. The euthanized animals were necropsied and tissues were collected (full list for Groups 1 to 4, including bone marrow, and bone marrow only for Group 5).Selected organs were weighed. Tissues from the high dose and control groups were processed and histopathological examination was conducted. Bone marrow smears from the control, high dose and positive control groups were scored for the micronucleus test.

No significant test item-related effects were observed among clinical observations, body weights, food consumption, functional observational battery (indicating a lack of neurobehavioral toxicity), urinalysis, clinical pathology parameters, blood coagulation parameters, mortality, or macroscopic and histopathological findings. Small but statistically significant differences in some hematology results and organ weights were deemed to have low biological significance. Given that the positive control animals in the micronucleus test satellite group failed to respond to the positive control agent cyclophosphamide, the micronucleus test has been repeated in a separate study, TEH-153. For TEH-153, it was concluded that under the repeated dose conditions of the experiment, A4+ was negative for the production of elevated micronucleus counts and did not exhibit bone marrow toxicity. A no adverse effect dose level (NOAEL) of 2000 mg/kg was assigned for genotoxicity and bone marrow toxicity based on the results of the study, combined with those of TEH-147. Details on TEH-147 and TEH-153 can be found in their separate reports.

It was concluded that A4+ did not exhibit toxicity, including neurotoxicity, under the conditions of this study and a no adverse effect level (NOAEL) of 2000 mg/kg can be assigned.

COMPLIANCE STATEMENT

A 28-DAY REPEAT DOSE ORAL TOXICITY STUDY OF A4+ IN RATS

This study was conducted in compliance with OECD Principles of Good Laboratory Practice (ENV/MC/CHEM(98)17), with the following exceptions. Test item characterization and dose concentration analysis were not conducted in compliance with GLP as A4+ contains extracts of herbs for which no analytical standard is currently available. A different statistical method was used for micronucleated PCE's than that stated in the study plan.

The undersigned have reviewed and approved the report for final issuance.

Study Director:

Hugh A. Semple
Hugh Semple, DVM, PhD

March 29, 2011
Date

Test Facility Management:

Curtis Kuzyk

25/Mar/2011
Date

QUALITY ASSURANCE STATEMENT

A 28-DAY REPEAT DOSE ORAL TOXICITY STUDY OF A4+ IN RATS

Inspection Date	QAI #	Inspection Type	Phase	Date Reported to Study Director and Management
5-Feb-10	QAI0803	Study Plan review	Study Plan	5-Feb-10
16-Feb-10	QAI0800	Study -based	Dose Preparation	17-Feb-10
16-Feb-10	QAI0801	Study -based	Dosing	17-Feb-10
19-Feb-10	QAI0802	Study -based	Test Item Documentation	19-Feb-10
25-Feb-10	QAI0804	Study -based	Interim Raw Data Audit	1-Mar-10
8-Mar-10	QAI0806	Study -based	Dose Preparation	8-Mar-10
8-Mar-10	QAI0807	Study -based	Clinical Observations	8-Mar-10
8-Mar-10	QAI0808	Study -based	Body Weights	8-Mar-10
8-Mar-10	QAI0809	Study -based	Food Consumption	8-Mar-10
8-Mar-10	QAI0810	Study -based	Dosing	8-Mar-10
12-Mar-10	QAI0811	Study -based	Functional Observation Battery	12-Mar-10
16-Mar-10	QAI0815	Study -based	Necropsy	18-Mar-10
16-Mar-10	QAI0816	Study -based	Bone Marrow Flushing	18-Mar-10
17-Mar-10	QAI0817	Study -based	Smear Preparation	18-Mar-10
16-Mar-10	QAI0818	Study -based	Clinical Chemistry	18-Mar-10
16-Mar-10	QAI0819	Study -based	Coagulation	18-Mar-10
17-Mar-10	QAI0820	Study -based	Hematology	20-Mar-10
17-Mar-10	QAI0821	Study -based	Urinalysis	18-Mar-10
17-Jan-11 to 21-Jan-11	QAI0851	Raw Data Audit	Raw Data	23-Jan-11
17-Jan-11 to 21-Jan-11	QAI0852	Draft Report	Draft Report	23-Jan-11
3-Mar-11 to 7-Mar-11		Final Report	Final Report	7-Mar-11

The reported results in this final report accurately and completely reflect the raw data.

Quality Assurance Auditor

March 9/11
Date

A 28-DAY REPEAT DOSE ORAL TOXICITY STUDY OF A4+ IN RATS

1.0 GENERAL POINTS

1.1 Test Item

Sabell A4+

1.2 Nature and Purpose

The purpose of this study was to determine the toxicity of Sabell A4+ when given daily for 28 days in order to support an NHP product license and human clinical trials applications.

1.3 Study Plan No.

TEH-148 (Appendix A)

1.4 Supervisory Personnel

Head, Animal Care: Shawna Bast
Veterinary Care: Dr. Hugh Semple
Statistician: Michelle Hiltz
Head, Pathology/Data Management/Pharmacy: Marja Rynsewyn

1.5 Regulatory Compliance

This was a GLP study and was conducted in accordance with OECD GLPs and ToxTest Standard Operating Procedures with the exception of test item characterization and dose concentration analysis which was not conducted in compliance with GLP as A4+ contains extracts of herbs for which no analytical standard is currently available.

1.6 Test Method/Guideline

The design was a repeat dose oral toxicity study integrating a micronucleus genotoxicity test conducted under GLP according to OECD 407 (28-day repeated dose oral toxicity) and OECD 474 (integrated multiple dose micronucleus protocol) guidelines.

1.7 Schedule of the Study

Experimental starting date: February 13, 2010
First day of acclimation: February 10, 2010 (Groups 1-4), March 12, 2010 (Group 5)
First dosing date: February 16 & 17, 2010 (Groups 1-4), March 16, 2010 (Group 5)
Necropsy day: March 16 & 17, 2010
Experimental completion date: March 17, 2010

2.0 TEST ITEM INFORMATION

2.1 Test Item

- Name or Code: A4+
- Supplier: Sabell Corporation
- Appearance: Tan powder
- Purity: Supplied by the sponsor. Test item purity and stability and homogeneity were assessed and the reports are found in Appendices F and H.
- Batch Number: 1-2009
- Storage: Room temperature
- Retest Date: March 31, 2010
- Fate of Test Article: Returned to Sponsor upon completion of the studies.

2.2 Vehicle 1

- Name or Code: Nanopure water with 0.2%v/v of simethicone USP
- Supplier: In-house water, PCCA for simethicone (Lot #C128706 PCCA, exp. Sep. 2011, Store @ room temperature); see Appendix G for certificate of analysis.
- Storage: Room temperature
- Expiry Date: March 23, 2010

2.3A Reference Item 1 (Vehicle Control)

- Name or Code: Vehicle: Nanopure water with 0. 2%v/v of simethicone USP
- Supplier: In-house, PCCA for simethicone (Lot #C128706 PCCA, exp. Sep. 16, 2011, Store @ room temperature); see Appendix G for certificate of analysis.
- Storage: Room temperature
- Expiry Date: March 23, 2010

2.3B Reference Item 2 (positive control)

- Name or Code: Cyclophosphamide monohydrate
- Supplier: Sigma-Aldrich Canada Ltd.
- Batch Number: 068K1131; see Appendix G for certificate of analysis.
- Storage: 2-8°C
- Expiry Date: August 2011

2.3C Vehicle 2

- Name or Code: Sterile Water, sterile filtered, cell culture tested
- Supplier: Sigma-Aldrich Canada Ltd.
- Batch Number: RNBB1408; see Appendix G for certificate of analysis.
- Storage: Room temperature
- Expiry Date: November 2011

2.4 Formulation of the Test Item and Reference Item 2

2.4.1 *Test Item*

- Preparation: The dose materials were calculated and prepared assuming the test item was 100% pure. An appropriate amount of A4+ dried extract was finely ground with a mortar and pestle. Appropriate amounts were weighed into Falcon tubes, 0.002 mL of simethicone USP/mL dosing solution, and an appropriate volume of water was added to make sufficient quantities of test item suspension for administering for each day's dosing. The mixtures were vortexed to make a homogeneous suspension. The suspension was again mixed by a magnetic stirrer and stir bar immediately before taking up each dose for administration. Some dosage preparations were weighed out ahead of time, but were prepared daily. The suspension was stable for at least 6 hours after preparation.
- Storage conditions after formulation: room temperature
- Special handling requirements after formulation: The suspension was mixed with a magnetic stirrer and stir bar to make it homogeneous before administration.
- Fate of remaining dosing solutions: Discarded each day when dosing was finished
- Frequency of Preparations: Once on day of dosing

2.4.2 *Reference Item 2*

- Preparation: A 2 mg/ml cyclophosphamide solution was prepared on the day of dosing.
- Storage conditions after formulation: Room temperature if used within 2 hours, or if more than 2 hours, at 2 – 8° C.
- Fate of remaining dosing solutions: discarded after completion of draft report
- Frequency of Preparations: once on day of dosing

2.5 Fate of Test Item

Unused test item was transferred to study TEH-153.

2.6 Test Item Concentration Analysis

Determination of concentration of the test item in the dose material and the reference items was conducted. A 2 ml aliquot of the dose material was stored frozen at below -15° C and shipped on ice to Dr. Duff Sloley, Phytovox, for analysis. Samples were sent on Study Day 1 and Day 21. A sample of cyclophosphamide dosing solution that had been stored refrigerated from March 16, 2010 was sent for analysis on May 19, 2010.

The method used to analyze the samples, along with supporting data, is provided in Appendix E.

2.7 Retainer Sample

A 2 mg retainer sample of reference and test item and 2 mL of vehicle water will be stored at room temperature.

3.0 EXPERIMENT PROCEDURE

3.1 Test System and Environment

3.1.1 *Justification for Selection of Test System*
Standard species for test guidelines (OECD 407/474)

3.1.2 *Species, Strain, Supplier and Specifications*

- Species/Strain: Rats, Sprague-Dawley
- Supplier: Charles River Canada Inc.
- Number of Animals in the Study: 50
- Age at Initiation of Treatment: 8-10 weeks
- Sex: male and female
- Weight Range at Initiation of Treatment (grams, Groups 1-4):
  Male: 275.3-315.6 g
  Female: 188.7-211.9 g

3.1.3 *Environment and Husbandry*

The animal room environment was controlled with targeted conditions:

- Housing: Individual
- Temperature: 18 - 26°C
- Relative Humidity: 30 - 70%
- Air Changes: 10 - 15 per hour
- Light Cycle: ~12 hours light
- Caging: Shoebox cages with Bed-O-Cobs corn cob bedding (The Anderson's, Inc.), and metal tube environmental enrichment

3.1.4 *Diet and Water*

- Diet: Certified Pico Rodent Diet (LabDiet) – Irradiated
- Water: *ad libitum* reverse osmosis UV sanitized water

The study director reviewed the feed and bedding for contaminants and found none present.

3.2 Acclimation Procedures

- Animal Health Procedure: Rats were observed daily and body weights taken on the day of randomization. A health status report was generated prior to animals being released to the study (randomization).
- Acclimation Period: Animals were acclimated to rack and position for 6 days prior to the start of the study (Groups 1-4) and for 4 days (Group 5). Animals in Group 5 arrived on Study Day 24 and were dosed on Day 28.
- Allocation to Treatment Group: Groups 1-4 were allocated with a computer generated randomization program using the SAS PROC PLAN procedure to minimize differences in body weight between treatment groups. This was a staggered study so that the end of in-life data could be collected over 2 study days. These groups were randomized across the two days of the staggered study. Group 5 animals were manually randomized to rack and position and were not randomized to Group by body weights.
- Identification of the Animals: tail tattoo, except for Group 5 animals, which received temporary identification with a marker.
- Identification Numbers: Specified in raw data
- Identification of the Cage: Cage card

3.3 Treatment

3.3.1 *Experimental Design*

This study involved administration of three dosage levels of A4+ plus vehicle control to male and female rats daily for 28 days (with the exception of Treatment Group 5, which received a single dose of cyclophosphamide) as per Table 1, followed by euthanasia and necropsy.

Table 1. Study Design with Dosing Details

Treatment Group	Treatment	No. of Animals per Group		Dosage Level (mg/kg BW)	Dosage Concentration (mg/ml)	Dosage Volume (ml/kg BW)
		Male	Female			
1	Vehicle Control	5	5	0	0	6
2	Low dose A4+	5	5	125	20.81	6
3	Mid dose A4+	5	5	500	83.25	6
4	High dose A4+	5	5	2000	333	6
5	Positive Control (cyclophosphamide)	5	5	20	2	10

3.3.2 *Rationale for Dose Selection*

The doses of test item (A4+) were calculated as large multiples of the human dose and are the same as for the 7 day repeated dose study in which little evidence of toxicity was observed. The dose of cyclophosphamide was the standard positive control dose for rats used in micronucleus studies (Krishna & Hayashi, 2000).

3.3.3 *Route and Method of Administration*

Vehicle control and A4+:

- Route: oral
- Method: gavage
- Rate of Dosing: bolus
- Volume Administered: 6 mL/ kg BW
- Dosing was completed before 12:00 PM, except for Study Day 28, which was scheduled to accommodate conducting necropsys 18 to 24 hours after the last dose.

Cyclophosphamide:

- Route: intraperitoneal
- Method: injection
- Rate of Dosing: bolus
- Volume Administered: 10 mL/ kg BW

3.3.4 *Frequency and Duration of Treatment*

Vehicle Control and A4+:

- Frequency: Once daily
- Number of administrations: 28

Cyclophosphamide:

- Frequency: Once
- Number of administrations: Once, on Day 28

3.4 Examinations Performed During the Test Period

3.4.1 *Observations*

Groups 1-4:

Animals were observed twice daily, with one observation prior to dosing and the second 3-6 hours post-dosing, with particular attention to possible toxic effects in the post-dosing observation; clinical observations were made before the first dosing, then weekly thereafter.

Group 5:

Animals were observed twice on Study Day 28, with one observation prior to dosing and the second 3-6 hours post-dosing, with particular attention to possible toxic effects in the post-dosing observation; clinical observations were made before dosing.

3.4.2 *Body Weight*

Groups 1-4:

Body weights were taken on all animals on Study Day 1 and weekly thereafter prior to dosing and on Study Day 29 prior to necropsy.

Group 5:

Body weights were taken on Study Day 28 prior to dosing. These weights were used to calculate dose.

3.4.3 *Food Consumption*

Groups 1-4:

Food consumption was measured weekly, starting with the first day of dosing. Note: Animals were fasted overnight during urine collection and before necropsy, so last food consumption determination was completed before fasting began on Day 28.

Group 5:

Food consumption was not necessary.

3.4.4 *Functional Observational Battery*

Functional Observational Batteries (FOB) were done on all animals in Groups 1-4 during the last week of the study.

The FOB's were conducted according to a test facility SOP. The test included a cage-side assessment, handling assessment, open field and neuromuscular measurements (foot splay and fore- and hind-limb grip strength). The observers had to properly identify from a test facility SOP, the behavioral changes according to the Adult FOB key, and record them on the appropriate form. The order of the animals from the 4 groups (4 treatments X 2 sexes) being tested was randomized and counterbalanced to minimize the effect of time of day when testing was done. The animals were already randomized to rack placement and therefore they were tested in rack order. This met the requirement for randomization and counterbalancing and reduced the risk of errors.

For reference, the Functional Observational Battery measurement scale used is as follows:

ADULT RAT FUNCTIONAL OBSERVATIONAL BATTERY (FOB) LEGEND

BRIEF HOME-CAGE OBSERVATIONS (PART I)

(quick assessment (5-10 sec); observer's presence may affect activity)
Posture (rank): Posture is defined as the animal's position in the home cage when first approached by the observer.

0. normal (sitting normally, curled up often asleep, standing or sitting alert, grooming, rearing, walking around the cage)
1. non-normal lying (lying on side or back)
2. agitated posture (rapid motions including jumping, running, rolling, digging)

Involuntary movements
Tremors (descriptive): rhythmical, repetitive flexing of muscles resulting from alternating contractions of opposing muscle groups.

0. none (no tremors, includes mild shivers that are not abnormal)
1. mild to moderate movements
2. severe movements on selected muscle groups
3. severe whole body tremors

Convulsions: clonic (descriptive): characterized by contraction of the voluntary muscles followed by relaxation and contraction again.

0. none
1. clonic: a coordinated, asymmetrical convulsion with natural, purposeful-like movements, sometimes preceded by excitement ("paddling" motion of the forelegs of the animal)
2. clonic symmetrical: repetitive symmetrical jerks or twitches of the limbs
3. running excitement: often accompanied by mild clonus or leading to severe convulsion
4. repetitive movements of mouth and jaws (smacking)
5. popcorn: animal repeatedly jumps in the air
6. asphyxial: bout of severe clonic-tonic convulsions resulting in difficult respiration or death

Convulsions: tonic (descriptive): characterized by prolonged muscular tension of a set of voluntary muscles

0. none
1. contraction of extensors such that limbs are rigid and extended
2. opisthotonous (seizure where the head, body, and limbs are rigidly arched backward)
3. emprosthotonus (seizure where the head, body, and limbs are extended forward)

Wasting (quantal): flanks are concave in appearance and the animal's ribs may be visible

0. no (appears normal)
1. yes (appears emaciated/dehydrated)

Palpebral closure (descriptive): record the degree of eye opening; the drooping eyelid is usually associated with a decrease in sympathetic activity

0. eyelids wide open (normal)
1. eyelids completely shut (normal if sleeping)
2. eyelids slightly drooping
3. ptosis (drooping eyelids approximately half-way)

HANDLING OBSERVATIONS (PART II)

Ease of removal (rank): the ease with which the animal is removed from its home cage
0. normal (easy to remove, slight resistance to being picked up, may rear and follow observer's hand; with or without vocalizations)
1. very easy (no reaction, lethargic)
2. freezes (with or without vocalizations)
3. difficult (runs around cage, hard to grab, with or without vocalizations)
4. very difficult (tail and throat rattles, with or without vocalizations, may attack hand, hyper-reactive)

Ease of handling (rank): responsiveness to being handled after removal
0. normal (easy to handle, alert, limbs may be pulled up against body, with or without vocalizations)
1. nonreactive (rat is totally limp)
2. freezes (rigid in hand with or without vocalizations)
3. hyper-reactive (squirming, twisting, attempting to bite, with or without vocalizations)

Salivation (rank): evidenced by wetness around mouth/chin
0. normal (no observable saliva around mouth)
1. slight (small amount of saliva around mouth)
2. moderate
3. severe (mouth and muzzle wet with saliva)

Piloerection (quantal): uniform standing of the fur on end caused by fear, cold, or pharmacological effect of a drug
0. no (appears normal)
1. yes (ruffled fur, increasingly puff ball-like appearance)

Muscle tone (rank): evaluated by pushing plantar surface of each hindpaw several times with the index finger (determines resistance to passive flexion); if no resistance, grasp between thumb and index finger, and extend and flex several times
0. normal, slight resistance
1. completely flaccid, no return, hypotonia
2. slightly flaccid, rapid or very slow return
3. moderate resistance
4. extreme resistance, board like, hypertonia

Eyes
Exopthalmus (rank): bulging of the eye(s), usually associated with sympathetic stimulation
0. none
1. slight
2. moderate
3. severe

Red crusty deposits (rank): red crusty deposits around the eye(s)
0. none
1. slight
2. moderate
3. severe

Conjunctivitis (rank): inflammation of conjunctiva (mucous membrane which lines the eyelids and is reflected into the eyeball)
0. none
1. slight
2. moderate
3. severe

Ocular exucate (rank): secretion directly from eye (usually transparent and yellow)
0. none
1. slight
2. moderate
3. severe

Opacity (rank): loss of transparency of the lens
0 none
1. slight
2. moderate
3. severe

Lacrimation (rank): secretion of tears; includes chromodacryorrhea (reddish conjunctival exudates without the presence of blood cells)
0 none
1. slight
2. moderate
3. severe

Fur appearance (descriptive)
0. normal
1. barbered (patches of fur missing)
2. slightly soiled
3. very soiled, crusty

Mouth and Nose Deposits (rank): red crusty deposits around the nose or mouth, and/or nasal discharge
0. none
1. slight
2. moderate
3. severe

OPEN FIELD OBSERVATIONS (PART III)

Posture (rank): Posture is defined as the animal's position in the home cage when first approached by the observer.
0. normal (sitting normally, curled up often asleep, standing or sitting alert, grooming, rearing, walking around the cage)
1. non-normal lying (lying on side or back)
2. agitated posture (rapid motions including jumping, running, rolling, digging)

Involuntary movements

Tremors (descriptive): rhythmical, repetitive flexing of muscles resulting from alternating contractions of opposing muscle groups.

0. none (no tremors, includes mild shivers that are not abnormal)
1. mild to moderate movements
2. severe movements on selected muscle groups
3. severe whole body tremors

Tremors (rank): *also need to score severity*

0. none
1. slight
2. moderate
3. severe

Clonic convulsions (descriptive): characterized by contraction of the voluntary muscles followed by relaxation and contraction again.

0. none
1. clonic: a coordinated, asymmetrical convulsion with natural, purposeful-like movements, sometimes preceded by excitement ("paddling" motion of the forelegs of the animal)
2. clonic symmetrical: repetitive symmetrical jerks or twitches of the limbs
3. running excitement: often accompanied by mild clonus or leading to severe convulsion
4. repetitive movements of mouth and jaws (smacking)
5. popcorn: animal repeatedly jumps in the air
6. asphyxial: bout of severe clonic-tonic convulsions resulting in difficult respiration or death

Convulsions: clonic (rank): *also need to score severity*

0. none
1. slight
2. moderate
3. severe

Tonic convulsions (descriptive): characterized by prolonged muscular tension of a set of voluntary muscles

0. none
1. contraction of extensors such that limbs are rigid and extended
2. opisthotonous (seizure where the head, body, and limbs are rigidly arched backward)
3. emprosthotonus (seizure where the head, body, and limbs are extended forward)

Convusions: tonic (rank): *also need to score severity*

0. none
1. slight
2. moderate
3. severe

Abnormal motor movements

Writhing (rank): twisting or wave-like undulatory movement of part or all of the body; usually involves a flattening of the abdominal wall, accompanied by asymmetrical stretching and extending of the body and hindlimbs

0. none
1. slight
2. moderate
3. severe

Circling (rank): repeated movement of the animal in a circular manner
0. none
1. slight
2. moderate
3. severe

Bizarre behaviors (descriptive) *allow for more than one observation*
0. none
1. hallucinatory-like (animal appears to be responding to objects not present)
2. upright walking (walking on hindlimbs only)
3. aimless wandering (slow, plodding movements around the open field with no apparent purpose)
4. retropulsion (walking backward)
5. spatial disorientation (walking, stumbling into objects)
6. other (describe in detail)

Stereotypic behaviors (descriptive) *allow for more than one observation*
0. none
1. head flicking (head shaking or backward flip of head)
2. head searching: stereotyped, repetitive turning of the head from side to side, as though searching the environment
3. compulsive biting (biting of objects in open field)
4. compulsive licking (licking objects in open field)
5. self-destructive biting (biting of itself, usually the toes, with bleeding)
6. prancing forelimbs: restless shifting from one forelimb to the other, with slight turning of the body from side to side

Gait (descriptive): animal's manner of movement
0. normal
1. ataxia, excessive sway, rocks, or lurches as the animal proceeds forward
2. body drags and stomach makes contact with the surface
3. forelimbs dragging, unable to support weight
4. motionless

Total gait score (rank): ability to locomote despite abnormalities in gait
0. normal
1. somewhat impaired
2. totally impaired

Rearing (rank): animal rears on hind legs, *count number of rears in timed open field only
0.0-5 rears
1.6-10 rears
2.more than 10 rears

Vocalizations (quantal): record the presence of spontaneous vocalizations during testing in open field

0. no vocalizations
1. vocalizations

Arousal level (rank): level of alertness in the open field
0. normal (alert, vibrissae movements, exploratory movements)
1. depressed arousal (stupor, no responsiveness, or some head or body movements with periods of immobility)
2. moderate (some attention to surroundings, sniffing, some periods of inattention – less than normal though)
3. hyper-aroused (hyper-alert, excited tense sudden darting or freezing)

Urination (rank): based on approximate number of pools following 2-minute testing period
0. none
1. slight (1-2 pools, small dribbles)
2. some (3-5 pools, small dribbles)
3. many pools (>5 pools, small dribbles)
4. polyuria (overlapping pools, or very large pools)

Urination/characteristics (descriptive): based on the characteristics of the urine pools
0. normal in color
1. very light in color
2. very dark in color
3. other (describe in detail)

Defecation (rank): based on number of fecal boluses on paper following 2-minute testing period
0. none
1. slight (1-2 boluses)
2. some (3-5 boluses)
3. many boluses (>5)

Defecation/characteristics (descriptive): based on type of fecal boluses
0. normal
1. very dry
2. very soft
3. bloody
4. liquid stool/diarrhea
5. other (describe in detail)

SENSORY AND NEUROMUSCULAR OBSERVATIONS (PART IV)

Approach response (rank): response is measured by approaching the animal head-on with a blunt object (e.g. eraser end of a pencil); object is held approximately 3 cm from the face of the animal for approximately 4 seconds, and the response of the animal is rated
0. normal (animal slowly approaches and sniffs at the object)
1. no reaction from the animal
2. animal pulls away slightly or jumps and turns away from object
3. animal freezes
4. animal jumps at object, attacks, and bites

Startle response (rank): response is measured by depressing a clicker behind the head of the animal being tested, and the response of the animal is rated

0. normal (animal flinches or flicks ears)
1. no reaction
2. animal jumps, flips, or shows a violent or bizarre reaction

Tail pinch (rank): measured by pinching the animal's tail with forceps approximately 5 cm from the tip, and response is rated

0. normal response (animal immediately turns, might bite at the site)
1. analgesia (no response from the animal)
2. indifference (animal sluggishly turns to look, may sniff the site, or walk away from the stimulus)
3. freezing (animal freezes, with or without vocalizations)
4. exaggerated response (animal turns rapidly, may jump, bite or attack)

Pupil size (rank)

0. normal (pupil appears to be normal size)
1. constricted (pupil is smaller than normal)
2. dilated (pupil appears to be larger than normal)

Pupil response (quantal): bring a pen light in from the side of the animal's head and note the changes to the pupil size

0. normal response (constriction of pupil)
1. lack of response

3.4.5 *Urinalysis*

Timed collections on Groups 1-4 animals were done during overnight fast, prior to necropsy.

Urine was analysed macroscopically (ie colour and turbidity, manual inspection) and microscopically (manually with an Olympus BH2 Microscope) according to a test facility SOP.

Chemical analysis for urine pH, leukocytes, nitrite, protein, glucose, ketones, urobilinogen, bilirubin and blood was measured on a Urisys 1100 Urine test strip reader with Chemstrip 10A testing strips (Roche Diagnostics, 201 Armand Frappier Blvd. Laval, PQ).

Urine specific gravity was measured using a Sper Digital Refractometer 300036 (Manufacturer: Sper Scientific Ltd. 7720 East Redfield, Suite 7, Scottsdale, Arizona 85260, USA, supplier: Anachemia Science, Anachemia Canada Inc., 15006-116 Ave. Edmonton AB T5M 3T4)

3.4.6 *Euthanasia*

Exsanguination was done under anesthesia with isoflurane.

3.4.7 *Blood Collection*

Groups 1-4:

Blood was collected on Study Day 29 prior to euthanasia via venipuncture of the abdominal vena cava and assayed for the following parameters:

Clinical Chemistry:

The parameters in Table 2 below were evaluated on a Hitachi 912 Automatic Analyzer:

Table 2. Clinical chemistry parameters analyzed.

Parameter Analyzed	Method of Analysis	Reagent Manufacterer
Alanine Aminotransferase	IFCC without pyridoxal (UV test)	Roche
Albumin	Bromocresol green	Roche
Albumin/Globulin Ratio	Calculated (ALB/GLOB)	N/A
Alkaline Phosphatase	ALP-IFCC liquid	Roche
Aspartate Aminotransferase	IFCC without pyridoxal	Roche
Calcium	o-Cresolphtalein complexone	Roche
Chloride	Ion Selective Electrode with dilution	Roche
Cholesterol	CHOD-PAP (enzymatic colorimetric)	Roche
Creatine Kinase	CK-liquid IFCC	Roche
Creatinine	Jaffe (kinetic) rate-blanked and compensated	Roche
Globulin	Calculated (total protein – albumin)	N/A
Glucose	GOD-PAP	Roche
Sorbitol dehydrogenase	Enzymatic – NADH to NAD^+	Catachem
Phosphorus	Molybdate	Roche
Potassium	Ion Selective Electrode with dilution	Roche
Sodium	Ion Selective Electrode with dilution	Roche
Total Bilirubin	Jendrassik	Roche
Total Protein	Biuret	Roche
Triglycerides	GPO-PAP (enzymatic colorimetric)	Roche
Urea Nitrogen	Urea kinetic (UV)	Roche

Hematology:

The following parameters were evaluated on an Abbott Cell-Dyn® 3700 CS using Abbott reagents:

- Red Blood Cell count and morphology
- White Blood Cell count
- Differential White Blood Cell Count (Manual).
- Hematocrit
- Hemoglobin
- Mean Cell Hemoglobin
- Mean Cell Volume
- Mean Cell Hemoglobin Concentration
- Platelet count

Blood taken on Study Day 29 was assayed for coagulation parameters:

Coagulation Panel:

The following parameters were evaluated on a Coagamate® XM using Somagen reagents:

- Prothrombin time (PT)
- Partial Thromboplastin time (PTT)

Group 5:
Blood collection was not necessary. Blood was discarded after exsanguination.

3.4.8 *Necropsy*

All **Groups 1-4** rats were euthanized by exsanguination under isoflurane anesthesia on study day 29 following final blood collections and underwent a necropsy under the supervision of a Board Certified Veterinary Pathologist. All animals underwent a necropsy. The following tissues were examined and collected into 10% neutral buffered formalin from all animals from Groups 1-4. A femoral bone marrow flush on all Groups 1-5 animals was collected according to micronucleus assay protocols and processed for future examination.

- Brain
- Spinal Cord
- Stomach
- Duodenum
- Jejunum
- Ileum
- Cecum
- Colon
- Rectum
- Liver
- Kidneys
- Adrenals
- Spleen
- Muscle
- Sciatic Nerve
- Heart
- Thymus
- Thyroids
- Trachea
- Lungs (inflated with formalin)
- Prostate
- Seminal Vesicles
- Epididymides
- Testes
- Uterine Horns
- Ovaries
- Oviducts
- Vagina
- Urinary Bladder
- Mesenteric Lymph Nodes
- Mandibular Lymph Nodes
- Sternum with marrow
- Bone marrow via flush

3.4.9 *Organ Weights*

The following organs from Groups 1-4 were dissected, trimmed free of fat and weighed for each rat. Paired organs were weighed together.

Liver, kidneys, adrenals, testes or ovaries, epididymes or uterine horns, thymus, spleen, brain, heart, lungs (weighed before inflation with formalin). The brain-to-body-weight ratio was calculated using the last total body weight measured.

Organs were not collected from Group 5 animals.

3.4.10 *Histology*

Histological examination was conducted on tissues from the high dose and vehicle control groups only. Therefore the tissues from the high dose and control animals only were sent to the test site for histological processing. The slides were returned to the test facility and examined by a Board Certified Veterinary Pathologist.

3.4.11 *Femur Collection and Bone Smear Preparation*

One femur was collected from each animal At least one femur was collected from each animal at 18-24 hours after dosing for Groups 1-4 and at 24±4 hours after dosing for the positive control animals in Group 5. Bone marrow was collected via flushing the marrow with fetal bovine serum, followed by gentle flushing with a Pasteur pipette, allowing the large particles to settle,

then decanting the supernatant into a separate tube; a smear was then prepared and stained with Wright's Giemsa. The slides were labeled with animal number but not group number so that the slides were read blind.

3.4.12 *Scoring for Micronucleated Polychromatic Erythrocytes*
Bone marrow slides were scored blindly (without knowledge of treatment).

The ratio of polychromatic erythrocytes to total erythrocytes was determined by examining at least 200 erythrocytes per animal.

The number of micronucleated PCE's (MNPCE) in 2000 PCE/animal was then determined. The unit of analysis was PCE and not the number of micronuclei per PCE, as a PCE may contain more than one micronucleus.

4.0 STATISTICAL ANALYSES

The Statistical report can be found in Appendix C.

All statistical analyses were performed separately for each sex using SAS Release 9.2 for Windows XP. Statistical procedures were selected based on the distribution of the data and the validity of the assumptions. Statistical significance was declared when $p \leq 0.05$.

Body weights were analyzed using a repeated measures analysis of variance. The model included treatment group (1-4), study day (7, 14, 21, 28, and 29) and treatment group by study day interaction as fixed effects as well as the body weights from study day 1 (pre-dose) as a covariate. In addition, body weights on study day 1 (pre-dose) were compared between treatment groups (1-4) using an analysis of variance.

Average daily food consumption was analyzed using a repeated measures analysis of variance. The model included treatment group (1-4), time period (study days 1 to 7, 7 to 14, 14 to 21, 21 to 28), and treatment group by time period interaction included as factors in the model. Average daily food consumption (g/day) for each individual during each time period was calculated by dividing the total individual food consumption (in grams) by the number of days within the time period.

Clinical chemistry, hematology and coagulation parameters from study day 29 were compared between groups (1-4) using an analysis of variance model. The exception to this was TBIL, BASO, and ABS BASO which were analyzed using Cochran-Mantel-Haenszel's row mean score statistic due to low variability in the data. SDH was analyzed both including and excluding an outlier.

The number of polychromatic erythrocytes (PCE) in 200 erythrocytes per animal and the number of micronucleated PCEs (MNPCE) in 2000 PCE per animal were compared between groups (1, 4, and 5) using an analysis of variance. MNPCE was normalized using a natural log-transformation of the value +1. The ratio of the number of PCE per 200 erythrocytes and the ratio of the number of MNPCE per 2000 PCE were also calculated for each animal and summarized by sex and group. The influence of an outlier was also investigated for female MNPCEs.

Categorical urinalysis and FOB parameters were compared between groups (1-4) using Fisher's exact test, while ordinal urinalysis and FOB parameters were compared between groups (1-4)

using Cochran-Mantel-Haenszel's row mean score statistic. Specific gravity (from urinalysis) and average foot splay, temperature, and max forelimb and hindlimb grip strengths (from FOBs) were compared between groups using a one-way analysis of variance. The max forelimb grip strength was normalized using a natural log transformation.

Organ weights were analyzed as absolute weights and as a proportion of total body weight using a one-way analysis of variance model to compare treatment groups (1-4).

For statistical analysis of body weight, food consumption, clinical chemistry, hematology, and coagulation parameters, micronucleus parameters, continuous urinalysis and FOB parameters, and organ weights, if the model revealed statistical significance ($p \leq 0.05$), Tukey-Kramer adjusted comparisons were used to determine if pairwise differences existed.

5.0 QUALITY ASSURANCE

The study plan was inspected. Specific procedures and data from this study were inspected. Other relevant procedures and data were also inspected periodically. The final report was reviewed to assure that it accurately described the methods, procedures and observations and that the results accurately and completely reflected the raw data. Reports on these activities were made to the Study Director and to Management

Lead Quality Assurance (located at test facility) verified the study plan and inspected the final report for compliance with the OECD Principles of GLP, including verification that the Principal Investigator contributions and evidence of quality assurance at the test site had been properly included. Lead QA prepared a Quality Assurance Statement relating to work undertaken at the test facility and referenced quality assurance statements from the test site.

Test Site Quality Assurance inspected study-related work at their site and reported any inspection results promptly to the Principal Investigator, test site management, Study Director, test facility management and lead Quality Assurance. Test site QA provided a QA statement relating to the quality assurance activities at the test site.

6.0 ARCHIVES

The following materials will be maintained in the archives of the test facility:

- Study plan and (if applicable) amendment(s).
- Raw data and final report
- Blood smears
- Formalin fixed tissue, blocks and slides
- Test item and reference item retainer samples

Note: Labile specimens were stored until acceptance of the draft report and then disposed of.

7.0 RESULTS

7.1 Clinical Observations

Detailed clinical observations can be found in Appendix B, Table B1.

Two males, W10 of the mid dose group and W17 of the high dose group exhibited elevated lung sounds consistent with mild aspiration, and both animals returned to normal lung sounds over a few days. Neither experienced elevated body temperature.

One mid dose male (W16) and three high dose males (W11, W15 and W20) exhibited alopecia on the limbs.

High-dose group female W29 had green diarrhea at the time of euthanasia.

7.1.1 *Interpretation*

Aspiration is a common complication when animals are administered test item via gavage. Two cases of mild aspiration is a very low incidence and was unlikely to have affected the study.

Alopecia is a very mild occurrence and at the low incidence observed, is not likely to be test item-related.

7.2 Body Weights

Detailed individual animal body weights can be found in Appendix B, Table B2. Summary statistics for body weights can be found in Appendix C, Table 1.

There were no statistically significant intergroup differences in body weights over the course of the study, but body weights increased over the course of the study for both sexes.

7.2.1 *Interpretation*

A4+ administration at any of the doses did not affect body weight. Increases in body weights of the animals were due to normal growth.

7.3 Food Consumption

Detailed food consumption data can be found in Appendix B, Table B3. Summary statistics for food consumption can be found in Appendix C, Table 2.

There were no intergroup differences in food consumption for either sex over the course of the study, nor were there any group by time period interactions. For both sexes, however, there were significant differences between time periods. For the females, food consumption in Weeks 2-4 of the study exceeded that during Week 1, and in Weeks 3 and 4, exceeded that in Week 2. For the males, food consumption in Weeks 2-4 of the study exceeded that during Week 1, and in Week 4, exceeded that in Week 2.

7.3.1 *Interpretation*

None of the doses of A4+ affected food consumption over the course of the study. Slight increases in food consumption in the later weeks of the study were due to normal growth of the animals.

7.4 Functional Observational Battery

Detailed FOB data can be found in Appendix B, Tables B4a-d. Summary data for FOB's can be found in Appendix C, Table 11 and statistical analysis results can be found in Table 12.

There were no significant intergroup differences in FOB measurements among the animals of either sex, with the exception of tail pinch response, where the males of the control group had more abnormal responses (2 indifference and 3 exaggerated) than those of the low dose group (4 normal and one freezing).

7.4.1 *Interpretation*

The differences in tail pinch response were deemed not biologically significant given that the abnormal responses occurred only in the control group and no other intergroup differences were observed. It was concluded that none of the doses of A4+ affected the neurobehavioural parameters measured in the functional observation battery.

7.5 Urinalysis

Detailed urinalysis data for Day 29 of the study can be found in Appendix B, Table B5. Summary data for urinalyses can be found in Appendix C, Table 9 and statistical analysis results can be found in Table 10.

There were no significant intergroup differences in urinalysis parameters among the animals of either sex.

7.5.1 *Interpretation*

A4+ did not affect any of the urinalysis parameters.

7.6 Clinical Pathology

Detailed clinical pathology data for Day 29 of the study can be found in Appendix B, Tables B6a-b. Summary data for clinical pathology parameters can be found in Appendix C, Table 3 and statistical analysis results can be found in Table 4.

There were no significant intergroup differences in clinical pathology parameters among the animals of either sex.

7.6.1 *Interpretation*

A4+ did not affect any of the clinical pathology parameters under the conditions of the study.

7.7 Hematology

Hematology values and morphology cell observations for Day 29 of the study can be found in Appendix B, Table B7. Summary data for hematology parameters can be found in Appendix C, Table 5 and statistical analysis results can be found in Table 6.

The high dose females had significantly higher mean segmented neutrophil counts than the rest (0.21 vs. 0.11, 0.12 and 0.12) and higher mean absolute segmented neutrophil counts than the two lower dose groups but not the controls (1.55 vs. 0.73 and 0.84 $x10^9$/L).

The high dose females had significantly lower mean lymphocyte counts than the controls or low dose females (0.76 vs. 0.86 and 0.84). Also, the mid dose males had significantly lower lymphocyte counts than the low dose males (0.79 vs. 0.88 $x10^9$/L).

7.7.1 *Interpretation*

Although statistically significant, the differences in cell counts were relatively small, and were deemed of low biological significance since all of the counts were close to the normal range (Giknis and Clifford, 2006).

7.8 Blood Coagulation

Blood coagulation values can be found in Appendix B, Table B8. Summary data for coagulation parameters can be found in Appendix C, Table 7.

There were no significant intergroup differences for coagulation parameters among the animals of either sex.

7.8.1 *Interpretation*

A4+ did not affect blood coagulation under the conditions of the study.

7.9 Organ Weights

Organ weight data can be found in Appendix B, Table B9. Summary data for organ weights can be found in Appendix C, Table 13, and statistical analysis results can be found in Table 14.

The only significantly different organ weight was in the males, where the controls had a significantly greater mean lung to body weight ratio than the mid dose males (0.00370 vs 0.00321).

7.9.1 *Interpretation*

Given that that absolute lung weight ratios from the affected groups differed by less than 5% and no other parameters and groups differed significantly, this result was deemed to be a statistical anomaly and not biologically significant. Therefore, A4+ did not affect organ weights under the conditions of the study.

7.10 Micronucleus

Micronucleus data for the control, high dose and positive control groups can be found in Appendix B, Table B10. Summary statistics for Groups 1, 4 and 5 can be found in Appendix C, Table 8 and in Table 3 below.

The number of polychromatic erythrocytes (PCE) in 200 erythrocytes did not differ between groups for males, however they did differ significantly between groups for females. Specifically, the control and high dose females had significantly higher numbers of PCEs than females in the positive control group.

The number of micronucleated PCEs (MNPCE) in 2000 PCE did not differ significantly between groups for either sex. There was an outlier in the females, ID# W45 in the control group with a MNPCE of 27, however the removal of this observation did not change the results (i.e. still no statistical difference between the MNPCE for females).

Table 3. Summary statistics for integrated micronucleus test

Group	Variable	Females		Males	
		Mean	Std Dev	Mean	Std Dev
Vehicle Controls (n=5)	Polychromatophilic erythrocytes (PCE)/200 erythrocytes	84.000	8.155	88.800	18.089
	PCE to total red blood cell ratio (PCE TOT RBC RATIO)	0.422	0.042	0.448	0.091
	Micronucleated PCE (MNPCE)/2000 PCE	9.200	10.109	6.400	3.362
	MNPCE PCE RATIO	0.005	0.005	0.003	0.002
High Dose (n=5)	PCE/200 erythrocytes	88.200	9.203	88.600	9.503
	PCE TOT RBC RATIO	0.444	0.046	0.446	0.050
	MNPCE/2000 PCE	5.400	3.647	5.800	4.919
	MNPCE PCE RATIO	0.003	0.002	0.003	0.002
Positive Controls (n=5)	PCE/200 erythrocytes	71.200	4.324	85.600	17.615
	PCE TOT RBC RATIO	0.358	0.022	0.430	0.088
	MNPCE/2000 PCE	6.800	2.280	14.000	8.276
	MNPCE PCE RATIO	0.003	0.001	0.007	0.004

7.10.1 *Interpretation*

Only in the females was there a significantly lower mean PCE count in the positive controls compared with the vehicle controls, indicating bone marrow toxicity.

Even though there was no significant reduction in PCE counts in the males or elevated MNPCE counts in either sex in the high dose group compared with the vehicle controls, it cannot be concluded that there was no toxicity because the positive control animals did not give significantly different results either. The failure of the positive controls to cause reduced PCE counts and more importantly, elevated MNPCE counts, is puzzling. The standard positive control protocol for Sprague-Dawley rats (i.e. 20 mg/kg ip 24 hours prior to sacrifice) was followed. The concentration of cyclophosphamide in the dosing solution used has been confirmed. A response to cyclophosphamide was observed in the females (PCE count), indicating that the animals were likely properly dosed. Intraperitoneal injection is known to be subject to some missed targeting (i.e. some injections end up in the gastrointestinal tract or outside the peritoneal space) but this is usually less than 15% of injections (Steward et al., 1968). As well, older rats such as those used for this study tend to be less responsive to cyclophosphamide, however elevated counts are nevertheless usually observed (Hamada et al., 2003). Therefore, a certain cause for the failure of the positive controls has not been determined, and the micronucleus portion of the study must be deemed inconclusive.

Note: In the study plan, a reference to expected results from the negative control being "nearly the same as naturally occurring micronucleated PCE's (approximately 1-2 micronucleated PCE's per 1000 PCE's, Shelby *et al.*, 1989)" was inadvertently inserted. This reference related to

expected data from mice. Krishna and Hayashi (2000) give higher ranges for rats (Males, 1.1-6.4, Females, 0.8-4.9 MNPCE/1000 PCE). The means for all of the vehicle control groups fall within the expected ranges.

7.11 Mortality

Macroscopic findings can be found in Appendix D, Part B. No animal deaths occurred during the study. Therefore, a mortality table was not created.

7.11.1 *Interpretation*

A4+ administration did not result in any animal deaths under the conditions of the study.

7.12 Macroscopic Findings

No abnormalities were observed during necropsy. Therefore, a macroscopic findings table was not created.

7.12.1 *Interpretation*

A4+ administration did not result in any abnormal macroscopic findings under the conditions of the study.

7.13 Histopathologic Findings

Histopathologic findings are reported in Appendix D, Part C. The Principal Investigator's Report on histology (processing of tissues to slides) is found in Appendix I.

Control group female rats W41, W42 and 45 each had a diffuse but very mild infiltrate of neutrophils in the submucosal connective tissue and to a lesser extent the inner muscular layer of the uterus. The liver of rat W42 had a single localized area of inflammation in the parenchyma of the liver with loss of several hepatocytes. There were no other microscopic abnormalities in either these rats or any of the other control group animals.

In the high dose group, male rats W15, W17 and W20, and female rat W25 all had a local area in the heart section examined in which the sarcoplasm of myocardial fibers was missing and in the interstitium of which there were occasional mononuclear cells. In rat W15 this was a single area in the right ventricular wall, in W17 and W25 it was at the tip of the left ventricle and in W20 within the body of the left ventricular muscle. Separate from the lesion in the ventricle of W17, a small accumulation of less than ten mononuclear cells was seen elsewhere in the myocardial interstitium.

Female W25 had a diffuse, mild, uniform infiltrate of neutrophils in the submucosal connective tissue of the uterine wall. This extends to the perivascular spaces of the vessels of the inner muscular layer of the uterus.

Male W17 had occasional parenchymal clusters of cells in the liver and clusters of epithelioid cells around various airways and vessels throughout the lungs.

There were no other microscopic abnormalities in either these rats or any of the other high dose group animals.

7.13.1 *Extended Histopathological Examination of Myocardial Tissues*

In the original examination of the study tissues, microscopic lesions were described in the hearts of some of the high dose group rats. In order to further determine the frequency and extent of such lesions, serial sections of heart were examined from each of the rats in all groups. Serial sections of each heart were prepared at 100 μm intervals for a total of ten sections, stained with hematoxylin and eosin, and examined microscopically.

Local areas of myocardial damage were found in four of the high dose rats in the original set of sections examined. Other than the area of inflammation in the wall of the right ventricle of rat W15, the same lesions were not seen in the additional set of sections. No additional lesions were found in any of the high dose rats except for the local area of fibrosis and mild inflammation seen in rat W15 at levels 3 – 5 inclusive.

Other lesions were found by the pathologist in the extended set of sections. These can be grouped as follows:

Incidental findings: In low dose group female W34, the arterial lesion seen is an incidental finding that is very unlikely to be related to the test article.

Mild focal inflammation:
Epicarditis: Control group rat W01 had mild localized epicarditis over the base of the right ventricle in all sections. This is very unlikely to have had any clinical effects.

Endocarditis: Low dose group male W23 had a local endocarditis. This is very unlikely to have had any clinical effects.

Myocarditis: Control group female W45, mid dose group females W28 and W32, mid dose group males W04, W10, W12 and W16, and high dose group male W15 each had small foci of mild interstitial inflammation in the hearts. None of these would have had any clinical effects.

Mild focal degeneration: usually very small numbers (<10) of cardiomyocytes were affected in these areas. This includes control group male W07, low dose group males W18 and W22 and mid dose group males W04, W05 and W12.

7.13.2 *Interpretation*

Infiltrates of neutrophils were present in the uteri of three rats from the control group, and one from the high dose group. Given that the group with the most findings is the control group, then this finding is unlikely to be related to A4+ administration.

Local areas of myocardial damage were found in four of the high dose group rats in the initial histopathological examination. As this change was not observed in any of the control animals, it could not be concluded that there was no A4+ treatment-related change. Therefore, the extended examination of all study hearts was conducted as described above and lesions were found in all groups.

Very mild focal lesions were seen in the hearts of animals from all four groups (Controls, 1F, 2M; Low dose, 1F, 3M; Mid dose, 2F, 5M; High dose, 1F, 3M). The pathologist considered whether the changes were A4+ related and found that there was no clear dose responsiveness, the inflammatory and degenerative changes were not necessarily related to each other, and all of the changes were mild and would not be clinically evident. Furthermore, similar lesions were

encountered in the control group rats. Although there are more of these changes than would be expected in a normal population of rats, the pathologist concluded that there did not appear to be any clear connection to the experimental treatment.

8.0 DEVIATIONS

8.1 Study Events

16-Feb-2010	Animals W41 (gr 1), W45 (grp 1), W27 (grp 4) and W38 (grp 4) received 1.1ml instead of the required 1.2ml.
18-Feb-2010	The amount of A4+ test item in the container is more than the amount stated on the container resulting in -1.040g recorded of the Test and Reference Item Storage, Inventory and Handling Form.
6-Mar-2010	Two different lot numbers of simethicone was used for dose preparations in the study.
9-Mar-2010	Cohort B's food consumption for time period 3 was lower than expected and the pattern also inconsistent with weight gain. Assumption made that balance was not tared and corrections made accordingly.
12-Mar-2010	Unable to perform grip strength on Cohort A due to grip strength meter not working. Grip strength was completed the next day, March 13.
17-Mar-2010	Animal W04 (male, grp 3, cohort B) had clotted Na Citrate tube and therefore unable to run PT and APTT.
2-Feb-2011	It was determined that only one lot number of simethicone (C128706) was used. It was confirmed by the supplier that their product was mislabeled.

8.2 Study Plan

16-Feb-2010	The test item retainer sample was stored at room temperature as per Study Director instruction. This was followed by an amendment.
23-Feb-2010	Animals were not observed 3-6 hours post dose as per study plan, all animals were alive the next day.
1-Mar-2010	A 2ml aliquot of dose material prepared on study day 14 was not collected and sent for concentration analysis as stated in the study plan. This task was completed one week later on Study Day 21.
12-Mar-2010	Group 5 animals were identified with a temporary marker rather than tattoo as specified in the study plan.
15-Mar-2010	Samples for dose verification for Study day 28 were not shipped to analytical lab as required in the study plan.
16-17-Mar-2010	SDH was not run on Study day 29 because controls were not available, and was run on frozen serum on March 25 when controls arrived.
13-14-May-2010	Room temperature where reagents and test item were stored exceeded room temperature range.
16-May-2010	Double processed tissue culture water was used in dose preparation instead of sterile water USP for injection as specified in the study plan.
22-July-2010	Study plan Amendment #2 states that the micronucleated PCE counts were to be compared using Fisher's exact test. The method used was analysis of variance.

None of the deviations were deemed to have materially affected the study.

9.0 CONCLUSIONS

No significant test item-related effects were observed among clinical observations, body weights, food consumption, functional observational battery (indicating a lack of neurobehavioral toxicity), urinalysis, clinical pathology parameters, blood coagulation parameters, mortality, or macroscopic and histopathological findings. Statistically significant differences in some hematology results and organ weights were deemed to have low biological significance.

Given that the positive control animals in the micronucleus test satellite group failed to respond to the positive control agent cyclophosphamide, the results cannot be accepted and this test has been repeated in a separate study, TEH-153. For TEH-153, it was concluded that under the repeated dose conditions of the experiment, A4+ was negative for the production of elevated micronucleus counts and did not exhibit bone marrow toxicity. A no adverse effect dose level (NOAEL) of 2000 mg/kg was assigned for genotoxicity and bone marrow toxicity based on the results of the study, combined with those of TEH-147. Details on TEH-147 and TEH-153 can be found in their separate reports.

It was concluded that A4+ did not exhibit toxicity, including neurobehavioral toxicity, under the conditions of this study and a no adverse effect level (NOAEL) of 2000 mg/kg can be assigned.

10.0 REFERENCES

Giknis, MLA and Clifford, CB (2006) Clinical Laboratory Parameters for Crl:CD(SD) Rats. Charles River Laboratories.

Hamada S, Nakajima K, Serikawa T and Hayashi M (2003) The effect of aging on the results of the rat micronucleus assay, *Mutagenesis* 18:273-275.

Krishna G and Hayashi M (2000). In vivo rodent micronucleus assay: protocol, conduct and data interpretation, *Mutat. Res.* 455:155-166.

Shelby MD, Gulati DK, Tice RR and Wojciechowski JP (1989) Results of tests for micronuclei and chromosomal aberrations in mouse bone marrow cells with human carcinogens 4-aminobiphenyl, treosulphan, and melphalan, *Env. Mol. Mutagen.* 13:339-342.

Steward JP, Ornellas EP, Beernink KD, Northway WH (1968) Errors in the technique of intraperitoneal injection of mice, *Appl. Microbiol.* 16:1418-1419.

Reference # 21

ToxTest®

FINAL REPORT

A Repeated Dose Mammalian Erythrocyte Micronucleus Study of A4+ in Mice

TEH-153
Sabell Corporation

FINAL REPORT

STUDY TITLE: **A REPEATED DOSE MAMMALIAN ERYTHROCYTE MICRONUCLEUS STUDY OF A4+ IN MICE**

STUDY PLAN NO.: TEH-153

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Study Initiation Date: August 23, 2010

Study Completion Date: March 29, 2011

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APPENDICIES

SUMMARY

The intent of this study was to evaluate the genotoxic potential of Sabell A4+ based upon its ability to induce micronuclei in rodent polychromatophilic erythrocytes (PCE). Groups of Balb/c mice (n=5 per sex per group, except for the positive control group, which had 8 per sex) were gavaged on three consecutive days with one of threedosages of test item (Group 2, 125 mg/kg, Group 3, 500 mg/kg, and Group 4, 2000 mg/kg BW orally) or vehicle control (Group 1, water containing 0.2% v/v simethicone), or injected intraperitoneally once with positive control (Group 5, cyclophosphamide, 40 mg/kg BW). No abnormalities were observed during clinical observations. Animals were euthanized 24 hours after the final or in the case of the positive control group, only dosing, both femurs were collected, and the marrows were flushed with fetal bovine serum. From the suspended cells, bone marrow smears were made. The high dose group and both negative and positive control group smears were scored for PCE/200 erythrocytes and for micronucleated PCE (MNPCE)/2000 PCE.

Statistical analysis of the bone marrow scores revealed that the positive control compound cyclophosphamide reduced PCE scores similarly in both sexes, an indicator of bone marrow toxicity. No changes in PCE scores were associated with high dose A4+ test item.

MNPCE scores, the primary indicator of genotoxicity in this test, were significantly higher in the positive control animals of both sexes than in either the high dose A4+ treated animals or the negative controls. The A4+ treated animals of both sexes has similar low MNPCE scores to the control animals with no significant differences between groups.

It was concluded that under the repeated dose conditions of the experiment, A4+ was negative for the production of elevated micronucleus counts and did not exhibit bone marrow toxicity. A no adverse effect dose level (NOAEL) of 2000 mg/kg was assigned for genotoxicity and bone marrow toxicity based on the results of this study, combined with those of TEH-147.

COMPLIANCE STATEMENT

A REPEATED DOSE MAMMALIAN ERYTHROCYTE MICRONUCLEUS STUDY OF A4+ IN MICE

This study was conducted in compliance with OECD Principles of Good Laboratory Practice (ENV/MC/CHEM(98)17) with the following exceptions. Test item and dose concentration analysis were not characterized in compliance with GLP. A study plan amendment was not issued to accommodate changes to the number of groups' bone marrow slides read, changes to the statistical method used for micronucleated PCE's, inclusion of statistical analysis of body weights and analysis of cyclophosphamide quantitatively rather than qualitatively.

The undersigned have reviewed and approved the report for final issuance.

Study Director:

Hugh Semple, DVM, PhD — Date

Test Facility Management:

Curtis Kuzyk — 25/Mar/2011
Date

QUALITY ASSURANCE STATEMENT

A REPEATED DOSE MAMMALIAN ERYTHROCYTE MICRONUCLEUS STUDY OF A4+ IN MICE

Date	QAI Number	Inspection Type	Phase	Date reported to Study Director and Management
17-Aug-10	QAI0832	Study Plan Review	Study Plan	18-Aug-10
1-Sept-10	QAI0833	Study-based	Dose Preparation	1-Sept-10
1-Sept-10	QAI0834	Study-based	Dosing	1-Sept-10
1-Sept-10	QAI0835	Study-based	Clinical Observations	1-Sept-10
2-Sept-10	QAI0837	Study-based	Bone Marrow Flushing	2-Sept-10
2-Sept-10	QAI0838	Study-based	Smear Preparation	2-Sept-10
4-6-Jan-11	QAI0849	Raw Data Audit	Raw Data	6-Jan-11
4-6-Jan-11	QAI0850	Draft Report	Draft Report	6 Jan-11
3-Mar-11 to 7-Mar-11		Final Report	Final Report	7-Mar-11

The reported results in this final report accurately and completely reflect the raw data.

Quality Assurance Auditor

March 9, 2011
Date

A REPEATED DOSE MAMMALIAN ERYTHROCYTE MICRONUCLEUS STUDY OF A4+ IN MICE

1.0 GENERAL POINTS

1.1 Test Item

Sabell A4+

1.2 Nature and Purpose

The purpose of this study was to determine the genotoxicity of A4+ via a repeated dose micronucleus test in order to support an NHP product license and human clinical trials applications. This study was a follow-up study from TEH-147, in which single doses of A4+ did not produce elevated micronucleus counts.

1.3 Study Plan No.

TEH-153 (Appendix A)

1.4 Supervisory Personnel

Head, Animal Care: Shawna Bast
Veterinary Care: Dr. Hugh Semple
Head, Pathology/Data Management/Pharmacy: Marja Rynsewyn

1.5 Regulatory Compliance

This was a GLP study and was conducted in accordance with OECD GLPs (with the exception of test item characterization, dose concentration analysis), and ToxTest Standard Operating Procedures.

1.6 Test Method/Guideline

The design is a mammalian erythrocyte micronucleus study to be conducted under GLP according to OECD 474 guidelines.

1.7 Schedule of the Study

Experimental starting date: August 25, 2010 (manual randomization to group upon arrival)
First day of acclimation: August 25, 2010
Dosing dates: August 30-September 1, 2010
Necropsy day: September 2, 2010
Experimental completion date: September 2, 2010

2.0 TEST ITEM INFORMATION

2.1 Test Item

- Name or Code: A4+
- Supplier: Sabell Corporation
- Appearance: Tan powder
- Purity: Supplied by the sponsor and added to Appendix E. The sponsor assumed responsibility for characterization including identity, purity and stability of the bulk test item under the test conditions used in this study. Homogeneity and stability of the formulated test item are found in Appendix F.
- Batch Number: 1-2009
- Storage: Room temperature
- Retest Date: September 22, 2010
- Fate of Test Item: Returned to sponsor.

2.2 Vehicle 1

- Name or Code: Nanopure water with 0. 2%v/v of simethicone USP
- Supplier: In-house water, PCCA for simethicone (Lot #12806 PCCA, exp. Sep. 16, 2011, Store @ room temperature); see certificate of analysis in Appendix G.
- Batch Number: TR-383/1 (Nanopure Water), RCS -807-TI (Simethicone)
- Storage: Room temperature
- Expiry Date: October 18, 2010

2.3 Reference Item 1

- Name or Code: Vehicle: Nanopure water with 0. 2%v/v of simethicone USP
- Supplier: In-house, PCCA for simethicone (Lot #12806 PCCA, exp. Sep. 16, 2011, Store @ room temperature); see certificate of analysis in Appendix G.
- Batch Number: TR-383/2, 4, 5
- Storage: Room temperature
- Expiry Date. October 18, 2010

2.4 Reference Item 2 (positive control)

- Name or Code: Cyclophosphamide monohydrate
- Supplier: Sigma-Aldrich
- Appearance: white powder
- Lot Number: 079K1569; see certificate of analysis in Appendix G.
- Storage: 2-8°C
- Retest Date: July 2012

2.5 Vehicle 2

- Name or Code: Sterile filtered water, cell culture tested
- Supplier: Sigma
- Lot Number: RNBB2491; see certificate of analysis in Appendix G.
- Storage: Room temperature
- Expiry Date: January 2012

2.6 Formulation of the Test Item

- Preparation: The dose materials were calculated and prepared assuming the test item was 100% pure. An appropriate amount of A4+ dried extract was finely ground with a mortar and pestle. Appropriate amounts were weighed into Falcon tubes, 0.002 mL of simethicone USP/mL dosing solution, and an appropriate volume of water was added to make sufficient quantities of test item suspension for administering for the day of dosing. The mixtures were vortexed to make a suspension. The suspension was mixed with a stirring bar immediately before taking up each dose for administration.
- Storage conditions after formulation: room temperature
- Special handling requirements after formulation: The suspension was mixed to make it homogeneous before administration.
- Fate of remaining dosing solutions: Discarded after completion of the draft report.
- Frequency of Preparations: Once on day of dosing

2.7 Formulation of Reference Item 1

- Preparation: The dose materials were calculated and prepared assuming the reference item was 100% pure. Simethicone USP was added to nanopure water to a concentration of 0.002 mL/mL of dosing solution at an appropriate volume to make sufficient quantities of reference item for the day of dosing. The mixtures were vortexed to make an emulsion. The emulsion was mixed with a stirring bar immediately before taking up each dose for administration.
- Storage conditions after formulation: Room temperature
- Special handling requirements after formulation: Stir the emulsion with a stirring bar to make it homogeneous before administering.
- Fate of remaining dosing solutions: Discarded each day when dosing finished.
- Frequency of Preparations: Once on day of dosing (three days).

2.8 Formulation of Reference Item 2

- Preparation: prepared a 4 mg/ml cyclophosphamide solution on day of dosing
- Storage conditions after formulation: room temperature during dosing, then 2-8 °C after dosing has been completed
- Fate of remaining dosing solutions: discarded after completion of draft report
- Frequency of Preparations: once on last day of dosing

2.9 Fate of Test Item

Unused test item was returned to the sponsor.

2.10 Fate of Reference Item 2

All reference item was used in this study so none was retained.

2.11 Test and Reference Item Concentration Analysis

Determination of concentration of the test and reference items in the dose material was conducted. A 2 ml aliquot of each dose material was stored at below -15°C and shipped on ice to Dr. Duff Sloley, Phytovox, Inc. for analysis by reverse phase liquid chromatography with positive mode electrospray mass spectrometric detection (LC/MS). The cyclophosphamide solution was

intended to be analysed qualitatively only due to its instability, however, a standard curve was nevertheless developed for the analysis and the sample was shown to have sufficient stability to be analysed quantitatively. The method of analysis is described in Appendix D.

3.0 EXPERIMENT PROCEDURE

3.1 Test System and Environment

3.1.1 *Justification for Selection of Test System*

Standard species for test guideline (OECD 474)

3.1.2 *Species, Strain, Supplier and Specifications*

- Species/Strain: Balb/c mice
- Supplier: Charles River Canada Inc.
- Number of Animals in the Study: 56
- Age at Initiation of Treatment: 6-8 weeks
- Sex: male and female
- Weight Range at Initiation of Treatment (grams): The weight variation of animals was minimal and did not exceed ± 20% of the mean weight for each sex.
  Male: 16.2-22.2g
  Female: 16.1-19.1g

3.1.3 *Environment and Husbandry*

The animal room environment was controlled with targeted conditions:

- Housing: group housing (4-5 animals per cage)
- Temperature: 18 - 26°C
- Relative Humidity: 30 - 70%
- Air Changes: 10 - 15 per hour
- Light Cycle: ~12 hours light
- Caging: Shoebox cages with environmental enrichment – Nestlets (Ancare, Lot# 1015090700), Enviro-dri (Shepherd Specialty Papers, Lot# ED0410)
- Bedding: Anderson's Bed-O-Cobs, Lot # 092109

3.1.4 *Diet and Water*

- Diet: *ad libium* Certified Rodent Chow – Irradiated (Certified Pico Rodent Diet Lot MAY24102A from LabDiet)
- Water: *ad libitum* reverse osmosis UV sanitized water

The study director has reviewed the feed, and bedding for contaminants and found none present.

3.2 Acclimation Procedures

- Animal Health Procedure: A health status report was generated prior to animals being released to the study.
- Acclimation Period: Animals were acclimated to rack and position for five days.
- Allocation to Treatment Group: Mice were manually randomized to group on the day of arrival.
- Identification of the Animals: temporary marking and tail tattoo

- Identification Numbers: Specified in raw data
- Identification of the Cage: Cage card

3.3 Treatment

3.3.1 *Experimental Design*

This study involved administration of three doses of A4+ plus vehicle control once on three consecutive days, as well as a positive control substance, cyclophosphamide, to male and female mice once on the last dosing day for A4+, as per Table 1, followed 24 hours later by euthanasia and necropsy.

Table 1. Study Design with Dosing Details

Treatment Group	Treatment	No. of Animals per Group		Dosage Level (µg/g BW)	Dosage Concentration (mg/ml)	Dosage Volume (µL/g BW)
		Male	Female			
1	Reference Item 1 (vehicle control)	5	5	0	0	6
2	Low dose A4+	5	5	125	20.81	6
3	Mid dose A4+	5	5	500	83.25	6
4	High dose A4+	5	5	2000	333	6
5	Cyclophosphamide (positive control, in water)	8	8	40	4.0	10

3.3.2 *Rationale for Dose Selection*

The A4+ doses were calculated as large multiples of the human dose. The cyclophosphamide dose is the standard dose for a micronucleus assay positive control.

3.3.3 *Route and Method of Administration*

3.3.3.1 *A4+ and Reference Item 1*

- Route: oral
- Method: gavage
- Rate of Dosing: bolus
- Volume Administered: 6 µL/g BW for A4+, Reference Item 1

3.3.3.2 *Cyclophosphamide*

- Route: intraperitoneal
- Method: injection
- Rate of Dosing: bolus
- Volume Administered: 10 µL/g BW for cyclophosphamide 3

3.3.4 Frequency and Duration of Treatment

- Frequency: Once daily for three days (Groups 1-4); once on Study day 3 for Group 5.
- Number of administrations: 3 for Groups 1-4; 1 for Group 5

3.4 Examinations Performed During the Test Period

3.4.1 *Observations*
Daily clinical observations (post dose and prior to euthanasia)

3.4.2 *Body Weight*
Body weights were taken on all animals on study day 1 prior to dosing. These weights were used to calculate dose.

3.4.3 *Euthanasia*
CO_2 asphyxiation and cervical dislocation

3.4.4 *Femur Collection and Bone Smear Preparation*
Both femurs were collected from each animal between 18 and 24 hours following the final treatment for Groups 1-4 and not earlier than 24 hours or later than 26 hours after dosing for Group 5. Bone marrow was collected into fetal bovine serum (see certificate of analysis in Appendix G); a smear was then prepared and stained with Wright's Giemsa. The slides were labeled with animal number but not group number so that the slides were read blind.

3.4.5 *Scoring for Micronucleated Polychromatic Erythrocytes*
Bone marrow slides were scored blindly (without knowledge of treatment) for groups 1, 4 and 5 (negative control, high dose and positive control groups) only, and the slides for groups 2 and 3 were held to be read only if evidence of toxicity was observed in the counts from Group 4 (high dose).

The ratio of polychromatic erythrocytes to total erythrocytes was determined by examining 200 erythrocytes per animal.

The number of micronucleated PCE's (MNPCE) in 2000 PCE/animal was then determined. The unit of analysis was PCE and not the number of micronuclei per PCE, as a PCE may contain more than one micronucleus.

The results were recorded on the Micronucleus Score Sheet.

4.0 STATISTICAL ANALYSES

The Statistical report can be found in Appendix C.

- The statistical analyses were performed using SAS Release 9.2 for Windows XP.
- Statistical procedures were selected based on the distribution of the data and the validity of the assumptions.
- Statistical significance was declared when $p \leq 0.05$.
- The number of polychromatic erythrocytes (PCE) in 200 erythrocytes per animal and the number of micronucleated PCEs (MNPCE) in 2000 PCE per animal were compared between sexes and groups using an analysis of variance.
- MNPCE was normalized using a natural log-transformation of the value +1. If the model revealed statistical significance ($p \leq 0.05$), Tukey-Kramer adjusted comparisons were used to determine if pair-wise differences existed between groups.

- The ratio of the number of PCE per 200 erythrocytes and the ratio of the number of MNPCE per 2000 PCE were also calculated for each animal and summarized by sex and group.

5.0 ARCHIVES

The following materials will be maintained in the archives of the test facility:

- Study plan and (if applicable) amendment(s).
- Raw data and final report
- Bone marrow smear slides
- Test item retainer sample
- Copies of analytical results from Phytovox

6.0 RESULTS

6.1 Dose Verification

Using the rutin content of the Lot# 1-2009 A4+ source material (Appendix E) and the rutin content in the dose verification samples (Appendix D), the measured amounts of A4+ powder per mL dosing suspension were calculated and presented in Table 2 below. The target values are also included for comparison. The measured concentration of cyclophosphamide in the positive control solution is found in Appendix D.

Table 2. Dose verification by comparison of measured and target concentrations of test item and control for each treatment.

Treatment Group	Treatment	Dosage Level (µg/g BW)	Target Dosage Concentration (mg/ml)	Measured Dosage Concentration (mg/ml)
1	Reference Item 1 (vehicle control)	0	0	0
2	Low dose A4+	125	20.81	23
3	Mid dose A4+	500	83.25	95
4	High dose A4+	2000	333	328
5	Cyclophosphamide (positive control, in water)	40	4.0	4.07

6.1.1 *Interpretation*

The measured concentrations of A4+ powder fell close to the target concentrations. Therefore the dose suspensions were correctly formulated.

6.2 Clinical Observations

One group 3 female (W177) and one Group 4 male (W188) showed signs of aspiration after dosing. W177 appeared to recover before the study concluded but W188 still had increased lung sounds at the end of the study.

6.2.1 *Interpretation*
The data from W188 (Table B2, Appendix B) fell in the middle of the range of PCE and MNPCE values for the group, therefore it was deemed that any aspiration that occurred did not affect the results, so the data from this animal was kept in the study.

6.3 Body Weights

Detailed individual animal body weights can be found in Appendix B, Table B1. Summary statistics for body weights are found in Appendix C, Table 1. The females had a weight range of 16.1-19.1 g, with a mean of 17.7 g. The males had a weight range of 16.2-22.2 g with a mean of 19.6 g. All weights were within ±20% of the mean body weight. There were no significant group differences for body weights.

6.3.1 *Interpretation*
Body weights of all animals were within acceptable limits.

6.4 Bone Marrow Scores

Detailed individual animal bone marrow scores can be found in Appendix B, Table B2. Summary statistics for polychromatic erythrocytes (PCE), micronucleated PCE's (MNPCE), and the ratios of PCE per 200 erythrocytes and MNPCE per 2000 PCE by sex and group (1, 4, 5 only) are presented in Table 2, Appendix C and in Table 3 below .

The number of polychromatic erythrocytes (PCE) in 200 erythrocytes differed significantly between groups for females ($p=0.0005$) and males ($p<0.0001$). Specifically, group 1 and 4 females had significantly higher numbers of PCEs than females in group 5 ($p=0.0021$ and $p=0.0016$, respectively). There was no significant difference between females in group 1 and females in group 4 in terms of PCEs ($p=0.9909$). For the males, groups 1 and 4 had significantly higher numbers of PCEs than group 5 ($p<0.0001$ and $p=0.0043$, respectively). In addition, group 1 males had significantly higher numbers of PCEs than group 4 males ($p=0.0129$).

The number of micronucleated PCEs (MNPCE) in 2000 PCE differed significantly between groups for females and males (both $p<0.0001$). Group 1 and 4 females had significantly lower numbers of MNPCEs than group 5 females (both $p<0.0001$), while groups 1 and 4 females did not differ significantly ($p=0.9619$). Similarly, group 1 and 4 males had significantly lower number of MNPCEs than group 5 males (both $p<0.0001$) and group 1 and 4 males did not differ significantly ($p=0.7336$).

Table 3. Summary statistics for the number of PCE per 200 erythrocytes, ratio of PCE to total erythrocytes, the number of MNPCE per 2000 PCE, and the ratio of MNPCE to total PCE by sex and group.

GROUP	Variable	Females		Males	
		Mean	Std Dev	Mean	Std Dev
1 (Vehicle control) (n=5)	PCE/200 RBC's	101.400	11.371	127.800	13.255
	PCE to total RBC ratio	0.507	0.057	0.639	0.066
	MNPCE/2000 PCE's	4.400	2.302	2.600	0.548
	MNPCE to PCE_RATIO	0.002	0.001	0.002	0.001
4 (High) (n=5)	PCE/200 RBC's	102.400	15.043	102.000	11.979
	PCE_TOT_RBC_RATIO	0.512	0.075	0.510	0.060
	MNPCE/2000 PCE's	5.000	3.000	3.600	2.408
	MNPCE_PCE_RATIO	0.003	0.002	0.002	0.001
5 Cyclophosphamide (Positive Control) (n=8)	PCE/200 RBC's	72.000	10.994	74.875	12.124
	PCE_TOT_RBC_RATIO	0.360	0.055	0.374	0.061
	MNPCE/2000 PCE's	19.625	4.373	18.875	2.416
	MNPCE_PCE_RATIO	0.010	0.002	0.010	0.001

6.4.1 *Interpretation*

The assay performed as expected, producing negative control counts of MNPCE consistent with the results from the negative controls reported by Krishna and Hayashi (2000). Their mean negative control assay range for MNPCE/1000 PCE was 0.4-3.8 for males and 0.6-3.6 for females. Approximately 1-2 micronucleated PCEs per 1000 PCEs was also reported by Shelby *et al.* (1989). In this study, the means were in the middle of those ranges. Furthermore, the means from the high dose A4+ treatment group for both sexes were similar and not statistically different from controls, which is the most important indicator that under the conditions of the study, A4+ did not show any genotoxicity.

Similarly, the positive control ranges for MNPCE/1000 PCE reported by Krishna and Hayashi (2000) were 7.7-42.7 for the males and 8.0-44.7 for the females. In this study, the means for both sexes were within these ranges. For both males and females, the positive control (Group 5) means were significantly greater than the means for the treatment vehicle negative controls (Group 1) and for the A4+ high dose treatment group (Group 4), indicating that elevated numbers of MNPCE were detected where they were expected to occur, but not with the highest dose of A4+.

Therefore, no evidence of genotoxicity of A4+ was uncovered at the highest dose administered.

A secondary endpoint of the micronucleus test is the number of PCE per 200 erythrocytes. Reduced PCE numbers in a treatment group compared with controls may indicate bone marrow toxicity. In this study, the male high dose PCE counts were significantly lower than the negative

control counts. This result is puzzling because the high dose group PCE counts were almost identical to those in the TEH-147 study which preceded this one. As well, they are almost identical to the female control and high dose A4+ counts, which are similar to the results in TEH-147. In this case, it would appear that the PCE counts in the males are slightly higher than normally expected and the high dose treatment counts are normal, rather than low in terms of PCE per 200 erythrocytes (which would normally be interpreted as evidence of bone marrow toxicity).

In both sexes, PCE levels were significantly lower in the positive control group than in the negative control high dose A4+ treatment groups, indicating successful induction of bone marrow toxicity by the cyclophosphamide.

Therefore, no evidence for bone marrow toxicity of A4+ in the females and weak evidence for bone marrow toxicity in the males was uncovered, however, the cyclophosphamide treatment was toxic to bone marrow in both sexes. Therefore, it was concluded that A4+ at the highest dose was not toxic to bone marrow under the conditions of the study

7.0 DEVIATIONS

7.1 Study Plan Deviation

2-Sept-2010	Method of euthanasia differed from study plan in that cervical dislocation followed CO_2 asphyxiation.
30-Aug-2010	Reference Item 2 solution used in dosing was stored in a freezer instead of a fridge.
30-Aug-2010	Test and Reference Item retainer samples were not collected and archived at the time of the study.
10-Sept-2010	Only the high dose and control group bone marrow slides were read.
10-Sept-2010	Cyclophosphamide was analysed quantitatively not qualitatively.
10-Sept-2010	A different statistical method was used to analyse the micronucleated PCE's than that stated in the study plan.
10-Sept-2010	Statistics were performed for body weights but were not required.

7.2 Study Plan Event

2-Feb-2011	The lot number of simethicone used for dose preparations was recorded as C12806 and should have been C128706. This was confirmed by the supplier. The product had been mislabeled.

These deviations were deemed to not have a negative impact on the study.

8.0 CONCLUSIONS

Under the repeated dose conditions of the experiment, A4+ was negative for the production of elevated micronucleus counts and did not exhibit bone marrow toxicity. With the results of this study, combined with those of TEH-147, a no adverse effect dose level (NOAEL) of 2000 mg/kg may be assigned for genotoxicity and bone marrow toxicity.

9.0 REFERENCES

Krishna G and Hayashi M (2000). In vivo rodent micronucleus assay: protocol, conduct and data interpretation, *Mutat. Res.* 455:155-166.

Shelby MD, Gulati DK, Tice RR and Wojciechowski JP (1989) Results of tests for micronuclei and chromosomal aberrations in mouse bone marrow cells with the human carcinogens 4-aminobiphenyl, treosulphan, and melphalan, *Env. Mol. Mutagenesis* 13:339–342.

Reference # 22

ToxTest®

FINAL REPORT

A Mammalian Erythrocyte Micronucleus Study of A4+ in Mice

TEH-147
Sabell Corporation

FINAL REPORT

STUDY TITLE: **A MAMMALIAN ERYTHROCYTE MICRONUCLEUS STUDY OF A4+ IN MICE**

STUDY PLAN NO.: TEH-147

STUDY DIRECTOR:
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Study Initiation Date: February 2, 2010

Study Completion Date: September 2, 2010

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APPENDICIES

SUMMARY

The intent of this study was to evaluate the genotoxic potential of Sabell A4+ based upon its ability to induce micronuclei in rodent polychromatophilic erythrocytes (PCE). Groups of Balb/c mice (n=5 per sex per group) were gavaged with escalating dosages of test item (125 mg/kg, 500 mg/kg, and 2000 mg/kg BW orally), vehicle control (water containing 0.2% v/v simethicone) or injected intraperitoneally with positive control (cyclophosphamide, 40 mg/kg BW), or a second vehicle control (sterile water). No abnormalities were observed during clinical observations. Animals were euthanized 24 hours post-dosing, both femurs were collected, and the marrows were flushed with fetal bovine serum. From the suspended cells, bone marrow smears were made. The smears were scored for PCE/200 erythrocytes and for micronucleated PCE (MNPCE)/2000 PCE.

Statistical analysis of the bone marrow scores revealed that only the positive control compound cyclophosphamide reduced PCE scores in the males, an indicator of bone marrow toxicity. No changes in PCE scores were associated with A4+ test item.

MNPCE scores, the primary indicator of genotoxicity in this test, were significantly higher in the positive control animals of both sexes than in any of the A4+-treated animals or the treatment negative controls. Therefore, under the conditions of this experiment, the micronucleus test was considered negative for A4+ genotoxicity.

Because of the poor aqueous solubility of A4+, absorption of the product is likely to be slow. Therefore, the results of an acute micronucleus protocol may not be sufficient to predict the genotoxicity potential in humans. It is recommended that a second micronucleus assay be integrated into a repeated dose oral toxicity study.

COMPLIANCE STATEMENT

A MAMMALIAN ERYTHROCYTE MICRONUCLEUS STUDY OF A4+ IN MICE

This study was conducted in compliance with OECD Principles of Good Laboratory Practice (ENV/MC/CHEM(98)17) with the following exceptions. Test item and dose concentration analysis were not characterized in compliance with GLP. Also, micronucleus scores were directly entered into a computer.

The undersigned have reviewed and approved the report for final issuance.

Study Director:

Hugh A. Semple — Sept. 2, 2010

Hugh Semple, DVM, PhD — Date

Test Facility Management:

Donna M. Day — August 4, 2010

Donna M. Day — Date

QUALITY ASSURANCE STATEMENT

A MAMMALIAN ERYTHROCYTE MICRONUCLEUS STUDY OF A4+ IN MICE

Date	QAI Number	Inspection Type	Phase	Date reported to Study Director and Management
01-Feb-10	QAI0789	study plan	study plan	02-Feb-10
11-Feb-10	QAI0795	study-based	dosing	12-Feb-10
11-Feb-10	QAI0796	study-based	clinical observations	12-Feb-10
12-Feb-10	QAI0797	study-based	bone marrow collection	12-Feb-10
12-Feb-10	QAI0798	study-based	bone marrow smear preparation and staining	12-Feb-10
12-Feb-10	QAI0799	study-based	dose preparation documentation	17-Feb-10
15-Mar-2010 to 17-Mar-2010	QAI0812	draft report	draft report audit	18-Mar-10
15-Mar-2010 to 17-Mar-2010	QAI0813	raw data	raw data audit	18-Mar-10
1-Sept-2010	QAI0836	final report	final report audit	1 Sept-2010

The reported results in this final report accurately and completely reflect the raw data.

Quality Assurance Auditor

Sept 1/10
Date

A MAMMALIAN ERYTHROCYTE MICRONUCLEUS STUDY OF A4+ IN MICE

1.0 GENERAL POINTS

1.1 Test Item

A4+

1.2 Nature and Purpose

The purpose of this study was to determine the genotoxicity of A4+ via a micronucleus test in order to support an NHP product license and human clinical trials applications.

1.3 Study Plan No.

TEH-147 (Appendix A)

1.4 Supervisory Personnel

Head, Animal Care: Shawna Bast
Veterinary Care: Dr. Hugh Semple
Head, Pathology/Data Management/Pharmacy: Marja Rynsewyn

1.5 Regulatory Compliance

This was a GLP study and was conducted in accordance with OECD GLPs (with the exception of test item characterization, dose concentration analysis and data entry for micronucleus scoring), and ToxTest Standard Operating Procedures.

1.6 Test Method/Guideline

The design is a mammalian erythrocyte micronucleus study to be conducted under GLP according to OECD 474 guidelines.

1.7 Schedule of the Study

Experimental starting date: February 4, 2010 (manual randomization to group upon arrival)
First day of acclimation: February 4, 2010
Dosing date: February 11, 2010
Necropsy day: February 12, 2010
Experimental completion date: February 12, 2010

2.0 TEST ITEM INFORMATION

2.1 Test Item

- Name or Code: A4+
- Supplier: Sabell Corporation
- Appearance: Tan powder

- Purity: Supplied by the sponsor and added to Appendix E. The sponsor assumed responsibility for characterization including identity, purity and stability of the bulk test item under the test conditions used in this study.
- Batch Number: 1-2009
- Storage: Room temperature
- Retest Date: March 31, 2010
- Fate of Test Item: Returned to sponsor or reallocated to another study.

2.2 Vehicle 1

- Name or Code: Nanopure water with 0. 2%v/v of simethicone USP
- Supplier: In-house water, PCCA for simethicone (Lot #C128706 PCCA, exp. Sep. 16, 2011, Store @ room temperature)
- Batch Number: TR-372
- Storage: Room temperature
- Expiry Date: February 20, 2010

2.3 Reference Item 1

- Name or Code: Vehicle: Nanopure water with 0. 2%v/v of simethicone USP (Lot #C128706 PCCA, exp. Sep. 16, 2011, Store @ room temperature)
- Supplier: In-house, PCCA for simethicone
- Batch Number: TR-372
- Storage: Room temperature
- Expiry Date: February 20, 2010

2.4 Reference Item 2 (positive control)

- Name or Code: Cyclophosphamide monohydrate
- Supplier: Sigma-Aldrich
- Appearance: white powder
- Lot Number: 079K1569
- Storage: 2-8°C
- Expiry Date: August 2012

2.5 Vehicle 2

- Name or Code: Sterile Water, USP, for injection
- Supplier: Sigma
- Lot Number: RNBB0279
- Storage: Room temperature
- Expiry Date: August 2011

2.6 Reference Item 3

- Name or Code: Sterile Water USP, for injection
- Supplier: Sigma
- Lot Number: RNBB0279
- Storage: Room temperature

- Expiry Date: August 2011

2.7 Formulation of the Test Item

- Preparation: The dose materials were calculated and prepared assuming the test item was 100% pure. An appropriate amount of A4+ dried extract was finely ground with a mortar and pestle. Appropriate amounts were weighed into Falcon tubes, 0.002 mL of simethicone USP/mL dosing solution, and an appropriate volume of water was added to make sufficient quantities of test item suspension for administering for the day of dosing. The mixtures were vortexed or mixed with a stirring bar to make a suspension. The suspension was again vortexed or mixed with a stirring bar immediately before taking up each dose for administration.
- Storage conditions after formulation: room temperature
- Special handling requirements after formulation: The suspension was mixed to make it homogeneous before administration.
- Fate of remaining dosing solutions: Discarded after completion of the draft report.
- Frequency of Preparations: Once on day of dosing

2.8 Formulation of Reference Item 1

- Preparation: The dose materials were calculated and prepared assuming the reference item was 100% pure. Simethicone USP was added to nanopure water to a concentration of 0.002 mL/mL of dosing solution at an appropriate volume to make sufficient quantities of reference item for the day of dosing. The mixtures were stirred with a stirring bar to make an emulsion.
- Storage conditions after formulation: Room temperature
- Special handling requirements after formulation: Stir the emulsion with a stirring bar to make it homogeneous before administering.
- Fate of remaining dosing solutions: Discarded after completion of the draft report.
- Frequency of Preparations: Once on day of dosing

2.9 Formulation of Reference Item 2

- Preparation: prepared a 4 mg/ml cyclophosphamide solution on day of dosing
- Storage conditions after formulation: room temperature
- Fate of remaining dosing solutions: discarded after completion of draft report
- Frequency of Preparations: once on day of dosing

2.10 Fate of Test Item

Unused test item was transferred to another study (TEH-148). When all scheduled studies have been finished, unused test item will be returned to the sponsor.

2.11 Fate of Reference Item 2

Unused reference item was retained by test facility for use in future studies.

2.12 Test and Reference Item Concentration Analysis

Determination of concentration of the test and reference items in the dose material was conducted. A 2 ml aliquot of each dose material was stored at below -15°C and shipped on ice to

Dr. Duff Sloley, Phytovox, Inc. for analysis. The methods of analysis are described in Appendix E.

2.13 Test Item Stability and Homogeneity

Test item stability and homogeneity under the conditions of the study were determined by Dr. Duff Sloley, Phytovox. The conditions of the determinations and methods of analysis are described in Appendix F.

3.0 EXPERIMENT PROCEDURE

3.1 Test System and Environment

3.1.1 *Justification for Selection of Test System*
Standard species for test guideline (OECD 474)

3.1.2 *Species, Strain, Supplier and Specifications*

- Species/Strain: Balb/c mice
- Supplier: Charles River Canada Inc.
- Number of Animals in the Study: 60
- Age at Initiation of Treatment: 6 weeks
- Sex: male and female
- Weight Range at Initiation of Treatment (grams): The weight variation of animals was minimal and did not exceed ± 20% of the mean weight for each sex.
  Male: 18.2-23.1g
  Female: 16.9-19.9g

3.1.3 *Environment and Husbandry*
The animal room environment was controlled with targeted conditions:

- Housing: group
- Temperature: 18 - 26°C
- Relative Humidity: 30 - 70%
- Air Changes: 10 - 15 per hour
- Light Cycle: ~12 hours light
- Caging: Shoebox cages with environmental enrichment
- Bedding: Anderson's Bed-O-Cobs, Lot # 091808

3.1.4 *Diet and Water*

- Diet: *ad libium* Certified Rodent Chow – Irradiated (Certified Pico Rodent Diet Lot AUG 17 09 3A from LabDiet)
- Water: *ad libitum* reverse osmosis UV sanitized water

The study director has reviewed the feed, and bedding for contaminants and found none present.

3.2 Acclimation Procedures

- Animal Health Procedure: A health status report was generated prior to animals being released to the study.

- Acclimation Period: Animals were acclimated to rack and position for seven days.
- Allocation to Treatment Group: Mice were manually randomized to group on the day of arrival.
- Identification of the Animals: temporary marking
- Identification Numbers: Specified in raw data
- Identification of the Cage: Cage card

3.3 Treatment

3.3.1 *Experimental Design*

This study involved administration of three doses of A4+ plus vehicle control as well as a positive control substance, cyclophosphamide, to male and female mice once as per Table 1, followed 24 hours later by euthanasia and necropsy.

Table 1. Study Design with Dosing Details

Treatment Group	Treatment	No. of Animals per Group		Dosage Level (µg/g BW)	Dosage Concentration (mg/ml)	Dosage Volume (µL/g BW)
		Male	Female			
1	Reference Item 1 (vehicle control)	5	5	0	0	6
2	Low dose A4+	5	5	125	20.81	6
3	Mid dose A4+	5	5	500	83.25	6
4	High dose A4+	5	5	2000	333	6
5	Water (Reference Item 3)	5	5	0	0	10
6	Cyclophosphamide (positive control, Reference Item 2)	5	5	40	4.0	10

3.3.2 *Rationale for Dose Selection*

The A4+ doses were calculated as large multiples of the human dose. The cyclophosphamide dose is the standard dose for a micronucleus assay positive control.

3.3.3 *Route and Method of Administration*

3.3.3.1 *A4+ and Reference Item 1*

- Route: oral
- Method: gavage
- Rate of Dosing: bolus
- Volume Administered: 6 µL/g BW for A4+, Reference Item 1
- Dosing was completed before 12:00 PM.

3.3.3.2 *Cyclophosphamide and Reference Item 3*

- Route: intraperitoneal
- Method: injection
- Rate of Dosing: bolus
- Volume Administered: 10 µL/g BW for cyclophosphamide and Reference Item 3

- Dosing was completed before 12:00 PM.

3.3.4 *Frequency and Duration of Treatment*

- Frequency: Once
- Number of administrations: 1

3.4 Examinations Performed During the Test Period

3.4.1 *Observations*

Daily clinical observations (post dose and prior to euthanasia)

3.4.2 *Body Weight*

Body weights were taken on all animals on study day 1 prior to dosing. These weights were used to calculate dose.

3.4.3 *Euthanasia*

CO_2 asphyxiation

3.4.4 *Femur Collection and Bone Smear Preparation*

Both femurs were collected from each animal no earlier than 24 hours after dosing, and no later than 26 hours post-dosing. Bone marrow was collected; a smear was then prepared and stained with Wright's Giemsa. The slides were labeled with animal number but not group number so that the slides were read blind.

3.4.5 *Scoring for Micronucleated Polychromatic Erythrocytes*

Bone marrow slides were scored blindly (without knowledge of treatment).

The ratio of polychromatic erythrocytes to total erythrocytes was determined by examining 200 erythrocytes per animal.

The number of micronucleated PCE's (MNPCE) in 2000 PCE/animal was then determined. The unit of analysis was PCE and not the number of micronuclei per PCE, as a PCE may contain more than one micronucleus.

The results were recorded on the Micronucleus Score Sheet.

4.0 STATISTICAL ANALYSES

The Statistical report can be found in Appendix C.

- The statistical analyses were performed using SAS Release 9.2 for Windows XP.
- Statistical procedures were selected based on the distribution of the data and the validity of the assumptions.
- Statistical significance was declared when $p \leq 0.05$.
- The number of polychromatic erythrocytes (PCE) in 200 erythrocytes per animal and the number of micronucleated PCEs (MNPCE) in 2000 PCE per animal were compared between sexes and groups using an analysis of variance.
- MNPCE was normalized using a natural log-transformation of the value +1. If the model revealed statistical significance ($p \leq 0.05$), Tukey-Kramer adjusted comparisons were used to determine if pair-wise differences existed between groups.

- The ratio of the number of PCE per 200 erythrocytes and the ratio of the number of MNPCE per 2000 PCE were also calculated for each animal and summarized by sex and group.

5.0 ARCHIVES

The following materials will be maintained in the archives of the test facility:

- Study plan and (if applicable) amendment(s).
- Raw data and final report
- Bone marrow smear slides
- Analytical results from Phytovox

6.0 RESULTS

6.1 Clinical Observations

No abnormalities were encountered during clinical observations.

6.2 Body Weights

Detailed individual animal body weights can be found in Appendix B, Table B1. The females had a weight range of 16.9 g to 19.9 g, with a mean of 18.7 g. The males had a weight range of 18.2 g to 23.1 g with a mean of 21.1 g. All weights were within ±20% of the mean body weight.

6.2.1 *Interpretation*

Body weights of all animals were within acceptable limits.

6.3 Bone Marrow Scores

Detailed individual animal bone marrow scores can be found in Appendix B, Table B2. Summary statistics for polychromatic erythrocytes (PCE), micronucleated PCE's (MNPCE), and the ratios of PCE per 200 erythrocytes and MNPCE per 2000 PCE by sex and group are presented in Table 2.

There were no significant differences between the treatment groups and the negative controls, nor were there significant differences among the treatment groups at different doses for the number of PCE's in 200 erythrocytes. Only in the males was there a difference between the positive control counts and those of the three treatment groups. The positive control males had significantly lower numbers of PCE's than those of the three treatment groups.

There were no significant differences between the treatment groups and the negative controls, nor were there significant differences among the treatment groups at different doses for the number of MNPCE in 2000 PCE. Positive control group females had significantly higher number of MNPCE than females in the control, low, mid and high dose groups and positive control group males had significantly higher numbers of MNPCE than all other groups.

The analysis of both PCE and MNPCE including both sexes showed significant differences between sexes; therefore data was not combined for further analyses.

Table 2. Summary statistics for the number of PCE per 200 erythrocytes, ratio of PCE to total erythrocytes, the number of MNPCE per 2000 PCE, and the ratio of MNPCE to total PCE by sex and group.

GROUP	Variable	Females		Males	
		Mean	Std Dev	Mean	Std Dev
1 (Vehicle control) (n=5)	PCE	94.200	11.520	94.400	14.241
	PCE to total RBC ratio	0.471	0.058	0.472	0.071
	MNPCE	3.600	2.510	4.200	1.643
	MNPCE to PCE_RATIO	0.002	0.001	0.002	0.001
2 (Low) (n=5)	PCE	103.200	9.550	101.800	8.012
	PCE_TOT_RBC_RATIO	0.516	0.048	0.509	0.040
	MNPCE	2.800	1.924	4.400	3.912
	MNPCE_PCE_RATIO	0.001	0.001	0.002	0.002
3 (Mid) (n=5)	PCE	96.800	10.895	111.800	11.345
	PCE_TOT_RBC_RATIO	0.484	0.054	0.559	0.057
	MNPCE	3.400	2.510	4.000	2.449
	MNPCE_PCE_RATIO	0.002	0.001	0.002	0.001
4 (High) (n=5)	PCE	87.200	5.891	102.000	10.840
	PCE_TOT_RBC_RATIO	0.436	0.029	0.510	0.054
	MNPCE	2.600	1.949	5.600	2.881
	MNPCE_PCE_RATIO	0.001	0.001	0.003	0.001
5 (Cyclophosphamide vehicle control) (n=5)	PCE	95.200	8.349	96.400	12.116
	PCE_TOT_RBC_RATIO	0.476	0.042	0.482	0.061
	MNPCE	3.800	2.280	4.400	1.140
	MNPCE_PCE_RATIO	0.002	0.001	0.002	0.001
6 Cyclophosphamide (Positive Control) (n=5)	PCE	95.400	15.143	76.800	15.611
	PCE_TOT_RBC_RATIO	0.477	0.076	0.384	0.078
	MNPCE	14.600	8.112	33.200	7.727
	MNPCE_PCE_RATIO	0.007	0.004	0.017	0.004

6.3.1 *Interpretation*

The assay performed as expected, producing negative control counts of MNPCE consistent with the results from the negative controls reported by Krishna and Hayashi (2000). Their mean negative control assay range for MNPCE/1000 PCE was 0.4-3.8 for males and 0.6-3.6 for females. In this study, the means were in the middle of those ranges. Furthermore, the means from the three A4+ treatment groups were similar and not statistically different from controls, which is the most important indicator that under the conditions of the study, A4+ did not show any genotoxicity.

Similarly, the positive control ranges for MNPCE/1000 PCE reported by Krishna and Hayashi (2000) were 7.7-42.7 for the males and 8.0-44.7 for the females. In this study, the mean for the males was in the middle of the range, while for the females, the mean was slightly below that range. The reason for the low mean in the females is that two of the five animals had low MNPCE scores, 3MNPCE/1000PCE, which is in the range of the negative control animals. The cause of this is not known, however, animals dosed by the intraperitoneal route occasionally have test item enter the cecum instead of the peritoneal cavity despite the use of proper technique. This resulted in the positive control scores narrowly missing significant difference from its own vehicle control in the females, even though the other relevant comparisons showed statistically significant differences. Nevertheless, for both males and females, the positive control means were greater than the means for the treatment vehicle negative controls (Group 1) and for any of the A4+ treatment groups, indicating that elevated numbers of MNPCE were detected where they were expected to occur.

A secondary endpoint of the micronucleus test is the number of PCE per 200 erythrocytes. Reduced PCE numbers in a treatment group compared with controls may indicate bone marrow toxicity. In this study, none of the A4+ treatments were significantly different from controls in terms of PCE per 200 erythrocytes. In the males, however, PCE levels were lower in the positive control group than in the three A4+ treatment groups, (but not significantly lower than either the treatment or cyclophosphamide vehicle negative controls), indicating bone marrow toxicity of the cyclophosphamide. Therefore, no evidence for bone marrow toxicity of A4+ was uncovered, however, the cyclophosphamide treatment appeared to be toxic to bone marrow in the males.

6.4 Dosing Concentration

Dosing solutions were subjected to verification of dose concentration by Phytovox Inc. Dose verification reports are found in Appendix D. Using the quantity of rutin (0.118 g rutin/g A4+ powder) in the A4+ powder and in the suspension, the quantity of powder in the suspensions versus the nominal concentrations was compared. The low dose suspension contained 22.8 mg/mL of powder (110% of nominal), the mid dose suspension contained 98.8 mg/mL of powder (119% of nominal) and the high dose suspension contained 46 mg/mL of powder (117% of nominal). The control solutions contained no rutin.

Cyclophosphamide could be measured quantitatively, so that was done. The measured concentration of cyclophosphamide was 3.8 mg/mL which was very close to the nominal concentration of 4 mg/mL. The sterile water solution contained no cyclophosphamide.

6.4.1 *Interpretation*

All of the concentrations were within acceptable limits.

7.0 DEVIATIONS

7.1 Study Plan Deviation

The lot number of cyclophosphamide used for the reference item 2 positive control was not the number that was specified in the study plan.

Reference item 2 (cyclophosphamide) was not stored at 2-8°C after 2 h as this period was during dosing.

None of the deviations were deemed to have a negative impact on the study.

8.0 CONCLUSIONS

Under the conditions of the experiment, A4+ was negative for the production of elevated micronucleus counts and did not exhibit bone marrow toxicity. If an acute micronucleus test is negative after 24 hours exposure, another cohort of animals should be examined 48 hours post dosing, or consideration should be given to testing for micronuclei after multiple dosing (Krishna and Hayashi, 2000). In the case of A4+, because the extract is poorly water soluble, oral absorption is likely to be slow. Therefore, the results of an acute micronucleus protocol may not adequately predict the genotoxicity potential of A4+ in humans. Therefore, integration of another micronucleus test into a repeated dose oral toxicity study is recommended.

9.0 REFERENCES

Krishna G and Hayashi M (2000). In vivo rodent micronucleus assay: protocol, conduct and data interpretation, *Mutat. Res.* 455:155-166.

Reference # 23

UNIVERSITY OF CALGARY

Evaluation of the Hepatoprotective and Behavioural Effects of the Herbal Compound A4+ in Liver Injury Models

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Evaluation of the Hepatoprotective and Behavioural Effects of the Herbal Compound A4+ in Acute Liver Injury Models

ABSTRACT:

The herbal preparation A4+ has been postulated to have beneficial effects on quality of life and liver injury in patients with hepatitis. The studies described in this report were designed to determine whether A4+ could attenuate the detrimental behavioural (*i.e.*, sickness behaviours) and biochemical effects associated with liver injury in two well characterized mouse models of liver injury.

The first model was bile duct ligation and resection (BDR). For this the mice were randomly divided into two groups: BDR surgery only (control) and BDR plus A4+ (160 mg/kg/day administered by oral gavage. Sickness behaviour was evaluated using two well established methods: (i) A social investigation paradigm and (ii) open field locomotor activity measurement to assess overall mobility.

Results: Levels of plasma ALT and total bilirubin levels were similar in control and A4+ treated BDR mice at 9 days post surgery, as was time spent in social investigation behaviour. By contrast, BDR animals administered A4+ were significantly more active in overall mobility for both ambulatory movements ($p = 0.03$) and enhancement of the number of horizontal movements ($p=0.04$), respectively.

The second model was Concanavalin A (ConA)-induced hepatitis. For this study, one group of mice were pre-treated with vehicle (control) and another group with A4+ (640 mg/kg). As in the first study, biochemical measurements were measured. In addition, livers were dissected and

processed for flow cytometry analysis (FACS) after staining of different cell surface markers to identify immune cell subsets and to identify cytokine production profiles of these cells using intracellular staining.

Results: Plasma ALT levels in the A4+ treated Con A mice were significantly ($p=0.04$) reduced compared to the control Con A mice. Pretreatment with A4+ had no significant effect on IFNγ cell recruitment and activation. In contrast, hepatic recruitment of IFNγ expressing NK cells to the liver were significantly ($p<0.05$) increased in the A4+ Con A treated animals compared to Con A controls.

Conclusions: The first study indicated that A4+ has beneficial behavioral modifying effects in a test which examined sickness-related immobility. These improvements occurred in the absence of significant changes in biochemical indices of liver damage.

In the second study A4+ treatment attenuated Con A hepatitis as reflected by a reduction in plasma ALT levels compared to vehicle-treated controls. Pretreatment with A4+ had no significant effect on IFNγ cell recruitment and activation. However, more hepatic NK cells expressed IFNγ in mice pretreated with A4+ which received ConA than in vehicle-treated mice.

Overall, the finding of positive changes in behavior, liver enzymes and some aspects of immune function in these animal models of hepatic injury indicate a potential clinical relevance for the treatment of patients with hepatic injury.

Evaluation of the Hepatoprotective and Behavioural Effects of the Herbal Compound A4+ in Acute Liver Injury Models

BACKGROUND:

A4+ is a product that consists of a combination of three herbal ingredients: Cordia lutea (80%), Annona muritata (10%) and Curcuma longa (10%). In anecdotal reports, this compound has been used for many years to treat individuals with a variety of different liver diseases, with a suggestion of beneficial outcomes in some of these people. In open-label clinical studies, it has been shown to confer benefit with regards to quality of life measurements and possibly biochemical indices of liver function to patients with hepatitis C. The mechanism of action of A4+ is not known, although it has been shown in preliminary studies to possess anti-oxidant properties.

The studies described in this report were designed to determine whether A4+ could exert potential beneficial effects in attenuating the widely recognized detrimental behavioural effects associated with liver injury (*i.e.*, sickness behaviours) in well-characterized models of acute liver injury.

Evaluation of the Hepatoprotective and Behavioural Effects of the Herbal Compound A4+ in Acute Liver Injury Models

A) Behavioural Studies:

Patients with liver diseases often exhibit a number of associated symptoms including fatigue, malaise, loss of interest in engaging in social activity and an inability to concentrate, which have been collectively termed sickness behaviours[1, 2]. In fact, patients often describe these symptoms as having the greatest detrimental impact upon their quality of life. Although there is a tendency to consider sickness behaviours in these patients as being inconsequential compared to the underlying disease itself, it is important to recognize that these symptoms are major causes of disability and work absenteeism, not to mention the high associated social and economic costs. Moreover, currently available therapies for most liver diseases have minimal impact on most of these sickness behaviours. Despite their high prevalence, these symptoms are often not considered or are disregarded by physicians who often target treatment of the specific physical manifestations of the liver disease. This issue is further compounded by the fact that the etiology of such symptoms, in the setting of liver diseases, is poorly understood and no therapies are currently available to specifically treat them.

METHODS:

Animal Model:

Male C57BL/6 mice with initial body weight 22-24 g were used in the present study. Animals were housed in an environmentally-controlled room with a 12-12-h light-dark cycle and allowed access to food and water *ad libitum*. The mice were randomly divided into two groups: bile duct

resection (BDR) surgery[3, 4] only (control) and BDR plus A4+ (160 mg/kg/day administered by oral gavage). Briefly, after 4 days of adaptation, surgery was performed under isoflurane inhalation anesthesia, the common bile duct was exposed through a midline abdominal incision, doubly ligated with 5-0 silk and sectioned between the ligatures. The incision was then closed by 5-0 silk sutures. Immediately after surgery, mice were treated with antibiotic ointment to the abdominal suture site to prevent infection. Behaviour studies were conducted one day before surgery and 5 and 9 days after the operation.

At the end of the experiments, blood was collected for ALT (an indicator of liver cell destruction or damage) and total bilirubin (marker of impaired liver cell biosynthetic capabilities) measurements (serum biochemical measurements were performed by Calgary Laboratory Services, Calgary, AB).

Sickness Behaviour Quantification:

Sickness behaviour was evaluated using two well established methods:

(i) A social investigation paradigm was used to examine loss of social interest[4]. Prior to observation, test and control mice were housed in individual cages for a period of 24 hours. Experiments were performed between 7:00 and 10:00 am on the day prior to surgery and then repeated on days 5 and 9 post-surgery. A 3-4 week old juvenile male C57BL/6 mouse was introduced into the home cage of the test mouse, and behaviour was assessed for a period of 10 minutes by two blinded observers. A number of facets of social exploratory behaviour were

examined, including the total time of social investigation, total time the test mouse remained immobile and the number of social interactions between the test mouse and a juvenile mouse.

(ii) Open field locomotor activity measurement was used to assess overall mobility[5]. Prior to observation, test mice were housed in individual cages for a period of 24 hours. Experiments were performed between 7:00 and 10:00 am on day 9 post-surgery. Locomotor activity was measured using an Optivarimax apparatus (Columbus Instruments, Columbus, OH) which records the ambulatory and horizontal movements of a mouse placed in an open field, as measured by the number of times infrared photoelectric beams are broken by the mouse during the observation period (5 min).

RESULTS:

(1) The effects of A4+ on plasma liver biochemistry levels in BDR mice at 9 days post-surgery:

As shown in Figure 1, the levels of plasma ALT and total bilirubin levels were similar in control and A4+ treated BDR mice at 9 days post surgery.

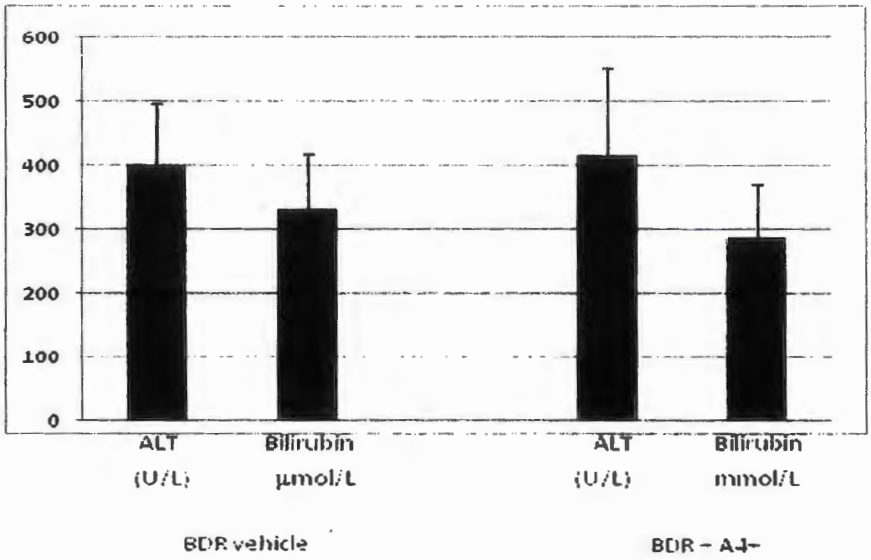


Figure 1. Plasma ALT and total bilirubin levels were not significantly different between control and A4+ treated BDR mice on day 9 post surgery. Bars indicate the means ± SEM of data from 7-8 mice/group.

(2) The effects of A4+ on sickness behaviours:

The times spent in social investigation behaviour of the control (BDR + vehicle) mice and A4+ treated (160 mg/kg/day by oral gavage) BDR mice were not significantly different at days 5 and 9 post surgery (Figure 2). Baseline activity was assessed just prior to surgery on the same day. There were also no significant differences in number of social interactions exhibited by the BDR + vehicle and BDR + A4+ groups of mice at days 5 and 9 post-BDR surgery (figure 3).

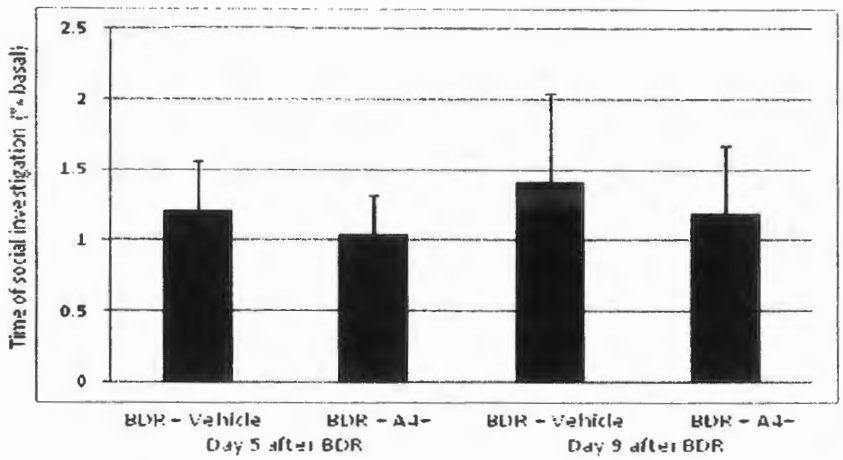


Figure 2. Time that the test BDR mouse spent in social investigation behaviour with the juvenile mouse (as % of basal activity as determined on day before surgery) at days 5 and 9 post-BDR surgery. Two groups of BDR mice were studied: BDR + vehicle and BDR + A4+ (160 mg/kg/day by oral gavage). Bars represent the means $\pm$ SD of data from 7-8 mice/group. BDR *vs* BDR + A4+ groups are not significantly different at day 5 or day 9.

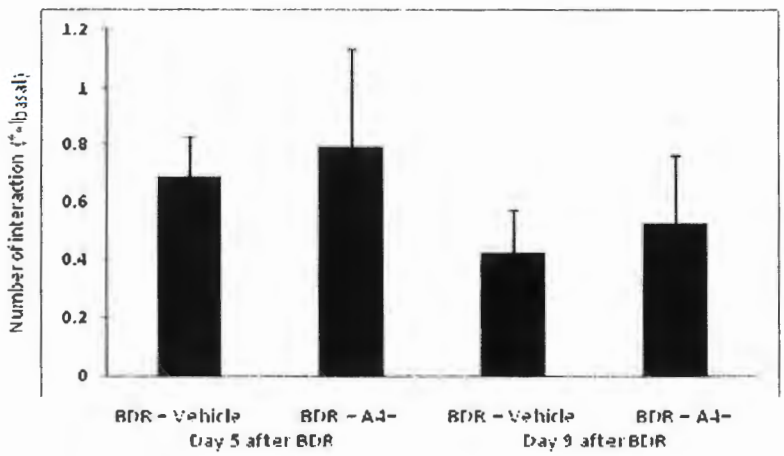


Figure 3. Number of social interactions exhibited by the test BDR mouse towards the juvenile mouse (as % of basal activity as determined on day before surgery) at days 5 and 9 post-BDR

surgery. Two groups of BDR mice were studied: BDR + vehicle and BDR + A4+ (160 mg/kg/day by oral gavage). Bars represent the means ± SD of data from 7-8 mice/group. BDR *vs* BDR + A4+ groups are not significantly different at day 5 or day 9.

In contrast to the social interaction experiments above, studies of the ambulatory movements revealed positive effects. BDR animals administered A4+ were more active in overall mobility. As shown in Figure 4, the number of ambulatory movements on day 9 post surgery were greater in the BDR A4+ treated animals compared to controls. In addition, there was an enhancement of the number of horizontal movements (Figure 5). The differences in mobility were significant at $p = 0.03$ (Figure 4) and $p = 0.04$ (Figure 5), respectively.

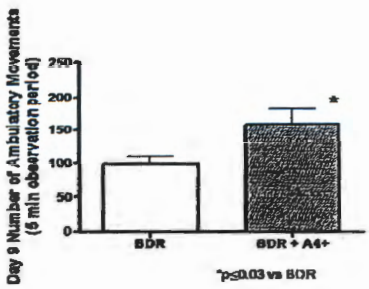


Figure 4. Number of ambulatory movements exhibited by day 9 BDR+vehicle mice *vs* day 9 BDR + A4+ (160 mg/kg/day) treated mice after being placed in an open field apparatus. Observation period was 5 minutes. Bars are the mean ± SD of data from 7 BDR+vehicle and 8 BDR+A4$^+$ treated mice per group. *p=0.03 *vs* BDR+vehicle group.

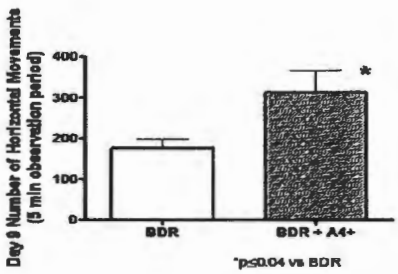


Figure 5. Number of horizontal movements exhibited by day 9 BDR+vehicle mice *vs* day 9 BDR + A4+ (160 mg/kg/day) treated mice after being placed in an open field apparatus. Observation period was 5 minutes. Bars are the mean $\pm$ SD of data from 7 BDR+vehicle and 8 BDR+A4^{+} treated mice per group. *p=0.04 *vs* BDR+veh group.

(B) Hepatoprotection Studies in an Acute Model of Liver Injury:

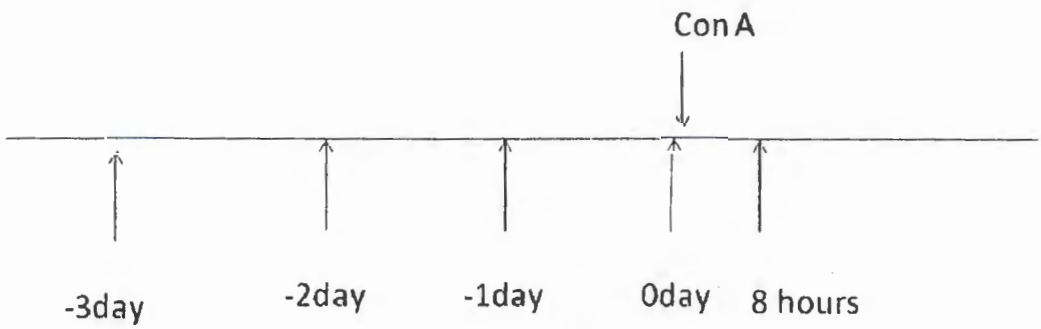
Concanavalin (Con) A-induced hepatitis is a well-characterized and widely used model of T cell-mediated hepatitis mimicking many aspects of human T cell-mediated liver disease, including autoimmune hepatitis and viral hepatitis[6, 7]. Con A-induced hepatitis is mediated by liver-infiltrating activated CD4(+) T cells. These cells produce mainly IFN-γ. Resident hepatic NKT cells produce mainly IL-4 (and to a lesser extent IFN-γ)[8]. Kupffer cells (which secrete TNF-α) and neutrophils have also been directly implicated in the pathogenesis of Con A-induced hepatitis. One group of mice were pre-treated with vehicle (control) and another group with

A4+ (640 mg/kg) by oral gavage. This dose of A4+ was found to be most effective by oral gavage in this model prior to Con A treatment (see methods below).

METHODS:

Animal Model:

Male C57BL/6 mice, body weighing between 22-24 g, were used for this study. Con A (13.5 mg/kg) or saline vehicle (control) was injected intravenously. Twelve hours after injection, mice were killed and blood was collected for measurement of ALT (an indicator of liver cell destruction or damage). Biochemical measurements were performed by Calgary Laboratory Services, Calgary, AB[7]. In addition, livers were dissected and processed for flow cytometery analysis (FACS) after staining of different cell surface markers to identify immune cell subsets and to identify cytokine production profiles of these cells using intracellular staining[6, 7].



A4+ was gavaged on day 3, 2, 1 and one hour before Con A injection, Mice were killed 8 hours later after Con A injection

RESULTS:

(i) Effects of A4+ on the severity of Con A-induced hepatitis as reflected by serum ALT levels.

As shown in Figure 6, plasma ALT levels in the A4+ treated Con A mice were significantly reduced compared to the control Con A mice.

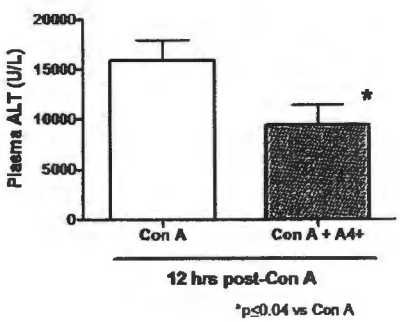


Figure 6. Plasma ALT levels were measured 12 hrs post-Con A (13.5 mg/kg iv) treatment in mice which received either saline vehicle (Con A) or 640 mg/kg A4+ (Con A + A4+) by oral gavage 3,2 and 1 days and 1 hr prior to Con A treatment. Bars are the means $\pm$ SD of data from 7 Con A plus vehicle mice and 9 Con A plus A4+ mice, per group. *p=0.04 *vs* Con A plus vehicle group.

(ii) Effects of A4+ on hepatic immune cell recruitment and activation (*i.e.*, IFNγ production) in Con A treated mice as measured by flow cytometry (FACS)

Pretreatment with A4+ had no significant effect on IFNγ production (Figure 7).

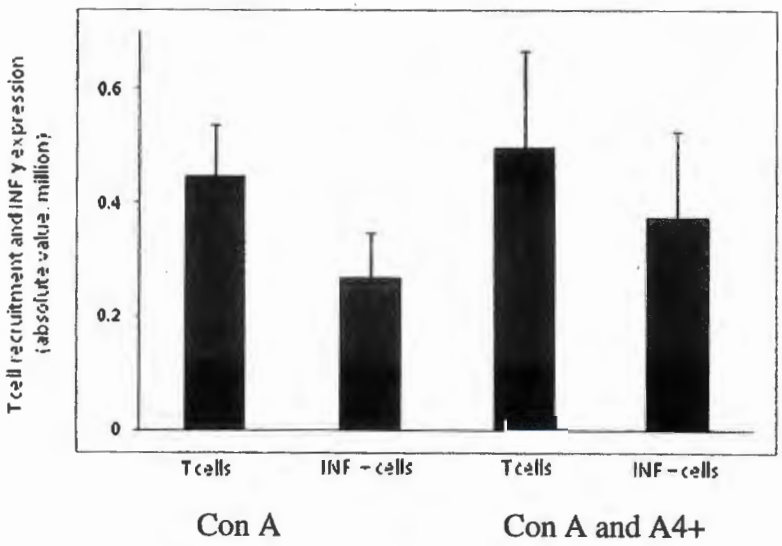


Figure 7. Hepatic T cell (determined as $CD3^+$ $NK1.1^-$ T cells by FACS) recruitment to the liver and activation (*i.e.*, IFNγ expression by FACS). Bars are the mean ± SD of data from n= 6 mice per group. T cell recruitment and $IFN\gamma^+$ T cells (in millions of cells per liver) were similar in ConA plus vehicle treated *vs* ConA plus A4+ treated groups.

In contrast, (Figure 8) hepatic recruitment of IFNγ expressing NK cells to the liver were significantly increased in the A4+ Con A treated animals compared to Con A controls.

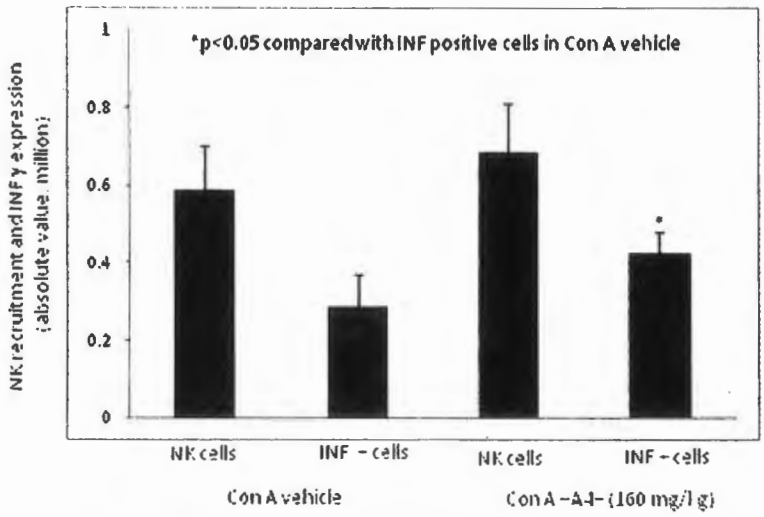


Figure 8. Hepatic NK cell (determined as $NK1.1^+$ $CD3^-$ cells by FACS) recruitment to the liver and activation (*i.e.*, IFNγ expression by FACS). Bars are the mean ± SD of data from n= 6 mice per group. NK cell recruitment (in millions of cells per liver) was similar in ConA plus vehicle treated *vs* ConA plus A4+ treated groups. $IFN\gamma^+$ NK cells (in millions of cells per liver) were significantly increased in ConA plus A4+ groups vs ConA plus vehicle treated groups; *$p<0.05$.

Data shown in Figure 9 below demonstrates that A4+ treatment did not significantly affect the numbers of NKT cells within the liver that expressed IFNγ after Con A treatment.

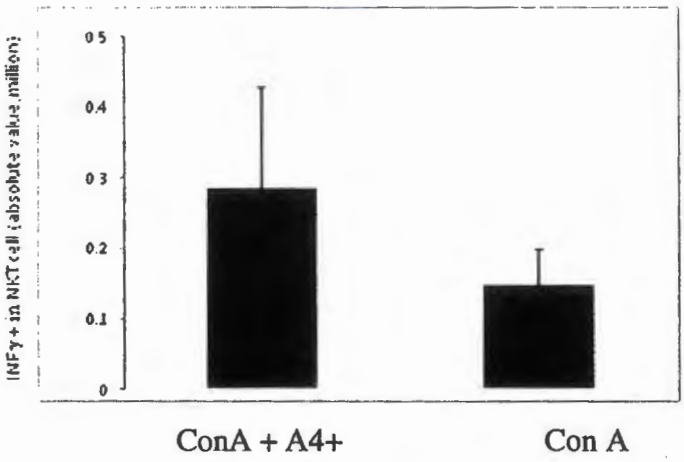


Figure 9. Hepatic NKT cell (determined as $NK1.1^+$ $CD3^+$ cells) IFNγ expression by FACS. Bars are the mean ± SD of data from n= 6 mice per group. $IFN\gamma^+$ NKT cells (in millions of cells per liver) were similar in ConA plus A4+ (640 mg/kg) group vs ConA plus vehicle treated group (*i.e.*, the 2 groups are not significantly different from each other).

CONCLUSIONS:

(A) Behavioral Assessments:

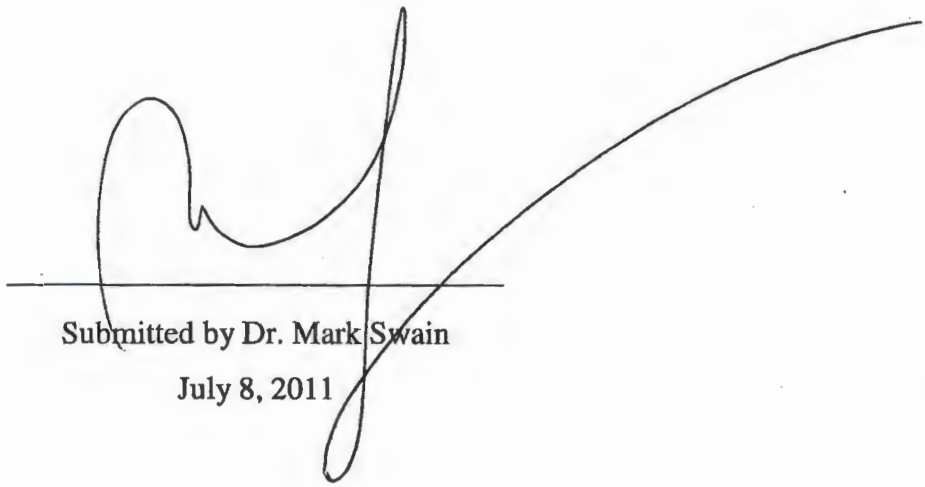
A4+ treatment resulted in no significant changes in plasma biochemical indices of liver injury in the BDR model. BDR mice treated with A4+ showed no beneficial effects with regards to sickness behaviour development as assessed by the social interaction test; however, BDR mice treated with A4+ did show a significant improvement in locomotor activity in the open field mobility assessment paradigm.

These observations suggest that A4+ has beneficial behavioural modifying effects in this model resulting in an overall behavioural activation in a test which examines sickness-related immobility. These improvements occurred in the absence of significant changes in biochemical indices of liver damage.

(B) Effects on Hepatic Injury Severity and Immune Cell Activation and Recruitment to the Liver in an Acute Hepatitis Model:

A4+ treatment attenuated Con A hepatitis as reflected by a reduction in plasma ALT levels compared to vehicle-treated controls. However, in general this reduction in plasma ALT levels induced by A4+ treatment was not paralleled by changes in hepatic recruitment of T cells or NK cells, or by changes in the activation (*i.e.*, IFNγ production) of T cells or NKT cells within the liver. However, more hepatic NK cells expressed IFNγ in A4+ pretreated mice which received ConA than in vehicle-treated mice. This finding may have potential clinical relevance as NK cell production of IFNγ has been implicated in the anti-viral and anti-tumor properties of NK cells within the liver in animal models[9-11].

Evaluation of the Hepatoprotective and Behavioural Effects of the Herbal Compound A4+ in Acute Liver Injury Models



Submitted by Dr. Mark Swain

July 8, 2011

REFERENCES:

1. Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW. From inflammation to sickness and depression: when the immune system subjugates the brain. Nat Rev Neurosci 2008;9:46-56.

2. Swain MG. Fatigue in liver disease: pathophysiology and clinical management. Can J Gastroenterol 2006;20:181-8.

3. Kerfoot SM, D'Mello C, Nguyen H, Ajuebor MN, Kubes P, Le T, Swain MG. TNF-alpha-secreting monocytes are recruited into the brain of cholestatic mice. Hepatology 2006;43:154-62.

4. D'Mello C, Le T, Swain MG. Cerebral microglia recruit monocytes into the brain in response to tumor necrosis factoralpha signaling during peripheral organ inflammation. J Neurosci 2009;29:2089-102.

5. Burak KW, Le T, Swain MG. Increased sensitivity to the locomotor-activating effects of corticotropin-releasing hormone in cholestatic rats. Gastroenterology 2002;122:681-8.

6. Ajuebor MN, Aspinall AI, Zhou F, Le T, Yang Y, Urbanski SJ, Sidobre S, Kronenberg M, Hogaboam CM, Swain MG. Lack of chemokine receptor CCR5 promotes murine fulminant liver failure by preventing the apoptosis of activated CD1d-restricted NKT cells. J Immunol 2005;174:8027-37.

7. Ajuebor MN, Wondimu Z, Hogaboam CM, Le T, Proudfoot AE, Swain MG. CCR5 deficiency drives enhanced natural killer cell trafficking to and activation within the liver in murine T cell-mediated hepatitis. Am J Pathol 2007;170:1975-88.

8. Tiegs G, Hentschel J, Wendel A. A T cell-dependent experimental liver injury in mice inducible by concanavalin A. J Clin Invest 1992;90:196-203.

9. Subleski JJ, Wiltrout RH, Weiss JM. Application of tissue-specific NK and NKT cell activity for tumor immunotherapy. J Autoimmun 2009;33:275-81.

10. van Dommelen SL, Tabarias HA, Smyth MJ, Degli-Esposti MA. Activation of natural killer (NK) T cells during murine cytomegalovirus infection enhances the antiviral response mediated by NK cells. J Virol 2003;77:1877-84.

11. Nakagawa R, Nagafune I, Tazunoki Y, Ehara H, Tomura H, Iijima R, Motoki K, Kamishohara M, Seki S. Mechanisms of the antimetastatic effect in the liver and of the hepatocyte injury induced by alpha-galactosylceramide in mice. J Immunol 2001;166:6578-84.

Reference # 24

HEPATOPROTECTIVE ACTION OF *CORDIA DICHOTOMA* AGAINST CARBON TETRACHLORIDE INDUCED LIVER INJURY IN RATS

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Key words: *Cordia dichotoma* Linn., Boraginaceae, carbon tetrachloride; serum enzyme levels; antioxidants; hepatoprotective activity.

ABSTRACT

The methanolic extract of the leaves of *Cordia dichotoma* at 300 mg/kg and 500 mg/kg were studied for hepatoprotective action in male Wistar rats by inducing liver damage using carbon tetrachloride (CCl_4). Silymarin was used as standard drug. The extract (300 mg/kg) significantly reduced the AST ($P<0.001$), ALT ($P<0.001$) and TBR levels ($P<0.01$) and at 500 mg/kg dose significantly reduced the AST ($P<0.001$), ALT ($P<0.001$), TBR ($P<0.01$) and lipid peroxide levels ($P<0.05$). The antioxidant parameters like glutathione and total antioxidant levels increased considerably although they were statistically insignificant. The hepatoprotective activity of *C. dichotoma* was comparable with silymarin (100mg/kg).

INTRODUCTION

Liver is the seat of xenobiotic metabolism. Various agents like drugs, alcohol, viruses and many other toxic agents damage the cells of liver. Since ancient periods many herbal medicines were used for treating the liver diseases. In modern medical practice also various herbal-based drugs like Silymarin from *Silybum marianum* and Phyllanthins which are polyphenols from *Phyllanthus amarus* have been successfully used [1]. Hence it becomes essential to explore the plant kingdom for development of new phytotherapeutic agents for liver diseases.

C. dichotoma Linn. (Boraginaceae) is an evergreen tree widely distributed in India and Srilanka [2]. It exhibits juvenomimetic [3] antifertility [4] and anti-inflammatory activities [5]. Traditionally it was being used as an astringent, anthelmentic, diuretic, demulcent, anti-diabetic and expectorant. The leaves of *C. dichotoma* are traditionally used for treatment of jaundice at Dandakaranya area, Andhra Pradesh in India.

The objective of the present study is to evaluate the protective effect of MCD against the toxicity of CCl_4 in rodents.

RESULTS

The effect of the extract on Aspartate amino transferase (AST), Alanine amino transferase (ALT), Alkaline phospatase (ALP), serum bilirubin(TBR), glutathione, lipid peroxide(MDA) and total antioxidants levels in CCl_4 induced liver damage in rats are summarized in Table 1. There was a significant increase in AST, ALT, ALP, serum bilirubin and lipid peroxide levels in the test group and a sharp decrease in Glutathione (GSH), total antioxidants (TAO) levels in rats treated with CCl_4 [6] alone when compared to normal rats. These parameters were positively altered on treatment with extract at 300 and 500mg/Kg rat. The therapeutic effects exhibited by these extracts were comparable to standard drug Silymarin (100 mg/kg).

Table 1. Effect of methanolic extract of leaves of *Cordia dichotoma* on serum enzymes Aspartate amino transferase (AST), Alanine amino transferase (ALT), and Alkaline phospatase (ALP), Total Bilurubin(TBR), Glutathione (GSH), lipid peroxide (MDA) and total antioxidants (TAO) levels in CCl_4 induced liver damage in rats.

Groups	AST(U/L)	ALT(U/L)	ALP(KAU/ml)	TBR(mg %)	MDA(nM/ml)	GSH(µM/ml)	TAO(µM/ml)
Normal	37.48(± 3.05)	53.38(±8.52)	83.63(±19.54)	0.26(±0.04)	1.12(± 0.16)	149.7(± 42.58)	18.13(± 3.52)
Control	60.72 (± 2.55)*	82.59 (±1.90)*	128.2 (±2.94)	1.29 (±0.02)*	2.54 (± 0.45)*	112.8 (± 15.59)**	13.82(±0.77)**
Standard	43.48 (± 1.13)	58.46 (±1.80)	89.41 (±20.17)	0.66 (±0.04)	1.41 (± 0.38)	144.2 (± 4.20)	17.93(±3.50)
MCD300	52.01 (± 1.25)*	68.85 (±1.12)*	114.1 (±5.77)	0.79 (±0.50)*	2.10(± 0.48)	132.4 (± 6.96)	15.22(±0.50)
MCD500	47.15 (± 1.08)*	63.86 (±0.67)*	102.5 (±23.33)	0.70 (±0.30)*	1.98 (± 0.26)*	139.4 (± 8.76)	16.99 (± 1.01)

Values are mean ± S.E.M. number of rats=6. Control group compared with normal group * $p<0.001$. Experimental groups compared with CCl_4 control group * * $p<0.05$.

The AST levels were increased to 60.72 U/L in CCl_4 treated rats and are considerably reduced on treatment with the extract at 300mg/Kg rat (52.01U/L) and 500mg/kg rat (47.15 U/L). A significant reduction on ($p<0.001$) was observed in AST levels when compared with toxic group. Dose dependency was exhibited by the extracts. The ALT levels of the rats treated with CCl_4 were found to be increased (82.59U/L) and there was a significant reduction to 68.85 U/L and 63.86 U/L by the extract at 300 and 500mg/kgrat respectively.

The raise in ALP levels due to induction of hepatotoxicity by CCl_4 were reduced, but the values were found to be statistically insignificant ($p>0.05$). The serum bilirubin levels of hepatotoxicity-induced rats (1.29 mg% units) were considerably reduced on treatment at both 300 (0.79 mg% units) and 500mg/kg (0.70 mg% units). The observed decrease in glutathione and total antioxidant levels due to induction of hepatotoxicity by CCl_4 were increased, but the values were found to be statistically insignificant ($p>0.05$).

The plant extract also exhibited satisfactory antioxidant property by reducing the lipid peroxides in CCl_4 induced hepatotoxic rats. The reduction in lipid peroxide level was found to be statistically significant ($p<0.05$) at 500mg/kg but insignificant ($p>0.05$) at 300.

DISCUSSION

The hepatotoxicity induced by CCl_4 is due to its metabolite $CCl_3^{\bullet}$, a free radical that binds to lipoprotein and leads to peroxidation of lipids of endoplasmic reticulum [7]. The ability of a hepatoprotective drug to reduce the injurious effects or to preserve the normal hepatic physiological mechanisms, which have been disturbed by a hepatotoxin, is the index of its protective effects. Protection of hepatic damage caused by carbon tetrachloride administration was observed by recording AST, ALT, ALP and serum bilirubin levels in treated, toxic and normal groups because serum transaminases, serum alkaline phosphatase and serum bilirubin have been reported to be sensitive indicators of liver injury [8]. The level of lipid peroxidase is a measure of membrane damage and alterations in structure and function of cellular membranes. The level of thiobarbituric acid reactive substance (TBARS) is an indirect measurement of lipid peroxidation. Lipid peroxide levels in tissue were found to be significantly elevated in CCl_4- challenged rats [9]. GSH is one of the most abundant tripeptide non-enzymatic biological antioxidant present in the liver. Its functions are concerned with the removal of free radical species such as hydrogen peroxide, superoxide radicals, alkoxy radicals, and maintenance of membrane protein thiols and as a peroxidation in a liver homogenate can proceed in a non-enzymatic way[10].

In our present study, the CCl_4 treated group exhibited increased serum enzyme levels. This indicated the damage of hepatocytes by CCl_4. There was a decrease in GSH and TAO levels which may be due to the oxidative stress induced by CCl_4. The reversal of serum enzyme levels would indicate that damaged hepatocytes were repaired and supported their regeneration.

The hepatoprotective properties of Silymarin have been related to the inhibition of lipid peroxide formation or scavenging of free radicals generated by microsomal ethanol oxidations. The extract of *C. dichotoma* also produced hepatoprotective activity similar to silymarin and so its action may also be due to inhibition of lipid peroxide formation. It also increased the total antioxidant levels to some extent. The hepatoprotective action combined with antioxidant activity may have a synergistic effect in preventing the initiation and progress of hepatocellular diseases [11].

MATERIALS AND METHODS

Plant Material

The leaves of *C. dichotoma* were collected from the campus of Kakatiya University, Warangal District, Andhra Pradesh, India. They were identified by Dr. Raju S. Vastavaya, Department of Botany, Kakatiya University, Warangal. Voucher specimens are being maintained in the herbarium (No.PLB-047) of University College of Pharmaceutical Sciences, Kakatiya University, Warangal. Fresh leaves were washed with tap water to make them free of dust and later dried under shade for 4 days. Dried leaves were ground to coarse powder and stored in an airtight container.

Extraction

The methanolic extract of *C. dichotoma* (MCD) was prepared by the maceration of leaf powder (1 Kg) with methanol (3L) at room temperature in a round bottom flask for 7 days with intermittent stirring. Then filtered to collect the extract and concentrated under reduced pressure using a rotary flash evaporator. The extract obtained was preserved in a desiccator to prevent degradation by moisture.

Drugs and Chemicals

Silymarin was obtained as a gift sample from Micro Labs (Hosur, Tamilnadu, India). ALT test kit, AST test kit, ALP test kit (Asclepius Immunotek Private Limited, Hyderabad, India), bilirubin test kit (M/s Excel Diagnostics Pvt. Ltd. Hyderadad, India), thiobarbituric acid (Hi Media Laboratories Ltd; Mumbai, India), 1, 1, 3, 3-tetraethoxy Propane (Sigma, St. Louis, USA), trichloro acetic acid (Qualigens Fine chemicals, Mumbai, India), 5-5^1-Dithiobis-2-Nitro benzoic acid (Hi Media Laboratories Ltd, Mumbai, India), sodium dihydrogen Phosphate (S.D. Fine chemicals; Mumbai, India), glutathione (Hi Media Laboratories Ltd; Mumbai, India), ascorbic acid (S.D. Fine chemicals; Mumbai, India), diphenyl picryl hydrazyl (Sigma, St. Louis, USA) are various chemicals and reagents which were used in different stages of the study. They were used as received without any further purification.

Animal

Studies were carried out using male Wistar albino rats (180–220g). They were obtained from the animal house of Mahaveera Enterprises (Reg. No.146/1999/CPCSEA), Ranga Reddy District, India. The animals were grouped and housed in polyacrylic cages (38 x 23 x 10 cm) with not more than six animals per cage and maintained under standard laboratory conditions (temperature 25 2°C) with dark and light cycle (12/12 h). They were allowed free access to standard dry pellet diet and water *ad libitum*. All procedures described were reviewed and approved by the Institutional animal ethical committee.

Animal grouping

Healthy albino rats were divided into 5 groups each containing six animals. Group 1, which served as normal, received 10 ml / kg body weight, orally (p.o) of 5% w/v gum acacia in water daily for seven days. Group 2 served as toxic and they received 1.5 ml/kg body weight of 25% v/v CCl_4 in olive oil on seventh day only. Group 3 received silymarin 100 mg/kg body weight of rats per day, Group 4 and 5 received MCD (300 and 500 mg/kg body weight) daily p.o for seven days respectively.

On the seventh day, 25%v/v CCl_4 in olive oil was given by oral route at a dose of 1.5 ml/kg body weight 30 minutes after the administration of silymarin and extracts. After 36 hours of administration of CCl_4 the rats were anaesthetized with thiopentone sodium (60 mg/ kg body weight i.p) and the blood samples were collected from common carotid artery by carefully opening the neck region.

Biochemical Studies

The blood samples were allowed to coagulate at room temperature for at least one hour. Serum was separated by centrifugation at 3000 rpm for 20 minutes and analyzed for AST, ALT, ALP, serum bilirubin, Glutathione, lipid peroxide and total antioxidants levels [6,12-16].

Estimation of AST Levels

Into a clean dry test tube, 0.25 ml of buffered substrate aspartate keto-glutarate (AKG) was taken and incubated at 37°C in a water bath for 30 minutes. Then 0.25 ml of dinitrophenyl hydrazine (DNPH) colour reagent was added, mixed well and allowed to stand at room temperature for 20 minutes. To this, 0.05 ml of serum was added and then 2.5 ml of 0.4 NaOH solution in water was added, mixed well and allowed to stand at room temperature for further 10 minutes for complete development of brown colour. Optical density of the solution was read in a spectrophotometer at 505 nm against blank.

Estimation of ALT levels

Into a clean dry test tube, 0.25 ml of buffered substrate Alanine ketoglutarate (AKG) was taken and incubated at 37°C in a water bath for 30 minutes. Then 0.25 ml of DNPH reagent was added, mixed well and allowed to stand at room temperature for 20 minutes.

To this, 0.05 ml of serum was added and then 2.5 ml of 0.4 M NaOH solution in water was added, mixed well and allowed to stand at room temperature for further 10 minutes for complete development of brown colour. Optical density of the solution was read in a spectrophotometer at 505 nm against blank. ALT levels were obtained by extrapolation from the standard curve and expressed in Karamen units (KU) per dl.

Estimation of Serum Alkaline Phosphatase levels

Into a clean dry test tube, 1.0 ml each of buffered substrate (phenyl phosphate) and distilled water were taken, mixed well. To this, 0.1ml of serum was added, mixed well and incubated at 37°C for 15 minutes in the water-bath. Then 2.0 ml of colour reagent was added and thoroughly mixed. Corresponding to each of the test samples, a control is run in which serum is added to the contents after the incubation period, all other additions being the same. A standard was run in the same way as for test in which 1.0 ml of 1% phenol standard was added in place of serum. Similarly, a blank was run to which buffered substrate, distilled water, and colour reagents were only added. Optical densities of blank, standard, control and test were measured at 510 nm in spectrophotometer against distilled water. Serum alkaline phosphatase activity was calculated based on the following formula and expressed in Kind and Anderson units per ml.

Serum Alkaline Phosphatase activity,

$$KA\ U/ml = \frac{(O.D.\ test - O.D.\ control)}{(O.D.\ S\tan dard - O.D.\ blank)\ X10)}$$

Estimation of Total Serum Bilirubin

Into a clean test tube, 1ml of Diazo A and 0.1ml of Diazo B reagents, 1.0 ml of activator, 2.5 ml of distilled water and 0.2 ml of serum were added (test solution). Corresponding to each test a control was run in the same way, the only difference being Diazo B was not added. The tubes were mixed well and kept in dark for 5 minutes. The optical density of test and control was read at 540 nm in a spectrophotometer against distilled water. Similarly the optical density of the artificial standard was read against distilled water. Serum bilirubin levels were calculated based on the following formula and expressed in mg per ml.

Total Serum Bilirubin in mg% =

$$\frac{\text{Optical density of test - Optical density of control}}{\text{Optical density of artificial standard}} X10$$

Estimation of Lipid Peroxides

The amount of lipid peroxidation products present in the serum samples/pleural fluid was estimated by the thiobarbituric acid reactive substances (TBARS) method, which measures the malondialdehyde (MDA) reactive products by using High Pressure Liquid Chromatography (HPLC). To 0.5 ml of serum/pleural fluid 0.5 ml of 30% trichloro acetic acid (TCA) was added to precipitate the proteins and vortexed for 30 sec. Clear supernatant was taken after centrifuging at 3000 rpm for 10 min. To the supernatant 50 µl of 1%TBA solution was added and the solution was heated for 1hr at 98°C. 20 µl of the mixture, which is pink in colour, was injected into HPLC.

The HPLC Conditions are Mobile phase :Methanol: Water (70:100) containing 250 µl of H_3PO_4 with 80 nM of NaOH; Column : Altec C18 (25 cm length, 4.6 mm diameter, 5 µsize); Wavelength was fixed at λmax 540 nm. The temperature : Ambient; Flow rate :1ml/ min.; Injection volume : 20µl. Standard graph was plotted using TEP (1, 1, 3, 3-tetra ethoxy propane).

Estimation of Glutathione

Glutathione forms a coloured complex with DTNB, which is measured spectrophotometrically. To 0.5 ml of citrated blood, 0.5 ml of 5% TCA solution was added to precipitate the proteins

and centrifuged at 3000 rpm for 20 minutes. To 0.1 ml of supernatant, 1 ml of sodium phosphate buffer and 0.5 ml of DTNB reagent were added. The absorbance of the yellow colours developed was measured at lmax 421 nm. The glutathione content was determined from standard graph by using pure glutathione.

Estimation of Total Antioxidant Status

For the estimation of total antioxidant status, we used a stable free radical α, α,-diphenyl–α-picryl hydrazyl (DPPH), at the concentration of 0.2 mM in methanol. 0.1ml of serum / pleural fluid was deproteinated by the addition of 1 ml of methanol, vortexed for 30 sec. Then centrifuged at 3000 rpm for 30 minutes to separate the proteins. To the clear supernatant 1.5 ml of methanol and 0.5 ml of DPPH solution were added, mixed thoroughly and absorbance was read at λmax 517nm against blank, prepared in an identical way but without the addition of serum / pleural fluid. Ascorbic acid was used as a reference standard. The standard graph was plotted using different concentrations of ascorbic acid and the antioxidant status values were expressed in terms of nM of ascorbic acid. Standard graphs were also prepared with known concentrations for tests where.

Statistical analysis

The results obtained were statistically analyzed by Student's T-test followed by Newman-Keul's multiple comparison tests.

REFERENCES

1. Scot Luper, N.D. (1998). A review of plants used in the treatment of liver disease, Part 1, 3(6),410-419.
2. Kirtikar, K. R. and Basu. B. D. (1975), Indian Medicinal Plants, Vol. II, Dehradun, India, Bishen mahendra pal singh, p. 842-844.
3. Neraliya, S. and Srivastava, U. S., (1997). Juvenomimetic activity in some Indian angiosperm plants. *Journal of Medicinal and Aromotic Plant sciences.* **19** (3), 677-681.
4. Choudhary, D. N., Singh, J. N., Verma, S. K. and Singh. B.P. (1990), Antifertility effects of leaf extracts of some plants in male rats. *Indian Journal of Experimental Biology.* **28** (8), 714-716.
5. Agnihotri, V. K., Srinvastava, S. D., Srivastava, S. K., Pitre, S., and Rusia, K. (1987). Constituents from the seeds of *Cordia obliqua* as potential anti-inflammatory agents. *Indian J. Phram. Sci.* **49**(2), 66-69.
6. Hewawasam, R. P., Jayatilaka kapw., Pathirana and Mudduwa, L. K. B. (2004), Hepatoprotective effect of Epaltes divaricata extract on carbon tetrachloride induced hepatotoxicity in mice. *Indian J. Med. Res.* , 30-34.
7. Recknagel, R. O., Glende, E. A. Jr, Dolak, J.A. and Walter, R. L. (1989). Mechanisms of carbon tetrachloride Toxicity. *Pharmacol Ther.* **43**,139-154.
8. Molander, D. W., Wroblewski. F. and La Due, J. S. (1955). Transaminase compared with cholinesterase and alkaline phosphatase an index of hepatocellular integrity. *Clinical Research Proceedings.* **3**, 20-24.
9. Tappel, A.C. (1973), Lipid peroxidation damage to cell components. *Federal Proceedings.***32**, 1870-1874.
10. Prakash, J., Gupta, S. K., Kochupillai, V., Singh, N., Gupta, Y. K. and Joshi, S. (2001). Chemopreventive activity of *Withania somnifera* in experimentally induced fibrosarcoma tumours in swiss albino mice. *Phytother Res.* **15**, 240–244.
11. Wilkinson, J. H. (1962). An introduction to diagnostic enzymology. In. Arnold. E. (Ed.). Academic Press, London, p.84.
12. Blois, M. S. (1958). Antioxidant determinations by the use of a stable free radical. *Nature.* **181**, 1199-1200.
13. Carbonneau, M.A., Peuchant, E., Sess, D., Canioni, P., Clerc, M. (1991). Free and bound MDA adduct by HPLC in serum and plasma. *Clinc.Chem.* **37** (8), 1423-1429.
14. Jendrassik. L. and Grof, P. (1938). Simplified photometric methods for. The determination of the blood bilirubin. *Biochem. Z.* **297**, 81-89.
15. Klind, P. R. M. and King, E. J. (1954). Estimation of plasma phosphatase by determination of hydrolysed phenol with amino-antipyrine. *Clin path.* 7(4), 322.
16. Reitman, S. and Frankel, S. (1957). Colorimetric methods for determining GOT and GPT. *Am J Clin Path.* **28**, 56.

Reference # 25

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Final Report for Sabell Corporation

October 11, 2011

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Part 1: Antiviral effects of A4+ plant extracts on hepatitis C virus (HCV) infected cells

Abstract

A significant number of plant products have important medicinal properties. There were reports that an extract from a plant native to Peru had beneficial effects in patients infected with hepatitis C virus (HCV). Extracts from this plant (A4+) as well as extract from its component parts – leaf, root, and flower – were tested for their antiviral effect in HCV-infected cells. Extracts from the whole plant were shown to inhibit HCV production from HCV-infected cells. Studies using extracts from the plant's component parts indicated the most potent antiviral extract was from the plant's leaf. None of the plant extracts of A4+ demonstrated cell toxicity by the MTT assay in cell cultures, they did not inhibit HBV replication, and they failed to show an effect on natural killer (NK) T-cell activity *in vivo*.

Introduction

Hepatitis C virus (HCV) causes a viral infection of the liver, which can lead to chronic hepatitis, cirrhosis, and hepatocellular carcinoma. HCV has a single-stranded positive-sense RNA genome that encodes 10 structural and non-structural proteins. These proteins effectively exploit the host cell to enable viral replication. As well, many of these proteins have the capacity to interfere with cell signaling during infection, interference that is crucial to the mechanisms by which the virus escapes host immune responses. As a result, most infected individuals become chronic HCV carriers. The current treatment for

HCV infection includes pegylated interferon alpha and ribavirin. The effectiveness of this therapy varies with HCV genotypes. This therapy is effective in 45-50% of genotype 1, 65-70% of genotype 3, and 85-90% of genotype 2 infections. Unfortunately, the hardest to treat genotype, genotype 1, is the most common genotype of HCV.

Hepatitis B virus (HBV) infects the liver and can cause chronic hepatitis, cirrhosis, and hepatocellular carcinoma. HBV is more prevalent than HCV but the availability of preventative HBV vaccine, along with antiviral drugs such as lamivudine, entecavir, and tenofovir has helped to decrease the number of new infections and treat many HBV-infected patients. This virus contains a double-stranded DNA genome and encodes its own DNA polymerase, among other proteins. During the course of infection, a covalently closed circular DNA (cccDNA) is synthesized in the nucleus and serves as the template for virus resurgence when therapy is discontinued. The stability of the cccDNA of HBV makes this chronic infection difficult to cure.

The search for new treatments against viral hepatitis, and the growing popularity of natural products for the treatment of illnesses, has encouraged scientists to look more closely at plant products. It is with this intent that the Sabell plant products (A4+ and its components) from Peru were studied for their antiviral properties against HCV and HBV. These plant products have previously been reported to benefit patients with viral hepatitis (Sabell reference).

Materials and Methods

The studies were carried out in the laboratory of D. Lorne Tyrrell in the Li Ka Shing Institute of Virology, University of Alberta.

The test products were supplied by Sabell Inc. as either tinctures or powders of plant extracts. Plant extracts received as tinctures in 70% ethanol solutions were tested in serial dilutions in the cell culture systems. Plant extracts received as powders were dissolved in 45% ethanol and added at 0.1, 0.5, 1, 5 and 10 ug/mL in cell culture media for the treatment of HBV or HCV-infected cells.

Cell culture assays for HBV activity

HepG 2.2.15 cells were used for the HBV studies. This cell line expresses HBV constitutively. Cells were seeded in 6 well plates at 1 x 10^6 cells/well and the cells were left to adhere overnight 12 – 14 hours. Plant extracts were added at specified concentrations. The media containing either the plant extracts or lamivudine (positive control) was changed and replenished every two days. The cells were treated for 6 days, at which time supernatant and cells were harvested for HBV quantification. Non-viral nucleic acid was digested with DNase I and RNase A followed by virion precipitation with PEG 8000. Viral nucleic acid was extracted by treatment with proteinase K and SDS overnight followed by phenol-chloroform extraction. DNA was precipitated with ethanol, dried, and resuspended in 10uL of water. The copies of HBV DNA were measured by quantitative polymerase chain reaction PCR (qPCR).

HCV – Cell culture assays

HCV antiviral studies were carried out using Huh7.5 cells and a tissue culture adapted strain of HCV, JFH-1. Cells were seeded in 6 well plates at 1 x 10^5 cells/well and were left to adhere overnight. After 14 hours, the media was changed and the virus was added at a multiplicity of infection of 3 viral genomes per cell and incubated for 4 hours. Cells were subsequently washed 3 times with media and fresh media was added to each well. Treatment with plant extracts was started immediately. Media was changed every 2 days and the plant extracts replenished. Viral RNA was extracted from tissue culture supernatant and cells on day 6. RNA was isolated from supernatants with the Roche High Pure Viral Nucleic Acid (Laval, Quebec) (extracellular virus, ECV), and by Trizol for intracellular virus (ICV) according to the manufacturer's protocol.

HCV – Western blot assays

Cells collected for western blots were lysed with 100-200uL RIPA lysis buffer (made in house), incubated for 5-10 minutes at room temperature, and protein quantified using Bio-Rad DC protein assay kit (Bio-Rad Laboratories (Canada) Limited in Mississauga, ON). Between 25-40 ug of protein was run on either a 10% or 15% SDS-polyacylamide gel (SDS-PAGE), transferred to nitrocellulose membrane and blocked with 5% milk/TBS-T. HCV NS3 antibody was supplied by Chemicon International in Temecula, California (Cat#MAB8691) and HCV core antibody was supplied by Affinity BioReagents in Nepean, Ontario (Cat#MA1-080).

HCV – Viral RNA quantitation

Viral nucleic acid quantification was done using quantitative PCR. ECV samples were compared to a standard curve of cloned HCV cDNA and ICV samples compared to an internal control, RNA for the housekeeping gene HPRT (ABI hu HPRT assay) (Streetsville, ON). HCV 150 primer 5'-TCTGCGGAACCGGTGAGTA-3' and HCV 382 primer 5'-GTGTTTCTTTTGGTTTTTCTTTGAGGTTTAGG-3' were used for amplification and HCV 315 5'-CACGGTCTACGAGACCTCCCGGGGCAC-3' was used as a probe. All reactions were performed using Taqman Universal PCR master mix.

Cell Toxicity assays

Cell viability during plant extract treatment was determined using an MTT assay (6). Cells were seeded at approximately 20% confluency in a 96 well plate and incubated overnight to permit cell adhesion. Fresh media was added containing the plant extract at the highest concentration (10 ug/ml) for 48 hours. Cells were washed with PBS and fresh media containing 0.5mg/mL MTT was added. Cells were then cultured for 16 hours. Media was aspirated and the cells were dissolved in 100uL 0.1N HCl/isopropanol. The absorbance of MTT reduced to purple formazan by viable cells was read at 570nm on a SpectraMax 384 Plus.

Results and Discussion

Hepatitis B studies

Interpretation of the results with HepG2.1.1.5 cells was difficult as cell confluency had a significant effect on the amount of HBV produced. However, once these issues were resolved, there was no significant antiviral effect in HepG2.1.1.5 cells treated with A4+ (Figure 1).

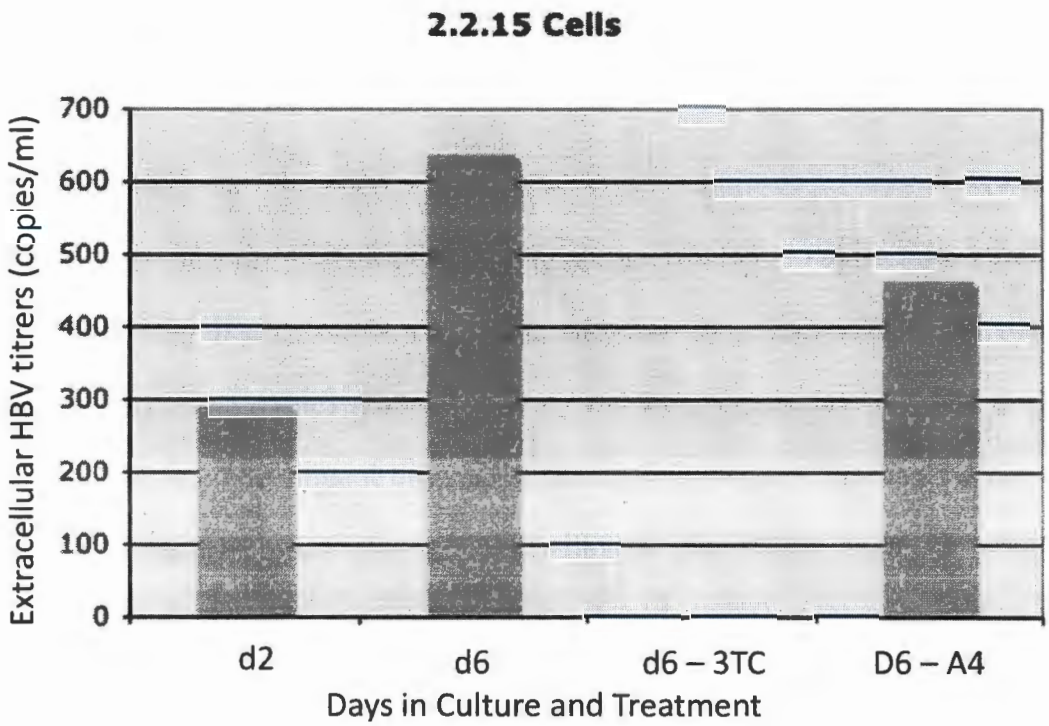

Figure 1. Antiviral treatment of HBV in HepG2.2.15 cells over 6 days. Samples of supernatant were collected for extracellular virus (ECV) measurement on day 6. 3TC (lamivudine) was tested at a concentration of 10ug/mL (positive control), and A4+ was tested at a concentration of 10ug/mL.

Conclusion: The *in vitro* HBV studies did not demonstrate an antiviral activity in the presence of A4+

Hepatitis C studies

The antiviral effect of the plant extract A4+ against hepatitis C virus was more promising than its effect against HBV. The A4+Leaf (A4+L) component of the A4+ powder demonstrated significant antiviral activity (as shown in Figures 2 and 3.) Intracellular and extracellular levels of HCV RNA were measured after 4 days of drug treatment in cell culture. Both the intracellular and extracellular HCV RNA levels were significantly decreased at concentrations of A4+ greater than 1 ug/mL (Figures 2, 3, blue bars). Of the components of A4+, A4+Flower (A4+F) and A4+Root (A4+R) did not show significant inhibition of HCV. However, the A4+L component showed the most potent antiviral activity as shown in Figures 2 and 3 (green). It is likely that the A4+ antiviral activity can be attributed to the A4+L component in the whole plant extract.

To further confirm the antiviral activity of the A4+L extract, viral protein levels were determined by Western blot. Cells were plated, infected, and treated with A4+, A4+F, A4+L, A4+R extracts as previously described. After treatment, cells were lysed with RIPA buffer to solubilize proteins and prevent their degradation. Protein concentrations were determined with a BioRad protein assay so the same amount of total protein could be added to each well. Antibodies specific to HCV core and NS3 proteins were used to visualize these proteins on the Western blot. A decrease in NS3 is seen in lanes A4+ and A4+L with the greatest being in observed in the A4+L lane (Figure 4A). Core protein also decreased in cells treated with A4+ and A4+L, but the high level of background made it more difficult to detect the differences (Figure 4B).

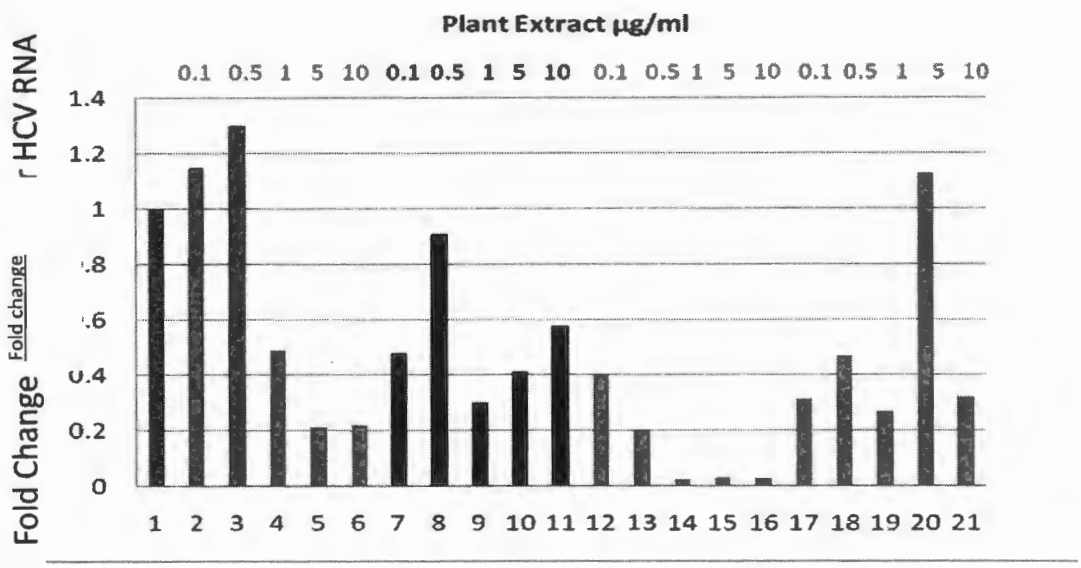

Figure 2. Levels of intracellular HCV RNA in plant extract treated cell cultures compared to ethanol treated control. Ethanol treated control cells (grey, lane 1); A4+ extract treated cells (blue, lanes 2-6); A4+F extract treated cells (red, lanes 7-11); A4+L treated cells (green, lanes 12-16) and A4+R treated cells (orange, lanes 17-21). This plot is representative of three repeats of this study.

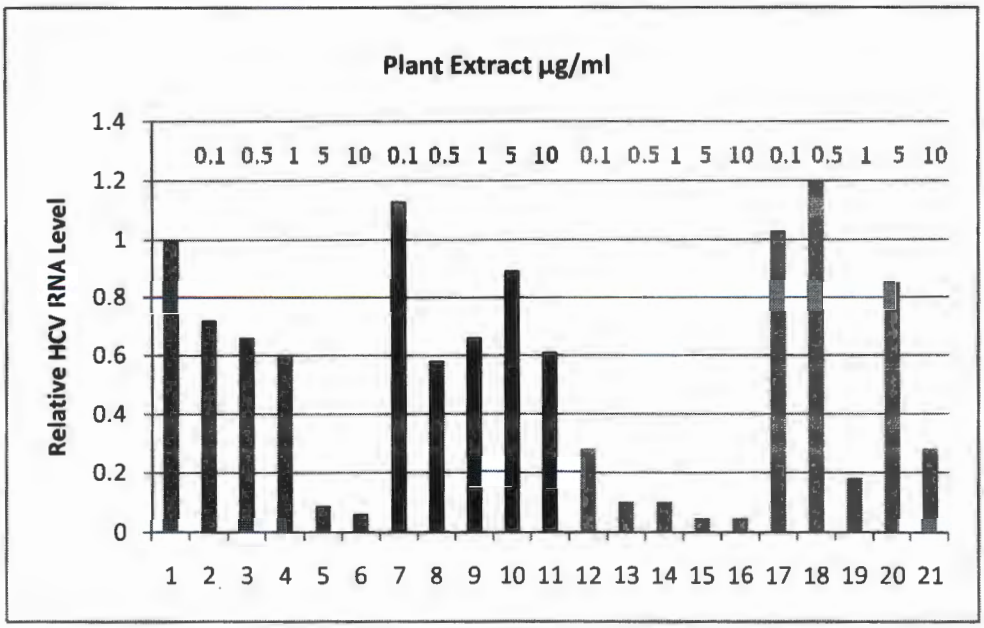

Figure 3. Levels of extracellular HCV RNA produced by plant extract treated HCV-infected cells in comparison to ethanol treated control. Ethanol treated control (grey, lane 1); A4+ extract treated cells (blue, lanes 2-6); A4+F extract treated cells (red,lanes 7-11); A4+L extract treated cells (green, lanes 12-16) and A4+R extract treated cells (orange, lanes 17-21). This plot is representative of three repeated experiments.

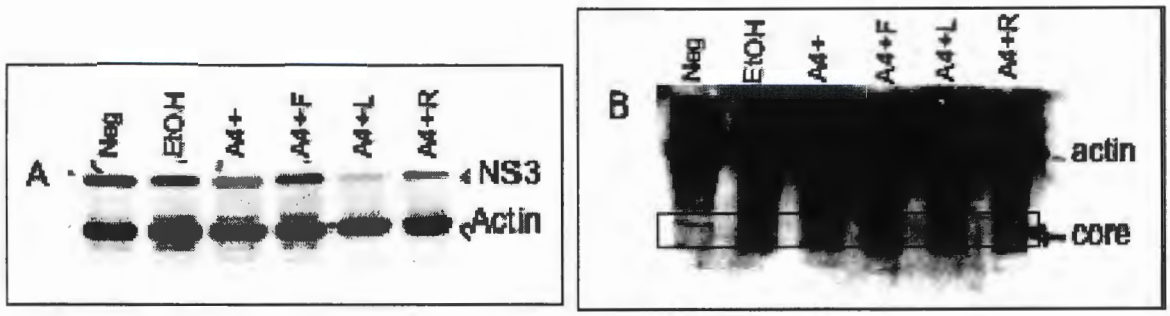

Figure 4. Western blots of HCV proteins from the plant extract treated HCV-infected cells. Each plant extract was used at a concentration of 10 ug/ml with treatment for 4 days. Figure 4A shows the Western blot of NS3 protein and Figure 4B shows the Western blot for core protein. This is a representative Western blot from three repeats for each HCV protein.

Acute Cytotoxicity Assay

All plant extracts were tested on HCV-infected cells to determine if they induced acute cytotoxicity. The MTT assay (6) was used to determine if the plant extracts decreased cell viability. In the assay, viable cells reduce MTT to an intracellular purple formazan dye. Formazan extracted from the cells is quantified on a spectrophotometer. In this acute study of plant extract toxicity, none of the extracts prepared in the standard concentrations of ethanol solvent demonstrated cell cytotoxicity.

Conclusions, Part 1

1. Screening of the test compounds were performed against HBV and HCV.
2. None of the plant extracts demonstrated antiviral activity against HBV in the cell culture system used.
3. Plant extracts A4+ and A4+L demonstrated antiviral activity against HCV.
4. The A4+L had the highest antiviral activity with concentrations of 1 ug/ml producing greater than 95% inhibition of intracellular virus production and 90% inhibition of extracellular virus production.
5. MTT assay for drug toxicity did not show acute cell toxicity for any of the plant extract preparations tested.

Part 2: Effect of Short-term exposure to A4+ plant extracts on Natural Killer Cell Activity

Introduction

Natural Killer (NK) cells are lymphocytes that play a key role in innate immunity. Their main function is the activation of apoptosis in cancerous cells or in cells infected with virus. Once NK cells become activated, they release a mixture of proteins against the cell membranes of adjacent target cells. The process is called degranulation. One component of the granules, perforin, perforates the target cell membrane allowing entry of a second component, granzyme B, which initiates apoptosis.

The health benefits of many natural products are often attributed to enhancing immunity by increasing NK activity. A preliminary study was completed to determine if the antiviral benefits of the A4+ plant extracts could be attributed to enhancement of NK activity.

Materials and Methods

Plant extracts were received as powders and were dissolved in 45% ethanol, and diluted in 20% sucrose. C57/B6 mice were given 100 uL of the resulting mixture of sucrose, ethanol, and plant extract at 1.3 mg/100 ug by gavage. Animals were treated daily for 14 days. Mice were then euthanized and their spleens were removed for NK cell preparation. A cell suspension was prepared in isolation media by passing the spleen through a fine mesh sieve. Cells were washed 2 times with fresh isolation media, and red blood cells were lysed with a single wash in RBC lysis buffer. NK cells were then purified using an Easy Sep Mouse NK Cell Enrichment Kit (from STEMCELL Technologies Inc. in Vancouver BC). Purified NK cells were incubated with YAC-1 target cells at a 1:5 ratio at 37C for 4 hours in the presence of a CD107a antibody (degranulation marker). The last 15 minutes of the incubation included NK antibodies CD3, CD45, NK1.1 and DX5 (BD Biosciences, Mississauga, Ontario). Cells were then fixed with 1% paraformaldehyde, permeabilized in 0.1% saponin, and resuspended in FACS buffer. The cells were

sorted with appropriate gating to detect total NK cells and the portion of NK cells that had undergone degranulation.

Results and Discussion

NK cells are a small population of lymphocytes that have an ability to target specific cells and induce cell death. Activated NK cells are detected with fluorescently labeled antibodies specific for CD107a, a protein found on the inner surface of the cytotoxic granule membrane, which becomes exposed on the NK cell surface after degranulation. In order to distinguish NK cells from others in the spleen, antibodies DX5, NK1.1, CD3 and CD45 were used to label NK cells. As cells pass through the FACS machine, the different labels are detected, allowing for determination of cell type and activation. Cells positive for both NK1.1 and CD107 represent activated NK cells undergoing degranulation.

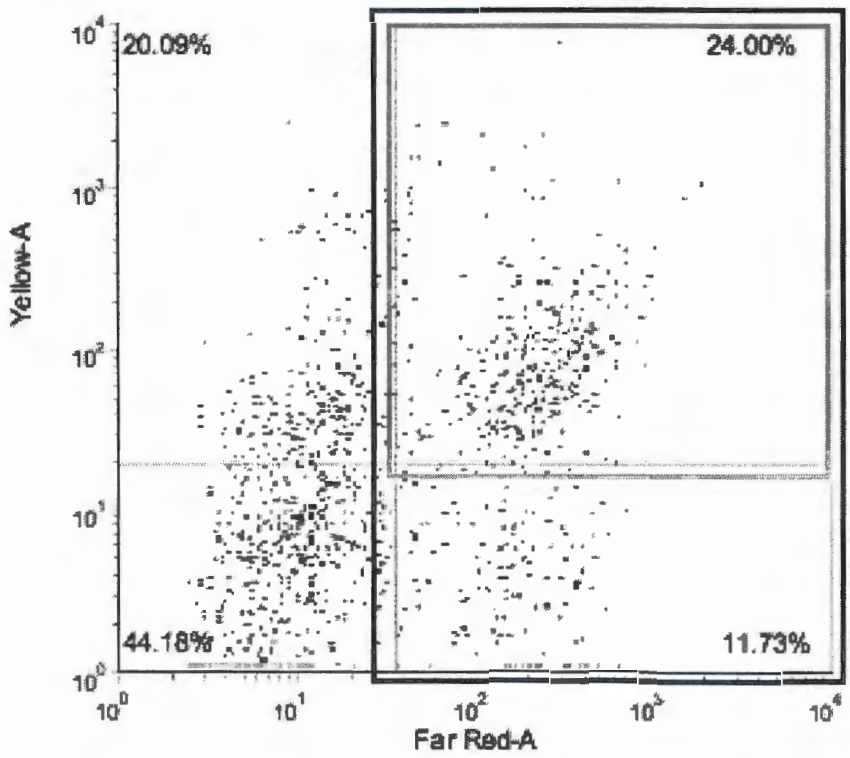

Figure 6. A FACS analysis of the negative control sample. Each dot represents a cell that has passed through the FACS detector. The x-axis is the fluorescent marker for NK cells, and the y-axis is the fluorescent marker for degranulation (CD107a). The cells within the blue box represent total NK positive cells, and the green box within the blue box are NK cells that have degranulated. The percentage of activated NK cell was calculated by dividing the NK+CD107a+ positive cells by the total NK population.

NK activity was approximately 12% higher with ethanol in the control treatment, compared to the comparable sucrose solution used for gavage. Neither treatment with A4+ or A4+L resulted in significant enhancement of NK activity. There may have been a tendency for decreased NK activity with A4+ or A4+L treatment, but the number of experiments was too low to show statistically significant differences. In this short term experiment the A4 extracts were not toxic to the mice.

Treatment	Exp 1	Exp 2	Exp 3	Average
Negative	54.2	56.9	56.0	55.7
Neg-Etoh	67.2	85.2	51.7	68.0
A4+	61.9	54.8	63.0	59.9
A4+L	64.6	15.8*	38.8	39.7

Table 1. Three repeats of NK assays showing the percentage of NK cells that have degranulated on exposure to the target YAC-1 cells.'

*This animal had aspiration during the oral gavage.

Final Conclusions and Future Work

In Part 1, we demonstrated that the A4+ plant extract had significant antiviral activity against HCV in cell culture. The studies were repeated three times and in each, the A4+L demonstrated the highest activity against HCV. There was no antiviral activity shown by A4+ plant extracts for HBV. In Part 2 we showed that A4+ did not enhance NK activity *in vivo.* Since the antiviral activity was only shown in lymphocyte free cell cultures, we conclude that the antiviral effect of A4+L on HCV is a direct antiviral effect and is not mediated though a NK immune enhancement.

Future work should focus on trying to identify the active ingredient in A4+L that inhibits HCV in cell culture. If this component can be identified, it could lead to novel antivirals for HCV. Such compounds could be tested in the chimeric mouse – an animal model that can support HCV infection and replication.

References

1. Chisari, Francis V., "Unscrambling hepatitis C virus-host interactions." Nature, 2005, **436**: 930.

2. Guidotti, Luca G., Chisari, Francis V., "Immunobiology and Pathogenesis of Viral Hepatitis." Annu. Rev. Pathol. Mech. Dis. 2006, **1**: 23-61.

3. Wohlfarth, Carolin., Efferth, Thomas. "Natural products as promising drug candidates for the treatment of hepatitis B and C." Acta Pharmacol Sin, 2009, **30** (1): 25-30.

4. Betts, Michael R., et al. "Sensitive and viable indentification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation." J of Immunol Methods, 2003, **281**: 65-78.

5. Do Santo, James P., "Natural killer cells: diversity in search of a niche." Nature Immunology, 2008, **9** (5): 473-510.

6. Mosmann T. "Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays." J Immunol Methods. 1983, **65**:55-63.

Reference # 26

Evaluation of the Anti-Oxidant, Anti-Inflammatory and Mucosal Protective Actions of A4+ and Its Constituents

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Abstract

A4+ is a product that consists of a combination of three herbal ingredients. In open-label clinical studies, it has been shown to confer benefit to patients with hepatitis. The mechanism of action of A4+ is not known. The studies described herein investigated the possibility that A4+ exerts anti-inflammatory effects, which could contribute to its beneficial effects in hepatitis. We also examined the relative anti-oxidant properties of A4+ and its constituents, as such properties could also contribute to its beneficial effects. Finally, we determined if A4+ was capable of protecting the stomach from damage induced by a nonsteroidal anti-inflammatory drug. These studies provided insights to the mechanism of action of A4+, as well as an indication of additional therapeutic utilities of this product.

Background and Significance

A4+ is a product consisting of a combination of three herbal constituents (see table below). It has been reported to produce beneficial effects in human hepatitis, but its mechanism of action is unknown. Moreover, the contributions of the individual constituents of A4+ to its activities are not completely clear. It is possible that A4+, or some of its constituents, may exert beneficial effects in other diseases characterized by inflammation and/or mucosal injury. The purpose of the studies described herein was to gain better understanding of the mechanism(s) of action of A4+, and of its constituents. Specifically, these studies examined whether or not A4+ and/or its constituents exert anti-oxidant actions (*in vitro*) and if they exhibited anti-inflammatory and/or gastric mucosal protective actions (*in vivo*).

Composition of A4+

Constituent	Percentage of Total
Cordia lutea	80
Annona muricata	10
Curcuma longa	10

Anti-Oxidant Activity of A4+ and its Constituents

Free radicals can contribute to tissue injury in many settings. Anti-oxidants, by scavenging free radicals, can exert protective effects against tissue injury. The anti-oxidant effects of A4+ and of its constituents were examined in a well established *in vitro* model.[1]

Methods

The anti-oxidant activity of A4+ and its constituents was evaluated using an *in vitro* assay in which a stable free radical (1,1-diphenyl-2-picrylhydrazyl; DPPH) was allowed to interact with the test substance. In the presence of free radical scavengers (i.e., anti-oxidants), this purple-colored substance, which strongly absorbs at 540 nm, is converted to a colourless compound, 1,1-diphenyl-2-pierylhydrazine.[1] This was detectable as a decrease in absorbance. A 20 µL aliquot of each compound was then be added to 180 µL of DPPH (in 95% ethanol; final concentration 100 µM) in a 96-well plate. Changes in absorbance at 540 nm were recorded every minute over a 10-min period using a plate scanner. The effects of nordihydroguaiaretic acid (NDGA), a potent anti-oxidant, were examined as a positive control.

Results

A4+ exhibited potent anti-oxidant activity. As shown in Figure 1, the positive control substance, NDGA, produced a concentration-dependent anti-oxidant activity, with marked

activity at concentrations greater than 10 uM. A4+ exhibited substantial anti-oxidant activity at concentrations greater than 1.6 ug/mL.

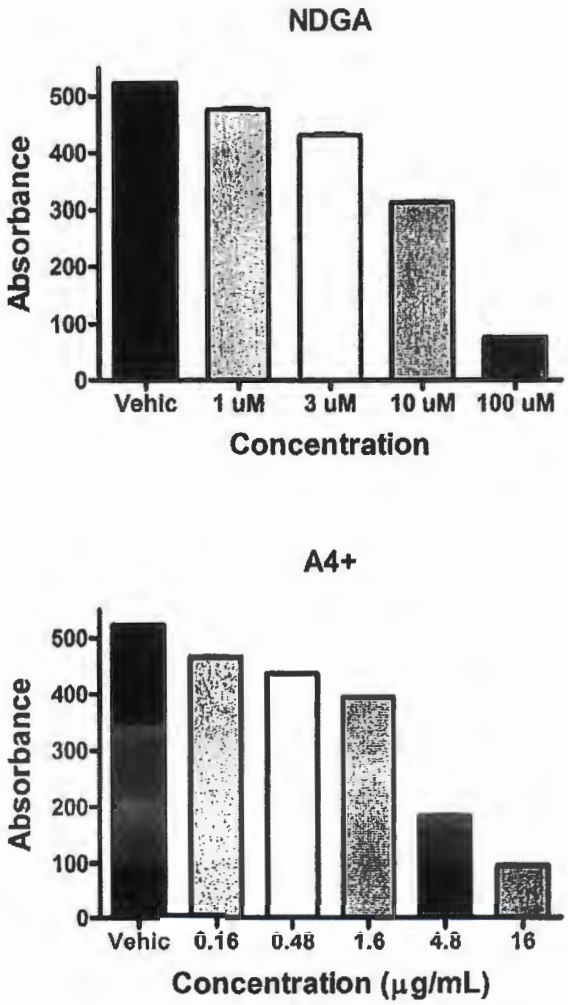


Figure 1: *In vitro* anti-oxidant activity of A4+ and of nordihydroguaiaretic acid (NDGA). NDGA was used as a positive control. Each bar shows the mean ± SEM of 10 separate experiments.

The anti-oxidant effects of the constituents of A4+ were then examined in the same assay. As shown in Figure 2, anti-oxidant activity was observed with Cordia and Annona , but not with

Curcuma. Unfortunately, concentrations of Annona and Curcuma greater than 1.6 ug/mL could not be tested because the substances were not maintained in solution. These results suggest that the anti-oxidant effects of A4+ can mainly be attributed to the Cordia and Annona, but not the Curcuma.

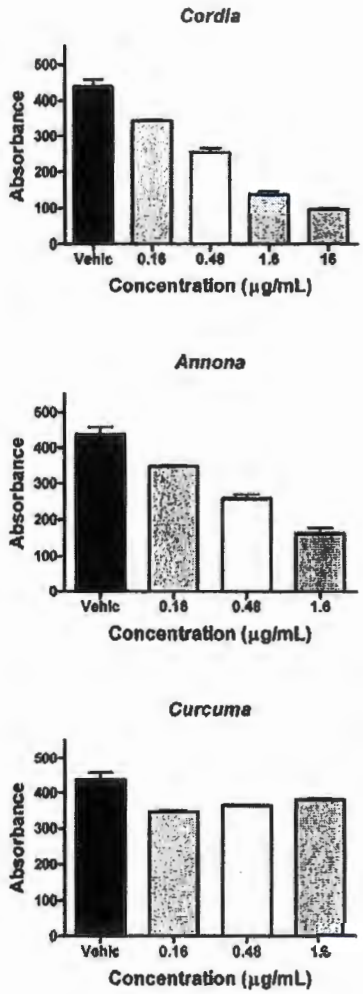


Figure 2: *In vitro* anti-oxidant activity of constituents of A4+. Each bar shows the mean ± SEM of 10 separate experiments. Note that Annona and Curcuma did not remain in solution at concentrations >1.6 ug/mL.

Effects of A4+ and its Constituents in a Model of Acute Inflammation

One of the most widely used *in vivo* models for assessing the anti-inflammatory actions of drugs is the "air pouch" model.[3] This model allows one to determine the effects of a drug on many different aspects of the inflammatory process, thereby allowing for determination of mechanism(s) of action. As the model has been used extensively over the past 30 years, there is a considerably body of data on a wide range of drugs.

In this study, the ability of A4+ and its constituents were evaluated to determine if they were capable of reducing the infiltration of leukocytes to a site of inflammation. We also determined if A4+ and its constituents could suppress the generation of some key inflammatory mediators (prostaglandin E_2 and leukotriene B_4).

Methods

A subdermal 'pouch' was created on the back of the rat by repeated injection of air.[4] The lining of the airpouch, after one week, is histologically similar to the synovial lining,[3] and therefore this model has been used extensively for the characterization of drugs developed for the treatment of arthritis. After the pouch had been created, an inflammatory event was triggered by injecting zymosan (1 mL of a 1% solution). The test compounds were administered either directly into the airpouch or orally 2 hours prior to injection of zymosan. The rats (n= 5 or 6) were euthanized 4 h after injection of zymosan and the exudates were withdrawn from the airpouch for analysis. The volume of the exudates were measured and aliquots were frozen for subsequent measurement of concentrations of inflammatory mediators by ELISA.[4] The

number of leukocytes in the exudates was determined using a Sysmex KX-21N Hematology Analyszer. In addition to a negative control (vehicle), indomethacin was included as a positive control in these experiments. Indomethacin is an anti-inflammatory drug used for the treatment of arthritis, ankylosing spondilitis and gout that has been extensively studied in this model.[4]

Results

The effects of A4+ and its constituents on infiltration of leukocytes in the airpouch model, and on the associated changes in prostaglandin E_2 and leukotriene B_4 concentrations in the exudates are summarized in the figures below (see legends for details).

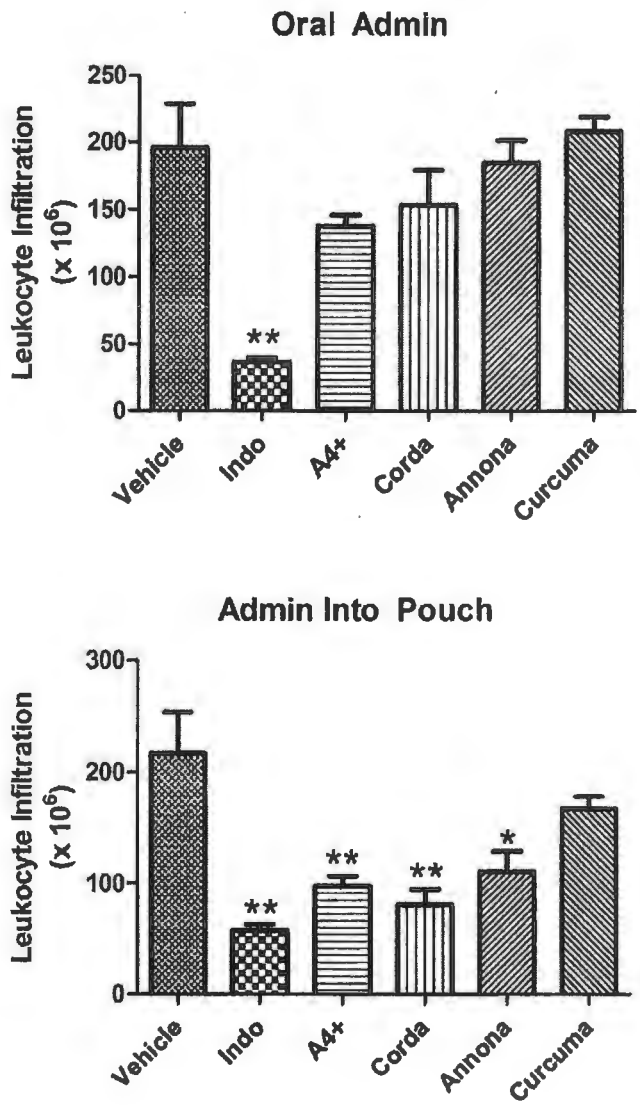


Figure 3: Effects of A4+ and its constituents on zymosan-induced leukocyte infiltration in the rat airpouch model. Administration of A4+ directly into the airpouch at a dose of 10 mg/kg markedly reduced leukocyte infiltration, to a similar extent as was observed with a nonsteroidal anti-inflammatory drug (indomethacin; 1 mg/kg). The Corda (8 mg/kg) and Annona (1 mg/kg) constituents of A4+ produced effects similar to A4+, but no significant effect was observed with Curcuma (1 mg/kg). When given orally 2 hours prior to the injection of zymosan into the airpouch, indomethacin produced a marked reduction of leukocyte infiltration, but A4+ (100 mg/kg) and its constituents (Cordia at 80 mg/kg, Annona and Curcuma each at 10 mg/kg) had

no significant effect. Results are shown as mean ± SEM (n= 5-6 rats/group). *p<0.05, **p<0.01 versus the vehicle-treated group (ANOVA and Dunnett's Multiple Comparison test).

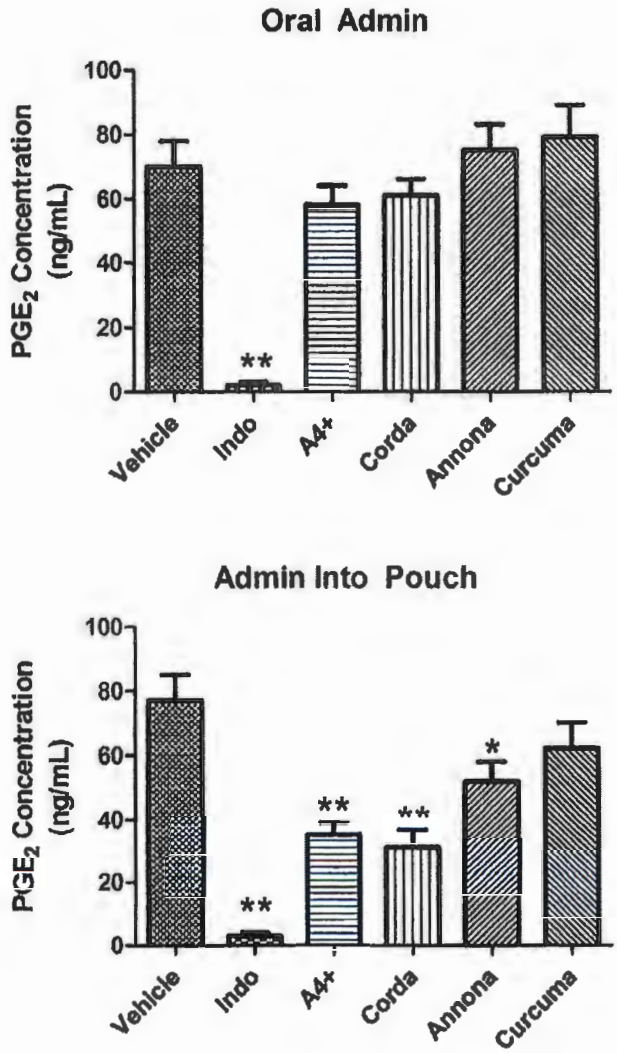


Figure 4: Effects of A4+ and its constituents on zymosan-induced prostaglandin E_2 production in the rat airpouch model. Administration of A4+ directly into the airpouch 2 hours prior to zymosan administration substantially reduced prostaglandin E_2 levels, albeit not to the extent as was observed with indomethacin (1 mg/kg). The Corda (8 mg/kg) and, to a lesser extent, the Annona (1 mg/kg) constituents of A4+ produced effects similar to A4+, but no significant effect was observed with Curcuma (1 mg/kg). When given orally 2 hours prior to the injection of zymosan into the airpouch, indomethacin produced a marked reduction of

prostaglandin E_2 levels, but A4+ and its constituents had no significant effect. A4+ was administered at 100 mg/kg, while Cordia, Annona and Curcuma were administered at doses of 80, 10 and 10 mg/kg, respectively. Results are shown as mean ± SEM (n= 5-6 rats/group). *p<0.05, **p<0.01 versus the vehicle-treated group (ANOVA and Dunnett's Multiple Comparison test).

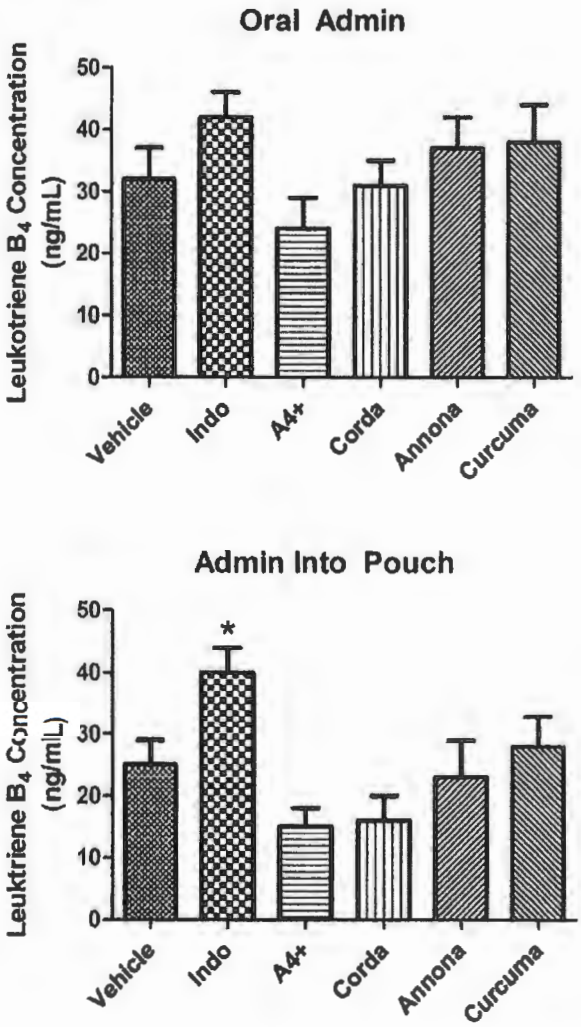


Figure 5: Effects of A4+ and its constituents on zymosan-induced leukotriene B_4 production in the rat airpouch model. Administration of A4+ or its constituents directly into the airpouch 2 hours prior to

zymosan administration had no effect on leukotriene B_4 levels. A4+ was administered at 10 mg/kg, while Cordia, Annona and Curcuma were administered at 8, 1 and 1 mg/kg, respectively. In contrast, indomethacin produced a significant increase in leukotriene B_4 levels. When given orally 2 hours prior to the injection of zymosan into the airpouch, A4+ and its constituents had no significant effect. Similarly, indomethacin (1 mg/kg) had no significant effect. A4+ was administered at 100 mg/kg, while Cordia, Annona and Curcuma were administered at doses of 80, 10 and 10 mg/kg, respectively. Results are shown as mean ± SEM (n= 5-6 rats/group). *$p<0.05$, **$p<0.01$ versus the vehicle-treated group (ANOVA and Dunnett's Multiple Comparison test).

Mucosal Protective Activity of A4+ and Its Constituents

Background

Ulceration in the gastrointestinal tract is a significant clinical problem. It is the major limitation to the use of nonsteroidal anti-inflammatory drugs (NSAIDs) for the treatment of disorders such as arthritis.[5] Thus, there is substantial clinical need for agents that can reduce the ulcerogenic effects of NSAIDs. At present there is no evidence that A4+ can protect the gastrointestinal mucosa from damage, but its reported beneficial effects in hepatitis do suggest that it can exert anti-inflammatory effects. As inflammation contributes significantly to the pathogenesis of ulcer formation, it is possible that A4+ and/or its constituents will exert protective effects in the GI tract. The experiments outlined below evaluated whether or not there is promise for A4+ as a mucosal-protective agent.

Methods

The potential mucosal-protective effects of A4+ and its constituents were assessed using three treatment protocols. In all cases, male Wister rats weighing 200-225 g were used and they were deprived of food, but not water, for 18-20 h prior to oral administration of indomethacin (20 mg/kg). The extent of hemorrhagic damage in the stomach was assessed, in a blind manner, 3 hours after indomethacin administration. This involved measuring the lengths of all lesions in mm, then summing those lengths to give a 'gastric damage score' for each rat.[6]

In the first study, the rats were treated orally with A4+ (100 mg/kg), Cordia (80 mg/kg), Annona (10 mg/kg) or Curcuma (10 mg/kg) 1 hour prior to indomethacin administration. As a positive control, another group of rats was treated orally with prostaglandin E_2 (0.1 mg/kg), since it has well characterized protective effects against indomethacin-induced gastric damage.[5] In addition to blind macroscopic scoring of the gastric lesions, tissue was excised and processed for measurement of gastric prostaglandin synthesis.[6] This allowed us to determine if the test substances interfered with the ability of indomethacin to suppress prostaglandin synthesis, which could be responsible for any observed protective effects. Each group consisted of 5 rats.

In the second study, groups of 5 rats each were treated orally with A4+ at a dose of 300 mg/kg at 1 hour, 2 hours or 4 hours prior to oral administration of indomethacin (20 mg/kg). The control group received vehicle 1 h prior to indomethacin. The rats were sacrificed 3 h after indomethacin administration and the extent of damage will be assessed, as described above.

In the third study, groups of 5 rats each were treated orally with A4+ at doses of 50, 300 or 1000 mg/kg 4 hours prior to oral indomethacin (20 mg/kg) administration The rats were sacrificed 3 h after indomethacin administration and the extent of damage will be assessed, as described above.

Results

As shown in figure 6, indomethacin administration resulted in extensive hemorrhagic damage in the stomach. Pretreatment, 1 hour prior to indomethacin administration, with A4+ (100

mg/kg) did not significantly affect the extent of indomethacin-induced damage. Similiarly, none of the three constituents of A4+ (each at 100 mg/kg) significantly affected the extent of indomethacin-induced damage. In contrast, pretreatment with prostaglandin E_2 reduced the extent of indomethacin-induced gastric damage by ~90%. Gastric prostaglandin E_2 synthesis was markedly suppressed (as compared to naïve controls) in all rats treated with indomethacin (by >95%). There were no significant differences in gastric prostaglandin synthesis among the treatment groups.

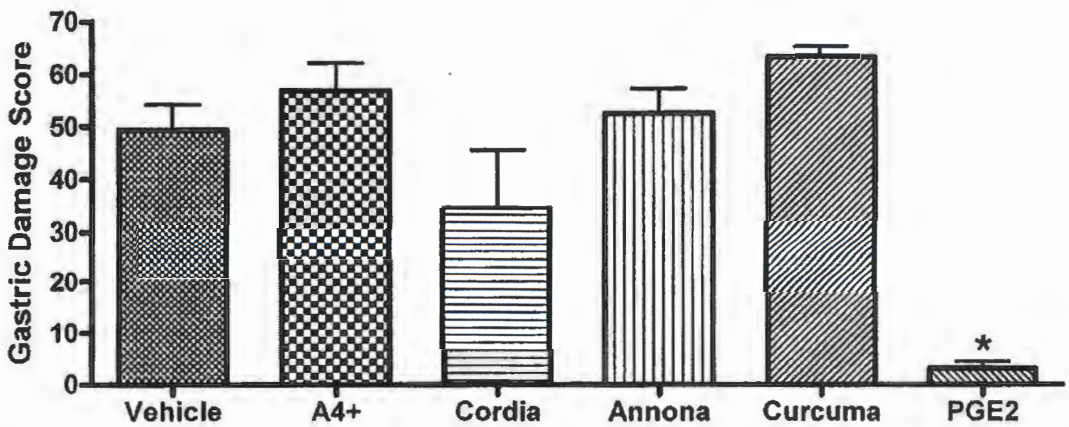


Figure 6: Indomethacin-induced gastric damage in rats pretreated with A4+ or its constituents (each at 100 mg/kg). Prostaglandin E_2 (0.1 mg/kg) significantly reduced the extent of damage, but A4+ and each of its constituents had no significant effect. *$p<0.05$ versus the vehicle-treated group. N=5 per group.

At a dose of 300 mg/kg, A4+ significantly reduced the severity of indomethacin-induced gastric damage when given 4 hours prior to the indomethacin, but not when a shorter pre-treatment time was used (Figure 7).

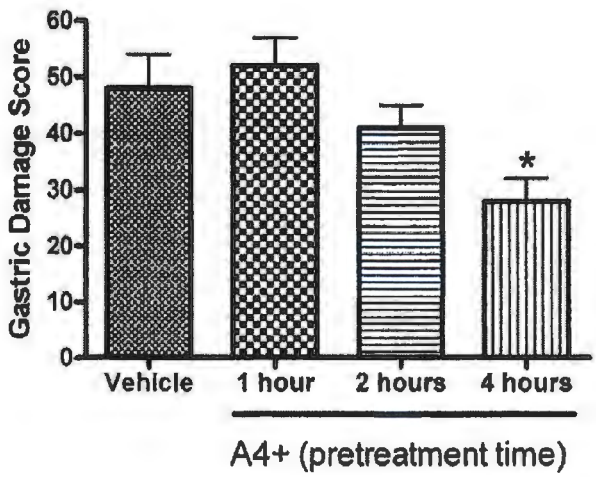


Figure 7: Time-dependent effects of A4+ on indomethacin-induced gastric damage in rats. Groups of 5 rats each received A4+ (300 mg/kg orally) 1, 2 or 4 hours prior to oral administration of indomethacin (20 mg/kg). *p<0.05 versus the vehicle-treated group.

With a 4-hour pretreatment time, A4+ given at 300 or 1000 mg/kg produced significant and dose-dependent reductions of the severity of indomethacin-induced gastric damage (Figure 8). The 50 mg/kg dose of A4+ had no significant effect.

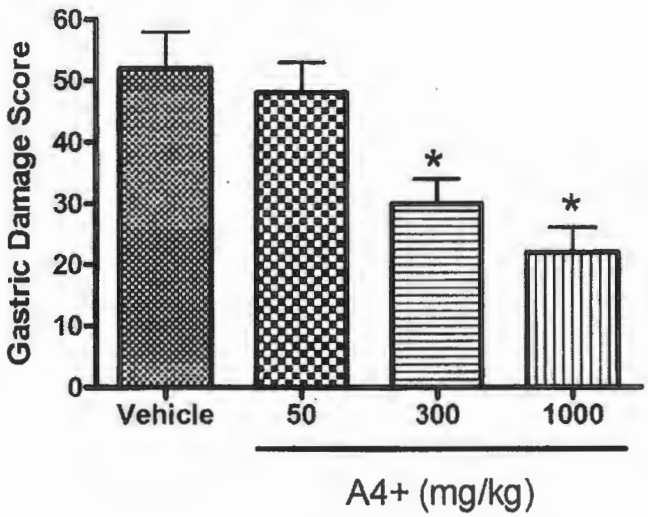


Figure 8: Dose-dependent effects of A4+ on indomethacin-induced gastric damage in rats. Groups of 5 rats each received A4+ (50, 300 or 1000 mg/kg orally) 4 hours prior to oral administration of indomethacin (20 mg/kg). *p<0.05 versus the vehicle-treated group.

These results demonstrate that A4+ is capable of significantly reducing the acute gastric damage induced by indomethacin, when given at a dose of 300 mg/kg or greater, and when given at least 4 hour prior to indomethacin administration. The need for a 4 hour pretreatment time likely reflects the time necessary for sufficient blood levels of A4+, or some of its constituents, to be achieved.

Conclusions

A4+ has very potent anti-oxidant activities that appear to be attributable to the Cordia and Annona components. This activity may contribute to the significant anti-inflammatory effects of A4+ in the zymosan-airpouch model in rats. A4+ was particularly effective when administered directly into the airpouch, reducing leukocyte infiltration and prostaglandin E_2 levels. A4+ was less effective when administered orally at 10-times the dose given directly into the pouch. It is possible that the pretreatment time used (2 h prior to zymosan) was insufficient for adequate blood levels A4+ to be achieved. Like the anti-oxidant activity, the Cordia and Annona components appeared to be the major contributors to the anti-inflammatory activity of A4+.

A4+ administration reduced the severity of gastric damage induced by subsequent administration of a potent nonsteroidal anti-inflammatory drug (indomethacin), but only at quite a high dose (≥300 mg/kg) and only when given at least 4 hours prior to the indomethacin. These results suggest that sufficient blood levels of A4+ much be achieved to observe the gastroprotective effect. It is possible that with repeat administration of A4+, a more potent effect would be seen. The anti-inflammatory and anti-oxidant effects of A4+ may contribute to its gastroprotective actions, as the pathogenesis of NSAID-gastropathy includes inflammatory events, some of which are mediated by reactive oxygen species.[1,5]

References

1. Vaananen *et al.*, Inflammation 1992; 16: 227-240.
2. Miller *et al.*, J Invest Dermatol 2001; 117: 725-730.
3. Edwards *et al.*, J Pathol 1981; 134: 147-156.
4. Chavez-Pina *et al.*, Br J Pharmacol 2007; 152: 930-938.
5. Wallace, Physiol Rev 2008; 88: 1547-1565.
6. Wallace *et al.* Gastroenterology 2007; 132: 261-271.
7. Wallace *et al.* FASEB J 2007; 21: 4070-4076.

Evaluation of the Anti-Oxidant, Anti-Inflammatory and Mucosal Protective Actions of A4+ and Its Constituents

Composition of A4+

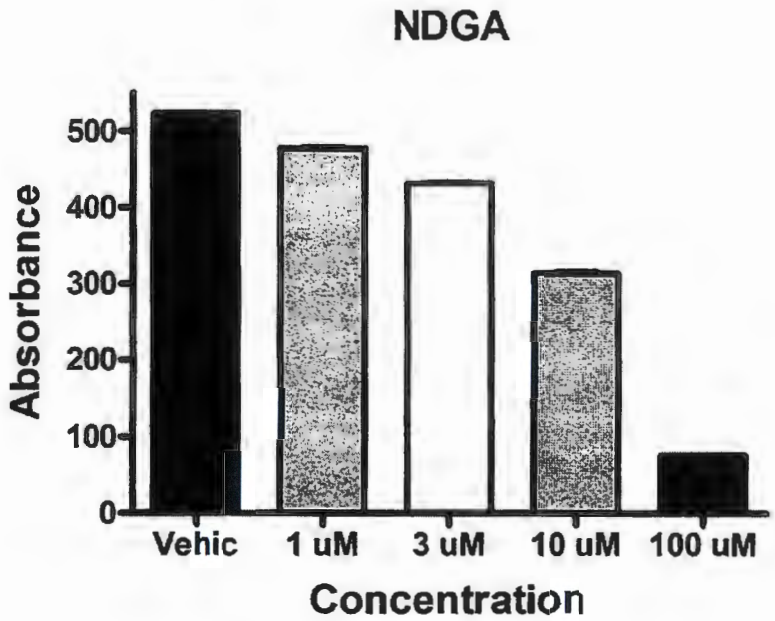
Constituent	Percentage of Total
Cordia lutea	80
Annona muricata	10
Curcuma longa	10

Anti-Oxidant Activity of A4+ and its Constituents

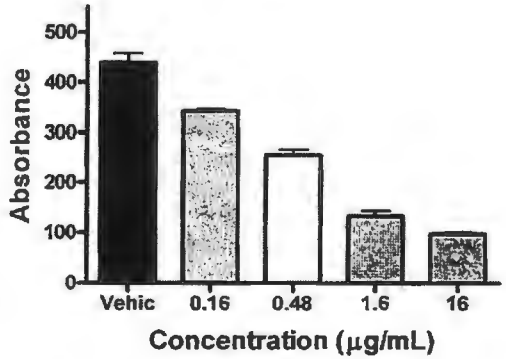
Anti-Oxidant Activity of A4+ and its Constituents

The anti-oxidant activity of A4+ and its constituents was evaluated using an *in vitro* assay in which a stable free radical (1,1-diphenyl-2-picrylhydrazyl; DPPH) was allowed to interact with the test substance.

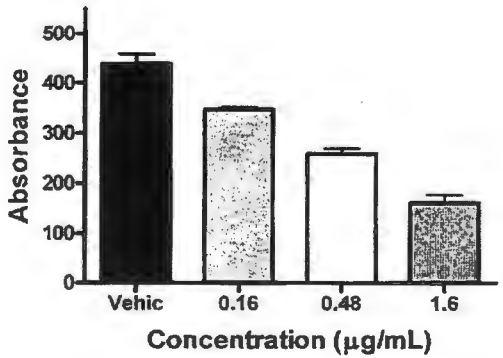
In the presence of an anti-oxidant, this purple-coloured substance is converted to a colourless compound. This was detectable as a decrease in absorbance (at 540 nm).


NDGA
Absorbance
500
400
300
200
100
0
Vehic
1 uM
3 uM
10 uM
100 uM
Concentration

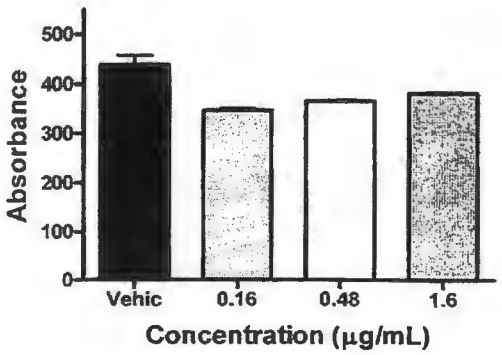
Cordia



Annona



Curcuma



These results suggest that A4+ is a potent anti-oxidant.

This property is mainly attributable to the Cordia component of A4+, and also the Annona component.

Effects of A4+ and its Constituents in a Model of Acute Inflammation

One of the most widely used *in vivo* models for assessing the anti-inflammatory actions of drugs is the "air pouch" model.

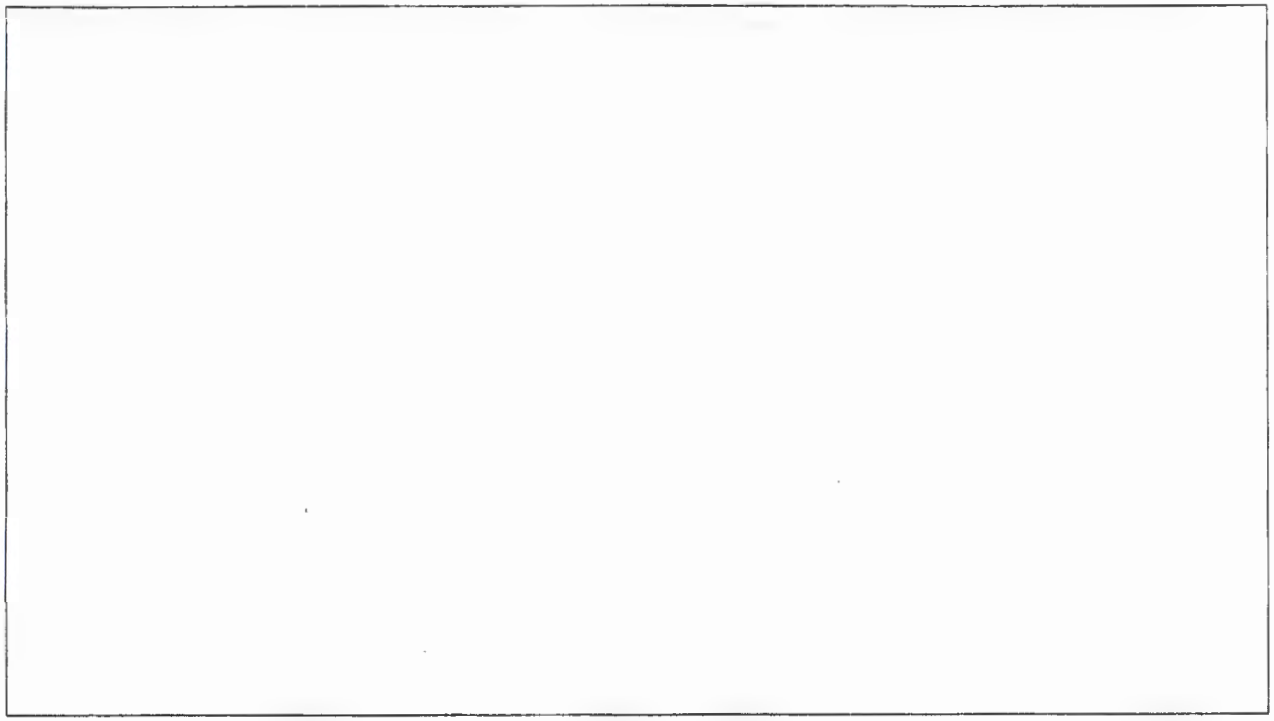
This model allows one to determine the effects of a drug on many different aspects of the inflammatory process, thereby facilitating determination of mechanism(s) of action.

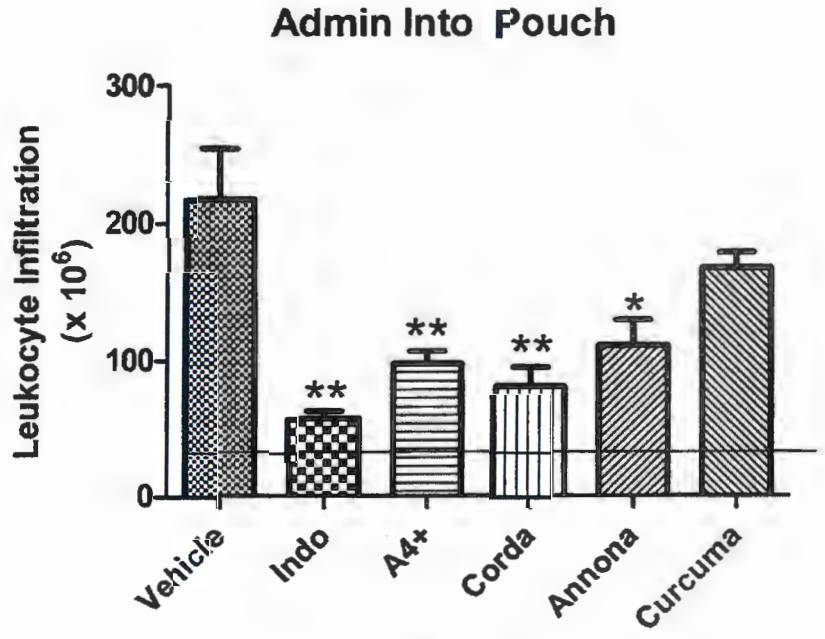
The test compounds were administered either directly into the airpouch or orally 2 hours prior to injection of zymosan.

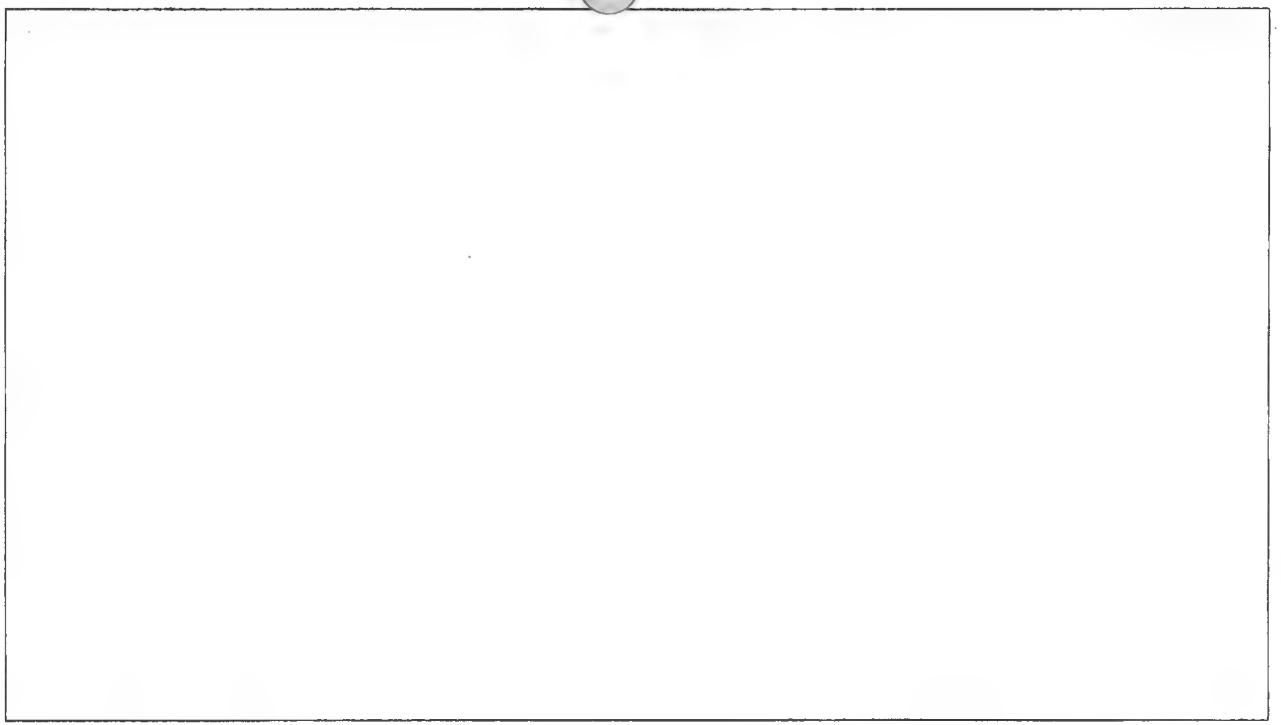
The rats (n= 5 or 6) were euthanized 4 h after injection of zymosan and the exudates were withdrawn from the airpouch for analysis.

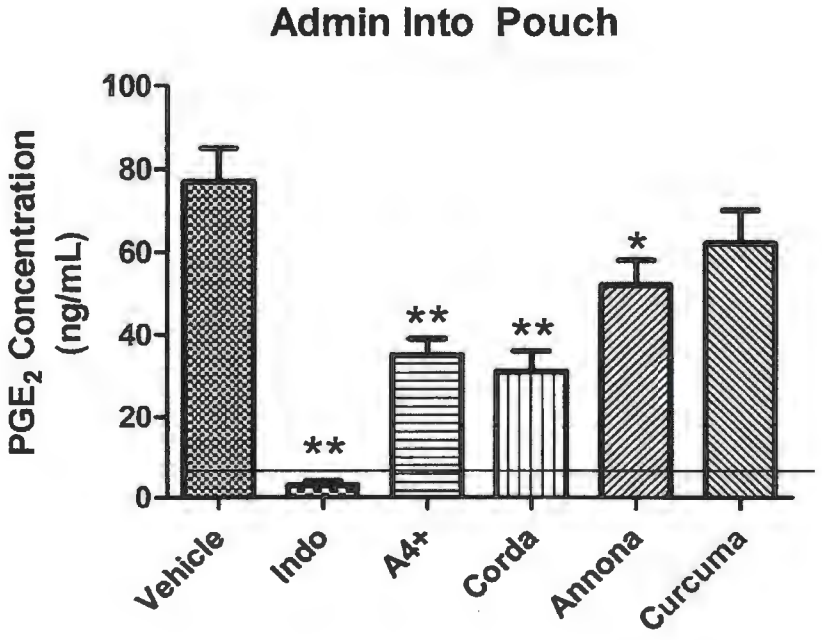
A4+ *directly into the airpouch* at a dose of 10 mg/kg
Corda (8 mg/kg)
Annona (1 mg/kg)
Curcuma (1 mg/kg)
Indomethacin (1 mg/kg)

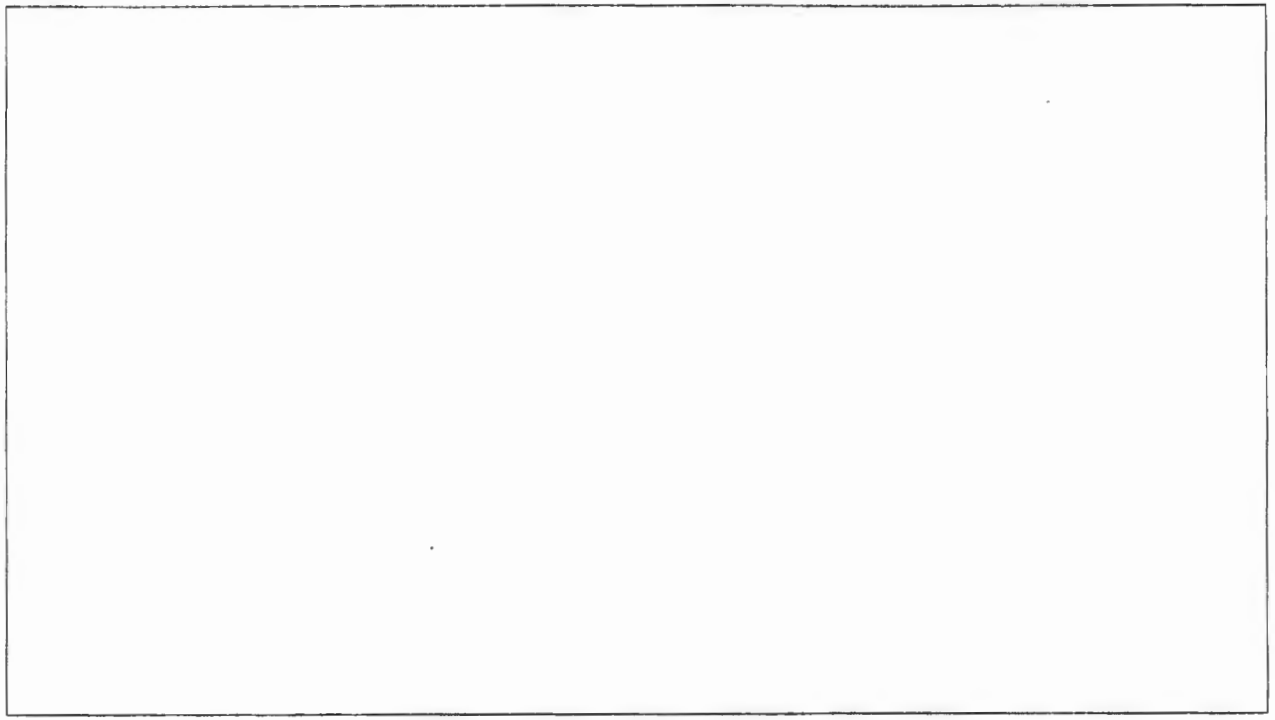
A4+ *orally* at a dose of 100 mg/kg
Corda (80 mg/kg)
Annona (10 mg/kg)
Curcuma (10 mg/kg)
Indomethacin (10 mg/kg)

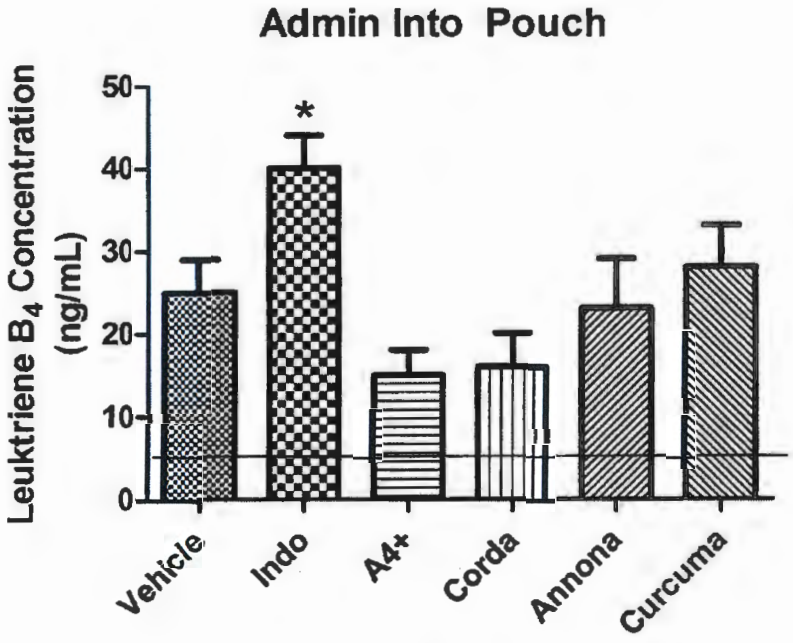



Admin Into Pouch
Leukocyte Infiltration
(x 10^6)
300
200
100
0
**
**
**
*
Vehicle
Indo
A4+
Corda
Annona
Curcuma




Admin Into Pouch
PGE2 Concentration (ng/mL)
100
80
60
40
20
0
**
**
**
*
Vehicle
Indo
A4+
Corda
Annona
Curcuma




Admin Into Pouch
Leuktriene B_4 Concentration (ng/mL)
50
40
30
20
10
0
*
Vehicle
Indo
A4+
Corda
Annona
Curcuma

Mucosal Protective Activity of A4+ and Its Constituents

Ulceration in the gastrointestinal tract is a significant clinical problem.

It is the major limitation to the use of nonsteroidal anti-inflammatory drugs (NSAIDs) for the treatment of disorders such as arthritis.

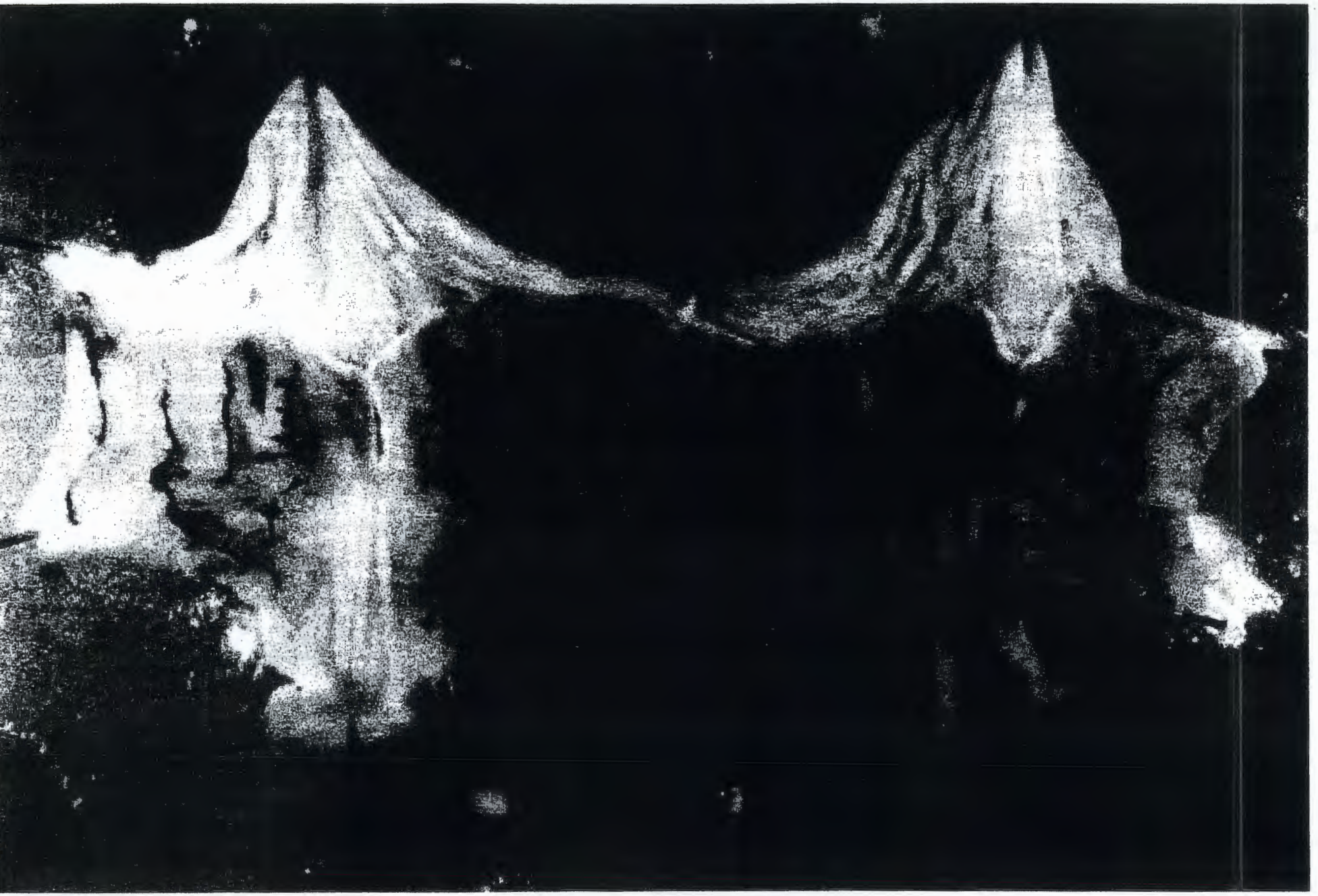
There is therefore a substantial clinical need for agents that can reduce the ulcerogenic effects of NSAIDs.

Study 1

Male Wister rats were used and they were deprived of food, but not water, for 18-20 h prior to oral administration of indomethacin (20 mg/kg).

Test drugs given orally 1 h prior to indomethacin.

The extent of hemorrhagic damage in the stomach was assessed, in a blind manner, 3 hours after indomethacin administration.



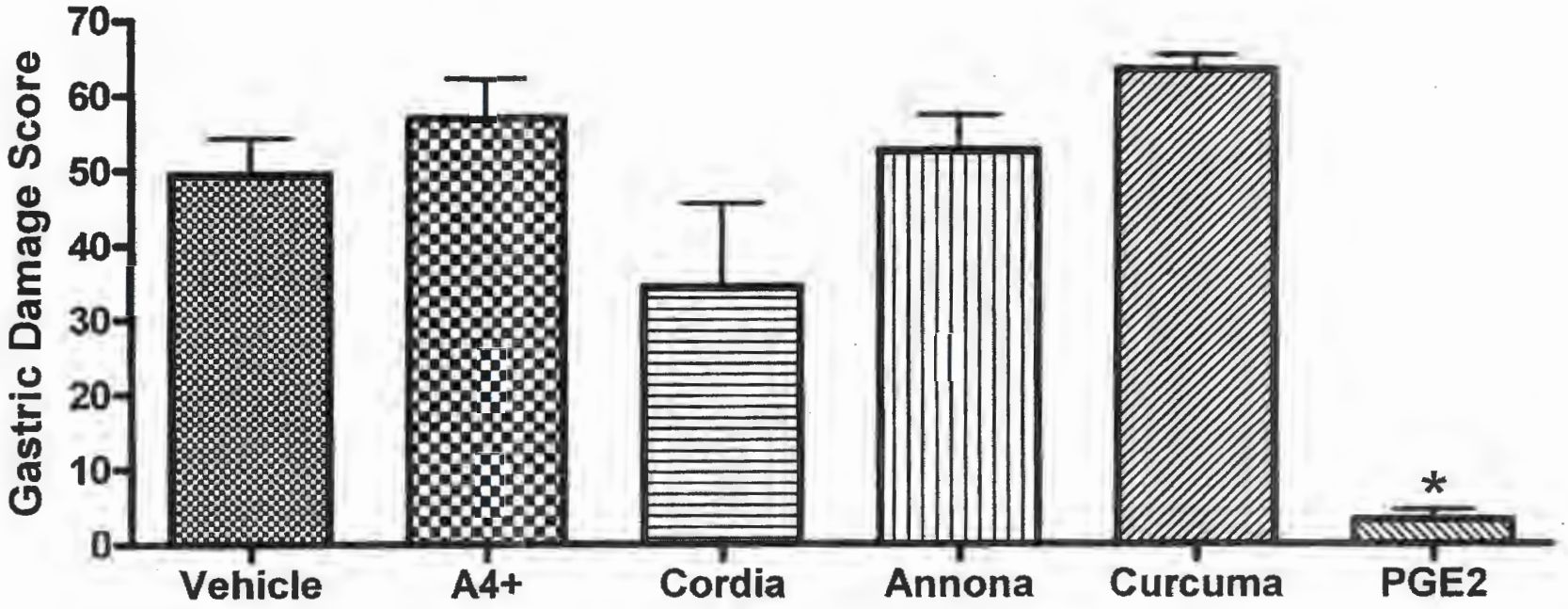
A4+ (100 mg/kg)

Cordia (80 mg/kg)

Annona (10 mg/kg)

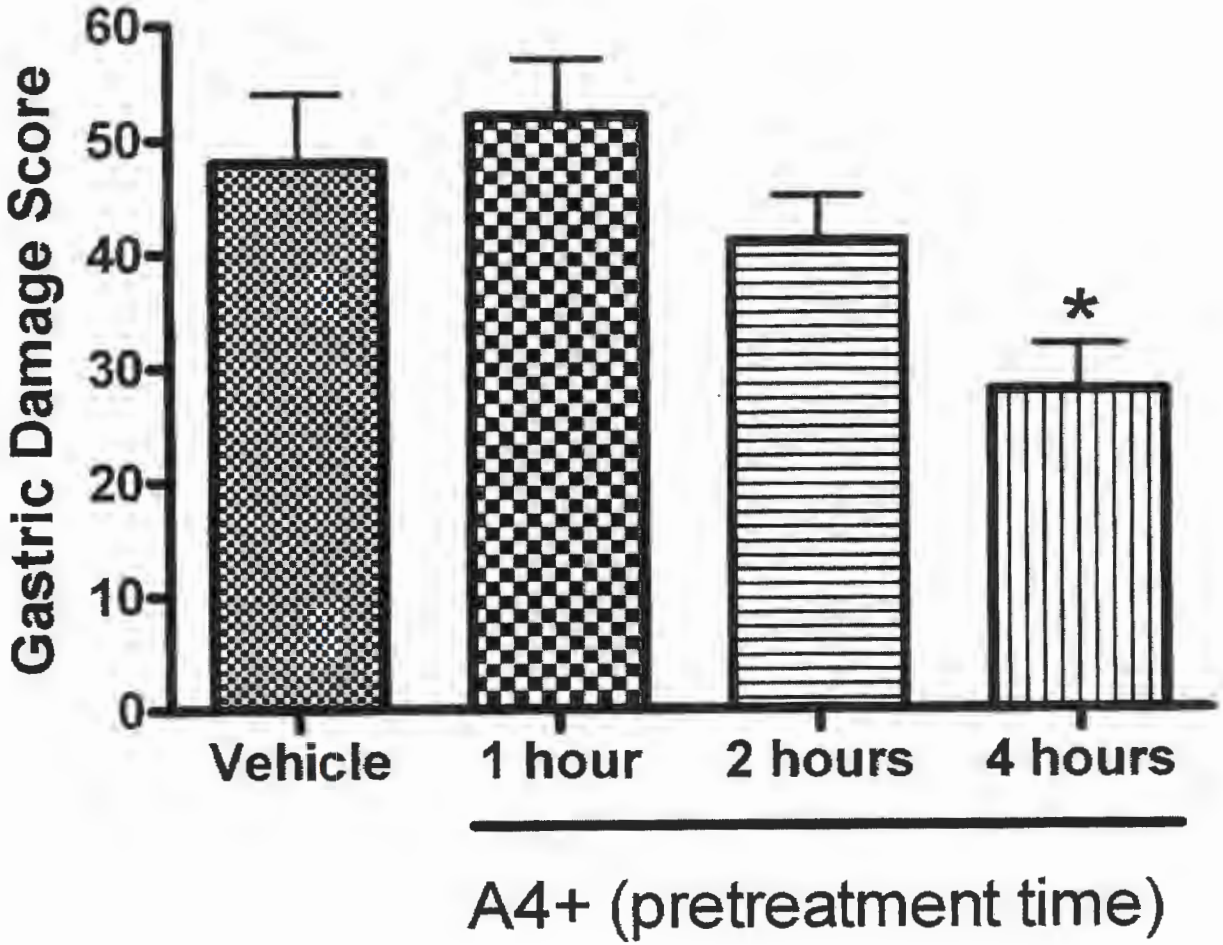
Curcuma (10 mg/kg)

Prostaglandin E_2 (0.1 mg/kg)


Gastric Damage Score
70
60
50
40
30
20
10
0
*
Vehicle
A4+
Cordia
Annona
Curcuma
PGE2

Study 2

Rats treated orally with A4+ at a dose of 300 mg/kg at 1 hour, 2 hours or 4 hours prior to oral administration of indomethacin.


Gastric Damage Score
60
50
40
30
20
10
0
*
Vehicle
1 hour
2 hours
4 hours
A4+ (pretreatment time)

Study 3

Rats were treated orally with A4+ at doses of

50, 300 or 1000 mg/kg

4 hours prior to indomethacin

SUMMARY

A4+ exhibits potent anti-oxidant activity

A4+ modestly reduces inflammation induced by zymosan (when given directly into the pouch)

A4+ can reduce indomethacin-induced gastric damage when given 4 h before, at doses ≥300 mg/kg

Mainly attributable to the Cordia and Annona constituents

FARNCOMBE

Farncombe Family Digestive Health Research Institute

McMaster University

CT470 – Final report

Study on the mitogenic action of 4 natural extracts in primary cultured rat hepatocytes

ADVANCELL
advanced in vitro cell technologies, s.l.

STATEMENT of the QUALITY ASSURANCE DEPARTMENT

REFERENCE

CT470

TITLE

Study on the mitogenic action of 4 natural extracts in primary cultured rat hepatocytes

TEST ITEM

Guava, Chamomile, Cat's claw and Papaya

The Quality Assurance Department has done the following inspection(s):

Inspection Number	Date	Checked Stage
IGC231	December 16th, 2005	Final Report

The final report reflects faithfully the project study

SIGNED | DATE | SIGNATURE + STAMP

Dr. Conxita de Castellarnau
Head of Quality Assurance Unit
ADVANCELL

15/MAR/06

ADVANCELL
advanced in vitro cell technologies, s.l.
Quality Assurance

QUALITY STATEMENT of the STUDY DIRECTOR

REFERENCE

CT470

TITLE

Study on the mitogenic action of 4 natural extracts in primary cultured rat hepatocytes

STATEMENT

I, the undersigned, hereby declare that this study was performed under my direction, according to the procedures herein described and this report constitutes a true and faithful account of the results obtained in the performance of this study.

In this study, ISO principles and procedures have been conducted according to ISO 9001:2000.

SIGNED BY	SIGNATURE and DATE
Lourdes Gombau Senior researcher – Study director ADVANCELL	15/MAR/06

REFERENCE

TITLE

Study on the mitogenic action of 4 natural extracts in primary cultured rat hepatocytes

TIMESCHEDULE

Protocol acceptance	December 2004
Final / Draft report	December 2005

CUSTOMER

Dr. José G Cabanillas Coral
Jiron San Miguel # 431, Urb. Cahuache San Luis
Lima 30
Perú

PERFORMED BY

Amparo Aranda	Dr. Lourdes Gombau

TEST LOCATION

Advanced in vitro cell technologies, s.l. (ADVANCELL)
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REPORT AUTHOR

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REPORT APPROVAL

REFERENCE: CT470

TITLE

Study on the mitogenic action of 4 natural extracts in primary cultured rat hepatocytes

TEST ITEMS

Guava, Chamomile, Cat's claw and Papaya

SUPERVISED BY	SIGNATURE and DATE
Dr. Mª José Gómez-Lechón Scientific Supervisor ADVANCELL	15/MAR/06
Dr. Conxita de Castellarnau Head of Quality Assurance Unit ADVANCELL	15/MAR/06
APROVED BY	**SIGNATURE and DATE**
Dr. Luís Ruiz Chief Executive Officer ADVANCELL	15/MAR/06
Dr. Lourdes Gombau Study Director ADVANCELL	15/MAR/06
APROVED by SPONSOR	**SIGNATURE and DATE**
Dr. José G Cabanillas Coral	

No legal requirements (nor technical) where applied to any protocol contained in this report.

3.1 Objective

The aim of this project was to perform an *in vitro* study to test the proliferative effect of 4 natural extracts (Guava, Chamomile, Cat's Claw and Papaya) on a human hepatic cell line.

3.2 Material and methods

Cell cycle analysis was performed in a human hepatoma derived cell line (HepG2 cells) and in rat primary cultured hepatocytes in order to set the proper conditions to assay compound's proliferative effect by flow cytometry. To do so, HepG2 cell cycle analysis was carried out after cells starvation for 48 hours and subsequent addition to the medium of 10% serum and a further 12 and 24-h incubation period. Primary cultured rat hepatocytes were analyzed under the same experimental conditions although they were not subjected to cell starvation to avoid a decrease in cell viability.
Obtained results pointed out primary cultured rat hepatocytes as the best alternative for proliferation studies. For this purpose, hepatocytes isolated from three different rats were incubated for a 48-h period with increasing concentrations (1, 4, 20 and 100 μg/ml) of these compounds, previously found non-cytotoxic. At the end of the incubation period medium was removed and monolayers washed with PBS. Finally, cells were incubated at 4ºC for 24 hours with a propidium iodide (PI) staining solution. PI intercalates into the major groove of double-stranded DNA and produces a highly fluorescent adduct that it is directly proportional to the DNA content. At the end of the incubation period cell lysates were recovered and finally analylzed by flow cytometry.

3.3 Main results and conclusions

Initial experiments were addressed to determine the cell type to be used in the proliferation studies. Whereas HepG2 cells did not show any differences in the cell percentage that are in the G0/G1 phase and those that are in the S or G2/M phase after a 12 or 24-h culture in the presence of serum cultured rat hepatocytes treated under the same experimental conditions for the same time periods did increase the percentage of cells in the S phase of the dyploid and the tetraploid cycle (DNA content > 4C). Based on these results, primary cultured hepatocytes were selected as the model cell system to perform the proliferation studies. Thus, hepatocytes isolated from three rat livers were exposed to increasing concentrations of the four natural extracts. Cells treated with compounds at concentrations of 1 and 4 μg/ml showed an increase of the cell percentage that was in the S phase of dyploid and tetraploid cycle.
In parallel to the proliferation study, it was achieved a cytotoxicity assay under the same experimental conditions. Results demonstrated no cytotoxicity for any compound at any tested concentration.
In summary, primary cultured rat hepatocytes exposed to Guava, Chamomile, Cat's Claw and Papaya at 1 and 4 μg/ml for a 48-h period resulted in an increase of DNA content. The mitogenic capacity showed by these compounds *in vitro* could open new expectations as potential mitogens *in* vivo.

Dr. José G Cabanillas Coral et al have performed clinical, biochemical, haematological, serological, anatomopathological and ecographic studies to 10 patients suffering from cronical hepatitis C. These patients received in their diet active ingredients of organic origin (A4+) as a unique treatment. Measurement of the mentioned parameters resulted in the following results:

- An important decrease in patient's symptomatology
- A moderate degree in the signs of hepatic illness
- A significative increase of the hepatic function
- A significative decrease of the enzymes that measure hepatocyte damage in the group of patients with necro-inflammatory lesions
- A decrease in the histopathological lesions detected and controlled by ecosonography
- In any case, it was observed an increase in patient's symptomatology, hematic alterations or any other complications.
- The evolution of patients with necro-inflammatory damage was more favorable than that obtained in patients with a higher degree in hepatic fibrosis.

Hepatic regeneration is of vital importance in the prognosis of patients suffering from acute or chronic hepatic injury and it occurs through growth-factors and cytokine-mediated proliferation of differentiated hepatocytes (1,2). Based on this, the project aims to investigate the proliferative effect of 4 natural extracts on a cell line of hepatic origin. A human hepatoma derived cell line (HepG2) with a high proliferative background and primary cultured rat hepatocytes with no capacity to proliferate but with the capability to leave the G0/G1 state and enter the S and G2/M phase were used for this purpose (2-4).

5 OBJECTIVE

The aim of this project was to test *in vitro* the proliferation capacity of 4 natural extracts provided by Dr.Cabanillas' lab by means of a cell line of hepatic origin.

6 MATERIAL AND METHODS

6.1 Test compound

Dr. Cabanillas' Laboratories provided Advancell with one batch of the following compounds, Guava, Chamomile, Cat's Claw and Papaya in their solid form. A 5 mg/ml stock solution was prepared by dissolving 5 mg of each compound in 0.7% EtOH/distilled water. With the exception of Papaya all the other compounds were solubilized in this solution. The use of other organic solvents (DMSO, methanol or 10%, 50% or pure ethanol) did not improve its solubility.
Solutions were freshly prepared for each assay. Working concentrations of these compounds were obtained by diluting the former solutions in culture medium.

6.2 Equipment

- Equipment for cell isolation
- Cell culture equipment (CO_2, hood, cell incubator, etc)
- Microcentrifuge Eppendorf model 5410
- Centrifuge Jouan model CR412
- Spectrophotometer Shimadzu model UV-240
- Fluorometer, Molecular Devices Spectra MAX Gemini XS
- Elisa reader, SLT Lab Instruments 340 ATTC
- Flow cytometer EPICS Profile II

6.3 Methodology

6.3.1 Isolation of rat hepatocytes

Four male Sprague Dawley rats were provided by CRIFFA (Sta. Perpètua de Mogoda, Barcelona) and subsequently estabulated at the animal facilities of *Centro de Investigación del Hospital La Fe (Valencia)* until subjected to hepatocyte isolation.

Animal handling, sacrifice and disposal was carried out in accordance with National regulations and the principles for the guide of care and use of laboratory animals published by the US National Institute of Health.

Isolation of adult rat hepatocytes is based on the method of Berry and Friend (5), which consists of *in situ* perfusion of the whole liver with a solution containing collagenase as disintegrating enzyme. Briefly, after anaesthetizing the animal with an intraperitoneal injection of tiobarbital, an abdominal laparotomy is performed and the cava vein is cannulated with a 1mm-diameter needle. Using a peristaltic pump adjusted to a flow rate of 18-20 ml/min, a balanced salt solution is injected to clean the organ, after which a collagenase solution is added for liver disintegration. The cell suspension obtained using this process is filtered and centrifuged and, after a couple of washings to remove collagenase, the cells are resuspended in the appropriate culture medium.

Technical data from rat hepatocyte isolation:

Rat R-2236

Race: Sprague Dawley

Sex: Male

Perfusion time with collagenase: 10 min

Collagenase concentration: 44 mg in 200 ml of perfusion buffer (Krebs-Ringer/ EGTA/ NAME)

Viability: 86%

Rat R-2243

Race: Sprague Dawley

Sex: Male

Perfusion time with collagenase: 10 min

Collagenase concentration: 44 mg in 200 ml of perfusion buffer (Krebs-Ringer/ EGTA/ NAME)

Viability: 79%

Rat R-2247

Race: Sprague Dawley

Sex: Male

Perfusion time with collagenase: 10 min

Collagenase concentration: 44 mg in 200 ml of perfusion buffer (Krebs-Ringer/ EGTA/ NAME)

Viability: 89%

Rat R-2250

Race: Sprague Dawley

Sex: Male

Perfusion time with collagenase: 10 min

Collagenase concentration: 44 mg in 200 ml of perfusion buffer (Krebs-Ringer/ EGTA/ NAME)

Viability: 92%

6.3.2	Hepatocyte culture

Rat isolated hepatocytes were resuspended in Ham's F-12/Lebovitz L-15 (1:1), medium supplemented with 2mM glutamine, 170μg/ml sodium selenite, 2% newborn calf serum, 50mU/ml penicillin, 50μg/ml streptomycin, 0.2% bovine serum albumin and 10nM insulin and subsequently seeded at a density of 80,000 viable cells/cm^2 on previously fibronectin-coated plastic dishes (6,7). Compound cytotoxicity was assessed in cells seeded on 96 well/plates. Cell cycle analysis and metabolic characterization was performed in cells grown on 3,5 cm dishes.

After resuspension, a cell aliquot is removed and viability determined by cell counting using the trypan blue exclusion method. Trypan blue uptake is the result of cell membrane alteration and in consequence of cell death. Thus, to quantify cell viability, 0.4% trypan blue in saline is added to the cells and these are immediately loaded in a Neubauer chamber. Viable cells are counted in 5 different fields under the optical microscope as non-blue cells.

$$\% \text{ Viability} = \frac{\text{Number of non-blue cells}}{\text{Number of total cells}} \times 100$$

6.3.3 Determination of the metabolic capacity of cultured hepatocytes

Rat primary cultured hepatocyte were metabolically characterized to ascertain their proper functionality. The assays employed to assess this metabolic status were the ECOD activity and the testosterone metabolism (see section 11-List of appendixes).

6.3.3.1 *Measurement of ECOD activity*

This technique is based on the O-deethylation of 7-ethoxycoumarin resulting in the formation of 7-hydroxycoumarin, which is then readily estimated fluorimetrically. To do so, cells were seeded on 35 mm dishes at a density of $8x10^4$ viable cells/cm^2. After culture stabilization, monolayers were washed with PBS and subsequently incubated with medium containing 800 μM 7-ethoxycoumarin for 1 hour. Finally, medium was harvested and deconjugated by treatment 2h at 37ºC with the enzymes β-glucuronidase/arylsulfatase and generated 7-hydroxycoumarin quantified fluorimetrically. Protein quantification from cellular lysates was used to normalize data (8,9).

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6.3.3.2 *Quantification of testosterone metabolism*

To assess the expression of CYP450, the activity of this enzyme was tested as testosterone hydroxylation. Hepatocytes were cultured on a 3.5 cm dish. After culture stabilization, cells were incubated for 60 min with 1 ml of culture medium containing 200μM testosterone. Metabolites (2β-hydroxytestosterone, 6β-hydroxytestosterone, 15β-hydroxytestosterone, 16α-hydroxytestosterone, 16β-hydroxytestosterone, 7α-hydroxytestosterone and androstenedione) were extracted and analysed by HPLC. Protein quantification from cellular lysates was used to normalize data. (8,9)

6.3.4 Cytotoxicity assessment by the MTT assay

The MTT assay is based on the ability of mitochondrial succinate dehydrogenase to transform the MTT substrate, a tetrazolium salt, into an insoluble blue formazan. The amount of colored product formed is directly related to the activity of the mitochondria, which is a clear indicator of cell viability.
To do so, rat isolated hepatocytes seeded on 96 well-plates were incubated with the compounds under the same experimental conditions than those carried out for cell proliferation studies. At the end of the incubation period, medium was removed and wells washed twice with PBS. Subsequently a MTT solution was added to the wells and incubated at 37ºC during 2 additional hours. After incubation, MTT was discharged and the formazan precipitated inside the wells re-dissolved in DMSO. Blue-formazan produced by metabolically active cells was quantified by measuring the absorbance at 550 nm in an ELISA reader. Non-treated cells were used as positive controls of viability (8,9).

6.3.5	Assays to select the model cell type for proliferation studies

A human derived cell line (HepG2) and primary cultured rat hepatocytes were subjected to the following experimental conditions:

1) HepG2 cells were seeded on ten 35 mm dishes at 100,000 cells/plate. 24-h later, medium was removed from 2 plates and monolayers washed with PBS and immediately frozen in liquid nitrogen. Cell cycle analysis in these cells set the culture basal conditions. The remaining plates were deprived for an additional 48-h period in serum free medium. During this period of time two other plates were treated as described above at 24-h incubation as well as at the end of the 48-h starvation period. Lasting plates were incubated with medium containing 10% serum. Half of these plates were frozen at 12 h and the other half at 24-h of serum exposure. Finally, propidium iodide staining solution was added to thawed monolayers and incubated O/N at 4ºC. Cell lysates were recovered by pipeting several times on the monolayer and nuclei analyzed by flow cytometry.

2) Primary cultured rat hepatocytes were seeded on 35 cm dishes at 80,000 viable cells/cm^2. At the end of culture recovery (24-h) cells were incubated either in the absence or presence of medium containing 2% serum for two additional periods (12 hours and 24 hours). Finally, monolayers were stained with propidium iodide solution as described above and subsequently analyzed by flow cytometry.

6.3.6	Incubations for cell cycle analysis in natural extract treated cells

Primary cultured rat hepatocytes isolated from 3 independent assays were seeded on 3,5 cm dishes as described elsewhere (see former sections). 24-h later cells were incubated with increasing concentrations of the compounds at 1, 4, 20 and 100 μg/ml (each experimental condition by duplicate) in serum free medium. After an exposure period of 24 h, medium was renewed and incubated for an additional 24-h period. At the end of the incubation, medium was withdrawn and cells were washed with PBS and monolayer stained with propidium iodide solution O/N for flow cytometry analysis.

Untreated cells were used to analyze cell cycle under culture basal conditions.
Cells treated with 2% serum were used as positive controls of proliferation.

The compounds have been stored in Advancell, until the devolution of the leftovers to DR. JOSÉ G CABANILLAS CORAL at the end of the project.

Samples generated in Advancell:

- Will be stored in our facilities for 3 years. ☐
- Are sent to the client, because accepts their devolution. ☐

Data obtained during the study will be stored at ADVANCELL for 3 years and ADVANCELL will provide the sponsor with the final report, a CD copy with all the information (data, graphics, images...).

8.1	Selection of the model cell type for proliferation studies

The cell cycle of two cell lines of hepatic origin (a human hepatoma derived cell line (HepG2) and primary cultured rat hepatocytes) was analyzed with the purpose of selecting one of them for proliferation studies.
HepG2 cells because of their tumor cell behavior proliferate rapidly in culture. This characteristic that could be beneficial to study cell proliferation in the presence of natural extracts could also quench the effect of these potential mitogens.
With the purpose of arresting the HepG2 cells at G0/G1 phase of the cell cycle, cells were starved for 48-h. After this period, cells were incubated with medium containing 10% serum to reactivate the cell cycle.
As expected, cell deprivation for 24-h revealed a decrease of the cell percentage that is in the S phase (table 1). This percentage remains invariable when deprivation goes for another 24-h. After the starvation period, cell incubation with medium containing 10% serum for 12-h slightly increased the S phase against the G0/G1 phase. This distribution remained stable when cells were incubated for an additional 12-h period. Data demonstrated that HepG2 cells were not a useful model for proliferation studies.

Primary cultured rat hepatocytes present a heterogenous population with a diploid and a tetraploid population. It is technically difficult to distinguish, on the basis of DNA content, between the cells that are in the G2/M phase of the diploid cycle and those that are in the G0/G1 phase of the tetraploid cycle. Proliferation in the tetraploid subpopulation was estimated by the percentage of nuclei having a DNA content >4C (2).
Taking into consideration the polyploidy of these cells, primary cultured rat hepatocytes were incubated in the absence/presence of medium containing 2% serum for two periods of time (12 and 24-h). At the end of the former period, a 1.2 and 3.5-fold increase of the population of tetraploid cells was observed at 12 and 24-h, respectively. This increased was also detected for the population having a DNA content >4C. Thus, this population increased 2.4 and 12-fold after incubation periods of 12 and 24-h, respectively (Table 2).

Despite primary cultured rat hepatocytes do not divide in culture, data contain herein demonstrate the usefulness of primary hepatocytes against HepG2 cells to study potential mitogens.

Table 1. **Cell cycle** analysis in HepG2 cells incubated under different experimental conditions

Experimental Conditions	**% G0/G1**	**% S**	**% G2/M**
Basal	22	63	15
24-h starvation	31,2	31,2	37,7
48-h starvation	29,6	34,2	36,2
12-h 10% serum	21,3	44,6	34,1
24-h 10% serum	21,6	47,1	31,3

Table 2. Cell cycle analysis in primary cultured rat hepatocytes incubated under different experimental conditions

Experimental Conditions	% Diploid Nuclei (2C)	% Tetraploid Nuclei (4C)	% Nuclei (>4C)
12-h in the absence of serum	54,8	44,8	0,33
24-h in the absence of serum	83,8	16,2	---
12-h 2% serum	46,5	52,7	0,8
24-h 2% serum	41,5	57,2	1,2

8.2	Mitogenic effect of 4 natural extracts on primary cultured rat hepatocytes

Four natural extracts provided by the sponsor (Guava, Chamomile, Cat's Claw and Papaya) were used to investigate their mitogenic effect on primary cultured rat hepatocytes. To do so, rat hepatocytes isolated from 3 independent assays were incubated with increasing concentrations of these compounds (1, 4, 20 and 100 μg/ml) for a 48-h period. At the end of this period, monolayers were treated as described in section 6.3.6 for flow cytometry analysis.
In parallel to the proliferation study, cell viability was also tested under the same experimental conditions. As shown in Figure 1 no cytotoxicity was observed for any compound at any tested concentration in any culture.

Cell cycle analysis of the three different cultures exposed to the natural extracts resulted in an increase of the cell population with higher DNA content. Thus, cells treated for 48-h with concentrations of 1 and 4 μg/ml of these compounds increased the number of cells that left the G0/G1 phase and correlated with an increase of cell population that enters into the S phase of the diploid and tetraploid cycle. In contrast, compounds incubated at concentrations of 20 and 100 μg/ml had minor or no effect on cell proliferation.

Figure 1. Cell viability study in primary cultured rat hepatocytes exposed to Guava, Chamomile, Cat's Claw and Papaya.

[illegible] ree [illegible] nt [illegible] es of [illegible] patocytes [illegible] at R-2[illegible]3; Panel A, Rat R-2247 [illegible] P[illegible] nd R[illegible] R-2250 Panel C[illegible] ve[illegible] re inc[illegible] d w[illegible] Gu[illegible] va, Ch[illegible] momi[illegible], Cat's [illegible] and Papaya [illegible] 1, 4, [illegible]0 and 100 µg/m[illegible] for a [illegible]-h[illegible] the end of the [illegible] tin[illegible] ce[illegible] y w[illegible] te[illegible] the e[illegible] rim[illegible] tal [illegible] ons by [illegible] T[illegible]

[illegible] d ce[illegible] p[illegible] ve [illegible] ontr[illegible] vi[illegible] y

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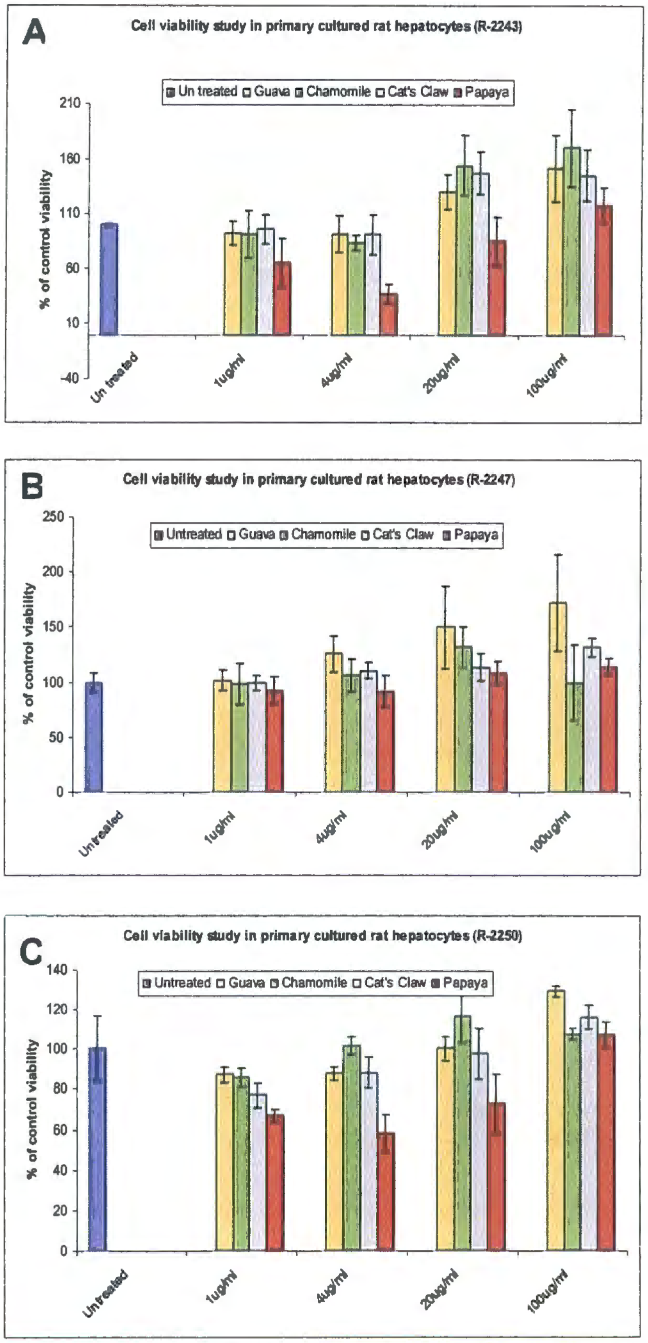

Table 3. Cell cycle analysis of primary cultured rat hepatocytes (R-2243) exposed to 4 natural extracts.

Exp Cond	%G0/G1	%S Diploid C	%4C + G2/M	% ≥ 4C Tetraploid C
Control	50	7	15	6,5
2% Serum	57	14	16	10,2
100 µg/ml Guava	71	5	18	3,3
20 µg/ml Guava	46	11,5	13	12,2
4 µg/ml Guava	36	15,5	13	20,8
1 µg/ml Guava	36	16	11	18,8
100 µg/ml Chamomile	66	6,5	16	6,6
20 µg/ml Chamomile	62	6	16	5,4
4 µg/ml Chamomile	41	16,5	9	14,8
1 µg/ml Chamomile	35	16	7	19
100 µg/ml Cat's Claw	66	6,5	15	5,2
20 µg/ml Cat's Claw	60	9,5	15	7,5
4 µg/ml Cat's Claw	42	16	12	16,6
1 µg/ml Cat's Claw	44	14,5	11	14,7
100 µg/ml Papaya	69	5,5	18	3,7
20 µg/ml Papaya	49	14,5	15	11,3
4 µg/ml Papaya	36	17,5	11	18,7
1 µg/ml Papaya	38	16,5	11	18,6

Table 4. Cell cycle analysis of primary cultured rat hepatocytes (R-2247) exposed to 4 natural extracts.

Exp Cond	%G0/G1	%S Diploid C	%4C + G2/M	% ≥ 4C Tetraploid C
Control	53,5	4,2	19,5	3,2
2% Serum	58,5	3,4	23	2,9
100 µg/ml Guava	61	2,7	16,5	6,4
20 µg/ml Guava	55,5	3,9	16,5	6,3
4 µg/ml Guava	51,5	4,5	18	8,4
1 µg/ml Guava	50	4,4	26,5	6,6
100 µg/ml Chamomile	54,5	2,8	27	2,3
20 µg/ml Chamomile	47	3,8	19	6,9
4 µg/ml Chamomile	50,5	5,2	22,5	5,8
1 µg/ml Chamomile	52,5	5,5	25	6,7
100 µg/ml Cat's Claw	59,5	3,2	22,5	5
20 µg/ml Cat's Claw	52,5	4,2	20,5	6,4
4 µg/ml Cat's Claw	48	5,9	21	5,8
1 µg/ml Cat's Claw	48	6,1	24	8,1
100 µg/ml Papaya	57	3,5	20,5	7,7
20 µg/ml Papaya	53	6	17	6,8
4 µg/ml Papaya	51,5	5,9	20	11,8
1 µg/ml Papaya	50,5	6,5	25,5	10

Table 5. Cell cycle analysis of primary cultured rat hepatocytes (R-2250) exposed to 4 natural extracts.

Exp Cond	%G0/G1	%S Diploid C	%4C + G2/M	% ≥ 4C Tetraploid C
Control	64	8,5	12	6,5
2% Serum	67	8,5	15,5	4,5
100 µg/ml Guava	67	5	15	3,9
20 µg/ml Guava	70	7,5	13,5	7,7
4 µg/ml Guava	66	11	13	13,1
1 µg/ml Guava	62	11,5	14	10,4
100 µg/ml Chamomile	68	5,5	13	4,3
20 µg/ml Chamomile	67	6,5	13,5	4,1
4 µg/ml Chamomile	65	9,5	14,5	7,5
1 µg/ml Chamomile	63	10,5	14,5	9,4
100 µg/ml Cat's Claw	64	8,5	15	5,1
20 µg/ml Cat's Claw	65	9	14,5	6,1
4 µg/ml Cat's Claw	64	11,5	14,5	11,6
1 µg/ml Cat's Claw	66	9,5	15,5	5,8
100 µg/ml Papaya	63	8	14	4,4
20 µg/ml Papaya	62	13	12,5	10,1
4 µg/ml Papaya	62	13,5	13,5	11,1
1 µg/ml Papaya	66	12,5	12	10,4

- Despite primary hepatocytes do not divide in culture, they were found to be a useful model to study compound's mitogenic effect

- Guava, Chamomile, Cat's Claw and Papaya did not result cytotoxic under the same experimental conditions used in the proliferation assay in any culture at any tested concentration.

- Primary cultured rat hepatocytes exposed to Guava, Chamomile, Cat's Claw and Papaya at 1 and 4 μg/ml for a 48-h period resulted in an increase of DNA content. The mitogenic capacity showed by these compounds *in vitro* could open new expectations as potential mitogens *in* vivo.

1. FF Mohammed and R Khokha. Thinking outside the cell: proteases regulate hepatoctte division. Trends Cell Biol 15:555-563, 2005

2. MJ Gómez-Lechón, JV Castell, I Guillén, E O'Connor, T Nakamura, R Fabra and R Trullenque. Effects of hepatocyte growth factor on the growth and metabolism of human hepatocytes in primary culture. Hepatology 5: 1248-1254, 1995

3. Zhang X, Xu LS, Wang ZO, Wang KS, Li N, Cheng ZH, Huang SZ, Nei DZ, Han ZG. ING4 induces G2/M cell cycle arrest and enhances the chemosensitivity to DNA-damage agents in HepG2 cells. FEBS Lett 570:7-12, 2004

4. Chenoufi N, Drenou B, Loreal O, Pigeon C, Brissot P, Lescoat G. Antiproliferative effect of diferiprone on the HepG2 cell line. Biochem Pharmacol 56:431-437, 1998

5. MN Berry, DS Friend. High yield preparation of isolated rat liver parenchymal cells. J. Cell.Biol.43:506-520, 1969

6. MJ Gómez-Lechón, MT Donato, JV Castell. Use of Cultured Hepatocytes to Investigate Drug Metabolism and Toxicity. In Vitro Toxicology 10:63, 1997

7. MJ Gómez-Lechón, X Ponsoda, R Bort, JV Castelll. The Use of Cultured Hepatocytes to Investigate the Metabolism of Drugs and Mechanisms of Drug Hepatotoxicity. ATLA 29:225, 2001

8. JV Castell, MJ Gómez-Lechón. *In vitro* alternatives to animal pharmaco-toxicology. Farmaindustria Ed. JV Castell and MJ Gómez-Lechón. Madrid, 1992.

9. M. J. Gómez-Lechón, T. Donato, X. Ponsoda, R. Fabra, R. Trullenque and J. V. Castell. Isolation, culture and use of human hepatocytes in drug research. IN VITRO METHODS IN PHARMACEUTICAL RESEARCH. ISBN 0-12-163390-X. Eds. J. V. Castell y M. J. Gómez-Lechón eds. pp. 129-154. Academic Press. London (1997)

11.1	Appendix 1: Metabolic characterization of cultured rat hepatocytes

Rat R-2236

1. ECOD ACTIVITY

ECOD activity in rat hepatocytes (R-2236) after 24-hours in culture

22.7 pmol/min x mg cell prot

Mean value 29.8 ± 10.4 pmol/min x mg cell prot (n=46) (Historical data)

2. TESTOSTERONE METABOLISM

	Enzymatic activity (pmols/mg cell protein x min)						
R-2236	15β	7α	6β	16α	16β	2β	Androstenedione
Mean value (24-hour culture)	1.3	17.3	0.8	50.8	0	12.3	44.2
Rat cultured hepatocytes (n= 50)*	2.1±1.8	20.3±18.1	4.0±3.0	74.5±90.4	1.6±2.9	33.1±30.1	52.3±39.4

(*) Historical data

Rat R-2243

1. ECOD ACTIVITY

ECOD activity in rat hepatocytes (R-2243) after 24-hours in culture

(sample lost) pmol/min x mg cell prot

Mean value 29.8 ± 10.4 pmol/min x mg cell prot (n=46) (Historical data)

2. TESTOSTERONE METABOLISM

	Enzymatic activity (pmols/mg cell protein x min)						
R-2243	15β	7α	6β	16α	16β	2β	Androstenedione
Mean value (24-hour culture)	0.6	21.5	1.7	32.7	0	1.8	13.9
Rat cultured hepatocytes (n= 50)*	2.1±1.8	20.3±18.1	4.0±3.0	74.5±90.4	1.6±2.9	33.1±30.1	52.3±39.4

(*) Historical data

Rat R-2247

1. ECOD ACTIVITY

ECOD activity in rat hepatocytes (R-2247) after 24-hours in culture

25.8 pmol/min x mg cell prot

Mean value 29.8 ± 10.4 pmol/min x mg cell prot (n=46) (Historical data)

2. TESTOSTERONE METABOLISM

	Enzymatic activity (pmols/mg cell protein x min)						
R-2247	15β	7α	6β	16α	16β	2β	Androstenedione
Mean value (24-hour culture)	1.1	11.0	2.5	22.8	0	9.7	76.9
Rat cultured hepatocytes (n= 50)*	2.1±1.8	20.3±18.1	4.0±3.0	74.5±90.4	1.6±2.9	33.1±30.1	52.3±39.4

(*) Historical data

Rat R-2250

1. ECOD ACTIVITY

ECOD activity in rat hepatocytes (R-2250) after 24-hours in culture

36.3 pmol/min x mg cell prot

Mean value 29.8 ± 10.4 pmol/min x mg cell prot (n=46) (Historical data)

2. TESTOSTERONE METABOLISM

	Enzymatic activity (pmols/mg cell protein x min)						
R-2250	15β	7α	6β	16α	16β	2β	Androstenedione
Mean value (24-hour culture)	0.7	8.3	3.9	9.3	0	3.6	26.5
Rat cultured hepatocytes (n= 50)*	2.1±1.8	20.3±18.1	4.0±3.0	74.5±90.4	1.6±2.9	33.1±30.1	52.3±39.4

(*) Historical data

Study on the mechanisms of hepatoprotection of 4 natural extracts by using rat primary cultured hepatocytes

ADVANCELL
advanced in vitro cell technologies, s.l.

STATEMENT of the QUALITY ASSURANCE DEPARTMENT
REFERENCE
CT438
TITLE
Study on the mechanisms of hepatoprotection of 4 natural extracts by using rat primary cultured hepatocytes
TEST ITEMS
Guava, Chamomile, Cat's claw and Papaya

Quality Assurance Department has done the following inspections		
Inspection Number	Date	Checked Stage
IGC 240	FEB/28/2006	Final Report

The final report reflects faithfully the project study		
SIGNED BY	DATE	SIGNATURE & STAMP
Dr. Conxita de Castellarnau Head of Quality Assurance Unit ADVANCELL	15/MAR/06	ADVANCELL Quality Assurance

QUALITY STATEMENT of the STUDY DIRECTOR

REFERENCE

CT438

TITLE

Study on the mechanisms of hepatoprotection of 4 natural extracts by using rat primary cultured hepatocytes

STATEMENT

I, the undersigned, hereby declare that this study was performed under my direction, according to the procedures herein described and this report constitutes a true and faithful account of the results obtained in the performance of this study.

In this study, ISO principles and procedures have been conducted according to ISO 9001:2000.

SIGNED BY	SIGNATURE and DATE
Lourdes Gombau Senior researcher – Study director ADVANCELL	15/MAR/06

TITLE

Study on the mechanisms of hepatoprotection of 4 natural extracts by using rat primary cultured hepatocytes

TIME SCHEDULE

Protocol acceptance	December 2004
Final / Draft report	December 2005

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Amparo Aranda	
Lourdes Gombau	

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requirement expires once the final report of the study has been approved.

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REFERENCE: CT438	
TITLE	
Study on the mechanisms of hepatoprotection of 4 natural extracts by using rat primary cultured hepatocytes	
TEST ITEMS	
Guava, Chamomile, Cat's claw and Papaya	
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2 LEGAL REQUIREMENTS

No legal requirements (nor technical) where applied to any protocol contained in this report.

3 SUMMARY

3.1 Objective

This study was addressed to investigate *in vitro* the mechanism of hepatoprotection of 4 natural extracts by using rat primary cultured hepatocytes.

3.2 Material and methods

To do so, cells were preincubated with increasing concentrations of these 4 compounds (4, 20, 100 and 500 μg/ml) for different time periods, in agreement with the assay conditions (see sections 6.3.5 and 6.3.6 for more details). At the end of the corresponding incubation periods, an oxidant agent (t-butyl hydroperoxide) was added to the cells in the presence of the compounds at the concentrations mentioned above. Subsequently, free radical formation, hydroperoxide production and reduced glutation (GSH) levels were quantified. In addition, a putative increase in albumin and transferrin synthesis at the expression level was also determined in rat hepatocytes incubated with the compounds for a 48-hour period. Used concentrations were below the maximal non-toxic concentration (MNTC).
In parallel, cell viability was quantified according to the different assay conditions by means of the mitochondrial MTT test.

3.3 Main results and conclusions

Cell viability was found altered when cells were incubated with Guava and Papaya at the highest concentration tested in this study (500 μg/ml) in medium containing 2% serum. In contrast, rat hepatocytes incubated with Guava, Chamomile and Cat's Claw at concentrations of 20 and 100 μg/ml did not resulted cytotoxic. In fact, cell viability was maintained in these conditions in the absence of serum.
All tested compounds resulted hepatoprotectors in a dose-dependent manner. Thus, rat hepatocytes incubated with increasing concentrations of the compounds in the presence of an oxidant agent (t-butyl hydroperoxide) resulted in a decrease of free radical formation and in consequence of hydroperoxide production. Moreover, cells exposed under the same experimental conditions showed a tendency to recover basal GSH levels when compound administration reached a concentration of 100 μg/ml.
Cells exposed to Guava, Chamomile and Cat's Claw at 4 μg/ml caused an increase of the mRNA levels of albumin and transferrin in comparison to untreated cells. This effect was also observed in cells treated with Papaya at 100 μg/ml.

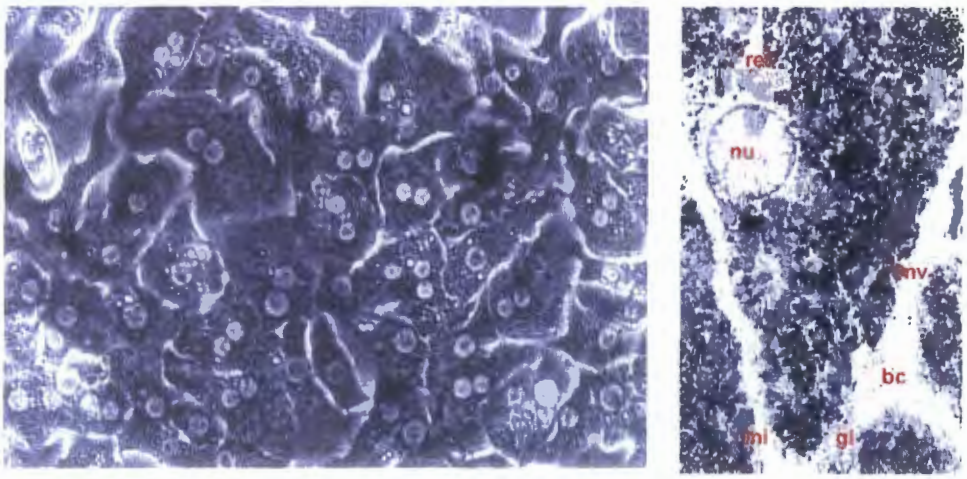
4 INTRODUCTION

Dr. José G Cabanillas Coral et al have performed clinical, biochemical, haematological, serological, anatomopathological and ecographic studies to 10 patients suffering from cronical hepatitis C. These patients received in their diet active ingredients of organic origin (A4+) as a unique treatment. Measurement of the mentioned parameters resulted in the following results:

- An important decrease in patient's symptomatology
- A moderate degree in the signs of hepatic illness
- A significative increase of the hepatic function
- A significative decrease of the enzymes that measure hepatocyte damage in the group of patients with necro-inflammatory lesions
- A decrease in the histopathological lesions detected and controlled by ecosonography
- In any case, it was observed an increase in patient's symptomatology, hematic alterations or any other complications.
- The evolution of patients with necro-inflammatory damage was more favourable that that obtained in patients with a higher degree in hepatic fibrosis.

Based on these data, the project aimed to investigate *in vitro* the mechanisms of action that underlie to these clinical results. As far as known, hepatocytes in culture are the model cell system more comparable to liver since primary cultured hepatocytes retain their characteristics at the morphological as well as at the functional level (see figure 1). Hepatocytes in culture exhibit a cell surface completely covered with microvilli. Cells show a large number of glycogen particles forming rosettes spread throughout the cytoplasm that guaranties high levels of intracellular glycogen, similar to those reported for fed human liver *in vivo*. Hepatocytes efficiently convert lactate into glucose or stimulate urea synthesis when the media is overloaded with ammonium ions. Hepatocytes synthesize and secrete plasma proteins (albumin, transferrin, fibronectin, fibrinogen, α_1-acid glycoprotein, etc) to culture medium. In addition, they maintain the expression of drug metabolizing enzymes (cytochrom P450 and conjugation enzymes: mainly glucuronil transferase and glutation transferase), although the metabolizing capacity decreases with time in culture (1-3).

Figure 1 Primary cultured hepatocytes analyzed by phase contrast microscopy (left panel) and electronic microscopy (right panel).
nu (nucleus), rel (endoplasmic reticulum), mv (microvilli), bc (bile canaliculum), gl (lipid droplets) and mi (mitochondria).



The rat hepatocyte model cell system was employed in this project to reproduce *in vitro* the hepatoprotector effect of the clinical tested compound (A4+) as well as of three different analogues. Measurement of their action on oxidative processes such as free radical formation, lipid peroxidation and reduced glutation levels were used to evaluate their putative protective role. Likewise, their effect on hepatocyte specific functions (albumin and transferrin production) were also tested at the expression level (mRNA).

5. OBJECTIVE

The main objective of this study was to test *in vitro* the hepatoprotector capacity of 4 natural extracts provided by Dr. Cabanillas Laboratories as well as to study the mechanism by which this action is exerted.

6. MATERIAL AND METHODS

6.1 Test compound

Dr. Cabanillas' Laboratories provided Advancell with one batch of the following compounds, Guava, Chamomile, Cat's Claw and Papaya in their solid form. A 5 mg/ml stock solution was prepared by dissolving 5 mg of each compound in 0.7% EtOH/distilled water. With the exception of Papaya all the other compounds were solubilized in this solution. The use of other organic solvents (DMSO, methanol or 10%, 50% or pure ethanol) did not improve its solubility.
Solutions were freshly prepared for each assay. Working concentrations of these compounds were obtained by diluting the former solutions in culture medium.

6.2 Equipment

- Equipment for cell isolation
- Cell culture equipment (CO_2, hood, cell incubator, etc)
- Microcentrifuge Eppendorf model 5410
- Centrifuge Jouan model CR412
- Spectrophotometer Shimadzu model UV-240
- Fluorometer, Molecular Devices Spectra MAX Gemini XS
- Lightcycler PCR, Roche

6.3 Methodology

6.3.1 Isolation of rat hepatocytes

Five male Sprague Dawley rats were provided by CRIFFA (Sta. Perpètua de Mogoda, Barcelona) and subsequently estabulated at the animal facilities of *Centro de Investigación del Hospital La Fe (Valencia)* until subjected to hepatocyte isolation.

Animal handling, sacrifice and disposal was carried out in accordance with National regulations and the principles for the guide of care and use of laboratory animals published by the US National Institute of Health.

Isolation of adult rat hepatocytes is based on the method of Berry and Friend (4), which consists of *in situ* perfusion of the whole liver with a solution containing collagenase as disintegrating enzyme. Briefly, after anaesthetizing the animal with an intraperitoneal injection of tiobarbital, an abdominal laparotomy is performed and the cava vein is cannulated with a 1mm-diameter needle. Using a peristaltic pump adjusted to a flow rate of 18-20 ml/min, a balanced salt solution is injected to clean the organ, after which a collagenase solution is added for liver disintegration. The cell suspension obtained using this process is filtered and centrifuged and, after a couple of washings to remove collagenase, the cells are seeded on fibronectin-coated plates.

Technical data from rat hepatocyte isolation:
Rat R-2222 Race: Sprague Dawley Sex: Male Perfusion time with collagenase: 10 min Collagenase concentration: 44 mg in 200 ml of perfusion buffer (Krebs-Ringer/ EGTA/ NAME) Viability: 89%

Rat R-2223 Race: Sprague Dawley Sex: Male Perfusion time with collagenase: 10 min Collagenase concentration: 44 mg in 200 ml of perfusion buffer (Krebs-Ringer/ EGTA/ NAME) Viability: 75%

Rat R-2224

Race: Sprague Dawley

Sex: Male

Perfusion time with collagenase: 10 min

Collagenase concentration: 44 mg in 200 ml of perfusion buffer (Krebs-Ringer/ EGTA/ NAME)

Viability: 88%

Rat R-2243

Race: Sprague Dawley

Sex: Male

Perfusion time with collagenase: 10 min

Collagenase concentration: 44 mg in 200 ml of perfusion buffer (Krebs-Ringer/ EGTA/ NAME)

Viability: 79%

Rat R-2250

Race: Sprague Dawley

Sex: Male

Perfusion time with collagenase: 10 min

Collagenase concentration: 44 mg in 200 ml of perfusion buffer (Krebs-Ringer/ EGTA/ NAME)

Viability: 92%

6.3.2	Hepatocyte culture

Rat isolated hepatocytes were resuspended in Ham's F-12/Lebovitz L-15 (1:1), medium supplemented with 2mM glutamine, 170μg/ml sodium selenite, 2% newborn calf serum, 50mU/ml penicillin, 50μg/ml streptomycin, 0.2% bovine serum albumin and 10nM insulin and subsequently seeded at a density of 80,000 viable cells/cm^2 on previously fibronectin-coated plastic dishes (1,2).

After resuspension, a cell aliquot is removed and viability determined by cell counting using the trypan blue exclusion method. Trypan blue uptake is the result of cell membrane alteration and in consequence of cell death. Thus, to quantify cell viability, 0.4% trypan blue in saline is added to the cells and these are immediately loaded in a Neubauer chamber. Viable cells are counted in 5 different fields under the

peroxidation and albumin and transferrin expression. To do so, rat isolated hepatocytes seeded on 96 well-plates were incubated with the compounds under the same experimental conditions than those carried out in the corresponding assay. At the end of the incubation period, medium was removed and wells washed twice with PBS. Subsequently a MTT solution was added to the wells and incubated at 37ºC during 2 additional hours. After incubation, MTT was discharged and the formazan precipitated inside the wells re-dissolved in DMSO. Blue-formazan produced by metabolically active cells was quantified by measuring the absorbance at 550 nm in an ELISA reader. Non-treated cells were used as positive controls of viability (5-7).

6.3.5 Quantification of free radical formation

Rat primary cultured hepatocytes seeded on 96 well/plates (1 compound/plate) were concomitantly preincubated for 40 min with 5-chloromethyl-2',7'-dichlorodihydrofluorescin (DCFH-DA) a fluorescence probe, and with Guava, Chamomile, Cat's claw and Papaya at 4, 20, 100 and 500 μg/ml. Owing its non-polar and non-ionic structure, DCFH-DA diffuses through the cell membrane and in the presence of reactive oxygen species is oxidized to its fluorescent form (8). The reason of preincubating cells with the compounds was to protect them from the oxidative agent (t-butyl hydroperoxide), to which they were subsequently subjected. At the end of the preincubation period, cells were exposed to t-butyl hydroperoxide (t-BH) in the presence of the compounds and immediately after fluorescence was measured at a wavelength of 485 nm (excitation) and 527 (emission). Finally, cultures were further incubated at 37ºC and fluorescence was measured every 30 min for 2 hours.
Quercetin, a well-known antioxidant was used as a positive control.
Cells incubated with t-butyl hydroperoxide in the absence of the compounds were correlated with a 100% of free radical formation.

6.3.6 Incubations to quantify lipid peroxidation and reduced glutation levels

To evaluate both parameters, rat isolated hepatocytes were seeded on 24 well/plates at a density of 80,000 viable cells/cm^2. One hour after culture stabilization, cells were preincubated for 24 hours with each compound at 4, 20, 100 and 500 μg/ml. At the end of the preincubation period, cells were exposed to 250 μM t-butyl hydroperoxide in the presence of the compounds at the concentrations mentioned above. 24 h later, conditioned media were harvested and after centrifugation at 1200 rpm for 5 min, supernatants were recovered to determine malondialdehyde (MDA) formation. Previously washed cell monolayers were immediately frozen for protein and GSH quantification.
Untreated cells or t-BH treated cells were used as controls of basal and induced oxidation, respectively.

6.3.7 Measurement of lipid peroxidation

This parameter was evaluated by measuring malondialdehyde (MDA) production in the culture medium. (5-7). To do so, conditioned medium from cells incubated as described in section 6.3.6, were collected and centrifuged at 1200 rpm for 5 min to remove cell debris. Harvested supernatants were incubated with buffer containing 7% SDS, 0.1N HCl, 1% phosphotungstic acid and 0.67% thiobarbituric acid (TBA) in a boiling bath for 60 min in the dark. Thereafter, samples were subjected to butanol extraction and after centrifugation at 3000 rpm for 10 min, the organic phase (upper) containing the MDA-TBA adduct was read in a fluorometer at 530nm (excitation wavelength) and 595 nm (emission wavelength).

MDA diluted in medium was used to prepare the standard curve.
Untreated cells or t-BH treated cells were used as controls of basal and induced oxidation, respectively.
Protein quantified from cell monolayers was employed to normalize data.

6.3.8	Determination of GSH levels

GSH quantification was based on the fluorimetric reaction of thiols with o-phthaldialdehyde (OPD) in deproteinated samples (5-7). Cells incubated for 24 hours with compound's concentration of 4, 20 and 100 µg/ml were sonicated and homogenized in a buffer containing 5% trichloroacetic acid and 2mM EDTA for 1-2 sec. 1M NaOH, 0.1M sodium phosphate buffer and an OPD solution were sequentially added to aliquots of the supernatants obtained by centrifugation of the homogenates at 3000 rpm for 30 min. Thereafter, samples were sonicated for 1-2 sec and after 30 min of incubation in the dark, the fluorescence was read at 360 nm (excitation wavelength) and 450 nm (emission wavelength). 10 mM GSH diluted in homogenization buffer was employed to prepare the standard curve.
Untreated and t-BH treated cells were correlated with basal and reduced levels of glutation.
Protein quantified from cell monolayers was employed to normalize data.

6.3.9	Incubations to evaluate albumin and transferrin production at the transcriptional level

Rat cultured hepatocytes seeded on 60 m/m dishes at the regular density (see former sections for details) were incubated with increasing concentrations of the compounds (1, 4, 20 and 100 µg/ml). After a time exposure of 48 hours, the medium was removed and the monolayers washed with PBS and subsequently kept frozen until mRNA extraction was performed.

Untreated cells correlated with basal synthesis of albumin and transferrin.
Cells treated with 25 ng/ml EGF, a known inducer of albumin synthesis (9), were used as positive control.

6.3.10	Isolation and purification of total RNA

Total RNA was extracted from the culture plates using TRIZOL, following the recommendations of the manufacturer (GibcoBRL). The cDNA was prepared by the reverse transcription of total RNA using high fidelity reverse transcriptase and the guidelines issued by the manufacturer (M-MLV reverse transcriptase, Invitrogen Life Technologies) (10,11).

6.3.11	Preparation of cDNA standards of CYP isoenzymes

Standard reference samples of each CYP cDNA were prepared by PCR amplification run to saturation (between 32 and 35 PCR cycles), using the appropriate primers. The resulting cDNAs were purified by column chromatography (High pure PCR product purification kit), and eluted with TE buffer. The samples showed a unique band in agarose gel electrophoresis (10,11).

6.3.12	Quantitative PCR

A quantitative RT-PCR assay has been developed to measure the mRNA content of the major CYPs, in human liver samples as well as in cultured human hepatocytes. The technique is highly specific, rapid and sensitive enough to quantify low abundant mRNAs. The PCR primers were selected to specifically match each CYP mRNA, to have a very close annealing temperature, and to render PCR products of similar sizes. The PCR conditions were designed to facilitate the simultaneous measurement of the various human liver CYPs in a single run. The measured CYP mRNA content was normalised against the ß-actin mRNA content of the samples. Each RT-sample was twice measured in the quantitative PCR (10,11).

7 STORAGE of SAMPLES and DATA

The compounds have been stored in Advancell, until the devolution of the leftovers to DR. JOSÉ G CABANILLAS CORAL at the end of the project.

Samples generated in Advancell:

- Will be stored in our facilities for 3 years. ☐
- Are sent to the client, because accepts their devolution. ☐

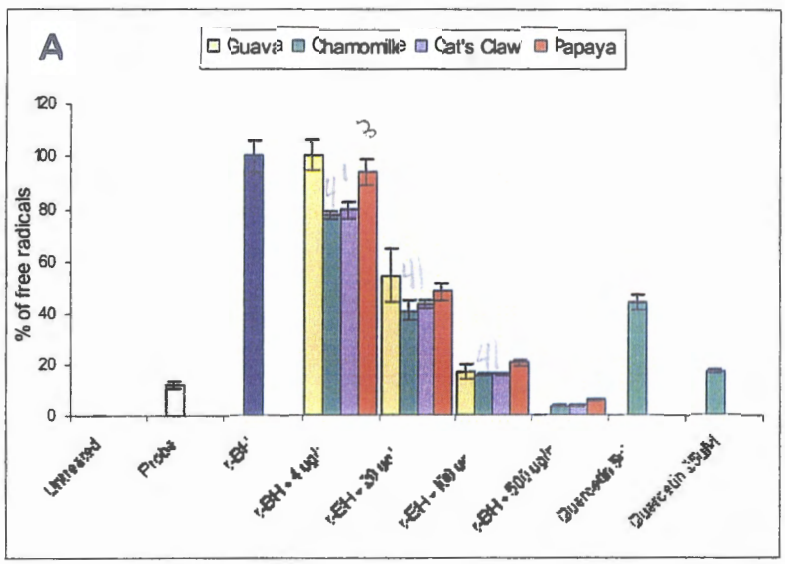
Data obtained during the study will be stored at ADVANCELL for 3 years and ADVANCELL will provide the sponsor with the final report, a CD copy with all the information (data, graphics, images...).

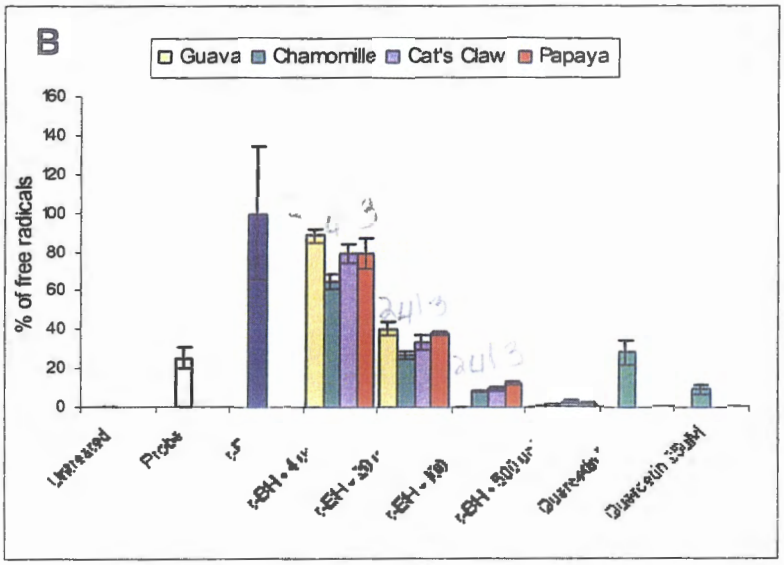
8 RESULTS and DISCUSSION

8.1 Free radical formation

Figure 2 shows the results of quantifying free radical formation in rat hepatocytes obtained from two different hepatocyte isolations (rat 2222 Panel A and rat 2224 Panel B). Both panels demonstrate that free radical production was markedly reduced when the oxidant agent t-BH was concomitantly incubated with the tested compounds. This protective effect occurs in a dose dependent manner and is comparable to that shown by Quercetin, a well-known antioxidant. Although only concentrations of 100 and 500 µg/ml totally blocked the oxidant capacity of t-BH, the concentration of 20 µg/ml already diminished t-BH oxidative effect by 40-50%. A viability test was not performed in parallel with this assay since incubations are performed for a short period of time, a total of 2 h between the preincubation and the incubation period (see section 6.3.5 for details).

Figure 2. Quantification of the oxidative stress in rat hepatocytes treated with potential antioxidants.
Two independent cultures of rat hepatocytes (rat R-222 Panel A and rat R-2224 Panel B) were concomitantly preincubated for 40 min with the fluorescent probe, 5-chloromethyl-2' 7'-dichlorodihydrofluorescin (DCFH-DA) and with Guava, Chamomille, Cat's Claw and Papaya at 4, 20 100 and 500 µg/ml. At the end of the preincubation period, cells received an oxidative stimulus (250 µM t-butyl hydroperoxide) in the presence of the compounds. Fluorescence was read immediately after as well as 60 min later at 485 nm (excitation) and 527 nm (emission).
Non-treated cells were used to quantify basal oxidation
Cells treated with t-BH in the absence of the compounds were correlated with a100% of free radical formation

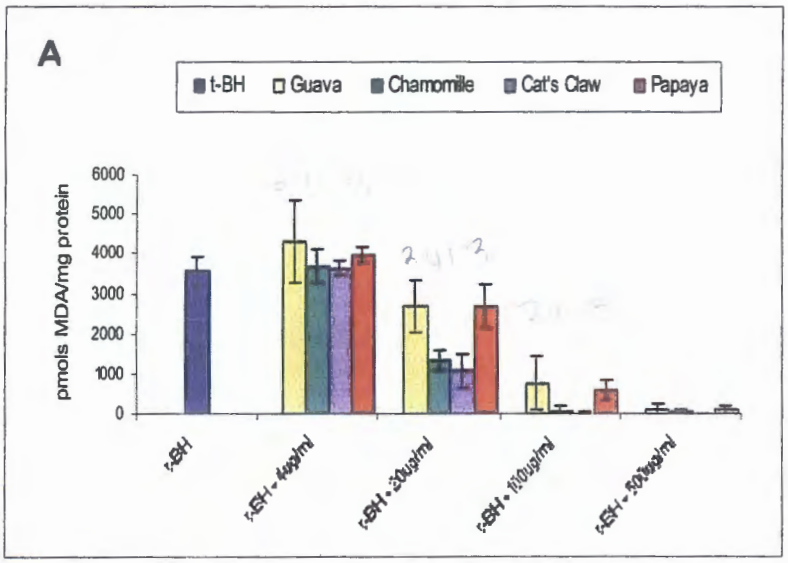


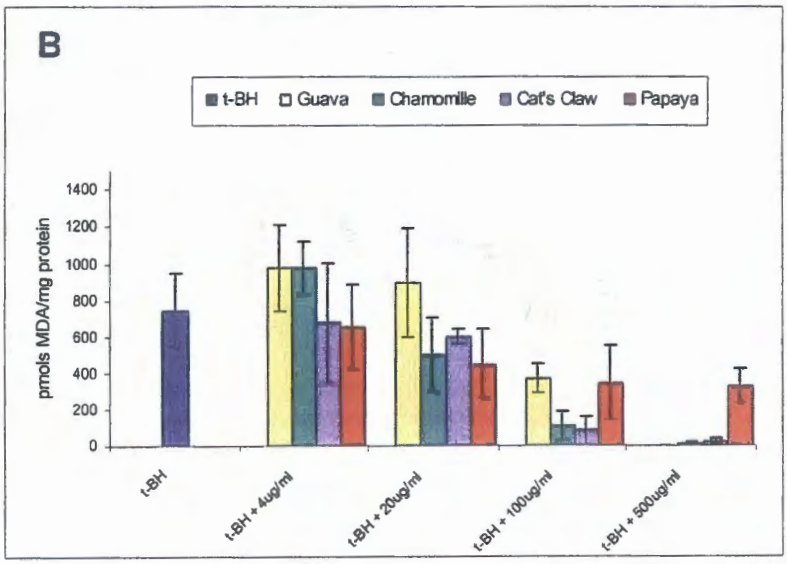


8.2	Measurement of lipid peroxidation by malondialdehyde (MDA) quantification

Data obtained in this section correlated with the results obtained in the previous section (8.1). Thus, lipid peroxidation quantified as MDA production decreased when t-BH was incubated with increasing concentrations of the compounds (Figure 3). As in the previous assay, any compound showed a better antioxidant capacity than the others, since MDA production decreased in two independent cultures (Panel A and B) in a quite comparable manner. These data corroborate the antioxidant properties of tested compounds in these cells.

Figure 3. Measurement of lipid peroxidation in rat hepatocytes exposed to different natural extracts.
Rat hepatocytes from two independent cultures (rat R-2222 Panel A and rat R-2224 Panel B), were preincubated with Guava, Chamomille, Cat's Claw and Papaya at 4, 20, 100 and 500 μg/ml. At the end of the preincubation period, cells received an oxidative stimulus (250 μM t-butyl hydroperoxide) in the presence of the compounds. Quantification of the MDA release to the medium was determined by its capacity to bind to TBA to generate a fluorescent compound.





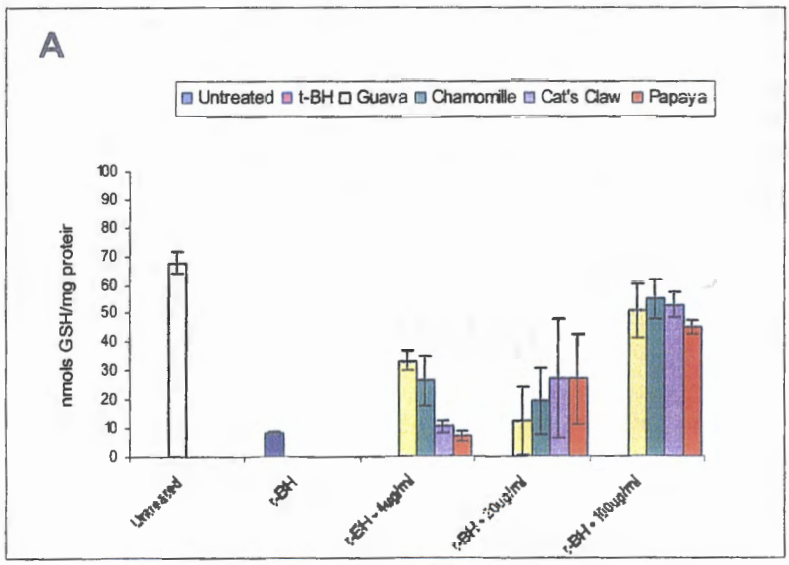
8.3 Determination of GSH

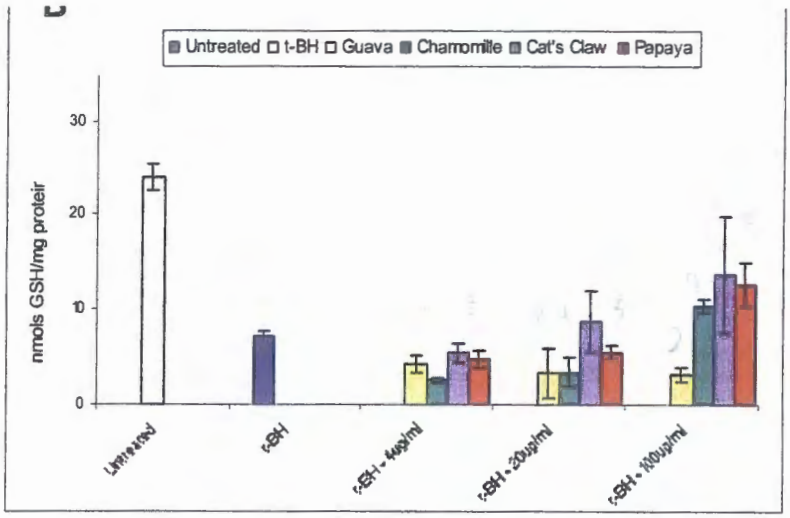
Figure 4 shows the effects of Guava, Chamomille, Cat's Claw and Papaya on the GSH depletion caused by t-BH in 2 independent cultures of rat hepatocytes (rat R-2222 (Panel A) and rat R-2223 (Panel B)). Data indicated a tendency to maintain basal GSH levels in cells incubated with increasing concentrations of the compounds. Hepatocytes incubated with a concentration of 100 μg/ml reached the closer levels to untreated cells. In contrast, cells exposed to 500 μg/ml decrease their GSH content probably due to the cytotoxicity caused by Guava and Papaya at this concentration (see figure 5). In the case of Chamomille and Cat's Claw, although cytotoxicity was not apparent at this concentration a decrease of the GSH levels could be an early event of a cytotoxic process.

Figure 4. Determination of GSH levels in rat hepatocytes exposed to different compounds

Two independent cultures of rat hepatocytes (rat R-2222 Panel A and rat R-2223 Panel B) were preincubated with Guava, Chamomile, Cat's Claw and Papaya at 4. 20 and 100 μg/ml. At the end of the preincubation period. cells received an oxidative stimulus (250 μM t-butyl hydroperoxide) in the presence of the compounds and the GSH content was quantified in all the different experimental conditions.

Non-treated or t-BH treated cells were used as controls of basal and induced GSH levels, respectively.

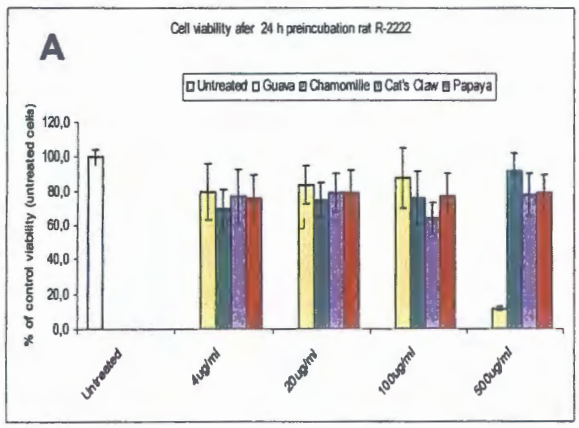


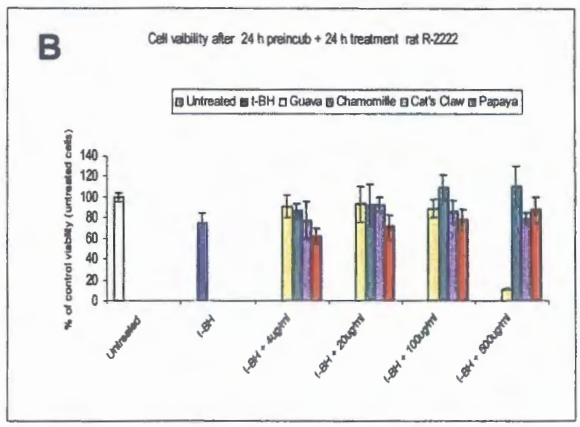


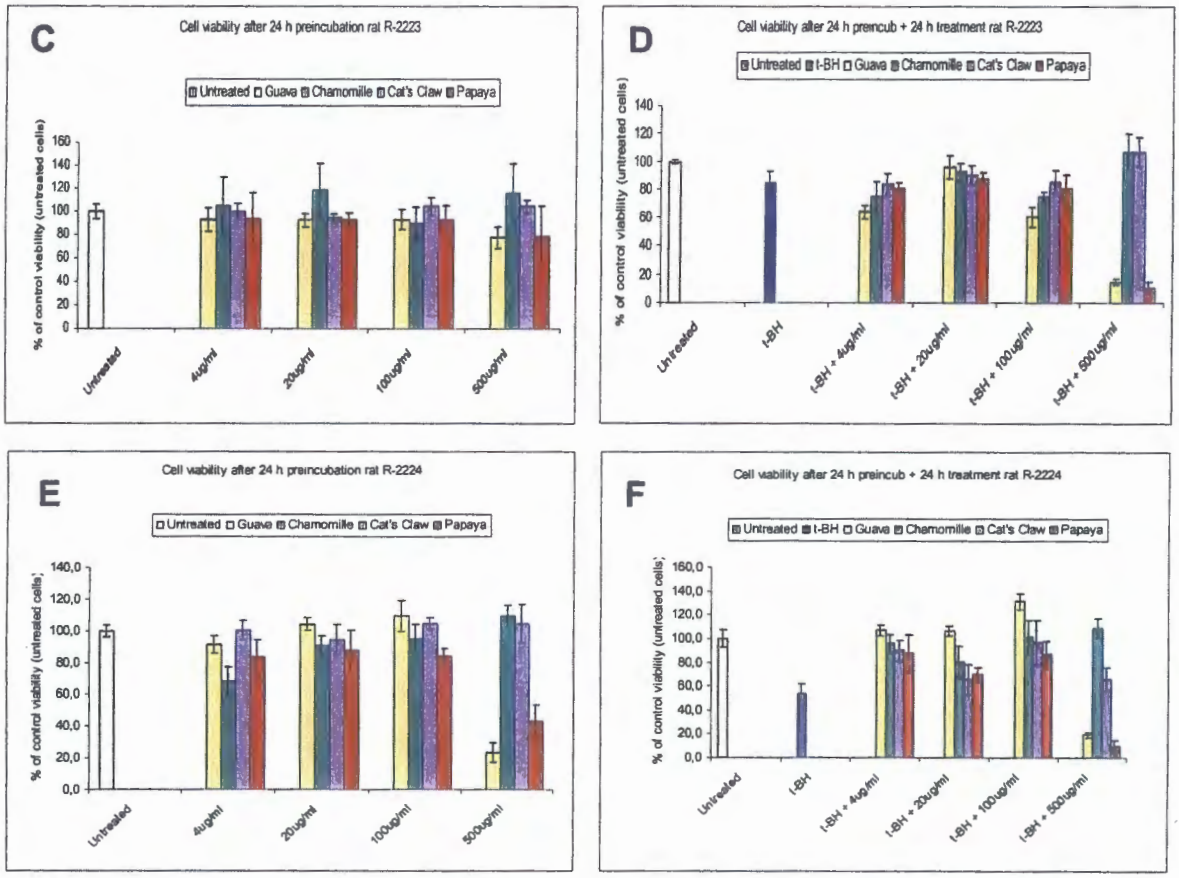
8.4 Viability studies for lipid peroxidation and glutation assays

Viability tests were carried out in parallel with the assays performed for measuring lipid peroxidation and glutation levels under the same experimental conditions. In all tested cultures, Guava decreased cell viability in 80% when incubated at 500 μg/ml. Under the same conditions, Papaya only resulted cytotoxic in 2 out of 3 cultures. No apparent toxicity was observed at any tested concentration for the other two compounds (Chamomille and Cat's Claw).

Figure 5. Compound's cytotoxicity in rat primary cultured hepatocytes
Three independent cultures of rat hepatocytes (rat R-222, Panels A and B: rat R-2223. Panels C and D and rat R-2224 Panels E and F) were preincubated for 24 h with Guava, Chamomille, Cat's Claw and Papaya at 4, 20, 100 and 500 μg/ml At the end of the preincubation period, cell viability was tested by means of the MTT test. In the same way, viability was also quantified at the end of each assay (24 h preincubation + 24 h t-BH treatment under all the experimental conditions) by using the same test.
Non-treated cells were used as positive controls of viability







8.5 Effect of compounds on albumin and transferrin expression level

Hepatocytes in culture maintain their capacity to synthesize and secrete plasmatic proteins (α_1-antitrypsin, α_2-macroglobulin, α_1-antichymotrypsin, fibronectin, fibrinogen, albumin, etc) into the medium (1-3). This rate of synthesis can be altered by several physiological processes as hepatic regeneration (12), inflammation (13) or even by the action of several drugs (14,15). With the purpose of studying whether these compounds could alter plasmatic protein synthesis, albumin and transferrin production was determined at the transcriptional level in two independent cultures of rat hepatocytes (R-2243 and R-2250). Cells were exposed to increasing concentrations of Guava, Chamomile, Cat's Claw and Papaya (1, 4, 20 and 100 μg/ml) for 48 hours. At the end of this period samples were subjected to total RNA isolation and subsequently to PCR quantification (see methodology for details).
Despite that high variability is due to the low number of tested rats (standard deviation is the mean of 4 samples of 2 independent cultures), hepatocytes incubated with Guava, Chamomile and Cat's Claw at a concentration of 4 μg/ml tend to increase albumin and transferrin mRNA levels above basal expression. Hepatocytes treated with Chamomile also increase albumin mRNA at 4μg/ml, whereas transferrin expression is only above control cells when Chamomile concentration reaches a value of 100 μg/ml.

Papaya treated cells although do not respond to low concentrations of the compound (1, 4 and 20 μg/ml), it results in an increase of the expression levels of both proteins at the higher concentration tested in this assay (100 μg/ml).

Figure 6. Effect of 4 natural extracts (Guava, Chamomile, Cat's Claw and Papaya) on the expression levels of albumin.
Two independent cultures of rat hepatocytes (R-2243 and R-2250) were incubated for a 48-hour period in the presence of 4 natural extracts: Guava (Panel A), Chamomile (Panel B), Cat's Claw (Panel C) and Papaya (Panel D) at concentrations of 1 4, 20 and 100 μg/ml. At the end of this period, samples were subjected to total RNA isolation and subsequently to PCR quantification of albumin mRNA. Samples were also measured for ß-actin expression to normalize data.
Untreated cells were used as positive control of basal synthesis of albumin
Cells treated with 25 ng/ml EGF were employed as control of albumin induction

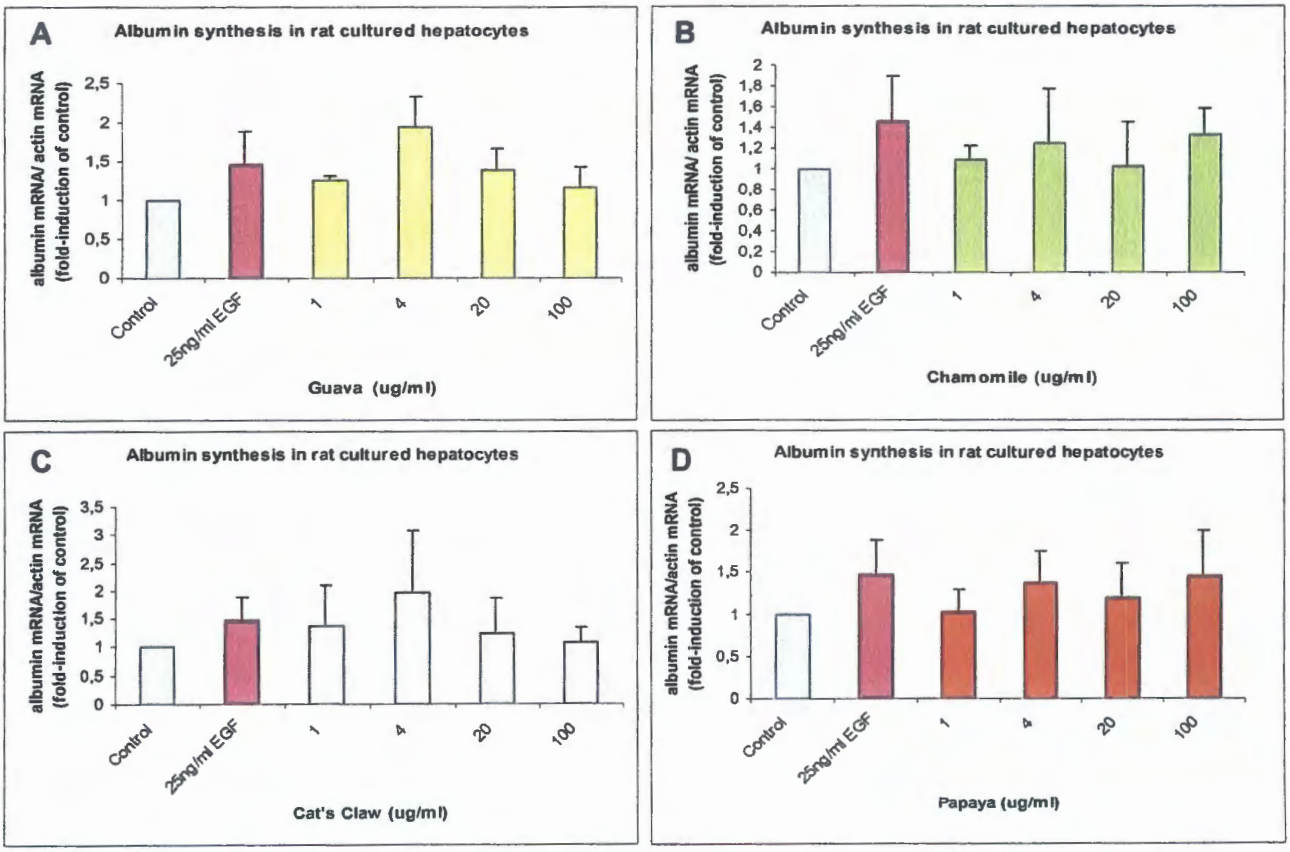
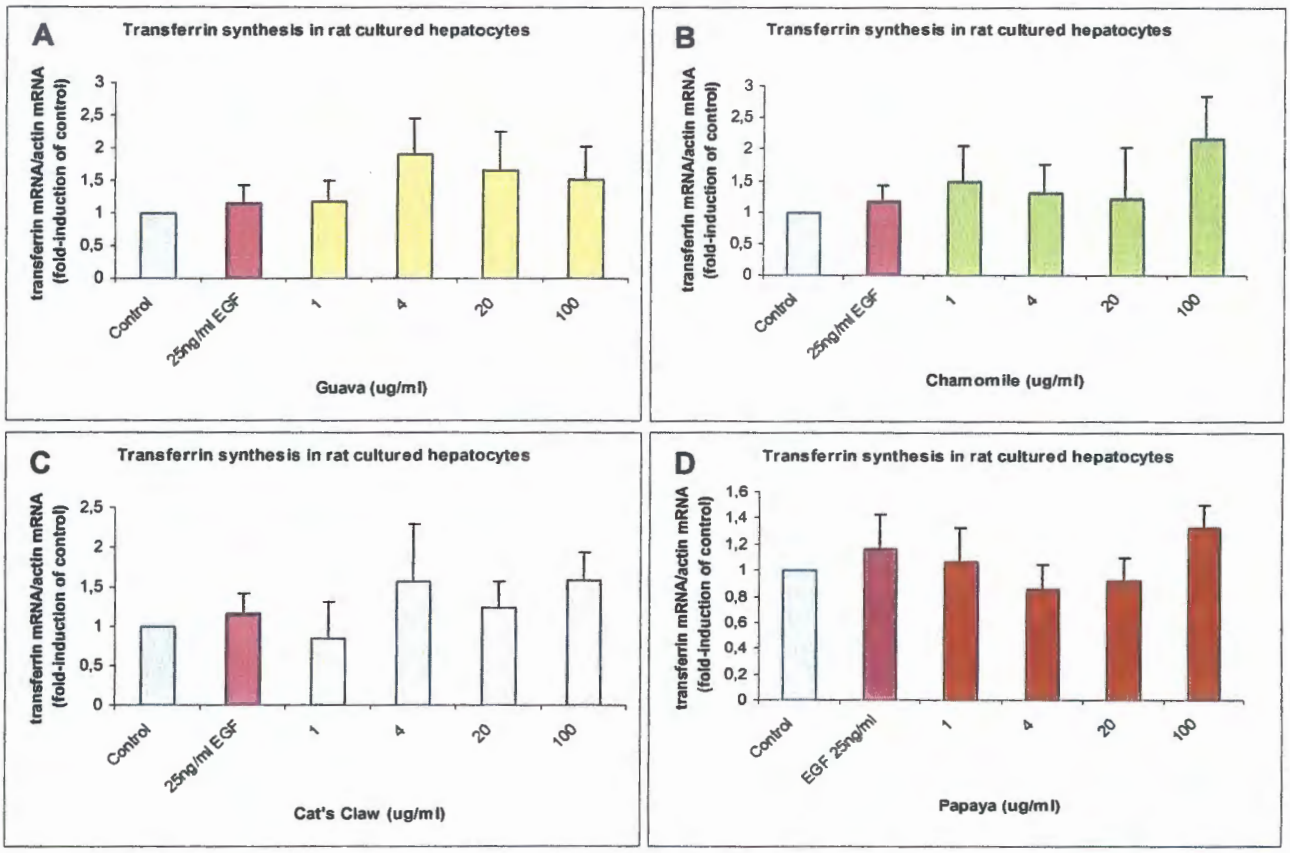


Figure 7. Effect of 4 natural extracts (Guava, Chamomile, Cat's Claw and Papaya) on the expression levels of transferrin.
Two independent cultures of rat hepatocytes (R-2243 and R-2250) were incubated for a 48-hour period in the presence of 4 natural extracts: Guava (Panel A), Chamomile (Panel B), Cat's Claw (Panel C) and Papaya (Panel D) at concentrations of 1, 4, 20 and 100 μg/ml. At the end of this period, samples were subjected to total RNA isolation and subsequently to PCR quantification of transferrin mRNA. Samples were also measured for ß-actin expression to normalize data.
Untreated cells were used as positive control of basal synthesis of transferrin.
Cells treated with 25 ng/ml EGF were also assayed to quantify whether or not EGF (a known albumin inducer) alters transferrin expression in rat hepatocytes.



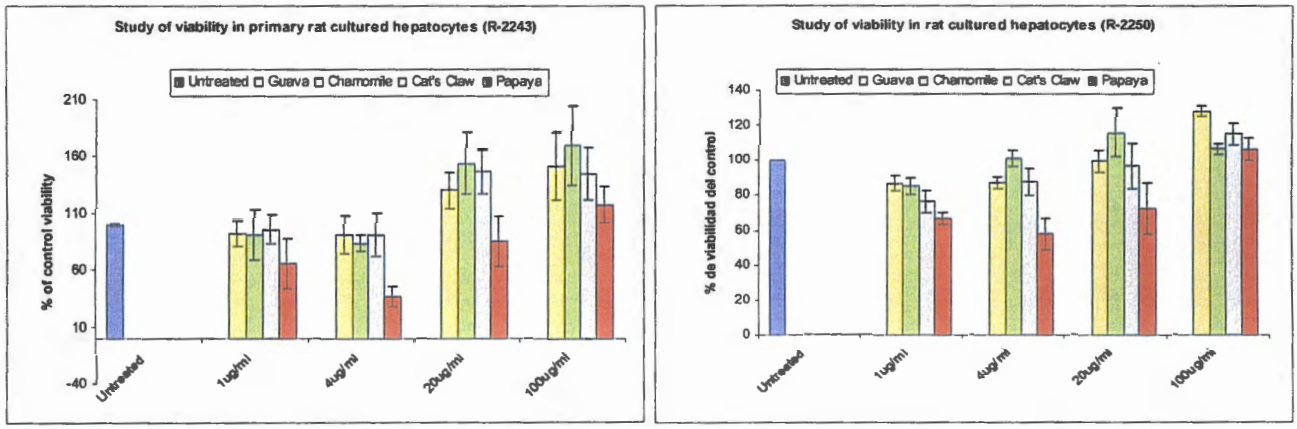
8.6 Viability studies for albumin and transferrin expression assays

Rat hepatocytes obtained from two different isolations were exposed to Guava, Chamomile and Cat's Claw for a 48-hour period at the concentrations detailed in Figure 8 (Panels A and B). As shown in this figure, cells incubated with Guava, Chamomile and Cat's Claw did not decrease cell viability at any tested concentration in any culture. Even more, concentrations of 20 and 100 μg/ml increase cell viability 30 and 50% of control, respectively. In contrast, cells incubated with Papaya at 1, 4 and 20 μg/ml diminished cell viability approximately 40% in both cultures. Only, when Papaya was administered at 100 μg/ml, cell viability was comparable to that obtained for untreated cells.

These data are in agreement with those obtained at the transcriptional level (see section 8.5). Unlike the other 3 compounds, Papaya is only able to increase albumin and transferrin mRNA at 100 μg/ml.

But how to compare these results with those found in a former experiment of viability (Figure 5) where compounds if cytotoxic have only effect at high concentration (500 μg/ml)?
A possible explanation is that although both experiments were performed for the same period of time (48 hours), they were not carried out under identical experimental conditions. Whereas, in the former assay, compounds were added to medium containing 2% serum, in the rare one, the assay was performed in serum free medium to avoid collateral effects. While in the first experiment we observed how the viability of cells decrease with increasing concentrations of some of these compounds, in the second assay we appreciated how cells exposed to compound concentrations below the MNTC can favor hepatocyte viability when these cells grow in serum free medium. Since primary hepatocytes do not divide in culture, it is unexpected an increase in the number of cells by the effect of these compounds. In contrast, it is quite possible that hepatocytes in the absence of serum decrease their viability and only the hepatocytes that grow in the presence of high concentrations (20 and 100 μg/ml) of Guava, Chamomile and Cat's Claw tend to maintain 100% of cell viability.

Figure 8. Compound's cytotoxicity in rat primary cultured hepatocytes
Two independent cultures of rat hepatocytes (rat R-2243, Panel A and rat R-2250, Panel B) were incubated for 48 h with Guava, Chamomille, Cat's Claw and Papaya at 1, 4, 20 and 100 μg/ml. At the end of the incubation period, cell viability was tested by means of the MTT test.
Non-treated cells were correlated with 100% of viability



9 CONCLUSIONS

» The compounds Guava and Papaya resulted cytotoxic in 2 out of 3 cultures of rat hepatocytes when they were added at a concentration of 500 μg/ml in medium containing 2% serum. In contrast, the other two compounds (Chamomille and Cat's Claw) did not affect hepatocyte viability at any tested concentration when incubated for the same period of time (48 hours).

» Rat hepatocytes incubated in the absence of serum decrease their viability with time in culture. However, cells incubated in the presence of increasing concentrations (20 and 100 μg/ml) of Guava, Chamomile and Cat's Claw helped to maintain culture viability in a mean percentage of 20 and 40%, respectively.

» All compounds showed antioxidant capacities since they were able to block free radical formation and in consequence lipid peroxidation. In addition, hepatocytes incubated with a concentration of the compounds of 100 μg/ml maintained GSH levels closer to GSH basal levels. The antioxidant properties of these compounds can exert in the liver a hepatoprotector role.

» Guava, Chamomile and Cat's Claw at 4 μg/ml tend to increase albumin synthesis at the transcriptional level in rat cultured hepatocytes. However, higher concentrations (20 and 100 μg/ml) of these compounds decrease albumin expression at the basal level. This behavior is also applicable to transferrin expression in cells exposed to Guava and Cat's Claw whereas hepatocytes incubated with Chamomile only increased transferrin mRNA at 100 μg/ml. Papaya was the single compound that induced both, albumin and transferrin expression, at the highest tested concentration (100 μg/ml).

10 REFERENCES

1. MJ Gómez-Lechón, MT Donato, JV Castell. Use of Cultured Hepatocytes to Investigate Drug Metabolism and Toxicity. In Vitro Toxicology 10:63, 1997

2. MJ Gómez-Lechón, X Ponsoda, R Bort, JV Castelll. The Use of Cultured Hepatocytes to Investigate the Metabolism of Drugs and Mechanisms of Drug Hepatotoxicity. ATLA 29:225, 2001

3. M.J.Gómez-Lechón and J.V. Castell. Primary culture of human hepatocytes. In: Cell and Tissue Culture: Laboratory Procedures. Ed. J.B. Griffiths, A. Doyle and D.G. Newell. ISBN: 0471928526. John Wiley & Sons Ltd. Baffins Lane, England, 1998; 12B: 15.1-15.7

4. MN Berry, DS Friend. High yield preparation of isolated rat liver parenchymal cells. J. Cell.Biol.43:506-520, 1969

5. M.J.Gómez-Lechón and J.V. Castell. In Vitro Toxicity Testing. In: Cell and Tissue Culture: Laboratory Procedures. Ed. J.B. Griffiths, A. Doyle and D.G. Newell. ISBN: 0471928526. John Wiley & Sons Ltd. Baffins Lane, England, 1998; 12B: 5.6

6. JV Castell, MJ Gómez-Lechón. *In vitro* alternatives to animal pharmaco-toxicology. Farmaindustria Ed. JV Castell and MJ Gómez-Lechón. Madrid, 1992.

7. M. J. Gómez-Lechón, T. Donato, X. Ponsoda, R. Fabra, R. Trullenque and J. V. Castell. Isolation, culture and use of human hepatocytes in drug research. IN VITRO METHODS IN PHARMACEUTICAL RESEARCH. ISBN 0-12-163390-X. Eds. J. V. Castell y M. J. Gómez-Lechón eds. pp. 129-154. Academic Press. London (1997)

8. Lautraite S, Bigot-Lasserre D, Bars R and Carmichael N. Optimisation of cell-based assays for medium thoughput screening of oxidative stress. Toxicol in vitro 17:207-220 (2003).

9. Miyazaki M, Akiyama I, Sakaguchi M, Nakasshima F, Okada M, Katoka K and Huh NH. Improved conditions to induce hepatocytes from rat bone marrow cells in culture. Biochem Biophys Res Commun 298:24-30, 2002

10. C Rodríguez-Antona, R Jover, MJ Gómez-Lechón, JV Castell. Quantitative RT-PCR Measurements of Human Cytochrome P-450s: Application to Drug Induction Studies. *Archives Biochem Biophys* **376**:109, 2000

11. C Rodríguez-Antona, MT Donato, E Pareja, MJ Gómez-Lechón, JV Castell. Cytochrome P450 mRNA Expression in Human Liver and Its Relationship with Enzyme Activity. *Archives Biochem Biophys* **393**:308, 2001

12. Wang J, Clark JB, Rhee GS, Fair JH, Reid LM and Gerber DA. Proliferation and hepatic differentiation of adult-derived progenitor cells. *Cells Tissues Organs* 173:193-203 (2003).

13. Kang YH, Berthiaume F and Yarmush ML. Long-term stable cultures of rat hepatocytes: an *in vitro* model to study acute and chronic hepatic inflammation. *Tissue Eng* 8:681-693 (2002).

14. Isozaki M, Ito K, Masubuchi Y and Horie T. Plasma retinol binding protein for monitoring the acetaminophen-induced hepatotoxicity. *Drug Metab Pharmacokinet* 17:540-545 (2002).

15. Ito K, Iwatsubo T, Kanamitsu S, Ueda K, Suzuki H and Sugiyama Y (1998). Prediction of pharmacokinetic alterations caused by drug-drug interactions: metabolic interactions in the liver. *Pharmacol Rev* **50**, 387-412

11.	LIST OF APPENDIXES
11.1	Appendix 1: Metabolic characterization of rat cultured hepatocytes

Rat R-2222

1. ECOD ACTIVITY

ECOD activity in rat hepatocytes (R-2222) after 24-hours in culture

19.4 pmol/min x mg cell prot

Mean value 29.8 ± 10.4 pmol/min x mg cell prot (n=46) (Historical data)

2. TESTOSTERONE METABOLISM

	Enzymatic activity (pmols/mg cell protein x min)						
R-2222	15β	7α	6β	16α	16β	2β	Androstenedione
Mean value (24-hour culture)	3.7	16.2	4.9	114.4	10.1	18.7	73.8
Rat cultured hepatocytes (n= 50)*	2.1±1.8	20.3±18.1	4.0±3.0	74.5±90.4	1.6±2.9	33.1±30.1	52.3±39.4

(*) Historical data

Rat R-2223

1. ECOD ACTIVITY

ECOD activity in rat hepatocytes (R-2223) after 24-hours in culture

24.3 pmol/min x mg cell prot

Mean value 29.8 ± 10.4 pmol/min x mg cell prot (n=46) (Historical data)

2. TESTOSTERONE METABOLISM

	Enzymatic activity (pmols/mg cell protein x min)						
R-2223	15β	7α	6β	16α	16β	2β	Androstenedione
Mean value (24-hour culture)	3.0	32.7	3.0	1.3	0.2	0.2	20.0
Rat cultured hepatocytes (n= 50)*	2.1±1.8	20.3±18.1	4.0±3.0	74.5±90.4	1.6±2.9	33.1±30.1	52.3±39.4

(*) Historical data

Rat R-2224

1. ECOD ACTIVITY

ECOD activity in rat hepatocytes (R-2224) after 24-hours in culture

20.6 pmol/min x mg cell prot

Mean value 29.8 ± 10.4 pmol/min x mg cell prot (n=46) (Historical data)

2. TESTOSTERONE METABOLISM

	Enzymatic activity (pmols/mg cell protein x min)						
R-2224	15β	7α	6β	16α	16β	2β	Androstenedione
Mean value (24-hour culture)	1.6	11.5	3.0	54.3	0.6	13.2	63.8
Rat cultured hepatocytes (n= 50)*	2.1±1.8	20.3±18.1	4.0±3.0	74.5±90.4	1.6±2.9	33.1±30.1	52.3±39.4

(*) Historical data

Rat R-2243

1. ECOD ACTIVITY

ECOD activity in rat hepatocytes (R-2243) after 24-hours in culture

Loss sample pmol/min x mg cell prot

Mean value 29.8 ± 10.4 pmol/min x mg cell prot (n=46) (Historical data)

2. TESTOSTERONE METABOLISM

	Enzymatic activity (pmols/mg cell protein x min)						
R-2243	15β	7α	6β	16α	16β	2β	Androstenedione
Mean value (24-hour culture)	0.6	21.5	1.7	32.7	0	1.8	13.9
Rat cultured hepatocytes (n= 50)*	2.1±1.8	20.3±18.1	4.0±3.0	74.5±90.4	1.6±2.9	33.1±30.1	52.3±39.4

(*) Historical data

Rat R-2250

1. ECOD ACTIVITY

ECOD activity in rat hepatocytes (R-2250) after 24-hours in culture

36.3 pmol/min x mg cell prot

Mean value 29.8 ± 10.4 pmol/min x mg cell prot (n=46) (Historical data)

2. TESTOSTERONE METABOLISM

	Enzymatic activity (pmols/mg cell protein x min)						
R-2250	15β	7α	6β	16α	16β	2β	Androstenedione
Mean value (24-hour culture)	0.7	8.3	3.9	9.3	0	3.6	26.5
Rat cultured hepatocytes (n= 50)*	2.1±1.8	20.3±18.1	4.0±3.0	74.5±90.4	1.6±2.9	33.1±30.1	52.3±39.4

(*) Historical data

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