



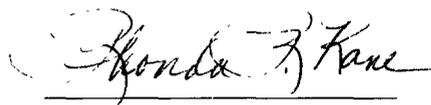
Memorandum

Date: December 16, 2002
From: Consumer Safety Officer, Division of Standards and Labeling Regulations, Office of Nutritional Products, Labeling and Dietary Supplements, HFS-821
Subject: 75-Day Premarket Notification of New Dietary Ingredients
To: Dockets Management Branch, HFA-305

9062 02 DEC 16 P 3:39

Subject of the Notification: Gold Root Extract (GRE)
Firm: PHYTOS, Inc.
Date Received by FDA: February 12, 2002
90-Day Date: May 13, 2002

In accordance with the requirements of section 413(a) of the Federal Food, Drug, and Cosmetic Act, the attached 75-day premarket notification and related correspondence for the aforementioned substance should be placed on public display in docket number 95S-0316 as soon possible since it is past the 90-day date. Thank you for your assistance.


Rhonda R. Kane, M.S., R.D.

Attachments

95S-0316

RPT118

PHYTOS



Rhonda Kane
Consumer Safety Officer
Division of Standards and Labeling Regulations
Office of Nutritional Products, Labeling, and Dietary Supplements (HFS-820)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
HFS 821
5100 Paint Branch Parkway
Room 4D0008
College Park, MD 20740

Date: December 13, 2002

From: Philip E. Wolfson MD

To: Office of Nutritional Products, Labeling, and Dietary Supplements

Subject: **Your redaction of Pre-market Notification for a New Dietary Ingredient-
Gold Root--*Heliopsis longipes* Both of our submissions**

Dear Ms. Kane:

**As per our telephone conversations, and regarding both of our submissions,
there is no material that cannot be disclosed.**

Sincerely your,

A handwritten signature in black ink that reads "Philip E. Wolfson MD". The signature is written in a cursive style and includes a small "uw" monogram at the end.

Philip E. Wolfson, MD
President & CEO



APR 30 2002

Philip Wolfson, MD
President and CEO
PHYTOS
6 Crest Road
San Anselmo, California 94960

Dear Dr. Wolfson:

This is to inform you that the notification, dated February 7, 2002, you submitted pursuant to 21 U.S.C. 350b(a)(2) was received and filed by this office of the Food and Drug Administration (FDA) on February 12, 2002. The notification concerns a dried extract of the substance "*Heliopsis longipes*" S. F. Blake (Asteraceae) or "Gold Root Extract (GRE)," that you assert is a new dietary ingredient. You intend to market the new ingredient as a lozenge or chewing gum containing 5 mg to 50 mg GRE per piece.

The term "dietary supplement" is defined in 21 U.S.C. 321(ff). 21 U.S.C. 321(ff) provides that the term means a product (other than tobacco) intended to supplement the diet that bears or contains a vitamin, a mineral, an herb or other botanical, an amino acid, a dietary substance for use by man to supplement the diet by increasing the total dietary intake, or a concentrate, metabolite, constituent, extract, or combination of any of the above ingredients. 21 U.S.C. 321(ff) further states that dietary supplements are intended for ingestion in a form described in 21 U.S.C. 350(c)(1)(B)(i) or in compliance with 21 U.S.C. 350(c)(1)(B)(ii), are not represented as conventional food or as a sole item of a meal or the diet, and are labeled as a dietary supplement

Based on the information in your notification, GRE does not appear to meet the statutory definition of a dietary supplement contained in 21 U.S.C. 321(ff) because it is not "intended for ingestion." Therefore, GRE cannot be marketed as a dietary supplement. We explain the basis for FDA's determination below.

Your notification states that the gum or lozenge containing GRE "generates a characteristic acerbic or lemon-like taste, with a pleasant, tingling sensation accompanied by salivation." In addition, you state that: "salivation is an important element in oral hygiene as saliva tends to wash the mouth of food and contaminants; promotes a balanced ecology of the oral cavity including the gums and teeth; and salivation refreshes the mouth." You further state that "GRE's stimulation of salivation makes our products useful and beneficial for oral and dental hygiene, alleviating or ameliorating the negative sensations and perception of dry mouth." References included in the notification describe GRE's oral local anesthetic effects and the traditional use of *Heliopsis longipes* for relief of toothache pain by local application of the

root to the affected tooth and gum. Your notification also refers to, but does not substantiate with written documentation, pilot clinical studies conducted by Phytos that demonstrate GRE impregnated gum's effect to produce "consistently large increase in salivation". Other references describe the antimicrobial and insecticidal properties of GRE.

An article that is delivered orally, but that exerts its effect prior to being swallowed (for example, a gum or lozenge that stimulates salivation) is not "intended for ingestion." As stated above, the definition of dietary supplement in 21 U.S.C. 321(ff) states that a dietary supplement is a product "intended for ingestion." The term "ingestion" has been addressed by the court in United States v. Ten Cartons, Ener-B Nasal Gel, 888 F. Supp. 381, 393-94 (E.D.N.Y.), aff'd, 72 F.3d 285 (2d Cir. 1995), which states:

The ordinary and plain meaning of the term "ingestion" means to take into the stomach and gastrointestinal tract by means of enteral administration. See Stedman's Medical Dictionary (4th Lawyer's Ed. 1976) (defining ingestion as the "introduction of food and drink into the stomach."); Webster's Third New International Dictionary (1976) (defining ingestion as "the taking of material (as food) into the digestive system.")....

The interpretation of the term "ingestion" to mean enteral administration into the stomach and gastrointestinal tract is also supported by the language of the statutory sections immediately preceding and following section 350(c)(1)(B)(ii). Section 350(c)(1)(B)(i) states that the vitamin must be intended for ingestion in tablet, capsule or liquid form. Each of these forms denotes a method of ingestion that involves swallowing into the stomach. Section 350(c)(2) states that a food is intended for ingestion in liquid form under section 350(c)(1)(B)(i) "only if it is formulated in a fluid carrier and is intended for ingestion in daily quantities measured in drops or similar small units of measure." This elaboration of "liquid form" also denotes ingestion by swallowing the fluid.

Therefore, a product like yours that does not appear to be a dietary supplement under the Act and that is represented to affect the structure or function of the body of man appears to be a drug within the meaning of 21 U.S.C. 321(g)(1)(C). As such, it is subject to regulation under the drug provisions of the Act. If you wish GRE to be evaluated as a drug, you should contact FDA's Center for Drug Evaluation and Research, Office of Compliance, HFD-310, 7520 Standish Place, Rockville, Maryland 20855.

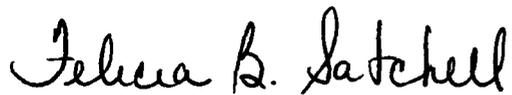
For the reasons discussed above, the Agency concludes that *Heliopsis longipes* gum and lozenge preparations do not meet the definition of a dietary supplement. Introduction of such products into interstate commerce is prohibited under 21 U.S.C. 331(a) and (v). In addition, because the Agency concluded that the subject of your notification cannot be marketed as a dietary supplement, FDA did not review the evidence of safety information you submitted on *Heliopsis longipes* extract gum or lozenges.

Page 3 – Dr. Phillip Wolfson

Your notification will be kept confidential for 90 days from the date of its receipt. After May 13, 2002, your notification will be placed on public display at FDA's Dockets Management Branch in docket number 95S-0316. However, any trade secret or otherwise confidential commercial information in the notification will not be disclosed to the public. You may wish to identify in writing specifically what information you believe is proprietary by contacting this office before May 13, 2002. Nevertheless, our Center's Freedom of Information Officer has the authority to make the final decision about what information in the notification should be redacted before it is posted at Dockets.

Should you have any questions concerning this matter, please contact me at (301) 436-2371.

Sincerely yours,



Felicia B. Satchell

Director

Division of Standards

and Labeling Regulations

Office of Nutritional Products, Labeling

and Dietary Supplements

Center for Food Safety

and Applied Nutrition

PHYTOS



Division of Standards and Labelling Regulations
Office of Nutritional Products, Labelling, and Dietary Supplements (HFS-820)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
200 C St. SW.
Washington, DC 20204

Date: February 7, 2002

From: Philip E. Wolfson MD

To: Office of Nutritional Products, Labelling, and Dietary Supplements

Subject: Premarket Notification for a New Dietary Ingredient-*Heliopsis longipes*

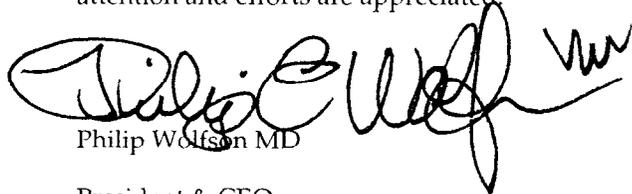
Pursuant to Section 413(a)(2) of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. 350b (a)(2)), Phytos wishes to inform the Food and Drug Administration of our intention to market a new dietary ingredient; a dry extract powder of the root of the Mexican plant *Heliopsis longipes* (Gold Root). Accordingly, three copies of this notification are submitted for your reference.

Also attached please find the supporting materials for safety and pertinent product information. They included the:

- NLEA Proximate analysis of the extract.
- Summary of toxicity studies
- Photocopies of the references cited and translations where appropriate.

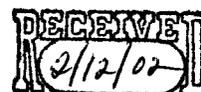
Based on the information submitted, we anticipate that FDA will agree with PHYTOS that the new dietary ingredient of the root of *Heliopsis longipes* can reasonably be expected to be safe under the recommended conditions of use.

Please do not hesitate to contact me if you have any further questions concerning this matter. Your attention and efforts are appreciated.



Philip Wolfson MD

President & CEO



PHYTOS

**Name/Address**

Dr. Philip Wolfson MD
President & CEO
PHYTOS
6 Crest Road, San Anselmo, CA 94960, 415-339-9026, Fax 415-339-9031

Notifying party: Philip Wolfson MD, President & CEO - Phytos

Name of New Ingredient

Dry extract of Gold Root - *Heliopsis longipes* S.F.Blake (Asteraceae)

Description of dietary supplement that contains the new dietary ingredient:

When a Gold Root Extract (GRE) containing product is placed in the mouth, it generates a characteristic acerbic, or lemon like taste, with a pleasant, tingling sensation accompanied by salivation. GRE will be a component of a lozenge, or chewing gums. The gum will be of a composition sufficient for chewing. Suitable gums include gum arabic, chicle gum, guar gum, or gum base as used in current commercial chewing gum and lozenges.

Salivation is an important element in oral hygiene (refs. are provided in the addendum), as saliva tends to wash the mouth of food, and contaminants; promotes a balanced ecology of the oral cavity including the gums and teeth; and salivation refreshes the mouth. GRE's stimulation of salivation makes our products useful and beneficial for oral and dental hygiene, alleviating or ameliorating the negative sensations and perception of dry mouth.

Level of the new dietary ingredient

The amount of GRE is from 5 mg to 50 mg per piece (dose), depending on the specific product, with current preparations containing between 25 and 60 mg.

Conditions of use

One lozenge or chewing gum piece every 2 hours or as needed.

History of use

Heliopsis longipes S.F.Blake (Asteraceae) is a herbaceous plant found in a remote region of Guanajuato State, Mexico. The roots of *H. longipes* have been used primarily as a spice or flavoring and for stimulation of salivation. The roots were also chewed to relieve toothache, based on the tingling sensation producing a localized anesthesia. Extracts from the roots of *H. longipes* have been used historically for the treatment of colds and pneumonia, and as an alcohol extract used as an anesthetic for tooth extraction. In vitro studies have demonstrated that an extract of these roots possesses antimicrobial properties.

Constituents of the Plant

Heliopsis longipes roots are known to contain a bioactive alkamide (an isobutylamide), affinin, identified as *N*-isobutyl-2*E*, 6*Z*, 8*E*-decatrienamide or *N*-isobutyldeca-*trans*-2,*cis*-6,-*trans*-8-trienamide. Isobutylamides are found in other plants such as Echinacea and are generally considered to be safe for human use.

An NLEA proximate analysis of GRE is provided in the appended materials.

PHYTOS



Safety

To ensure safety PHYTOS has ensured that extraction and standardization meet both GMP and GLP standards.

Standardization

Phytos has prepared a GC/MS standard for the affinin constituent of the plant. This is being used to standardize the quantity of GRE from batch to batch of raw material, thus providing for quality control in the production of our products. Phytos has prepared a chromatographic fingerprint of the plant, thus protecting against adulteration in the QC of our products.

Extraction

Clean dry roots are macerated in a circulating alcohol/water solution. After 2 days time the residual solid is filtered off and the liquid phase is spray dried using maltodextrin as the carrier. Quality control and GMP procedures are applied at all stages of the extraction process.

Toxicology

In the literature appended, there is some toxicological information about *H. longipes* and affinin. In addition, Phytos has conducted its own safety and efficacy studies.

Animal Studies

Phytos has provided its own independent laboratory assessment of GRE including mutagenicity, lethality and 28 day, 2 dosage rodent feeding trial. Reports are provided in the documentation enclosed. No morbidity or mortality occurred, i.e. there were no negative findings.

Human Studies

Phytos has conducted its own open label, and double-blind pilot clinical studies, the latter with IRB approval from the University of the Pacific School of Dentistry, and in collaboration with Bastyr University's IRB. These pre-publication studies have demonstrated that GRE impregnated gum, causes a consistently large increase in salivation as compared with active placebos. No adverse effects were reported or observed.

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Reference List



Extract analysis report: Nutritional Labeling Education Act Abbreviated Nutrient Package (Proximate)

Toxicology reports: a) Bacterial Reverse Mutation Screen
b) Acute Oral Toxicity Study in Rats
c) GRE 28 day study

Martinez M, 1959 Plant Utiles De La Flora Mexicana Ediciones Botas
English translation of same.

Martinez M, 1990 Las Plantas Medicinales De Mexico Ediciones Botas
English translation of same.

Romero et.al
Estudios preliminares de los efectos antibacterianos, insecticidas y toxicologicos da la Raiz Del Chilcuan (*Heliopsis longipes*). Veterinaria 1989 20:151-156
English translation of same.

Ogura M, et.al.
Ethnopharmacologic studies. I. Rapid solution to a problem--oral use of *Heliopsis longipes*--by means of a multidisciplinary approach. J Ethnopharmacol. 1982 Mar;5(2):215-9.

Gutierrez-Lugo M.T. et.al.
Antimicrobial and cytotoxic activities of some crude drug extracts from Mexican Medicinal Plants. Phytomedicine 1996 2 (4): 341-347

Molina-Torres J, et.al.
Antimicrobial properties of alkamides present in flavouring plants traditionally used in Mesoamerica: affinin and capsaicin. J Ethnopharmacol. 1999 Mar;64(3):241-8.

Acknowledgment of receipt of *Heliopsis longipes* voucher specimen by the California Academy of Sciences



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Extract analysis report:

Nutritional Labeling Education Act Abbreviated Nutrient Package (Proximate)



Food, Beverages, and Dietary Supplements
Contract Laboratory Services

Committed to Uncompromising Science and Customer Support

January 30, 2002

David Hoffmann, F.N.I.M.H.
Chief Herbalist
PHYTOS
325 Turney Street
Sausalito CA 94965

ALPHA Job # 12495

Sample Identification:

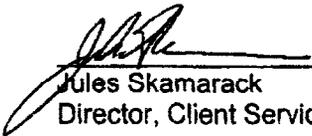
Alpha Sample #: 02-0316 Gold Root Dry Extract Heliospis Lougipes, Powder
Date Received: 01-18-02, Time Received: 01:00 PM

ALPHA Method:

PB100: NLEA Abbreviated Nutrient Package (Proximate)

Results:

ALPHA Sample # 02-0316	
Proximate	(per 100 grams)
Moisture, g	3.11
Ash, g	4.26
Protein, g	6.54
Fat, g	1.39
Carbohydrates (Total), g	84.7
Others	
Calories	378
Calories from fat	13


Jules Skamarack
Director, Client Services



PHYTOS



Toxicology reports: a) Bacterial Reverse Mutation Screen

b) Acute Oral Toxicity Study in Rats

c) GRE 28 day study



PROPRIETARY PROTECTION

**COMPOUND: BACTERIAL REVERSE MUTATION SCREEN:
PLATE INCORPORATION METHOD FOR LIQUIDS/SOLIDS**

CLIENT STUDY NUMBER: 2002-C

· PROJECT NUMBER: 01-10-001

Next Century Incorporated
Delaware Technology Park
3 Innovation Way, Suite 220
Newark DE 19711

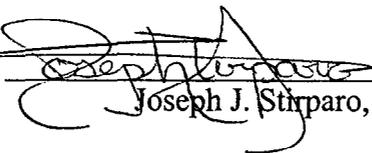
CERTIFICATION

We, the undersigned, declare that this report presents an accurate evaluation of all data obtained from this study.

This study was not conducted under Good Laboratory Practices. Relevant guidelines and GLPs were used as a reference for the conduction of the screening assay to determine the proper methodology and technique for the bacterial reverse mutation test. Furthermore, several sections required for regulatory submission (e.g., Quality Assurance Statement, GLP Compliance Statement, etc.) have been omitted.

Signature/Approval

Technical Personnel:  11/19/01
Kristen Schiavone Date

Study Director:  11/23/01
Joseph J. Stirparo, B.S. Date

PROPRIETARY PROTECTION

Study Designation

Bacterial Reverse Mutation Assay in *Salmonella typhimurium*: Plate
Incorporation and/or Preincubation Method for Liquids/Solids

Project Number: 01-10-001
Client Study Number: 2002-C
Test Code: 413
Author: Joseph J. Stirparo, B.S.

Test Guidelines

The assay was neither designed nor conducted in compliance with Good
Laboratory Practices.

Study Initiation/Completion Dates

Experimental Start Date: 10-12-01
Experimental Termination Date: 10-18-01

Compound/Test Article Identification

Name(s)/Identification(s): Gold Root Extract
CAS Registry Number (if applicable): N/A
Strength: N/A
Composition: N/A
Purity: N/A
Known Impurities: N/A
Physical Characteristics: Light Brown Powder

Testing Facility

Next Century Incorporated
Delaware Technology Park
3 Innovation Way, Suite 220
Newark DE 19711 ~ U.S.A. for Phytos Incorporated
6 Crest Road
San Anselmo, CA 94960
(hereinafter Sponsor)

Study Personnel

Study Director: Joseph J. Stirparo, B.S.
Management: Li Jie Fu, Ph.D.
Primary Technician: Kristen Schiavone
Quality Assurance Auditor: Debbie A. Vick



PROCEDURAL SUMMARY

The objective of the study was to evaluate the test article/substance, Gold Root Extract, for the ability to induce mutations, and thereby determine the mutagenic potential of the test article/substance, either in the presence or absence of exogenous metabolic activation systems (e.g., mammalian microsomal enzymes) at the histidine locus in the genome of two strains of *Salmonella typhimurium*.

The test article/substance, Gold Root Extract, was evaluated in a bacterial reverse mutation screen employing *Salmonella typhimurium* strains TA98 and TA100 both in the presence and absence of an exogenous metabolic activation system. The test article/substance was evaluated using the plate incorporation assay. Molten agar (45 to 48°C) was utilized as the medium for transference of the test article/substance, metabolic activation system (or appropriate sham control), and the bacterial culture to the agar plate. *Salmonella typhimurium* tester strains were obtained from Bruce N. Ames Ph.D. through the Children's Hospital of Oakland Research Institute, Oakland CA.

The solvent, diluent and negative control used in this assay was Distilled Water. Test article/substance concentrations of 5, 10, 50, 100, 500, 1000, 2500, and 5,000 were evaluated with concurrent positive and negative (solvent) controls. Duplicate treatments at the aforementioned concentrations were performed. All test article/substance concentration levels were assessed with respect to negative (solvent) controls in order to determine test article/substance mutagenicity and/or toxicity. Phenotypes for sensitivity to UV light, histidine growth dependence, resistance to ampicillin and sensitivity to tetracycline were determined for each strain.

Accepted scientific procedures were used in the preparation of solutions/suspensions, and dilutions of the test article. Demonstration of the uniformity, concentration, or stability of the test article in solvents or diluents was not conducted. In the absence of visible evidence to the contrary, solutions or mixtures were believed to be uniform, stable, and have the calculated concentration at the time of the assay performance. Further assessment was considered unnecessary to achieve the objectives of the study.

Only trials meeting the following criteria of acceptability are presented in this report. The mean revertants per plate in the presence or absence of exogenous metabolic activation systems was within the following ranges: *Salmonella typhimurium* strain TA98: 5-40; TA100: 60-190. Mean positive control values represented at least a three-fold increase over the respective mean of the concurrent negative (solvent) control value for each tester strain and condition. Bacterial titers were approximately 1×10^9 . A minimum of five analyzable concentration levels was required for test article classification. A plate was to be rejected if contamination, test article precipitation, or conditions exist that prevented an accurate counting of revertant colonies.

Data was evaluated using scientific judgment taking into account both statistical and biological significance. The guidelines below were used in the classification of the test article/substance:

A test article was classified as POSITIVE (i.e., mutagenic) if the average number of revertants in any strain at any test article concentration was at least two times greater than the average number of revertants in the concurrent vehicle control and there was a concentration-related increase in the mean revertants per plate in that same strain.

A test article was classified as NEGATIVE (i.e., nonmutagenic) if there were no test article concentrations with an average number of revertants that was at least two times greater than the average number of revertants in the concurrent vehicle control and there was no positive concentration-related increase in the mean revertants in that same strain. In consultation with the Sponsor, negative results may be confirmed as needed.

Results not meeting criteria for positive or negative classification were evaluated and reported as EQUIVOCAL.

RESULTS AND DISCUSSION

The test article/substance Gold Root Extract was evaluated in a bacterial reverse mutation screen employing *Salmonella typhimurium* strains TA98 and TA100 in the presence and absence of exogenous metabolic activation (Aroclor[®] induced rat liver S9). The test article/substance was evaluated using the plate incorporation assay. Distilled water was selected as the test article/substance solvent, diluent, and negative control.

Tester strains demonstrated appropriate phenotypic characteristics (Table 3). The mean numbers of revertants observed in the negative control were within the acceptable range as outlined by historical and published control data for each strain used in this study. Mean positive control values, documented as the number of revertant colonies per plate, demonstrated greater than a three-fold increase over the means of their respective negative controls. Concentrations of 5, 10, 50, 100, 500, 1000, 2500, and 5,000 µg/plate of test article/substance were assessed in reference to negative controls. Each test article/substance concentration, negative and positive control was plated in duplicate both in the presence and absence of exogenous metabolic activation.

No evidence of substance-related precipitate was observed. Test substance related toxicity, as evidenced by the reduction of the microcolony background lawn and/or as a concentration related reduction in the mean number of revertants per plate, was observed in all tester strains (Tables 1 and 2). No test article/substance concentration resulted in a mean number of revertants that were four times greater than the mean of the concurrent controls in this assay (Tables 1 and 2).

RECORDS RETENTION

The following records will be returned to the Sponsor or maintained at Next Century Incorporated as per the Sponsor's request: raw data, the final report, the procedure, and any amendments. Next Century archives a verified, copy of any original documents returned to the Sponsor

CONCLUSION

Under the conditions of this study, no evidence of mutagenic activity was detected in *Salmonella typhimurium* strains TA98 and TA100. Based on the findings outlined, the test article/substance – Gold Root Extract – is concluded to be negative for the induction of mutagenicity in the bacterial reverse mutation screen.

TABLE 1: OBSERVATIONS IN *Salmonella typhimurium* TA98

Concentration ($\mu\text{g}/\text{plate}$)	Revertants per Plate		Mean	SD	Score Codes
	1	2			
WITHOUT EXOGENOUS METABOLIC ACTIVATION					
0	20	30	25.0	7.07	TO, PO
5	34	27	30.5	4.95	TO, PO
10	25	22	23.5	2.12	TO, PO
50	11	21	16.0	7.07	TO, PO
100	19	18	18.5	0.71	TO, PO
500	26	23	24.5	2.12	TO, PO
1000	18	31	24.5	9.19	TO, PO
2500	24	35	29.5	7.78	TO, PO
5000	32	28	30.0	2.83	TO, PO
2-NF (25 μg)	$\sim 2932^1$	$\sim 1908^1$	~ 2420.0	724.08	TO, PO
WITH EXOGENOUS METABOLIC ACTIVATION					
0	27	33	30.0	4.24	TO, PO
5	19	51	35.0	22.63	TO, PO
10	15	33	24.0	12.73	TO, PO
50	30	29	29.5	0.71	TO, PO
100	21	33	27.0	8.48	TO, PO
500	28	36	32.0	5.66	TO, PO
1000	16	40	28.0	16.97	TO, PO
2500	21	22	21.5	0.71	TO, PO
5000	29	27	28.0	1.41	TO, PO
2AA (2.5 μg)	$\sim 3792^1$	$\sim 3064^1$	~ 3428.0	514.77	TO, PO

¹ $\frac{1}{4}$ of plate counted and multiplied by four to obtain colony counts

TABLE 2:OBSERVATIONS IN *Salmonella typhimurium* TA100

Concentration ($\mu\text{g}/\text{plate}$)	Revertants per Plate		Mean	SD	Score Codes
	1	2			
WITHOUT EXOGENOUS METABOLIC ACTIVATION					
0	106	104	105.0	1.41	TO, PO
5	76	102	89.0	18.38	TO, PO
10	106	88	97.0	12.73	TO, PO
50	132	100	116.0	22.63	TO, PO
100	92	111	101.5	13.44	TO, PO
500	98	112	105.0	9.90	TO, PO
1000	98	99	98.5	0.71	TO, PO
2500	118	93	105.5	17.68	TO, PO
5000	112	139	125.5	19.09	TO, PO
NAAZ (2 μg)	~3,172 ¹	~3,184 ¹	~3178.0	8.485	TO, PO
WITH EXOGENOUS METABOLIC ACTIVATION					
0	104	188	146.0	59.40	TO, PO
5	95	174	134.5	55.86	TO, PO
10	119	158	138.5	27.58	TO, PO
50	124	148	136.0	16.97	TO, PO
100	122	171	146.5	34.65	TO, PO
500	154	182	168.0	19.80	TO, PO
1000	192	189	190.5	2.12	TO, PO
2500	173	167	170.0	4.24	TO, PO
5000	146	180	163.0	24.04	TO, PO
2AA (2.5 μg)	~2,036 ¹	~3,208 ¹	~2622.0 ¹	828.73	TO, PO

¹ ¼ of plate counted and multiplied by four to obtain colony counts

TABLE 3: PHENOTYPE VERIFICATION OF TESTER STRAINS

STRAIN	DEPENDENT GROWTH		GROWTH CHARACTERISTICS			
	D-Bio	D-Bio/His /Trp	Crystal Violet	UV Light	Ampicillin	Tetracycline (optional)
TA98	-	+	S	-	R	S
TA100	-	+	S	-	R	S

S = Strain is sensitive; R = Strain is resistant; + = Growth; - = No Growth

N/A = Not assessed (or NA)

NOTE: *Salmonella* strains require histidine for growth.

STRAIN GENOTYPES/PHENOTYPES

Strain	Gene Locus	Excision Repair	LPS	R-factor (pKM101)	pAQ1 Plasmid
<i>S. typhimurium</i> TA98	<i>hisD3052</i>	Δ <i>uvrB</i> *	<i>rfa</i>	Present	Absent
<i>S. typhimurium</i> TA100	<i>hisG46</i>	Δ <i>uvrB</i> *	<i>rfa</i>	Present	Absent

LPS = lipopolysaccharide; Δ = deletion; *rfa* = deep rough mutation

REFERENCES

- Ames, B.N., McCann, J. and Yamasaki, E. (1975) Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-micromosome mutagenicity test. *Mutation Research* 31: 347-364.
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- Gatehouse, D., Haworth, S., Cebula, T., Gocke, E., Kier, L., Matsushima, T., Melcion, C., Nohmi, T., Venitt, S. and Zeiger, E. (1994) Recommendation for the performance of bacterial mutation assays. *Mutation Research* 312: 217-233.
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APPENDIX A

Abbreviations Used in Tables

Presence of Test Article Precipitate

Formation of a precipitate by the test article will be documented using the following key:

- P0 **No precipitate**, no precipitate observed.
- P1 **Microscopic precipitate**, precipitate present, which does not interfere with background lawn evaluation or automated colony counting.
- P2 **Non-interfering precipitate**, precipitate present that is visible to the naked eye that does not interfere with automated colony counting.
- P3 **Interfering precipitate**, precipitate present that requires plate to be counted by hand.
- P4 **Heavy interfering precipitate**, precipitate present that prevents accurate colony counting and obscures the background lawn requiring plate rejection (R).

Appearance of the Background Lawn

Evidence for test article toxicity to the bacteria will be documented by recording the appearance of the background lawn using the following key:

- T0 **Normal**, background microcolony lawn appears normal.
- T1 **Slightly reduced**, background microcolony lawn is noticeably thinner.
- T2 **Moderately reduced**, background lawn is markedly thinner resulting in an increase in the size of microcolonies compared to the vehicle control plate(s).
- T3 **Severely reduced**, background lawn is distinguished by an extreme thinning resulting in an increase in the size of the microcolonies compared to the vehicle control plate(s). Microcolonies may be seen readily by the unaided eye and are greatly enlarged relative to controls.
- T4 **Absent**, plate(s) are distinguished by a complete lack of any microcolony lawn over a majority of the area of the plate(s).

Positive Controls

STRAIN	POSITIVE CONTROLS (in ABSENCE of metabolic activation system)
<i>S. typhimurium</i> strain TA98	25 µg 2-Nitrofluorene (2NF) [CAS No.: 607-57-8]
<i>S. typhimurium</i> strain TA100	2 µg Sodium azide (NAAZ) [CAS No.: 26628-22-8]
	(in PRESENCE of metabolic activation system)
<i>S. typhimurium</i> strain TA98	2.5 µg 2-Aminoanthracene (2AA) [CAS No.: 613-13-8]
<i>S. typhimurium</i> strain TA100	2.5 µg 2-Aminoanthracene; (2AA) [CAS No.: 613-13-8]

Additional Observations

Additional observations relevant to the appearance of the plates will be noted in the study records including:

N - Absence of any noteworthy observation SD – Standard Deviation



product safety labs

2394 Route 130 • Dayton, New Jersey 08810 • www.productsafetylabs.com

732-254-9200 • 800-425-0002 • FAX-732-254-6736

Thursday, November 01, 2001

Philip Wolfson
PHYTOS INC.
6 Crest Rd
San Anselmo, CA 94960

Dear Dr. Wolfson:

Enclosed please find the following FINAL REPORT for Gold Root Extract:

Study #11325 - P203 Acute Oral Toxicity Study in Rats

Invoice will be sent under separate cover.

Thank you for your confidence in Product Safety Labs. Please contact Daniel J. Merkel, our Study Director, for comments and/or questions.

Sincerely,

A handwritten signature in cursive script that reads 'Tammy Paleopanidis'.

Tammy Paleopanidis

Enc.



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732-254-9200 • 800-425-0002 • FAX-732-254-6736

ACUTE ORAL TOXICITY STUDY IN RATS - LIMIT TEST

TEST METHOD NO.: P203

STUDY NUMBER: 11325

SPONSOR: PHYTOS, INC.
6 Crest Road,
San Anselmo, California 94960

TEST SUBSTANCE IDENTIFICATION: Gold Root Extract

TEST SUBSTANCE DESCRIPTION: Yellow powder

DATE RECEIVED: September 19, 2001

PSL REFERENCE NO.: 010919-8D

DATES OF TEST: September 26 - October 10, 2001

NOTEBOOK NO.: 01-47: pages 208-213

1. PURPOSE

To provide information on health hazards likely to arise from a short-term exposure to Gold Root Extract by the oral route.

2. PROCEDURE

A group of Sprague-Dawley derived, albino rats was received from Ace Animals, Inc., Boyertown, PA. The animals were singly housed in suspended stainless steel caging with mesh floors. Litter paper was placed beneath the cages and was changed at least three times per week. The animal room was temperature controlled and had a 12-hour light/dark cycle. The animals were fed Purina Rodent Chow #5012 and filtered tap water was supplied *ad libitum* by an automatic watering system.

Following acclimation to the laboratory, a group of animals was fasted for approximately 19 hours by removing feed from their cages. After the fasting period, ten rats (five male and five female) were selected for test based on health and initial bodyweights. Individual doses were calculated based on these bodyweights, taking into account the specific gravity (determined by PSL) of the test substance. The test substance was administered as a 45% w/w suspension in distilled water. Each animal received 5,000 mg/kg of the test substance by intubation using a stainless steel ball-tipped gavage needle attached to an appropriate syringe. After administration, each animal was returned to its designated cage. Feed was replaced approximately 4 hours after dosing.

The animals were observed for mortality, signs of gross toxicity and behavioral changes at approximately one hour post dosing and at least once daily for 14 days. Bodyweights were recorded prior to initiation and at termination. All animals were euthanized by CO₂ inhalation at termination.

3. RESULTS

Individual bodyweights and doses are presented in Table 1. Cage-side observations are presented in Table 2.

Apart from one female that exhibited an abnormal gait 2 hours after administration, all animals survived, gained weight and appeared active and healthy. There were no other signs of gross toxicity, adverse pharmacologic effects or abnormal behavior.

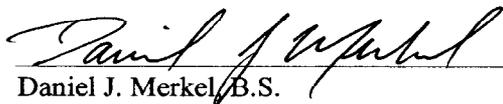
4. CONCLUSION

Under the conditions of this study, the single dose acute oral LD₅₀ of Gold Root Extract is greater than 5,000 mg/kg of bodyweight in male and female rats.

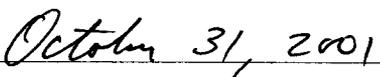
SIGNATURES

Gold Root Extract

We the undersigned declare that the methods, results and data contained in this report faithfully reflect the procedures used and raw data collected during the study.



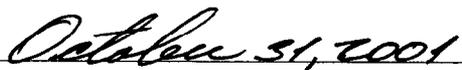
Daniel J. Merkel, B.S.
Study Director



Date



Frank Fielder, B.S.
Quality Assurance Supervisor



Date

TABLE 1: INDIVIDUAL BODYWEIGHTS, DOSES AND MORTALITY

Animal No.	Sex	Bodyweight (g)		Dose ¹
		Initial	Day 14	ml
6349	M	233	361	2.3
6350	M	232	347	2.3
6351	M	239	355	2.3
6352	M	252	369	2.5
6353	M	246	374	2.4
6354	F	175	236	1.7
6355	F	171	239	1.7
6356	F	179	244	1.7
6357	F	178	250	1.7
6358	F	186	249	1.8

¹ Administered as a 45% w/w suspension in distilled water. Specific Gravity - 1.139 g/ml.

TABLE 2: INDIVIDUAL CAGE-SIDE OBSERVATIONS

<u>Animal Number</u>	<u>Findings</u>	<u>Day of Occurrence</u>
<u>MALES</u>		
6349 - 6353	Active and healthy	0-14
<u>FEMALES</u>		
6354 - 6357	Active and healthy	0-14
6358	Abnormal gait Active and healthy	0 (2 hrs) 0 (1 hr), 0(3 hrs)-14

TABLE 3: INDIVIDUAL NECROPSY OBSERVATIONS

<u>Animal Number</u>	<u>Tissue</u>	<u>Findings</u>
<u>MALES</u>		
6349-6353	All tissues/organs	No gross abnormalities
<u>FEMALES</u>		
6354-6358	All tissues/organs	No gross abnormalities



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January 28, 2002

Dr. Philip Wolfson
PHYTOS INC.
6 Crest Rd
San Anselmo, CA 94960

Dear Dr. Wolfson:

At the request of Dr. Ted Farber, I am writing to provide you with a preliminary description of the results of the 28 day study on Gold Root Extract. The study was conducted with three test groups (low, mid and high dose level) and 1 negative control group. The test compound was incorporated into the diets of the animals assigned to the test groups at levels of 130, 1,300 and 13,000 ppm.

As you know, the "in-life" portion of the study ended last week. The amount of information we have at this time is limited. What I can tell you is that animals from all test groups survived, gained weight and appeared active and healthy during the 28 day study. Gross necropsy findings at terminal sacrifice were unremarkable. Evaluation of the following parameters are still pending; food consumption, bodyweight gain, organ to bodyweight ratios, clinical chemistry, hematology and histopathology. Once we have compiled this data, we will be provide you with a final report describing our findings in more detail.

Please feel free to contact me if you have any questions or need additional information.

Sincerely,
PRODUCT SAFETY LABS


Gary Wnorowski
Laboratory Director

PHYTOS



Martinez M, 1959 Plant Utiles De La Flora Mexicana
English translation of same.

Ediciones Botas

ROBERT BYE

PROF. MAXIMINO MARTINEZ

Useful Plants of the Mexican Flora.

ANDRES BOTAS
EDICIONES BOTAS

1959

CHILCUAN⁴

Other common names: Chilcuague, Pelitre; Chilmecatli (in Aztec: chil, *chile*, and mecatle, *filament*, alluding to the filiform roots, spicy taste).

Botanical name: *Heliopsis longipes* (A. Gray) Blake. Composite family. For a long time, the plant was erroneously confounded with *Erigeron affinis*; precisely identified by Dr. S. F. Blake.

215 20, 22, and 24 Ichthyomethia Ichthyometia

⁴ Translator's note: translation only on the section "CHILCUAN"

[Caption]

CHILCUAN

Heliopsis longipes

A wild plant whose roots, when chewed, produce a copious flow of saliva and leave a spicy taste. Recently studied as an insecticidal plant.

Herbaceous, with semilegnious stems in its lower part and between 20 and 70 cm in height. The leaves are opposed, oval-shaped, between 2 and 4 cm in length, serrated, and with short petioles. The flower heads are largely pedunculated and yellow in color. The roots measure between 15 and 30 cm in length and 3 cm in width, frequently less. The plant almost dries out in winter, but buds vigorously during the rainy season, from July to September.

The plant has been observed from San Luis de la Paz to Xichú and in the vicinity of Hacienda de La Mesa, and in Palmillas, Vergel, Macula, Ahorcados, Charco Azul, and Santa Catarina, all located in the State of Guanajuato. Also in the South of San Luis Potosí and in the North of Querétaro. Grows at an altitude between 1,825 and 2,250 m.

The usable part is the root. Chewing a piece of about two mm numbs the tongue, produces a spicy flavor, and triggers abundant secretion of saliva. These properties exist in the cortex of the root, and not in the center of the same.

The root is commonly used as a condiment and added to beans instead of chile; it is also added to alcoholic beverages to give them a special flavor and chewed to relieve molar pain. In medicine, the alcoholic tincture can be used as a stimulant, and the extract as an anesthetic. In certain cases, it may possibly be useful against hyperchlorhydria.

Prof. Miguel Cordero analyzed Chilcuán around 1903 and obtained the following results:

Liquid Fat.....	2,042
Resinous Substances, one of them similar to pyrethrin.....	3,362
Alkaloid	[blank]

MAXIMINO MARTINEZ

217

Glucose	2,270
Early gummy stages.....	5,420
Dextrin and similar substances	1,420
Hygroscopic water.....	8,509
Mineral salts	8,530
Cellulose, wood and loss	67,991

(The alkaloid was isolated afterwards and called "affinin").

In 1945, Acree, Jacobson, and Haller conducted another analysis after isolating a main active agent, i.e. the alkaloid referred to by Cordero, and whose chemical composition is N-isobutyl-2,6,8-decatrienoamide (C¹⁴H²³NO). Other investigators found that Chilcuán exhibits insecticidal properties. Flies are killed when drinking milk mixed with the ground root. The smoke produced when burning [the root] allegedly chases away flies.

References: Francisco Hernández - De Historia Plantarum Novae Hispaniae [History of the Plants of New Spain]. Vol. III. Ed. Matritense, 1790.

Francisco Ximénez – De la yerba llamada Chilmeatl. Los Cuatro Libros de la Naturaleza [The Herb Called Chilmeatl. The Four Books of Nature], 121 (Reprint), Mexico, 1888.

Anales del Inst. Med. Nacional [Annals of the National Medical Institute]. Vol. VI, 237, Mexico, 1905.

Juan Manuel Noriega – Curso de Historia de Drogas [Course in Drug History]. 419, Mexico, 1902.

Elbert L. Little - *Heliopsis longipes*. Journ. Wash. Acad. Sci. 38. 1948 and Bol. Soc. Botánica de México [Bulletin of the Botanical Society of Mexico], Number 7, Mexico, October 1948. These and other bibliographical notes are cited in this work.

Acree, Fred, Jr., Jacobson, Martin, and Haller, H. L.- "An Amide Possessing Insecticidal Properties from the Roots of *Erigeron affinis* D.C." Journal of Organic Chemistry 10: 236-242. 1945.

Acree, Fred, Jr., Jacobson, Martin, and Haller, H. L.- "The structure of Affinin, the Insecticidal Amide from *Erigeron affinis* D.C." Journal of Organic Chemistry 10: 449-451. 1945.

Jacobson, Martin, Acree Fred Jr., and Haller H. L.- "Correction of the Source of "Affinin" (N-isobutyl-2,6,8-decatrienoamide)". Journal of Organic Chemistry 12: 731-732. 1947.

Roark R. C.- "Some Promising Insecticidal Plants". Economic Botany 1947.

Maximino Martínez – Plantas Medicinales de México (Medicinal Plants of Mexico), 1959

PROF. MAXIMINO MARTINEZ

PLANTAS UTILES DE LA FLORA MEXICANA

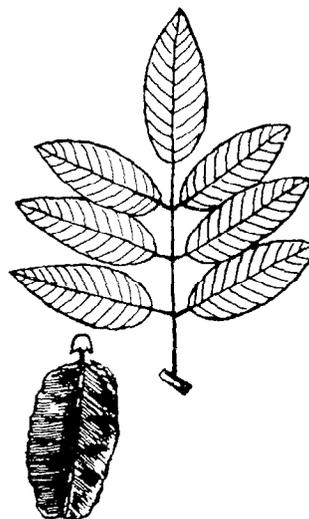


1959

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Es un árbol bien conocido de Tamaulipas y San Luis Potosí a Yucatán y Quintana Roo. Alcanza de 15 a 18 metros de altura o algo más, con tronco hasta de un metro de diámetro. Corteza agrietada, de color ceniciento. Sus hojas se componen de 7 a 9 hojuelas ásperas, oblongas o elípticas y agudas, de 4 a 12 cm. de largo. Flores de color rosado algo violáceo; fruto de 2.5 a 7.5 mm. con unas seis semillas o menos,



CHIJOL
Piscidia piscipula.

de color moreno. La madera es moreno amarillenta con peso específico de 0.87, durable y muy resistente a la humedad, llegando a petrificarse. Se usa en carpintería, construcciones, durmientes, etc. La corteza es irritante y de olor desagradable y contiene una sustancia (picidina) narcótica y analgésica, usada contra neuralgias, insomnio y tos ferina. Las hojas y la corteza, machacadas y arrojadas en el agua, atontan a los peces.

Hay otras tres especies de *Ichthyomethia* en México:

Ichthyomethia grandiflora (Don. Sm.) Blake, en Oaxaca, *I. mollis* (Rose) Blake, en Sinaloa y Sonora; *Ichthyomethia americana* Moc. et Sessé, en Michoacán y Guerrero. "Tatungo", "zatzumbo", "coquile", "co-

lorín de peces", "mata pez".

Bibliografía: Blake.—Revision of *Ichthyomethia*. Journ. Wash. Acad. Sci. IX. p. 241, 1919.

P. C. Standley. Trees and Shrubs of México. Contr. U. S. N. Herb. XIII, p. 511. Washington, 1922.

M. Martínez.—Plantas Medicinales de México. p. 428. 1959.

CHILCUAN

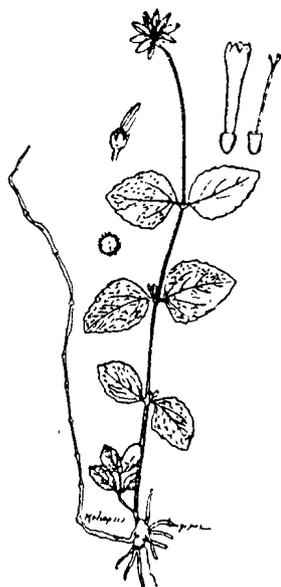
Otros nombres vulgares: Chilcuague, pebtie, chilmeatl (en lengua azteca: chil, de chil, y meatl, hilo, haciendo alusión a las raíces filiformes, de sabor picante).

Nombre botánico: *Heliopsis longipes* (A. Gray) Blake. Familia de las Compositas. Durante mucho tiempo se confundió con *Erigeron affinis*, y fué el Dr. S. F. Blake quien la identificó exactamente.

215 20, 22 y 25

Ichthyomethia

,, *Ichthyomethia*



CHILCUAN
Heliopsis longipes.

Es una planta silvestre, cuyas raíces, al masticarse, producen un copioso flujo de saliva y dejan un sabor picante. Recientemente se ha estudiado como planta insecticida.

Es herbácea, con los tallos semileñosos en su parte inferior y de 20 a 70 cm. de altura. Las hojas son opuestas, ovadas, de 2 a 4 cm. de largo, aserradas y con pecíolos cortos. Las cabezuelas son largamente pedunculadas y de color amarillo. Las raíces miden de 15 a 30 cm. de largo por 3 mm. de ancho, frecuentemente menos. La planta casi se seca en el invierno, pero brota con vigor en la estación lluviosa, de julio a septiembre.

Se ha observado de San Luis de la Paz hacia Nichú y cerca de la hacienda de La Mesa, y en Palmillas, Vergel, Macula, Ahorcados, Charco Azul y Santa Catarina, todas localidades del Estado de Guanajuato. También al sur de San Luis Potosí y al norte de Querétaro. Vive entre 1,825 y 2,250 metros de altitud.

La parte utilizable es la raíz. Cuando se mastica un pedazo de unos dos milímetros,

adormece la lengua, se percibe un sabor picante y se produce abundante secreción de saliva. Esas propiedades existen en la corteza de la raíz y no en el eje de ella.

Vulgarmente se usa como condimento, añadiéndola a los frijoles en vez de chile; también se agrega a bebidas alcohólicas para darles mayor fuerza; se usa masticarla contra el dolor de muelas. En medicina puede usarse la tintura alcohólica como estimulante y el extracto como anestésico. Posiblemente en ciertos casos sea aprovechable como sialagogo para combatir la hiperclorhidria.

El Prof. Miguel Cordero analizó el chilcuan por el año de 1903 y obtuvo estos resultados:

Grasa líquida	2.042
Substancias resinosas, una de ellas semejante a la piretrina	3.363
Alcaloide	-

Glucosa	2.720
Principios gomosos	5.120
Dextrina y análogos	1.420
Agua higroscópica	8.509
Sales minerales	8.530
Celulosa, leñosa y pérdida	67.991

(El alcaloide se ha aislado posteriormente, llamándolo afinina).

En 1945 Acree, Jacobson y Haller hicieron otro análisis, habiendo aislado un principio activo (afinina), o sea el alcaloide de que habla Cordero, y cuya composición es N-isobutyl 2,6,8, decatrienoamida (C₁₄ H₂₃ N O). Otros investigadores han encontrado en el chilcuan propiedades insecticidas. Las moscas mueren si beben la leche mezclada con la raíz molida. Se dice que el humo que produce al quemarse ahuyenta los mosquitos.

- Bibliografía:** Francisco Hernández.—De Historia Plantarum Novae Hispaniae. Vol. 111, 383. Ed. Matritense, 1790.
Francisco Jiménez.—De la yerba llamada Chilmeacatl. Cuatro Libros de la Naturaleza, 121 (Reimpresión). México, 1888.
Anales del Inst. Med. Nacional. Vol. VI, 237. México, 1905.
Juan Manuel Noriega.—Curso de Historia de Drogas. 419. México, 1902.
Elbert L. Little.—*Heliopsis longipes*. Journ. Wash. Acad. Sci. 38, 1948 y Bol. Soc. Botánica de México, número 7, México, octubre de 1948. En este trabajo se citan estas y otras notas bibliográficas:
Acree Fred, Jr., Jacobson, Martin y Haller, H. L.—“An Amide Possessing Insecticidal Properties from the Roots of *Erigeron affinis* D. C.”. Journal of Organic Chemistry 10: 236-242. 1945.
Acree Fred, Jr., Jacobson, Martin and Haller H. L.—“The structure of Affinin the insecticidal Amide from *Erigeron affinis* D. C.”. Journal of Organic Chemistry 10: 449-451. 1945.
Jacobson Martin, Acree Fred Jr. and Haller H. L.—“Correction of the Source of “Affinin” (No. isobutyl-2, 6, 8-decatrienoamide)”. Journal of Organic Chemistry 12: 731-732. 1947.
Roark R. C.—“Some Promising Insecticidal Plants”. Economic Botany 1947.
Maximino Martínez.—Plantas Medicinales de México, 1959.

CHILE

Nombres vulgares: Chile verde, chile serrano, chile morita, chilitipin, etc., según las especies y variedades. Ik (en Yucatán).

Nombre botánico: *Capsicum annuum* L., *Capsicum frutescens* L. y otros, con numerosas variedades y formas.

Es una de las plantas más importantes en el país por su uso tan generalizado como condimento desde tiempos muy remotos. A pesar de la introducción de los condimentos europeos, el chile se emplea de prefe-

PHYTOS



Martinez M, 1990 Las Plantas Medicinales De Mexico
English translation of same.

Edicionas Botas

MAXIMINO MARTINEZ

MEDICINAL PLANTS OF MEXICO

SIXTH EDITION

1990

CHILCUAN³

Other common names: Chilcuague, Pelitre or Peritre. In the Aztecan language Chilmecatl (from chili, *chile*, and mecatl, *filament*, alluding to the filiform roots and their spicy taste).

Botanical name: *Heliopsis longipes* (A. Gray) Blake. Composite family.

Places of Vegetation: San Luis de la Paz, Xichú, Palmillas, Vergel, Macula, Ahorcados, Charco Azul, and Santa Catarina, all located in the State of Guanajuato. Also in the South of San Luis Potosí and North of Querétaro.

A herbaceous, perennial plant, between 20 and 70 cm. Opposed leaves, oval-shaped, between 2 and 4 cm, serrated and with small petioles; yellow flower heads with a large peduncle. Roots 15 to 30 cm in length and 2 mm wide, with a dark brown cortex covering a ligneous and yellowish center.

Parts used: Roots. When chewed, the roots produce a spicy flavor, numb the tongue, and cause abundant secretion of saliva. These effects are obtained with the cortex of the root, not with its center.

[Caption]

CHILCUAN
Heliopsis longipes

³ Translator's note: translation of section on "CHILCUAN"

**Missing Page 11 of English translation of
*Medicinal Plants of Mexico***

Its action on other secretions, as mentioned above, and its action on all functions of the organism also remains to be studied; and finally, the main active agent(s) remain to be isolated (Dr. E. Novoa).

References: Francisco Hernández: *De Historia Plantarum Novae Hispaniae* (History of the Plants of New Spain). III. Ed. Matritense. 1790.

Francisco Ximénez. *Los Cuatro Libros de la Naturaleza* (The Four Books of Nature), p. 121. Morelia Reprint, 1888.

Anales del Instituto Nacional (Annals of the National Institute). VI. p. 237. Mexico, 1905.

Acree, Jacobson, et al. An Amide Possessing Insecticidal Properties from the Roots of *Erigeron affinis* D.C. *Journ. Organic Chemistry* X, p. 236-242. 1945. The structure of Affinin, the insecticidal amide from *Erigeron affinis* D.C. *Ibid.* 449-451; Correction of the source of affinin. *Ibid.* XII. p. 831-732 [sic]. 1947. (until 1947, Chilcuán was incorrectly identified as *Erigeron affinis*. Blake clarified that it was *Heliopsis longipes*).

Elbert E. Little. *Heliopsis longipes*. *Journ. Wash. Acad. Sci.* XXXIII. 1948; *Boletín de la Soc. Botánica de México* (Bulletin of the Botanical Society of Mexico), Number 7. October 1948. Other bibliographical notes are cited in this work.

Maximino Martínez. *Algunas Plantas Útiles de la Flora Mexicana* (Some Useful Plants of the Mexican Flora). Mexico, 1959.

M A X I M I N O M A R T I N E Z

LAS
PLANTAS MEDICINALES
DE
MEXICO

SEXTA EDICIÓN



1990

MAXIMINO MARTINEZ

113

naces con diez centigramos de 1.40 gramos de extracto hidroalcohólico, observándose en algunos casos cólicos intestinales, estado nauseoso y vómitos. Además de sus efectos purgantes se le encontraron marcados efectos diuréticos en enfermos del corazón y de los riñones a la dosis de 0.30 a 0.40 gramos de extracto hidroalcohólico en píldoras de 0.10 gramos repartidos en 24 horas. Por otra parte, ejerce acción sobre el sistema nervioso, el aparato circulatorio, locomotor y respiratorio. Hay que advertir que estos efectos de la planta sólo se verifican con el extracto hidroalcohólico o con el polvo de la raíz, la tintura es ineficaz, lo mismo que el cocimiento.

*Referencias: Datos para la Materia Médica Mexicana, 3a. parte, pág. 93. México, 1900.

CHILCUAN

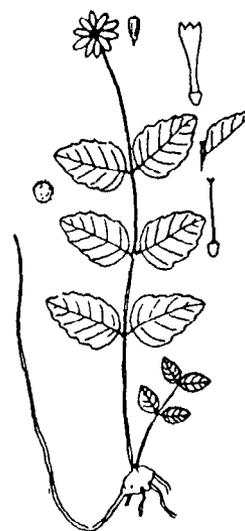
Otros nombres vulgares: Chilcuague, pelitre o peritre. En la lengua azteca chil-mecatl (de chili, chile y mecatl, hilo, aludiendo a las raíces filiformes y al sabor picante de éstas).

Nombre botánico: *Heliospis longipes* (A. Gray) Blake. Familia de las Compuestas.

Lugares de vegetación: San Luis de la Paz, Xichú, Palmillas, Vergel, Macuala, Ahorcados, Charco Azul y Santa Catarina, todos del Estado de Guanajuato. También al sur de San Luis Potosí y norte de Querétaro.

Es planta herbácea, perenne, de 20 a 70 cm. Hojas opuestas, ovadas, de 2 a 4 cm., aserradas y con pecíolos cortos; cabezuelas amarillas con largo pedúnculo. Raíces de 15 a 30 cm. de largo por unos 2 mm. de ancho, con corteza morena que cubre a un eje leñoso y amarillento.

Partes usadas: Las raíces. Al masticarse producen un sabor picante, adormecen la lengua y provocan abundante secreción de saliva. Esos efectos se obtienen con la corteza de la raíz y no con su eje.



CHILCUAN.
Heliospis longipes.

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of *Medicinal Plants of Mexico***

PHYTOS



Romero et.al

Estudios preliminares de los efectos antibacterianos, insecticidas y toxicologicos da la Raiz Del Chilcuan (*Heliopsis longipes*). *Veterinaria* 1989 20:151-156

English translation of same.

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Preliminary Studies of the Antibacterial, Insecticidal, and Toxicological Effects of Chilcuán root (*Heliopsis longipes*)

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SUMMARY

The root of Chilcuán (*Heliopsis longipes*) is used empirically as an oral anesthetic in humans and against cattle worms. In this study, the antibacterial, insecticidal, and toxicological effects of the total alcoholic extract of Chilcuán root (EAT) were investigated. The antibacterial effects of EAT were tested *in vitro* against *Escherichia coli* and *Staphylococcus aureus*; EAT exhibited a bactericidal activity on both bacteria, the first one being more resistant to small doses (0.01 mg), whereas the effect was the opposite at higher doses (1.0). The insecticidal effects were also quantitatively examined *in vitro* against *Oestrus ovis* larvae and qualitatively against *Gasterophilus app*; the stage II *Oestrus* larva had a higher incidence of survival in the culture medium and was more sensitive to EAT (1.0 mg/ml), which produced a mortality of 43% after 24 hours vs. 3% for the reference sample; under the same conditions, the stage III larva exhibited a mortality of 50% vs. 40% for the reference sample. On the other hand, EAT (400 mg) was capable of producing the detachment of the *Gasterophilus* larvae of equine gastric mucosa *in vitro*. Finally, the toxic effects were determined by calculating the intraperitoneal 50% lethal dose of EAT in rats, which was 566 mg/kg. These results, although they are the first ones to show the antibacterial effects of EAT and its insecticidal action on fly larvae affecting domestic animals, can not be attributed to any compound in particular, and may even be caused by different compounds; for that reason, it is necessary to purify the extract and test the different fractions.

INTRODUCTION

In Mexico, medicinal plants are commonly used for therapeutic purposes, both for humans and animals. Although this practice is old, very common, and effective in many cases, so far, nobody has yet attached the necessary importance to the experimental study of the therapeutic properties attributed to these plants.

Chilcuán root (*Heliopsis longipes*), formerly classified as *Erigeron affinis*⁹, is used in rural areas as a local anesthetic administered orally to humans, and against cattle worms¹⁰. The therapeutic use of this root dates back to the days before the Spanish Conquest and was described by the Surgeon General of the Indies, Francisco Hernández⁶. Affinin (N-isobutyl-2,6,8-decatrionoamide), which exerts insecticidal effects similar to those exhibited by pyrethrins against domestic flies and mosquitos, has been isolated from this root^{1,2,7,8}. From *Heliopsis scabra*, another plant of the same genus, a compound, the so-called "scabrin", has been isolated which, in addition to insecticidal effects against domestic flies, has been found to exert antibacterial effects against both gram-positive and gram-negative bacteria. During preliminary tests, this compound was found to exert a toxic effect against rats at a dose between 50 and 300 mg/kg.¹³

In the case of Chilcuán, in addition to the aforementioned insecticidal effects, this effect of affinin has only been found against bean grub³. This present study resumes a number of studies aimed at the experimental analysis of the antibacterial, insecticidal, and toxicological effects of the total alcoholic extract (EAT) of the Chilcuán root.

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MATERIAL AND METHODS

Collection. Roots of plants collected in the Sierra del Pinal, Guanajuato, Mexico, were used and analyzed in the herbarium of the School of Sciences of UNAM (National Autonomous University of Mexico).

Obtention of the Alcoholic Extract. The root was desiccated, ground and extracted in an Soxhlet extractor with absolute ethyl alcohol at 70° C; extraction continued until alcohol appeared colorless in the funnel. Afterwards, the alcohol was removed through evaporation until two equal weights of extract were obtained. This EAT was used to prepare the working dilutions.

Experiment 1. The antibacterial effects were tested in vitro against *Escherichia coli* and *Staphylococcus aureus* bacteria. The bacteria were placed in Petri dishes with nutrient agar, after sterility testing for 48 hours. At the time of sowing, lots comprising 25 dishes were prepared for each EAT dose (0.0, 0.01, 0.1, and 1.0 mg) which was impregnated on a filter paper disk 6.3 mm in diameter and placed in the center of each dish. The dishes were incubated in a stove at 37° C, and the growth inhibition areas were measured at 24, 48, and 72 hours after sowing. To measure the growth inhibition area, the perimeter of the halo surrounding the disk without bacterial growth was traced on graph paper.

Experiment 2. The insecticidal effects were examined against the larval stages of two types of arthropods. The first of these experiments was carried out with larvae of the *Oestrus ovis* fly, which were collected in the residue directly from the head of cattle. The larvae were separated by development stage and distributed in Petri dishes (15 dishes with 10 stage II larvae and 15 dishes with 10 stage III larvae each) containing 15 ml of a culture medium consisting of 25% nutrient agar, 12.5% defibrinated ovine blood and 62.5% of a 0.85% NaCl solution; plus 100,000 U of procaine penicillin per liter⁴.

The treatments were carried out in triplicate for each group; these treatments consisted in adding a suspension of the extract to obtain a final concentration of 0.0, 0.01, 0.1, and 10.0 mg/ml of EAT in each dish, respectively. The Petri dishes were maintained in a bacteriological stove at 37° C, and the number of dead/alive larvae was counted after 24, 42, and 54 hours. The lack of movement of the respiratory signs and the absence of response to mechanical stimuli was used as a criterion for determining the death of the larvae.

Experiment 3. The second of the experiments aimed at determining the insecticidal activity was carried out against *Gasterophilus app.* larvae. To two sections of equine stomach, maintained in a Ringer solution, to whose mucosa these living larvae were attached, 20 ml of a solution containing 400 mg of EAT were added. The degree of attachment and the viability of the larvae was evaluated.

Experiment 4. For the purpose of analyzing the toxicological aspects, a first test was carried out during which the dose was determined at which the first signs of toxicity occurred in 5 groups of 4 rats each upon administration of 0.0, 0.1, 1.0, 10.0, and 100.00 mg of EAT per kilogram of weight, respectively. The results of this pilot experiment showed that the first signs of toxicity in the rats appeared at a dose of 100.00 mg of EAT per kilogram of weight. Based on the results of this test, the EAT concentrations were chosen to determine the intraperitoneal 50% lethal dose (i.p. LD₅₀) in rats. Five groups with 20 animals were randomly formed: 11 Wistar females, 4 Wistar males, and 5 Long-Evans males in each group. Each of the groups was treated with 0.0, 100, 200, 400, and 800 mg of EAT per kilogram of body weight. The number of alive and dead animals after application of the treatments was recorded for a period of seven days¹¹. Once LD₅₀ had been obtained, the value was empirically tested in a group of 10 Wistar females.

Statistical Analysis. Experiments 1 and 2 were subject to multiple regression variance analysis; averages were separated by using Tukey's honestly significant minimum difference method; path diagrams were also drawn to determine at what percentage the variables were responsible for the overall effect. The LD₅₀ results were analyzed by using the Reed and Muench method and graphically represented on a probit-log scale. The results are shown as the average +/- standard deviation.

RESULTS

Experiment 1. Tables 1 and 2 in Figure 1 show the growth inhibition areas of *E. coli* and *S. aureus* produced by EAT at different times. The multiple linear regression results showed that the variable that has the most significant impact on the variation in the size of the inhibition area was the treatment (93.7%); bacteria type also impacts this variation (5.0%), with *S. aureus* being more sensitive than *E. coli* to the small EAT dose, while this effect is reversed at higher doses. Time had very little impact on the size of the inhibition area (0.5%).

X-axis: Hours
 Y-axis: Inhibition Area (mm²)
 Tratamiento: Treatment

Figure 1: Inhibitory effect of EAT on bacterial growth [illegible]

TABLE 1

INHIBITION OF GROWTH (mm²) OF E.COLI

TREATMENT (mg)	24	48	72 hours
0.0	13.3 +/- 16.2	10.3 +/- 14.0	4.7 +/- 9.9
0.01	31.8 +/- 38.9	25.5 +/- 35.4	17.4 +/- 24.6
0.1	90.3 +/- 85.0	71.4 +/- 65.7	56.0 +/- [illegible]
1.0	258.0 +/- 149.0	218.0 +/- 169.0	153.0 +/- 149.0

TABLE 2

INHIBITION OF GROWTH (mm²) OF S.AUREUS

TREATMENT (mg)	24	48	72 hours
0.0	6.7 +/- 13.9	4.7 +/- 11.6	3.3 +/- 9.4
0.01	83.4 +/- 67.4	74.8 +/- 64.6	66.0 +/- 53.0
0.1	135.8 +/- 71.3	125.0 +/- 70.6	115.0 +/- 69.1
1.0	136.9 +/- 70.3	127.0 +/- 67.5	115.0 +/- 50.7

Experiment 2. The results are shown in Tables 3 and 4. The most striking result in Table 3 is the higher mortality of the larvae when treated with 1.0 and 10.0 mg/ml; treatments with lower doses do not show significant differences compared with the reference group. Table 4 shows that the larvae III experienced high mortality in all treatments, including the reference group. The path diagram showed that the variable having the most significant impact on larvae mortality was time (81.3%), followed by the treatment (16.7%); larva type had a very small impact (2.0).

TABLE 3

SURVIVAL OF STAGE II LARVAE

TREATMENT	NUMBER OF LARVAE			
	0	24	42	54 hours
0.0 (mg/ml)	10	9.7 +/- 0.6	5.0 +/- 1.0	5.0 +/- 2.0
0.01	10	7.7 +/- 0.6	6.3 +/- 0.6	5.7 +/- 0.6
0.1	10	8.7 +/- 1.2	6.3 +/- 0.6	5.7 +/- 1.2
1.0	10	5.7 +/- 1.2	4.0 +/- 1.0	2.7 +/- 2.1
10.0	10	3.3 +/- 0.6	2.0 +/- 1.0	1.7 +/- 1.2

TABLE 4
SURVIVAL OF STAGE III LARVAE

TREATMENT	NUMBER OF LARVAE			
	0	24	42	54 hours
0.0 (mg/ml)	10	6.0 +/- 1.7	3.3 +/- 1.6	2.3 +/- 1.2
0.01	10	4.7 +/- 1.2	2.7 +/- 1.5	1.3 +/- 0.6
0.1	10	4.3 +/- 0.6	2.7 +/- 0.6	2.3 +/- 0.6
1.0	10	5.0 +/- 1.7	1.7 +/- 0.6	0.3 +/- 0.6
10.0	10	4.0 +/- 1.0	1.7 +/- 0.6	1.9 +/- 1.0

Experiment 3: Exposure of the larvae to the EAT-containing solution for thirty minutes or more lead to their detachment from the gastric mucosa; however, after three hours, the larvae were still alive and moving.

Experiment 4: The values of LD₅₀ are depicted in Table 5 and show that deaths only occurred with high doses of EAT (400 and 800 mg/kg). The table also shows that the Long-Evans rats were more susceptible than the Wistar ones. The LD₅₀ value obtained was 566.0 mg/kg for all animals together; for the Long-Evans males, it was 400.0 mg/kg, and 682.0 mg/kg for the Wistar animals. Table 5 shows the results of the empirical LD₅₀ test. Toxic symptoms appear in acute form in all animals and preceded death in five out of the ten animals used in the experiment.

TABLE 5

LD₅₀ VALUES OF
CHILCUAN ROOT EAT

DOSE mg/ml	ANIMALS PER LOT	ALIVE	DEATHS	CUMULATIVE % ALIVE	CUMULATIVE % DEAD
0.00	11 female W	11	0	100	0
	4 female W	4	0	100	0
	5 male LE	5	0	100	0
	20 TOTAL	20	0	100	0
100.0	11 female W	11	0	100	0
	4 female W	4	0	100	0
	5 male LE	5	0	100	0
	20 TOTAL	20	0	100	0
200.0	11 female W	11	0	100	0
	4 female W	4	0	100	0
	5 male LE	5	0	100	0
	20 TOTAL	20	0	100	0
400.0	11 female W	9	2	85.7	14.3
	4 female W	3	1	83.3	16.7
	5 male LE	2	3	50.0	50.0
	20 TOTAL	14	6	76.9	23.1
800.00	11 female W	3	8	23.1	76.9
	4 female W	2	2	40.0	60.0
	5 male LE	1	4	12.5	87.5
	20 TOTAL	6	14	23.1	76.9

TABLE 6

EMPIRICAL TEST OF THE LD₅₀ VALUE
(682.0 mg/kg) OF EAT

Animal Weight (g)	Latency Time	Duration of Toxic Symptoms	Observations
301	2 min	2 minutes	Lack of coordination, contractions, diarrhea, asphyxiation. Death.
277	4 min	5 minutes	Lack of coordination, pain, contractions, rigidity, respiratory and cardiac arrest. Death.
265	3 min	27 minutes	Very irritable, [illegible], pain.
315	3 min	52 minutes	Diarrhea, prostration, [illegible] for 24 hours.
236	4 min	47 minutes	Pain, contractions, prostration, diarrhea, [illegible] for 48 hours.
258	2 min	4 minutes	Contractions, prostration, diarrhea, respiratory arrest. Death.
231	3 min	35 minutes	Pain, [illegible]
204	5 min	15 minutes	Pain, [illegible].
238	2 min	2 minutes	Rapid death.
225	2 min	3 minutes	Pain, prostration, contractions, lack of coordination, rigidity, diarrhea, respiratory and cardiac arrest. Death.

CONCLUSION

The results shown herein are the first to describe an antibacterial effect of an EAT of the Chilcuán root and an insecticidal effect on parasitic insect larvae of domestic species. Even though the antibacterial effect of a compound isolated from the root of *Heliopsis scabra* has already been reported, the discovery of this effect in the EAT of the Chilcuán root is relevant since it is commonly used in this manner against cattle worms; for that reason, it was decided to use EAT for this study. The use of a total extract, however, while permitting the experimental evaluation of established popular treatments, is not sufficient to attribute the effects observed to a specific compound. In view of the foregoing, all of the effects of EAT described herein can not be attributed to affinin, even though affinin has already been proven to exert insecticidal effects.

As a result, these experiments must only be considered preliminary steps in the isolation of the compound(s) exerting bactericidal and insecticidal properties, or both, of the root of *Heliopsis longipes*, whose toxicity must be proven in test animals.

For the moment being, as shown in this study, EAT exerts more of a bactericidal effect on *E. coli* than on *S. aureus* during the first 24 hours, even though this effect was more sustained in the latter case. The antibacterial effects must be tested with a larger spectrum of both gram-positive and gram-negative bacteria.

The insecticidal effect on *Oestrus ovis* larvae could not be assessed in a satisfactory manner since no medium was available which would permit good survival of the larvae; in spite thereof, stage II larvae were found to be more sensitive to EAT, which was also the most resistant to the medium used. Subsequent experiments will require a suitable culture medium for larvae maintenance.

Even though the acute insecticidal effect of the EAT on *Gasterophilus* larvae did not lead to mortality, the exposure time was similar to that in case the EAT was acting on parasites *in vivo*, and this time was sufficient to cause the detachment of the larvae from the gastric mucosa, which represents sufficient action to permit the removal of the same through the peristaltic movements of the animal.

Finally, the LD₅₀ of the EAT in rats was approximately equivalent to the dose required for an insecticide against *Oestrus ovis* and *Gasterophilus*, as a result of which its use as such is not recommended; prior thereto, it is necessary to at least partially purify the component(s) responsible for this effect and once again assess its toxicity.

SUMMARY:

Chilcuan (*Heliopsis longipes*) is a Mexican plant, the root of which has been used traditionally as an oral cavity anesthetic in humans, and as an insecticide in cattle myiasis. In this work, the antibacterial, insecticidal and toxicological effects of an alcoholic extract of Chilcuan root (EAT) were investigated. Four experiments were performed: 1) Antibacterial effects of EAT were tested on agar cultures of *Escherichia coli* and *Staphylococcus aureus* to which 0.0, 0.01, 0.1 or 1.0 mg doses of EAT were added. 2) Insecticidal effects were examined against *Oestrus ovis* larvae (larval stages II and III) cultivated *in vitro*, by adding enough EAT to the culture medium in order to get a final concentration of 0.0, 0.01, 0.1 or 10.0 mg/ml. 3) Insecticidal properties were also tested against *Gasterophilus app* larvae, by mixing 400 mg EAT with the culture medium containing a section of equine stomach to which these sort of larvae were attached. 4) Toxic effects of EAT were evaluated by determining its intraperitoneal 50% lethal dose (i.p. LD₅₀) in rats. Results of experiment 1 showed that EAT exerts antibacterial effects on both *E. coli* and *S. aureus*. However, the antibacterial action of EAT against *S. aureus* was greater at lower doses (0.01 mg), while the growth-inhibition area was large in *E. coli* cultures at higher doses (1.0 mg). Results of experiment 2 indicate that *O. ovis* larvae stage II survived longer in the culture medium, displaying higher susceptibility to antilarval effects of EAT (1.0 mg/ml); stage III larvae survived poorly in the culture medium, making it difficult to assess the antilarval effect of EAT. In experiment 3, detachment of *Gasterophilus* larvae was observed. Lastly, i.p. LD₅₀ of EAT was found to be 566.0 mg/kg b.w. in rats. Further investigation is needed to isolate the active ingredients present in Chilcuan root.

CREDITS

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Estudios preliminares de los efectos antibacterianos, insecticidas y toxicológicos de la raíz del Chilcuán (*Heliopsis longipes*)

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RESUMEN

La raíz del Chilcuán (*Heliopsis longipes*), es utilizada empíricamente como anestésico bucal en humanos y contra las gusaneras del ganado. En este trabajo se analizaron los efectos antibacterianos, insecticidas y tóxicos del extracto alcohólico total (EAT) de la raíz del Chilcuán. Los efectos antibacterianos fueron probados *in vitro* sobre *Escherichia coli* y *Staphylococcus aureus*; el EAT mostró actividad bactericida sobre ambas bacterias, siendo más resistente a pequeñas dosis (0.01 mg) la primera, pero invirtiéndose el efecto a dosis mayores (1.0 mg). Los efectos insecticidas fueron probados también *in vitro*, cuantitativamente sobre *Oestrus ovis* y cualitativamente sobre *Gasterophilus* spp, la larva de *Oestrus* en segundo estadio, tuvo mayor sobrevivencia en el cultivo y fue más sensible al EAT (1.0 mg/ml) que produjo una mortalidad del 43% a las 24 horas vs 3% del testigo; la larva 3, en las mismas condiciones tuvo una mortalidad de 80% vs 40% del testigo. Por otro lado, el EAT (400 mg) fue capaz de producir el desprendimiento de las larvas de *Gasterophilus* de la mucosa de estómagos de equino *in vitro*. Por último, los efectos tóxicos se determinaron calculando la dosis letal 50% del EAT por vía intraperitoneal en ratas que fue de 566 mg/kg. Estos resultados aunque son los primeros en mostrar efectos antibacterianos del EAT y actividad insecticida sobre larvas

de moscas que afectan a los animales domésticos, no pueden ser atribuidos a ningún compuesto en particular, e incluso cada uno puede ser debido a compuestos diferentes, por lo que se hace necesario purificar el extracto y probar las diferentes fracciones.

INTRODUCCIÓN

En México es común el uso de las plantas medicinales en la terapéutica, tanto en humanos como en animales. A pesar de que esta práctica es antigua, ampliamente diseminada y efectiva en muchos casos; hasta ahora no se le ha dado la importancia debida al estudio experimental de las propiedades terapéuticas asignadas a estas plantas.

La raíz del Chilcuán (*Heliopsis longipes*), anteriormente clasificada como *Erigeron affinis*,⁹ es utilizada en el medio rural como anestésico local a nivel bucal en humanos y contra las gusaneras del ganado.¹⁰ El uso terapéutico de esta raíz es anterior a la conquista y fue descrita por el protomédico de las Indias, Francisco Hernández.⁸ De esta raíz se ha aislado la afinina (N-isobutil-2,6,8-decatrienamida), que tiene efectos insecticidas semejantes a los que tienen las piretrinas, contra la mosca doméstica y los mosquitos.^{12,7,8} De *Heliopsis scabra*, otra planta del mismo género, se ha aislado un compuesto, el "scabrin", al que además de los efectos insecticidas contra la mosca doméstica, se le ha encontrado efectos antibacterianos sobre bacterias Grami positivas y negativas. Este compuesto en pruebas preliminares ha mostrado efectos tóxicos en ratas a dosis entre 80 y 300 mg/kg.¹³

Para el caso del chilcuán, además de las valoraciones insecticidas mencionadas, sólo se ha probado ese efecto de la afinina contra el gorgojo del frijol.³ En el presente trabajo se resumen varios estudios encaminados al análisis experimental de los

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efectos antibacterianos, insecticidas y toxicológicos del extracto alcohólico total (EAT) de la raíz del Chileuán.

MATERIAL Y MÉTODOS

Colecta. Se utilizaron raíces de plantas colectadas en la Sierra del Pinal, Guanajuato, México y fueron determinadas en el herbario de la Facultad de Ciencias de la UNAM.

Obtención del extracto alcohólico. La raíz fue desecada, molida y sometida a extracción en un extractor Soxhlet con alcohol etílico absoluto a 70 C; la extracción fue mantenida hasta que el alcohol apareció incoloro en el embudo. Posteriormente se eliminó el alcohol por evaporación hasta obtener dos pesos iguales del extracto. A partir de este EAT se hicieron las diluciones de trabajo.

Experimento 1. Los efectos antibacterianos fueron probados *in vitro* contra las bacterias *Escherichia coli* y *Staphylococcus aureus*. Las bacterias fueron sembradas en cajas de Petri con agar nutritivo, después de una prueba de esterilidad de 48 horas. Al tiempo de la siembra, se hicieron lotes de 25 cajas para cada dosis del EAT, (0.0, 0.01, 0.1 y 1.0 mg) que se impregnó en un disco de papel filtro de 6.3 mm de diámetro y se colocó al centro de cada caja. Estas fueron incubadas en una estufa de cultivo a 37 C y se midieron las áreas de inhibición del crecimiento a las 24, 48 y 72 horas después de la siembra. Para medir el área de inhibición del crecimiento se calcó sobre papel albanene milimétrico, el perímetro del halo que rodeaba al disco en donde no había crecimiento bacteriano.¹²

Experimento 2. Los efectos insecticidas fueron probados en los estados larvarios de dos tipos de artrópodos. El primero de estos experimentos se realizó con las larvas de la mosca *Oestrus ovis*, las cuales fueron colectadas en el resto directamente de cabezas de ovinos. Las larvas fueron separadas por su estadio de desarrollo y repartidas en cajas de Petri (15 cajas con 10 larvas en el estadio 2 y 15 cajas con 10 larvas en el estadio 3 cada una), las cuales contenían 15 ml de un medio de cultivo, compuesto por: 25% de agar nutritivo, 12.5% de sangre de ovino desfibrinada y 62.5% de solución de NaCl al 0.85%; adicionado con 100,000 U de penicilina procainica por litro.⁴

Los tratamientos se hicieron por triplicado para cada grupo, estos consistieron en adicionar una suspensión del extracto para dar una concentración final de 0.0, 0.01, 0.1, 1.0 y 10.0 mg/ml

del EAT en cada caja respectivamente. Las cajas de Petri fueron mantenidas en una estufa bacteriológica a 37 C y se hicieron conteos de larvas vivas y muertas a las 24, 42 y 64 horas. Se tomó como criterio para considerar la muerte de las larvas, la falta de movimiento en los estigmas respiratorios y la ausencia de respuesta a los estímulos mecánicos.

Experimento 3. El segundo de los experimentos que valoró la actividad insecticida se realizó sobre larvas de la mosca *Gasterophilus spp.* Se tomaron dos trozos de estómago de equino, mantenidos en una solución de Ringer, con larvas vivas fijadas a la mucosa y se le agregaron 20 ml de una solución que contenía 400 mg de EAT. Se valoró el grado de la fijación y la viabilidad de las larvas.

Experimento 4. Para analizar los aspectos toxicológicos, primero se realizó una prueba en la que se determinó la dosis a la cual se presentaron los primeros signos de toxicidad en 5 grupos de 4 ratas cada uno, cuando se les administró 0.0, 0.1, 1.0, 10.0 y 100.0 mg de EAT por kg de peso respectivamente. Los resultados de este experimento piloto, mostraron que los primeros signos de toxicidad en las ratas se presentaron a la dosis de 100.0 mg de EAT por kg de peso. Con base en los resultados de esta prueba se seleccionaron las concentraciones del EAT para la determinación de la dosis letal 50% (DL₅₀) por vía intraperitoneal en ratas. Se formaron aleatoriamente cinco grupos con 20 animales: 11 hembras Wistar, 4 machos Wistar y 5 machos Long-Evans en cada grupo. Cada uno de los grupos fue tratado con 0.0, 100, 200, 400 y 800 mg de EAT por kg de peso corporal. El número de animales vivos y muertos después de la aplicación de los tratamientos se recabó durante siete días.¹¹ Una vez obtenida la DL₅₀ se probó empíricamente en un grupo de 10 hembras Wistar.

Análisis Estadístico. Los experimentos 1 y 2 se sometieron a un análisis de varianza regresión múltiple. En ellos se realizó la separación de medias por el método de Tukey de diferencia mínima significativa honesta; asimismo se efectuaron diagramas de trayectorias, para conocer el porcentaje de participación de las variables en el efecto total. Los resultados de la DL₅₀ fueron analizados por el método de Reed y Muench, graficados en escala probit-log.³ Los resultados se muestran como la media \pm la desviación estándar.

RESULTADOS

Experimento 1. En los Cuadros 1 y 2 y en la Figura 1, se muestran las áreas de inhibición del crecimiento de *E. coli* y de *S. aureus* producidas

por el EAT a diferentes tiempos. Los resultados de la regresión lineal múltiple, mostraron que la variable que explica en mayor parte la variación en el tamaño del área de inhibición fue el tratamiento (93.7%), el tipo de bacteria también influye en esta variación (5.0%), siendo más sensible *S. aureus* que *E. coli* a pequeñas dosis del EAT, pero invirtiéndose este efecto a dosis mayores. El efecto del tiempo fue poco importante en el tamaño del área de inhibición (0.5%).

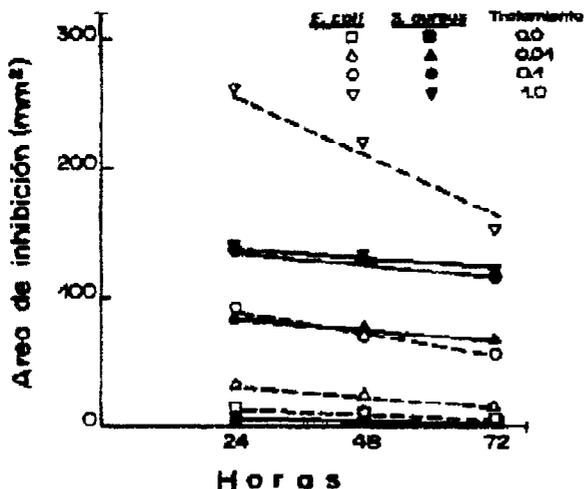


Figura 1. Efecto inhibitorio del crecimiento bacteriano del EAT. Observase que a dosis mayores, *S. aureus* es más susceptible al EAT; sin embargo, este efecto no se incrementa proporcionalmente a dosis mayores como en el caso de *E. coli*.

CUADRO 1

INHIBICIÓN DEL CRECIMIENTO (mm²) DE *E. coli*

TRATAMIENTO (mg)	24	48	72 horas
0.0	13.3 ± 10.2	10.3 ± 14.0	4.7 ± 9.9
0.01	31.8 ± 30.9	25.5 ± 35.4	17.4 ± 24.0
0.1	90.3 ± 85.0	71.4 ± 66.7	50.0 ± 59.2
1.0	250.0 ± 140.0	210.0 ± 100.0	153.0 ± 140.0

Experimento 2. Los resultados aparecen en los Cuadros 3 y 4. Lo más sobresaliente en el Cuadro 3, es la mayor mortalidad de las larvas en los tratamientos de 1.0 y 10.0 mg/ml, los tratamientos por debajo de estas dosis no muestran diferencias significativas con el grupo testigo. En el Cuadro 4 se puede observar que la larva 3 tuvo una alta mortalidad en todos los tratamientos incluyendo al grupo testigo. El diagrama de trayectorias

CUADRO 2

INHIBICIÓN DEL CRECIMIENTO (mm²) DE *S. aureus*

TRATAMIENTO (mg)	24	48	72 horas
0.0	6.7 ± 13.0	4.7 ± 11.6	3.3 ± 9.4
0.01	83.4 ± 67.4	74.0 ± 64.8	60.0 ± 53.0
0.1	125.0 ± 71.3	125.0 ± 70.5	115.0 ± 60.1
1.0	130.0 ± 70.3	127.0 ± 67.3	115.0 ± 54.7

mostró que la variable que explica en mayor medida la mortalidad de las larvas fue el tiempo (81.3%), siguiéndole el tratamiento (16.7%); el tipo de larva tuvo un efecto muy pequeño (2.0%).

CUADRO 3

SOBREVIVENCIA DE LARVAS EN ESTADIO 2

TRATAMIENTO	NÚMERO DE LARVAS			
	0	24	42	54 horas
0.0 (mg/ml)	10	9.7 ± 0.6	8.0 ± 1.0	5.0 ± 2.0
0.01	10	7.7 ± 0.6	6.3 ± 0.6	3.7 ± 0.9
0.01	10	8.7 ± 1.2	6.3 ± 0.6	6.7 ± 1.2
1.0	10	5.7 ± 1.2	4.0 ± 1.0	2.7 ± 2.1
10.0	10	3.3 ± 0.8	2.0 ± 1.0	1.7 ± 1.2

CUADRO 4

SOBREVIVENCIA DE LARVAS EN ESTADIO 3

TRATAMIENTO	NÚMERO DE LARVAS			
	0	24	42	54 horas
0.0 (mg/ml)	10	6.0 ± 1.7	3.3 ± 1.5	2.3 ± 1.2
0.01	10	4.7 ± 1.2	2.7 ± 1.5	1.9 ± 0.6
0.1	10	4.3 ± 0.8	2.7 ± 0.8	2.3 ± 0.9
1.0	10	5.0 ± 1.7	1.7 ± 0.8	0.9 ± 0.6
10.0	10	4.0 ± 1.0	1.7 ± 0.6	1.0 ± 1.0

Experimento 3. La exposición por 30 o más minutos de las larvas a la solución que contenía el EAT, provocó su desprendimiento de la mucosa gástrica; aunque después de 3 horas las larvas continuaron vivas y con movimiento.

Experimento 4. La valoración de la DL_{50} se muestra en el Cuadro 5 y en él se puede observar que las muertes se presentaron sólo en las dosis altas del EAT (400 y 800 mg/kg). También se desprende que las ratas Long-Evans fueron más susceptibles que las Wistar. La DL_{50} obtenida fue de 566.0 mg/kg para el conjunto de todos los animales, mientras que para los machos Long Evans fue de 400.0 mg/kg y para los Wistar de 682.0 mg/kg. En el Cuadro 6 se presentan los resultados de la prueba empírica de la DL_{50} , los signos tóxicos se presentaron en forma aguda en todos los animales y antecedieron la muerte en 5 de los 10 animales experimentales.

CUADRO 5

VALORACIÓN DE LA DL_{50}
DEL EAT DE LA RAÍZ DEL CHILCUÁN

DOSES mg/ml	ANIMALES POR LOTE	VIVOS	MUERTOS	% ACUMULADO DE VIVOS	% ACUMULADO DE MUERTOS
0.0	11 ♀ W	11	0	100	0
	4 ♂ W	4	0	100	0
	5 ♂ LE	5	0	100	0
	20 TOTAL	20	0	100	0
100.0	11 ♀ W	11	0	100	0
	4 ♂ W	4	0	100	0
	5 ♂ LE	5	0	100	0
	20 TOTAL	20	0	100	0
200.0	11 ♀ W	11	0	100	0
	4 ♂ W	4	0	100	0
	5 ♂ LE	5	0	100	0
	20 TOTAL	20	0	100	0
400.0	11 ♀ W	9	2	81.7	14.3
	4 ♂ W	3	1	75.0	25.0
	5 ♂ LE	2	3	40.0	50.0
	20 TOTAL	14	6	70.0	37.5
800.0	11 ♀ W	3	8	27.3	72.7
	4 ♂ W	2	2	50.0	75.0
	5 ♂ LE	1	4	20.0	80.0
	20 TOTAL	6	14	30.0	86.7

DISCUSIÓN

Los resultados aquí mostrados, son los primeros que describen un efecto antibacteriano del EAT de la raíz del Chilcuán y un efecto insecticida sobre las larvas de insectos parásitos de especies

CUADRO 6

PRUEBA EMPÍRICA
DE LA DL_{50} (682.0 mg/kg) DEL EAT

Peso del animal (g)	Tiempo de latencia	Duración de signos tóxicos	Observaciones
301	2 min	3 minutos	Incoordinación, contracciones de vena, zofia. Muerte.
277	4 min	5 minutos	Incoordinación, dolor, contracciones rígidas, paro respiratorio y cardíaco. Muerte.
285	3 min	27 minutos	Muy sensible, eufemia, dolor.
315	3 min	52 minutos	Dilatación, posturación. Ataje por 24 horas.
236	4 min	47 minutos	Dolor, contracciones, posturación, dilatación, hipobagia por 48 horas.
258	2 min	4 min	Contracciones, posturación, énfasis, paro respiratorio. Muerte.
231	3 min	35 minutos	Dolor, inquietud.
204	3 min	15 minutos	Dolor, eufemia.
238	2 min	2 minutos	Muerte rápida.
228	2 min	3 minutos	Dolor, posturación, contracciones incoordinación, rigidez, énfasis, paro respiratorio y cardíaco. Muerte.

domésticas. Aun cuando ya se había comunicado el efecto antibacteriano de un compuesto aislado de la raíz de *Hehopsis scabra*, el hallazgo de este efecto en el EAT de la raíz del Chilcuán cobra relevancia, ya que es de esta forma como se utiliza comúnmente contra las gusaneras del ganado; razón por la cual se resolvió utilizar el EAT en este trabajo. La utilización de un extracto total si bien permite la valoración experimental de los tratamientos popularmente establecidos, por otro lado, es insuficiente para poder asignar a un determinado compuesto los efectos observados. Por lo anterior, no se puede atribuir a la afinina todos los efectos del EAT aquí descritos, aun cuando ya se ha demostrado que tiene efectos insecticidas.

Consecuentemente, estos experimentos se deben considerar sólo como los pasos preliminares para el aislamiento del o los compuestos con propiedades bactericidas e insecticidas (o ambas) de la raíz de *Hehopsis longipes*; de los que habrá que probar su toxicidad en animales experimentales.

Por el momento, según se muestra en este trabajo, el EAT ejerció un mayor efecto bactericida sobre *E. coli* que sobre *S. aureus* durante las primeras 24 horas, aunque este efecto fue sostenido por mayor tiempo para el segundo caso. Es necesario probar los efectos antibacterianos con un mayor espectro de bacterias tanto Gram positivas como negativas.

El efecto insecticida sobre las larvas de *Oestrus ovis* no pudo ser valorado satisfactoriamente, por

no tener un medio que permitiese una buena sobrevivencia de las larvas; aún así, se encontró una mayor sensibilidad de la larva 2 al EAT, que además fue la más resistente al medio que se usó. En experimentos posteriores será necesario contar con un medio de cultivo adecuado para el mantenimiento de las larvas.

Aún cuando el efecto insecticida del EAT sobre las larvas de *Gasterophilus* en forma aguda, no produjo mortalidad, el tiempo de exposición semejó al que estaría al EAT actuando sobre los parásitos *in vivo*, y este tiempo bastó para provocar el desprendimiento de las larvas de la mucosa gástrica, acción suficiente para permitir la salida de las mismas por los movimientos peristálticos del animal.

Finalmente, la LD_{50} del EAT en ratas, aproximadamente equivalente a la dosis requerida como insecticida sobre *Oestrus ovis* y *Gasterophilus*, por lo que no se recomienda su uso como tal; antes, es necesario purificar por lo menos parcialmente, el o los compuestos responsables de este efecto y valorar nuevamente su toxicidad.

SUMMARY

Chilcuan (*Heliopsis longipes*) is a Mexican plant, the root of which has been used traditionally as an oral cavity anaesthetic in humans, and as an insecticide in cattle myiasis. In this work, the antibacterial, insecticidal and toxicological effects of an alcoholic extract of Chilcuan root (EAT) were investigated. Four experiments were performed: 1) Antibacterial effects of EAT were tested on agar cultures of *Escherichia coli* and *Staphylococcus aureus* to which 0.0, 0.01, 0.1 or 1.0 mg doses of EAT were added. 2) Insecticidal effects were

examined against *Oestrus ovis* larvae (larval stages II and III) cultivated *in vitro*, by adding enough EAT to the culture medium in order to get a final concentration of 0.0, 0.01, 1.0 or 10.0 mg/ml. 3) Insecticidal properties were also tested against *Gasterophilus* spp larvae, by mixing 400 mg EAT with the culture medium containing a section of equine stomach to which these sort of larvae were attached. 4) Toxic effects of EAT were evaluated by determining its intraperitoneal 50% lethal dose (i.p. LD_{50}) in rats. Results of experiment 1 showed that EAT exerts antibacterial effects on both *E. coli* and *S. aureus*. However, the antibacterial action of EAT against *S. aureus* was greater at lower doses (0.01 mg), while the growth-inhibition area was larger in *E. coli* cultures at higher doses (1.0 mg). Results of experiment 2 indicate that *O. ovis* larval stage II survived longer in the culture medium, displaying higher susceptibility to antilarval effects of EAT (1.0 mg/ml); stage III larvae survived poorly in the culture medium, making it difficult to assess the antilarval effects of EAT. In experiment 3, detachment of *Gasterophilus* larvae was observed. Lastly, i.p. LD_{50} of EAT was found to be 566.0 mg/kg b.w. in rats. Further investigation is needed to isolate the active ingredients present in Chilcuan root.

AGRADECIMIENTOS

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PHYTOS



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Short Communication

Ethnopharmacologic studies. I. Rapid solution to a problem — oral use of *Heliopsis longipes* — by means of a multidisciplinary approach

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One goal in studying plants alleged by indigenous practitioners to be useful for the alleviation of human illness is to isolate the principle(s) responsible for the stated effect so that it (they) can be determined safe and effective. A knowledge of the exact pharmacological effects of active principles may then lead to improved application of the ethnomedical preparation. Under ideal circumstances, problems of this type are best solved by a multidisciplinary group of scientists, each contributing their specific expertise.

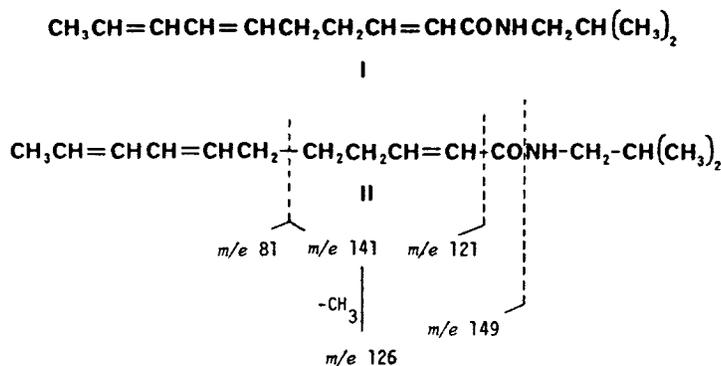
At this time we would like to report on a recent ethnopharmacological problem that was solved in our laboratories involving a plant alleged to be used by indigenous practitioners and people for the palliative treatment of toothache. The personnel involved in the study included a physician, a botanist, a biologist, two pharmacognosists, one organic chemist and one pharmacologist. Starting with less than 30 g of dried plant material, a chemical compound was isolated in sufficient quantity to determine that it was the active principle. The structure was determined, the compound was studied for acute toxicity (LD_{50}), and several additional pharmacological tests were conducted. Less than one week was required to complete the entire study.

We were made aware that a Mexican plant, claimed to be effective for the alleviation of toothache pain, was being used extensively in the rural areas of Mexico. Relief was claimed to be rapid following local application of a piece of the root of the plant to the tooth and gum involved. In Mexico,

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the plant is commonly known as *peritre del pais*, *chilcuague* or *chilcuan*, and was identified, by comparison with herbarium specimens, as *Heliopsis longipes* (A. Gray) Blake, a member of the Compositae. Because of the rapid resolution of the problem all of the literature applicable to the plant was not acquired until the experimental work was completed. According to the literature, the roots of *H. longipes* have been used popularly in Mexico for the treatment of toothache [1, 2] and in the preparation of an insecticidal powder [2]. Acree and co-workers [3, 4] isolated the active principle in 1.9 per cent yield from the dried roots of *H. longipes*, and showed it to be the isobutylamide of an unsaturated C₁₀ acid; the compound was named affinin [3, 5]. Affinin was subsequently shown to be *N*-isobutyldodeca-2-*trans*-6-*cis*-8-*trans*-trienamide (I) [6 - 8], and is identical with spilanthol [9, 10], the pungent principle of several *Spilanthes* species [11 - 14].

The source of affinin was originally named as *Erigeron affinis* DC., but in 1946 Little [15] indicated that Mexican *Peritre del pais* was actually *Heliopsis longipes* (A. Gray) Blake. This led Jacobson *et al.* [4] to correct the plant source and to recommend that the name affinin *not* be used for the active principle. Subsequently, several investigators have implied that *H. longipes* is synonymous with *E. affinis*, and this error may be traced to Martinez's Catalogue of 1937 [16]. When Fisher [17] monographed the genus *Heliopsis* he clearly indicated that *Erigeron affinis* DC. was a misidentification of *Heliopsis longipes* (A. Gray) Blake, since the two binomials are not synonymous.



Extraction and identification of the active principle

A commercial sample of the dried stem of *H. longipes* (29.8 g), obtained from a native market in Mexico, was extracted with two 200-ml aliquots of 50 per cent ethanol by stirring the mixture for several minutes each time over a steam bath, filtering, combining the two filtrates, and reducing the volume to approx. 100 ml. This aqueous ethanolic extract was partitioned with 3 × 100 ml volumes of ethyl acetate and the combined ethyl acetate extracts were reduced to dryness *in vacuo* to yield 1.4 g of residue (A). The aqueous fraction was frozen and lyophilized to give 4.2 g of a dry powder (B).

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A sample of fraction A, which was shown by bioassay to contain a highly active analgesic principle (*vide infra*), was dissolved in a minimum volume of ethyl acetate and applied to several silica gel PF₂₅₄ preparative-layer (2 mm) chromatography plates and developed twice with a mixture of chloroform-methanol (98:1). The major component of the mixture was visualized under ultraviolet light at R_F 0.25. This band was removed from the plate, extracted from the silica gel with hot methanol, and crystallized from methanol to yield 0.102 g of crystalline material having m.p. 22 - 23 °C, IR ν_{\max} 3380, 3075, 3020, 1675, 1632, 1550, 980, 948 and 760 cm^{-1} ; UV λ_{\max} 277 nm; NMR δ ppm 0.90 (6H, d, $J = 7$ Hz, $-\text{CH}(\text{CH}_3)_2$), 1.75 (3H, d, $J = 7$ Hz, 10-H_3), 1.78 (1H, m, $-\text{CH}(\text{CH}_3)_2$), 2.25 (4H, m, 4-H_2), 3.08 (2H, t, $J = 6$ Hz, N-CH_2), and 5.10 - 6.80 (6H, m, olefinic H) NH not observed; MS m/e 221 (M^+ , 12.5), 149 (8.0), 142 (8.0), 141 (67.2), 126 (23.5), 121 (5.5), 103 (8.0), 98 (24.4), 93 (8.0), 81 (100), 79 (31), 69 (20.3), 68 (34.0), 57 (22.5), 56 (14.0), 55 (23.0), 53 (35.0), 43 (33.0), and 42 (15.0). The infrared (IR) [18], ultraviolet (UV) [18] and nuclear magnetic resonance (NMR) [1] data are in agreement with those reported previously for affinin (I) and the mass spectrum is in accord with the fragmentation shown in II.

Biological evaluation of extracts and affinin

Initially, we determined that the application of a piece of the stem to the tongue produced an intense numbing effect that persisted for about 45 - 60 minutes. Using the inhibition of acetic acid writhing effect in mice [19] as a bioassay for analgesic activity, the ethyl acetate (A) and water (B) fractions were tested. Fraction B was found to have an ED_{50} of 426.98 mg/kg in this assay. On the other hand, fraction A exhibited an ED_{50} of 19.04 mg/kg. All samples were administered by gavage, with distilled water serving as the solvent for fraction B and 10 per cent ethanol for fraction A. Morphine (HCl) routinely has shown an ED_{50} of 2.0 mg/kg (p.o.) in this assay in our laboratory. The data are presented in Table 1.

Affinin, in aqueous solution and administered orally at doses ranging from 2.5 to 10.0 mg/kg, exhibited an ED_{50} of 6.98 mg/kg.

No gross manifestations of pharmacologic activity were noted with any doses of fraction B (50.0 - 400.0 mg/kg, p.o.). Mice receiving 25.0 mg/kg (p.o.) of fraction A showed some initial depression followed by mild tremors. Normal activity resumed after 5-10 minutes, interrupted by short, infrequent periods of minor tremor and a decrease in motor activity. Animals receiving 50 mg/kg of fraction A exhibited marked tremors, especially of the head and forelimbs, within 2 - 5 minutes following dosing. Tremors continued for about 20 minutes. An initial increase in respiration and heart rate followed by severe depression was also observed; within 30 minutes 60 per cent of the mice had died. The remaining mice survived, but exhibited depressed motor activity for more than 2 hours.

The only manifestation of toxicity observed in mice receiving 7.5 mg/kg (p.o.) affinin was an initial mild depression, followed by resumption of

TABLE 1

Analgesic evaluation of *Heliopsis longipes* extracts and affinin (I)

Fraction	Dose (mg/kg)	No. animals utilized	Mean No. writhes	S.D.	S.E.	Percentage inhibition	p	Significance	ED ₅₀ (mg/kg)
A	1	5	41.8	2.17	0.97	2.0	0.636	n	19.04
	2	5	40.2	3.30	1.65	5.7	0.321	n	
	5	5	27.8	3.86	1.93	34.9	0.002	s	
	10	5	24.6	1.67	0.75	42.3	0.001	s	
	25	5	21.2	6.42	2.87	50.3	0.001	s	
	50	*							
10% ethanol		5	42.7	2.52	1.45				
B	50	5	39.4	2.97	1.33	16.2	0.020	s	426.98
	75	5	34.4	2.79	1.25	26.8	0.002	s	
	100	5	32.0	3.74	1.87	31.9	0.002	s	
	200	5	30.7	3.40	1.70	34.5	0.001	s	
	400	5	23.3	16.1	8.07	50.5	0.026	s	
H ₂ O		5	47.0	5.00	2.24				
Affinin	2.5	5	39.4	7.16	3.20	12.1	0.142	n	6.98
	5.0	5	30.6	7.13	3.18	31.7	0.003	s	
	7.5	5	21.8	9.98	4.47	51.3	0.001	s	
	10.0	5**	14.3	3.21	1.85	68.0	0.001	s	
10% ethanol		5	44.8	1.92	0.86				

*3 animals (60%) died within 30 minutes after dosing; remaining animals were not used due to their severe depression.

**2 animals (40%) died within 30 minutes after dosing.

normal motor activity within 10 minutes. Animals given 10 mg/kg (p.o.) were more severely depressed, 40 per cent of the mice dying within 40 minutes following dosing. No toxicity was observed at doses lower than 7.5 mg/kg (p.o.).

A 1.0 per cent aqueous solution of affinin failed to elicit a local anesthetic effect when applied to rabbit cornea. At 15.0 mg/kg (p.o) affinin also failed to exhibit an anti-inflammatory effect in the carrageenin-induced rat pedal edema assay [20].

The study of indigenous drugs used in traditional medicine will become increasingly important for the developing nations as petroleum costs increase. This paper, using the example of *Heliopsis longipes*, a drug used orally as an analgesic, emphasises the need for collaboration between scientists to evaluate effectively the potential of these drugs and their active constituents.

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Antimicrobial and cytotoxic activities of some crude drug extracts from Mexican Medicinal Plants

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Summary

31 crude extracts derived from 28 plants highly valued as anti-infective agents in Mexican folk medicine have been screened for antimicrobial activity against four bacteria, a yeast and two molds. The results of the quantitative study indicated that the extracts derived from five species (*Mabouya depressa*, *Heliopsis longipes*, *Datura lanosa*, *Cnidiosculus tehuacanensis* and *Helianthella quinquevarvis*) possessed significant antiseptic properties, therefore supporting the ethnomedical uses of these species. The cytotoxic activity was assayed against three cell lines HT-29 (Colon adenocarcinoma), MCF-7 (Breast carcinoma), A-549 (Lung carcinoma) and only the extract of *Helianthella quinquevarvis* possessed significant activity against the MCF-7 cell line.

Keywords: antimicrobial activity, cytotoxic activity, Mexican medicinal plants, brine shrimp bioassay.

Introduction

In Mexico, as well as in other parts of the world, traditional healers prescribe herbal preparations for the treatment of different types of infectious diseases and cancer ailments. Considering that folk medicine is a good lead for discovering new drugs with antimicrobial and antitumor properties, the potential for finding useful new agents from these medicinal species is very promising (Mitscher et al., 1984, 1987; Hamburger and Hostettman, 1991).

In this context, the aim of the current work was to investigate the antimicrobial and cytotoxic activities of 31 extracts derived from 28 Mexican plants. Most of these species are highly valued as anti-infective agents in Mexican folk medicine (Martinez, 1989; Diaz, 1976).

For some of the plant species examined in this study, anti-infective and cytotoxic properties have been described previously. Thus *Lepidium virginicum* inhibits the growth of *Escherichia coli*, *Salmonella typhi* A and *Shigella boydii* (Avila et al., 1993) and *Heliopsis longipes* the growth of *E. coli* and *Staphylococcus aureus* (Romero et al., 1989).

The extract of *Plumbago scandens* had antimicrobial activity against *S. aureus*, *Bacillus subtilis*, *Klebsiella pneumoniae* and *Candida albicans* (Hoffman et al., 1993). The antimicrobial activity of this species has been traced to the naphthoquinone plumbagin which inhibits the growth of several microorganisms including some fungi and the gram negative bacteria *K. pneumoniae* and *S. aureus* (Goncalves et al., 1968). Finally, *Ptelea trifoliata* demonstrated antimicrobial properties against *Mycobacterium smegmatis* and *C. albicans* (Mitscher et al., 1975). Bioactivity guided fractionation of the active extract led to the isolation of preletinium chloride as the active principle (Mitscher et al., 1975). Other compounds isolated from *P. trifoliata* possessed marked antimicrobial and cytotoxic properties (Chineau et al., 1976; Rideau et al., 1979).

From a chemical point of view the composition of some of these species has been previously described. Phytochemical investigations of *P. trifoliata* have allowed the isolation of different types of secondary metabolites including several alkaloids and coumarins (Prolova et al., 1964; Kowalska, 1966; Mulvey et al., 1969; Reish et al., 1969, 1972).

1973, 1975 a, b, 1978; Korosi, 1976; Novak et al., 1970; Szendrei et al., 1973, 1974; Rideau et al., 1979; Petit-Paly et al., 1989). From *Swietenia humilis* six limonoids were isolated (Okorie et al., 1971; Segura-Cortez et al., 1993). Several diterpenoids were obtained from *Kohleria deppiana* (Nogiers et al., 1994) and *Cigarilla mexicana* (Mata et al., 1988, 1989). Chemical analysis of *Anredera scandens* has revealed the presence of a retrochalcone (Calzada et al., 1990), α -spinasterol and β -amyriin were isolated from *Conyza flagitoides* (Domínguez et al., 1972) and *Datura lanosa* was found to contain several tropane alkaloids (Bye et al., 1991). Plumbagin was isolated from *Plumbago scandens* (Goncalves et al., 1968; Bhattacharyya and De-Caballo, 1986). Chromans were obtained from *Helianthella quinquenervis* (Herz and Kulanthavel, 1984). Tetragalloylquinic acid derivatives and some triterpenoids were isolated from *Galphimia glauca* (Wagner and Dorsch, 1992; Toscano et al., 1993). Finally, from *Matopium brownii* several flavonoids were detected using paper chromatography (Young, 1979).

Materials and methods

Plant material

Authenticated voucher specimens (in parenthesis) of some of the species investigated were deposited in the National Herbarium (MEXU) Institute of Biology, UNAM, Mexico, D.F. The others were deposited at the herbarium of the Institute of Ecology (XAL), Xalapa, Veracruz. Plants were collected in different parts of Mexico: *Conyza flagitoides* (DC.) Hieron. (Bye and Linares 18865), *Lepidium virginicum* L. (Bye and Linares 18872), *Acalypha phloides* Cav. (Bye and Linares 18780) and *Bunchosia lindaniana* Juss. (Bye and Linares 18677) from the state of Mexico. *Illex rubra* S. Wats (Bye et al. 18004), *Elytaria imbricata* (Vahl) Pers. (Bye et al. 18015), *Cardiospermum corindum* L. (Bye et al. 18016), *Ptelea trifoliata* L. (Bye et al. 18029), *Perityle batopilensis* Powell (Bye et al. 18030), *Plumbago scandens* L. (18013), *Datura lanosa* Barclay ex Bye (Bye et al. 13619), *Dyssodia papposa* (Venz.) Hitchc. (Bye et al. 18238), *Geranium niveum* S. Wats (Bye et al. 18054) and *Helianthella quinquenervis* (Hook.) A. Gray (Bye et al. 18058) from the state of Chihuahua. *Cigarilla mexicana* (Zucc. et Martius ex DC.) Aiello. (Bye and Linares 17982) and *Heliopsis longipes* A. Gray Blake (Bye and Linares 18938) from the state of San Luis Potosí. *Pteridium arachnoides* (Kaulf.) Maxon (Palacios-Rios and V. Rico-Gray 3503) and *Cnidocaulis barbaceus* I. M. Johnston (J. I. Calzada, 366-7) from the state of Veracruz. *Anredera scandens* L. Moq. (Bye and Linares 18177) from Distrito Federal. *Commelina erecta* L. (Bye and Linares 17984) from the state of Tlaxcala. *Galphimia glauca* Cav. (Bye and Meraz, 18105) from the state of Guerrero. *Kohleria deppiana* (Schlecht and Cham.) Fritsch (Bye and Linares 17742),

Tournefortia hirsutissima L. (Bye and Linares 17744), *Cnidocaulis tehuacanensis* Breckon (J. A. Zavala 63) and *Swietenia humilis* Zucc. (Bye and Linares 18824) from the state of Puebla. *Malmiea depressa* (Baill.) R. E. Fries (Anaya 1993-2), *Matopium brownii* (Jacq.) Urban (Anaya 1993-1) and *Stemorrhynchos lanceolatus* (Aubl.) L. C. Richard (Anaya 1993-3) from the state of Quintana Roo.

Preparation of crude drug extracts

Biological (microbiological and cytotoxicity) studies were performed with the extracts of the dried plants prepared as follows: 100 g of the finely ground material were macerated at room temperature with methanol (500 ml) or chloroform (500 ml). The resulting extracts were evaporated under vacuum to a thick residue.

Microorganisms

The microorganisms used in the present study were obtained from ATCC: *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 10536), *Pseudomonas aeruginosa* (ATCC 27853), *Candida albicans* (ATCC 10231), *Aspergillus niger* (ATCC 16888) and *Trichophyton mentagrophytes* (ATCC 9129).

Antimicrobial assay

Tryptic soy agar (DIFCO) was used for bacterial cultures and Sabouraud agar (DIFCO) supplemented with glucose (4%) for yeast and molds. Tryptic soy broth (DIFCO) and Sabouraud-glucose agar (DIFCO) were used for MIC determinations.

The crude extracts were evaluated for qualitative antimicrobial activity using two different diffusion assays: a gradient plate diffusion procedure (Cooper, 1972) and the disc diffusion assay (Vanden Berghe and Vlietinck, 1991; Rios et al., 1989; Linton, 1983). Determination of minimum inhibitory concentration (MIC) was accomplished by the dilution technique (Vanden Berghe and Vlietinck, 1991; Sahm and Washington, 1991).

The gradient plate diffusion procedure was performed as follows: an amount of extract, enough to obtain a concentration of 1 mg/ml in agar, was dissolved in 0.5 ml of methanol. This solution was poured into a Petri dish which was placed on a tilted surface, in order to obtain solidification with a concentration gradient. After solidification 10 ml of the same agar medium was added and left to solidify horizontally. The surface of the agar medium was inoculated (by stroke) with an appropriate suspension of microorganism, containing 10^6 bacteria/ml, in the case of the bacteria. For fungi a suspension of spores with a turbidity equivalent to Mc Farland tube N° 0.5 was prepared (Shadomy and Pfaller, 1991).

The disc diffusion assay was performed as follows: two

layer plates were seeded with a suspension containing 10^6 bacteria/ml of an overnight broth culture. Disc papers having a diameter of 13 mm were placed on the seed layer and impregnated with 100 μ l of test solution to obtain concentrations of 500 and 1000 μ g per disc. The crude extracts were assayed as methanolic solutions of 10 mg/ml and 5 mg/ml. The plates were then incubated at 37 °C for 24 h except for *C. albicans*, *A. niger* and *T. mentagrophytes*, which were incubated at 28 °C.

For each organism a positive control was employed: ampicillin sodium salt (Sigma) (1 μ g/disc) for *S. aureus*, *E. Coli* and *P. aeruginosa*; streptomycin sulfate (Sigma) (10 μ g/disc) for *B. subtilis*; amphotericin B (Sigma) (15 μ g/disc) for *C. albicans* and *A. niger*, and for *T. mentagrophytes*, griseofulvin (Sigma) (8 μ g/disc).

The diameters of the resulting inhibition zones were measured. As each experiment was performed in triplicate, the means of the diameters for inhibition zones were determined.

For quantitative antimicrobial assays, each extract was dissolved in methanol (10 mg/ml). Different volumes of this solution were added aseptically to 1 ml of tryptic soy broth and 1 ml of bacterial culture (10^6 bacteria/ml) to produce concentrations ranging from 1000 μ g to 10 μ g/ml. After overnight incubation the MIC was determined. A series of three determinations were run for each organism.

To test the quantitative antimicrobial activity in the case of *T. mentagrophytes*, the agar dilution method was used (Sahn and Washington, 1991; Vanden Berghe and Vlietinck, 1991). The plant extract was dissolved in methanol

Table 1. Screening for antimicrobial activity of crude drug extracts derived from 28 medicinal plants by the gradient agar plate diffusion procedure.

Species	Family	Part used ^a	Microorganisms ^b						
			1	2	3	4	5	6	7
<i>Acalypha phleoides</i>	Euphorbiaceae	WP	-	-	-	-	-	-	-
<i>Anacardium scandens</i>	Basellaceae	RZ	-	-	-	-	-	-	-
<i>Bunchosia lindneriana</i>	Malpighiaceae	SB	-	-	-	-	-	-	-
<i>Bunchosia lindneriana</i>	Malpighiaceae	LF	-	-	-	-	-	-	-
<i>Cardiospermum cordatum</i>	Sapindaceae	FT	-	-	-	-	-	-	-
<i>Ciguarrilla mexicana</i>	Rubiaceae	LF	-	-	-	-	-	-	-
<i>Cnidocaulis herbacea</i>	Euphorbiaceae	WP	-	-	-	-	-	-	-
<i>Cnidocaulis tabacensis</i>	Euphorbiaceae	WP	-	-	-	-	-	+	-
<i>Commelina arcta</i>	Commelinaceae	WP	-	-	-	-	-	-	-
<i>Coryza filaginoides</i>	Asteraceae	WP	-	-	-	-	-	-	-
<i>Datura lanosa</i>	Solanaceae	LF	++	+++	-	+	-	+	-
<i>Dryssodia papposa</i>	Asteraceae	WP	-	-	-	-	-	++	-
<i>Elytaria imbricata</i>	Asteraceae	WP	-	-	-	-	-	-	-
<i>Galphimia glauca</i>	Malpighiaceae	WP	-	-	-	-	-	-	-
<i>Geranium ulveum</i>	Geraniaceae	FL	+	-	-	-	-	++	-
<i>Helianthella quinquevervis</i>	Asteraceae	RT	-	-	-	-	-	+++	+
<i>Heliopsis longipes</i> [*]	Asteraceae	RT	+++	-	-	-	-	++	-
<i>Ilex rubra</i> S. Wats	Aquifoliaceae	WP	-	-	-	-	-	-	-
<i>Kobleria jaysonii</i>	Commelinaceae	AP	-	-	-	-	-	-	-
<i>Lepidium virginicum</i>	Cruciferaeae	WP	-	-	-	-	-	-	-
<i>Malmia depressa</i>	Annonaceae	SB	-	-	-	-	-	-	-
<i>Malmia depressa</i> [*]	Annonaceae	SB	-	-	-	-	-	+++	-
<i>Metopium brunei</i>	Anacardiaceae	WD	++	-	-	-	-	-	-
<i>Metopium brunei</i>	Anacardiaceae	SB	-	-	-	-	-	-	-
<i>Periyle batopilensis</i>	Asteraceae	WP	-	-	-	-	-	-	-
<i>Plumbago scandens</i>	Plumbaginaceae	LF	-	-	-	-	-	-	-
<i>Psalea trifoliata</i>	Rutaceae	SB	-	-	-	-	-	+++	-
<i>Psidium arachnoidium</i>	Derrisadriaceae	WP	-	-	-	-	-	-	-
<i>Stenonrynchus lanceolatus</i>	Orchidaceae	WP	+++	+++	-	-	-	+	+
<i>Sivastonia humilis</i>	Meliaceae	SD	-	-	-	-	-	-	-
<i>Thunbergia hirsutissima</i> [*]	Botaginaceae	SB	-	-	-	-	-	++	-

^a (WP) Whole plant; (RZ) Rhizome; (SB) Stem bark; (LV) Leaves; (FT) Fruit; (FL) Flowers; (RT) Roots; (AP) Aerial parts; (WD) Wood; (SD) Seeds.

^b (1) *Staphylococcus aureus*; (2) *Bacillus subtilis*; (3) *Escherichia coli*; (4) *Pseudomonas aeruginosa*; (5) *Aspergillus niger*; (6) *Trichophyton mentagrophytes*; (7) *Candida albicans*.

(-) Culture growth more than 3/4 of the Petri dish. (+) Culture growth of 3/4 of the Petri dish diameter. (++) Culture growth of 1/2 of the Petri dish diameter. (+++) Culture growth of 1/4 of the Petri dish diameter.

^{*} Chloroform extract.

Table 2. Antimicrobial activity of some crude drug extracts derived from eleven medicinal plants by the diffusion disc agar plate and dilution methods.

Species	Inhibition zones (mm) ^a						MIC (µg/ml) Microorganism ^b		
	1000 µg			500 µg			1	2	3
	1	2	3	1	2	3			
<i>Cnidococcus toboucanensis</i>			19			16			400
<i>Datura lanosa</i>	21	26	19	18	21	17	230	80	> 1000
<i>Dysodia papposa</i>			17			13			1000
<i>Geranium niveum</i>	14		13	13		13	> 1000		> 1000
<i>Helianthella quinqueveneris</i>			31			21			300
<i>Heliopsis longipes</i>	26		20	22		14	400		80
<i>Malmes depressa</i> (stem bark)	13	17	13	13	13	13		450	1000
<i>Malmes depressa</i> (stem bark)*	17	21	33	15	17	22	400	160	300
<i>Metopium brownii</i> (wood)	15			13			> 1000		
<i>Ptelea trifoliata</i> (wood)			17			13			750
<i>Ptelea trifoliata</i> (stem bark)			15			13			750
<i>Stemotrychus lanceolatus</i>	13	15	13	13	13	13	> 1000	1000	> 1000
<i>Tournefortia bicucullata</i> *			20						750

^a Disc diameter 13 mm. ^b (1) *Staphylococcus aureus*; (2) *Bacillus subtilis*; (3) *Trichophyton mentagrophytes*. * Chloroform extract.

Table 3. Results of Brine shrimp lethality and cytotoxic activity.

Plant Material	Brine shrimp lethality LC ₅₀ (µg/ml)	ED ₅₀ (µg/ml) Cell line		
		HT-29 ^a	MCF-7 ^b	A-549 ^c
<i>Ilex rubra</i>	40.63	29.33	49.13	31.28
<i>Ptelea trifoliata</i> (Stem bark)	63.06	ND	ND	ND
<i>Datura lanosa</i>	63.66	ND	ND	ND
<i>Dysodia papposa</i> *	40.01	30.25	48.24	50.46
<i>Geranium niveum</i>	88.46	29.33	84.65	49.89
<i>Parityle batopilensis</i>	815.21	> 100	> 100	> 100
<i>Anradant scandens</i>	430.82	> 100	> 100	> 100
<i>Coryza filaginoides</i>	342.05	30.98	> 100	> 100
<i>Heliopsis longipes</i>	58.65	ND	ND	ND
<i>Helianthella quinqueveneris</i>	6.90	> 100	13.10	36.46
<i>Metopium brownii</i> (wood)	214	> 100	> 100	> 100
<i>Metopium brownii</i> (Stem bark)	104	> 100	> 100	> 100
<i>Malmes depressa</i> (Stem bark)*	62.46	20.96	20.86	20.57

^a Colon adenocarcinoma, ^b Breast carcinoma, ^c Lung carcinoma, * Chloroformic extract. Roots: (AF) Aerial parts; (WD) Wood; (SD) Seeds.

and an aliquot of this solution (enough to obtain concentrations ranging from 1000 to 10 µg/ml) was mixed with dextrose Sabouraud agar (which was maintained at 45 °C) and with a suspension of spores of *T. mentagrophytes* (10⁶ spores/ml). Control agar plates were always used in parallel and treated similarly. After incubation at 28 °C for 7 days, the agar plates were observed. MIC represents the lowest concentration of plant extract at which complete inhibition occurs.

Brine shrimp and cytotoxic bioassays

The brine shrimp assay was performed as previously described (Meyer et al., 1981; McLaughlin, 1991; Wah, 1993). Cytotoxicity against three human solid tumor cells

was measured at the Cell Culture Laboratory, Purdue Cancer Center, Purdue University, for the A-549 (Lung carcinoma), MCF-7 (Breast carcinoma) and HT-29 (Colon adenocarcinoma), with adriamycin as a positive control in seven day assays (Anderson et al., 1991).

Results and Discussion

Thirty one crude plant extracts obtained from medicinal plants used in Mexican folk medicine as anti-infective agents were tested against several standard microorganisms. The selected microorganisms were predictive of potential applications against human diseases caused by bacteria, yeasts and molds.

The extracts were first evaluated by the gradient plate diffusion assay and the results are summarized in Table 1. The test microorganisms showed strong differential sensitivity to the plant extracts. The most sensitive was *T. mentagrophytes* which was inhibited by 10 (32%) of the extracts. *S. aureus* was inhibited by five extracts (16%) while *B. subtilis* and *C. albicans* by two (6%). As with most plant extracts the Gram negative bacteria were not affected by any of the test extracts. The extracts of *D. lanosa* and *S. lanceolatus* showed the widest spectrum of activity.

The active extracts from Table 1 were thereafter evaluated by the agar disc diffusion method and the zones of inhibition are given in Table 2. In this assay the active extracts were only tested against the susceptible microorganism according to the results indicated in Table 1. Also, the minimal inhibitory concentration (MIC) of these active extracts was determined and summarized in Table 2.

According to the results shown in Table 2, *Cnidiosculus tehucanensis*, *Datura lanosa*, *Helianthella quinquevenis*, *Heliopsis longipes* and *Malmia depressa* have the potential to generate novel anti-infective agents. In the case of *M. depressa* the active principle seems to be of a lipophilic nature since the chloroform extract exhibits better activity than the methanol extract. In addition, the use of these species as anti-infective agents in folk medicine can be explained on the basis of the present results.

The remaining species from Table 2 were not active at concentrations $\leq 1000 \mu\text{g/ml}$ in the tests performed in this investigation. Further work may show that many of these species contain active compounds, as has been the case of *Ptelea trifoliata* (Mitscher et al., 1975). The antimicrobial activity previously described for *Ptelea trifoliata*, *P. scandens* and *L. virginicum* could not be reproduced in the present investigation. These differences could be attributed to a variation in the content of active secondary metabolites due to geographic variations.

The brine shrimp (*Artemia salina* Leach) lethality assay is considered a useful tool for preliminary assessment of cytotoxicity (Meyer et al., 1981; Anderson et al., 1991; McLaughlin, 1991). Therefore, the 31 extracts listed in Table 1 were tested using this bioassay. Of these extracts only 13 (42%) exhibited a significant activity (Table 3). The remaining crude extracts not listed in Table 3 exhibited LC_{50} values higher than $1000 \mu\text{g/ml}$. The cytotoxicity of some these extracts was evaluated in three different cell lines: A-549 (Lung carcinoma), MCF-7 (Breast carcinoma) and HT-29 (Colon adenocarcinoma). From the results summarized in Table 3 it is clear that only the methanol extract of *Helianthella quinquevenis* displayed significant cytotoxicity effect against the MCF-7 cell line (ED_{50} 13.10 μml). Therefore *Helianthella quinquevenis* may be considered a candidate for the discovery of cytotoxic and/or anticancer agents.

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Antimicrobial properties of alkamides present in flavouring plants traditionally used in Mesoamerica: affinin and capsaicin

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Abstract

The bioactive amides affinin and capsaicin isolated respectively from *Heliopsis longipes* roots and *Capsicum spp* fruits, were assayed for activity against *Escherichia coli*, *Pseudomonas solanacearum*, *Bacillus subtilis* and *Saccharomyces cerevisiae* suspension cultures. The alkamide affinin inhibited growth of *E. coli* and *S. cerevisiae* at concentrations as low as 25 [Image] g/ml. Higher concentrations of affinin were necessary to inhibit growth of *P. solanacearum* and *B. subtilis*. However, high concentrations of capsaicin only retarded the growth of *E. coli* and *P. solanacearum*, whereas growth of *B. subtilis* was strongly inhibited and that of *S. cerevisiae* was initially enhanced. Results are discussed in relation to previous reports concerning crude extract and to the molecular structures of the bioactive compounds.

Author Keywords: Affinin; Capsaicin; Alkamides

Article Outline

1. Introduction

2. Methodology

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2.3. Growth of microorganisms

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Acknowledgements

References

1. Introduction

Affinin (*N*-isobutyl-2*E*,6*Z*,8*E*-decatrienamide) and capsaicin (*N*-({4-hydroxy-3-methoxyphenyl)-methyl}-8-methyl-6*E*-nonamide) are two tissue specific alkamides present in the roots of *Heliopsis longipes* (chilmecatl) and the fruits of *Capsicum* spp. (chili) respectively. Both plants have been used traditionally as flavouring components and medicinal agents by inhabitants of Mesoamerica. In Mexico, *Capsicum* species have been used by several cultures in such high frequency as to be considered an important component of diet. *Capsicum* together with pepper (*Piper* spp) are the most important species cultivated today (For a recent review on alkamides present in *Piper* spp D'angelo, et al., 1997).

Affinin is the main bioactive component present in the roots of *Heliopsis longipes*. These roots have been used in Mexico as a spice, insecticide and in traditional medicine since the Nahuatl civilizations. The distribution of this species is restricted, being endemic to the Sierra Gorda in the central area of Mexico. From this species a large supply of roots were obtained which provided an abundance of the insecticidal amide devastating the wild population of *H. longipes* (Fisher, 1957). More recently, in a general screening of traditionally used Mexican medicinal plants, it was observed that *H. longipes* extracts possessed significant antiseptic properties, therefore supporting the ethnomedical uses of this species (Gutierrez-Lugo et al., 1996).

Affinin is the main alkamide present in the five species of the Helianthese containing olefinic alkamides: *Wedelia parviceps* Blake, *Acmella ciliata* H.B.K., *A. oleracea* L., *A. oppositifolia* (Lam.) Jansen and *H. longipes* (Gray) Blake. It is present in higher quantities in the roots of *H. longipes* than in any other species containing alkamides (Molina-Torres, et al., 1996). Even though alkamides are characteristic and distributed among several species of the Asteraceae,

similar structures can be found in the Aristolochiaceae, the Piperaceae and the Rutaceae (Greger, 1984). It is possible that this distribution of similar structures is reflective of the protective function of the alkamides.

Pharmacological information on *Capsticum* species is available (Virus and Gebhart, 1979 and Monserenusorn, et al., 1982, Cordell and Araujo, 1993 and Surh and Lee, 1996). Recently the capsaicin receptor has been cloned from mouse dorsal root ganglion sensory neurons (Caterina, et al., 1997). However, information about the antimicrobial activity of capsaicin is scarce and available information is obscured by the use of crude extracts containing different amounts of active components (Cichewicz and Thorpe, 1996). Capsaicin is the main constituent of a family of compounds denominated capsaicinoids which possess the same amide structure but vary in chain saturation, substitution and length (Constant, et al., 1995).

The chemical structures of these families of amides, alkamides and capsaicinoids, have some similarities. Both are amides of medium length fatty acids, differing mainly in the chain unsaturation and the amine moiety. The Asteraceae family contains most of the reported alkamides including acetylenic and olefinic structures (Greger, 1984; Christensen and Lam, 1991 and Christensen, 1992). The majority of these compounds contain the 2*E* unsaturation present in affinin. Alkamides are found mainly in roots throughout the life of the plant. Capsaicin and the capsaicinoids differ from the mentioned alkamides in the following structural aspects and consequently in their biological activity: (a) Neither capsaicin nor the accompanying capsaicinoids contain the 2*E* unsaturation; (b) the tissue specificity is associated with the placentas in the fruits; and (c) the amine moiety of the amide is the same for all the capsaicinoids; *N*-4-hydroxy,3-methoxy, phenylmethyl amide. These differences may be related to different biosynthetic pathways and functions. Capsaicin and the capsaicinoids are restricted to the genus *Capsticum* (Cordell and Araujo, 1993).

Capsticum annuum extracts have been found to have activity against the cercaria of *Schistosoma mansoni* which is estimated to have infected over 200 million people world-wide, however it is not possible to determine if this activity is related to the presence of capsaicinoids or to other compounds, as the toxicity was assayed with crude fruit extracts (Frischkorn, et al., 1978). Affinin and other isobutylamides from Asteraceae are active against this parasite (Johns, et al., 1982).

Alkamides and capsaicinoids produce specific responses in taste and some neurophysiological responses (Kadir, et al., 1989), but there is no specific information on their respective toxicity towards microorganisms. This stimulated our interest in comparing the toxicity of these structurally related amides over a range of lower organisms.

2. Methodology

H. longipes (Gray) Blake (Asteraceae) specimens, as authenticated by Dr J. Rzedowski, Instituto de Ecología, Pátzcuaro, Michoacán, were collected in Sierra Gorda in the state of Guanajuato, Mexico at an altitude of between 2000 and 2500 meters above sea level. Voucher specimens (*H. longipes* JMT, IED) were deposited at the above mentioned institution. Affinin was isolated from plant roots as described previously (Molina-Torres, et al., 1995). Capsaicin was purchased from Sigma (8-methyl-*N*-vanillyl-6-noneamide cat. M-8147). This reagent analysed by GC/MS mostrate to include: capsaicin 74.63%, dihydrocapsaicin 15.79%, nordihydrocapsaicin and

nonivamide 4.43%.

2.1. Affinin extraction and purification

Dry roots were ground and extracted with absolute ethanol in a continuous extraction system (Tecator, Soxtec System HT 1043 Extraction Unit) for 2 h at 80°C. The ethanolic extract was freed from solvent in a rotary evaporator at 60°C under reduced pressure. This crude extract was then purified by TLC (20×20 cm silica gel on polyester plates Sigma cat. T-6770) with a solvent system of hexane:ethyl acetate (2:1 v/v). After development, plates were air dried and visualized under UV light. A dark band with R_f 0.5 was collected, reextracted with ethyl acetate, freed from solvent under a stream of nitrogen and further purified by HPLC on a Microbondapak C18 column eluted with acetonitrile:water 40:60 as described by (Bauer, et al., 1988). Purity was monitored by CG (Hewlett Packard 5890 Gas Chromatograph Series II) equipped with Ultra-2, 30 m capillary column and a coupled mass selective detector (Hewlett Packard 5972 series). This purification procedure was enough for affinin to showed up as a single peak in the chromatogram with mass spectra in agreement with (Yasuda, et al. (1980).

2.2. Microorganisms

The microorganisms used in the present study were: *E. coli* (DH5 [Image] Difco ATCC 25922), *P. solanacearum*, *B. subtilis* (ssp Kodiak) and *Saccharomyces cerevisiae* (wild strain 288C). All isolates were obtained from a collection located at CINVESTAV, U. Irapuato.

2.3. Growth of microorganisms

Liquid cultures were grown at 37°C in PDB medium with vigorous agitation in a rotary shaker at 280 rpm. All inocula were taken from cultures growing exponentially in the same medium. Growth was followed by determining turbidity at 650 nm in a Cary-Varian E3 spectrophotometer. Amides, affinin or capsaicin, when used were added to the flasks at a specific concentration as aliquots in ethanolic solution. The ethanol was then evaporated and the residue dissolved in the culture medium with warming and the addition of 500 [Image] g/ml of Triton X-100 in all cases as described by Kass (1968). At the concentration used the detergent had no effect on the growth rate of the bacteria. Flasks containing 15 ml of PDB and the different concentrations of the amide, were inoculated with 0.3 ml of the microorganism culture. Aliquots were collected at defined intervals and microorganism growth followed at 650 nm in a Cary-Varian 3E spectrophotometer. Graphical results presented in Fig. 1, Fig. 2, Fig. 3 and Fig. 4 represent the average of at least four replicates and the bars indicate the standard deviation of these values.

[Image] (6K)

Fig. 1. Effect of natural alkamides on the growth of *E. coli* liquid cultures. (a) Affinin: [Image] Control (0 [Image] g/ml); [Image], 25 [Image] g/ml; [Image], 50 [Image] g/ml; [Image] 75 [Image] g/ml. (b) Capsaicin: [Image] Control (0 [Image] g/ml); [Image], 200 [Image] g/ml; [Image], 300 [Image] g/ml.

[Image] (6K)

Fig. 2. Effect of natural alkamides on the growth of *P. solanacearum* liquid cultures. (a) Affinin: [Image] Control (0 [Image] g/ml); [Image], 50 [Image] g/ml; [Image], 75 [Image] g/ml; [Image], 150 [Image] g/ml. (b) Capsaicin: [Image] Control (0 [Image] g/ml); [Image], 75 [Image] g/ml; [Image], 150 [Image] g/ml;

[Image], 300 [Image]g/ml.

[Image] (6K)

Fig. 3. Effect of natural alkaloids on the growth of *B. subtilis* liquid cultures. (a) Affinin: [Image] Control (0 [Image]g/ml); [Image], 50 [Image] g/ml; [Image], 75 [Image]g/ml; [Image], 150 [Image]g/ml. (b) Capsaicin: [Image], Control (0 [Image]g/ml); [Image], 50 [Image]g/ml; [Image], 75 [Image]g/ml; [Image], 150 [Image]g/ml.

(7K)

Fig. 4. Effect of natural alkaloids on the growth of *S. cerevisiae* liquid cultures. (a) Affinin: [Image], Control (0 [Image]g/ml); [Image], 25 [Image]g/ml; [Image], 50 [Image]g/ml; [Image], 75 [Image]g/ml. (b) Capsaicin: [Image], Control (0 [Image]g/ml); [Image], 25 [Image]g/ml; [Image], 150 [Image]g/ml; [Image], 300 [Image]g/ml.

3. Results

The effect of the purified alkaloids on the growth of *E. coli* is presented in Fig. 1. The presence of affinin (Fig. 1(a)) had an effect at concentrations as low as 25 [Image]g/ml. At this concentration after eight hours the inhibition was close to 90%, and other concentrations assayed (50 and 75 [Image] g/ml) almost completely inhibited growth. However, capsaicin (Fig. 1(b)) at concentrations up to 200 or 300 [Image]g/ml only retarded the bacterial growth. The effect of affinin on growth of *Pseudomonas solanacearum* (Fig. 2(a)) was less inhibitory and it was necessary to increase the concentration of this amide to 150 [Image]g/ml in order to observe total inhibition. There was a proportional effect of the amide concentration in the media on growth inhibition. The inhibitory effect of capsaicin was much lower (Fig. 2(b)), since growth was reduced only about 20% with a concentration of 300 [Image]g/ml. In *B. subtilis* cultures, growth was reduced by concentrations of affinin (Fig. 3(a)) as low as 50 [Image]g/ml and totally inhibited with 150 [Image] g/ml. Capsaicin had a stronger inhibitory effect towards *B. subtilis* starting from 25 [Image] g/ml, the minimum concentration assayed (Fig. 3(b)). Finally, *S. cerevisiae* showed a response to the presence of affinin (Fig. 4(a)) similar to that observed in *E. coli* but over a longer time. The effect of capsaicin on *S. cerevisiae* was not clear (Fig. 4(b)), as short term cellular growth was stimulated at concentrations as high as 150 and 300 [Image] g/ml. Capsaicin has no effect on the long term (24 h) growth of this microorganism concentrations as high as 300 [Image]g/ml (data not shown).

4. Discussion

Different *Capsicum* spp. tissue (fruits or leaves) and *H. longipes* root extracts have previously been assayed for antimicrobial activity with a variety of treatments (Cichewicz and Thorpe, 1996, Gutierrez-Lugo et al., 1996), however the presence or absence of many different compounds may have affected the results. The study of purified components may help us to understand at least partially the mechanisms of interaction.

E. coli and *P. solanacearum*, both Gram negative bacteria, showed similar behavior even though the latter was less sensitive to the presence of affinin. The effect of capsaicin on both microorganisms was not significant even at concentrations as high as 300 [Image]g/ml. *B.*

subtilis, a Gram positive bacteria, demonstrated a similar response to affinin as *P. solanacearum* but surprisingly the lowest concentrations of capsaicin 25 [Image]g/mg, completely inhibited its growth.

In recent screening reports, crude extracts of *H. longipes* roots did not show any activity in plate diffusion tests against either *E. coli* or *B. subtilis* (Gutierrez-Lugo et al., 1996). Similarly, an absence of inhibition was observed with fruit extracts of *Capsicum annum* against *B. subtilis* (Cichewicz and Thorpe, 1996). Our results are difficult to compare with those in which discs were used as an experimental model, since the effect will depend on the diffusion of the active compound. Affinin is a hydrophobic molecule, and consequently will not passively migrate in an aqueous medium. Liquid medium is therefore a better choice, keeping in mind that it is important to properly emulsify all components in the medium.

The presence of the 2*E* unsaturation has been associated with the toxicity of alkamides towards insects (Jacobson, 1954). This may also be true for the toxicity results we observed here against bacteria and fungi since the main isomer in unsaturated amides has configuration *E* in this position. However, it is still necessary to confirm the specific toxicity of this isomerism.

It is interesting to compare the structure of affinin with 3-decynoyl-*N*-acetylcysteamine (NAC) (Helmkamp, et al., 1968) and 1-diazo-4-undecyn-2 one (DUO) (Henderson, et al., 1994), the synthetic specific inhibitors of the [Image]-hydroxydecanoyl thioester dehydratase enzyme (FabA). This enzyme is responsible for the reversible introduction of a double bond in the unsaturated fatty acids of *E. coli*, a vital component for the bacterial growth. The active form of the inhibitor is the 2,3-alene. The alene structures of NAC and DUO, together with affinin and capsaicin are presented in Fig. 5.

[Image] (3K)

Fig. 5. Natural alkamides and synthetic inhibitors of the [Image]-hydroxydecanoyl dehydratase. Affinin (*N*-isobutyl-2*E*,6*E*,8*E*-decatrienamide), capsaicin (*N*-{(4-hydroxy-3-methoxyphenyl)-methyl}-8-methyl-6*E*-nonenamide), NAC (3-decynoyl-*N*-acetylcysteamine) in the alene form (2,3-decanodienoil-*N*-acetylcysteamine), DUO (1-diazo-4-undecyn-2 one) in the alene form (1-diazo-3,4-undecenodien-2 one).

In vivo NAC displayed a strong antibacterial activity against *E. coli*, *Salmonella typhumurium* and *P. fluorescens* at a concentration of 10^{-4} M, but had only a slight and transient effect on the growth of *S. cerevisiae* (Kass, 1968). In the present study, the effect of affinin on growth of *S. cerevisiae* under the influence of 25 [Image]g/ml (10^{-4} M) was transient, as cultures recovered within 24 h, but this was not the case with concentrations of 50 [Image]g/ml or more where the inhibitory effect was irreversible (Fig. 4(a)). The activity of these synthetic inhibitors has been attributed to the specific recognition of chain length and irreversible binding of the alene to the catalytic site (Leason, et al., 1996). Affinin, and other olefinic alkamides, having structural similarities would be recognized by the active site but would not be able to bind irreversibly as the acetylenic inhibitors.

The inhibitor NAC does not affect the growth of mammalian hepatoma cell cultures (Kass, 1968) due to the absence of the anserobic path for unsaturated fatty acid biosynthesis in mammals. Similarly, the root extracts of *H. longipes* did not show cytotoxic activity on human solid tumor cells (Gutierrez-Lugo et al., 1996). Other 2*E* unsaturated alkamides present in pepper (*Piper* spp.) (Banerji, et al., 1974 and Mcferren and Rodriguez, 1998), which has alkamides as

flavouring components, may show similar antibacterial properties.

The antifungal activity of affinin or any other alkamide has not been reported previously. Recently however, this activity has been observed in sulphur containing amides from two species of *Glycosmis*. These amides contain a 2E unsaturation as in the alkamides (Greger et al., 1996).

5. Conclusions

The alpha unsaturated natural alkamides may have more than one mechanism of interaction with different tissue. They are toxic to microorganisms, showing some differences in toxicity between Gram positive and Gram negative bacteria. The toxicity mechanism of these compounds in other lower organisms such as the insects has been suggested to be a neural stimulating effect although the toxicity of crude root extracts against mammals is low (Romero et al., 1989) as was observed with *Capsicum* extracts. It has been observed that amides may give sweet, bitter or tasteless sensation depending on the acidic and amine constituents (Belitz et al., 1983). However amides in plants are largely responsible for the 'hot' taste in fruits, such as *Capsicum* spp., *Piper* spp., and in roots of many species in the tribe Hellantheae (Greger, 1984). These plants have been used for centuries by different cultures in America, Europe and Asia. However, a structural relation with a specific enzymatic inhibition and toxicity requires further study including other organisms as cold blood vertebrates as some unsaturated amides have been observed to be toxic to fish (Mcferren and Rodriguez, 1998 and Johns et al., 1982).

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**Acknowledgment of receipt of *Heliopsis longipes* voucher specimen by the
California Academy of Sciences**



January 20, 2000

Dr. Philip E. Wolfson
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Dear Dr. Wolfson:

On behalf of the Botany Department I wish to thank you very much for the two herbarium specimens of *Heliopsis longipes* that you recently. Although we have an excellent collection of specimens from Mexico, the collections you presented us are our first of this particular species for us although we have many specimens of other species of Mexican *Heliopsis*. So, your collections are a very important addition to our herbarium.

Sincerely yours,

Bruce Bartholomew
Collections Manager