

.1' PN 1136-854  
96448 8.068

FINAL REPORT

COVERING PERIOD: JUNE 1<sup>st</sup> 1989 DECEMBER 31<sup>st</sup> 1991

SUBMITTED TO THE OFFICE OF THE SCIENCE ADVISOR  
U.S. AGENCY FOR INTERNATIONAL DEVELOPMENT

CHARACTERIZATION OF THE ACTIVE PRINCIPLES, MECHANISM OF ACTION AND  
POSSIBLE MUTAGENIC ACTIVITY OF WOUND HEALING PLANTS

PRINCIPAL INVESTIGATOR : ABRAHAM J. VAISBERG, Ph.D.  
DEPARTAMENTO DE MICROBIOLOGIA  
UNIVERSIDAD PERUANA CAYETANO HEREDIA

CO-INVESTIGATORS: ALFONSO ZAVALETA, M.D., Ms.C.  
UNIVERSIDAD PERUANA CAYETANO HEREDIA

GERALD B. HAMMOND, Ph.D.  
UNIVERSITY OF MASSACHUSETTS, DARTMOUTH

HOLGER MALDONADO, Ms.C.  
UNIVERSIDAD PERUANA CAYETANO HEREDIA

IRMA D. FERNANDEZ, Bs.C., LIC.  
UNIVERSIDAD PERUANA CAYETANO HEREDIA

PROPOSAL NUMBER 8.068  
PROJECT NUMBER 936-5542.02  
GRANT NUMBER 936-5542-G-00-918-00

PROJECT DURATION: JUNE 1<sup>st</sup> 1989 THROUGH DECEMBER 31<sup>st</sup> 1991

REC'D IN R&D/R

JUL 13 1995

CC: BOSTIA

## TABLE OF CONTENTS

Page

1. EXECUTIVE SUMMARY	3
2. RESEARCH OBJECTIVES	4
3. MATERIALS AND METHODS	4
3.1. Collection of Plant Material	4
3.2. Preparation of Extracts for the Pharmacological Screening	6
3.3. General Pharmacological Evaluation and Mouse Lethality.	6
3.4. Recording of Blood Pressure.	6
3.5. Effect on Smooth Muscle from the Ileum and Uterus of the Rat.	6
3.6. Effect on Capillary Permeability in Rats.	7
3.7. Determination of the Cicatrizant Activity of the Extracts In Vivo	7
3.8. Cell Proliferation and Toxicological Studies of the Plant Extracts.	8
3.9. Testing the Possible Mutagenic Effect of the Extracts.	8
4. RESULTS	9
4.1. Pharmacological Screening.	9
4.2. Determination of the Cicatrizant Activity of the Plant Extracts In Vivo.	9
4.3. Effect of the Extracts from the Plants on the Blood Pressure.	9
4.4. Acute toxicity screening of the Plant Extracts.	10
4.5. Effect on Capillary Permeability in Rats.	10
4.6. Effect on Smooth Muscle from the Ileum and Uterus of the Rat.	10
4.7. Cell Proliferation Studies of the Extracts from <i>Peperomia galioides</i> and <i>Anredera diffusa</i>	10
4.8. Studies on the Possible Mutagenic Effect of the Extracts from <i>Peperomia galioides</i> and <i>Anredera diffusa</i>	10
4.9. Studies on the isolation and characterization of the active principles from the plant extracts.	10
4.10. Studies on the Isolation and Characterization of the Active Principles from the Plant Extracts.	11
5. IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER.	21
6. PROJECT ACTIVITIES/OUTPUTS	21
7. PROJECT PRODUCTIVITY	23
8. FUTURE WORK	23
9. LITERATURE CITED	23

## 1. EXECUTIVE SUMMARY

Pharmacological preparations prescribed for the treatment of wounds in the process of healing are either antiseptics, antibiotics or protective ointments. While this is so with XX<sup>th</sup> Century Medicine, Popular medicine in our country has been employing plant preparations as wound healing agents on superficial wounds and in some cases even on internal wounds like gastric ulcer.

The purpose of our work was to obtain conclusive evidence on whether some of these plants accelerate the wound healing process or not. For this reason we have tested in animal models the wound healing activity of nine plant species that come from the coast, the mountains and the jungle of Perú.

Our results showed that the extracts from three of the plants under study: *Peperomia galioides*, *Anredera diffusa*, and *Jatropha curcas* accelerated significantly the wound healing process. Furthermore, we have also shown that the extracts from the first two plants have no mutagenic activity. The study of the possible mutagenic activity of the third one will be done in the future.

Through this project we have strengthened the research capacity of our laboratories and have been able to train students and professionals from Lima and from the Provinces in the techniques required to study natural products. Furthermore, we have consolidated the formation of a multidisciplinary team covering the all the aspects of this type of research. Dr. Gerald B. Hammond, Associate Professor at the University of Massachusetts, Dartmouth, is an important part of this team.

## 2. RESEARCH OBJECTIVES

Traditional flora has been an important source for the discovery of new medicines. It is estimated that about 80% of the world's population still rely on traditional medicine practices which are based on the use of plants and the knowledge of their healing properties. Approximately 25% of all pharmaceutical products used in the U.S.A. today contain ingredients originally derived from wild plants (Cordell, 1990; Akhtar, 1991).

The abundance and diversity of the flora that exists in the Amazon jungle and in the mountains of Peru, make research on natural products a promising area of scientific endeavor. Our research was conducted in order to improve human well-being while enhancing economic growth and conserving biodiversity. With these in mind, our objectives were to study in animal models the cicatrizant activity of the different plant extracts, used by traditional medicine, in order to obtain conclusive evidence on whether they accelerate the wound healing process or not. We further selected the most active ones for the characterization of their active principles, study their mechanisms of action and find out if they have mutagenic activity.

In this context, the discovery and introduction of cicatrizant agents in the pharmacopeia is important for development for the following reasons:

- a) The acceleration of the healing process will reduce the time of hospitalization and outpatient costs.
- b) The extraction and commercialization of these compounds will mean the creation of new industries that will utilize the natural resources of the area and will also create new working opportunities for the local people.
- c) By proving the medicinal value of these plants we will contribute to the rationale for preserving biological diversity since by showing the economic value of the forest we would increase the likelihood of conservation.

Previous work done on plants with medicinal potential in our country have been isolated trials by botanists, chemists or pharmacologist. These researchers investigated only part of the problem, in some cases isolating and purifying from the extracts as many compounds as possible and without an activity-directed isolation procedure.

What we have accomplished is to form a multidisciplinary team covering all the aspects involved in the research. As could be seen from our results, we have validated the wound healing activity of three of the plants used by traditional medicine. For two of these plants we have also showed that they do not have mutagenic activity. Also through the general screening of the studied plants we have identified other important activities that will require follow up in the future.

The possible mutagenic activity of the active extracts has not been addressed previously by any investigator.

## 3. MATERIALS AND METHODS

### 3.1. Collection of Plant Material

Ten trips were organized in order to collect the following plant specimens for our work:

*Plants from the coast and the Mountains*

Mentzelia cordifolia Dombey. This bush belonging to the Loasaceae family and commonly known as "Anhuaraté" was found in the dry sheltered hillsides of the Cordillera Negra in the Department of Ancash, Province of Carhuaz, District of Jungay at 2,700 m of altitude.

Muehlenbeckia tamnifolia, a bush belonging to the Poligonaceae family and commonly known as "Pumahuascan" was found on the sides of the small streams of the Cordillera Negra in the Department of Ancash, Province of Carhuaz, district of Pucallaca at 2633 m of altitude.

Peperomia galioides. This succulent herb from the Piperaceae family commonly known as "Congona" was found growing between rocks in sheltered areas of the ravine of Vicus in the Department of Ancash, Province of Carhuaz, District of Marcará at 3,900 m of altitude. This plant was also found growing as an epifita over the rocks and trees of "tara" at the Lomas de Lachay area, at 700 m of altitude in the Department of Lima, Province of Chancay.

Mutisia acuminata var. *acuminata* R et. P. This bush from the Asteraceae family commonly known as "Chirchircuma" was found on the hillsides of the Rimac valley in the Department of Lima, Province of Huarochirí, District of Surco at 2,000 m of altitude.

Anredera diffusa. The latter plant belongs to the family Basellaceae and commonly known as "Lloto" and was collected from the area of Ollantaytambo, Department of Cusco, at 2,800 m of altitude.

Jatropha curcas, this tree belonging to the Euphorbeaceae family is commonly known as "piñon blanco" and grows at sea level. It was collected in the Department of Lima, province of Lima.

*Plants from the Jungle.*

Eleutherine bulbosa, a bush belonging to the Iridaceae family commonly known as "Piri-Piri" was collected from the vicinity of the rivers Amazonas and Nanay.

Himathanthus sucuba, a tree belonging to the Apocinaceae family commonly called by the natives "Bellaco-Caspi" was collected from the vicinity of the rivers Amazonas and Nanay.

Spondias mombin, a tree belonging to the Anacardiaceae family and commonly called by natives "Ubos" was collected from the vicinity of the rivers Amazonas and Nanay.

These last three species were collected by Dr. Franklin Ayala from the Herbarium Amazonense of the Universidad Nacional de la Amazonia, in Iquitos.

Complete voucher specimens for the taxonomical identification of the plants were prepared. Voucher specimen from the plants from the coast and the mountains have been deposited at the Museo de Historia Natural Javier Prado. For the plants from the jungle, voucher specimens were prepared by Dr. Ayala and one from each plant remains at the Herbarium Amazonense for future reference.

### 3.2. Preparation of Extracts for the Pharmacological Screening

Plant extracts were made following the methodology used by the local people in order to test their biological activity. The following preparations were made and used:

From Peperomia galioides and Mutisia acuminata: aqueous extract of the stems and the leaves of the plant.

From Mentzelia cordifolia and Muehlenbeckia tamnifolia : a filtrate, that resulted after the decoction of the woody pulverized stems in distilled water.

From Anredera diffusa: a filtrate, that resulted after the decoction of the leaves in distilled water.

From Spondias mombin (fruits): a filtrate, that resulted after the decoction of the fruits in distilled water.

From Spondias mombin (cortex), Himatanthus sucuba, and Eleutherine bulbosa: a filtrate, that resulted after the decoction of the woody pulverized cortex in distilled water.

From Jatropha curcas, the latex from the plants obtained after slashing the cortex was used.

Filtrates from the plant preparations were lyophilized and kept as a powder at  $-20^{\circ}\text{C}$ . For the pharmacological screening milligram quantities of this powder were dissolved in distilled water or saline and used.

### 3.3. General Pharmacological Evaluation and Mouse Lethality.

Experiments were performed on groups of male Balb\c mice whose weights oscillated between 20-25 g. Mice were housed in groups of five and were allowed food and water ad libitum. After several days of adaptation, 10 mice per group were injected intraperitoneally with 0.1 ml/10g (weight) of various doses of a given plant crude extract (between 0.1 and 1.0 mg/g weight for all except for Anredera diffusa. For the latter we used doses between 0.1 and 3.0 mg/g weight of the mouse). Mice were observed for the next 72 hours and the symptomatology and time of death was recorded. LD50 values were calculated by probit analysis (Finney, 1971).

### 3.4. Recording of Blood Pressure.

Male albino rats weighing 300-400 g were anesthetized by i.p. injection of sodium pentobarbital (50-60 mg/kg) prior to surgical procedures. The left common carotid artery was cannulated with polyethylene tubing filled with heparinized saline and connected to a Stathan p23-ID transducer for recording arterial blood pressure. All recordings were made on a Grass Polygraph Model 79D (Grass Instrument Co. Quincy, Massachusetts). The crude or purified extracts were injected into the tail vein of the mice.

### 3.5. Effect on Smooth Muscle from the Ileum and Uterus of the Rat.

Rat ileum preparations: we used segments of 3 to 3.5 cm (n=20). These segments were suspended in an isolated organ bath containing 10 ml of tyrodes solution at  $37^{\circ}\text{C}$ , to which 100% Oxygen was bubbled. Muscle responses were registered with a grass transducer on a P7 grass polygraph.

Rat uterus preparations (De Jalon et al., 1954): longitudinal sections of the uterine horns of 10 non-pregnant rats were cut longitudinally

obtaining two segments of approximately 3.5 cm. These segments were suspended in an isolated organ bath containing 10 ml of DeJalon solution at 37°C, to which 100% Oxygen was bubbled. Both spontaneous and provoked contractions were registered with a Grass transducer on a P7 Grass polygraph.

### 3.6. Effect on Capillary Permeability in Rats.

A capillary permeability test will be carried out according to the method of Milles and Wilhelm (Miles and Wilhelm, 1955) adapted to rats. All experiments will use 300-400 g adult male albino rats. The extracts to be tested will be injected intradermally onto marked sections on the animal's depilated back 90 minutes before an i.v. infusion of 2 ml of 1% Evans Blue dye solution. Twenty minutes after dye injection, the animal will be sacrificed and the dorsal skin removed. The blue spots on the inner surface of the skin will then be measured. At least 10 animals will be used per each test sample and control group, and duplicate injections will be made on the same animal's back. Statistical analysis of differences in the stain diameters will be calculated by the Wilcoxon signed rank sum test (Winer, 1971) which can be used in studies with a large range of observed values.

### 3.7. Determination of the Cicatrizant Activity of the Extracts *In Vivo*

The method used to determine the tensile strength of the wounds was a modification of the one reported by Howes, Sooy and Harvey (Howes et al., 1929). The protocol for a typical "in vivo" cicatrization test was as follows: Male mice (Strain A) of 2-3 months of age whose weights oscillated between 25-30 grams were maintained in a room at 22-25 C and received food and water ad libitum. Before making the wounds, the back of the mice was shaved with surgical clippers at the level of the scapular waist (an area that could not be scratched by the mouse). This same area was then depilated using Opilca (Hoescht). 48 hours later the weight of the mice was measured and they were distributed in groups at random so as the average and the standard error of the average of the weights of the groups were homogeneous. Each mouse was placed in a separate cage with a card that had the following information: number of the animal, initial weight, starting hour and date, duration of the experiment, final weight, wound breaking strength (WBS), observations if any and treatment it received. The latter information was recorded on the card after measuring the WBS of the mouse and right before the statistical analysis was made. All this information was also recorded in a notebook that was utilized at the time the different treatments were administered. The purpose of this procedure was to prevent the operator from making subjective errors when measuring the WBS. Next, mice were anesthetized with vapors of diethyl ether and a 1 cm incision was made perpendicular to the axis of symmetry of the animal and the two borders of the wound were stitched together at its center. Treatment was started immediately, and every 12 hours the compound being tested was applied to the wound, for this purpose 0.05 ml of the solution being tested was applied slowly and directly to the wound by means of a micropipete. The controls received only the solvent in which the compound was diluted. 48 hours later, mice were sacrificed with an ether overdose and the WBS were quantitated by fixing one of the borders of the wound (after

cutting the stitch) while applying a measurable force to the other one.

Once all the WBS were measured and recorded on their respective cards, we recorded the treatment received by each mouse on the same cards. The data was then subjected to statistical analysis using the Student's-t-Test. Values are significant when  $P < 0.05$  (Winer, 1971).

The percentage of activity was calculated according to the following formula:

$$\% \text{ Activity} = \frac{\text{WBSt} - \text{WBSc}}{\text{WBSc}} \times 100$$

WBSt = Average of the force necessary to open the wound of a treated mouse.

WBSc = Average of the force necessary to open the wound of an untreated mouse (Control).

### 3.8. Cell Proliferation and Toxicological Studies of the Plant Extracts.

For this purpose, Balb/c 3T3 cells grown on 25 cm<sup>2</sup> were trypsinized, resuspended in growing medium (Dulbecco's Modified Eagles Medium containing 10% fetal bovine serum, 50 ug/ml gentamycin) and the number of live cells was determined by the trypan blue exclusion method using an hemocytometer to count them (Kruse and Patterson, 1973). Then 100 ul of media containing 2000 cells was plated on each well of a 96 well plate and incubated at 37 C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. 24 hours later, the extracts were added. For this purpose, a two-fold dilution of the extracts in growing media was prepared and 100 ul of each dilution was inoculated into different wells of the plate. The plate was then incubated at 37 C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air for 48 hours. The amount of cells in each well was determined using the method of Skeham and collaborators (Skeham et al., 1990).

### 3.9. Testing the Possible Mutagenic Effect of the Extracts.

For this purpose we used the method of Ames (Maron and Ames, 1983). This method utilizes several specially constructed mutants of Salmonella typhimurium selected for their sensitivity and specificity in being reverted from a histidine requirement back to prototrophy by a wide variety of mutagens.

This Salmonella test has been validated for the detection of carcinogens as mutagens by studying a large number of organic chemicals which have been tested in the conventional animal carcinogenicity tests (McCann et al., 1975).

The strains that we used were : TA1535, TA97a, TA98, TA100 and TA102. These strains have been provided by Dr. Bruce N. Ames.

The method is as follows: 0.1 ml of an overnight nutrient broth culture of the bacterial tester strain, 0.1 ml of the compound to be tested and if required, 0.5 ml of S-9 mix (rat liver homogenate fraction) (Garner et al., 1972), were mixed together and then 2 ml of

molten top agar at 45 C was added, mixed rapidly and poured on minimal glucose agar plates that contained trace amounts of histidine. The plates were left to harden for several minutes, then inverted and cultured at 37 C. Two days later the colonies on the plates were counted.

In this experiment, besides the plant compounds which will be tested at different concentrations, we had as controls: i) plates without any chemical which gave us the spontaneous reversion rate of the tester strain; and ii) Plates with a known mutagen as a positive control for the experiment.

The plant compounds will be considered mutagenic if the number of revertants is two fold the number for the spontaneous reversion.

## 4. RESULTS

### 4.1. Pharmacological Screening.

The results of the pharmacologic screening of the plant extracts under study is summarize in Table 1. These results will be described in more detail in the corresponding sections.

### 4.2. Determination of the Cicatrizant Activity of the Plant Extracts *In Vivo*.

In Table 2 we show the results from the experiments in which we tested the cicatrizant effect of the crude preparations, described above from plants from the coast, the mountains and the jungle. The concentrations used were 100 mg/ml of lyophilized extract for all except for *Anredera*. The latter was used at a concentration of 200mg/ml. These results indicated that the preparations obtained from *Peperomia galioides*, *Anredera diffusa* and *Jatropha curcas*, had significant ( $p < 0.05$ ) cicatrizant activity. The extracts from *Spondias mombin*, *Mentzelia cordifolia*, *Mutisia acuminata*, *Muehlenbeckia tamnifolia*, and from *Eleutherine bulbosa* had no cicatrizant activity. In our earlier experiments extracts from *Himathanthus sucuba* showed significant cicatrizant activity, but we have been unable to confirm this result. In several assays that we have performed since then the results showed that it had no significant cicatrizant activity.

### 4.3. Effect of the Extracts from the Plants on the Blood Pressure.

In Table 1 we show the results from experiments in which we tested the effect of the crude liquid preparations, described above on the blood pressure of rats after intra venous administration of 0.1ml/rat, bolus. The concentration of extract used varied between plant species from 5% to 100%. The results indicated that the preparation obtained from *Mutisia acuminata* (100%) and *Himathanthus sucuba* (20%) had no effect; the preparations obtained from *Anredera diffusa* (2%) and *Peperomia galioides* (5%), produced hypertension in the rats; while the preparations from *Mentzelia cordifolia* (50%), and *Spondias mombin* (20%), produced hypotension.

#### 4.4. Acute toxicity screening of the Plant Extracts.

In Table 3 we show the results from the acute toxicity screening of the extracts after intraperitoneal administration of 0.1 to 1 mgr of extract per gram of weight of the mouse. Peperomia galioides, Himathanthus sucuba, Eleutherine bulbosa and Anredera diffusa were not toxic in this range. In a different experiment it was shown that the LD50 for Anredera diffusa was 2.18 mgr/gr of mouse (fiduciary limits: Superior 2.95 and inferior 1.61)

#### 4.5. Effect on Capillary Permeability in Rats.

In Table 4 we show the results from these experiments and as we can observe, all the extracts except the one from Peperomia galioides increase capillary permeability in rats.

#### 4.6. Effect on Smooth Muscle from the Ileum and Uterus of the Rat.

In Table 5 we show the results from these experiments and we observe that the extracts from the plants that have wound healing activity, Peperomia galioides and Anredera diffusa, have relaxant activity in both preparations.

#### 4.7. Cell Proliferation Studies of the Extracts from *Peperomia galioides* and *Anredera diffusa*

In Table 6. we observe the results of an experiment in which we tested the effect of different concentrations of the extracts on the proliferation of mouse 3T3 fibroblasts. The results showed that neither of the extracts tested had any effect on the stimulation of proliferation of the cells. This experiment is a preliminary one, and what would be important to test in the future is the effect of the purified active principles at the cellular level.

#### 4.8 Studies on the Possible Mutagenic Effect of the Extracts from *Peperomia galioides* and *Anredera diffusa*

For this purpose we used the method of Ames. The strains of Salmonella typhymurium that we used were : TA1535, TA98, TA100, TA 97a and TA 102. These strains were provided by Dr. Bruce N. Ames.

As shown on Tables 7 and 8, the extracts from Peperomia galioides and Anredera diffusa had no mutagenic activity.

#### 4.9. Studies on the isolation and characterization of the active principles from the plant extracts.

We are in the process of isolating in order to characterize the active principle(s) from the Peperomia and Anredera plants. During the fractionation of the extracts we are using the "in vivo" cicatrizant test to follow up on the active sub-fractions.

#### 4.10. Studies on the Isolation and Characterization of the Active Principles from the Plant Extracts.

In Table 9, we show the phytochemical analysis of the plants we studied.

In order to obtain more material from the plants, 95% ethanol extracts were prepared from Peperomia galioides and Anredera diffusa. After drying, each of the extracts was partitioned between water and dichloromethane to give fractions F2 and F3 respectively. Then the dichloromethane fraction (F3) was partitioned between hexane and 90% methanol to give fractions F4 and F5 respectively. We tested for the wound healing activity in the sub-fractions as we went along in the isolation procedure, and for Peperomia galioides we followed the activity to fraction F5 (90% MeOH) and for Anredera diffusa the wound healing activity remains in fraction F2.

From Peperomia galioides we have been able to isolate a prenylated phenolic compound that has not been reported previously.

With Dr. Hammond, we are at this time continuing our work on the isolation and characterization of the active principles from these two plants and have just started doing the same with Jatropha curcas.

TABLE 1

## PHARMACOLOGIC SCREENING OF THE PLANTS UNDER STUDY.

PLANT SPECIES	CICATRIZANT ACTIVITY	BLOOD PRESSURE	DL50 mgr/gr	CAPILAR PERM.	MUSC. ILEON	MUSC UTERO
<u>Peperomia galioides</u>	+	INCREASE	N T	NE	R	R
<u>Mentzelia cordifolia</u>	-	DECREASE	N D	INCREASE	N E	N E
<u>Mutisia acuminata</u>	-	N E	N D	INCREASE	N E	C
<u>Himathanthus sucuba</u>	-	N E	N T	INCREASE	R	R
<u>Spondias mombin</u>	-	DECREASE	0.869	INCREASE	C	R
<u>Eleutherine bulbosa</u>	-	N D	N T	INCREASE	R	R
<u>Muelenbeckia tamnifolia</u>	-	N D	0.372	INCREASE	C	C
<u>Anredera diffusa</u>	+	INCREASE	N T	INCREASE	R	R
<u>Jatropha curcas</u>	+	DECREASE	N D	INCREASE	C	R

Abbreviations: N T = Not toxic  
 N D = Not done  
 N E = No Effect  
 R = Relaxant  
 C = Contracturant

TABLE 2

TEST OF THE CICATRIZANT ACTIVITY OF THE PLANTS PREPARATIONS

PLANT SPECIES	WBS $\pm$ Sd CONTROL	WBS $\pm$ Sd EXTRACT	% ACTIVITY
<u>Peperomia galioides</u>	26.93 $\pm$ 7.83	39.28 $\pm$ 7.53	45.9*
<u>Mentzelia cordifolia</u>	27.30 $\pm$ 0.08	27.90 $\pm$ 0.39	2.2
<u>Mutisia acuminata</u>	26.36 $\pm$ 3.65	31.93 $\pm$ 6.44	21.1
<u>Himathanthus sucuba</u>	25.60 $\pm$ 4.09	39.50 $\pm$ 7.34	54.3*
<u>Spondias mombin</u> (fruit)	31.50 $\pm$ 2.04	34.55 $\pm$ 3.36	9.7
<u>Eleutherine bulbosa</u>	31.50 $\pm$ 2.04	38.95 $\pm$ 4.21	23.6*
<u>Muelenbeckia tamnifolia</u>	31.50 $\pm$ 2.04	35.90 $\pm$ 4.17	14.0
<u>Anredera diffusa</u>	25.14 $\pm$ 2.04	36.47 $\pm$ 3.95	45.1*
<u>Jatropha curcas</u>	28.0 $\pm$ 4.40	36.70 $\pm$ 5.70	31.1*

Abbreviation: WBS = Wound breaking strength in grams.

Mice were anesthetized with vapors of diethyl ether and a 1 cm incision was made perpendicular to the axis of symmetry of the animal and the two borders of the wound were slitched together at its center. Treatment was started immediately, and every 12 hours the compound being tested was applied to the wound. For this purpose 0.05 ml of the suspension being tested was applied slowly and directly to the wound. For Anredera diffusa the concentration of lyophilized extract tested was 200 mg/ml, for Jatropha curcas 100 mg of latex/ml, and for all the others it was 100mg/ml. The controls received only the solvent in which the compound was diluted. 48 hours later, mice were sacrificed with an ether overdose and the WBS were quantitated by fixing one of the borders of the wound (after cutting the stitch) while applying a measurable force to the other one. For more details on the methodology see original project.

\* Statistical analysis using the Student's-t-test showed that the difference between the control group and the treated group was significant ( $p < 0.05$ ).

TABLE 3

## ACUTE TOXICITY SCREENING OF LIQUID EXTRACTS FROM THE PLANTS

PLANT EXTRACT	DL50 mg/g weight	Superior	inferior
<u>Peperomia galioides</u>	N T	0	0
<u>Mentzelia cordifolia</u>	N D	-	-
<u>Mutisia acuminata</u>	N D	-	-
<u>Himatanthus sucuba</u>	N T	0	0
<u>Spondias mombin</u> (fruit)	0.869	1.12	0.67
<u>Eleutherine bulbosa</u>	N T	0	0
<u>Muehlenbeckia tamnifolia</u>	0.372	0.49	0.27
<u>Anredera diffusa</u>	2.18	2.95	1.61
<u>Jatropha curcas</u>	N D	-	-

Abbreviations: N T = Not toxic  
N D = Not done

Experiments were performed on groups of male Balb/c mouse whose weights oscillate between 20-25 g. Mice will be housed in groups of five and were allowed food and water ad libitum. After several days of adaptation, 10 mice per group were injected intraperitoneally with 0.1 ml/10 g (weight) of various increasing doses of a given plant crude extract (between 0.1 and 1.0 mg/g weight for all except for Anredera diffusa. For the latter we used doses between 0.1 and 3.0 mg/g weight of the mouse). Mice will be observed for the next 48 hours and the symptomatology and time of death will be recorded. LD50 values will be calculated by probit analysis.

TABLE 4

## EFFECT ON CAPILLARY PERMEABILITY IN RATS

PLANT EXTRACT	EFFECT	DOSES OF EXTRACT in mg
<u>Peperomia galioides</u>	NO EFFECT	1.25 - 5.0
<u>Mentzelia cordifolia</u>	INCREASE	2.50 - 5.0
<u>Mutisia acuminata</u>	INCREASE	2.50 - 5.0
<u>Himathanthus sucuba</u>	INCREASE	2.50 - 5.0
<u>Spondias mombin</u> (fruit)	INCREASE	2.50 - 5.0
<u>Fleutherine bulbosa</u>	INCREASE	1.25 - 5.0
<u>Muelenbeckia tamnifolia</u>	INCREASE	1.25 - 5.0
<u>Anredera diffusa</u>	INCREASE	1.25 - 10.0
<u>Jatropha curcas</u>	INCREASE	12.50 - 25.0 *

\* 100 ul of a 12.5 - 25% dilution of the latex was injected.

For each of the samples tested and the control, we used 10 adult male albino rats of 300-400 g weight. The extracts to be tested were injected intradermally onto marked sections on the animal's depilated back 90 minutes before an i.v. infusion of 2 ml of 1% Evans Blue dye solution. Twenty minutes after dye injection, the animal was sacrificed and the dorsal skin removed. The blue spots on the inner surface of the skin were then measured. Duplicate injections were made on the same animal's back.

TABLE 5

EFFECT ON SMOOTH MUSCLE FROM THE ILEUM AND THE UTERUS OF THE RAT				
PLANT EXTRACT	ILEUM		UTERUS	
	EFFECT	DOSES mg/ml bath	EFFECT	DOSES mg/ml bath
<u>Peperomia galioides</u>	R	1.50	R	0.25-1.50
<u>Mentzelia cordifolia</u>	N E	0.50-1.50	N E	0.25-3.75
<u>Mutisia acuminata</u>	N E	0.50-1.50	C	2.00-6.00
<u>Himatanthus sucuba</u>	R	1.50-3.00	R	0.75-1.75
<u>Spondias mombin</u> (fruit)	R	2.00	R	2.00
<u>Eleutherine bulbosa</u>	R	0.50-3.50	R	1.00-3.75
<u>Muelenbeckia tamnifolia</u>	C	0.50	C	0.25-0.75
<u>Anredera diffusa</u>	R	0.20-5.20	R	10.00
<u>Jatropha curcas</u>	C	1.00-20	R	2.5-10*

Abbreviations: N E = No Effect  
 R = Relaxant  
 C = Contracturant  
 \* =  $\mu$ l of the latex/ml bath

Rat ileum preparations: we used segments of 3 to 3.5 cm (n=20). These segments were suspended in an isolated organ bath containing 10 ml of tyrodes solution at 37°C, to which 100% Oxygen was bubbled. Muscle responses were registered with a grass transducer on a P7 grass poligraph.

Rat uterus preparations: longitudinal sections of the uterine horns of 10 non-pregnant rats were cut longitudinally obtaining two segments of approximately 2-5 cm. These segments were suspended in an isolated organ bath containing 10 ml of DeJalon solution at 37°C, to which 100% Oxygen was bubbled. Both spontaneous and provoked contractions were registered with a Grass transducer on a P7 Grass poligraph.

TABLE 6.

EFFECT OF EXTRACTS FROM *Peperomia galioides* AND *Anredera diffusa* ON THE PROLIFERATION OF 3T3 FIBROBLAST

CONCENTRATION mg/ml	<u>Peperomia galioides</u> No. Cells	<u>Anredera diffusa</u> No. Cells
4.000	33,951	28,578
2.000	31,762	32,071
1.000	29,633	30,011
0.500	31,304	29,533
0.250	30,031	30,767
0.125	34,031	30,866
0.063	32,419	31,324
0.031	34,250	31,065
0.016	29,672	29,434
0.008	31,742	32,638
0.004	32,439	32,936
0.002	31,085	30,667

For details on the methodology see text.

TABLE 7

EVALUATION OF THE MUTAGENIC ACTIVITY OF *Peperomia galioides*

CONCENTRATION OF EXTRACT mg/plate	NUMBER OF REVERTANTS PER PLATE									
	TA 97A		TA 98		TA 100				TA 1535	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0.1000	176	186	34	46	155	150	228	273	27	33
0.0100	159	185	42	44	168	159	275	278	22	25
0.0010	164	175	40	40	175	186	246	287	28	30
0.0001	180	184	39	47	159	167	288	296	25	32
0.0000 (ER)	156	179	43	44	170	183	285	295	25	29
A O	1900		1050							
S A					1977		1583			
MIT C									910	

## Abbreviations:

E R = Spontaneous Revertants (within the limits reported by Maron & Ames (16))

A O = Acridine Orange Positive control for

S A = Sodium Azide

M C = Mitomycin C

The method is as follows: Mix in a test tube 0.1 ml of an overnight nutrient broth culture of the bacterial tester strain, 0.1 ml of the different concentrations of *Peperomia* extracts and 0.5 ml of S9 mix (rat liver homogenate fraction). Incubate for 10 to 15 minutes at 37°C, and then mix with the top agar. mix rapidly and pour on minimal glucose agar plates that contain trace amounts of histidine and biotin. The plates were left to harden one hour in the dark, then inverted and cultured at 37 C. Two days later the colonies on the plates will be counted.

The compound was tested with and without S9 mix.

Plates with acridine orange, Sodium azide or Mitomycin C were our positive controls for the experiment.

TABLE 8

EVALUATION OF THE MUTAGENIC ACTIVITY OF *Anredera diffusa*

CONCENTRATION OF EXTRACT mg/plate	NUMBER OF REVERTANTS PER PLATE									
	TA 97A		TA 98		TA 100		TA 102		TA 1535	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0.1000	132	164	44	45	175	185	289	293	21	23
0.0100	159	158	49	54	182	189	265	298	24	28
0.0010	145	150	38	44	179	196	296	297	25	23
0.0001	148	170	39	37	169	173	288	300	25	29
0.0000 (ER)	156	175	48	50	178	189	287	299	25	29
A O	1900		1050							
S A					1977		1583			
MIT C									910	

## Abbreviations:

E R = Spontaneous Revertants (within the limits reported by Maron & Ames (16))

A O = Acridine Orange Positive control for

S A = Sodium Azide

M C = Mitomycin C

The method is as follows: Mix in a test tube 0.1 ml of an overnight nutrient broth culture of the bacterial tester strain, 0.1 ml of the different concentrations of *Peperomia* extracts and 0.5 ml of S9 mix (rat liver homogenate fraction). Incubate for 10 to 15 minutes at 37°C, and then mix with the top agar. Mix rapidly and pour on minimal glucose agar plates that contain trace amounts of histidine and biotin. The plates were left to harden one hour in the dark, then inverted and cultured at 37°C. Two days later the colonies on the plates will be counted.

The compound was tested with and without S9 mix.

Plates with acridine orange, Sodium azide or Mitomycin C were our positive controls for the experiment.

TABLE 9

## PHYTOCHEMICAL ANALYSIS OF THE WOUND HEALING PLANTS

TESTED FOR:	PLANTS STUDIED							
	I	II	III	IV	V	VI	VII	VIII
ALKALOIDS	-	-	-	-	-	-	-	+++
TANNINS	+	+	+	+	-	+	-	+++
SAPONINS	-	-	-	-	-	-	-	+++
TRITERPENES	+	+	-	+*	+*	+*	+	+++
FLAVONOIDS	+	-	-	-	ND	ND	ND	+++
ANTROQUINONES	-	-	-	-	ND	-	ND	ND
CUMARINS	+	+	+	+	ND	+	ND	++
LEUCOANTOCIANINS	-	-	+	-	ND	ND	ND	ND
SESQUITERPENELACTONES	+	-	-	+	ND	ND	ND	-
CARDIOTONIC GLYCOSIDES	-	-	-	-	ND	-	ND	-
PHENOLIC COMPOUNDS	+	+	-	+	+	+	+	ND

- I: Peperomia galioides  
 II: Mentzelia cordifolia  
 III: Mutisia acuminata  
 IV: Spondias mombin (cortex)  
 V: Spondias mombin (fruit)  
 VI: Himathantus sucuba  
 VII: Eleutherane bulbosa (cortex)  
 VIII: Anredera diffusa

ND: Not done

\* Could be triterpenes or steroids.

For the purpose of these analysis, 50 g of dried sample of the plant was extracted with 95% ethanol in a Soxhlet extractor for 2-4 hours. The alcoholic extract was concentrated and divided in three parts. Each of these parts was treated differently and used to test for the presence of the compounds mentioned above.

## 5. IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER.

Traditional medicine in our country has been employing plant preparations, like the ones we are studying, as cicatrizant agents for superficial wounds and in some cases for internal wounds such as gastric ulcers. These plant extracts are frequently employed without knowledge of their specific biological activity or possible toxicity and/or mutagenic activity.

Through this project we have been able to validate the traditional use of three of the plants under study, by showing that they have significant wound healing activity on superficial wounds. Furthermore, we have also shown that the extracts from two of these plants have no mutagenic activity. The study of the possible mutagenic activity of the third one will be done in the future.

The project has permitted us: a) to strengthen our laboratories (Departments of Microbiology, Chemistry and Physiological Sciences), making it possible to do our present research and to facilitate the development of new projects. The following equipment has been obtained: a lyophilizer with a high capacity vacuum pump, a -85°C freezer, a CO<sub>2</sub> incubator, an analytical balance, an automatic autoclave, a pH meter, a stereoscopic microscope and a Coulter counter. All of which are important for our actual and future research with natural products.

b) To train students and professionals from Lima and from the Provinces in the techniques required to study natural products.

c) To consolidate the formation of a multidisciplinary team covering all the aspects of the research. In this local team participated: (From Lima) Lic. Fernandez as botanists, Dr. Zavaleta as pharmacologist, and Ms.C. Maldonado as chemist. From Iquitos: Dr. Franklin Ayala as botanist. From Cusco Biologist Yesela Moscoso as pharmacologist. From Huaraz Lic. Hugo Loli as botanist. This local team has been strengthened by the participation of Dr. Gerald B. Hammond, presently at the University of Massachusetts, Darmouth. His collaboration was essential for the studies on the characterization of the principles from the active extracts.

## 6. PROJECT ACTIVITIES/OUTPUTS:

### Publications:

- 1.- Salas J., Tello V., Zavaleta A., Villegas L., Salas M., Fernandez I., Vaisberg A. Actividad Cicatrizante del latex de *Jatropha curcas* (1994). Rev. Biol. Trop. 42: 323-326.
- 2.- Isolation and Biological Activity of a Prenylated Phenolic compound from *Peperomia galioides* M. Allen, A. Vaisberg and G. B. Hammond. Manuscript in preparation.
- 3.- Isolation of Sinoacutine from the Leaves of *Croton lechleri*. Carlin, A. Vaisberg A.J., and G. B. Hammond. *Planta Medica*, accepted for publication (manuscript # 31/05 95).

### Abstracts:

- 1.- Vaisberg, A.J., Hammond, G.B. Investigation of the Wound Healing Properties of Some Peruvian Plants and Their Possible Utilization as therapeutic agents. Paper presented at the Iowa Academy of Sciences 102nd Session, Des Moines, Iowa. April 1990. Abstract: 73
- 2.- Villegas L., Bushby J., Salas M., Fernandez I., Maldonado H., Zavaleta A., Vaisberg A. Estudio Farmacológico de Plantas Medicinales Peruanas. Abstract presented at the VI Jornada Científica de la Universidad Peruana Cayetano Heredia. September, 1990.
- 3.- Maldonado H., Castagnetto J., Zavaleta A., Fernandez I., Vaisberg A. Estudio Fitoquímico de Plantas Cicatrizantes Peruanas. Abstract presented at the VI Jornada Científica de la Universidad Peruana Cayetano Heredia. September, 1990.
- 4.- Villegas L., Bushby J., Salas M., , Fernandez I., Maldonado H., Zavaleta A., Vaisberg A. Estudio Farmacológico de Jatropha curcas (Piñón). Abstract presented at the VI Jornada Científica de la Universidad Peruana Cayetano Heredia. September, 1990.
- 5.- Vaisberg A.J. and G.B. Hammond. Investigation of the wound healing properties of some Peruvian plants and their possible utilization as therapeutic agents. Paper presented at the American Chemical Society Northeast Regional Meeting XXI at the University of Massachusetts Amherst, June 23-26, 1991. Abstract: 35.
- 6.- Villegas L., Fernandez I., Maldonado H., Salas M., Zavaleta A., Vaisberg A. Evaluación Farmacológica de 12 Plantas Medicinales Peruanas Conocidas Como Cicatrizantes. Abstract presented at the VII Jornada Científica de la Universidad Peruana Cayetano Heredia. Rev. Med. Herediana 3: 84S, 1992.
- 7.- Moscoso Y., Villegas L., Salas M., Maldonado H., Fernandez I., Zavaleta A., Vaisberg A. Estudio Fitoquímico y Farmacológico de la Anredera diffusa. Abstract presented at the VII Jornada Científica de la Universidad Peruana Cayetano Heredia. Rev. Med. Herediana 3: 84S, 1992.
- 8.- Torres-Vadillo R., Vaisberg A. Evaluación de la Actividad Mutagénica de Productos Naturales Peruanos con Propiedades Cicatrizantes. Abstract presented at the XI Congreso Nacional de Biología. Tacna, Perú. Setiembre 1994.

#### Conferences and Meetings:

- 1.- Seminar on Medicinal Plants. Organized by the Consejo Nacional de Ciencia y Tecnología (CONCYTEC).  
Topic presented: Investigation of Natural Products with Wound Healing Activity.
- 2.- Pontificia Universidad Católica del Perú. Second International Course on Phytochemistry. January, 1990.  
Topic presented: Evaluation of Active Cicatrizant and Carcinogenic Principles.
- 3.- Sociedad Peruana de Farmacología y Terapéutica Experimental: Homenaje al Instituto Sanitas. Post graduate Course: Advances in Pharmacologic Investigation. September, 1990.  
Topic presented: Studies on Plants with Cicatrizant Activity.
- 4.- University of Massachusetts, Dartmouth, Department of Chemistry. Conference, September, 93.  
Topic Presented: Biological Characterization of Bioactive Principles from Peruvian Plants.

## 7. PROJECT PRODUCTIVITY

The project accomplished most but not all of its goals. Because of time constraints, we were unable to complete the isolation and characterization of the active principles and the work that derivated from that.

## 8. FUTURE WORK

With Dr. Hammond, we are at this time two small grants that allow us the continuation of our work on the isolation and characterization of the active principles from *Peperomia galioides*, *Anredera diffusa*, and *Jatropha curcas*.

We also plan to follow some of the results obtained during the pharmacologic screening of the plants, like the effect on blood pressure.

## 9. LITERATURE CITED

- Akhtar Husain. 1991. Economic aspects of exploitation of medicinal plants, 125-140. In: The Conservation of Medicinal Plants. Akerele O., Heywood V., Synge H. (Eds). Cambridge University Press. Cambridge.
- Cordell G.A. 1990. 23<sup>rd</sup> Great Lakes Regional Meeting of the American Chemical Society. May 30-June 1. Abstract N<sup>o</sup> 140.
- De Jalon, Bayo , De Jalon (1954). Farmacoter. Act. 3, 313.
- Finney, D.J. (1971) Statistical Methods in Biological Assay. 2nd Edition. Griffen Press. London.
- Garner, R.C., Miller, E.C., Miller, J.A. (1972). Cancer Res. 32, 2058-2066.
- Howes E.L., Sooy J.W., Harvey S.C. (1929). J.A.M.A. 92, 42
- Kruse, P.F.Jr., Patterson, M.K.Jr. Editors (1973) Tissue Culture: Methods and Applications. Academic Press. New York.
- Maron D.M., Ames, B.N. (1983). Mutation Research 113, 173-215.
- McCann, J., Choi, E., Yamasaki, E., Ames, B.N.(1975). Proc. Natl. Acad. Sci. USA. 72, 5135-5139.
- Miles, A.A. and Wilhelm, D.L. (1955). Br. J. Exptl. Path. 36, 71.
- Skeham Ph., Storeng R., Scudiero D., Monks A., McMahon J., Vistica D., Warren J. T., Bokesch H., Kenney S., Boyd M.R. (1990). Journal of the National Cancer Institute 82, 1107-1112
- Winer, B.J. (1971). Statistical Principles in Experimental Design. McGraw-Hill. Kogakusha. L.T.D.