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Brunfelsia Hopeana - Pharmacologic Screening: Isolation And Characterization Of Hopeanine

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BRUNFELSIA HOPEANA - PHARMACOLOGIC SCREENING: ISOLATION AND CHARACTERIZATION OF HOPEANINE

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BRUNFELSIA HOPEANA - PHARMACOLOGIC SCREENING; ISOLATION AND CHARACTERIZATION OF HOPEANINE

Abstract of the Dissertation

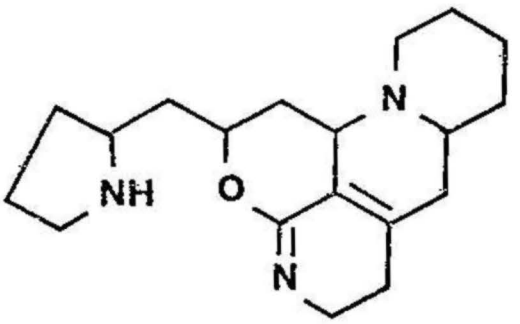
The genus Brunfelsia (Fam. Solanaceae) comprises some 40 species of shrubs native to South America and West Indies. Brunfelsia hopeana (common name: Manaca root) and related species are recognized in folk medicine in South America as being useful as diuretic, antirheumatic, antisyphilitic agents and as narcotic hallucinogens. In view of the interesting folkloric uses and a paucity of chemical and pharmacological investigation of B. hopeana, a systematic phytochemical and pharmacological investigation on B. hopeana was undertaken in our laboratory.

Hippocratic screening of the whole root powder and extracts of B. hopeana administered intraperitonially to rats, indicated that the whole root had CNS depressant activity and that the chloroform extract which contained the basic or 'alkaloidal' fraction concentrates this activity. The chloroform extract at an oral dose level of 100 mg/Kg, was equally effective (w/w) as phenylbutazone in reducing carrageenin-induced pedal edema in rats.

In addition, the extract also exhibited significant in vitro selective toxicity against SV 3T3 cells at a dose level of 2000 µg/ml. Based on these results, detailed studies on the isolation and characterization of the constituents of the chloroform extract were carried out.

A large-scale extraction of the plant material provided significant amounts of the chloroform extract for further study. Isolation of the constituents of the extract, was carried out using Column- and Preparative Thin-Layer Chromatography. Three alkaloids designated as Alkaloid I, Alkaloid II, and Alkaloid III and in addition a previously isolated coumarin compound, Scopoletin, were obtained from the chloroform extract. The major 'alkaloidal' component was Alkaloid III. The structure of this compound was elucidated with the help of UV, IR, PMR, C-13 NMR and Low- and High Resolution Mass Spectroscopy.

Based on these data, the following structure was assigned to Alkaloid III.



A survey of the literature to date showed that the structure of Alkaloid III was unique and new; being reported for the first time. The name 'Hopeanine' was therefore given to this compound. Alkaloid I and Alkaloid II were obtained in small amounts and hence only partial characterization of these two constituents could be achieved.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to Dr. Madhukar G. Chaubal for his encouragement and guidance throughout this study. I am indebted to Prof. Emery Gellert, University of Wollongong, Australia, for his guidance in my work and for generating interest in me, in the study of natural products. Appreciation is also expressed to Dr. Marvin H. Malone for his guidance in the pharmacological work and preparation of this thesis. I would also like to thank Dr. Donald K. Wedegaertner for helpful discussion in spectral analysis, Dr. John K. Brown and Dr. Donald M. Pace, for their suggestions from time to time.

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Finally, this thesis is dedicated to my mother, G. Thangam Ammal, who has been my unflickering guiding light.

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INTRODUCTION

THE GENUS BRUNFELSIA

Plants of the genus Brunfelsia belong to the family Solanaceae. This family comprises 85 genera and about 1800 species of plants (1). The family is distributed primarily in tropical South America where there are reportedly 38 endemic genera. The Solanaceae are a family of considerable economic importance and include food plants such as the potato, egg plant, tomato and strawberry tomato. The tobacco plant (fumitory) and such drug plants as henbane, belladonna, atropa and stramonium also belong to this family. In addition, ornamentals from many genera such as Petunia, Salpiglossis, Schizanthus, Lycium, Solanum, Streptosolen, Cestrum, Datura, Solandra, Browallia, Nierembergia also belong to this family (2).

The genus Brunfelsia was named after Otto Brunfels, a botanist of Metz (16th century) (3). The genus comprises approximately 40 species of shrubs native to South America and the West Indies, and to some extent distributed in the Malayan Peninsula (4). Plants of this genus have a rich folkloric medical history and have attracted attention in recent years both from ethnobotanic and chemotaxonomic points of view (5).

Folkloric Medicinal History of the Genus Brunfelsia

Chiric-Caspi and Chiric Sanango (Brunfelsia) are the most common names for the several species of the genus Brunfelsia that appear to have been important hallucinogens among certain South American Indian tribes (5). The use of the name "borrachero," which means "intoxicator," indicates that the natives of Colombia, Ecuador and Peru recognize the shrub's narcotic properties and the special care taken in its cultivation seems to suggest a religious or magic place in tribal rites. Recent evidence (5) has pointed to the use of several species of Brunfelsia either as the source of an hallucinogenic drink, as among the Kachinaua of Brazil, or as an additive to other hallucinogenic drinks, as among the Jívaro and Kofán Indians of Ecuador. The species thus employed are B. grandiflora and B. chiricaspi. All species, however, enter into folk medicine, being used especially to reduce fevers and as antirheumatic agents. B. uniflora and B. hopeana have been included in the Brazilian Pharmacopoeia (5).

Annotations on labels of several herbarium specimens collected in eastern Colombia and Peru indicate that these species of Brunfelsia cultivated in Indian dooryards are considered to be both narcotic and medicinal (6). Other collections from Bolivia, Brazil, Colombia, Peru, Ecuador also indicate a broad spectrum of therapeutic uses, ranging from "yellow fever" to "snake bite" (6). The

infusion of the scraped bark of Brunfelsia produces a sensation of chill and coldness (6). The lowland Quichwas on the Rio Napo of Ecuador utilize the plant medicinally as a remedy for rheumatism and it is reported that "they take it if they have a burning sensation in the lower part of their back. They place their hands in the area of the kidneys. Upon making a drink from the leaves in hot water, they become excessively chilled after drinking." A collection of Brunfelsia maritima from Mocoa in Southern Colombia, indicates that the shrub, reportedly toxic, is likewise called "borrachera" in this locality (7).

BRUNSFELSIA HOPEANA

A survey of the literature indicates that the species Brunsfelsia hopeana (Benth) to be most often used in native medicine under the name of "manaca." The species was also named Franciscea hopeana by Pohl for Emperor Franz II of Austria during whose reign he traveled through Brazil (3). Brunfelsia hopeana first appeared in The Dispensatory of the United States (USD) in 1892 (8a) under the name Manaca. It was continuously listed in USD until 1960. In addition, in 1926 only, two preparations, under the titles "Fluid Extractum Manacae, N.F."and "Elixir Manaca Compositum, N.F.," were listed in the USD (8b). National Formulary (N.F.)V lists the dried root of manaca (9a), Elixir Manacae Compositum (9b) and Fluid Extractum Manacae (9c). Franciscea uniflora Pohl and Brunfelsia

uniflora appear to be the scientific synonyms for this plant (3, 8b). B. hopeana is recognized by various other common names. According to Mortius (1829), in Brazil, it is known as "manacan" and as "geratacaca" and "camgamba." In Paraguay it is called "mercurio vegetal" (10). Other names include: "gerataca" or "jerataca" (which in the language of the Indians signifies a remedy against snake bite), "umbura-pauma" (tree of cures), bloom of the Lent, Christmas bloom and Santa Maria (11). According to Theodore Peckolt (12), the more frequently used name "manaca," originates from an ancient tribe, which named the shrub after a very lovely maiden called Manaja. Other names include, Paraguay jasmine and good night.

Folkloric Medicinal History of Brunfelsia hopeana

B. hopeana was employed by the natives of South America, before the arrival of Portuguese, as a remedy against arrow poison (11). In 1867, Edmon, who lived on the Amazon, reported that the flowers of manaca or franciscea were applied to indolent ulcers and that a decoction of the root was regarded as a sovereign remedy for rheumatism. It was also believed to be useful in small doses as an emetic, cathartic, diaphoretic and diuretic and in large doses as an acrid poison (10). "It was employed not only in rheumatism, but also in syphilis and other diseases in which mercury is indicated" (13). Other sources (10) indicate that the root acts as an irritant of the stomach and bowels, causing vomiting, purging

and abortion in pregnancy and even death.

A thesis on the toxic plants of Brazil (14) reports that the roots of manaca were employed by the Indians to produce strong delirium resembling persistent madness. Some tribes in the interior Amazon use this extract as an arrow poison (11). Egon (11) claims the successful use of manaca in cases of rheumatism and as a profuse diaphoretic agent and pain reliever. A decoction of the root has been employed as an irrigant for eczema and syphilitic tumors. It is considered a most valuable alternative in rheumatic arthritis (15). The leaves of the plant were used by primitive people as an antidote for snakebites (11).

B. hopeana is one of the fascinating drug plants and has the potential for being added to the list of authentic hallucinogenic plants. The evidence for the narcotic use of the plant is real, but not corroborated by a good body of evidence and observation (7, 16). The Kofán Indians of Amazonian Colombia and Ecuador, as well as the Jívaro of Ecuador, occasionally add the leaves and bark of cultivated B. hopeana to their "yaje" or "natema" drink which is prepared basically from Banisteriopsis. This use of Brunfelsia and its narcotic properties is well known in the Colombian Putumayo, where the shrub is called "borrachero" ("intoxicant") amongst the non-Indian population (17, 18). Table 1 gives the folkloric medicinal uses of B. hopeana.

Table I: Folkloric Medicinal Uses of Brunfelsia hopeana

Plant Parts (Preparation)	Alleged use	Areas of use (Reference)
Root Infusion	Diuretic, anti-rheumatic; anti-syphilitic	Brazil (7, 17)
Root Power	Yellow fever, snake bite; febrifuge, antirheumatic	Bolivia, Brazil, Colombia, Ecuador, and Peru (6, 7)
Root Power	Purgative	Brazil (11)
Leaves	Arrow poison	Brazil (11)
Root Decoction	To induce abortion	Africa (11)
Root	Emetic, cathartic; diuretic, diaphoretic; antisyphilitic; skin and mucous membrane infections	Amazon (11)

A Literature Survey of Phytochemistry and Pharmacology of B. hopeana

Pharmacology. B. hopeana has attracted the attention of pharmacologists from very early times. As early as 1882, Brewer (19) investigated the pharmacological actions of B. hopeana. According to Brewer, one or two fluidrachms or 4-8 g of the fluid extract of the roots of B. hopeana in man, produced a restlessness which could be allayed by walking and which was followed by a peculiar sense of fatigue. From these observations and from experiments on frogs and kittens, Brewer concluded that the fluid extract a) acted primarily on the spinal cord resulting in the stimulation of motor centers, b) stimulated salivary and gastric secretions, c) stimulated the intestine, liver and the kidneys, and d) depressed the "cardiac reflex center" and "respiratory reflex center." The total duration of action was three and a half hours.

The most intensive phytochemical and pharmacological investigation of B. hopeana root to date was carried out by Brandl (20). Brandl, investigating the pharmacological actions of B. hopeana on various laboratory animals, reported that the plant possessed tetanus toxin-like activity. He stated that "the primary symptom of intoxication was severe tremors in the head which lasted for a short time, followed by more rapid contraction of some facial muscles. Laboratory animals got restless, passed urine and feces, showed intense salivation and strong lacrimation." At

lethal doses, there was generalized muscle contraction, resembling "epilepsy." During this period "there was periodic cessation of respiration about five or six times, ultimately resulting in respiratory failure, heart continuing to beat in regular rhythm." Martin Costa (21) observed that oral administration of a dilute alcoholic extract of manaca root and intravenous injection of aqueous extract, produced effects quite similar to that of digitalis, "increasing the strength, but decreasing the number of contractions of the heart, with a concomitant increase in arterial tension."

According to Capanema (11), drinking the tea or infusion of B. hopeana produced excessive salivation and ulceration of buccal mucosa. He also reported of a man who took a cup of the root decoction which produced copious sweating, vertigo, general loss of sensitivity, incomplete paralysis of facial muscles, totally disturbed vision, trouble in walking, and rheumatic pains. An intense pain was also felt in the knee joints. After several hours, the symptoms disappeared and there was a reduction in the swelling of the knee joints. Freire de Cisneiros (22) reported that B. hopeana had a "characteristic" action on the heart.

Phytochemistry. B. hopeana has also attracted the attention of phytochemists from very early times. The presence of alkaloids and the isolation of basic materials have been reported in older literature, but the characterization

of the components has been insufficient. It is also quite probable that there has been some confusion among the various plants which are natively known as "manaca" and this has contributed to the uncertainty as to the chemical composition of manaca (10, 23, 24, 25).

In 1884, Lenardson (26) analyzed manaca and from the microscopic structure, he believed that manaca belonged to the Apocyanaceae family. Besides fat, resin, ash, starch and various other compounds, Lenardson reported the isolation of an alkaloid, "manacine," and a fluorescent compound. "Manacine" was assigned a molecular composition of $C_{15}H_{22}N_4O_5$. The fluorescent compound was thought to be gelseminic acid, i.e. the coumarin derivative, scopoletin. The alkaloid "manacine" was described by Lenardson as below:

> "Manacine is a weak base, has slight bitter taste, dialyzes readily, but has not been obtained in crystals. It dissolves readily in water, alcohol, and methyl alcohol, is insoluble or nearly so in other liquids, is easily decomposed in solution with separation of a brown resin, the hydrochloric acid solution being more stable than others, and is precipitated from its more or less concentrated solution by the reagents for alkaloids, but not by alkalies. The precipitate by phosphomolybdic acid dissolves in potash with a blue color. Characteristic color reactions were not observed."

A few years later, Lascelle Scott (27) reported the presence of an alkaloid, "francisceine," but was unable to assign a formula to it. Pammel (28) and Wehmer (29) remarked on the use of B. hopeana as an arrow poison and stated that it contained two alkaloids, "mandragorine" and "manacine."

The most intensive phytochemical and pharmacological investigation of manaca was carried out by Brandl (20). He isolated two alkaloids "manacine" (to which he assigned the formula $C_{22}H_{32}N_2O_{10}$) and "manaceine" ($C_{15}H_{24}N_2O_9$). He further remarked that in aqueous solution, manacine seemed to decompose to manaceine and a fluorescent compound which he believed might be identical with esculetin. Although Brandl assigned a molecular formula to certain of his materials, it is apparent that none were isolated in pure crystalline form.

Later Peckolt (12), claimed to have isolated from manaca, an amorphous substance of pale yellow color called "brunfelsine."

Various other sources (6, 18) have predicted the presence of atropine-like alkaloids in manaca, based on its pharmacological properties and taxonomic relationships to solanaceous plants such as Atropa, Hyoscyamus and Datura.

It is thus apparent that so far only preliminary chemical investigations of B. hopeana have been carried out. While the older literature mentions the isolation of alkaloidal components such as "francisceine," "manacine" and "brunfelsine," none of these seem to have been isolated in pure form and satisfactorily characterized (17). In a more recent report on the investigation of B. hopeana, B. pauciflora and B. brasiliensis, the presence of alkaloids was not mentioned, but the isolation of scopoletin, was again reported (30).

SCOPE OF THE PRESENT INVESTIGATIONS

The lack of knowledge concerning the chemical constituents of B. hopeana, coupled with reports of interesting pharmacological activity and folkloric use, suggested that a detailed phytochemical and pharmacological investigation of this plant would prove to be fruitful.

So far only one constituent of this plant--a coumarin derivative, scopoletin--has been satisfactorily characterized. Although the presence of alkaloidal constituents have been reported, these have not been isolated in satisfactory pure crystalline form. Hence the molecular formulas and physical constants assigned to these isolated fractions may be questioned as to their validity.

The investigation reported here is divided into the following phases.

a) Preliminary phytochemical screening
b) Development of a suitable extraction procedure and preparation of various extracts
c) Pharmacologic screening of the extracts
    i) Hippocratic screening
    ii) Cytotoxic activity
d) Specific pharmacological screening of the active fraction from step (c), based on folkloric use and the observed results of hippocratic screening
e) Large scale extraction to obtain the active fraction(s) for phytochemical investigation.

f) Isolation of the constituents of the active fraction by chromatographic methods.

g) Structure determination of the constituents by spectroscopic techniques.

h) Pharmacological screening of the isolated chemically pure constituents.

EXPERIMENTAL

PLANT MATERIAL

Brunfelsia hopeana root coarse powder[1] and whole root[2] were reduced to 40 mesh powder in a Wiley Mill. Herbarium specimens of all were prepared and have been kept for reference in the Pharmacognosy Laboratory, School of Pharmacy, University of the Pacific, Stockton. This 40 mesh powder was used in all the extraction procedures.

PRELIMINARY PHYTOCHEMICAL SCREENING

One hundred g powder was continuously percolated with about 1200 ml of cold methanol (Analytical Reagent Grade; all solvents used in those investigations were Analytical Reagent grade). The percolate was concentrated to 50 ml. The extract was designated as extract B and tested for presence of alkaloids according to the following method (31):

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[1]Lot #1404, BJM 001, received 7/8/68, S. B. Penick & Co., New York, NY.

[2]Lot # Sec. 9, # 53i, received 10/3/75, Wide World of Herbs Ltd., New York, NY.

a) Preliminary alkaloid test: To 2.0 ml of extract B, 2.0 ml of 2N HCl was added, filtered with the aid of Celite. The filtrate was divided into two equal parts in separate test tubes. A few drops of Mayer's reagent was added to one of the test tubes. A definite turbidity was observed. This gave preliminary evidence that alkaloids were present in the plant.

b) Confirmatory alkaloid test: To about 20 ml of the original extract B, 5.0 ml of 2N HCl was added, slightly warmed with stirring, and filtered with the aid of Celite. The filtrate was rendered alkaline with sufficient NH_4OH. The alkaline solution was transferred to a separatory funnel and extracted with two 20-ml portions of chloroform. These two chloroform extracts were combined and evaporated to dryness under vacuum ($< 45^{o}$). A small portion of the dry extract was treated by 2.0 ml of 2N HCl, stirred and filtered. The filtrate was divided into two equal parts in separate test tubes. A few drops of Mayer's reagent was added to one of the test tubes. A definite turbidity was observed. A few drops of Wagner's reagent was added to the other test tube. Again a definite turbidity was observed. This confirmed the presence of alkaloids.

Development of Extraction Procedure

Coarse root powder (20-40 mesh) was subjected to the following series of extraction procedures:

Cold Extraction: Three percolators were packed with 100 g each of the root powder and subjected to:

a) Percolation with methanol
b) Percolation with methanol:water (50:50)
c) Percolation with water

Hot Extraction: The hot extractions were carried out in Soxhlet extractors (31a):

a) Extraction with methanol
b) Extraction with methanol:water (50:50)

In all the above cases, percolation and extraction were continued to exhaustion i.e. until the extract showed a negative test with Mayer's reagent.

Thin-Layer Chromatography (TLC)

All the extracts were concentrated to a small volume and the residue subjected to TLC using the following system:

Plates: Silica Gel 60 (0.25 mm), without fluorescent indicator

Solvent: Butanol:acetic acid:water (4:1:5)

The plates were developed for a length of 16 cm, dried at 100^{o} and sprayed with Dragendorff's reagent (Munier and

Macheboeuf modified) (32). The hot and cold aqueous extracts did not show any Dragendorff-positive spots. Both cold and hot methanol as well as methanol:water (50:50) extracts showed Dragendorff-positive spots, indicating the presence of alkaloids, and were further treated as below:

The extracts were diluted with water, acidified with 2N HCl and filtered with the aid of Celite. The filtrate was basified with NH_4OH and extracted with chloroform. The chloroform layer was dried over anhydrous sodium sulfate and then evaporated to dryness. The residue was taken up in minimum amount of chloroform and TLC performed in the same system as above. The Dragendorff-positive spots observed before were seen again.

PHARMACOLOGICAL SCREENING

Hippocratic Screening

The hippocratic screening procedure in unanesthetized rats, as developed by Malone and Robichaud (33), was used throughout this study. The main purpose was to determine which extract should receive prime consideration in phytochemical work.

Non-fasted male albino rats (150-200 g, Sprague-Dawley strain)[1] were used. Food and water were allowed

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[1]Simonsen Laboratories, Gilroy, California.

ad libitum before and two hours after intraperitoneal injection.

Test materials (whole root powder and extracts) were suspended in freshly prepared aqueous 0.25% agar[2] by trituration in a glass mortar just prior to actual administration.

The coarse root powder 20-40 mesh of B. hopeana was reduced to 200-mesh powder in a Norton Grinding Mill, using a porcelain jar and Burundum chips[3] before dosing the animals with whole root for hippocratic screening.

Based on the results of the initial phytochemical screen, as described in the preceding pages, an extraction scheme was developed as described in the Figure 1. Those extracts which were subjected to hippocratic screening are asterisked.

The test samples were administered in log-dose sequence as intraperitonial injections with a constant dosage volume of 5 ml/Kg. Sterile disposable syringes of 1-or 3-ml capacity with 25 gauge, 3/8-inch or 20 gauge 1-inch needles were employed. Details of the procedures and worksheets for recording observations over the seven-day period were those given by Malone and Robichaud (33).

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[2]Agar (Lot #0140-01), Difco Laboratories, Michigan.

[3]Chemical Processing Div., formerly U.S. Stoneware Inc., Akron, Ohio.

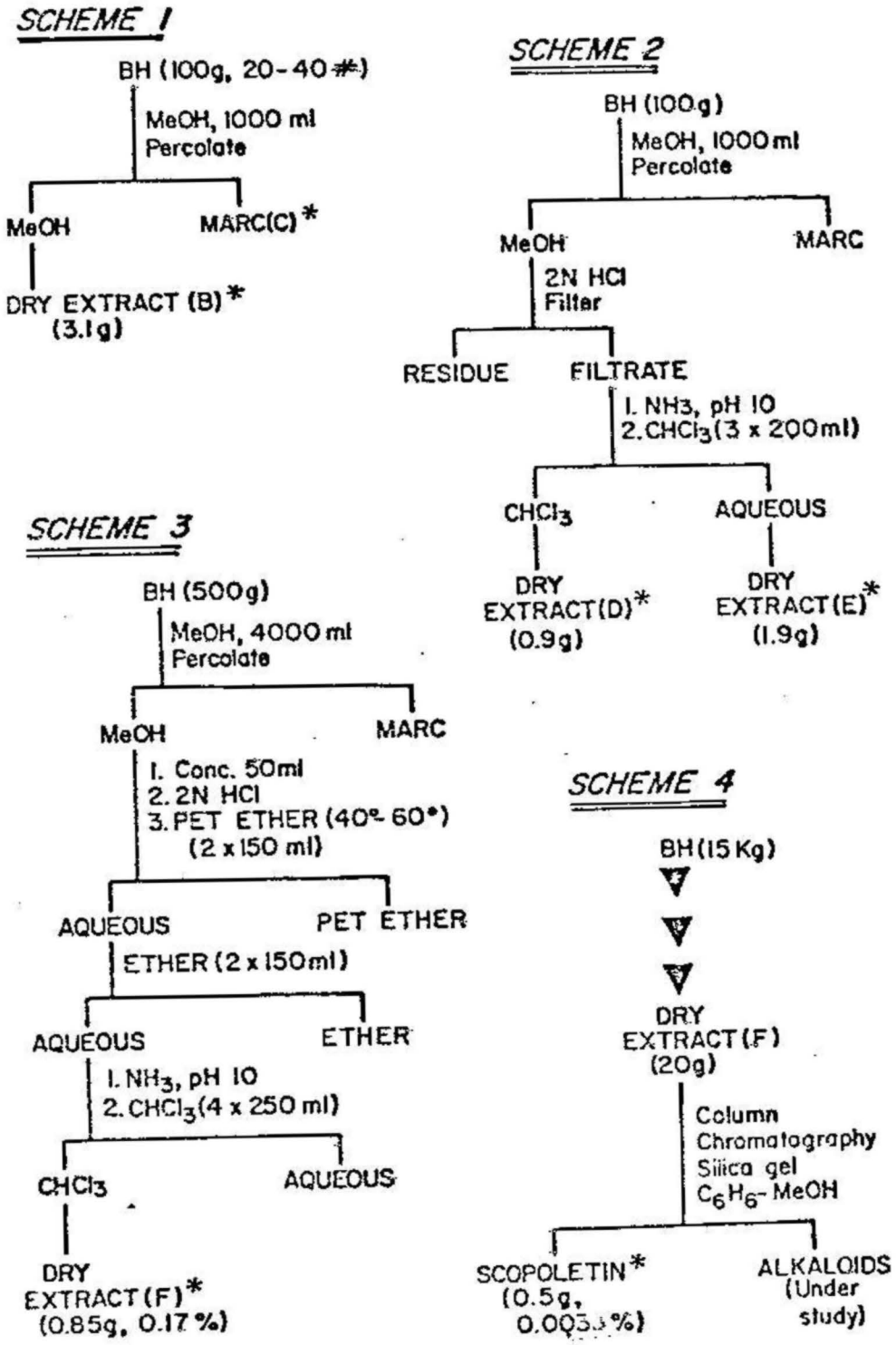

Fig. 1. Schemes of extraction of powdered root of Brunfelsia hopeana

Cytotoxic Activity

In 1962, the Cancer Chemotherapy National Service Center (CCNSC) issued a number of protocols for screening chemical agents and natural products against animal tumors and tumor cells in culture (34). The KB (Eagle) cell culture system is normally employed for screening plant extracts because it is useful as a "pre-screen" in showing concentrates that also show *in vivo* activity (35). In the present investigation, the effect of the plant extracts on normal and transformed cells in culture was studied. The index used for determination of cell growth was cell count. No attempt was made to determine the effect of the extract on protein synthesis. Because of the nonavailability of KB cells, SV 3T3 and 3T3 cells were used in the present investigation. Basal modified Eagle's medium (36) was used for growing the cells.

Stock solutions of the extracts B and F (Fig. 1) with a concentration of 2000 µg/ml were prepared using the following procedure. Ten mg of the dry extract was dissolved in 2.2 ml of 95% ethanol. The solution was then diluted to 5.0 ml with normal saline to give a final concentration of 2000 µg/ml.

The extract B was studied for growth inhibitory property at dose levels of 0, 50, 100, 200 and 400 µg/ml. The following scheme was employed (0 = a petri plate)

	3T3					SV 3T3				
	c	a	b	d	e	c	a	b	d	e
Day 1	0	0	0	0	0	0	0	0	0	0
Day 2	0	0	0	0	0	0	0	0	0	0
Day 3	0	0	0	0	0	0	0	0	0	0
Day 4	0	0	0	0	0	0	0	0	0	0
Day 5	0	0	0	0	0	0	0	0	0	0
Day 6	0	0	0	0	0	0	0	0	0	0

a ..... 50 µg/ml concentration of extract B

b ..... 100 µg/ml concentration of extract B

d ..... 200 µg/ml concentration of extract B

e ..... 400 µg/ml concentration of extract B

c ..... control concentration of extract B

The stock culture of 3T3 and SV 3T3 cells were suspended in 150 mls of basal modified Eagle's medium. Four ml of the medium were transferred to each of the 60 dishes. Three tenths of a ml of the solution was taken separately and the cells counted. The cells were then incubated for 24 hours and the extract was added to the dishes and incubated again.

For counting the cells, each dish was removed, trypsinized, and the contents transferred to a sample bottle and diluted to 10 ml with saline. The sample bottle was placed in a Coulter counter unit which estimated the number of cells in 100 µl of the solution. The total cell population was then computed.

The extract F (Fig.1) obtained was also used to study its growth inhibitory property. The experimental set up was as described above under extract B except that a single dose level of 2000 µg/ml was employed.

Antiinflammatory Screening

In view of the folkloric uses of Brunfelsia hopeana as an antirheumatic agent and based on the observed results of hippocratic screening, extract F was also subjected to antiinflammatory evaluation. Carrageenin-induced pedal edema in rats was used as the experimental model (37) and the effect of the extract F in reducing inflammation was compared to the known antiinflammatory effect of phenylbutazone.

Approximately seven-week old, male albino, Sprague-Dawley rats weighing between 120 and 150 g were allowed at least six days in our rat quarters(after shipment was received)to allow for acclimatization before experimentation. Daily body weights were checked to document whether or not normal growth patterns were present. Rat quarters consisted of an 8.5 meter x 5.2 meter room kept at constant temperature (21^{o}) and humidity and supplied with natural illumination from a 3.4 meter x 0.8 meter frosted window. Cages were positioned to approximate uniform lighting. Artificial lights were kept off, except during actual experimentation. All animals were offered a diet of commercially prepared food pellets[4] and tap water ad libitum. All drugs were

[4]Purina Rat Chow. Crude protein not less than 30%, crude fat not less than 4.5%, crude fiber not less than 6.0%, ash not less than 9%, plus vitamin and mineral supplement.

suspended in 0.25% agar and orally administered using a constant 10 ml/Kg dosage volume. The extract F and phenylbutazone[5] suspensions in agar were each made up daily just prior to use. Sodium pentobarbital[6] solution was made up every two days and refrigerated at 4° after use. A 1% solution of carrageenin[7] in 0.9% saline was used as the phlogistic agent.

A total of 30 male rats were used in the study. A line was drawn across the top edge of the lateral malleolus of both hindpaws using an indelible pencil lightly wetted with water. Hind paw volumes were then determined to this mark using the apparatus shown in Fig. 2. The opposite page describes the working procedure for calibration of the plethysmograph. Once the machine was calibrated, each hind paw was dipped thrice, from which an average could be calculated. It was necessary to check the calibration of the instrument every 20 minutes.

The rats were taken off food at -1 hr and orally dosed with the test drugs. The control groups received only the vehicle (aqueous 0.25% agar) at the constant dosage volume. One hour after oral administration of the test

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[5]Phenylbutazone (SN 20911) Ciba Pharmaceuticals, New Jersey.

[6]Sodium pentobarbital (Lot #816-1739), Abbott Laboratories.

[7]Carrageenin (Ident. #312503), Sea Plant Chemical Corp., Massachusetts.

INSTRUCTIONS FOR OPERATION OF PLETHYSMOGRAPH

(Beckman Type RS Dynograph)

(Measurement of Rat Pedal Edema)

Revised by Radhakrishnan Iyer and Jeffrey S. Bohrman, August 1976

1. Flip toggle switch A to "arterial." Depends on type of transducer you are using as to whether you flip to venous or arterial.

2. Flip toggle switch B to "negative."

3. Flip toggle switch C to "normal."

4. Flip toggle switch D to "average."

5. Turn knob E to "1 mv/cm." (approximate setting)

6. Turn knob F to "x.1 mv/cm." (approximate setting)

7. Flip toggle switch G to "out."

8. Depress button H to "1 mm/sec."

9. Turn knob I to "operate" and allow at least 30 minutes, but preferably, 60 minutes for machine warm-up.

10. Adjust mercury level in S shaped tube with the mercury level in the reservoir using the syringe which is filled with distilled water.

11. Close the screw clamp which regulates the flow of water from the syringe into the transducer.

12. Flip toggle switch B to "off" and turn knob N and position pen 6½ cm. from top of paper. The knob N should be in the middle - approximately.

13. Flip toggle switch B back to "negative" and turn knob O and position the pen 6½ cm. from the top of paper. The knob O should be in the middle - approximately.

14. Immerse the calibration rod to the 1 ml. mark.

15. Turn knob O (should be approximately in the middle) and again position pen to 6½ cm. from top of paper.

16. Immerse the calibration rod to the 2 ml. mark. This should position the pen 7 cm. from the top of paper. If not, adjust to 7 cm. by adjusting the mercury level in S shaped tube. The mercury level should be as described in Step 10. If not, restart the calibration from Step 10.

17. Continue to immerse calibration rod in 1 ml. increments, turning knob F slightly to keep pen positioned at 0.5 cm. intervals.

18. Withdraw calibration rod. The pen should come to rest approximately 6 or 6½ cm. from top of paper.

19. Keep repeating steps 14 to 18 until linearity is achieved; each 1 ml. of rod equals to 0.5 cm. displacement on the paper.

IMPORTANT NOTE: Each person has different technique of operating, thus to prevent disparity in results, only the investigator is to operate the machine.

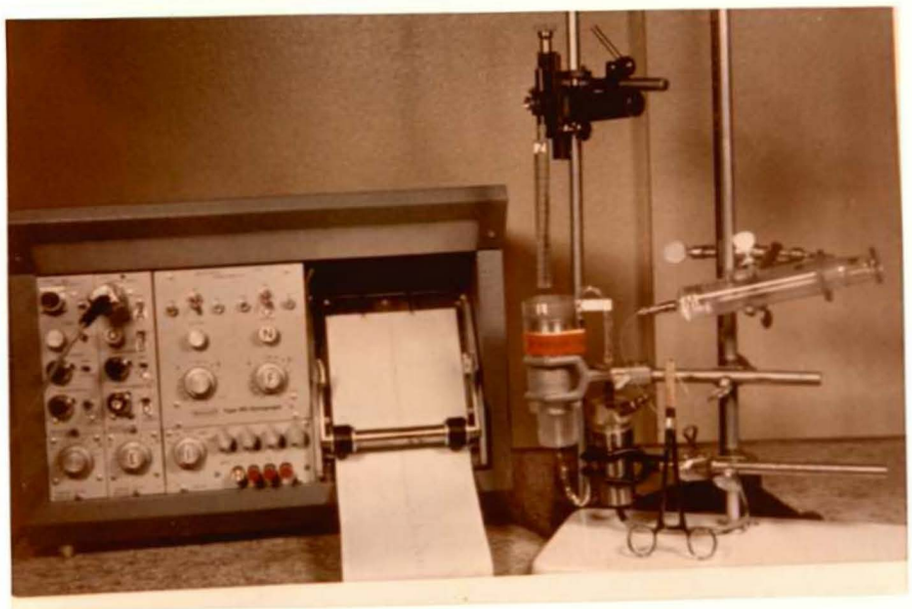

Fig. 2. Plethysmograph: Beckman Type RS dynograph connected to a Statham strain gauge pressure transducer (P23BB, 0-50 mm).

drugs, the rats received a subplantar injection of 0.1 ml suspension of 1% carrageenin in 0.9% saline in the right hind paw. At +5 min, recording of the paw volume was made after the carrageenin injection. This reading served as the actual '0' min reading and changes in paw volume with time was calculated relative to this value. The paw volumes were measured at time intervals of +30, +60, +90, +120, +150, +180, +210, +240, and +300 minutes.

Extracts D and F (see Fig. 1) had shown interesting activity. Therefore it was desirable to undertake a detailed phytochemical investigation of the extracts D and F. For this purpose a large-scale extraction of the crude drug was carried out as described in the following section.

LARGE SCALE EXTRACTION

Based on the results of the preliminary experiments, two types of extraction procedures were carried out as described below (see also Fig. 1).

Method 1. Two Kg of powdered drug was macerated with cold methanol for 24 hr in a glass percolator (length 21" and ID 8") and then continuously percolated with cold methanol in the dark. Percolation was carried out till a 200 ml volume of the percolate on concentration gave a negative test with Mayer's reagent. The percolate (42 liters) was concentrated under vacuum at a temperature less than 45^{o}. The concentrated syrupy liquid (640 ml) corresponded to a weight of 127 g of dry extract (10-ml of an aliquot of

the syrupy liquid when stripped completely of all the solvent corresponded to 1.9853 g of the dry residue).

The concentrate was diluted with water to double the volume and acidified with 800 ml of 2N HCl, kept at 5^{o} for 24 hours and filtered. The filtrate was recycled three times to get a fairly clear filtrate. The residue was suspended in dilute HCl, digested thoroughly by warming to less than 45^{o} and filtered. The washings were combined with the main filtrate and the combined filtrate was made alkaline with 28% NH_4OH to pH 10. The filtrate (2 liters) was divided into five portions. Each portion was extracted with $CHCl_3$ (3 X 200 ml). The chloroform extracts were combined, dried over sodium sulfate and the chloroform removed in vacuo to yield 8.5 g of a dark gummy residue. This extract was designated as extract D.

Method 2. Seven Kg of powdered drug was loaded in three glass percolators (length 21" and ID 8") and macerated with cold methanol, in dark, for 48 hours. The macerated drug was next percolated to exhaustion with cold methanol. Percolation was continued till a 500 ml aliquot on concentration yielded a negative test with Mayer's reagent. A total of 128 liters of the percolate was collected over a total period of 25 days. The percolate was concentrated in a rotary evaporator at a temperature less than 45^{o} to a final volume of one liter (6-ml aliquot on complete removal of all solvent corresponded to a weight of 2.74 g, representing 450 g of methanolic extract). The

total extract was diluted with two liters of distilled water and acidified with 2N HCl to a pH of 2.0. The aqueous acid solution was extracted with about three liters of petroleum ether (40-60°) and later followed by extraction with about five liters of ether. The ether layer was reextracted with 2N HCl and the aqueous layer combined with the acidic aqueous extract. The whole aqueous extract was carefully basified with 28% NH_4OH to a pH of 9-10. The aqueous layer (seven liters) was divided into three portions and each portion extracted with chloroform (3 X 1 liters). The combined chloroform extract (20 liters) and petroleum ether extract were dried over anhydrous sodium sulfate, filtered and concentrated. The dry chloroform extract weighed 12 g. This extract was designated as extract F. The petroleum ether extract was designated as extract P (30 g).

During the extraction, considerable emulsion formation was encountered. This was partly overcome by a) addition of saturated sodium chloride solution to the separatory funnel and b) by slight application of hot air on the outside of the separatory funnel with the aid of a hair dryer.

SEPARATION AND PURIFICATION OF CONSTITUENTS OF EXTRACTS

On the basis of preliminary phytochemical and hippocratic screening, the two extracts F and P (Fig. 1) were thought to be of interest for detailed examination. Separation and purification of constituents of these two extracts

were therefore attempted.

Chromatographic methods have been shown to be suitable for separation and purification of constituents of crude plant extracts (38). Of the chromatographic methods, TLC and Column Chromatography (CC) were chosen as the two most likely to succeed.

Column chromatographic separation yields amounts of substances sufficient for characterization and testing. In order to develop proper CC systems, TLC was first investigated in detail, since results of TLC can often be applied to CC (38). Also, CC separation could be followed by TLC checks.

Thin-Layer Chromatography (TLC)

The extracts were spotted using capillaries drawn from melting-point tubes, on precoated plates.[8] The spotted plates were transferred to glass chambers[9] previously saturated with solvent vapor. All solvents were reagent grade.[10] After development for a distance of 16 cm, the plates were removed from the chambers and air dried. The developed spots were visualized by three methods - exposure to short wavelength UV, spraying with Dragendorff's reagent and exposure to iodine vapors. These visualization

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[8]E. Merck, Darmstadt, Germany.

[9]Desaga, Heidelberg, Germany.

[10]ACS reagents, Matheson, Coleman and Bell, Norwood, Ohio.

procedures were carried out on each plate in succession, in the order given above. Spots observed after each exposure were marked on the plates by a stylus.

The absorbents (plate coatings) were of 250 micron thickness and contained F-254 fluorescent indicator. The absorbents and developing solvents used are given below:

Silica Gel 60

a)	Butanol:acetic acid: water	40:10:50
b)	Cyclohexanone:chloroform:diethylamine	50:40:10
c)	Ethylacetate:n-propanol:ammonia	40:30:3
d)	Chloroform:diethylamine	90:10
e)	Methanol:carbon tetrachloride: acetic acid	28:92:1
f)	Chloroform:methanol	85:15
g)	Chloroform:methanol:diethylamine	86:4:10
h)	Chloroform:methanol:acetic acid	85:5:10
i)	Benzene:methanol	80:20
j)	Chloroform:methanol:diethylamine	80:10:10

Aluminum Oxide

a)	Cyclohexanone:chloroform:acetic acid	45:45:10
b)	2-Butanone:methanol:ammonia	60:30:10
c)	Methanol:carbon tetrachloride: acetic acid	28:12:1
d)	Chloroform:methanol:acetic acid	25:65:10
e)	Chloroform:methanol:acetic acid	5:80:15

Column Chromatography

Extract D. A glass chromatographic column (5 X 50 cm) was packed with a slurry of 400 g alumina (activity II) in chloroform. Extract D (8.5 g) was dissolved in minimum methanol and about 20 g of alumina were added to the solution with stirring. The slurry was evaporated to dryness in vacuo and the resulting yellow powder was applied to the top of the packed column. Development was carried out utilizing the gradient elution technique beginning with chloroform and followed by chloroform containing increasing amounts of methanol. Two hundred ml fractions were collected, concentrated and examined by TLC on silica gel GF uniplates[11], using chloroform:methanol:diethylamine (86:4:10) as the solvent system. The plates were dried, examined under short wavelength UV and sprayed with Dragendorff's reagent. Table II gives the result of the chromatography.

Fractions 4 and 5 which yielded Dragendorff-positive spots on TLC were further rechromatographed on silica gel column.

A 2 X 25 cm glass column was packed with a slurry of 20 g silica gel 60[12] in benzene. Fractions 4 and 5 from alumina column were combined, dissolved in benzene and applied to the top of the packed column. Gradient

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[11] Analtech, Inc., Canoga Park, California.

[12] EM Laboratories, Elmsford, NY.

Table II: Column chromatographic separation of extract D

Fraction (no.)	Solvent (s) (ratio)	Elution volume (ml)	Weight of Dry Residue (g)	Remarks
1	chloroform (100.0)	500	0.6793	--
2	chloroform	200	0.0664	White crystals
3	chloroform	300	0.3460	
4	chloroform:methanol (98:2)	1000	0.3645	Alkaloidal mixture
5	chloroform:methanol (98:2)	2200	0.6433	Alkaloidal mixture
6	chloroform:methanol (95:5)	1000	-	--
7	chloroform:methanol (92:8)	1500	1.5534	--
8	chloroform:methanol (88:12)	1000	-	--
9	chloroform:methanol (80:20)	1000	-	--
10	chloroform:methanol (75:25)	3000	0.600	--
11	chloroform:methanol (50:50)	1000	-	--
12	methanol	1500	0.400	--

Total volume of eluate: 14.200 liters

Total weight of residue: 4.6529 g

elution was started with benzene and followed by increasing amounts of methanol and finally with methanol. Fifteen-ml fractions were collected and monitored by TLC on silica gel GF uniplates, using chloroform:methanol:diethylamine (86:4:10) as solvent. The spots were visualized as before. Table III gives the result of the chromatography. Attempts to obtain crystalline material from these fractions were not successful.

Fractions 2 and 3 from the chromatography of extract D on alumina, gave white crystals on addition of methanol. This compound gave a positive Liebermann-Burchard test (39) and also a positive Salkowski test (39). This compound was therefore presumed to be a sterol. It was later found to be present in the extract P also and was isolated as below:

Extract P. The sterol suspected to be present in the extract D was found to be present in a substantial amount in the extract P. An attempt was therefore made to isolate this sterol by column chromatography of the extract P.

A 5 X 50 cm glass column was packed with a slurry of 400 g silica gel (activity II) in petroleum ether (40-60^{o}). Twenty g of extract P was dissolved in sufficient methanol and 20 g silica gel added. The slurry was evaporated to dryness and the resulting yellow powder applied to the top of the packed column. Gradient elution was started beginning with petroleum ether. Five hundred-ml fractions were collected. The fractions were concentrated and examined on silica gel GF uniplates using three solvent

Table III: Column chromatographic separation of fractions 4 & 5 from alumina column

Fraction (no.)	Solvent(s) (ratio)	Elution volume (ml)	Weight of Dry Residue (g)	Remarks
1	Benzene:methanol (99:1)	100	0.060	--
2	Benzene:methanol (98:2)	100	0.050	--
3	Benzene:methanol (98:2)	100	0.082	Alkaloidal mixture
4	Benzene:methanol (97:3)	200	0.130	Alkaloidal mixture
5	Benzene:methanol (88:12)	300	0.054	Alkaloidal mixture
6	Methanol	100	-	--
7	Methanol:2%HCl (95:5)	100	0.206	Alkaloidal mixture

Total volume of eluate: 1000 ml

Total weight of residue: 0.582 g

systems-benzene:petroleum ether (20:80), benzene:petroleum ether (40:60) and chloroform. The plates after development were dried and visualized as before. Table IV gives the result of this chromatography.

Fractions 10 and 11 from the above chromatography mainly contained the sterol but could not be induced to crystallize even after prolonged standing. These were therefore combined and rechromatographed over alumina. However again no separation or crystallization occurred. Therefore the combined extracts were again rechromatographed on silica gel. A 2 X 25 cm glass column was packed with a slurry of silica gel in benzene. One g of the combined fractions was dissolved in a minimum amount of benzene and applied to the top of the chromatographic column. Elution was started with benzene. The first 1000 ml of the fraction contained the sterol. The eluate on complete removal of all solvent left a colorless gum behind. This gum was crystallized from aqueous methanol as colorless plates. The crystalline substance was identified as β-sitosterol.

Extract F. Before large-scale chromatography was undertaken, it was thought desirable to find the ideal conditions for the chromatographic separation of the constituents of the extract F. Earlier experiments on extract D had established that aluminum oxide was not the preferred adsorbent for separation. Hence it was thought necessary to evaluate the merits of other adsorbents like silica gel along with various combinations of solvents for elution. To facilitate this investigation, a series of small glass

Table IV: Column chromatograhic separation of extract P

Fraction (no.)	Solvent(s) (ratio)	Elution volume (ml)	Remarks
1	Petroleum ether 100	1000	-
2	Petroleum ether:benzene (80:20)	1000	-
3	Petroleum ether:benzene (60:40)	1000	-
4	Petroleum ether:benzene (60:40)	2000	-
5	Petroleum ether:benzene (20:80)	1000	-
6	Benzene 100	1000	-
7	Benzene:chloroform (80:20)	1000	-
8	Benzene:chloroform (60:40)	1000	-
9	Benzene:chloroform (40:60)	500	-
10	Benzene:chloroform (20:80)	500	sterol
11	Benzene:chloroform (20:80)	1000	sterol
12	Methanol 100	1000	-

Total elution volume 12.00 liters

microcolumns (1 X 10 cm) were prepared and 200 mg samples each of extract F were run.

On the basis of TLC on silica gel GF uniplates and alumina GF uniplates, the following adsorbents and solvent combinations were selected for this investigation:

a) A glass microcolumn was packed with a slurry in benzene, of 10 g of alumina (neutral and low adsorptive capacity) prepared according to the procedure of Johns and Lamberton (40). Extract F was dissolved in minimum methanol and 3.0 g of alumina added. The slurry was evaporated to dryness and the dry powder applied to the top of the packed column. Elution was started with benzene and then followed by benzene with increasing amounts of methanol. Ten ml fractions were collected and examined on silica gel GF uniplates with chloroform:acetone:diethylamine (50:40:10).

b) A similar alumina chromatographic column was set up, but elution was started with benzene, followed by chloroform and finally with methanol.

c) A glass microcolumn was packed with 10 g of silica gel (activity II) in benzene. Extract F was dissolved in minimum methanol and three g of silica gel (activity II) added. The slurry was evaporated to dryness and the dry powder was applied to the top of the packed column. Elution was started with benzene, followed by chloroform and methanol in that order. Ten-ml fractions were collected and monitored by TLC using silica gel GF uniplates.

d) A similar chromatographic column was set up

as above, but elution was started with benzene containing increasing proportion of methanol.

e) A glass microcolumn was packed with a slurry of silica gel (deactivated) in benzene according to the procedure of Johns and Lamberton (40). Extract F was dissolved in a minimum amount of methanol and three g of silica gel (deactivated) added. The slurry was evaporated to dryness and the dry powder was applied to the top of the packed column. Elution was started with benzene, followed by chloroform and methanol in that order. Ten-ml fractions were collected and monitored by TLC using silica gel GF uniplates.

f) A similar chromatographic column was set up as above, but the elution was started with benzene, followed by benzene containing increasing proportion of methanol.

From these experiments, it was concluded that CC of extract F on silica gel (deactivated) using benzene-methanol as eluent would yield the desired separation of the constituents. Since further quantities of extract F were necessary, a second large-scale extraction of powdered B. hopeana root was carried out according to the procedure outlined on page 26 . In this case 15 Kg of root powder was used and therefore quantities of solvents used in extraction had to be increased in proportion. At the end of the extraction, a red gummy extract F weighing 23 g was obtained. This quantity of the extract was then subjected to CC as below:

A 5 X 50 cm chromatographic column was packed with a slurry of 400 g of deactivated silica gel 60 in benzene using the procedure of Johns and Lamberton (40). Extract F was dissolved in minimum amount of methanol and about 30 g deactivated silica gel added. Removal of the solvent in vacuo gave a dry yellow powder which was applied to the top of the packed column. Gradient elution of the column was carried out beginning with benzene and followed by benzene containing increasing amounts of methanol and finally with methanol containing 1% HCl (98.2). Five hundred-ml fractions were collected. The fractions were concentrated and monitored by TLC as before for extract D. The individual fractions were combined on the basis of their TLC patterns. The results were presented in Table V.

Isolation of Scopoletin

The residue from fractions 2 and 3 crystallized as white solid on addition of benzene-methanol (95.5). This solid was recrystallized three times from benzene-methanol (95.5) to yield an analytically pure sample. This substance was later identified as scopoletin, a known coumarin derivative.

Using the chromatographic procedure described above, a partial separation of the components had been achieved. However, several of the eluate fractions from the CC were found to be mixtures of several components when monitored by TLC. Preparative TLC was used to further separate the components of these eluates.

Table V: Column chromatographic separation of extract F

Fraction (no.)	Solvent(s) (ratio)	Elution volume (ml)	Weight of Residue (g)	Remarks
1	Benzene	4000	0.200	
2	Benzene:methanol (98:2)	3000	0.524	Scopoletin
3	Benzene:methanol (96:4)	3000	1.611	Scopoletin
4	Benzene:methanol (94:6)	3000	0.453	
5	Benzene:methanol (92:8)	5000	3.400	
6	Benzene:methanol (90:10)	4000	2.365	Alkaloids
7	Benzene:methanol (88:12)	3000	0.550	Alkaloids
8	Benzene:methanol (85:15)	3000	0.876	Alkaloids
9	Benzene:methanol (80:20)	4000	0.940	Alkaloids
10	Benzene:methanol (70:30)	2000	0.408	
11	Benzene:methanol (50:50)	3000	0.697	
12	Methanol	3000	3.492	

Total eluate volume = 40 liters

Total weight of residue = 15.516 g

Preparative Thin-Layer Chromatography (Isolation of Alkaloids)

Fractions 6, 7, 8 and 9 were found to be the major alkaloid-bearing fractions. Three Dragendorff-positive (alkaloidal) spots were observed when these were monitored by TLC on silica gel GF uniplates. These three spots were initially simply designated as Alkaloid I, Alkaloid II and Alkaloid III. From the intensity of the Dragendorff-positive spots, it was concluded that alkaloid III was probably present in the highest quantity. Attempt was therefore made to isolate alkaloid III in a pure form using preparative thin-layer chromatography on alumina plates. The solvent system for this chromatography was selected after extensive experimentation with various combinations of solvents so as to give good separation between the components. The solvent systems which were found suitable for the desired separation along with the Rf values of the alkaloids are present in Table VI.

Isolation of Alkaloid III. A series of preparative, precoated aluminum oxide plates (150 F-254 PLC, 150 microns thick)[14] were activated for 10 minutes at 100°. The plates were allowed to cool to room temperature and stored in a desiccator before use. The residue from fractions 8 and 9 (Table V) were combined, weighed, and dissolved in minimum

[14]Scientific Products, Menlo Park, California.

Table VI: Rf values of the alkaloids I, II, III[a]

Solvent System	Rf Values		
	Alkaloid I	Alkaloid II	Alkaloid III
Chloroform:methanol (95:5)	0.62	0.56	0.37
Acetone:chloroform:methanol (5:90:5)	0.55	0.42	0.21
Benzene:chloroform:methanol (5:90:5)	0.66	0.56	0.38

[a]Adsorbent: Precoated aluminum oxide plates (type E), F_{254}, abrasion resistant, 20 X 20 cm.

of chloroform:methanol (95:5). The solution was uniformly applied 2.0 cm from the base of the plate with the aid of a streaker.[15] Up to 60 mg of the sample was applied to each plate. The plates were air dried and allowed to develop in benzene:chloroform:methanol (5:90:5) for a distance of 16 cm. After the first development, the plates were air dried and redeveloped in the same solvent system. After two developments, the plates were air dried and examined under short wavelength UV. The band corresponding to Rf 0.37 was carefully scraped off, loaded in a small glass column, and eluted with chloroform:methanol (80:20). From fractions 8 and 9, 0.250 g of the crude alkaloid III was obtained. The preparative TLC was repeated on this material using the same conditions as above to yield 0.150 g of pale yellow gum. This material could not be induced to crystallize even though it appeared as a single spot when examined by TLC in several solvent systems. To obtain crystalline material, an attempt was made to prepare the hydrochloride of the free base as detailed below.

To a solution of the free base in anhydrous methanol, few drops of concentrated HCl were added and the solvent removed in vacuo. The resulting colored material was dissolved in minimum anhydrous methanol and hot benzene was added till cloudiness appeared. The solution was allowed

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[15]Kontes Glass Company, Vineland, New Jersey.

to stand for a few days. However, no crystals separated. This led to the conclusion that additional purification was required. The free base was recovered from the hydrochloride as specified below.

To the boiling solution of the hydrochloride in methanol, a few drops of ammonia was added. The solvent was removed in vacuo to yield a colored gummy material. A small glass column of deactivated silica gel (prepared as before) was set up in chloroform and the colored gum was applied to the top of the column as a solution in chloroform. Elution was started with chloroform followed by increasing proportion of methanol. Ten ml fractions were collected and monitored by TLC on silica gel GF uniplates, using chloroform:acetone:diethylamine as the developing solvent and Dragendorff reagent as the detecting agent. Elution with chloroform:methanol (85:15) gave single spot fractions. Concentration and complete removal of the solvent in vacuo left behind a light yellow gum. This residue crystallized from acetone-petroleum ether to yield 0.060 g of pale yellow crystals of a substance which was designated as alkaloid III.

Isolation of Alkaloid I: Fraction 6 (Table V) was observed to contain this alkaloid and was therefore subjected to preparative TLC procedures as outlined for alkaloid III. The solvent for development however was a mixture of acetone:methanol:chloroform (5:5:90). The

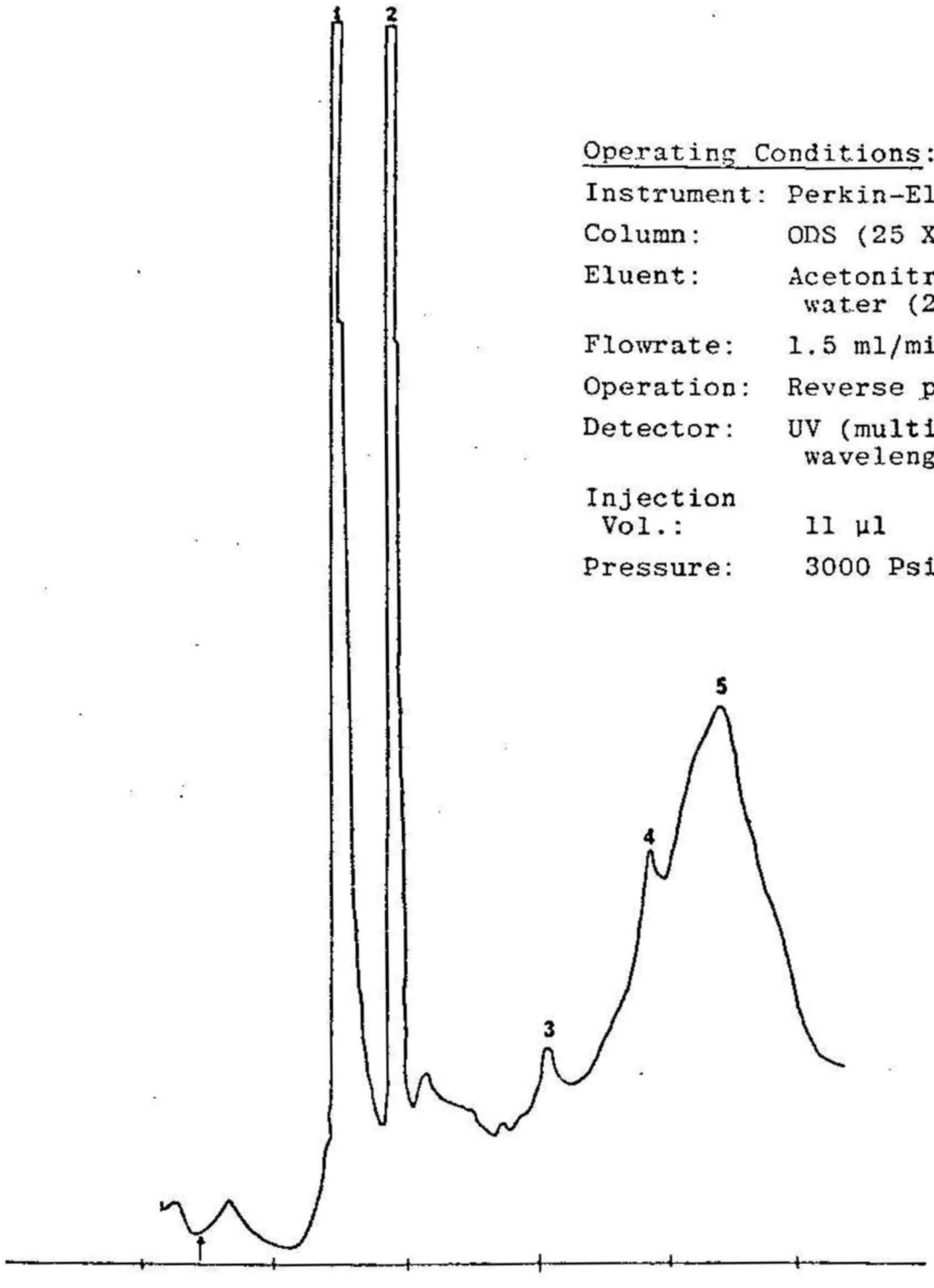
band at Rf 0.55 was due to alkaloid I and was scraped off and eluted by chloroform:methanol (90:10) as previously described. Removal of the solvent yielded a pale orange residue. In order to further purify this residue, it was rechromatographed by preparative TLC using acetone: methanol:chloroform (35:5:160) to obtain a pale yellow gum. Although monitoring by TLC showed only one spot, no crystallization could be achieved. This pale yellow gum was therefore rechromatographed by CC on silica gel (deactivated) with chloroform:methanol (90:10) as eluent. Removal of solvent from eluate gave a colorless gum. Addition of acetone-petroleum ether to this gum gave white crystals of Alkaloid I (0.020 g).

Similar procedures were carried out on eluate fraction 7 (Table V), since it contained Alkaloid II. Only 1-2 mg of pure substance was recovered.

High Pressure Liquid Chromatography

A qualitative study of the purity of the fraction 8, obtained from the chromatography of extract F, was carried out using a Perkin-Elmer high pressure liquid chromatography equipment. The conditions of the chromatography are as described below:

Column dimension:	25 X 0.26 cm
Adsorbent:	ODS
Elution solvent:	Acetonitrile:water (20:80)
Flow rate:	1.5 ml/min
Operation:	Reverse phase



Operating Conditions:

Instrument:	Perkin-Elmer
Column:	ODS (25 X 0.26)
Eluent:	Acetonitrile: water (20:80)
Flowrate:	1.5 ml/min.
Operation:	Reverse phase
Detector:	UV (multi-wavelength)
Injection Vol.:	11 µl
Pressure:	3000 Psi

Fig. 3. High pressure liquid chromatogram of a fraction of impure hopeanine (peak 5 corresponds to hopeanine)

Detector:	UV (multi wavelength)
Injection volume:	11 µl
Operating pressure:	3000 Psi.

After the injection of the sample, the chromatogram was carried out and the eluents collected as they emerged in separate flasks. The eluents were concentrated and examined by TLC. By this procedure it was concluded that the fraction 8 contained at least six components and that on a high pressure liquid chromatogram (run under the above conditions) Alkaloid III corresponded to peak 5 (Fig. 3). However this equipment was not available for further experimentation.

Spectral Analysis

The identity and characterization of the pure compounds were achieved by using various physico-chemical (spectral) methods of analysis, melting point determination and whenever possible derivatization. The instruments employed and conditions for spectral analysis were as given below:

a) UV - Perkin-Elmer UV-VIS 202; compounds dissolved in spectral grade methanol.

b) IR - Perkin-Elmer IR 137 and 337B; spectra obtained as KBr pellet (solid compounds) or dissolved in spectral grade CCl_4.

c) ^{1}H NMR - JOEL, 100 MHz spectrophotometer. Tetramethylsilane (TMS) as internal standard. Chemical shifts reported in δ units.

d) ^{13}C NMR - Bruker, WH-90. Pulse flip angle 33^{o}, repetition rate 0.7 seconds, spectral width 6024 Hz and computer limited resolution of 0.735 Hz.

e) Mass Spectrum (MS) -

Low Resolution - UCB, MS-12 operating at 70 ev.

High Resolution - UCB, SSL double focussing

Chemical Ionization - Dupont, 21491 B EI/CI.

RESULTS AND DISCUSSION

PRELIMINARY CHEMICAL SCREENING

Preliminary phytochemical screening was undertaken to confirm the reported presence of alkaloids in Brunfelsia hopeana. Definite turbidity was observed when the methanolic and chloroform extracts of the whole root were tested with Mayer's reagent.

In order to estimate the number and nature of the alkaloids present in the plant, so that a suitable extraction procedure could be developed for isolation of the constituents, a series of extracts obtained by extraction of the root powder with hot and cold methanol, aqueous methanol, and water were subjected to TLC on silica gel. On spraying with Dragendorff's reagent, the hot and cold methanol and aqueous methanol extracts showed the presence of two or possibly three alkaloids as noted by the appearance of discrete Dragendorff-positive orange spots on the TLC plate. The hot and cold aqueous extracts did not show any Dragendorff-positive spots. The nature of the alkaloids was predicted by testing the chloroform extracts. These again showed the presence of orange spots on the TLC. This indicated that the alkaloids were probably secondary or tertiary in nature. No evidence for the presence

of quarternary alkaloids could be found. When Mayer's reagent was added to a solution of the aqueous extract at alkaline pH, no turbidity was observed. These are therefore probably absent in the root of B. hopeana.

HIPPOCRATIC SCREENING

In the present investigation, the powdered crude drug and its various extracts (Fig. 1) were subjected to hippocratic screening. Malone and Robichaud (37) state that "the goal of Hippocratic screen is to determine one lethal dosage, one completely ineffective dosage and at least three effective log dosages between these two levels." They also noted that "the amount of test material needed to complete an evaluation should be small; yet for a natural product program, the screen must be designed to utilize equally well either chemically pure or grossly crude material in order that extract fractionation may be followed pharmacologically. If a pure chemical or crude material is not active intraperitonially in agar suspension at 100 mg/Kg and 1 gm/Kg, respectively, it will eventually prove to be not feasible commercially and therapeutically."

Observations after intraperitonial injection were recorded on the hippocratic screen work sheets described by Malone et al. (Fig. 4) (37). All references to time refer to the time after injection when the specified symptoms were first seen. Practical difficulties in suspending the test samples in agar prevented the administration of dosages

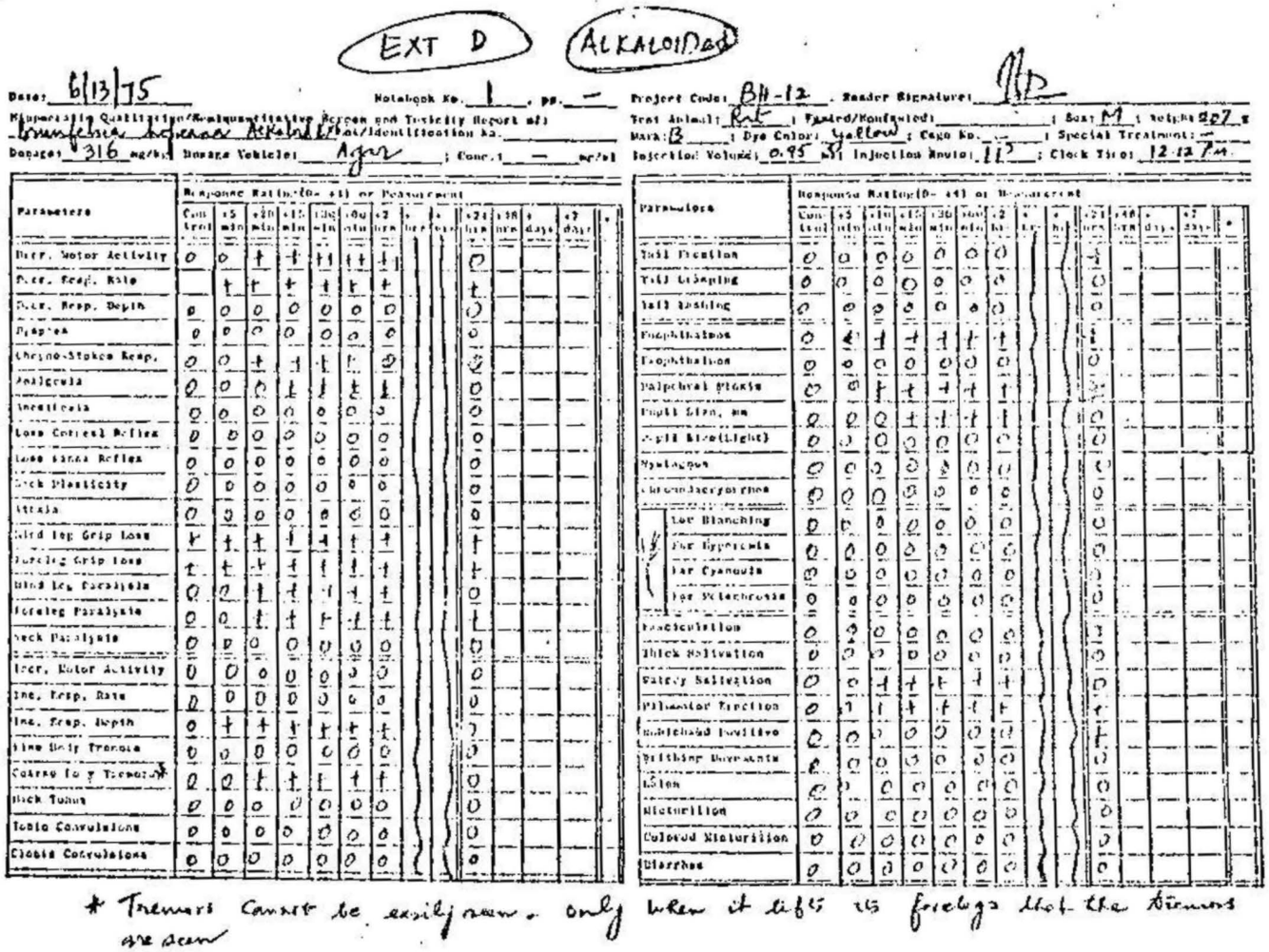
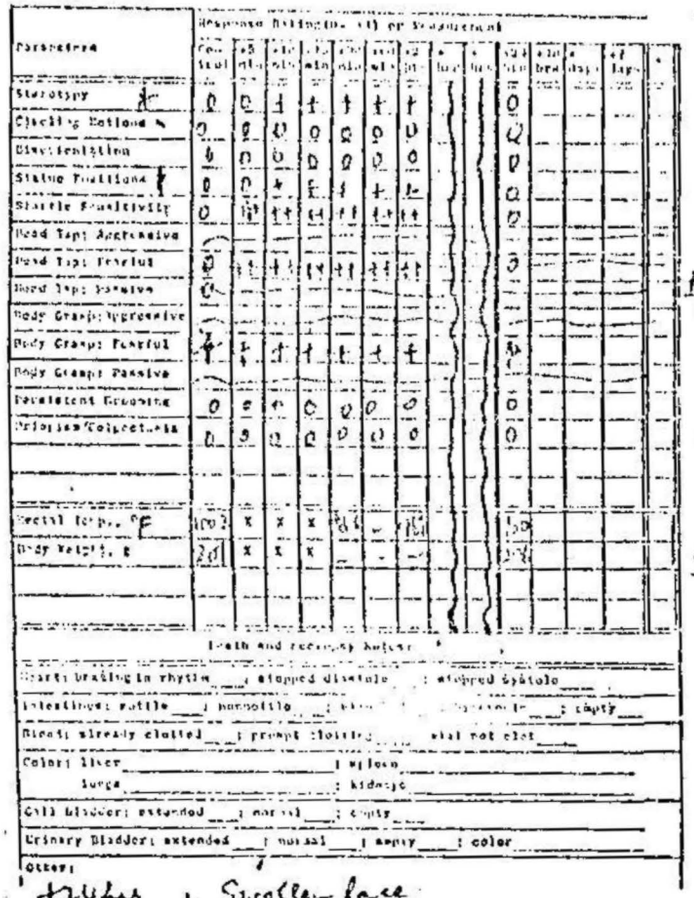


Fig. 4. Illustrative standardized work-sheet used for hippocratic screening, of extract D obtained from Brunfelsia hopeana root



Remarks:

1. Marked tendency to close the eye (Ptosis). — blinking. (quick closing & opening of eye)
2. Twitch head.

145 mins

*3. Animal sits in a corner, eyes almost completely closed with head lifted up. once in a while jerks its body & head.

4. Tendency to keep ears clamped.

5. Twitch head.

Stereotypy & Stature Position *

* Sitting in a corner slowly raises its head at the to high level, seem to gasp for breath, then slowly brings its head down. clear blinking of the eye. + discomfort near strong pinch motor erection.

+24hrs

1. Swollen face
2. Very back & Tail erection.
3. Ptosis still seems apparent.
4. Decrease in Respiratory rate

After 7hrs

Appears normal

Fig. 4. (continued)

higher than 1 gm/Kg. Results of the screen on the whole root and its various extracts are given below, followed by summarization of the observations.

Powdered Whole Root

100 mg/Kg: +10 Minutes: slight decrease in motor activity and mild analgesia lasting for about an hour. +2 Hours: Animal appeared grossly normal.

562 mg/Kg: +10 Minutes: decrease in motor activity, mild respiratory depression, and mild analgesia lasting for about two hours. +30 Minutes: slight tail erection, increase in pupil size, pilomotor erection, and startle sensitivity. Personality of the animal appeared grossly normal.

1000 mg/Kg: +10 Minutes: decrease in motor activity, loss of grip strength (hind legs followed by forelegs), mild analgesia, and mild respiratory depression lasting for about two hours. +30 Minutes: fine body tremors and increase in pupil size. Personality of the animal changed from normal to passive. +24 Hours: animal appeared grossly normal.

Summary. The minimum dose producing definite symptomology was found to be 100 mg/Kg. When animals were dosed

between 100 mg/Kg and a nonlethal dose of 1000 mg/Kg, the following symptoms were found to be dose-related:

a) decrease in spontaneous motor activity
b) respiratory depression
c) mild analgesia
d) hypersensitivity to sound
e) increase in pupil size

Extract B

178 mg/Kg: +10 Minutes: loss of grip strength (hind-legs followed by forelegs), slight enophthalmos, and pilomotor erection lasting for about two hours. +15 Minutes: decrease in motor activity, decrease in respiratory rate, increase in pupil size, and watery salivation lasting for about two hours. A personality change from normal to passive was observed. +24 Hours: some loss of grip strength still present (hind legs only); otherwise animal appeared grossly normal.

Summary. The minimum lethal dose was observed to be 1000 mg/Kg. All symptoms noted with the whole root dose of 1000 mg/Kg could be seen at a dose level of 178 mg/Kg, indicating concentration of activity. At the lethal dose level of 1000 mg/Kg, Cheyne-Stokes respiration and total paralysis were noted. Autopsy revealed that death could have occurred due to respiratory failure, since the

heart was beating in a coordinated fashion.

Marc C

100 mg/Kg: No gross symptoms were apparent.

1000 mg/Kg: +15 Minutes: slight decrease in respiratory rate and some increase in pupil size; otherwise animal was grossly normal.

Summary. At dose levels of 1000 mg/Kg, this material induced no grossly apparent symptomology. This indicated that the activity associated with the whole root had been efficiently extracted by menthanol.

Extract D

100 mg/Kg: +10 Minutes: decrease in motor activity, mild analgesia and decrease in respiratory rate. +15 Minutes: loss of grip strength (hindlegs followed by forelegs), fine body tremors, increase in pupil size, and a personality change from normal to passive. +30 Minutes: some stereotypic behavior, increased depth of respiration, enophthalmos, and palpebral ptosis. All of the above symptoms persisted for approximately two hours. +24 Hours: some loss of grip strength (hind legs) still present; otherwise grossly normal.

316 mg/Kg: +10 Minutes: decrease in motor activity, decrease in respiratory rate, and decrease

in respiratory depth lasting for about two hours. +15 Minutes: mild analgesia, enophthalmos, palpebral ptosis, watery salivation, pilomotor erection lasting for about two hours. Some stereotypic behavior characterized by the animal sitting in a corner with its forelegs lifted up and scratching the mouth and facial region. Hypersensitivity to sound and touch was also present. +30 Minutes: mild body tremor, hypothermia (2^{o} fall in temperature), and pilomotor erection lasting for approximately two hours. +24 Hours: loss of grip strength, slight tail erection, some ptosis, and a decrease in respiratory rate were still present.

Summary. Responses qualitatively similar to those previously seen but more pronounced were observed, at doses indicated, namely

a) decrease in motor activity
b) respiratory depression
c) mild analgesia
d) hypersensitivity to sound
e) increase in pupil size
f) salivation
g) lacrimation
h) hypothermia
i) pilomotor erection

Extract E

1000 mg/Kg: +15 Minutes: slight decrease in respiratory rate and some increase in pupil size; otherwise the animal was grossly normal. Even at this dose level the material induced no apparent symptomology.

Extract F

178 mg/Kg: +10 Minutes: slight decrease in motor activity. +15 Minutes: mild analgesia, increase in respiratory depth with decrease in rate, increase in pupil size. +30 Minutes: decrease in motor activity more pronounced, pilomotor erection, some hypothermia, change in personality from normal to passive. These symptoms persisted for about two hours. +24 hours: animal grossly normal.

316 mg/Kg: +10 Minutes: slight decrease in motor activity, and grip loss (hindlegs followed by forelegs). +15 Minutes: mild analgesia, increase in respiratory depth, fine body tremors, increase in pupil size, some stereotypic behavior and hypothermia (4° fall in temperature). +30 Minutes: pronounced decrease in motor activity, pilomotor erection, diuresis, change in personality from normal to passive, pronounced hypothermia

(5^{o} fall in temperature), and stereotypic behavior. Also noted were pinkish coloration of facial region and a tendency to hold back its ears. All of the above symptoms persisted for about two hours. +24 Hours: some grip loss (mainly of the hind legs) and fine body tremors were still present; otherwise grossly normal.

Summary. The minimum lethal dose was found to be 562 mg/Kg. When animals were dosed between 100 mg/Kg and 562 mg/Kg, the following symptoms were found to be dose-related:

a) decrease in spontaneous motor activity
b) decrease in respiratory rate with increase in depth
c) analgesia
d) fine body tremors
e) increase in pupil size
f) pilomotor erection
g) lacrimation
h) hypothermia

Lethality at dose level of 562 mg/Kg occurred between one and two days, hence the exact cause of death could not be ascertained.

Scopoletin

100 mg/Kg: No symptoms were apparent; animal remained

grossly normal.

At this dose level no symptoms were apparent. According to Malone and Robichaud this observation indicates no significant phamacological activity.

Discussion

As pointed out in the introduction, Brandl (20) in 1885, had published on the gross pharmacology of a product "manacine," isolated from manaca (B. hopeana) root. In spite of the interesting folkloric medicinal use of this plant, this is the only pharmacologic study published so far.

The present hippocratic screen of the crude drug powder and its extracts showed interesting pharmacological activity. The extraction procedure concentrated the activity especially in the extract F. This extract produced a qualitatively unique central nervous system depressant action characterized by a reduction in motor activity, foreleg and hind leg grip loss and hypothermia. The symptoms noted within the lower doses of the whole root and the extracts were as follows:

a) decrease in motor activity

b) foreleg and hind leg grip loss

c) mild analgesia

d) mild hypothermia

Further separation and purification of extract F yielded Alkaloids I, II, III and scopoletin. Scopoletin had no significant pharmacological activity. Presumably

the alkaloids would concentrate the activity of the extract F. However the low recovery of total quantities of pure compounds precluded their use in hippocratic and anti-inflammatory screens.

ANTIINFLAMMATORY SCREENING

The folkloric medicinal uses of Brunfelsia and the signs and symptoms observed during hippocratic screening indicated that extract F should be further examined for its antiinflammatory action. The procedure of Van Arman et al. (37) was selected for this screening. Inflammation was induced in the rat paw by injection of carrageenin and the degree of inflammation was measured by a method similar to that described by Winter and Nuss (41). The reduction of inflammation on oral administration of an antiinflammatory agent can be conveniently observed in this method.

The Table VII shows the mean injected paw volumes of the control and treatment groups as measured by the plethysmograph, at the specified time intervals. The difference between the mean paw volume at a specific time and the mean volume at +5 minutes is a measure of the degree of inflammation.

The significance of differences between test groups was assessed by Dunnett's 't' test (42, 43). It is seen that (Table VIII), there exists a significant difference ($p < 0.05$) in the degree of inflammation between the control animals and the animals which received the extract F

Table VII: Effects of drug treatment on carrageenin-induced hind paw swelling on rats

Treatment (Dosage)[a]		Mean hind paw volume, ml ± 1 S.E.[b]											
		0	+5	+30	+60	+90	+120	+150	+180	+210	+240	+270	+300
Control		0.71	0.97	1.11	1.21	1.26	1.47	1.58	1.62	1.63	1.55	1.49	1.43
(5 ml/Kg)	±	0.03	0.03	0.06	0.07	0.08	0.09	0.07	0.07	0.07	0.07	0.06	0.06
Chloroform		0.77	0.90	1.04	1.03	0.95	1.06	1.19	1.27	1.29	1.29	1.26	1.23
Extract (F)	±	0.04	0.04	0.05	0.06	0.06	0.05	0.07	0.06	0.06	0.07	0.05	0.05
(100 mg/Kg)													
Phenlybutazone		0.76	0.91	1.06	1.13	1.14	1.12	1.18	1.27	1.34	1.27	1.33	1.23
(100 mg/Kg)	±	0.03	0.03	0.05	0.06	0.06	0.06	0.06	0.06	0.07	0.05	0.07	0.10

[a] A constant dose volume of 10 ml/Kg was utilized for the oral administration.

[b] Each treatment group represents ten animals.

Table VIII: Effects of drug treatment on carrageenin-induced hind paw swelling on rats

Treatment (Dosage)	Mean Paw Volume, ml. ± 1 S.E.[a] (Min.)									
	+30	+60	+90	+120	+150	+180	+210	+240	+270	+300
Control (5 ml/Kg)	0.15 ±0.03	0.24 ±0.05	0.29 ±0.06	0.50 ±0.08	0.61 ±0.07	0.64 ±0.06	0.65 ±0.07	0.58 ±0.07	0.52 ±0.05	0.63 ±0.06
Chloroform Extract (F) (100 mg/Kg)	0.14 ±0.03	0.16 ±0.04	0.16 ±0.04	0.17[b] ±0.04	0.29[b] ±0.05	0.37[b] ±0.05	0.40[b] ±0.04	0.45 ±0.03	0.36[b] ±0.03	0.32[b] ±0.02
Phenylbutazone	0.15 ±0.03	0.23 ±0.04	0.23 ±0.05	0.21[b] ±0.05	0.27[b] ±0.05	0.36[b] ±0.05	0.43[b] ±0.07	0.36[b] ±0.06	0.42 ±0.06	0.37[b] ±0.09

[a]Represents the mean differences between volume at a specific time, minutes post-injection, and the volume at +5 minutes. Each treatment group represents 10 rats.

[b]Significant difference between the effects seen in the control and treated animals at 0.05 level of probability.

treatment, at time intervals of +120, +150, +180, +210, +270, and +300 minutes. In case of the test group which received the phenylbutazone treatment such differences were apparent at time intervals of +120, +150, +180, +210, +240, and +300 minutes post injection. It is seen therefore that at equal oral dose levels of 100 mg/Kg, extract F is as effective as phenylbutazone in reducing carrageenin-induced pedal edema. In both instances, the onset of effects were apparent two hours after pedal injection and persisted for at least three hours thereafter.

CYTOTOXIC ACTIVITY

The results of the experiments on testing the cytotoxic activity of the extracts B and F are recorded in Tables IX and X. The plot of cell population VS time, extract F, is shown in Fig. 5. Analysis of the results obtained with extract B showed that no correlation could be derived between control and treated cells. Thus this total methanolic extract (Fig. 1) was of very little further interest in this regard.

Extract F showed some apparent differences between control and treated SV 3T3 cells. However, the data available could not be used to obtain significances of these apparent differences. The control and treated 3T3 cells did not show any apparent differences. Any further investigations were precluded due to lack of test materials.

Table IX: Effect of extract F on the growth of 3T3 and SV 3T3 cells in culture

	3T3		SV 3T3	
Day	c[a]	t[b]	c	t
1	178[c]	211	362	205
2	340	274	636	213
3	379	545	1960	443
4	623	400	11480	582
5	713	506	confluent	0

[a] c = Control group

[b] t = Treatment group

[c] Each of the values in the above table represents the cell count in 100 µl of solution as recorded by the Coulter Counter and is the mean of three readings per sample.

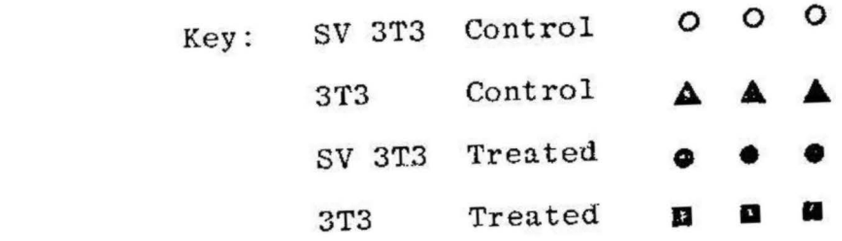
Table X: Effect of extract B on the growth of 3T3 and SV 3T3 cells in culture

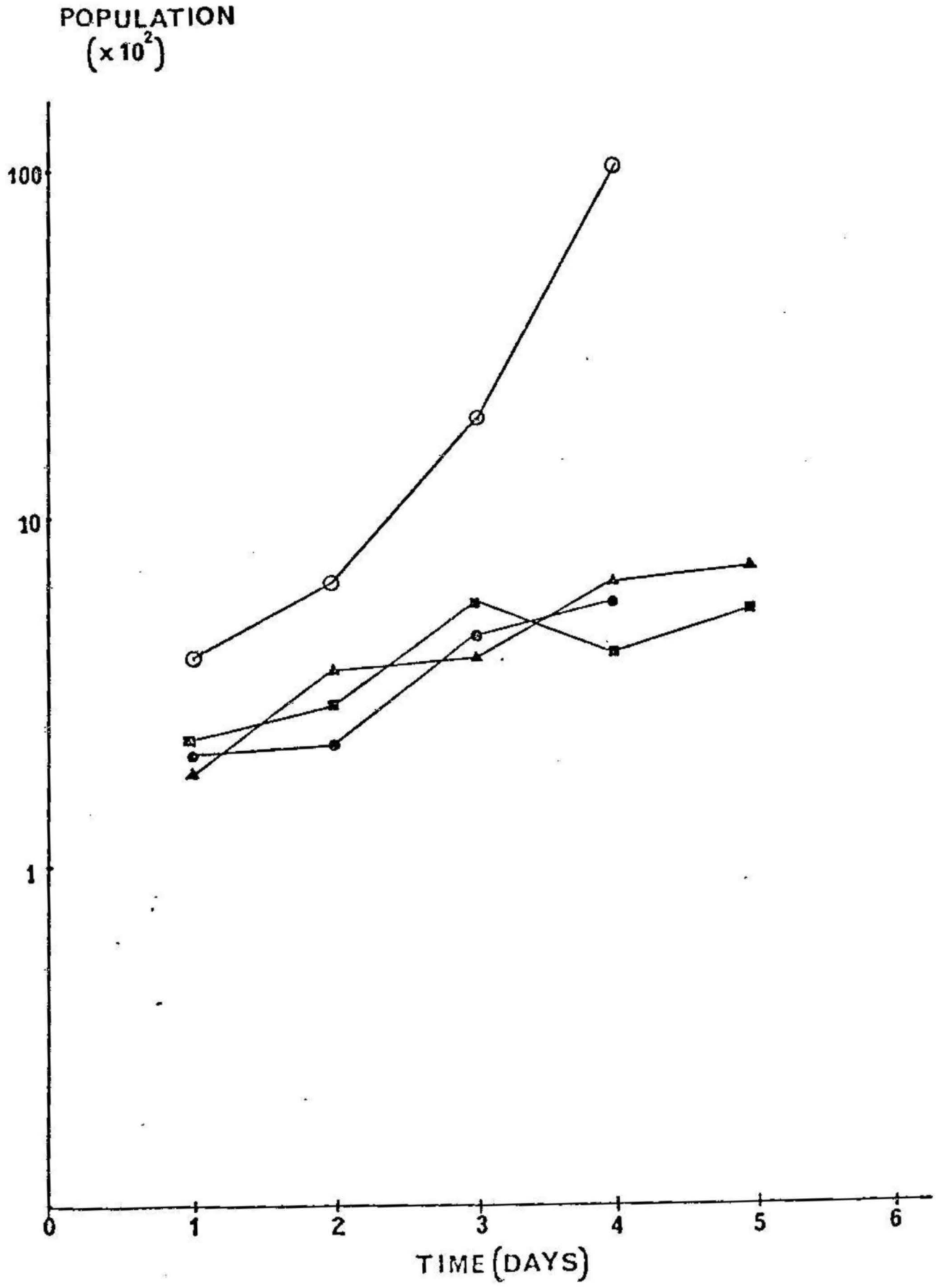
	3T3					SV 3T3				
Day	c	a	b	d	e	c	a	b	d	e[1]
1	594[a]	594	594	594	594	404	404	404	404	404
2	4541	2191	2110	1991	2002	1021	614	664	667	520
3	11625	9052	6639	10967	10460	1604	1041	1425	991	1022
4	----------confluent----------					1554	928	1276	1425	970
5	----------confluent----------					1990	2085	1451	1857	1602
6	----------					2150	1842	1656	1532	1856

[a]Each of the values in the above table represents the cell count in 100 µl of the solution as recorded by the Coulter Counter and is the mean of three readings.

[1]c,a,b,d and e are the treatments representing 0, 50, 100, 200, and 400 µg/ml concentration of the extract, respectively.

Fig. 5. Effect of extract F on the growth of normal and transformed cells, in culture.




POPULATION
(x 10²)
100
10
1
0
1
2
3
4
5
6
TIME (DAYS)

SEPARATION, PURIFICATION AND CHARACTERIZATION OF CONSTITUENTS

The crude screening procedure of Taylor (31) had shown that "alkaloids" were present in the crude extract. This procedure also indicated that the "alkaloids" were being extracted in extract F (Fig. 1). The TLC procedure had shown the presence of three Dragendorff- and iodine-positive spots in extract F. These were therefore designated as Alkaloid I, II and III (Fig. 6).

Since, in the present investigation, major attention was devoted to these three alkaloids, the CC procedures were developed to achieve their separation and purification. The other components were obtained serendipitiously.

β-Sitosterol

This ubiquitous plant sterol crystallized from the CC fractions 10 and 11 from extract P (Table IV). The identification of this constituent was based on the following data, which was identical to those of an authentic sample.[16]

a) m.p. 136-137°, mixed m.p. 136-138°

b) α_D -33° at 22°C

c) Positive Liebermann-Burchard and Salkowski reactions.

d) IR, UV and MS (Fig. 7)

---

[16]Supplied by Dr. H. Miles, University of Mississippi.

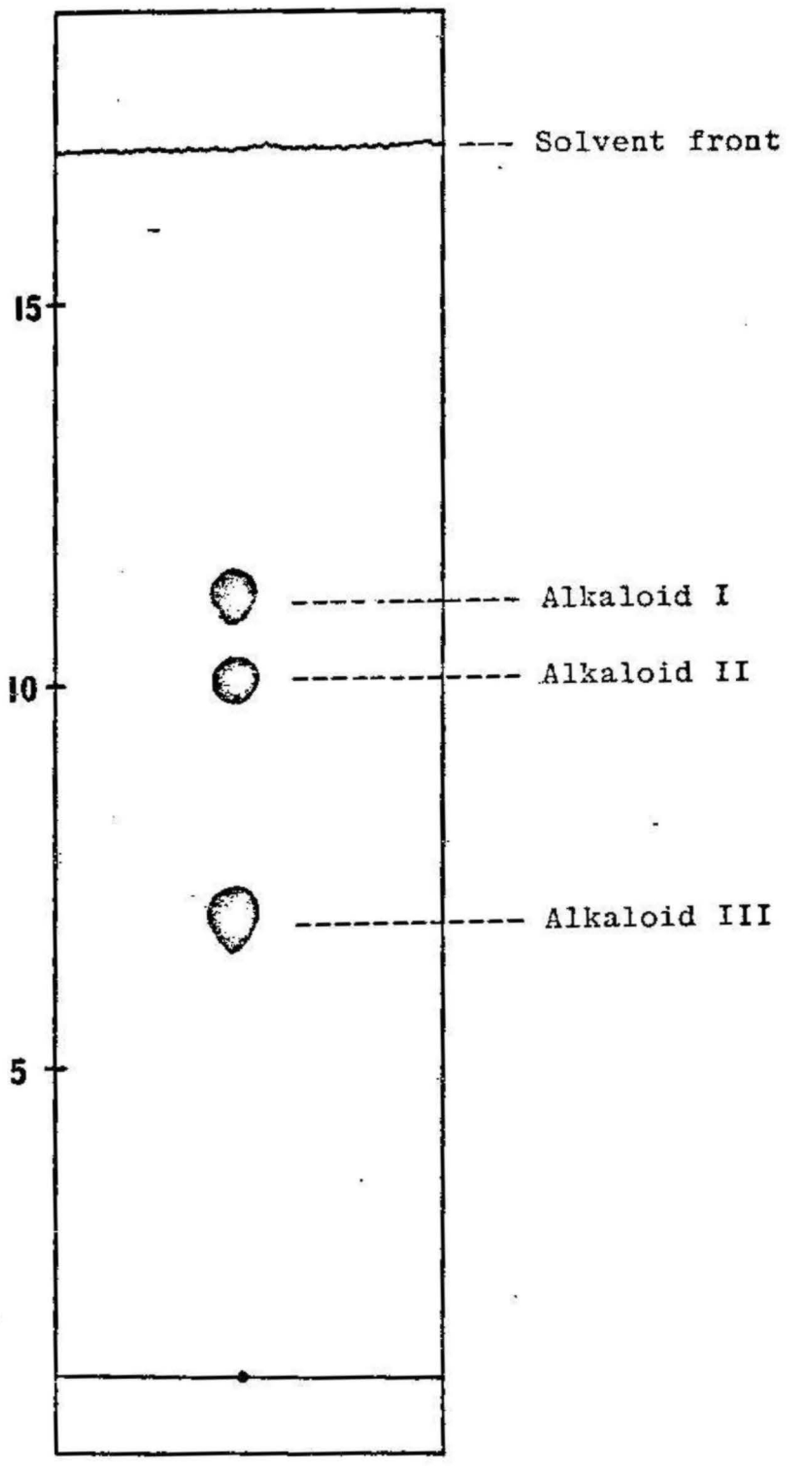


Fig. 6. Diagram of TLC separation of alkaloids from extract F. Alumina Type T, with F_{254}, 0.25 mm. Solvent system-Benzene:chloroform:methanol (5:90:5).

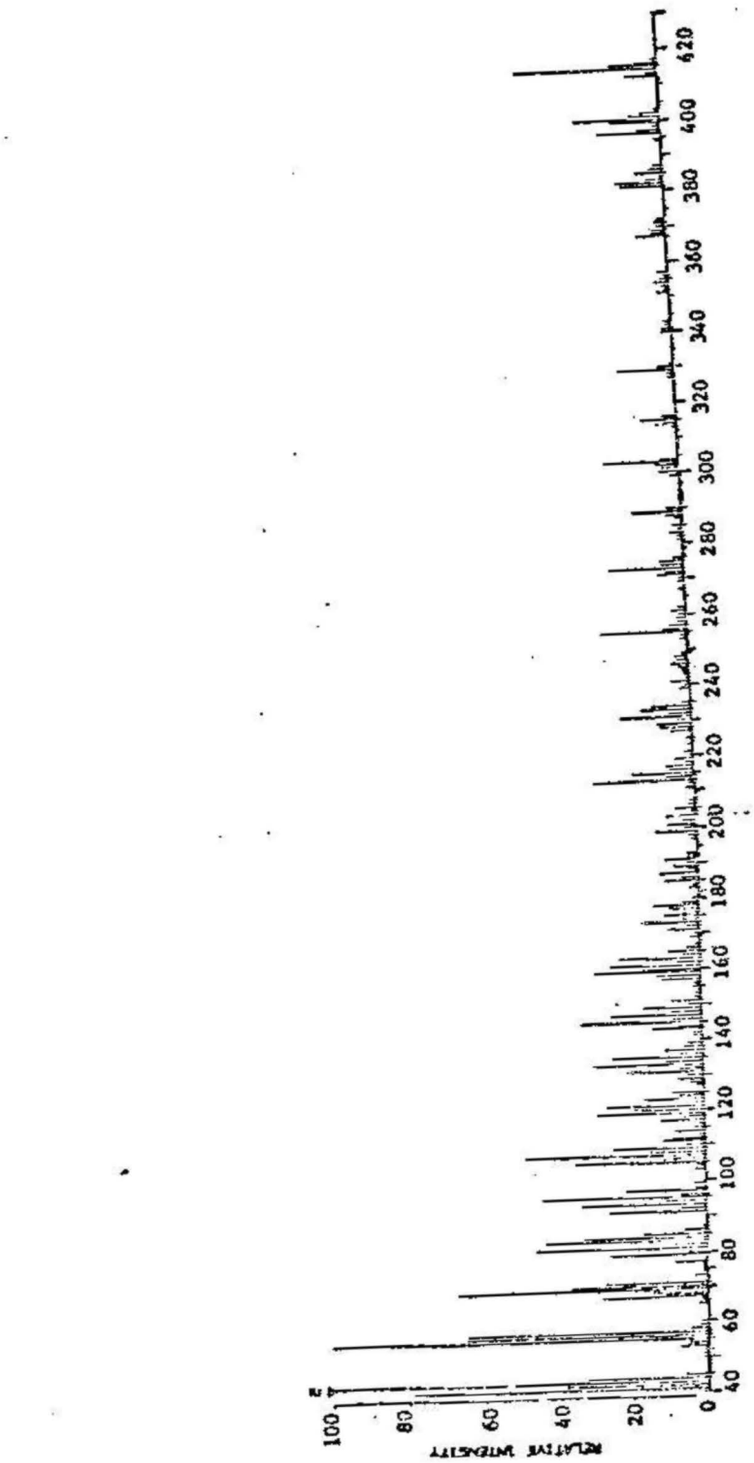


Fig. 7. Mass spectrum of β-sitosterol

Scopoletin

Fractions 2 and 3 (Table V) of CC of extract F yielded 0.5 g of white crystalline material (m.p. 204°). The physico-chemical characteristics of this compound were identical to those reported for scopoletin (44). Mors had previously isolated and characterized scopoletin from various species of Brunfelsia including B. hopeana. The spectra of scopoletin are presented in the following pages (Fig. 8, 9).

C-13 NMR. To our knowledge the ^{13}C NMR spectrum of the scopoletin had not been previously reported. Therefore this spectrum is discussed below:

The wide-band ^{1}H decoupled spectrum of scopoletin (Fig. 10) indicated the presence of 10 peaks, consistent with the presence of 10 different carbons in the molecule. The single frequency ^{1}H decoupled spectrum of scopoletin showed the presence of one quartet ($-CH_3$), four doublets (-CH), and five singlets (non-protonated carbons). For discussion's sake, the signals can be divided into four regions characteristic of different types of carbon atoms. The first region, from 0-50 ppm, characteristic of sp^3 carbon atoms bonded to carbon atoms; the second region, from 50-100 ppm, characteristic of sp^3 carbon atoms bonded to heteroatoms; the third region, from 100-150 ppm, characteristic of sp^2 carbon atoms not bonded to heteroatoms; the fourth region, from 150-200 ppm, characteristic

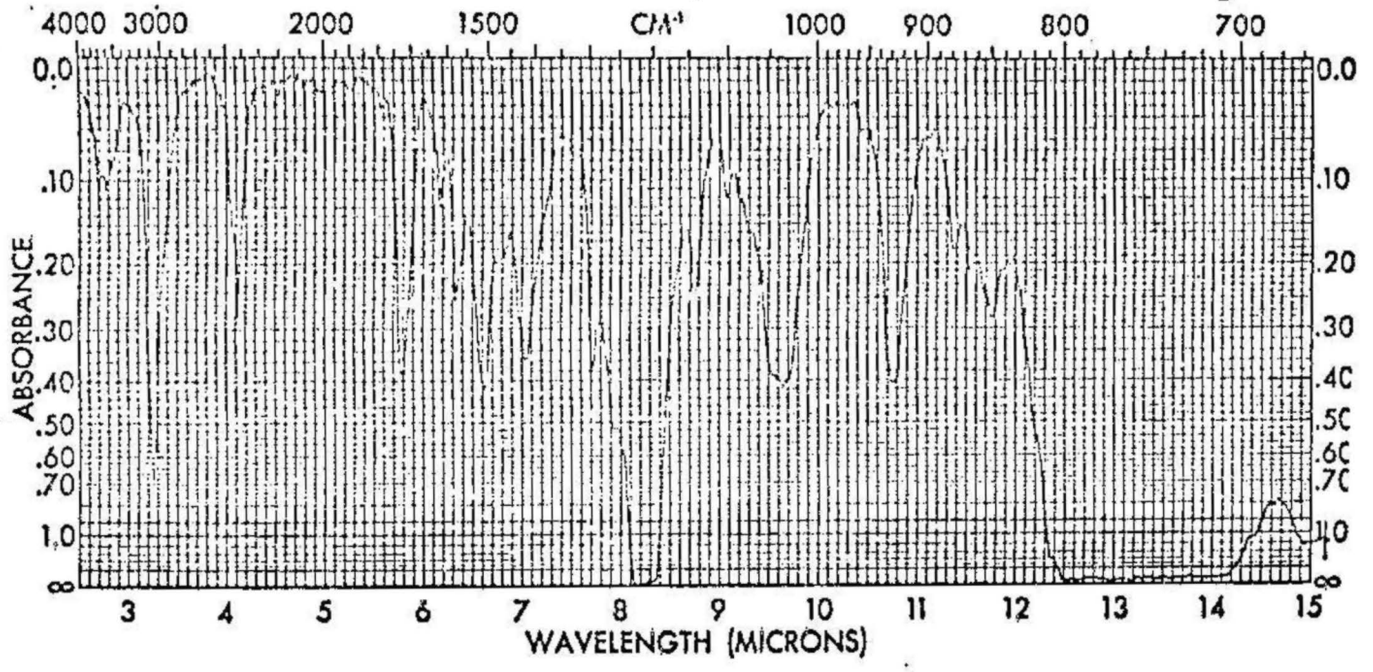


Fig. 8. IR spectrum of scopoletin in CCl_4

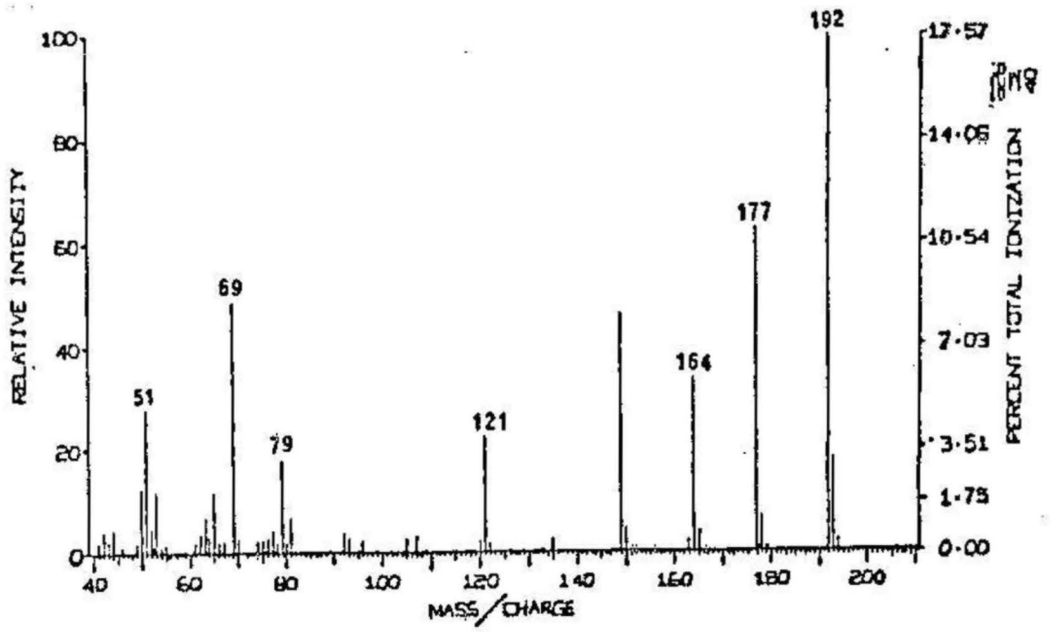
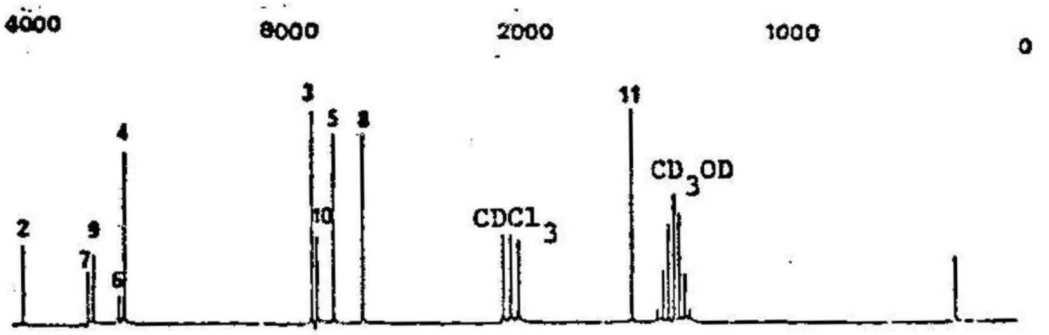


Fig. 9. Mass spectrum of scopoletin



a

Carbon #	Line #	Frequency	ppm	Area	Intensity
2	1	3690.789	183.164	47144.973	36736.111
7	2	3428.999	151.591	35319.035	25081.938
9	3	3403.996	150.486	26567.170	32105.150
6	4	3300.310	145.902	19902.721	13168.616
4	5	3275.308	144.797	246504.490	78680.649
3	6	2540.620	112.320	253678.248	97012.005
10	7	2523.031	111.539	47615.836	40435.337
5	8	2457.384	108.646	252595.319	87114.196
8	9	2342.867	103.575	259506.831	87034.986
	10	1787.668	79.030	150129.371	40688.494
	11	1755.312	77.600	174593.897	40253.690
	12	1723.691	76.202	155984.831	37477.604
11	13	1278.061	56.501	208191.770	97449.410
	14	1175.110	51.950	5003.205	7003.738
	15	1153.795	51.007	33297.466	23090.392
	16	1131.724	50.032	108075.230	44500.067
	17	1110.398	49.089	125583.454	58496.310
	18	1099.073	48.146	100906.715	48823.945
	19	1067.747	47.203	30543.946	22093.496
	20	1046.422	46.260	9600.462	5863.786
	21	0.000	0.000	64640.216	29968.302

b

Fig. 10. a. Wide-band ^{1}H decoupled ^{13}C NMR spectrum of scopoletin.

b. Computer print-out of the above spectrum.

of carbonyl carbons and aromatic carbons bound to oxygen (45).

In case of scopoletin, no signal is found in the region between 0 and 50 ppm, thus indicating that there are no sp^3 carbon atoms bound to carbon atoms. In the region between 50 and 10 ppm, one peak was observed at 56.5 δ indicating that it corresponds to an sp^3 carbon bonded to a heteroatom, in the present case, oxygen. In the single frequency ^1H decoupled spectrum, the peak at 56.5 δ appeared as a quartet. These two observations made it possible to attribute the signal at 56.5 δ to an $-OCH_3$ group.

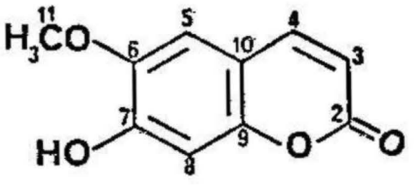
In the region between 100 and 150 ppm, six peaks were observed. Of these, the signals at 103.6, 108.6, 111.5 and 112.3 δ are part of sp^2 carbons not bonded to heteroatoms. The assignments of these shifts to carbons 8,5 and 10 (Table XI) were made using substituent chemical shift additivity principle (SCS principle) for hydroxy and methoxy substituents on the benzene ring. According to this principle, for a substituent such as methoxy or hydroxy, the ortho and para position δ values are shifted to higher field and that of the carbon of attachment to lower field, while the meta substituent is not much different from benzene (128.5 δ) (46). Carbons 5 and 8 appear as doublets in the single frequency ^1H decoupled spectrum and are ortho to the methoxy and hydroxy groups at 6 and 7 positions, respectively. These carbons will hence be

shifted -14.4 and -12.7, respectively, relative to benzene (46). Carbon 10 is para to the 7-hydroxy group and hence will be expected to appear at higher field. When these expectations are coupled with the values reported for coumarins (47, 48), it is possible to assign the peaks at 103.6, 108.6 and 111.5 δ to carbons 8,5 and 10 respectively. The peak at 112.3 δ can then be assigned to carbon 3 which is the α carbon of an α,β unsaturated ketone (lactone) system.

The signals at 144.8 and 145.9 also refer to sp^2 carbons. The one at 144.8 δ appears as a doublet in single frequency decoupled spectrum and evidently must be attributed to a carbon bearing a single hydrogen. Thus this signal can be assigned to carbon 4 which is also the β carbon of the α, β unsaturated ketone (lactone) system. The signal at 145.9 which appears as a singlet in the spectrum is then assigned to carbon 6.

Of the remaining three signals, the ones at 150.5 and 151.6 appear as singlets in the single frequency decoupled spectrum and are assigned to carbons 9 and 7, respectively, by comparison with the values reported for hydroxy coumarins (48). The signal at 163.2 is characteristic of carbonyl carbon in a five or six membered lactone system. Thus this peak could be assigned to the carbonyl group. Table XI lists the complete assignments for carbons in scopoletin.

Table XI: ^{13}C NMR data for scopoletin (1) (22.62 MHz, $CDCl_3$-CD_3OD)



1

Chemical Shift (ppm from TMS)	Signal multiplicity	Group	Assignment (carbon number)
163.2	s	O-C=O	2
151.6	s	HO-C=C	7
150.5	s	O-C=C	9
145.9	s	CH_3O-C=C	6
144.8	d	-C=C	4
112.3	d	-C=C	3
111.5	s	-C=C	10
108.6	d	-C=C	5
103.6	d	-C=C	8
56.5	q	$-OCH_3$	11

Alkaloid I

As pointed out in the experimental section the three "alkaloid" components were separated by CC and preparative TLC procedures, in pure crystalline form.

The spot corresponding to the Rf 0.55 (Table VI), on elution on preparative TLC gave 0.020 g of pure alkaloid I. The yield calculated on the basis of dried powdered root of B. hopeana was 1.4 mg/Kg.

Since the quantity of available pure compound was very small, characterization of this compound remains incomplete. It had a m.p. 178-180^{o}, and a molecular weight 315 (determined by chemical ionization mass spectrometry). The ^{1}H NMR spectrum in $CDCl_3$ shows (Fig. 11) no exchangeable protons. The PMR spectrum on gross analysis shows the possibility of an $-N-CH_3$ group (δ 3.4).

Alkaloid II

This compound from extract F crystallized from preparative TLC eluate of the spot corresponding to Rf 0.42 (Table VI). The total quantity of pure recrystallized substance obtained was 2 mg. This calculates to a yield of 0.14 mg/Kg of crude drug powder.

No further characterization of this constituent was possible.

Alkaloid III (Hopeanine)

This compound corresponding to the spot of RF 0.38 (Table VI) was recovered in pure crystalline form in

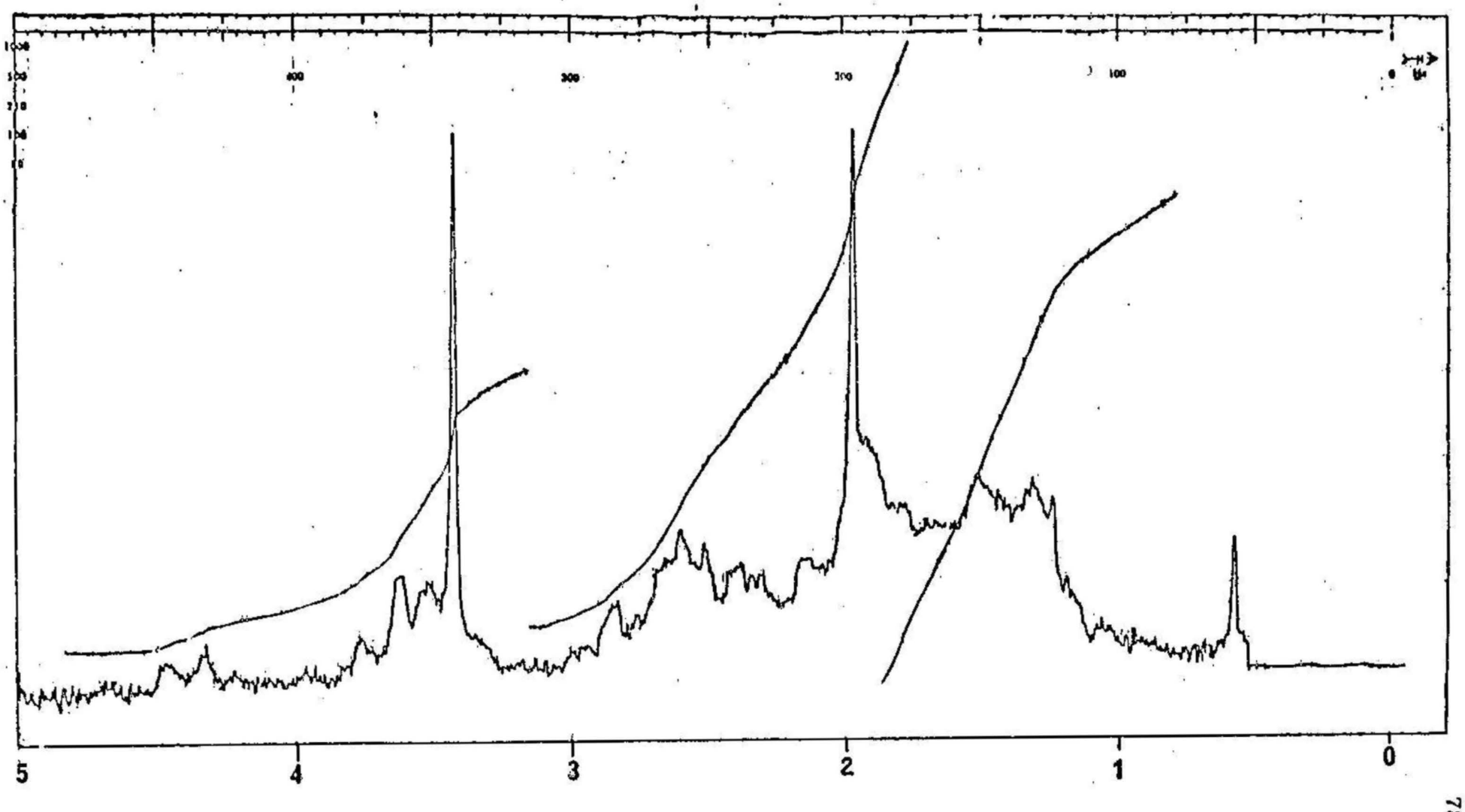


Fig. 11. 100 MHz ^{1}H NMR spectrum of Alkaloid I in $CDCl_3$

the total yield of 0.060 g calculating to a yield of 4 mg/Kg of dried root powder. The pale yellow crystals had a m.p. 164-5°. The purity of the compound was established by TLC on silica gel and alumina plates using various developing solvent systems (Table VI). In each case, only one single Dragendorff-positive spot was observed. The total amount of substance available, although small, made it possible to determine its structure by the use of special methods which are either non-destructive or require only very small samples. The many classical, destructive, chemical methods of analysis were precluded by the limited quantity available.

Structure of Alkaloid III (Hopeanine). As will be seen in the ensuing discussion, the compound initially designated as Alkaloid III is a new chemical entity, so far unreported in literature. Since it is obtained from Brunfelsia hopeana, alkaloid III was named "hopeanine." In the following discussion, this compound will be referred to as hopeanine.

The IR, UV and ^{1}H NMR (PMR) spectra of hopeanine are given in Figs. I2, 13 and 14, respectively. Only some gross information could be obtained by analyzing these spectra. The PMR spectrum was expected to give useful information regarding the number of protons and nature (environments) of each proton. However, the spectrum was complex and was of only limited use. The pertinent observations of spectral analysis are summarized below (49,50).

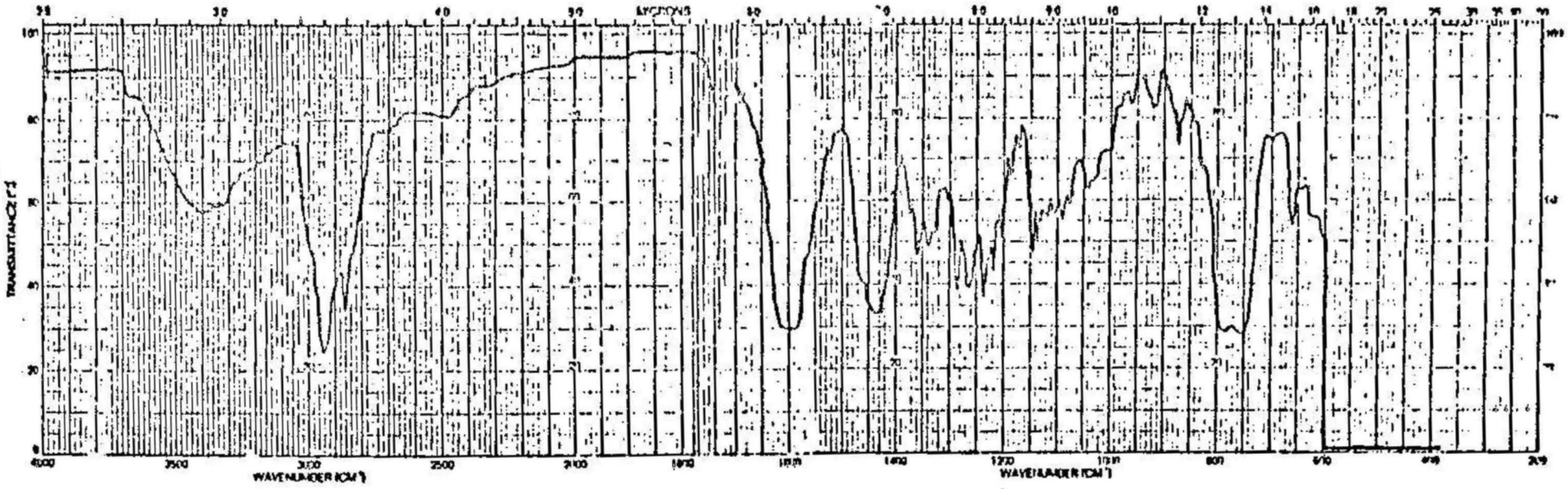


Fig. 12. IR spectrum of hopeanine in CCl_4

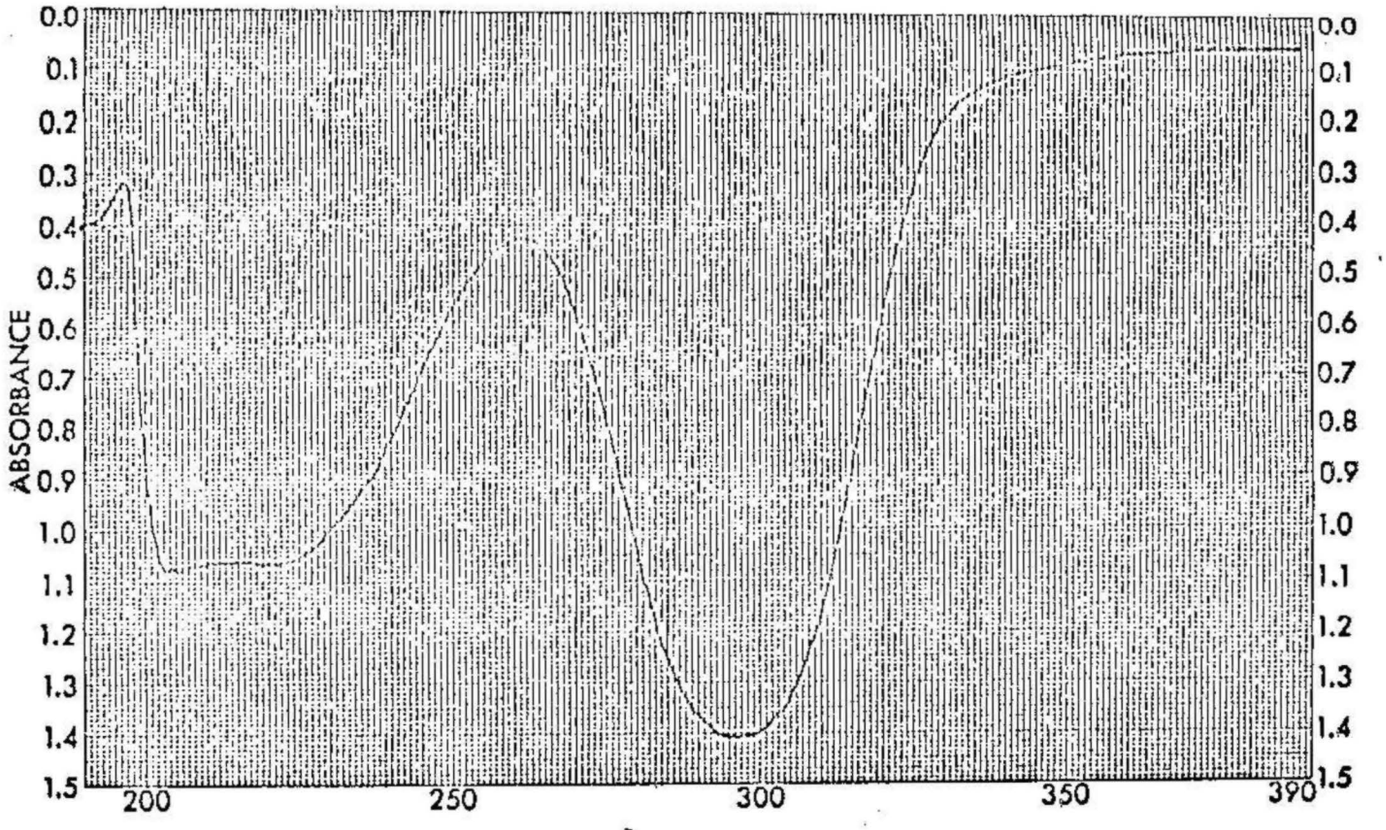


Fig. 13. UV spectrum of hopeanine in MeOH

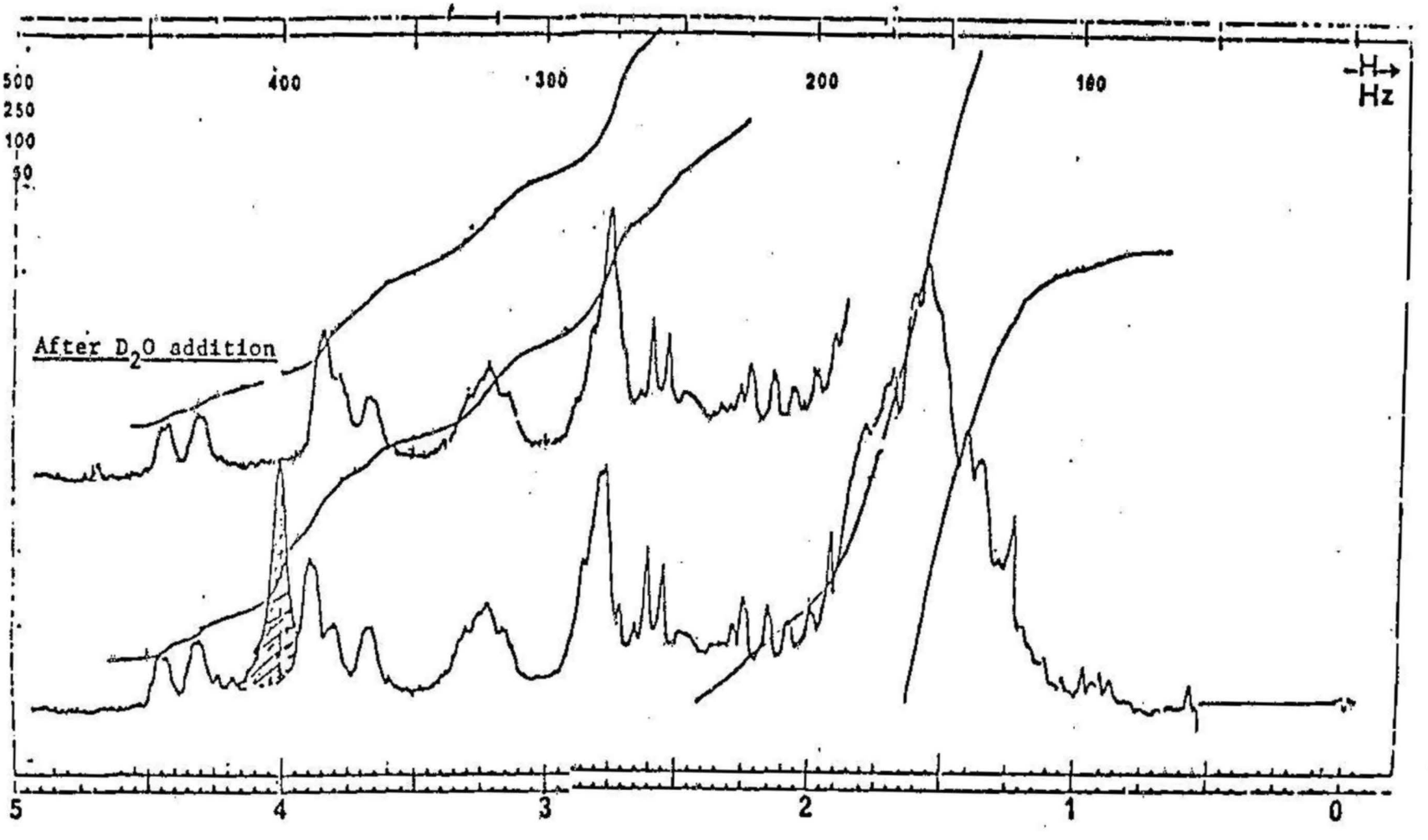


Fig. 14. 100 MHz ^1H NMR spectrum of hopeanine in $CDCl_3$

UV -- a. Strong, broad absorption around 295 nm (conjugate double bond system)

b. Plateau-like absorption around 215 nm

c. No bathochromic shift on addition of alkali (phenolic -OH absent)

IR -- a. Strong absorption around 3400 cm^{-1} (-NH or -OH group present)

b. Strong absorption at 2950 cm^{-1} (aliphatic -CH stretching)

c. Strong broad absorption around 1620 cm^{-1} (-C=N or -C=C present)

d. No absorption between 1640 to 1800 cm^{-1} (-C=O most likely absent)

PMR -- a. Total integration of spectrum - 448 divisions (29 or 30 protons)

b. No absorption between 6.7-8 δ (compound is non-aromatic or is fully substituted aromatic)

c. No absorption between 3.4-3.8 δ ($-OCH_3$ groups absent)

d. No absorption between 4 and 6 δ (allylic or vinylic proton absent)

e. Sharp, D_2O exchangeable, peak at 4 δ (-OH or -NH group present)

f. Signals for $C-CH_3$ or $N-CH_3$ not clear (possibly absent)

In order to more definitively characterize hopeanine, its molecular weight and molecular formula were determined. The classical method of determining molecular weights and elemental composition of a compound could not be used. Data obtained by low resolution and high resolution electron impact ionization (EI) mass spectrometry (MS) and chemical ionization (CI) MS and ^{13}C NMR was therefore used.

Low resolution MS (Fig. 15) showed apparent molecular ion peaks at 314 and 315 m/e. This suggested that the molecular weight of hopeanine was either 314 or 315. Based on the intensities of the M+1 peaks, various molecular formulas for the two possible molecular weights could be suggested. High resolution MS (Figs. 19, 20) gave a more precise measurement of the mass of the ions 314 and 315. The mass of the ion 314 was determined to be 314.220947 and that of 315 ion was found to be 315.227051. The possible molecular formulas for these weights are

M.W. 314.220947 = $C_{21}H_{30}O_2$; $C_{16}H_{30}N_2O_4$; $C_{19}H_{28}N_3O$

M.W. 315.227051 = $C_{21}H_{31}N_2O_4$; $C_{16}H_{31}N_2O_4$; $C_{19}H_{29}N_3O$

Analysis of the low resolution MS suggested at this stage that the M.W. of hopeanine was 314, since the intensity of the m/e peak for 314 was too high to be considered as M-1 peak of the 315 ion. On this basis the molecular formula $C_{16}H_{30}N_2O_4$ was tentatively assigned to hopeanine. This seemed to agree with the 29-30 protons suggested to

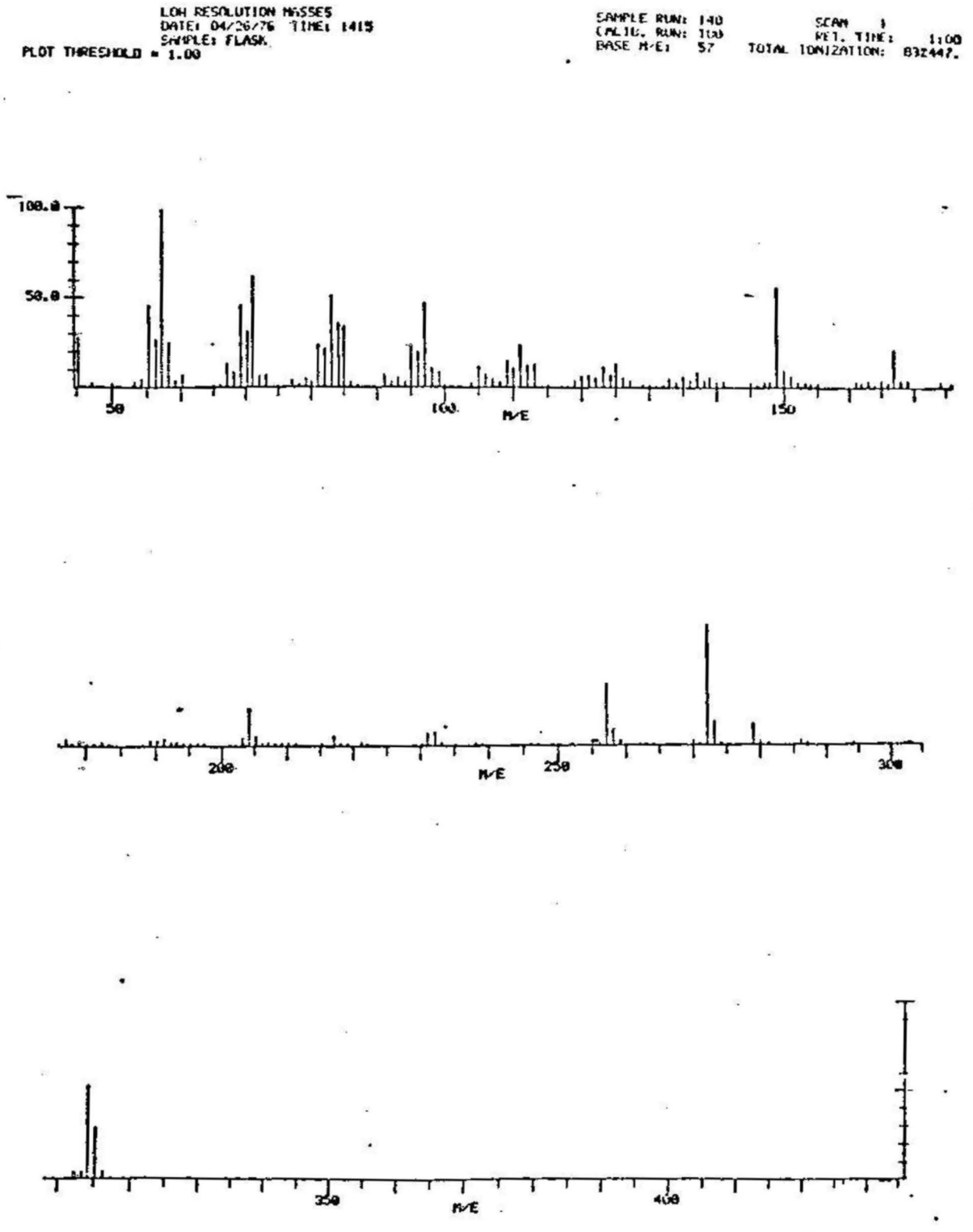


Fig. 15. Low resolution mass spectrum of hopeanine

LOW RESOLUTION MASSES SAMPLE RUN. 148 SCAN 1
DATE. 04/26/76 TIME. 1415 CALIB. RUN. T88 PCT TIME. 1.80
SAMPLE. FLASK Fa=3 BASE M/E. 57 TOTAL IONIZATION. 832417

INSTRUMENT. MS12 FORMULA. - WEIGHT. 0 080
ANALYST. S SUBMITTED BY. CHAUBAL ACCT. NO.. -

REQUESTED RESOLUTION. 2000 THRESHOLD. 1 MINIMUM WIDTH. 5
MEASURED SCAN TIME. 0.0 SEC SAMP. INTVL.. 0.400 MSEC. MINIMUM AREA. 15

1 EXPONENTIAL DOWN SCAN ACQUIRED
LOW MASS. 10 SCAN TIMES (SECS.) UP. 15.0 TOP. 5.0
HIGH MASS. 900 DOWN. 40.0 BOTTOM. 0.0
LIST THRESHOLD = 1.00 % RELATIVE ABUNDANCE.

PEAK NO.	NOMINAL MASS	% RA	% TI	INT
24	45	27.39	0.56	4624.
25	46	1.52	0.03	256.
26	47	2.61	0.05	440.
27	48	1.33	0.03	224.
29	50	2.51	0.05	424.
30	51	1.33	0.03	224.
32	53	3.27	0.07	552.
33	54	5.21	0.11	880.
34	55	46.87	0.95	7912.
35	56	28.06	0.57	4736.
36	57	100.00	2.03	16888.
37	58	25.64	0.52	4328.
38	59	4.27	0.09	720.
39	60	7.11	0.14	1200.
41	63	1.75	0.04	296.
42	66	1.56	0.03	264.
43	67	13.89	0.28	2344.
44	68	9.10	0.18	1536.
45	69	46.54	0.94	7856.
46	70	31.80	0.64	5368
47	71	62.27	1.26	10512.
48	72	6.40	0.13	1080.
49	73	7.54	0.15	1272.
50	74	1.47	0.03	248.
51	75	1.42	0.03	240.
52	76	1.33	0.03	224.
53	77	4.31	0.09	728.
54	78	2.27	0.05	384.
55	79	5.36	0.11	904.
57	80	3.79	0.08	640.
58	81	23.74	0.48	4008.
59	82	1.14	0.02	192.
60	82	21.61	0.44	3648.
61	83	51.37	1.04	8672.
62	84	35.26	0.71	5952.
63	85	34.27	0.69	5784.
64	86	3.22	0.07	544.
65	87	1.56	0.03	264.
67	91	7.58	0.15	1280.
68	92	3.13	0.06	528.
69	93	6.02	0.12	1016.
70	94	3.65	0.07	616.
71	95	23.93	0.49	4040.

PEAK NO.	NOMINAL MASS	% RA	% TI	INT
72	96	20.19	0.41	3408.
73	97	46.97	0.95	7928.
74	98	11.56	0.23	1952.
75	99	9.05	0.18	1528.
76	100	2.09	0.04	352.
77	101	1.42	0.03	240
80	104	2.65	0.05	448.
81	105	12.32	0.25	2080.
82	106	7.73	0.16	1304.
83	107	5.31	0.11	896.
84	108	3.79	0.08	640.
85	109	15.21	0.31	2568.
86	110	11.09	0.22	1872.
87	111	23.78	0.48	4000.
88	112	13.22	0.27	2232.
89	113	13.74	0.28	2320.
90	114	1.18	0.02	200.
91	115	1.80	0.04	304.
93	117	1.33	0.03	224.
94	118	1.33	0.03	224.
95	119	3.93	0.08	664.
96	120	6.48	0.13	1080.
97	121	7.30	0.15	1232.
98	122	5.64	0.11	952.
99	123	12.32	0.25	2080.
100	124	7.06	0.14	1192.
101	125	13.51	0.27	2280.
102	126	5.73	0.12	968.
103	127	4.31	0.09	728.
104	128	1.04	0.02	176.
105	129	2.04	0.04	344.
106	130	1.80	0.04	304.
107	131	1.42	0.03	240.
108	132	1.80	0.04	304.
109	133	5.26	0.11	888.
110	134	3.13	0.06	528.
111	135	6.68	0.14	1128.
112	136	3.93	0.08	664.
113	137	8.67	0.18	1464.
114	138	4.31	0.09	728.
115	139	5.88	0.12	992.
116	140	3.68	0.07	608.
117	141	3.46	0.07	584
121	145	2.51	0.05	424.
122	146	1.66	0.03	280
123	147	3.32	0.07	560.
124	148	3.84	0.08	648.
125	149	56.30	1.14	9504.
126	150	9.43	0.19	1592.
127	151	6.68	0.14	1128.
128	152	3.36	0.07	568.
129	153	3.32	0.07	560
130	154	2.51	0.05	424.
131	155	2.99	0.06	504.
135	158	1.18	0.02	200
138	161	3.68	0.07	608.
139	162	2.46	0.05	416.

Fig. 16. Computer print-out of the low resolution mass spectrum of hopeanine

PEAK NO.	NOMINAL MASS	% RA	% TI	INT
140	163	4.55	0.09	768.
141	164	2.27	0.05	384.
142	165	4.64	0.09	784.
143	166	2.46	0.05	416.
144	-167	21.42	0.43	3616.
145	168	4.41	0.09	744.
146	169	4.08	0.08	688.
150	174	3.22	0.07	544.
151	175	2.94	0.06	496.
152	176	2.04	0.04	344.
153	177	4.03	0.08	680.
154	178	1.85	0.04	312.
155	179	3.41	0.07	576.
156	180	2.18	0.04	368.
157	181	2.04	0.04	344.
158	182	2.42	0.05	408.
159	183	2.09	0.04	352.
163	188	1.18	0.02	200.
164	189	3.70	0.07	624.
165	190	3.46	0.07	584.
166	191	4.36	0.09	736.
167	192	2.80	0.06	472.
168	193	2.80	0.06	472.
169	194	1.99	0.04	336.
170	195	1.75	0.04	296.
171	196	2.09	0.04	352.
172	197	2.32	0.05	392.
175	201	1.52	0.03	256.
176	202	1.33	0.03	224.
177	203	4.69	0.10	792.
178	204	21.42	0.43	3616.
179	205	5.73	0.12	968.
180	206	1.80	0.04	304.
181	207	2.51	0.05	424.
182	208	2.13	0.04	360.
183	209	2.04	0.04	344.
184	210	1.80	0.04	304.
185	211	1.88	0.04	304.
188	215	1.56	0.03	264.
190	217	5.92	0.12	1000.
191	218	1.99	0.04	336.
192	219	1.66	0.03	280
194	221	2.42	0.05	408.
195	222	1.71	0.03	288.
196	223	1.23	0.02	208.
197	224	1.42	0.03	240.
198	225	1.52	0.03	256.
201	229	2.32	0.05	392.
202	230	1.99	0.04	336.
203	231	7.16	0.15	1208.
204	232	8.25	0.17	1392.
205	233	2.56	0.05	432.
206	234	1.42	0.03	240
207	235	1.99	0.04	336.
208	236	1.37	0.03	232.
209	237	1.09	0.02	184.
210	238	1.75	0.04	296.

PEAK NO.	NOMINAL MASS	% RA	% TI	INT
211	239	1.66	0.03	280.
216	245	1.14	0.02	192.
217	246	1.04	0.02	176.
218	247	1.18	0.02	200.
221	250	1.23	0.02	208.
223	252	1.71	0.03	288.
224	253	1.37	0.03	232.
226	255	3.70	0.07	624.
227	256	1.99	0.04	336.
228	257	34.93	0.71	5896.
229	258	9.81	0.20	1656.
230	259	3.79	0.08	640.
231	260	1.42	0.03	240.
232	261	1.23	0.02	208.
234	263	1.42	0.03	240.
235	264	1.37	0.03	232.
237	266	1.33	0.03	224.
238	267	1.28	0.03	216.
241	270	3.22	0.07	544.
242	271	2.32	0.05	392.
243	-272	67.58	1.37	11408.
244	273	13.68	0.23	2296.
245	274	1.90	0.04	320.
246	275	1.09	0.02	184.
249	278	1.14	0.02	192.
250	279	12.46	0.25	2104.
251	-280	3.08	0.06	520.
252	281	1.94	0.04	328.
254	285	1.04	0.02	176.
255	286	3.41	0.07	576.
256	287	1.75	0.04	296.
262	294	1.75	0.04	296.
263	295	1.33	0.03	224.
264	298	1.04	0.02	176.
269	306	1.18	0.02	200.
271	308	1.37	0.03	232.
272	309	1.09	0.02	184.
273	312	4.12	0.08	696.
274	313	4.27	0.09	720.
275	314	52.86	1.07	8912.
276	315	29.19	0.59	4923.
277	316	5.31	0.11	896.
278	317	1.09	0.02	184
281	320	1.28	0.03	216.
289	334	1.09	0.02	184.
291	336	1.33	0.03	224.

Fig. 16 (continued)

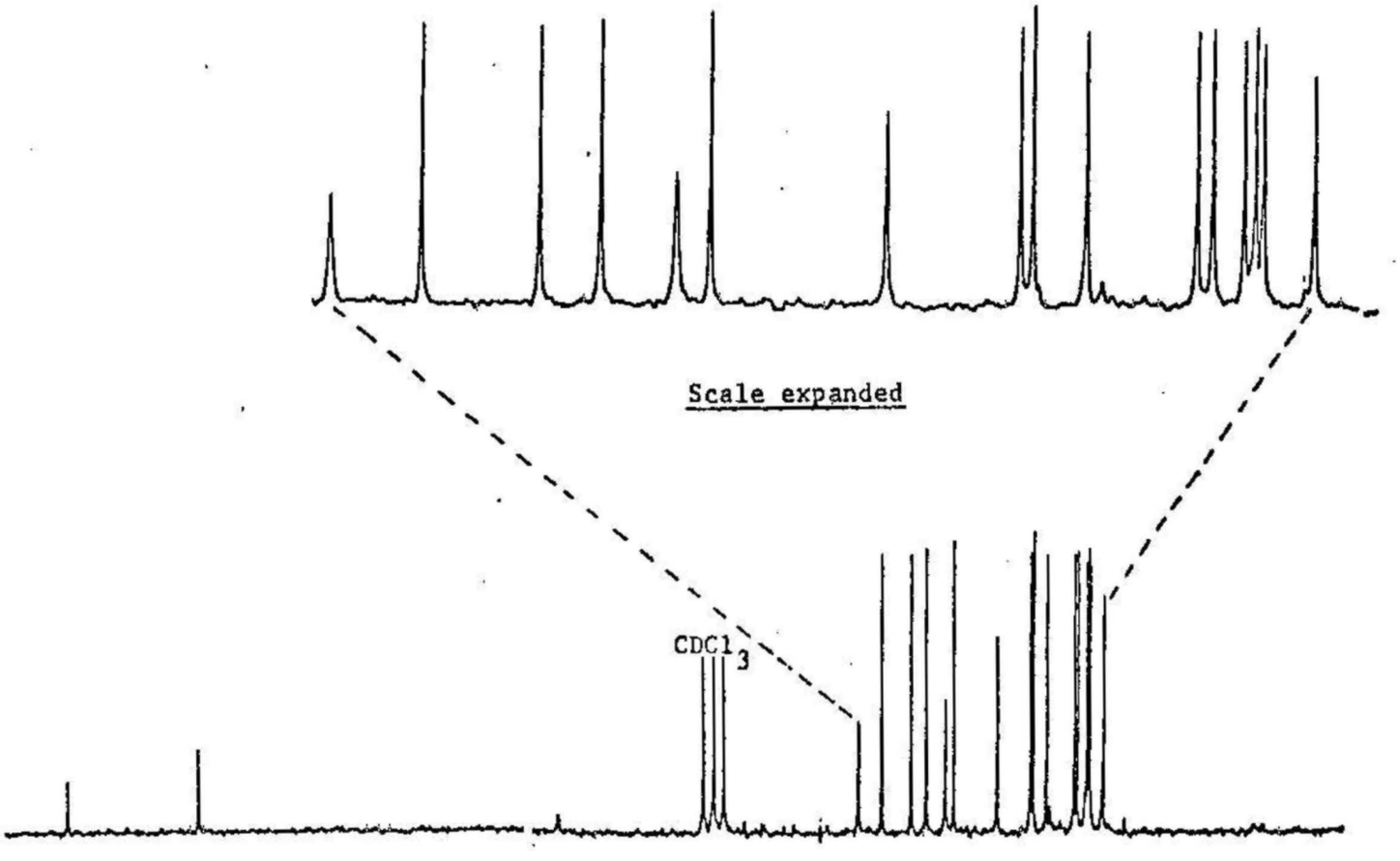


Fig. 17. Wide band ^{1}H decoupled ^{13}C NMR spectrum of hopeanine with scale expansion

Line	Freq.	ppm	Area	Intensity
1	3829.036	169.276	772.107	538.635
2	3411.350	150.811	808.316	890.704
3	1777.373	78.575	6858.131	1378.004
4	1745.017	77.144	8213.990	1966.501
5	1713.396	75.746	7219.586	1863.562
6	1284.679	56.793	6405.582	1181.192
7	1212.614	53.608	7174.413	2952.677
8	1118.487	49.446	7524.141	2946.709
9	1070.689	47.333	8318.344	3011.660
10	1010.389	44.667	7981.231	1407.549
11	983.916	43.497	8347.648	3086.300
12	844.932	37.353	7454.649	2078.029
13	738.304	32.639	7838.094	2968.289
14	733.157	32.411	587.389	229.890
15	728.009	32.184	8492.014	3184.417
16	722.862	31.956	197.965	237.778
17	685.359	30.298	8853.047	2939.898
18	672.122	29.713	232.254	267.784
19	599.585	26.462	8377.888	2950.735
20	586.819	25.942	9634.026	2973.742
21	561.817	24.837	9317.534	2864.999
22	552.257	24.414	10494.560	2996.001
23	546.374	24.154	10021.226	2833.753
24	511.077	22.594	539.522	216.994
25	505.194	22.333	8417.701	2497.857
26	22.796	1.007	1059.997	645.763
27	0.000	0.000	309.727	377.580

Fig. 18. Computer print-out of the wide band ^{1}H decoupled ^{13}C NMR spectrum of hopeanine

be present in the compound by the PMR spectrum (see above). As it turned out, this assumption was erroneous.

The wide-band ^{1}H decoupled C-13 NMR spectrum of the compound (Figs. 17, 18), besides being extremely helpful in giving information regarding some structural features of hopeanine also showed that there were at least 19 carbon atoms, with at least 28 hydrogens attached to these carbons, thus indicating that the molecular formula assumed above was in error.

The chemical ionization MS of hopeanine was able to resolve this problem. This spectrum showed that the M+1 ion was at 316. Therefore the unequivocal M.W. of hopeanine was 315 and not 314 as previously assumed. The non-nitrogen containing molecular formulas among the list shown above can be rejected. From the number of carbons observed in C-13 NMR and the fact that the formulas $C_{16}H_{31}N_2O_4$ and $C_{21}H_{31}N_2O_4$ represent even electron ions (51), the molecular formula $C_{19}H_{29}N_3O$ can be unambiguously assigned to hopeanine.

A great deal of structural information could be obtained from the wide-band ^{1}H decoupled C-13 NMR spectrum of hopeanine (Fig. 17). As indicated above, the presence of at least 19 carbon atoms was shown by this spectrum. Figure 18 represents a copy of the computer print-out of the analysis of the wide band ^{1}H decoupled spectrum of hopeanine, indicating the line number (peak #), frequency (Hz), parts per million (δ), area of peak and

intensity of each peak. It should be pointed out that the analysis by the computer takes into consideration every line (peak) observed, thus accounting for the listed 27 lines.

The single frequency proton off-resonance decoupled C-13 NMR allows assignments of peaks to various carbon environments such as non-protonated carbon and carbons attached to one, two, or three hydrogens (-CH, $-CH_2$, $-CH_3$). These environments are observed as singlets, doublets, triplets or quartets, respectively. Twelve triplets ($-CH_2$), four doublets (-CH) and three singlets (non-protonated carbons) were observed in the spectrum of hopeanine. Absence of quartets indicated absence of $-CH_3$ group. This is in agreement with the analysis of PMR which had indicated that C-methyl or N-methyl were probably absent (p. 83).

Further attempts were made to assign various carbon environments to the observed C-13 NMR peaks (signals) using literature reported δ values (46, 52). The signals appearing at 169.276 and 150.811 (singlets) were of special interest. These, according to literature (46, 52), may reflect unsaturated environments and location of such an environment in an unknown compound is significant. The UV and IR had indicated such an environment and its characterization would help in elucidating the structure of hopeanine. Tables XII and XIII list assignments of environments most likely to be associated with these two carbons.

Table XII: Some environments of carbons with a value of between 149-150 δ

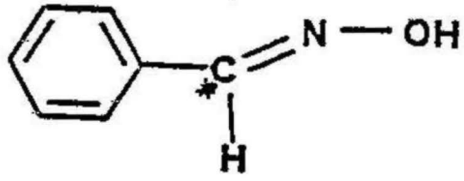
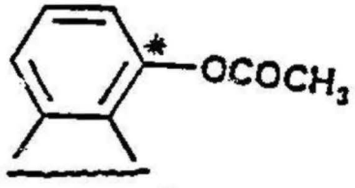
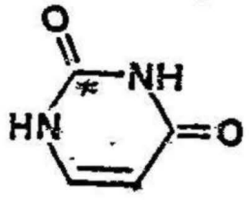
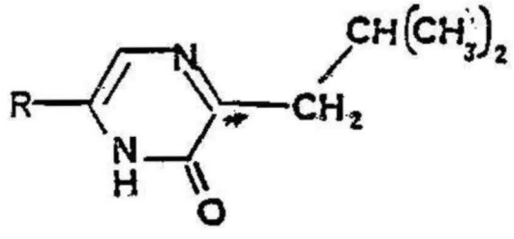
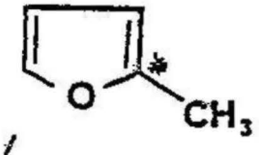
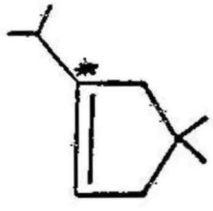
Structure	δ	Reference
	150.6	46
	149.6	46
	149.7	54
	152.8	46
	151.0	53
	152.0	52
	149.5	55

Table XII: (continued)

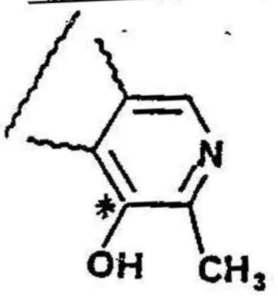
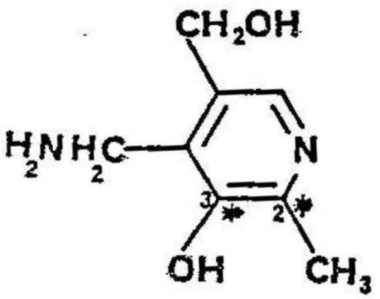
Structure	δ	Reference
	149.9	56
	150.6, 161.0	56

Table XIII: Some environments of carbons with a value between 168 - 172 δ

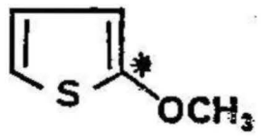
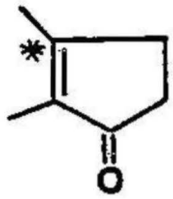
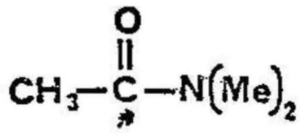
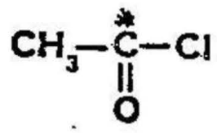
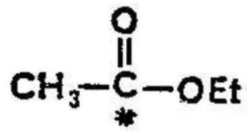
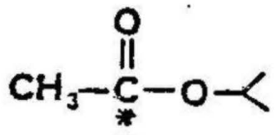
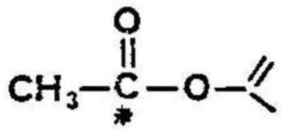
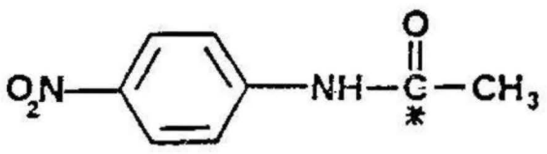
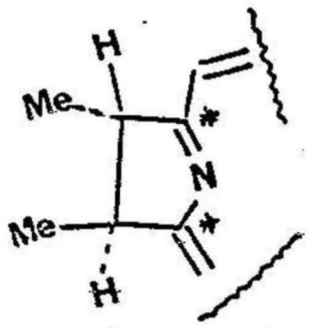
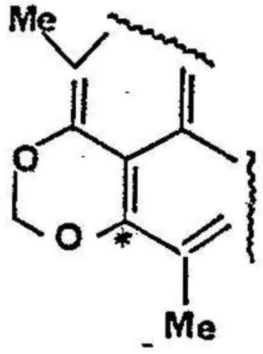
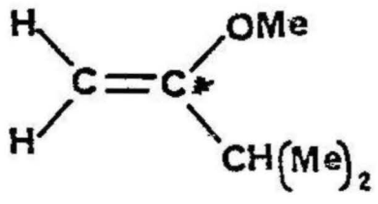
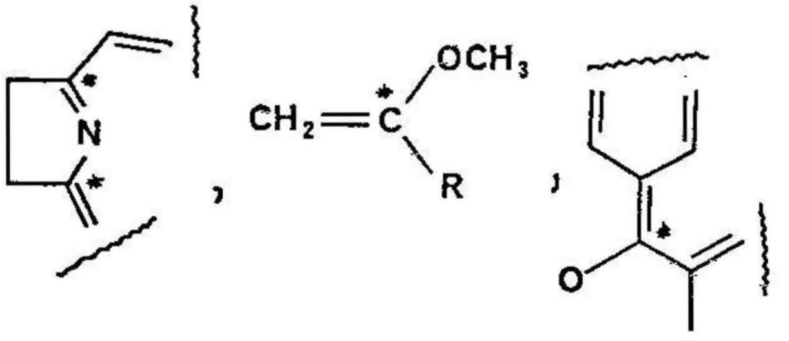
Structure	δ	Reference
	167.9	46
	168.9	46
	169.6	46
	169.9	46
	170.3	46
	169.8	46
	168.9	46
	169.2	46

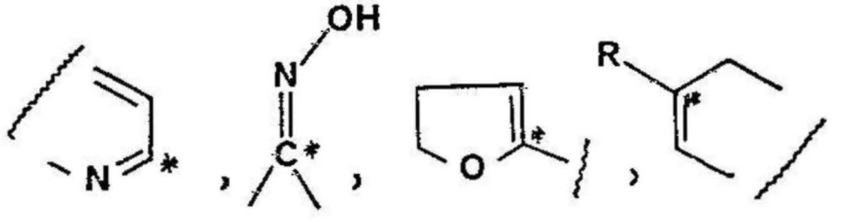
Table XIII: (continued)

Structure	δ	Reference
	168.5, 171.8	57
	168.2	46
	168.5	59
	168.9	58

It is evident that one can conceive of a variety of environments for the carbons whose δ values are 150.811 and 169.276. However, many of the environments could be eliminated based on the evidence of functional groups in the IR spectrum of hopeanine. For example, the δ value of 169.276 cannot be due to carbonyl group as no carbonyl absorption is evident in the IR spectrum. Thus one can limit the possible environments of the carbons to the following.



By similar arguments one can conceive of the following environments for the carbons with δ value of 150.811.



For a molecular formula $C_{19}H_{29}N_3O$, the corresponding parent hydrocarbon must have a composition $C_{22}H_{32}$. The corresponding saturated hydrocarbon can then be represented by a composition $C_{22}H_{46}$. Thus in hopeanine there is a

deficiency of 14 hydrogens or an equivalent of 7 unsaturation units (comprising rings and/or double bonds).

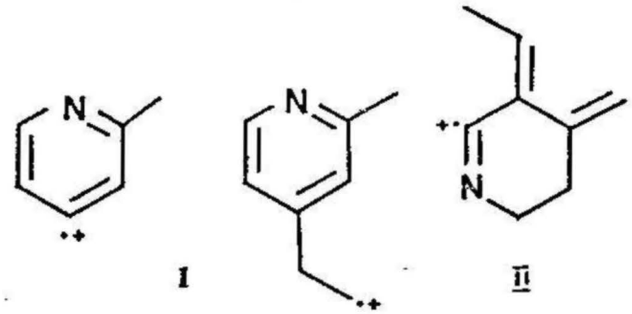
^{13}C NMR indicated that two of the unsaturations could be accounted for by C=N or C=C bonds. The remaining five unsaturation units may then be accounted for as representing five rings.

The shift values for the α carbon, in ethanol, 1-propanol, 2-propanol and 2-methyl-2-propanol are 57.3, 63.9, 63.7 and 68.7 δ, respectively (46). The wide band ^{1}H decoupled spectrum of hopeanine shows a peak at 56.793 δ. If this was attributed to a primary alcoholic group, then the carbinol carbon would be expected to appear as a triplet in the single frequency, off resonance decoupled spectrum. However, this peak was seen as a doublet. This rules out the possibility that the peak at 56.793 δ represents a $-CH_2$ attached to an -OH. Further, the α carbon in secondary and tertiary alcohols such as 2-propanol and 2-methyl-2-propanol, appears at much higher δ value than that observed. Thus the oxygen in the formula $C_{19}H_{29}N_3O$ is not part of a carbonyl or a hydroxy functional group. Hence the exchangeable hydrogen seen in the PMR spectrum of hopeanine is most likely to be part of an -NH group.

So far the molecular weight and formula of hopeanine had been determined with certainty. The spectral analyses also gave some indications as to a few functional groups present in the compound. Detailed structural determinations and assignment of final composite structure could be made

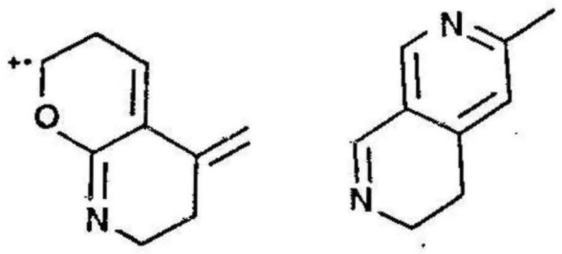
by the analysis of the high resolution mass spectrum, (Fig. 19) especially the available extensive computer print-out. A copy of selected m/e peaks and their computer analysis is shown in Fig. 20. The observed fragment ions were (Table XIV) assigned most probable structures and finally these structures were fit into a whole structure for hopeanine.

a) Fragments at m/e 92 (I) and m/e 120 (II) were even electron ions. The most suitable structure for these ions were



I II

b) Ion fragments at m/e 148 (III) was an even electron ion, while the ion at m/e 146 was an odd electron ion. The probable structures for these ions are



III IIIa

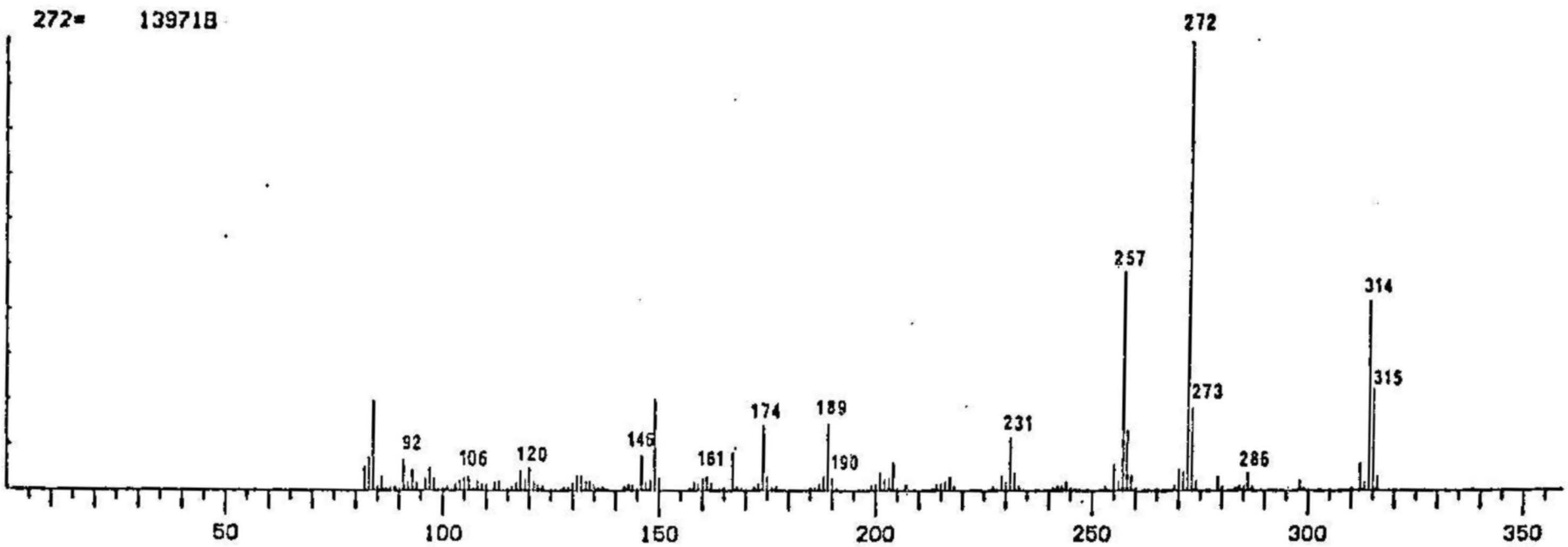


Fig. 19. High resolution mass spectrum of hopeanine

OBSMASS	INTENSITY	DELTA (PPM)	C	H	C13	O	N	S
92.049183	2969	-19.11	3	8	0	3	0	0
		10.62	6	6	0	0	1	0
		-1.89	2	9	1	0	1	1
126.080582	7032	-16.13	5	12	0	3	0	0
		6.20	8	10	0	0	1	0
148.100598	3653	-7.57	6	15	1	3	0	0
		10.53	9	13	1	0	1	0
		13.54	5	14	0	2	3	0
		-16.64	4	13	1	2	3	0
146.085037	11034	-4.36	9	10	0	0	2	0
		10.73	6	14	0	0	2	1
161.108926	4381	-5.90	10	13	0	0	2	0
		19.44	5	14	1	2	3	0
		15.04	7	17	0	0	2	1
		-12.70	6	10	1	0	2	1
174.051276	19956	-11.86	8	14	0	4	0	0
		3.52	11	12	0	1	1	0
		-19.60	6	12	0	3	3	0
		-4.20	9	10	0	0	4	0
		-2.78	7	15	1	1	1	1
		15.18	6	14	0	0	4	1
		-10.49	5	13	1	0	4	1
189.100769	20406	-3.49	8	15	0	4	1	0
		10.28	11	13	0	1	2	0
		-12.95	10	12	1	1	2	0
		-10.60	6	13	0	3	4	0
		4.89	7	16	1	1	2	1
190.167727	3560	1.05	8	16	0	4	1	0
		15.19	11	14	0	1	2	0
		-8.22	10	13	1	1	2	0
		-5.98	6	14	0	3	4	0
		9.42	7	17	1	1	2	1
231.145981	16144	4.67	11	21	0	4	1	0
		-14.67	10	20	1	4	1	0
		16.27	14	19	0	1	2	0
		-3.07	13	18	1	1	2	0
		-1.14	9	19	0	3	4	0
		-17.74	12	23	0	2	0	1
		11.52	10	22	1	1	2	1

Fig. 20. Computer print-out of selected peaks in the high resolution mass spectrum of hopeanine

OBSMASS	INTENSITY	DELTA (PPM)	C	H	C13	O	N	S
257.164307	68346	-6.21	13	23	0	4	1	0
		4.22	16	21	0	1	2	0
		-13.16	15	20	1	1	2	0
		-11.43	11	21	0	3	4	0
		17.34	13	25	0	1	2	1
		-0.04	12	24	1	1	2	1
272.176825	139718	5.91	18	24	0	2	0	0
		-10.51	17	23	1	2	0	0
		-8.88	13	24	0	4	2	0
		0.97	16	22	0	1	3	0
		-15.45	15	21	1	1	3	0
		15.97	11	23	1	3	4	0
		18.30	15	28	0	2	0	1
		1.68	14	27	1	2	0	1
		13.37	13	26	0	1	3	1
		-3.05	12	25	1	1	3	1
273.180664	26083	17.55	18	25	0	2	0	0
		1.19	17	24	1	2	0	0
		2.62	13	25	0	4	2	0
		-13.64	12	24	1	4	2	0
		12.63	16	23	0	1	3	0
		-3.73	15	22	1	1	3	0
		13.54	14	28	1	2	0	1
		-16.14	14	27	0	2	1	1
		8.62	12	26	1	1	3	1
286.193115	5277	0.59	15	26	0	2	0	0
		-15.03	18	25	1	2	0	0
		14.85	14	27	1	4	1	0
		-13.47	14	26	0	4	2	0
		-4.10	17	24	0	1	3	0
		-19.72	16	23	1	1	3	0
		18.16	12	25	1	3	4	0
		12.38	16	30	0	2	0	1
		-3.24	15	29	1	2	0	1
		7.68	14	28	0	1	3	1
314.220947	59542	11.58	21	30	0	2	0	0
		-2.65	20	29	1	2	0	0
		-1.23	16	30	0	4	2	0
		-15.46	15	29	1	4	2	0
		7.30	19	28	0	1	3	0
		-6.92	18	27	1	1	3	0
		8.05	17	33	1	2	0	1
		-17.71	17	32	0	2	1	1
		18.64	16	32	0	1	3	1
		3.81	15	31	1	1	3	1

Fig. 20. (continued)

OBSMASS	INTENSITY	DELTA	C	H	C13	O	N	S
315.227851	31837	17.88	21	31	0	2	0	0
		7.82	20	30	1	2	0	0
		4.24	16	31	0	4	2	0
		-9.94	15	30	1	4	2	0
		12.74	19	29	0	1	3	0
		-1.44	18	28	1	1	3	0
		13.52	17	34	1	2	0	1
		-12.19	17	33	0	2	1	1
		9.26	15	32	1	1	3	1
		-16.45	15	31	0	1	4	1

Fig. 20. (continued)

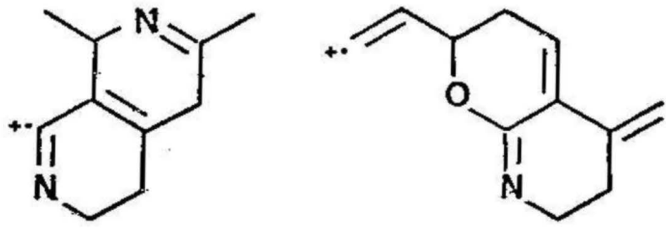
Table XIV. Important odd electron ions in the mass spectrum of hopeanine

m/e	Probable Composition
273	$C_{16}H_{23}N_3O$
190	$C_{11}H_{14}N_2O$
146	$C_9H_{10}N_2$

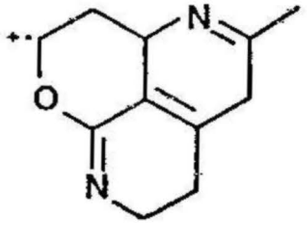
Important even electron ions in the mass spectrum of hopeanine

286	$C_{17}H_{24}N_3O$
272	$C_{16}H_{22}N_3O$
257	$C_{16}H_{21}N_2O$
231	$C_{14}H_{19}N_2O$
189	$C_{11}H_{13}N_2O$
174	$C_{11}H_{12}NO$
161	$C_{10}H_{13}N_2$
148	$C_9H_{10}NO$
120	$C_8H_{10}N$
92	C_6H_6N

c) The fragments at m/e 161 and m/e 174 were even electron ions and the likely structures for these fragments are

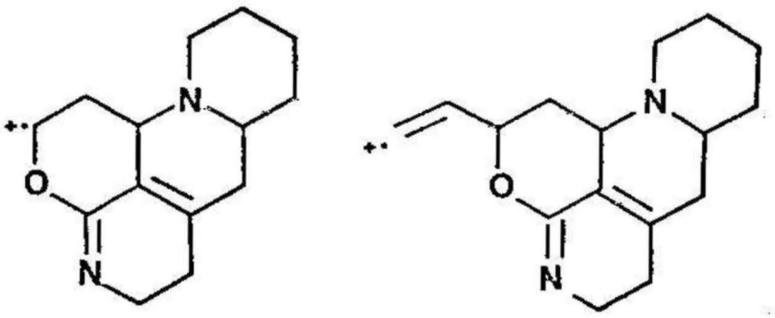


d) The fragment at m/e 189 (VII) was an even electron ion while the ion at m/e 190 was an odd electron ion with a composition $C_{11}H_{14}N_2O$.

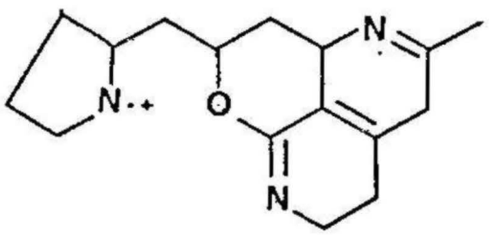


VII

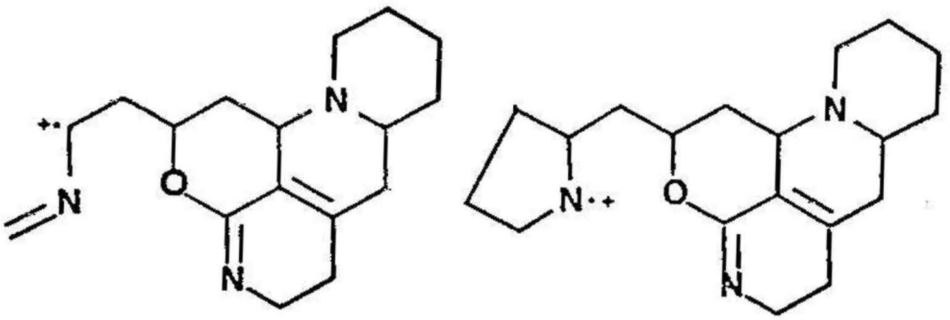
e) The fragments at m/e 231 and m/e 257 were even electron ions and the probable structures of these fragments are



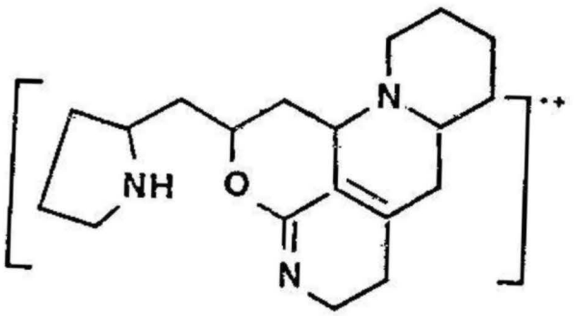
f) The fragment ion at m/e 272 was an even electron ion while the ion at m/e 273 was an odd electron ion with a composition $C_{16}H_{23}N_3O$



g) The fragments at m/e 286 and m/e 314 were even electron ions and the probable structures of these ions are



h) The fragment ion at m/e 315 was the molecular ion with a composition $C_{19}H_{29}N_3O$



A composite of these fragments and the most suitable structures assigned to these ion fragments is shown in Fig. 21.

The high resolution mass spectrum of the alkaloid is characterized by the presence a strong M-1 peak. Cyclic amines, aldehydes, secondary or primary alcohols usually show a significant M-1 peak in their mass spectrum. It is most likely that in the present case the M-1 peak represents the loss of proton from the -NH group. The fragmentation then proceeds in two competing directions, as shown in Figure 21. The loss of an m/e 42, C_3H_6 fragment gives

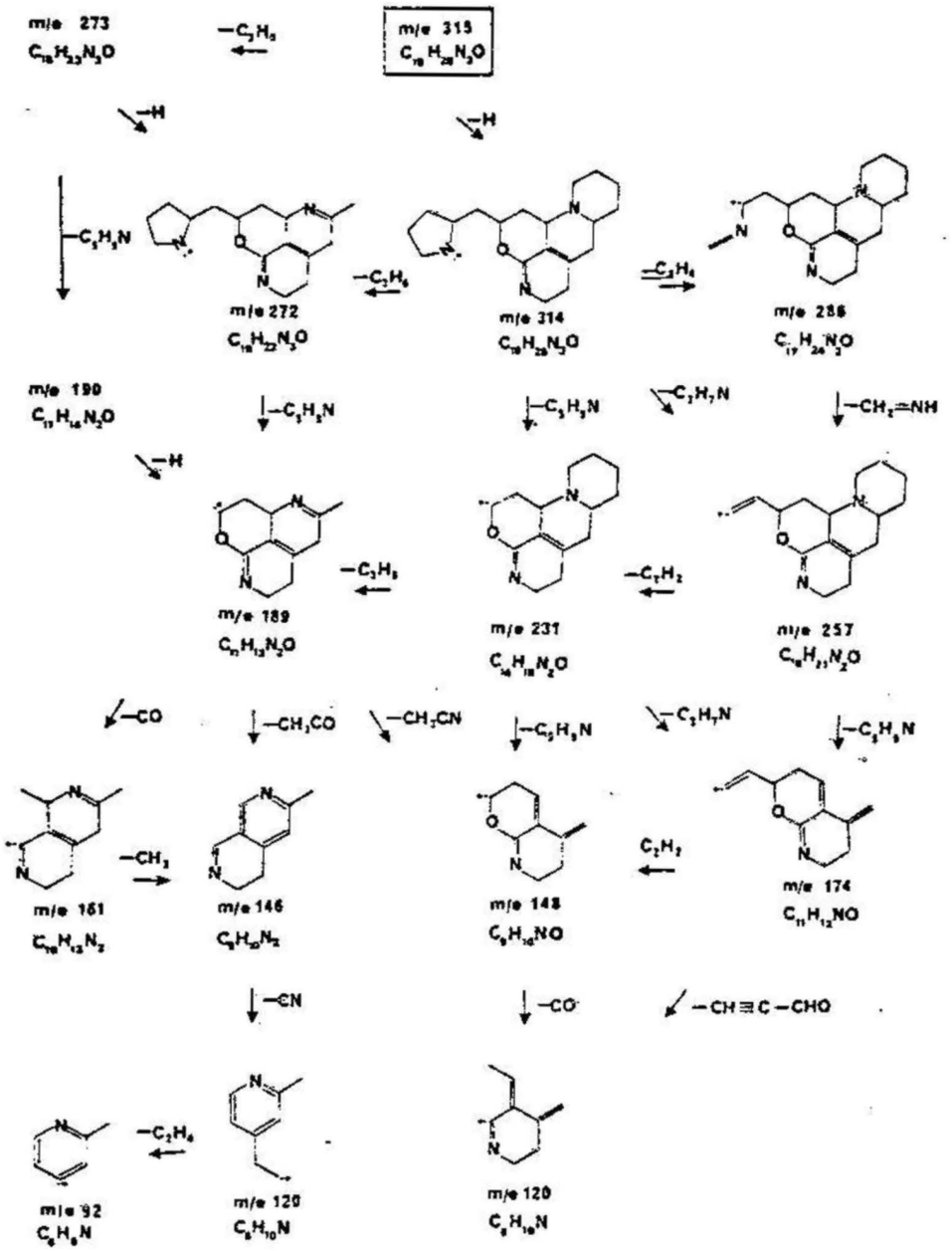


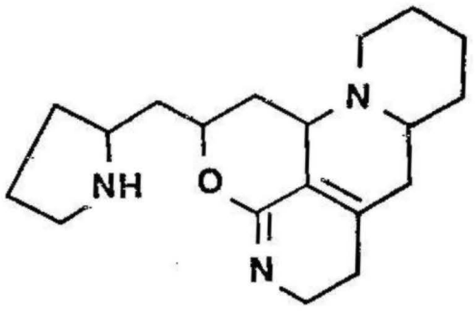
Fig. 21. Mass spectral fragmentation pathways of hopeanine

m/e 272, $C_{16}H_{22}N_3O$, the strongest peak after the base peak m/e 57. The fragment ion 272 is an even electron ion and yields the even electron ion m/e 189, $C_{11}H_{13}N_2O$ by loss of m/e 83, C_5H_9N. The neutral fragment C_5H_9N might probably represent a methylene pyrrolidine or piperidine group. In the other possible pathway, the m/e 314 ion could fragment by the loss of m/e 57 ion to yield the even electron ion, $C_{16}H_{21}N_2O$ of m/e 257. The latter could cleave by the loss of C_2H_2 fragment to yield the even electron ion $C_{14}H_{19}N_2O$, m/e 231. This latter fragment ion could also be produced by the direct loss of C_5H_9N fragment from m/e 314. Fragment ion m/e 231 can lose either m/3 42 to yield the ion 189 as above or lose another C_5H_9N fragment to form the even electron ion m/e 148, $C_9H_{10}N$. The ion m/e 189 can also fragment in two ways: a) Loss of m/e 28 (CO) would give m/e 161, $C_{10}H_{13}N_2$ or the loss of C_2H_3N (acetonitrile) would yield the above ion m/e 148. The ^{13}C satellites of all the fragments mentioned have been identified in the high resolution mass spectrum. However further confirmation of the above sequence of fragmentation reactions can be done only with the aid of the necessary metastable ions.

A number of other fragment ions can be explained on the basis of a retro-Diels-Alder reaction mechanism (51). These are -- m/e 174, $C_{11}H_{12}NO$ from m/e 257, $C_{16}H_{21}N_2O$ by the loss of C_5H_9N or fragmentation of m/e 161, $C_{10}H_{13}N_2$ and m/e 148, $C_9H_{10}NO$ to smaller fragment ions, e.g. m/e 146, 120,

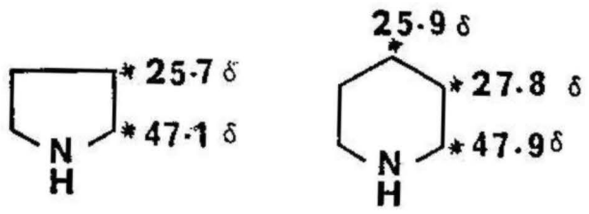
106, 94, 92, and m/e 78. These fragments represent a variety of pyridine derivatives.

A structure as shown below can now be assigned to hopeanine which helps explain the various spectral data discussed in these pages.



The presence of the α-substitued pyrrolidine ring as shown in the above structure was suggested from the analysis of the low resolution MS (Fig. 16). The two ions at m/e 69 (47%) and m/e 70 (32%) were quite prominent. These are characteristic of α-methyl pyrrolidines. The presence of the postulated neutral fragment C_5H_9N (Fig. 21) also supports this analysis. A second support to the presence of α-substituted pyrrolidine was obtained from the C-13 NMR (Fig. 16). Signals in the region of 47-49 δ were observed. Shift values for carbons α and β to the nitrogen in a pyrrolidine ring and those for carbons α, β and γ to the nitrogen in a piperidine ring have been reported

in the literature (46) as follows:



The structure of hopeanine proposed above, also seems to be consistent with biosynthetic considerations of the alkaloids of the Solanaceae family. The tropane group of alkaloids, found in many solanaceous plants, contain piperidine and pyrrolidine residues.

The pyrrolidine residue has been demonstrated to be derived from ornithine and the piperidine residue from lysine (60). The proposed hopeanine structure also incorporates pyrrolidine and piperidine units, although in a unique structure. It would be reasonable to assume that

hopeanine also would be biosynthesized from ornithine and lysine. At least the proposed structure does not exclude the possibility. Although various other structures could be written for hopeanine which would agree with some data, the one proposed above was the only one to agree with all the considerations.

In addition to determination of biosynthetic pathways of hopeanine, additional future research directions would be as follows:

> The unambiguous final proof of the structure of hopeanine remains to be elucidated. This can be obtained by unequivocal chemical synthesis. Other supportive evidence to the proposed structure could not be obtained due to paucity of material and lack of equipment. High resolution MS in the metastable mode and establishing the mother-daughter relationships would be helpful (51). The C-13 NMR of the acetate would also offer additional support. An attempt to acetylate 8 mg of hopeanine did indicate that reaction had occurred (tested by TLC); however the purified sample was too small to give a good, interpretable spectrum. X-ray crystallography could also provide valuable information. However, attempts at growing good crystals of hopeanine have not been successful to date; the compound is sensitive to air, light and moisture.

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