PHYTOCHEMICAL AND PHARMACOLOGICAL STUDIES OF THE BARK OF MITRAGYNAINERMIS (WILLD) O. KUNTZE (RUBIACEAE)

BY

AHMADU AUGUSTINE AJIBOGUN

JULY, 1999.

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BY

AHMADU AUGUSTINE AJIBOGUN

BEING

A THESIS SUBMITTED TO THE POST GRADUATE SCHOOL, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE (PHARMACEUTICAL CHEMISTRY).

DEPARTMENT OF PHARMACEUTICAL AND MEDICINAL CHEMISTRY,

FACULTY OF PHARMACEUTICAL SCIENCE,

AHMADU BELLO UNIVERSITY, ZARIA.

JULY, 1999.

DECLARATION

I hereby declared that this research work was carried out by me under the Joint supervision of Dr. A. K. Haruna And Dr .M Garba It has not been accepted in any previous publication for a higher degree elsewhere. The works of other investigators are acknowledged and referred to accordingly.



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AHMADU, AUGUSTINE AJIBOGUN, *B. Pharm., MPSN.*

DEPARTMENT OF PHARMACEUTICAL AND MEDICINAL CHEMISTRY

AHMADU BELLO UNIVERSITY, ZARIA.

CERTIFICATION

The dissertation entitled Phytochemical and Pharmacological studies of the bark of *Mitragyna inermis* (willd) O. Kuntze (Rubiaceae) by AHMADU AUGUSTINE meets the regulations governing the award of the degree of Master of science in pharmaceutical chemistry and is approved for the contribution to knowledge and Literary presentation.

Dr. A.K. HARUNA
B.Sc (Pharm.), M.Sc. Ph.D., MPSN
Department of Pharm. & Medicinal Chemistry
A.B.U., Zaria
Chairman, Supervisory Committee

14/XII/99
Date

Dr. M. GARBA
B.Sc. (Pharm) M.Sc. Ph.D., MPSN
Head, Department of Pharm. & Medicinal Chemistry
A.B.U., Zaria
Member, Supervisory Committee

14/12/91
Date

PROFESSOR J.A. OWOYALE
B.Sc (ABU) MSP, Ph.D., (Florida)
Department of Chemistry
University of Ilorin
External Examiner

14/12/99
Date

PROF. S.B. OJO
Dean, Postgraduate School,
A.B.U., Zaria

23/04/01
Date

DEDICATION

This research work is dedicated to:

Almighty God,

for His guidance, and protection.

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My sincere gratitude goes to my supervisor, Dr. A. K. Haruna for his patience, constructive criticism, and immense guidance through out this research work. I am also indebted to my co-supervisor and my head of Department. Dr. M. Garba for his words of encouragement and advice. My appreciation goes to my lecturers: Prof. M. Ilyas, Prof. A. Mustapha, and Dr. M. T. Odunola for their contribution to this work. Greeting to my colleagues in the department which include Mall. Yakasai, Mrs. Hassan, Ibrahim Sule and Umar Pateh for their moral support. No to forget my class mate like Ngwai Yakubu Ahmed Bala, Musa Abubakar, olowosulu and Giwa for their moral support.

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Finally I thank God for his mercy and guidance that saw me through this research wok.

ABSTRACT

The bark of *Mitragyna inermis* (Wild) 0. Kuntze was screened phytochemically and the results of the preliminary studies on the light petroleum ether and methanolic extract of the plant's bark revealed it to be rich in steroids, terpenes, flavonoids, tanins, alkaloids, saponins and carbohydrate.

Saponification of the light petroleum ether extract of the bark gave 0.84 g of unsaponifiable matter, while the saponified portion gave 84.5 mg. The unsaponfiable matter was subjected to gas chromatography and mass spectroscopy to yield azelaic acid, myristic acid, 2-methyl heptyl acetate and 9-oxo decanoic acid. These fatty acids and their derivatives were reported for the first time from this species.

The defatted marc was subjected to the usual extraction process for alkaloids to afford a portion referred to as "alkaloid extract". This portion gave positive tests for alkaloids. Column chromatography of the alkaloid extract and subsequent purification afforded an a morphous compound. Thin Layer Chromatography, chemical tests, UV, I.R. spectra of F_3 in comparison with spectra of known alkaloids, showed F_3 to be an oxindole alkaloid.

Pharmacological studies of the methanolic extract on isolated rabbit ileum showed a dose - dependent relaxation of rhythmic contractions of the tissue.

However, the extract did not block the contraction of acetylcholine indicating that the relaxation of the rab.bit ileum is not via the muscannic blockade on guinea pig ileum, it slightly reduced the histamine induced contraction from 75%, response to 64.02% response, indicating a weak anti histaminic activity. This suggests the beneficial use and explains some traditional claims of the plant in relieving abdominal pains and colics.

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.ABBREVIATIONS

Ach	=	Acetyl choline
AE	=	Alkaloidal Extract
$CeSo_4$	=	Cerium sulphate
C_6H_6	=	Benzene
$CHCl_3$	=	Chloroform
$(CH_3CO)_2O$	=	Acetic anhydride
Cu_2O	=	Copper (I) oxide
^{13}C-NMR	=	Carbon - 13. Nuclear Magnetic Resonance
$FeCl_3$	=	Ferric chloride
GC/M.S.	=	Gas chromatography coupled to Mass spectrometer.
HCl	=	Hydrochloric Acid
^{1}H-NMR	=	Proton magnetic resonance
H_2SO4	=	Sulphuric acid (dilute)
I. R	=	Infra red.
µg.	=	Microgram
ME	=	Methanolic extract
mg	=	Milligram
M.S.	=	Mass spectrometer or Mass spectra
M-P_2	=	Methylated saponifiable matter
Na_2CO_3	=	Sodium carbonate
NH_4OH	=	Ammonium hydroxide.
ng	=	Nanogram
P_1	=	Unsaponifiable matter
P_2	=	Saponifiable matter
PE	=	Petroleum ether extract
S.M.	=	Saponifiable matter
TLC	=	Thin layer chromatography
U.V	=	ultraviolet.

CHAPTER ONE

1.0 INTRODUCTION

About half of the world's medicinal compounds are derived or obtained from plants. The medicinal products from plants and other biota are in general more important in the developing countries than in industrialized nations, even where the focus is very much on chemical discovery and synthesis of pharmaceuticals, drug products from Biota are major contribution to human health services. Plants as sources of drugs and pharmaceuticals for man and other animals have been in existence time immemorial (Ghani, 1986).

Many plants possess therapeutic and pharmaceutical properties according to a survey by world health organisation (WHO), more than 33% of modern drugs and medicinal products are derived from plants (Ghani, 1986). The medicinal and pharmaceutical properties of these plants are due to the type of chemical substance they produce and store.

Medicinal plants are those which in one or more of its organ contain substances that can be used for therapeutic purposes or which are precursors for the synthesis of useful drugs (Sofowora, 1993). The living plant which may be regarded as a biosynthetic laboratory, produces or synthesizes a large variety of chemical compounds during its normal metabolic activities. These include compounds that are utilized as food by man and other animals and also other compounds that exert physiological effect on them. This second group of chemical substances often referred to as secondary metabolites gives plants their therapeutic and pharmaceutical properties Ghani (1986), the usual term used referring to these various chemical

substances present in plants is *constituents* and the constituents which possess pharmacological properties are called *active constituents*. These active constituents or secondary metabolites synthesized by plant include carbohydrates, terpenes, alkaloids, coumarins, glycosides (flavonoids, saponins, anthraquinones, cardiac, sterols), fixed and volatile oils, gums and mucillage, phenolic compounds. They dominate the chemical picture of drug plants giving them a unique chemistry which differs from plant to plant and are often found in remarkably high concentration, sometimes in great purity.

Phytochemistry is concerned with the chemical studies of these plant constituents. And phytochemical examination of these constituents have been made possible by improved methods of extraction, separation, isolation and characterization, Harbone (1973).

Folk-loric medicinal plants are of value to modern medicine in the following ways: Plants from the tropics are sometimes used as sources of direct therapeutic agents e.g. the alkaloid tubocurarine is extracted from the south American plant *Chindrodendrun tomentosum* and is widely used as a muscle relaxant in surgery, Lewis and Elwin-Lewis (1977). Similarly morphine (I) and atropine (II) isolated from opium and Aellodona plants respectively are still unsurpassed in their respective field, Hansel (1972).

Tropical plants can be used as sources of starting material for the synthesis of useful drugs, e.g. saponins that are chemically altered to produce sapogenins necessary for the manufacture of steroidal drugs. Previously, 95% of all steroids are obtained from the extracts of the genus Dioscorea. (Oldfield, 1984). Similarly, synthetic penicillins can be obtained from the natural penicillin (Hansel, 1972). Flora from the tropics can serve as a

sources of substances that can be used as models for new synthetic compounds e.g. cocaine from the coca plant *Erythroxylum coca* has served as a model for the synthesis of a number of local anaesthetics such as procaine.

Plants can also be used as taxonomic makers for the discovery of new compounds from phytochemical stand point e.g. liliaceae family is known to be rich in alkaloids, little was known for that of orchidaceae, plant of this family were investigated because of its close relationship to the liliaceae, and this led to the discovery that not only was the plant rich in alkaloid, but many of the alkaloid were unique and of medical importance Schultes (1973). The prospects of finding new drugs remains optimistic e.g. the discovery's of anti implantation agents O'Neill *et. al.*, (1987), immuno stimulants Wagner, *et. al.*, (1985) cardioactive agents (Aledansanmi and Ilesanmi, 1987). Information on ethnomedical uses of plants has provided an important lead in the discovery of the great number of clinically useful plant drug world wide (Farnsworth and Soejarto, 1991); hence the need to further study and document the ethnomedical uses of tropical plants with special emphasis on Nigerian plants by laboratory investigation, is more than justified.

1.1 LITERATURE REVIEW

1.1 *Mitragyna inermis*

1.1.1 Morphology of *Mitragyna inermis*

Mitragyna inermis (Willd) O. Kuntze also referred to as mitragyna africana Korth Dalziel (1955) is a specie in the family Rubiaceae. It Is a savannah specie. This bushy tree occur commonly in swampy savanna and is

usually recognized by the opposite pairs of rather thin leaves, the small heads of white or yellowish flowers and the small blackish heads of fruit which remain on the trees for a long time. It is known locally by the Hausa name "giyeya", Fulani, Koli or kwoli and in the Igbo dialect as "oroma". (Dalziel, 1955). The tree is usually 6 - 9 m high and 60 - 90 cm girth, but sometime as much as 15 m high and 2 m in girth. Usually with low branches and bushy crown. The bark is smooth, grey brown, with a few scales, slash, fibrous rapidly turning brown, branchlets with short hairs, leaves 6 - 12 cm long, 3.5 - 7.5 cm broad, broadly elliptic or ovate, rounded at base, accumunate at apex with short hairs, light green often with the stalk and mid-rib reddish pink. 6 - 9 pairs of lateral nerves. Stalk 3 - 3.5 cm long. Flower usually from July - November. White but turning yellow, densely crowded in balls about 3 cm in diameter. Stamens projecting, anthers grey, stigma reddish. Fruits from December - June very persistent in dense dark brown or blackish balls 12 - 18 mm in diameter. The individual fruits are club shaped about 5 mm long and contain winged seeds. It is widely found in West Africa viz. Ghana, Senegal, Nigeria and Ivory coast.

1.1.2 **Ethnomedicinal use**

A decoction of the leaves sometimes mixed with those of *Cassia tora* is widely used both externally and internally for fever and chills and for simple gastro intestinal problems. In Senegal and French guinea, a decoction of the leaves is given to women as a post partum treatment and is also used externally as a lotion or fumigation for rheumatic pains in the limbs or applied as a dressing for boils and eruptions Dalziel (1955).

In Ivory coast hot water extract of the bark is used orally in female to treat amenorrhea Bouquet and Debray (1974). In Senegal, the Hot water extract of the dried stem bark is used orally as a mixture with *Fagara zanthoxyloides, Kigelia africana,* and *Sterculia setigera* to treat epilepsy. Kerharo (1963).

Similarly in Senegal, the hot water extract of the dried bark is used as a cicatrizant and in gynaecology and as antitoxin Tignokpa, *et. al.*, (1986). In East Africa, a decoction of the bark is used orally as a Febrifuge Perrot *et. al.*, (1936). A decoction of the bark and leaf and sometime the root are used for gonorrhoea. The bitter roots are used in Peki for constipation. The tree is reported to be used as a cure for leprosy, as a diuretic and as an analgesic (Irvine, 1968). In Northern Nigeria, the decoction of the bark is used to treat abdominal colic (Personal communication).

1.1.3 **Biological Activities of the Plant**

The leaf of the plant has been reported to possess antiviral and cytotoxic activity Beuscher, *et. al.*, (1994). Similar cytotoxic activity of the stem bark was reported by Bishay, *et. al.*, (1986), while its antibacterial activity on *Sarcina lutea* was reported by Laurens, *et. al.*, (1985).

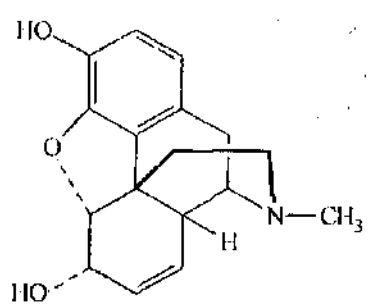
1.1.4 **Phytochemistry.**

The bark of *M. inermis* was reported to contain the steroids Daucosterol and β-sitosterol (III) Bishay *et. al.*, (1988).

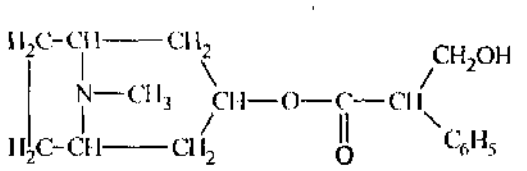
Scopoletin a coumarin was also discovered in the stem bark of this plant alongside the triterpenes quinovic acid and its derivatives, 3-oxoquinovic

acid, quinovic acid-3-O-α-L-rhamnoside and quinovic acid-3-O-β-D-glucoside Bishay *et. al.*, (1988).

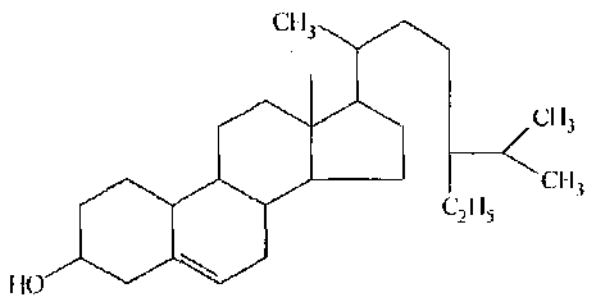
Since phytochemical studies on the plant has revealed the presence of sterols, terpenes especially triterpenes and alkaloids, its therefore of importance to discuss briefly the chemistry of terpenes, sterols and alkaloids.



(I)



(II)



(III)
β-sitosterol

1.2 **TERPENES**

These are most diverse and chemically interesting groups of natural products Hanson, (1972). Despite their structural diversity, they have a simple unifying feature used in their definition. Thus they can be defined as a group of natural products whose structure may be divided into isoprene units and based on these, the terpenes can be classified into monoterpenoids,

sesquiterpernoids, *diterpenoids*, *sesterpenoids*, *triterpenoids* and tetraterpenoids.

1.2.1 **Extraction of Terpenes**

A large number of terpenes are usually non-polar compounds and may therefore be separated from polar plant constituents by extraction with solvents like benzene or ether. Associated matters like lipids, esters and waxes may be removed by saponification in alcoholic alkali, followed by extraction with ether Robinson, (1969). Terpenoid glycosides however are usually insoluble in non-polar solvents they are most conviniently extracted from plants with 70 - 95% ethanol. Extraneous lipids are removed from the alcoholic solution by partition with benzene. Acidic terpenes when present as free acids are soluble in non polar solvents, but on saponification will pass into alkaline phase.

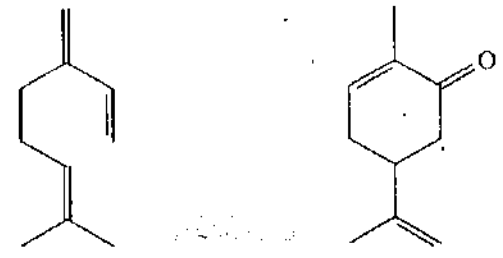
1.2.2 **Spectral Properties of Terpenes**

(i) Ultraviolet Spectroscopy (UV)

The main use of this technique in terpenoid chemistry is in the detection of conjugation. In simple acyclic dienes, λ_{max} is 217 - 218 nm while in heteroannular dienes λ_{max} is 230 - 240 nm, and for homoannular dienes is 256 - 265 nm Finar, (1991).

The absorption of the diene system is affected by substitution and Woodward (1942) proposed empirical rules which was later modified by Fieser in 1948 for calculating the λ_{max} from the molecular structure of a compound. A monoterpene myrcene (IV) based on these rules have a λ_{max} of

219 nm while the observed λ_{max} is 224. Similarly, carvone (V) has a calculated λ_{max} of 237 nm while the observed λ_{max} is 235 nm.



(IV) (V)

The position of the maximum is detected by the presence of basic chromophores and by its degree of substitution.

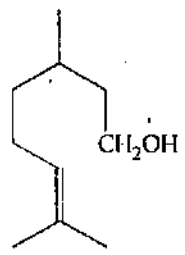
1.2.3 **Infra-red (IR) Spectroscopy**

This is of importance in terpenoid chemistry, there are two major regions of the spectrum which is of importance these are: 3650 - 2650 cm^{-1}, which is attributed to O-H stretching, apart from C-H stretching vibrations of terpenoids. These with judicious interpretation may be used to determine the presence of hydroxy groups and related species. A strong absorption in the second and most important region (1820 -1640 cm^{-1}) is usually attributed to carbonyl stretching: for saturated carbonyl (1750 - 1700 cm^{-1}) and unsaturated α, β- carbonyl 1700 - 1600 cm^{-1}.

Infra-red spectra is particularly useful for detecting the presence of isopropenyl group in terpenes usually between 895 - 885 cm^{-1} for example, the spectrum of citronenollol (VI) show a maximum at 890 cm^{-1} which corresponds to the presence of the isopropenyl group while that of isopropylidene is 850 - 790 cm^{-1}.

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Myrcene (IV) with λ_{max} 224 had no band at 890 cm^{-1}. This shows the complete absence of the isopropenyl group.



(VI)

The infra-red spectra is able to distinguish between heteroannular dienes and α, β-unsaturated ketones which cannot be distinguished by means of ultraviolet spectra.

(iii) **Nuclear Magnetic Resonance (NMR) Spectroscopy**

(a)^{1}H-NMR

This has been used to detect and identify double bonds, to determine the nature of end groups, the number of rings present and to ascertain the orientation of methyl groups present. For example, absicin (VII); a plant hormone and a monocyclic sesquiterpenoid has a ^{1}H-NMR spectrum as follows: methyl singlets at δ = 1.1 and δ = 1.17; two vinyllic methyl group singlets at δ 1.1 and 2.1; and a methylenic group adjacent to the carbonyl group at δ 2.41.

(ii) **^{13}C-NMR**

The ^{13}C-NMR is a more useful tool than ^{1}H-NMR for the elucidation of carbon skeleto of a compound (Stothers, 1972). This is because most of the carbons in compounds of moderate complexity give rise to separate signals, while the proton spectrum on the other hand permits the identification of only a few positions in the compound.

Table 1.1 shows C-13 shieldings in some acyclic terpenes and cyclic monoterpenes. For acyclic terpenes, from the table, the effect of substituents are essentially limited to the carbons of the isoprene units bearing that substituent. For example, C-1, C-4, and C-9 in the acyclic monoterpenes and C-1, C-8, C-13 and C-14 in farnesol and squalene exhibit comparable shieldings in each skeleton. The gamma effect of the carbonyl group is readily found by comparing the C-5 and C-10 shieldings of *cis*- and *trans*- citral. The sp^3 carbon *cis* to the formyl group is shielded by approximately 8 ppm.

The data for squalene showed that the shielding in non-terminal isoprene units differs from the terminal units in that C-1 and C-2 are shielded by -5 and -15 ppm respectively, and C-13 is deshielded by 1.5 ppm. The result for cyclic monoterpenes showed that in general 10 separate signals are resolved.

1.2.5 Mass Spectrometry (MS)

This is now being increasingly used as a means of elucidating the structure of terpenoids, thus it is possible to determine the molecular weight, molecular formula, the nature of various functional groups and the relative positions of double bonds. Since isoprene is the building unit of terpenoids, the following include the peaks which are usually observed in the spectra of terpenoids in general: 68 ($C_5H_8^+$), 67 ($C_5H_7^+$), 53 ($C_4H_5^+$), 51 ($C_4H_3^+$), 41 ($C_3H_5^+$), 39 ($C_3H_3^+$), 29 ($C_2H_5^+$) and 27 ($C_3H_3^+$). Paths that account for some of these fragments are:

TABLE 1.1

CARBON-13 SHIELDINGS IN SOME ACYCLIC TERPENES AND CYCLIC MONOTERPENES[a,b]

Compound	Structure	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10	C-11	C-12	C-13	C-14	C-15
Linalool	53	25.9	*131.0*	125.5	28.1	43.0	[72.9]	146.3	111.6	17.8	23.2					
Geraniol	54a	25.8	*131.5*	125.2	26.9	40.0	*137.6*	124.8	[59.0]	17.7	16.3					
cis-Citral	54b	25.6	*133.2*	123.4	27.5	32.8	*162.4*	129.0	[189.7]	17.7	24.7					
trans-Citral	54c	25.6	*132.6*	123.8	26.3	40.8	*162.4*	127.8	[190.3]	17.7	17.3					
Farnesol	55	25.7	*131.1*	125.3	27.2	40.0	*135.2*	124.6	25.9	40.1	*137.4*	125.0	[58.9]	17.7	(16.2[f]	(14.0[f]
Squalene	56	25.8	*130.9*	125.1	27.3	40.2	*135.1*	124.9	27.2	40.2	*134.9*	124.9	28.7	17.8	16.2	16.2
Menthane	57	*36.0*	33.4	30.2	44.4	30.2	33.4	22.8	*36.0*	19.3	19.3					
Menthol	58	*32.2*	45.9	[71.2]	50.7	23.8	35.3	22.6	*26.1*	16.3	21.3					
Cineole	59	[69.4]	32.0	23.3	33.4	23.3	32.0	27.7	[73.3]	29.1	29.1					
Limonene	60a	*133.5*	121.1	30.9	41.5	28.3	31.2	24.1	*150.0*	108.7	20.8					
Carvone	60b	*135.5*	144.1	31.6	43.0	43.4	[198.0]	15.7	*147.5*	110.6	20.5					
Pulegone	61	*31.7*	51.0	[201.4]	132.0	28.8	33.2	22.0	*141.1*	22.1	23.0					
α-Pinene	62	47.6	*144.4*	116.5	31.8	41.8	*38.3*	31.7	(23.1)	(26.8)	21.1					
β-Pinene	63	52.4	151.6	24.1	23.9	41.1	*41.1*	27.3	(22.0)	(26.1)	106.6					
Bornane[d]	64	*47.0*	36.8	28.7	46.3	28.7	36.8	*45.3*	19.2	19.2	15.8					
Bornylene[d]	65	(52.9)	139.8	134.3	52.7	24.8	31.9	(56.6)	19.9	19.7	13.4					
Camphor[d]	66	*57.4*	[216.7]	43.2	43.6	(27.3)	(30.2)	*46.8*	19.9	19.3	9.5					
Camphene[d]	67a	47.4	165.7	*42.0*	48.7	24.2[f]	29.2[f]	37.8	29.7[f]	26.1[f]	99.8					
Fenchone[e]	67b	*54.1*	[219.4]	*47.4*	45.8	25.6	32.3	42.1	22.2	23.9	15.3					

[a] δ_C, ppm from TMS. Original data converted using $\delta_C^{CS_2}$ 192.8. Italicized values denote carbons bearing methyl groups while carbons bearing oxygen are indicated by square brackets. Assignments for values in parentheses may be interchanged (see text).

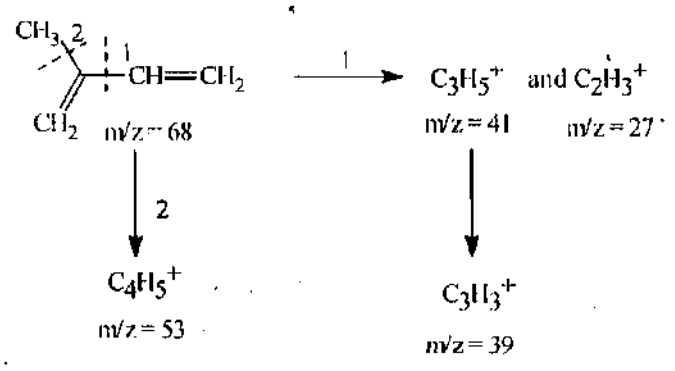
[b] Unless otherwise indicated data taken from Weigert *et al.* (74).

[c] Original assignments have been reversed.

[d] Grutzner *et al.* (45).

[e] Wenkert *et al.* (77).

[f] Assigned by off-resonance decoupling (34).



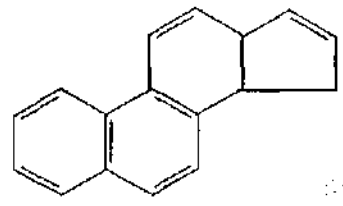
1.2.6 **Pharmaceutical Importance of Terpenes**

The volatile terpenes especially the monoterpenes have wide application in perfumery industries in production of perfumes; the sesquiterpenes have use in pharmaceutically practice especially with the introduction of the Chinese antimalaria Artemisinin which is an unusual sesquiterpenes lactone. It is used in the treatment of *Plasmodium falciparum* resistant malaria and also in cerebral malaria. The diterpenoids are also of current interest because of their potential as future drugs either as isolated from plants or as modified derivatives e.g. Forskohlin (Coleanol) a diterpene isolated by Indian workers from *Coleus forskohlii*. The preparation of the species have long been used in Hindu and Ayurvedic traditional medicine for treatment of heart disease, abdominal colic (Ammon and Muller. 1985) forskolin has been shown to have hypotensive, spasmolytic and cardiotonic effect.

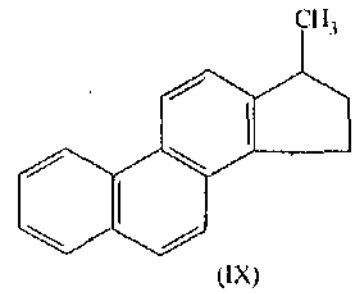
1.3 **STEROIDS**

Steroids form a group of structurally related compounds which are widely distributed in animals and plants. Included in the steroids are the sterols from which the name steroid is derived, Vitamin D, the bile acids and

the sex hormones. The steroid structure are based on the 1,2-cyclopentanophenanthrene skeleton (VIII).



(VIII)



(IX)

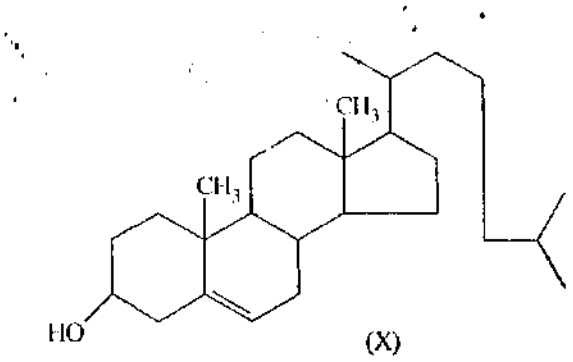
Steroids give among other products Diel's hydrocarbon (IX) on dehydrogenation with Selenium at 360 °C (Finar, 1991). Thus steroids can be defined as any compound which give Diel's hydrocarbon when distilled with Selenium.

1.3.1 STEROLS

The sterols are group of crystalline steroidal alcohol containing between 27 and 30 carbon atoms all possess a 3 β-hydroxyl group and an exocyclic double bond usually in 5, 6 position, together with a side-chain which exhibits various degrees of branching and unsaturation Dence (1980). The sterols can be classified into three:

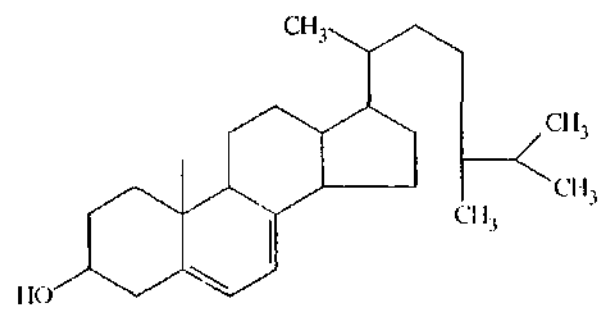
(i) *The Animal sterols*:- this is examplified with cholesterol (X). It is the principal mammalian sterols in human. Other example of animal steroids include bile acids sex hormones and the steroidal hormones of the Adrenal cortex. The animal sterols are also referred to as zoosterol.

(ii) *Plant sterols*:- The basic difference in structure of plant and animal sterols (Cholesterol) is the presence of an additional alkyl in the C-17 side chain of plant sterols.



(X)

Yeast sterols:- These sometime is classified as a plant sterol. Ergosterol (XI) is an important example of yeast sterol. Plant steroids e.g. cardiac glycosides, digitoxin occur generally largely in a conjugated form with one or more sugar residue including glucose. Xylose, galactose, rhamnose, fructose, digitalose etc.



(XI)

1.3.2 **Colour test for steroids**

These are simple chemical tests usually conducted to infer the presence of sterols.

a) *Salkowski test*: If concentrated H_2SO_4 is added to a solution of sterol in chloroform, a red colour is produced in the chloroform layer.

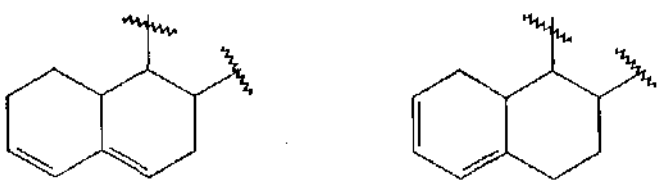
b) *Liebermann-Buchard reaction*: When the sample is in chloroform and a solution of concentrated H_2SO_4 in $(CH_3CO)_2O$ is added, valid test which is the progression of colour change from red to blue and finally bluish-green over a period of some minutes is an indication of steroid presence.

1.3.3 Spectral Properties of Steroids

Ultraviolet (UV) Spectrum

The UV absorption spectra of steroids are characteristics of the functional groups present. The observed maxima are in good agreement with those calculated based on Woodward's rule. Simple acyclic dienes have λ_{max} between 217 and 218 nm, while heteroannular and homoannular dienes have λ_{max} between 230 - 240 nm and 253 - 265 nm respectively.

Bathochromic shift observed in cholestan-2,4-diene (XIII) is as a result of the homoannular diene structure it has when compared to cholestan-3,5-diene (XII).



(XII) (XIII)

Infra-red (IR) Spectroscopy

Infra-red spectrum is valuable in elucidating the structures of unknown steroids. The most important groups are: hydroxyl group, carbonyl group, α, β- unsaturated carbonyl groups, alkene, caboxy acid group and ester group. A very important feature of steroid infra-red spectra however is the dependence of the absorption maximum of the keto group on its position in the nucleus, and in some cases whether the hydrogen atom at a ring junction is α or β Finar, (1991).

The absorption frequencies for saturated ketones (C=O stretching) are 3 C=O (5α and 5β), 1719 - 1712 cm^{-1}, 4 C=O (5α) 1712, 4 C=O (5β) 1713, C-(5α) 1714 - 1712, 6 C=O (5β) 1708 - 1706, 11 C=O (5β) 1710 - 1704.

When the functional group present is , α, β- unsaturated ketone both the carbonyl stretching frequency and the carbon-to-carbon double bond stretching frequency have the maximum frequencies in regions depending on the position of the carbonyl group in the molecule for example, Δ^1-3 ketone C=O 1684 - 1680, C=C 1609 - 1604, Δ^4-3 ketone, C=O 1681 - 1677 and C=C 1619 - 1615. If the nucleus of the steroids contain two keto groups, each show its own absorption frequency or one very close to it, in some cases, but different in others. For example, 3,17-diketo compound has two absorption frequency for the two ketonic group at 1719 and 1745 respectively, while 11,17-diketo compound has frequencies at 1713 and 1751 respectively.

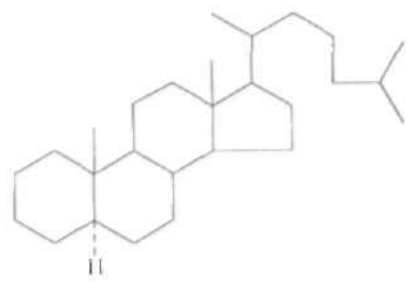
Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectroscopy has been used in structural studies of steroids. A number of steroids have been examined and certain regularities were observed. Steroids appear to have a finger print region which is characteristics of methine and methylene protons in the nucleus. Also, the vinylic protons has a definite δ value and hence these groups can be detected easily, and from the area of the proton peak the number of these groups present can be estimated. From the spectral data of known-steroids it is possible to determine the position of a double bond in an unknown steriod Shoolery, (1958). Also, the protons of the angular methyl groups at C-10 and C-13 in the steroidal skeleton have their own characteristic chemical shift. The actual shift depends on the presence or absence of the various functional groups such as C=C, C=O, thus a 3 keto group causes a shift of 0.15 ppm in the C-19 methyl line but has no effect on the position of C-18 methyl line.

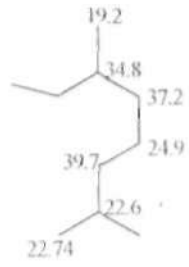
Hence, it may be possible to deduce both the nature and the position of a functional group in the molecule.

^{13}C-NMR

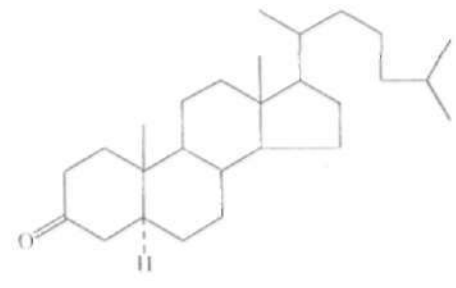
The introduction of ^{13}C-NMR was a major break through as information could be obtained about the features of a structure particularly the carbon skeleton (Verpoote, 1986). The spectrum of cholesterol twenty five separate signals were resolved and these have been assigned to specific carbon atoms Stother, (1972). While the proton spectrum on the other hand gives identification of only a few positions on the cholesterol skeleton. The ^{13}C shielding effect for cholestane and other related steroids (Table 1.2) the group exhibit signals at δC 21.0 ± 0.2 and 169.7 ± 0.2, for the methyl and carbonyl carbons respectively. Inspection of the data (Table 1.2) with the exception of ergosterol reveals the occurrence of a series of signals corresponding to those of 2,6-dimethyl octane (XV). The assignments for which follow from the substituent parameters for acyclic hydrocarbons (Sthoters, 1972).



(XIV)



(XV)



(XVI)

Twenty six of the twenty seven possible signals are resolved in the cholestane spectrum although only four pairs are separated by 0.1 ppm. Three of the signals shift appreciably upon introduction of a carbonyl group as in cholestan-3-one (XVI).

TABLE [illegible] J. 2

CARBON-13 SHIELDINGS IN CHOLESTANE-TYPE STEROIDS (43)[a]

C	72	73	74a	74b	74c	75a	75b	75c[b]	76	77	78	79	80
1	39.2	38.9	37.6	37.2	33.4	37.8	37.6	37.7	38.4	38.9	34.3	33.3	36.4
2	22.6	38.2	32.1	27.9	26.5	31.9	28.5	28.6	28.5	32.5	23.4	23.8	27.8
3	27.3	209.4	70.7	73.5	69.8	71.6	74.0	80.6	72.9	70.0	129.9	136.4	72.6
4	29.6	44.8	38.9	34.5	33.4	42.7	38.7	39.3	37.1	41.4	124.6	124.7	38.0
5	47.6	47.0	45.5	45.1	40.6	141.5	140.2	141.3	141.3	141.0	141.7	160.3	163.5
6	29.6	29.4	29.3	29.1	28.7	121.6	122.9	121.6	120.7	119.7	123.2	128.2	127.0
7	32.6	32.2	32.6	32.4	32.4	32.3	32.5	32.3	117.0	117.0	32.2	200.7	200.4
8	36.1	36.0	36.1	36.0	36.0	32.3	32.5	32.3	138.8	140.9	32.3	46.3	45.6
9	55.5	54.4	55.1	54.9	54.9	50.8	50.7	50.8	46.5	46.8	48.9	50.1	50.2
10	36.8	35.9	35.9	35.9	36.1	36.8	37.0	37.2	37.5	37.5	35.5	36.5	38.6
11	21.4	21.9	21.7	21.7	21.3	21.5	21.6	21.5	21.4	21.5	21.5	21.7	21.6
12	28.6	28.6	28.6	28.5	28.7	28.6	32.8	28.5	28.5	28.6	28.6	29.0	28.9
13	43.1	43.0	43.1	43.0	43.0	42.7	42.8	42.8	43.3	43.3	42.9	43.9	43.5
14	57.2	56.8	57.0	57.0	57.1	57.2	57.3	57.3	54.8	54.9	57.5	51.2	50.5
15	24.6	24.6	24.6	24.5	24.5	24.6	24.9	24.6	23.3	23.4	24.5	26.8	26.7
16	40.7	40.5	40.7	40.5	40.6	40.3	40.4	40.4	39.7	39.7	40.5	39.5	39.3
17	57.1	56.9	57.0	57.0	57.1	56.8	56.9	56.9	56.5	56.3	56.9	55.6	55.5
18	12.5	12.3	12.4	12.3	12.3	12.3	12.3	12.2	12.1	12.1	12.3	12.3	12.1
19	12.4	11.5	12.3	12.3	11.5	19.7	19.6	19.6	16.3	16.3	18.9	16.8	17.2
20	36.3	36.2	36.2	36.2	36.3	36.1	36.4	36.3	36.6	40.8	36.3	36.2	36.2
21	19.1	19.0	18.9	19.0	19.0	19.1	19.2	19.1	19.2	19.7	19.1	19.3	19.2
22	36.7	36.7	36.7	36.7	36.7	36.7	37.0	36.7	36.6	136.3[c]	36.8	36.7	36.7
23	24.5	24.3	24.4	24.4	24.4	24.4	24.6	24.4	24.3	132.5[c]	24.5	24.4	24.4
24	40.0	39.9	40.0	39.9	40.0	39.9	40.1	40.0	39.9	43.3	40.1	39.9	39.9
25	28.4	28.3	28.3	28.3	28.3	28.3	28.5	28.3	28.3	33.5	28.4	28.3	28.3
26	22.8	22.8	22.7	22.7	22.8	22.8	23.0	22.8	22.8	20.0	22.8	22.9	22.7
27	23.0	23.0	22.9	22.9	23.0	23.1	23.2	23.0	23.0	21.3	23.0	23.1	22.9
28										17.7			

[a] δ_C, ppm from TMS. Original data converted using $\delta_C^{CS_2}$ 192.8.

[b] Methoxyl carbon at δ_C 55.4.

[c] Original assignments reversed (see text).

Mass Spectrometry (MS)

This is a very useful tool for structural analysis of steroids. Steroids generally give an abundant molecular ion (M^+) hence, it is easy to determine the molecular weight and the molecular formula. Four peaks are usually observed: (a) $[M - R]^+$ where R is the side chain (b) $[M - (R + 42)]^+$, where mass 42 is C_3H_6 (c) $[M - 15]^+$, where 15 is due to loss of an angular methyl group (d) $[M - (R + 42 + 15)]^+$. Because of this fragmentation pattern it is possible to detect the presence of a steroidal nucleus (Finar, 1991).

Steroidal alcohols usually shows the presence of a peak at m/z, $[M - 18]^+$ due to the loss of a molecule of water and a peak at $[M - (18 + 15)]^+$ due to loss of a molecule of water and an angular methyl group.

Pharmaceutical Application of Steroid

The plant steroid e.g. stigmasterol is use as a starting material for the synthesis of steroid hormone which is used as replacement therapy in steroid hormone deficiency.

1.4 SAPONINS

These are group of plant containing glycosides and share in varying degrees two common characteristics.

a) They foam in aqueous solution; that is have the property of foaming in water.
b) Intravenous injection of their aqueous solution into animals results in haemolysisOlaniyi *et. al.*, (1993).

The aglycones of the saponins are called sapogenins. Plant material containing saponins have being used in many parts of the world for their detergent properties e.g. in Europe, the root of *Saponaria officinales* (Family Caryophyllaceae) and in South America.,the bark of *Quillaia saponaria* Family Rosaceae. Such plants contain a high percentage of glycosides known as saponins which are characterized by their property of producing a frothing aqueous solution Shafik *et. al.*, (1976). Saponins are toxic especially to cold blooded animals e.g. fish. by causing haemolysis of red blood cells.

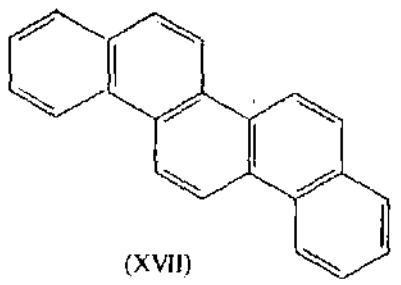
1.4.1 **Preparation of Saponins from Plant Material**

Due to the high molecular weight of saponins, their isolation presents some difficulties. They are usually extracted from the plant material by hot water or alcohol and after concentration of the extract, *in vacuo*, the saponins are precipitated with ether. In certain cases, lead acetate is used to precipitate acid saponins from their aqueous extract, while basic lead acetate is used to precipitate neutral saponins and the resulting lead salts of the saponins decomposed with sulphuric acid.

1.4.2 **Structure of Saponins**.

From a chemical point of view and according to the structure of the Sapogenin two kinds of Saponins are recognized (i) The steroidal (C-27) and the triterpenoid (C-30) Saponins. Both of these have glycosidic linkage at C-3, and probably have a common biogenetic origin via mevalonic acid and isoprenoid units. The steroid and triterpenoid Saponins can be distinguished by the fact that while the steroidal Saponins gives Diel's hydrocarbon on

dehydrogenation with Selenium at 360 °C, the triterpenoids gives mainly naphthalene or picine (XVII) derivatives

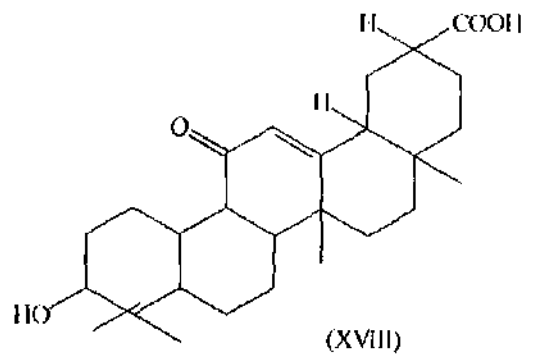


(XVII)

The sugar portion may consist of 1 to 5 sugar units and in some cases, the aglycone is not combined with sugar but with a closely related uronic acid.

Steroidal saponins are less widely distributed than the triterpenoid saponins. e.g. of steroidal Saponins include:

Digitonin which contains 2 glucose and 1 rhamnose as the sugar units and occurs in seeds of *Digitalis purpurea* and *D. lanata.*



(XVIII)

Pharmaceutical importance of Saponins

The wide occurrence of saponins in nature has evoked considerable interest in their uses and considerable data have accumulated in their physiological action and other properties. Saponins in general lower surface tension and possess emulsifying properties. They tend to alter the permeability of the wall hence exert a general toxicity in all organized tissues

their haemolytic and anti-lipemic activity and capacity to lower serum cholesterol level can be their important characteristic. The ability of saponins to reduce surface tensions have been utilized in making emulsion stabilizers Basu and Rastogi, (1967). Sapogenins, e.g. Diosgenin the steroidal sapogenin plays an important role in the development of steroid hormone in pharmaceutical industry as it can be easily converted to physiologically active steroids e.g. progesterone Dence, (1980).

Glycyrrhizin (Glycyrrhizic acid) (XVIII) the triterpenoid saponin of liquorice root has shown varied and interesting biological activity and patents have been taken in the preparation of glycyrrhizin as such or as its ammonium, sodium, potassium and colchicine salts. Yatsyn and Abubakinov, (1966). Glycyrrhizin has been used in the treatment of gastric ulcers and dermatitis Doruier, (1962).

1.5 ALKALOIDS

A precise definition of the term alkaloid (alkali-like) is some what difficult because there is no clear cut boundary between alkaloid and naturally occurring complex amines. Trease and Evans, (1985). Typical alkaloids are derived from plant sources. They are basic, they contain one or more nitrogen atoms (usually in heterocyclic ring) and they usually have a marked physiological action on man or animal. The name proto alkaloid or amino alkaloid is sometime applied to compound such as ephedrine and colchicine which lack one or more properties of the typical alkaloids Trease and Evans, (1985). Other alkaloids not conforming with the general definition are these derived from bacteria or animal sources together with synthetic alkaloids e.g.

Homatropine. In the plants, the alkaloids exist as salts, or as amine or N-oxide.

1.5.1 **Test for Alkaloids**

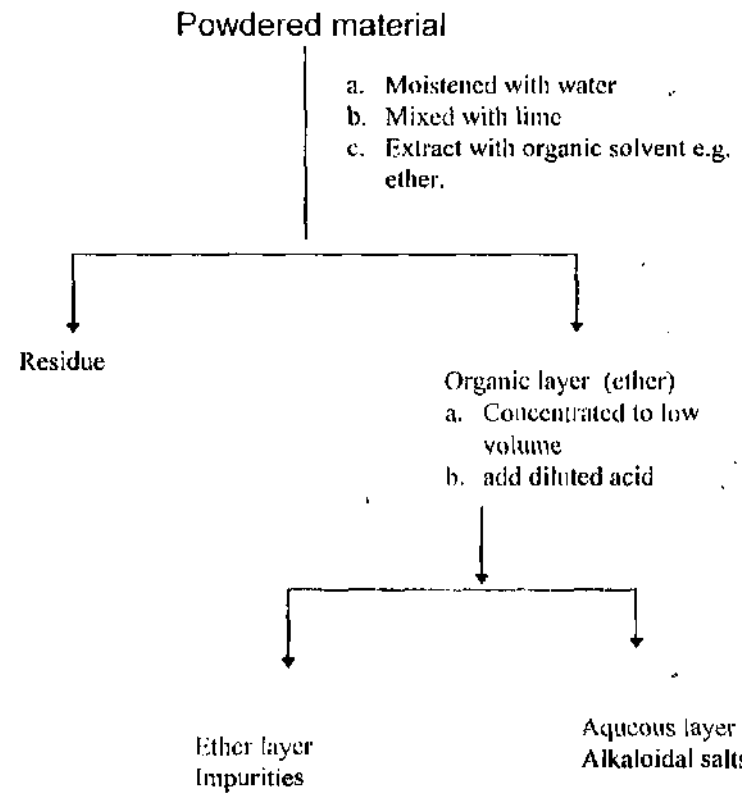
Most alkaloids are precipitated from neutral or slightly acid solution by: Mayer's reagent (which is potassium mercuric iodide solution), Wagner's reagent (solution of Iodine in potassium Iodide), solution of tannic acid, Hager's reagent (a saturated solution of picric acid), and Dragendorff's reagent (solution of potassium bismuth Iodide). These precipitates which may be amorphous are usually coloured: Cream or white to yellowish (Mayer's reagent), yellow (Hager's reagent), reddish-brown/orange-brown (Dragendoff's and Wagner's reagent): Caffeine and some other alkaloids do not give this test. However, these reagents also give positive test with proteins, but these can be taken care of if adequate extraction procedure for alkaloids is followed.

1.5.2 **Extraction of Alkaloids**

Alkaloids are usually found in the seeds, root, leaves and bark of the plant. They occur generally as salts of various plant acids such as acetic, oxalic, citric and tartaric acid: Extraction of an alkaloidal substance from plant utilizes the solubility properties of the alkaloid. The free alkaloidal base is usually liberated from the dried powdered drug with an alkali (NH_4OH or Na_2CO_3) and extracted with an organic solvent e.g. chloroform, ether and ethanol or methanol. The mixture of bases thus obtained is separated by chromatography. Olaniyi *et. al.*, (1993).

Trease and Evans, (1985) gave 2 methods of extraction of alkaloids.

(1) The powdered material is moistened with water and mixed with lime which combine with acids, tannins, and other phenolic substances and sets free the alkaloid (if they exists as salts). Extraction is then carried out with organic solvent such as ether. The concentrated organic liquid is then shaken with aqueous acid, and allowed to separate, alkaloidal salt will remain in the aqueous liquid while impurities remain in the organic layer.



Scheme 1.1: Profile for extraction of alkaloids.

(2). The powdered material is extracted with water or aqueous alcohol containing dilute acids. Pigments and other unwanted materials are removed by shaken with chloroform or other organic solvents. The free alkaloids are then precipitated by the addition of excess of sodium bicarbonate or ammonia and separated by filtration or extraction with organic solvents.

1.5.3 **Structural Elucidation of Alkaloids**

Since the isolation of the first alkaloids in the 19th century, the methods used in the identification and structure elucidation of such compounds have changed considerably, originally pure chemistry (e.g. the making of derivatives and performing degradative reaction) was the only available tools. However, over the past 40 years, a number of non-degradative spectral methods have been introduced, which had a great impact on natural products research Verpoote, (1986).

Application of such new methodology resulted in a number of structural revisions (Verpoote, 1986). Based on the isolation method used, it is possible to know whether the isolated compounds have an acidic, neutral or basic properties. The plant from which a compound has been isolated also adds important information as to which groups of compounds are likely to be found e.g. if the compound have been isolated from a plant belonging to the families of Apocyanaceae, Leguminaceae, or Rubiaceae, the compound is most likely to be an terpenoid - indole alkaloid. The strategy for identification of alkaloids can be summarised as in scheme 1.2.

General Strategy	Example
Isolation method	
↓	
Alkaloid	4,000 alkaloids are known
↓	↓
Chemotaxonomy (Plant genus)	Stychnus
↓	↓
Types of alkaloid	300 alkaloids are known
↓	↓
TLC + colour reactions	HR_f-values, Colour with $FeCl_3/HClO_4$, $FeSO_4/H_2SO_4$
UV	Chromophore
MS	Molecular weight
↓	↓
Identification of known alkaloid	Identification.

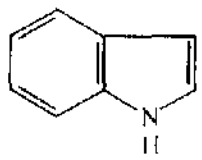
Scheme 1.2 Strategy for identification of non-alkaloids

UV, I.R, NMR, and Mass spectroscopy or combination of Gas chromatography and mass spectroscopy (GC-MS) gives a definite identification of the molecular weight and the fragmentation patterns. With these methods; known alkaloids can be identified, eventually [α],optical rotatory dispersion (ord), and circular dichroism (cd). may be necessary to confirm the stereochemistry. In the case of an unknown structure, other spectral methods such as ^{13}CnMr is usually needed for the structure elucidation.

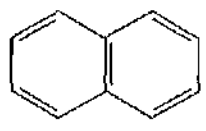
Spectral Methods

1.**UV.**

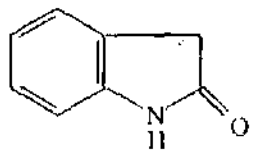
Considering indole alkaloid, the UV spectrum gives information about the aromatic part of the molecule Sangster and Stuart, (1965). The spectrum of indole (XXIII) has three bands at 200, 262 and 280-290 nm which is expected by analogy with naphthalene (XXIV) chromophore Scott, (1964). The 262 nm is modified in or absent from the spectra of many substituted indoles.



(XXIII)



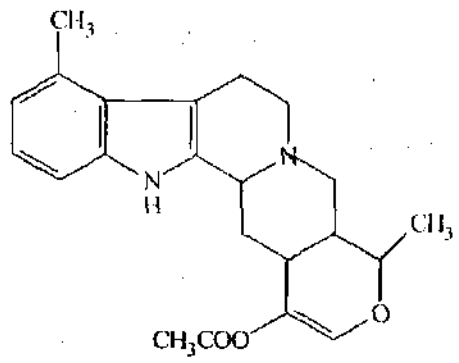
(XXIV)



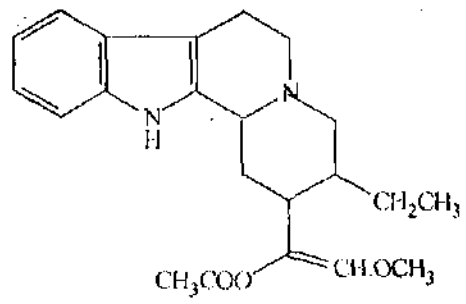
(XXV)

The oxindole chromophore (XXV) showed bands at 248 and 279 nm respectively Scott, (1964). Mitrajavine (XXVI) and Hursutine (XXVII) both indole alkaloid isolated from the leave of *Mitragyna Javanica*, and *Mitragyna hirsuta* Shellard *et. al.*, (1966) showed three bands at: 227, 265 and 292 nm for Mitrajavine and 226, 282 and 292 nm for Hursutine, while the oxindole

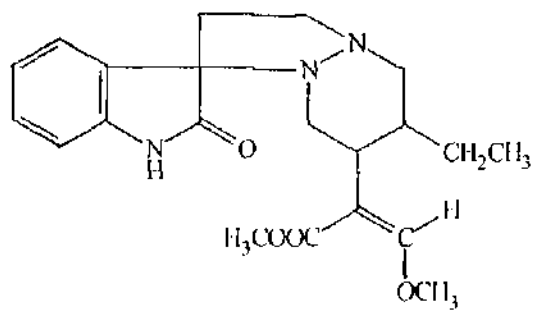
alkaloid, Rhynchophylline (XXVIII) isolated from the leaves of *Mitragyna stipulosa* showed 3 bands at λ_{max}:(EtOH) 208.3 nm, 243 nm and 282 nm. Beckett *et. al.*, (1963)



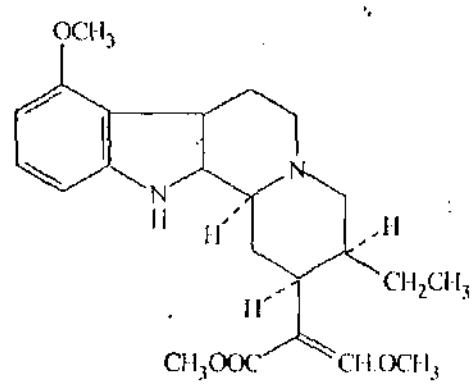
Mitrajavine
(XXVI)



Hursutine
(XXVII)



Rhynchophylline
(XXVIII)

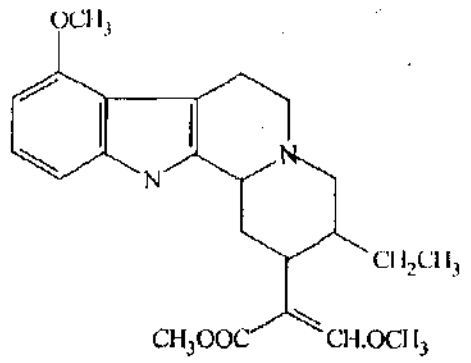


Mitragynine
(XXIX)

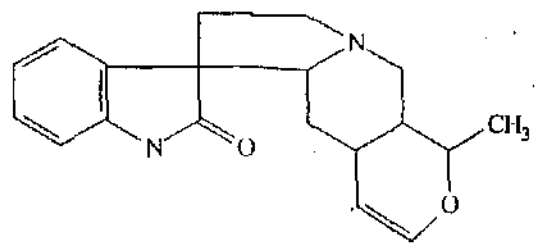
I.R

The IR spectra gives information about functional group because of the highly characteristic pattern of absorption. IR spectral are useful tools in the identification of known compounds by direct comparison of their spectra. Indole and oxindole alkaloids show characteristic IR absorption for the following functional groups: Indole nitrogen (N-H), ester oxindole carbonyl (C=O) absorption frequency and the presence of C=C double bond Bindra, (1973). The indole N-H group extends at 3365 cm^{-1} as in Mitragynine (XXIX) the presence of

C-H absorption is also evident in most indole and oxindole alkaloid. Speciophylline (XXXI) an oxindole alkaloid isolated from *mitragyna speciosa*, showed IR absorption of (N-H) stretching at 3220 cm^{-1}, C-H stretching at 2950 cm^{-1}, Ester/oxindole C=O 1713cm^{-1} and 1685 cm^{-1} while C=C at 1623 cm^{-1} (Beckitt *et. al.*, 1965).

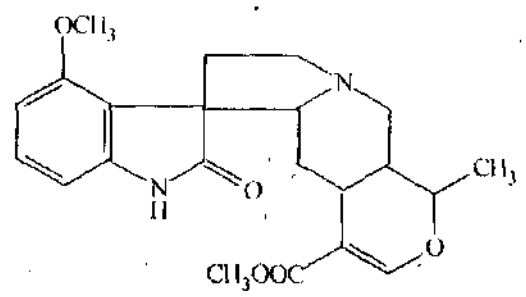


(XXX)



(XXXI)

Speciogynine (XXX) an indole alkaloid isolated from the same plant by the same author gave the following characteristic IR absorption bands for indole alkaloids: (N-H) stretching 3372 cm^{-1}, (C-H) stretching, 2950 cm^{-1}, ester/oxindole C=O, 1684 cm^{-1} and C=C band at 1614 cm^{-1}. For indole and oxindole alkaloids isolated from *Mitragyna Javanica* and *Mitragyna hirsuta*, the indole alkaloid showed only ester C=O absorption frequency, while the oxindole frequency was absent e.g. Mitrajavine (XXVI) showed ester C=O frequency at 1685 cm^{-1}, while oxindole carbonyl frequency is absent, while Pa7 (XXXII) an unnamed oxindole alkaloid showed both ester C=O frequency (1700 cm^{-1}) and oxindole C=O frequency at 1705 cm^{-1} all in Nujol. I.R., absorption bands between 1708 -1730 cm^{-1} indicates the presence of oxindole grouping Montiero, (1968).



Pa7
(XXXII)

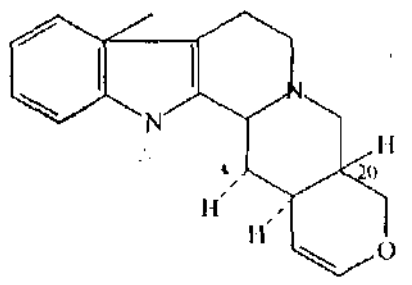
NMR spectrum of indole and oxindole alkaloids isolated from some species of mitragyna plants revealed the presence of indolic protons, aromatic protons, olefinic protons and methyl protons. Thus speciogynine (XXX) an indole alkaloid from the leaves of *M. speciosa* showed signals in the NMR spectrum at δ = 8.00 (for N-H) protons, δ = 7.41 for olefinic protons all singlet and two aromatic protons each showing multiplets at δ = 6.46(1H) and δ = 6.92 (2H) were observed Beckett *et. al.*, (1965). Similarly, the oxindole alkaloid speciophylline (XXXI) isolated from the same plant has NMR spectrum for the following protons: N - H, δ = 9.95, olefinic δ = 7.41 all singlet, aromatic protons: δ = 6.85-7.33 (4H) multiplets. The N-H protons disappeared upon deuteration. Both compounds all showed the presence of ester methoxy group protons (-CO-OCH_3) at δ = 3.38 for speciophyllinne and δ = 3.34 for speciogynine; and both appeared as singlet Beckitt *et. al.*, (1965). Similar observation of signals for the above protons were observed from the NMR spectrum of Hirsutine (XXVII) an indole alkaloid from the leaves of *M. hirsuta* Shellard *et. al.*, (1966); the N-H protons appeared at δ = 8.18 (singlet) and disappeared upon deuteration, while olefinic protons appeared at δ = 7.35 (singlet) and aromatic protons δ = 7.3 (4H) multiplet. The ester methoxy

group protons was observed at δ = 7.71 (singlet). The oxindole alkaloid named Pa7 (XXXII) isolated by the same author from the leaves of *M. javanica* gave NMR signals for N-H protons at δ = 8.80 (singlet) disappeared on addition of deuterium oxide, olefinic protons at δ = 7.47 (singlet), aromatic proton δ = 7.1(1H), δ = 6.6 (2H) both showed signal at δ = 3.56 (singlet). The ester methoxy protons showed signal at δ 3.56 (singlet).

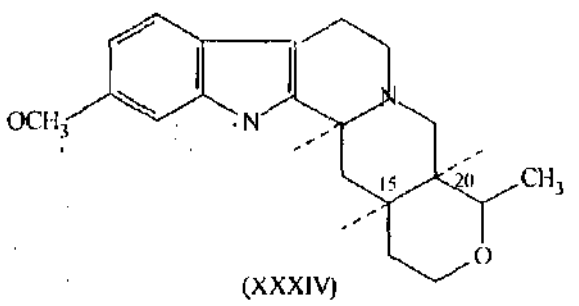
TLC

Among the methods that are useful in the identification and structure elucidation of alkaloids, TLC is an indispensable tool. Important information can be obtained from retention behaviour of a compound in TLC and colour reactions can give information about the presence of certain chromophore e.g. spraying of the plates with $FeCl_3$ in perchloric acid or $CeSO_4$ in concentrated H_2SO_4 gives characteristics colour for the indole alkaloids. Observation of a characteristic colour in a chromatogram immediately reduces the number of possible structure. Colour reactions also allow the rapid recognition of degradation products such as N-oxides as they usually give same colour reaction as the original compound, with however, a more polar character on TLC. Work by Phillipson and Shellard, (1966) on the relationship of retention behaviour in TLC and the configuration and aromatic substitution pattern of some heteroyohimbine and oxindole alkaloids. The stereochemistry of the D/E ring function in ajamalicine (XXXIII) type of alkaloid was found to strongly influence retention. Alkaloids with a *cis* D/E junction (H-15α, H-20α) e.g. reserpinine (XXXIV) have higher HRF value than those with a *trans* D/E junction (H-15α, H-20β) as in Ajamalicine (XXXIII)

those observation were used to obtain information on the stereochemistry of some new mitragyna alkaloids.

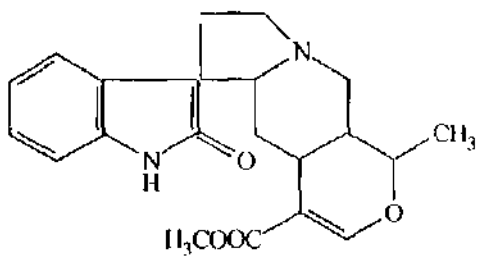


(XXXIII)

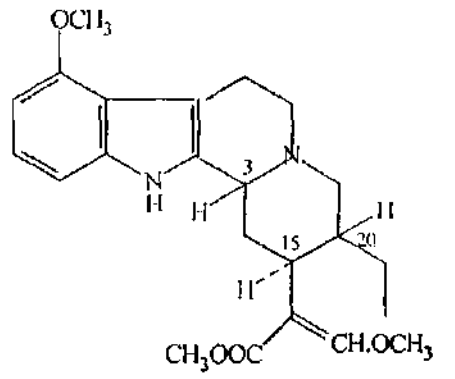


(XXXIV)

Speciogynine (XXXI) and speciocilliatine (XXXV) were known to be isomers of mitragynine (XXIX). From the TLC behaviour of these alkaloid, compared to some alkaloids of known structure, the stereochemistry of the alkaloid was determined to be *trans* D/E for speciogynine (XXXI) and *cis* D/E for speciociliatine (XXXV).



(XXXV)



(XXIX)

Hence TLC, on combination with colour reaction is a powerful tool for rapid identification of known compounds, classification of an unknown alkaloid into a certain group of compounds and obtaining information about the structural features e.g. stereochemistry basicity.

1.5.4 **Pharmacological Importance of Alkaloids**

Many drugs or agents in use today are either obtained from alkaloidal plants or are synthetic analogues of alkaloid. The group alkaloid has a lot of use in medicines as drugs, these includes:

a. *Narcotic analgesics*; since the discovery of morphine and its analogue by F.W. Serturner in 1805, morphine obtained from opium plants is still one agents in use as Narcotic analgesic.

b. *As Cardioactive drugs*; These include alkaloids that possess pharmacological activity on the heart muscle. The cinchona alkaloids quinidine and the rauwolfia alkaloid reserpine are some of the cardioactive drugs in use today.

c. *Antimalaria*; The chinchona alkaloids have long been use as antimalaria in ancient time. Quinine one of the alkaloid of cinchona plant is still an indispensable drug in use against some plasmodium strain resistance to the 4-amino quinollines.

d. *Uterine stimulants*; The ergot alkaloids e.g. ergometrine are still in use as uterine stimulant because of their oxytocic action (Manske, 1955).

e. *Local anaesthetics*; Cocaine and its derivative were one of the first local anaesthetic to be introduced, but due to the dependent effect of cocaine, the use of cocaine as local anaesthetic has become obsolete, it has rather served as a basis for synthesis of procaine a local anaesthetic.

f. *Mydriatic agents*; The tropane alkaloids e.g. Atropine (II) is still one of the myoriatic agents in use, synthetic derivatives like Honatropine is also in use.

g. *Respiratory stimulants*; The tobacco alkaloid, nicotine increases pulmunary ventilation thereby increasing respiratory stimulation. Other alkaloids include Atropine, the purine alkaloid caffeine (L) (Manske, 1955).

1.6 AIMS AND OBJECTIVE OF THE PROJECT

The objective of this project work is to further investigate the phytochemical constituents of the bark of the plant *M. inermis* which is widely used in Northern Nigeria and also to screen its extract pharmacologically using isolated tissue models.

CHAPTER TWO

2.0 EXPERIMENTAL

2.1 MATERIALS

Chemical/Reagents.

All solvents were distilled.

The silica gel for column chromatography is of mesh size (60-120) and that of thin layer chromatography is 0.25 mm thickness.

2.1.1 Instruments

(1) ***Melting point***:

All the value were uncorrected and the melting point value were determined using Gallenkamp Capillary and melting point apparatus.

(2) ***Ultraviolet spectroscopy*** (U.V)

pye- unicam Sp-8 100 UV spectrophotometer

(3) ***Infra-red spectroscopy***

pye- Unicam, Genesis Seried Fourier transform Infra-red spectrophotometer.

(4) Gas Chromatography/ Mass Spectroscopy (GC-MS).

Shimadzu QP-5000 GC-MS.

2.2 ANALYTICAL METHODS

(1) **Thin layer chromatography: (TLC)**

Technique: Ascending

Stationary phase: Silica gel 60 F_{254} pre-coated plates by MERCK of thickness 0.25 mm. Silica gel G. slurry made by dissolving 40 g of silica gel in 80 ml of distilled water and spread unto the plates (2 cm x 5 cm) using a spreader (Stahl, 1975), and activated at 100 °C for 1 hour.

Thickness: 0.25 mm for analytical TLC plates and activated at 110 °C for 1 hour before use.

Spotting and Development: The spots were applied manually using glass capillary tubes and the plates developed at room temperature using a Shandon chromatotank.

Mobile phase: Details in text.

Visualization: The developed chromatogram were dried in a fume cupboard and viewed under.

(1) U.V light for any fluorescent spots

(2) Exposed to Iodine vapour in a closed chamber

(3) Sprayed with suitable chromogenic reagents.

(II) **Column chromatography**:

Technique: Gradient Elution

Column: Sintered glass column (2 x 10 cm) packed by wet slurry method.

Adsorbent: Silica gel. (60-120 mesh size).

Eluting solvents: Details in text.

(III) **Melting point**

The melting point of the isolated compound was determined using Gallenkamp Capillary melting point apparatus and the value was uncorrected.

(IV) **Ultraviolet Spectroscopy**

The U.V. spectrum of the isolated compound was recorded in methanol (AnalaR grade).

(V) **Infra-red Spectroscopy**

The I.R. spectra of the isolated compound was recorded (i) as a thin film (ii) In nujol using Fourier transform infra-red spectrophotometer (FTIR).

(VI) Gas Chromatography/ Mass Spectroscopy (GC-MS).

2.3 SPRAY/VISUALIZATION REAGENTS

(i) Iodine vapour.

(ii) $FeCl_3$

(iii) Dragendorff's reagent (spray)

(iv) Antimony trichloride spray reagent.

2.4 EXTRACTION PROCEDURES

2.4.1 Collection and Drying of Plant Materials

Mitragyna inermis,. The plant material was collected from a bush near Basawa, Zaria between the months of November and January. It was authenticated at the herbarium, Biological Sciences Department, Ahmadu Bello University, Zaria. The bark was air-dried for several weeks before powdering.

2.4.2 Extraction

The air-dried powdered bark (300 g) was extracted exhaustively with petroleum ether (60-80 °C) Using cold maceration for several days. The combined extracts were concentrated at reduced pressure. This was kept at about 4 °C, the extract coded PE. The deffatted marc was air-dried and extracted by cold extraction using methanol for several days. The combined methanolic extracts were concentrated at reduced pressure to afford a dark brown gummy mass which was coded ME.

2.5 PRELIMINARY PHYTOCHEMICAL SCREENING

Small portions of PE and ME were used for phytochemical screening as summarised in below:

(1) Carbohydrate

(a) Molisch's test

A few drops of Molisch's reagent was added to each of the extract dissolved in water, followed by addition of 1 ml of concentrated H_2SO_4 down the side of the test tube. The mixture was allowed to stand for 2 mins., then diluted to 5 ml with water. Formation of a red, or dull violet at the interphase of the layer is taken as positive test.(Trease and Evans, 1983).

(b) Barfoed test

About 0.2 g of each of the extract was dissolved in water, filtered and 1 ml of the filtrate was mixed with 1 ml of Barfoed's reagent in a test tube heated on a

water bath for 2 mins. a red precipitae of Cu_2O is taken as positive test. (Brain and Turner, 1975).

(c) Reducing Sugars (Fehling's test)

About 0.2 g of each extract was dissolved in water, filtered, the filtrate was heated with 5 ml equal volumes of Fehling's solution A & B. Formation of a red precipitate, indicates the presence of reducing sugars. (Trease and Evans, 1983).

(d) **Combined Reducing sugar**

About 0.2 g of each extract was hydrolyzed by boiling with 5 ml of dil. HCl, and resulting solution neutralized with NaOH solution. Two drops of Fehling's solutions A & B was added and heated on a water bath for 2 mins. Formation of a reddish brown precipitate of Cu_2O indicates the presence of combined reducing sugars. (Trease and Evans, 1983).

(2) **Saponins**

Frothing test

About 0.5 g of each extract was shaken with water, in a test tube, frothing which persisted for 15 mins. or when warmed on water bath indicates the presence of saponins. Sofowora, (1993), Silva *et. al.*, (1998).

(3) **Flavonoids**

(a) **Shinoda test**

About 0.5 g of each extract was dissolved in Ethanol, warmed and filtered, three pieces of magnesium chips were added followed by a few drops of

concentrated sulphuric acid. An orange, pink red to purple will appear when flavones, flavonols, the corresponding 2,3 dihydro derivatives and or xanthones are present. (Farnsworth, 1966; Markham, 1982).

(b) **Ferric chloride test**

Each of the extract was boiled with water and filtered. To 2ml of the filterate, 2 drops of ferric chloride (freshly prepared) solution was added, a green, blue or violet colouration indicates the presence of phenolic hydroxyl group. Trease and Evans, (1983).

(c) **Sodium hydroxide test:**

2 mls of each of filtered extract was dissolved in 10% aqueous sodium hydroxide solution to give a yellow colour, a change in colour from yellow to colourless on addition of dilute HCl indicates the presence of flavonoids. (Trease and Evans, 1985).

(d) **Lead Acetate test**

A small quantity of each extract was dissolved in water and filtered to 5 ml of each of the filtrate. Three drops of lead acetate solution was added. A buff coloured precipitate indicates the presence of flavonoids. Brain and Turner, (1975).

(4) **Alkaloids**

0.5 g of each extract was stirred with 5 ml of 1% aqueous hydrochloric acid on a water bath and filtered. 3 mls of the filtrate was divided into three. To the first 1 ml, two drops of freshly prepared Dragendorff's reagent was added to

give a reddish-brownish precipitate, to the second, 1 drop of Mayer's reagent was added to give a white to yellowish precipitate or cream colour, to the third 1 ml, 1 drop of Wagner's reagent was added to give a brown or reddish-brown precipitate Trease and Evans, (1985); Silva *et. al.*, (1998).

(5) **Tannins**

A small quantity of each extract was boiled with water, and filtered. Two drops of ferric chloride was added to the filtrate. A blue black, green precipitate was taken as evidence for the presence of tannins. Trease and Evans, (1983).

(6) **Phlobatannins**

A small quantity of each extract was boiled with water and filtered. Five ml of 1% aqueous HCl was added to the filtrate, a red precipitate shows the presence of phlobatannins

(7) **Anthraquinones**

(a) **Free Anthraquinone**

Each of the extract was shaken with 10 ml of Benzene, the content was filtered, and 5 ml of 10% ammonia solution (NH_4OH) was added to the filtrate and the mixture was shaken. The presence of a pink, red or violet colour in the ammoniacal layer (lower phase) indicates the presence of free anthraquinones Trease and Evans, (1983).

(b) **Combined Anthraquinone**

Each of the extract was boiled with 10 ml of aqueous sulphuric acid and filtered hot. The filtrate was shaken with 5 ml benzene, the benzene layer separated and half its own volume, 10% NH_4OH was added. A pink, red or violet colouration in the ammonia phase (lower layer) indicates the presence of combined anthraquinone or anthraquinone derivative. Trease and Evans, (1983).

(8) **Steroidal Nucleus**

(a) **Salkowski test**

A little quantity of each of the extract (2 mg) was dissolved in 1 ml of chloroform and to it, 1 ml of concentrated H_2SO_4 was added down the test tube to form 2 layers. A red or yellow colour was taken as indication of the presence of sterols or methylated sterols. Silva *et. al.*, (1998).

(b) **Liebermann-Burchard's test**

1 ml of anhydrous acetic acid and 1 ml of chloroform were combined together with the extract and cooled in ice, then 1 drop of concentrated sulphuric acid was added down the side of the test tube. A violet or pink colour which changes gradually to blue and bluish-green is taken as positive test. *Silva et. al.*, (1998).

(9) **Cardenolides**

(a) **Legal test**

Solution I - 0.5% of a recently prepared sodium nitroprusside in water and solution II - 0.2N NaOH. About 2 mg of each plant extract was dissolved in 3 drops of pyridine, 1 drop of solution I was added followed by four drops of solution II one at a time. A deep red colour was taken as the presence of cardenolide aglycone. Silva *et. al.*, (1998).

(b) **Keller-Killani test**

0.5 g of each extract was dissolved in 2 ml of glacial acetic acid containing 1 drop of ferric chloride solution. A reddish- brown ring obtained at the interphase indicates the presence of a digitoxose sugar component characteristic of Cardenolide. Trease and Evans, (1983).

(10) **Alkaloids**

A little quantity of AE was dissolved in 3 ml of dilute hydrochloric acid and the solution divided into three test tubes and alkaloidal test carried using the following: Dragendorff's, Mayer's and Wagner's reagents.

2.5.1 CONFIRMATORY TEST FOR ALKALOID

A confirmatory test designed to remove non-alkaloidal compounds capable of eliciting "false positive reaction" was carried out for the extract which gave a preliminary positive test for alkaloids Sofowora, (1982). A modified form of thin layer chromatography described by Farnsworth and Euler, (1962) was

adopted. The methanolic extract (1.0 g) was treated with 40% calcium hydroxide, until the extract was distinctively alkaline with litmus paper, and extract twice with 10 ml portion of chloroform. The chloroform extracts were combined and concentrated at reduced pressure to about 5 ml. This was spotted on TLC plates and developed using four solvents of different polarities. The presence of alkaloids in the developed chromatograms was detected by spraying with freshly prepared Dragendorff's spray reagent. A positive reaction indicated by the appearance of an orange or darker coloured spots against a pale yellow back-ground on the chromatogram was taken as a confirmatory evidence that the plant extract contain alkaloid.

2.6 INVESTIGATION OF PETROLEUM ETHER EXTRACT (PE)

2.6.1 Saponification of Petroleum ether extract

The method of Mahrain *et. al.*, (1980) was adopted where 1.4 g of the extract was refluxed for 5 hrs with ethanolic potassium hydroxide (15 ml) and benzene (10 ml). The resultant mixture was diluted with water (20 ml), and concentrated at reduced pressure to distill off the organic solvent present. This was extracted with 2 x 25 ml portions of chloroform in a 100 ml separating funnel. The chloroform layer was collected, dried over anhydrous sodium sulphate and evaporated to give a light brown waxy material weighing 0.84 g. This was coded unsaponifiable matter (P1).

The aqueous layer (the saponifiable portion) was acidified with 50 ml of 5% dilute hydrochloric acid, until acidic to litmus, this was extracted with 2 x 25

ml portion of chloroform. The chloroform layer was washed with water, dried over anhydrous sodium sulphate to give a light yellow fraction, which was evaporated at reduced pressure to give a brownish residue (84.5 mg) with rancid odour. This was coded saponifiable matter (P2). fig. (2.1).

2.6.2 **TLC of Unsaponifiable Matter (P1)**

The unsaponifiable matter, from the petroleum ether was subjected to TLC using the solvent system below:

a) Benzene: Acetone (4 : 1)

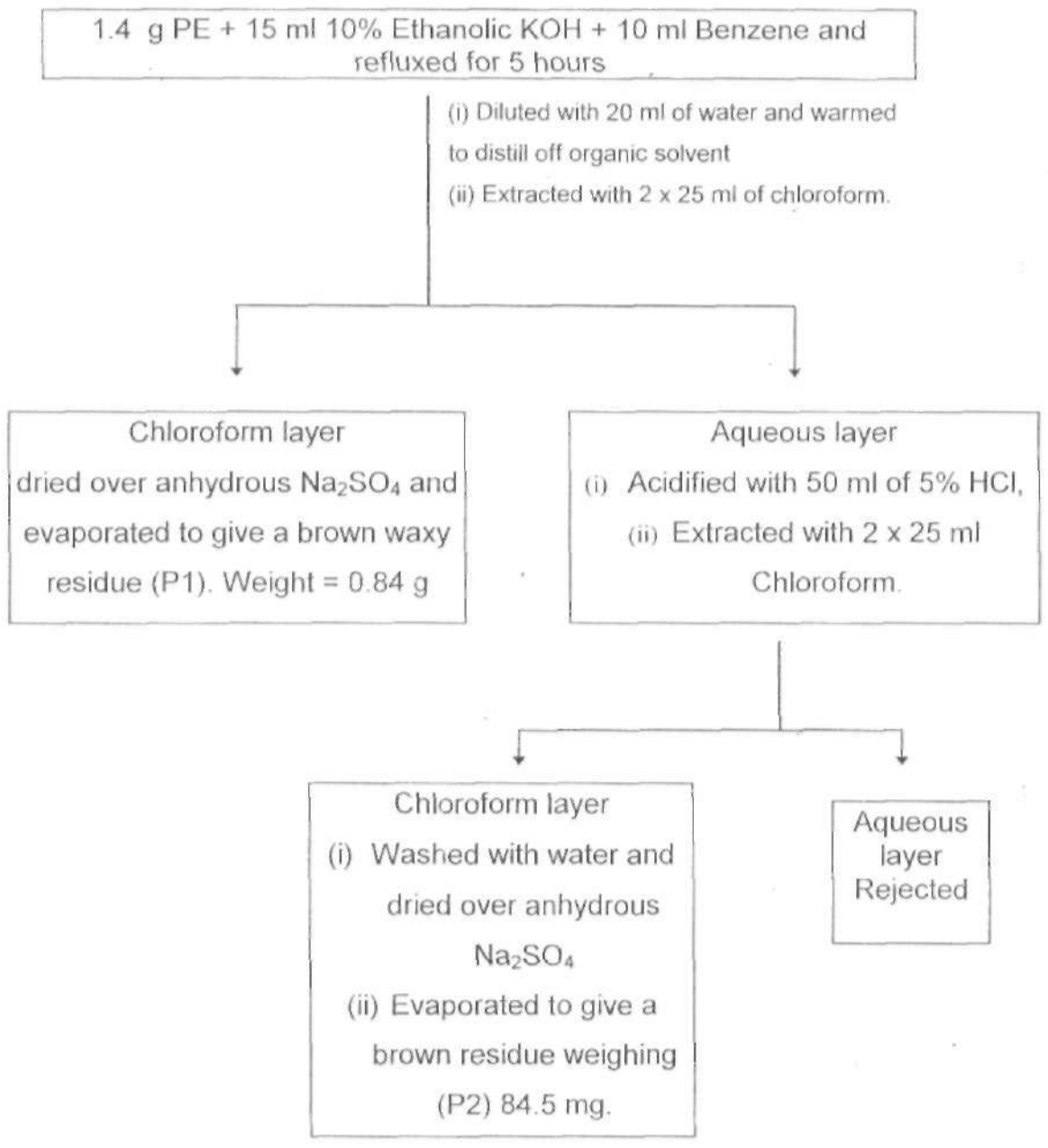
b) Benzene: Methanol (4 : 1)

Each plate was visualized under (i) Visible light (ii) U.V. light (iii) Antimony trichloride. The colour of spots and R_f values were noted.

2.6.3 **Methylation of Saponifiable matter (P_2)**

The saponifiable matter (P_2) was methylated using the method of Mahrain *et. al.* (1980) as follows: 45 mg of (P_2) was refluxed with absolute methanol (10 ml) and dilute sulphuric acid (1 ml) for 1 hour, after which the resulting mixture was diluted with water (10 ml) and extracted with chloroform (10 ml). The chloroform layer was evaporated at reduced pressure to afford a semi-solid residue weighing 34.5 mg which was coded (M-P_2). This was subjected to gas chromatography and mass spectroscopy using Shimadzu QP-5000 GC-MS.

.Fig 2.1: **Saponification of Petroleum Ether Extract**

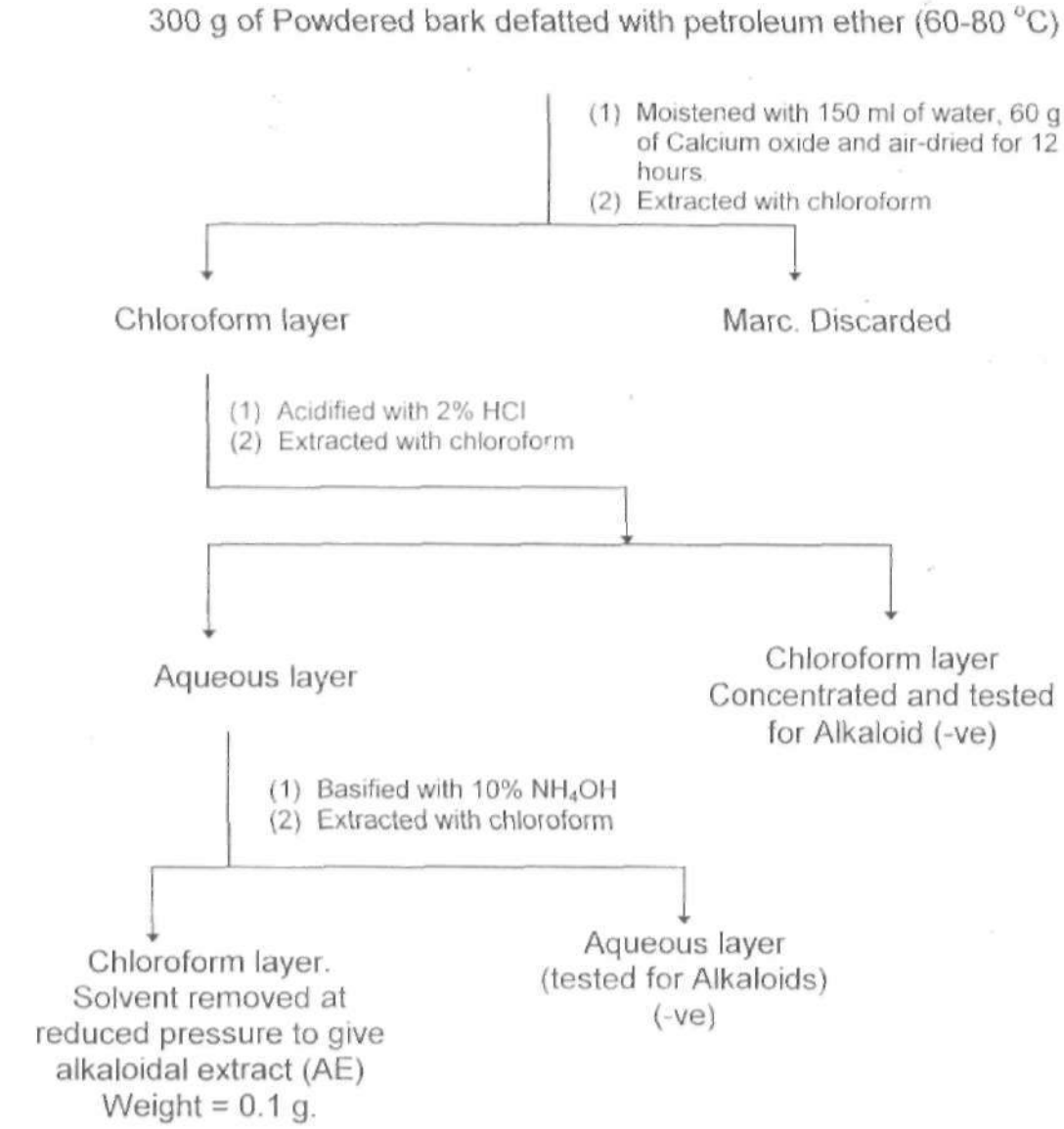


2.7 EXTRACTION OF ALKALOIDS

The methods of Badger *et. al.*, (1950), Brain and Turner, (1975) and Trease and Evans, (1985) were adopted as follow.

The defatted powdered bark (300 g) was moistened with 150 ml of water and 60 g of calcium oxide, and air-dried for 12 hours. This was transferred to a percolator and extracted with chloroform exhaustively. The combined chloroform fractions were transferred to a separating funnel and acidified with 2% HCl to pH 2. This was extracted again with chloroform. The aqueous layer was transferred to a beaker and precipitated with 10% ammonium hydroxide to pH 9. This portion was extracted with chloroform. The chloroform layer was drained off, evaporated at reduced pressure, to afford alkaloidal extract (AE) weighing 0.1 g. (See Fig. 2.2)

Fig 2.2. Extraction of Alkaloid.



AE was subjected to alkaloidal test

2.7.1 Thin Layer Chromatography of Alkaloidal Extract (AE)

A little quantity of AE was dissolved in few drops of chloroform, the resulting solution was spotted on a pre-coated Silica gel G plates. The spot was developed using the following solvent system.

i) Chloroform: Methanol (2:1)

ii) Chloroform: Methanol (3:1)

After the development of each plate with each of the above solvent systems, the plate was allowed to dry, then visualized under:

i) Visible light

ii) UV light

iii) Exposure to iodine in a closed chamber

iv) Spraying with freshly prepared Dragendorff's reagent.

In each of the plates, the R_f for the spots were recorded.

2.7.2 Column Chromatography

2.7.2.1 Sample Preparation

The alkaloidal extract (AE) (100 mg) was applied over a well-packed silica gel column. After introduction of sample into the column, it was eluted gradiently with petroleum ether, then the polarity was gradually increased using chloroform, then methanol as indicated in the Table 2.1.

Eluents were collected in 10 ml aliquots, and the progress of the separation was monitored by thin-layer chromatography, and similar fractions were pooled.

Table 2.1: Column Chromatography of Alkaloidal Extract.

Eluent No.	Fraction No.	Eluting solvent	No. of Spots		Remarks
1	1 - 3	Pet. Ether	-		
2	4 - 6	Pet. Ether: $CHCl_3$ (9:1)	-		
3	7 - 9	8 : 2	-		
4	10 - 12	7 : 3	-		
5	13 - 15	6 : 4	-		
6	16 - 18	5 : 5	-		
7	19 - 21	4 : 6	-		
8	22 - 24	3 : 7	-		
9	25 - 31	2 : 8	-		
10	32 - 37	1 : 9	1	Major	amorphous
12	38 - 43	$CHCl_3$	1	Major	"
13	44 - 48	$CHCl_3$; MEOH 9: 1	2		
14	49 - 51	8 : 2	2		
15	52 - 54	7 : 3	2		
16	55 - 57	6 : 4	-		
17	58 - 60	5 : 5	-		
18	61 - 63	4 : 6	-		
19	64 - 66	3 : 7	-		
20	67 - 70	1 : 9	-		
21	71 - 80	Methanol	-		

Fractions (32 - 37) gave a single major spot on TLC and was coded F_1.

Fractions (38 - 43) showed a single spot and was coded F_2. F_1 was subjected to

TLC using the solvent system chloroform: methanol (2:1) and (3:1) respectively. Similarly F_2 was subjected to TLC using the above solvent systems.

F_1 and F_2 were co-chromatograhped using the solvent system chloroform: methanol (2 : 1) and (3 :1) respectively, and the spots were visualized using one (i) UV light (ii) Iodine (iii) Dragendorff's spray reagent. The two fractions F_1 and F_2 were found to be similar and were therefore combined and coded F_3. This on evaporation afforded a faint yellow semi-solid. It was re-dissolved in chloroform several times and finally on evaporation afforded a white amorphous solid weighing 11 mg and melting point 212 - 214 °C., F_3 was found to be soluble in chloroform and acetone, slightly soluble in methanol but insoluble in benzene. F_3 was subjected to: (I) UV in methanol, (ii) I.R. (neat and in Nujol). F_3 was subjected to chemical tests and mixed melting point using pure sample of rhynchophylline.

CHAPTER THREE

3.0 RESULTS

3.1 DESCRIPTION OF THE EXTRACTS

(I) Petroleum ether extract (PE) yielded a light yellow oily material weighing 2.10 g.

(II) Methanolic extract (ME) yielded a dark brown gummy material weighing 28.185 g.

(III) Alkaloidal extract (AE) yielded a light or pale yellow material weighing 0.1g.

3.2 PHYTOCHEMICAL SCREENING

The petroleum ether extract was found to contain steroids and terpenes while the methanolic extract was found to contain carbohydrates, sugars, alkaloids, tanins, saponins, flavonoids, and cardenolides (see Table 3.1).

Table 3.1: Chemical Constituents of *Mitragyna inermis*

Group Constituent	Test	Observation	PE	ME
Carbohydrate				
General test	(i) Molisch's	Dull violet colour at the interface.	-	++
	(ii) Barfoed's	Red precipitate formed	-	+
Reducing sugar	Fehling's test	Red precipitate formed	-	+++
Combined reducing sugar		Reddish-brown precipitate formed	-	+++
Alkaloids	Dragendorff's	Reddish-brown colour/ppt	-	+++
	Wagner's reagent	reddish-brown	-	+++
	Mayer's reagent	White precipitate	-	+++
Tannins	Ferric chloride test	Blue black	-	++
Phlobatanins	Extract + 5ml of 1% dilute HCl	Red precipitate formed	-	-
Anthraquinones	(i) Free	No colour change	-	-
	(ii) Combined	No colour change in		

		Benzene layer	-	-
Steroidal nucleus	(i) Salkowski test	Reddish-brown colour at the interface	+++	++
	(ii) Liebermann-Buchard's test	Violet to blue to bluish-green	+++	-
Cardenollide aglycone test	(i) Legal test	Deep red colour formed	-	++
	(i) Keller-killani test	reddish-brown ring at the interphase	+	++
Saponins	Frothing test	frothing which persists on warming	-	+++
Flavonoids	(i) ferric chloride test	Green colour	-	++
	(ii) Shinoda's test	A pink coloured precipitate	-	+++
	(iii) Lead acetate test	A buff coloured precipitate	-	++
	(iv) NaOH test	Change in colour from yellow to colourless on addition of dilute HCl	-	+++

Key: - = absent; + = faintly present; ++ = moderately present; +++ = highly present.

Table 3.2: **Alkaloid Test for (A.E) Alkaloidal Extract**

Group Constituent	Test	Observation	INFERENCE (A.E)
Alkaloids	Dragendorff's	Reddish-brown colour/ppt	+++
	Wagner's	Reddish-brown precipitate	
		White precipitate	+++
	Mayer's		+++

3.3 **Thin layer chromatography of P.E.**

3.3.1 Thin layer Chromatography of P1

Solvent system: (I) Benzene: Acetone (4:1)

(ii) Benzene: methanol (4:1)

Visualization: Antimony trichloride sprayed and heated at 110 °C for 5 minutes. The colour of the spots and R_f values are as recorded in Tables 3.3 and 3.4.

Table 3.3 TLC of P1

Solvent system: Benzene: acetone (4:1)

Spot	Colour with antimony trichloride	R_f-value
1	Blue	0.09
2	Light yellow	0.25
3	Pink	0.53

Table 3.4: TLC of P_1

Solvent system: Benzene: methanol (4:1)

Spot	Colour with antimony trichloride	R_f-value
1	Brown	0.38
2	Blue	0.53
3	Light yellow	0.69
4	Pink	0.73

3.3.2 Thin-layer Chromatography of Alkaloidal extract (AE)

Solvent system: Chloroform: methanol (3:1)

Visualizing reagent: Dragendorff's spray.

Table 3.5: **Thin-Layer chromatography of alkaloidal extract (AE)**

Number of spot	Colour in UV	Colour with iodine	Colour with Dragendorff's reagent	R_f-value
1	Fluoresce	Faint-yellow	-	
2	-	Yellow	-	0.67
3	-	yellow	Orange colour	0.85

3.3.3 Thin-layer Chromatography of F_1(Fractions 32 - 37) and F_2 (Fractions 38 - 43)

Solvent system: Chloroform: methanol (2:1)

Chloroform: methanol (3:1)

Visualization: Iodine and Dragendorff's reagent.

Table 3.6: TLC Properties of F_1 & F_2.

Fraction	Colour in UV	Colour with iodine	Colour with Dragendorff's reagent	RF_1	RF_2
F_1	-	yellow	Orange	0.94	0.85
F_2	-	yellow	Orange colour	0.94	0.85

where RF_1 and RF_2 are the R_f values using the solvent system: chloroform: methanol (2:1) and (3:1) respectively.

3.3.4 **CO-TLC of F_1 and F_2**

Co-Thin-layer chromatography was run for fractions F_1 and F_2 using the solvent system: Chloroform:methanol (2:1) and (3:1) respectively. Visualization was done using iodine and Dragendorff's spray reagent, the result is summarized in Table 3.7.

Table 3.7: Co-TLC of F_1 and F_2

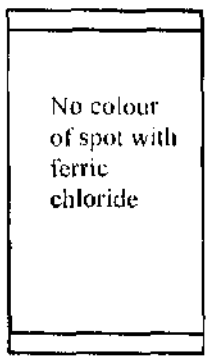
Fraction	No of spots	Colour in UV	Colour with iodine	Colour with Dragendorff's reagent	RF_1	RF_2
F_1	1	No colour	yellow	Orange	0.94	0.85
F_2	1	No colour	yellow	Orange colour	0.94	0.85

F_1 and F_2 were pooled together, evaporated at reduced pressure to yield a faint yellow semi-solid which was purified repeatedly in methanol and on evaporation at reduced pressure an amorphus powder coded F_3.

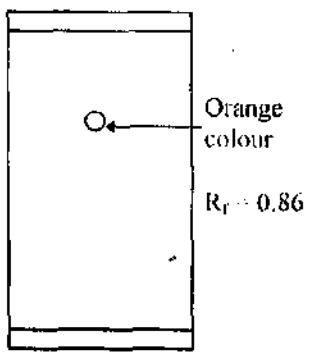
3.3.5. Thin-layer chromatography of F_3

Solvent system: Chloroform: methanol (3:1)

When sprayed with 10% ferric chloride



This was repeated but sprayed with Dragendorff's reagent



3.4 Chemical Test for F_3

Table 3.8

Reagent/Test	Observation	Inference
(1) Dragendorff's	Reddish brown	Alkaloid confirmed
(2) Mayer's	White precipitate	Alkaloid confirmed

3.5 Gas Chromatography and Mass Spectrometry (GC-MS) of (M-P2)

The methylated saponifiable matter (M-P2) was subjected to Gas chromatography and mass spectrometry (GC-MS). The Gas chromatography revealed the presence of seven well separated peaks on the chromatogram (coded peaks 1 - 7) , and minor peaks (which were not considered). Peak 5 with a retention time of 23.858 minutes have the highest peak height and area, thus indicating that it has the highest content (relative abundance) in the saponifiable portion of the petroleum ether extract, followed by peak 4 with retention time of 22.427 minutes, peak 2, 18.16 minutes, peak 7, 38.506 minutes, peak 6, 27.754 minutes and peak 1, 17.535 minutes. (See Fig. 3.1 and Fig. 3.2).

```
*** CLASS-5000 ***  Report No. -    1 Data : _DEFAULT.D56  99/06/28 11:43:55
Sample              : Methylated sample
ID                  : F-4
Sample Amount       : 1
Dilution Factor     : 1
Type                : Unknown
Operator            : Yemisi
Method File Name    : SAMPSON.MET
Vial No.            : 1
Barcode             :
```

Fig. 3.1

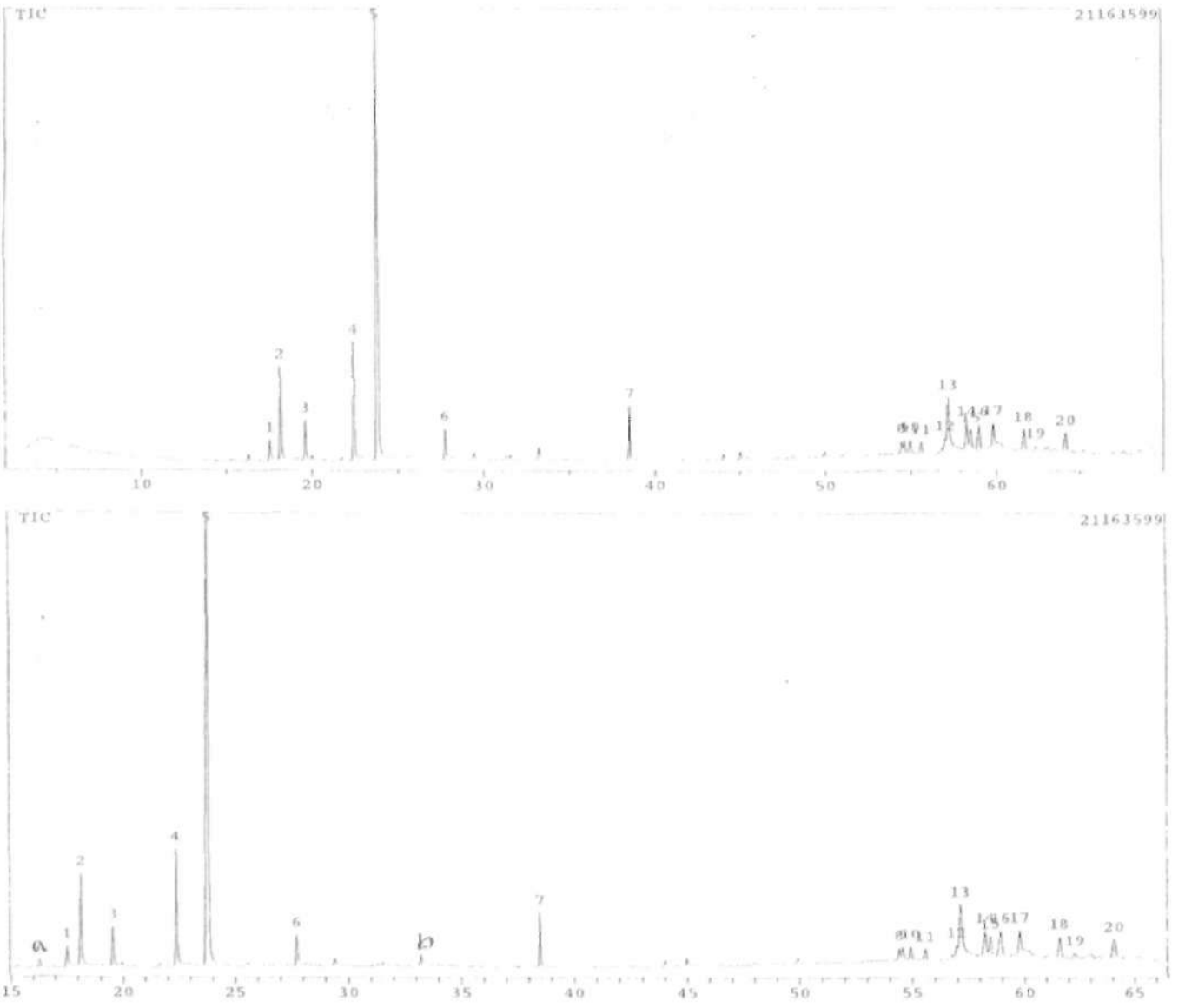


Fig 3.1

*** CLASS-5000 *** Report No. = 1 Data : _DEFAULT.D56 99/06/28 11:43:55
Sample : Methylated sample
ID : F-4
Sample Amount : 1
Dilution Factor : 1
Type : Unknown
Operator : Yemini
Method File Name : SAMPSON.MET
Vial No. : 1
Barcode :

**** Peak Report ****

PKNO	R.Time	I.Time - F.Time	Area	Height	A/H(sec)	MK	%Total	Name
1	17.535	17.442 - 17.775	6388179	1018259	6.274	V	1.59	
2	18.160	18.017 - 19.458	27880264	4392231	6.348	SV	6.96	
3	19.587	19.458 - 19.900	10628010	1926726	5.516	V	2.65	
4	22.427	22.292 - 23.650	32334812	5541909	5.835	SV	8.07	
5	23.858	23.650 - 38.367	165195551	20802958	7.941	SV	41.22	
6	27.754	27.592 - 28.050	7878228	1444153	5.455	TV	1.97	
7	38.506	38.367 - 43.967	7739597	2508948	3.085	S	1.93	
8	54.459	54.358 - 54.542	4443699	710670	6.253	V	1.11	
9	54.620	54.542 - 54.775	5462263	767161	7.120	V	1.36	
10	54.950	54.842 - 55.142	6192413	773664	8.004	V	1.55	
11	55.591	55.458 - 55.825	6040071	675716	8.939	V	1.51	
12	56.994	56.875 - 57.050	5920199	821701	7.205	V	1.48	
13	57.169	57.050 - 57.433	28158218	2729463	10.316	V	7.03	
14	58.255	58.050 - 58.367	15743896	1477411	10.656	V	3.93	
15	58.496	58.367 - 58.650	11791104	1204098	9.792	V	2.94	
16	58.966	58.775 - 59.125	15958941	1453722	10.978	V	3.98	
17	59.812	59.658 - 60.025	17004275	1462376	11.628	V	4.24	
18	61.616	61.433 - 61.775	11355035	1173838	9.673	V	2.83	
19	62.299	62.183 - 62.508	4644053	392922	11.819	V	1.16	
20	64.101	63.925 - 64.292	9982873	971981	10.271	V	2.49	
Total			400741880				100.00	

Fig. 3.2

Fig. 3.2

Fragmentation pattern of each of the separated peaks subjected to computer library search in the spectrometer and three compounds were suggested by the computer search.

Fig. 3.3 shows the fragmentation pattern of peak 2 with the following ions m/z 129, 111, 97, 87, 69, 55 and 43 (base peak). Computer search suggested three compounds: isopropyl-6-oxo-heptanoic acid ($C_{10}H_{18}O_3$), sec octyl ester ($C_{10}H_{20}O_2$) and 2-methylheptyl acetate ($C_{10}H_{20}O_2$).

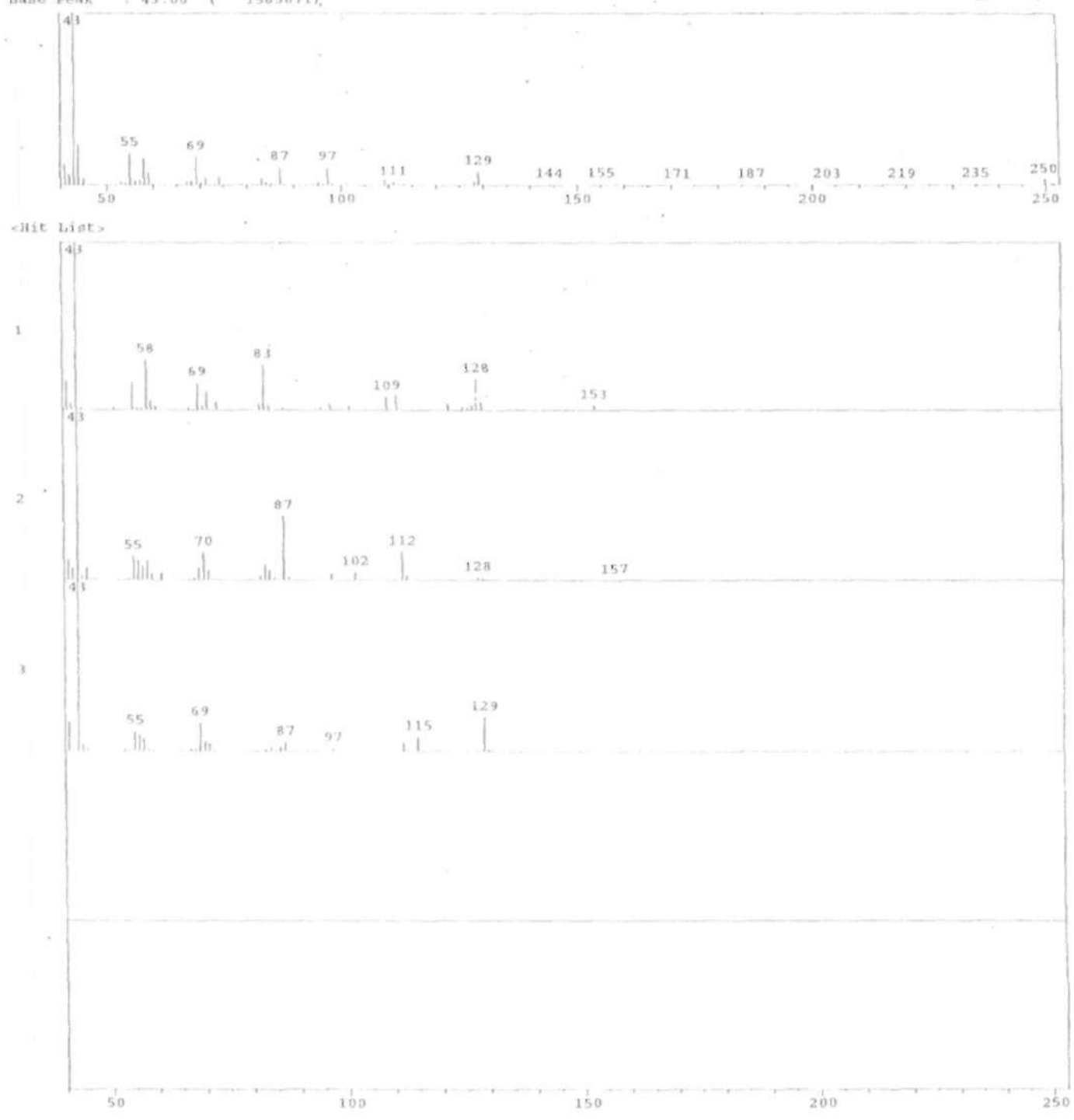
Fig. 3.4 shows the fragmentation pattern of peak 4 with the following ions: m/z 169, 125, 111, 97, 83, 69, 55 and 43 (base peak). Computer search suggested the following compounds: methyl-3-isopropyl-6-oxo heptanoate ($C_{11}H_{20}O_3$), 7-methyl-4-octyl acetate ($C_{11}H_{22}O_2$) and 9-oxo decanoic acid methyl ester ($C_{11}H_{20}O_3$).

Fig. 3.5 shows the separated peak five subjected to mass spectrometry, and the following ions were identified: m/z 185, 125, 111, 97, 83, 74, 55 (Base peak) and 43. Computer search suggest the following compounds: Nonanedioc acid monomethyl ester ($C_{10}H_{18}O_4$), nonanedioc acid dimethyl ester ($C_{11}H_{20}O_4$) and nonanoic acid-9-oxo-methyl ester ($C_{10}H_{18}O_3$).

Fig 3.6 showed the fragmentation pattern of peak 7. The mass spectrum showed the following ions and their m/z value: 227, 199, 143, 87, 74 (base peak) 55, and 43. Computer library search suggested three compounds namely: Decanoic acid methyl ester (capric acid) $C_{11}H_{22}O_2$, tetradecanoic acid methyl ester (myristic acid methyl ester), and dodecanoic acid methyl ester (lauric acid) $C_{13}H_{26}O_2$.

LIBRARY

Fig 3.3



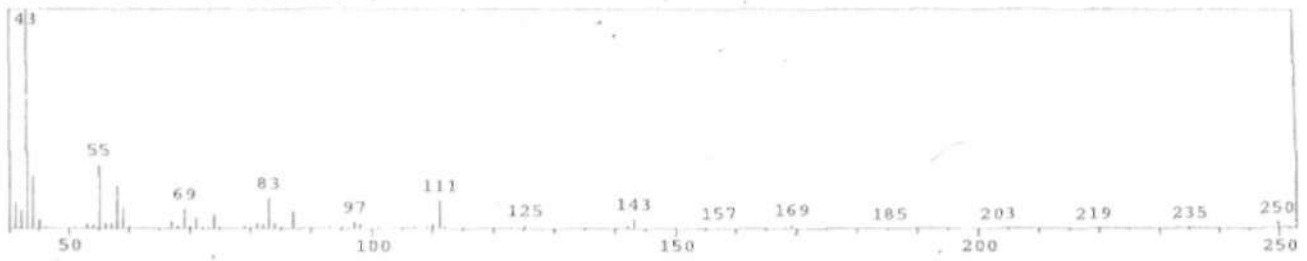
No	SI	Mol.Wgt.	Mol.Form./Compound Name	CAS No.	Entry	LIB#
1	83	186	C10H18O3	- -0	19376	1
			R-(+)-3-Isopropyl-6-oxoheptanoic acid			
2	82	172	C10H20O2	54515-77-4	15771	1
			Acetic acid, sec-octyl ester $$ sec-Octyl Acetate $$ 2-Methylheptyl acetate			
3	82	172	C10H20O2	- -0	15758	1
			2-Methyl-2-heptanol acetate			

Library Name
(1) NIST62.LIB

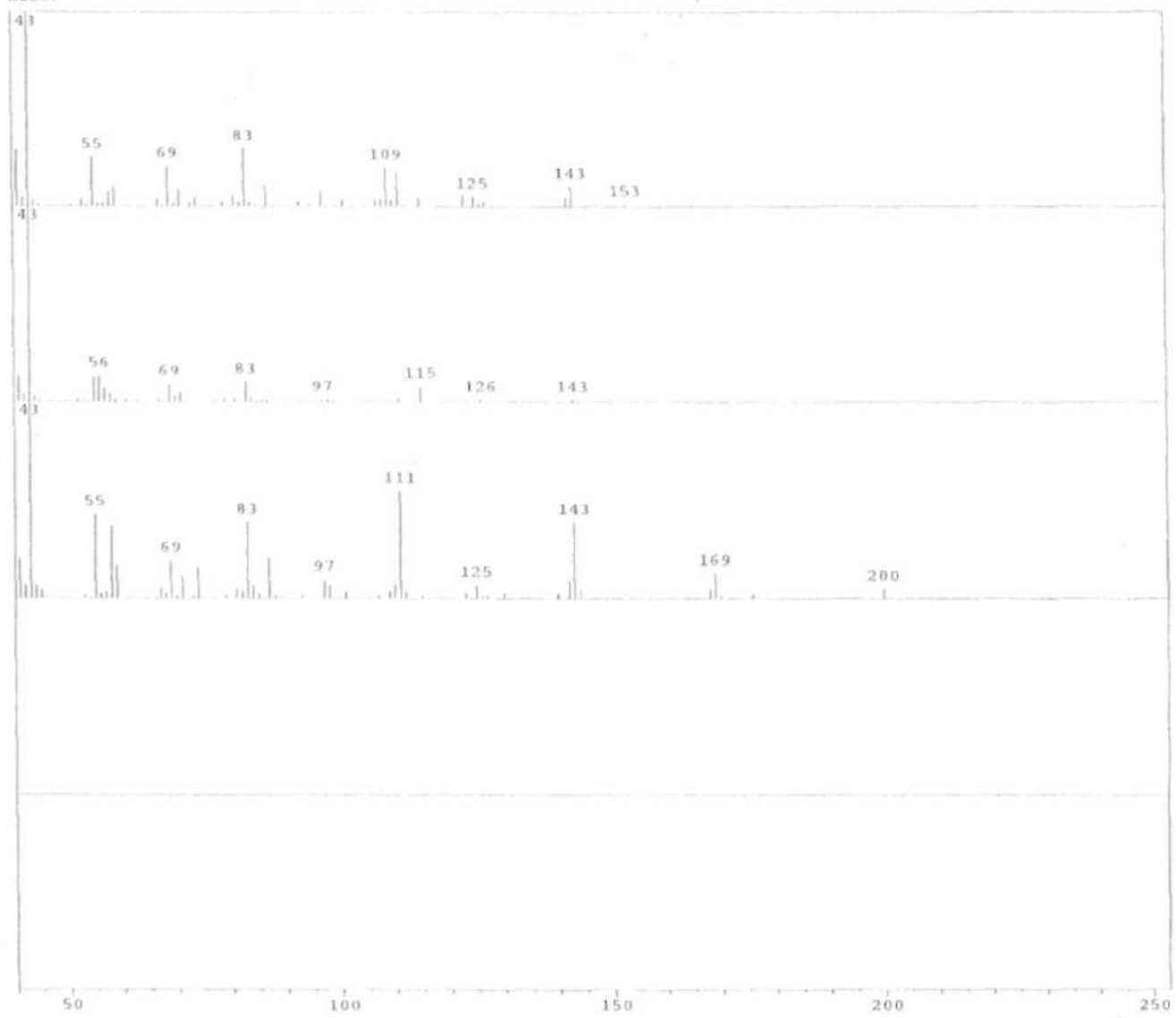
Fig. 3.3

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Data : _DEFAULT.D56
Mass Peak # : 98 Ret. Time : 22.431
Scan # : 2453
Base Peak : 43.05 (1775114)

Fig. 3.4



<Hit List>



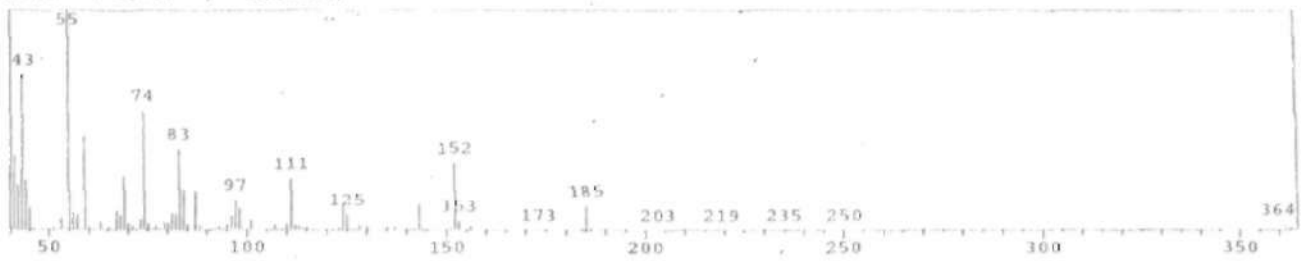
No	SI	Mol.Wgt.	Mol.Form./Compound Name	CAS No.	Entry	LIB#
1	86	200	C11H20O3	- -0	22894	1
			R-(+)-Methyl-3-isopropyl-6-oxoheptanoate			
2	83	186	C11H22O2	33933-81-2	19472	1
			4-Octanol, 7-methyl-, acetate $$ 7-Methyl-4-octyl acetate			
3	82	200	C11H20O3	2575-07-7	22901	1
			Decanoic acid, 9-oxo-, methyl ester			

Library Name
(1) NIST62.LIB

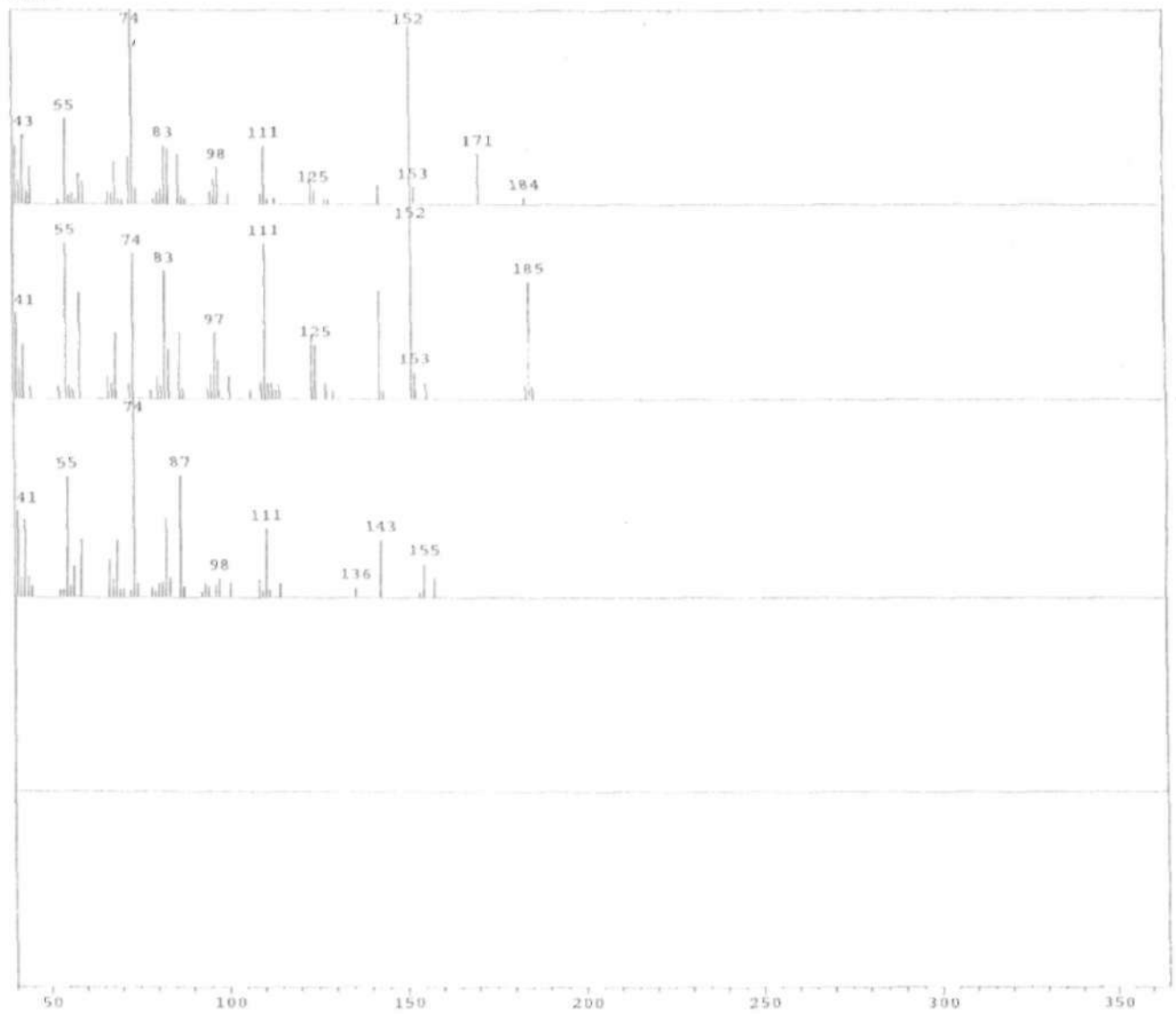
Fig. 3.4

Fig 3.5

<Unknown Spectrum>
Data : DEFAULT.D56
Mass Peak # : 125 Ret. Time : 23.858
Scan # : 2624
Base Peak : 55.00 (2908401)



<Hit List>



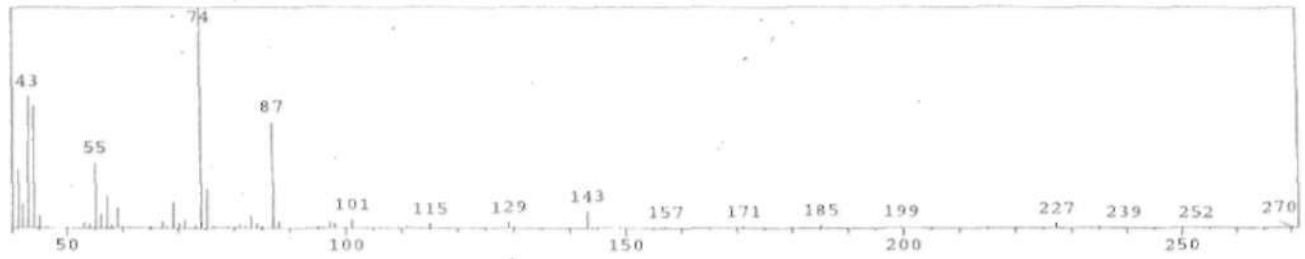
No	SI	Mol.Wgt.	Mol.Form./Compound Name	CAS No.	Entry	LIB#
1	82	202	C10H18O4	2104-19-0	23284	1
			Nonanedioic acid, monomethyl ester $$ Azelaic acid, monomethyl ester $$ Methyl hydrog			
2	80	216	C11H20O4	1732-10-1	26670	1
			Nonanedioic acid, dimethyl ester $$ Azelaic acid, dimethyl ester $$ Dimethyl azelate			
3	78	186	C10H18O3	1911-63-1	19385	1
			Nonanoic acid, 9-oxo-, methyl ester $$ Azelaaldehydic acid, methyl ester $$ Methyl az			

Library Name
(1) NIST62.LIB

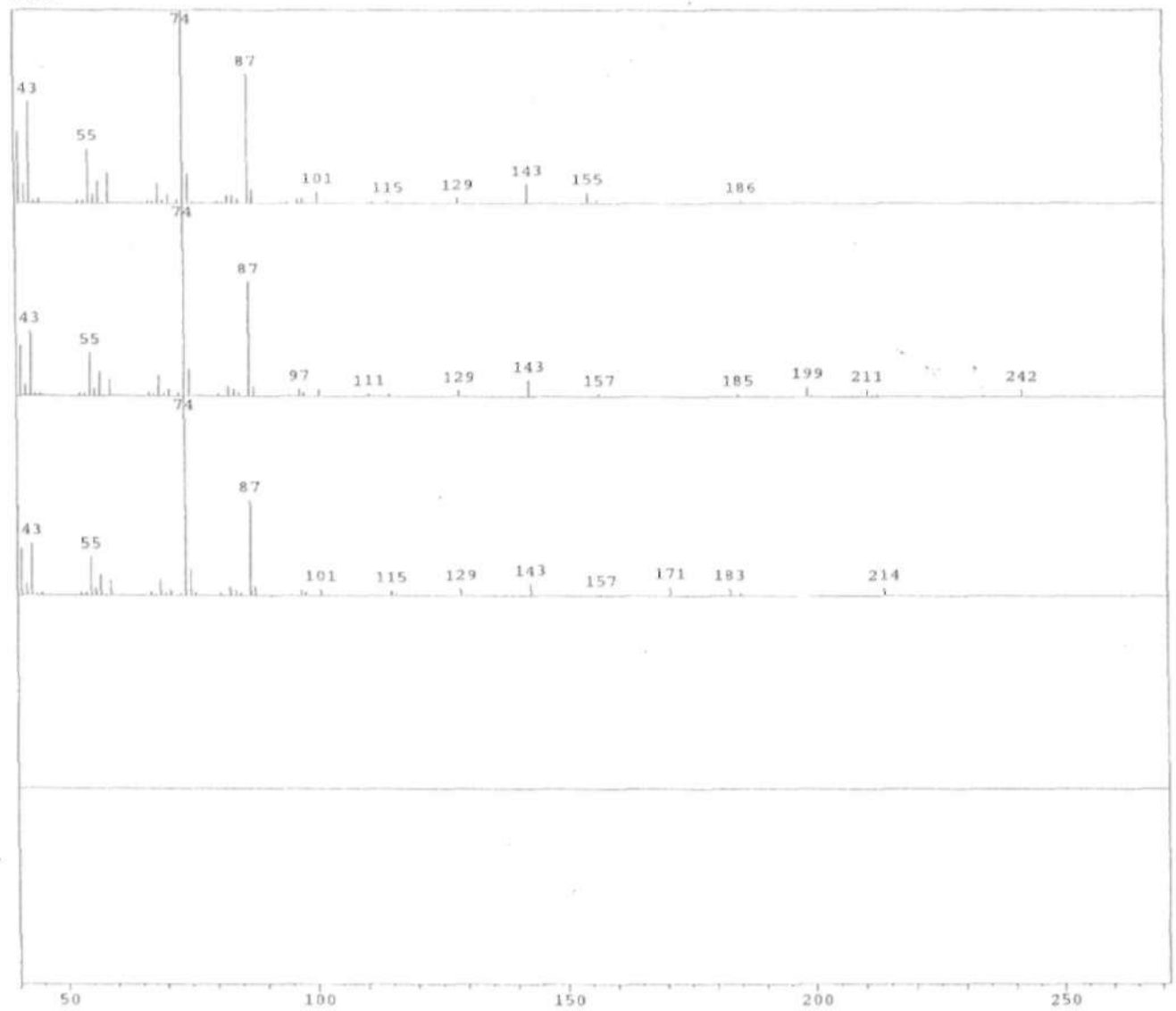
Fig. 3.5

Fig. 3.6.

<Unknown Spectrum>
Data : _DEFAULT.D56
Mass Peak # : 80 Ret. Time : 38.508
Scan # : 4382
Base Peak : 74.10 (603673)



<Hit List>



No	SI	Mol.Wgt.	Mol.Form./Compound Name	CAS No.	Entry	LIB#
1	90	186	C11H22O2	110-42-9	19451	1
			Decanoic acid, methyl ester $$ Capric acid methyl ester $$ Metholene 2095 $$ Methyl c			
2	89	242	C15H30O2	124-10-7	12399	1
			Tetradecanoic acid, methyl ester $$ Myristic acid, methyl ester $$ Metholeneat 2495 $			
3	88	214	C13H26O2	111-82-0	26365	1
			Dodecanoic acid, methyl ester $$ Lauric acid, methyl ester $$ Metholene 2296 $$ Methy			

Library Name
(1) NIST62.LIB

Fig. 3.6

3.7 Spectral properties of F_3

F_3 was subjected to the following spectral analysis.

(1) UV spectrum (see Fig. 3.7)

λ_{max} (methanol): 210 nm, 230 nm and 280 nm. λ_{min} 225 nm and 265 nm.

(2) I.R. spectrum (in cm^{-1}) neat (fig. 3.8)

3443.32 (w), 3149.9 (s), 2957.25 (s), 2855.65 (s), 1730.83 (s), 1433.89 (s), 1384.59 (s), 1273.86, 1121.86, 1121.90, 1072.83, 964.92, 825.53 and 742.55.

(3) I.R. spectrum (in cm^{-1}) in nujol (Fig. 3.9).

3434.25 (w), 3151.5 (s), 2954, 2854.46 (s), 1738.58 (w), 1598.11 (w), 1453.52, 1384.16, 1197.26, 1113.75 and 1034.37.

The alkaloid is identical from Co-TLC with Rhynchophylline (XXIX), and there was no depression of melting point when mixed with authentic sample of rhynchophylline.

ND Catalogue Number 648657

FIG 3.7
UV SPECTRUM OF
F3 in METHANOL
14-10-98

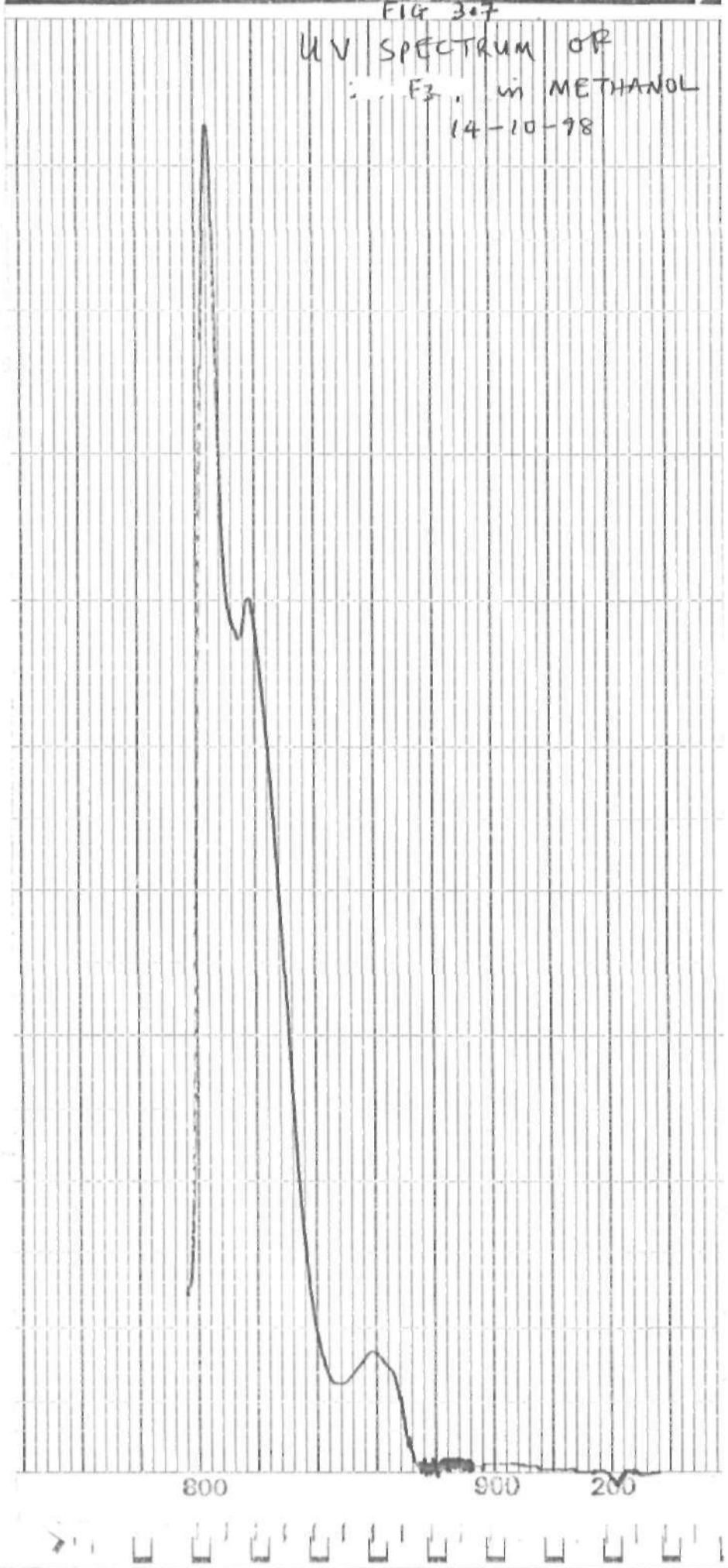


Fig. 3.8



Peak Report

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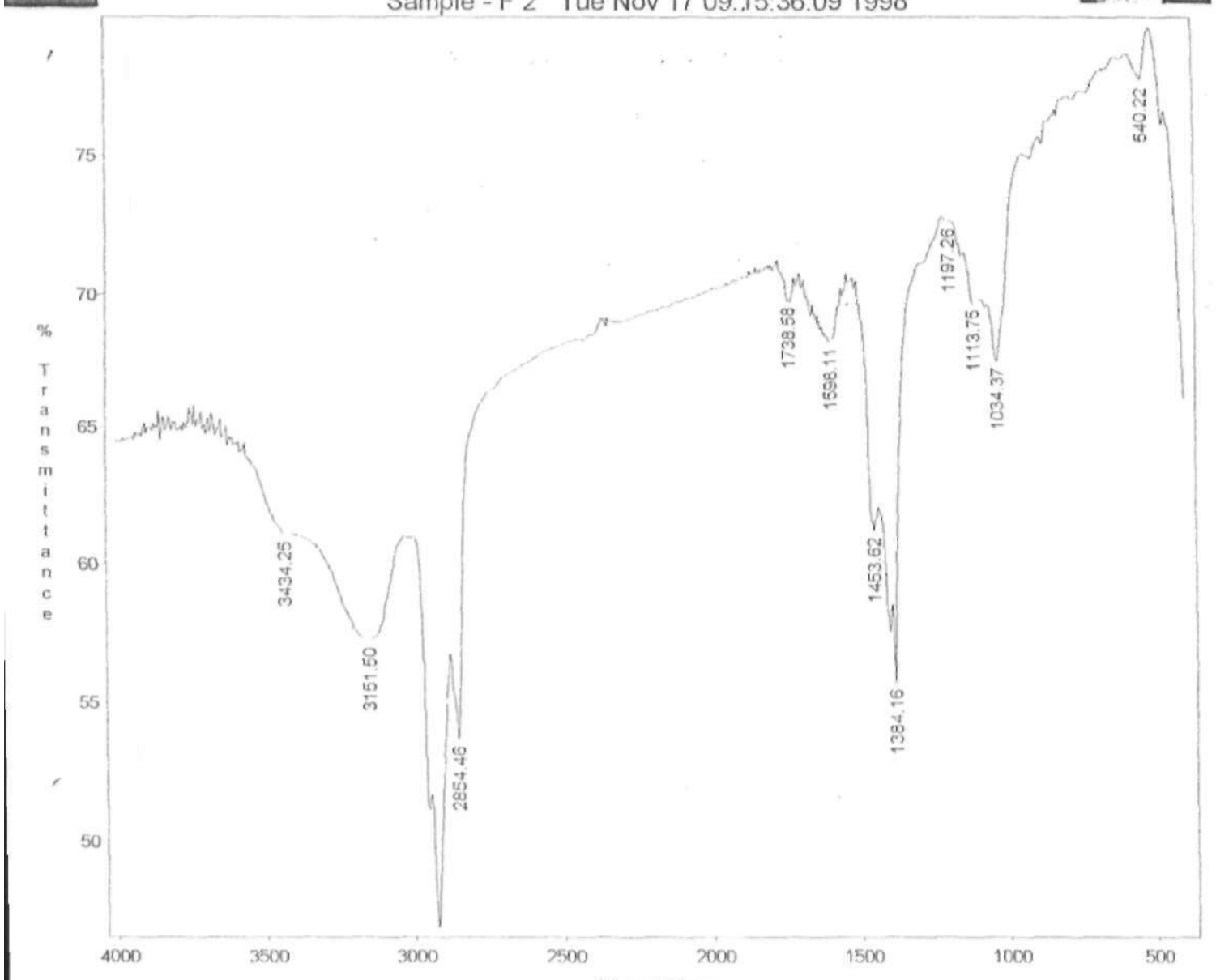
Title: Sample F-2 Thur.Oct 22 12:40:08:05 1998

Filter: Three Point Center of Gravity

cm-1	%T	cm-1	%T	cm-1	%T
3443.32	41.60	3149.90	36.70	2957.25	31.15
2925.63	28.27	2855.65	36.20	1730.83	42.17
1433.89	45.25	1384.59	34.72	1273.86	46.10
1121.90	50.94	1072.83	51.58	1039.78	55.97
964.92	61.12	825.53	63.94	742.55	63.42

Fig. 3.8

Fig. 3.9



Peak Report
File: c:\first\data\galaxy\lmp186.ras
Title: Sample - F 2 Tue Nov 17 09:15:36.09 1998
Filter: Three Point Center of Gravity

cm-1	%T	cm-1	%T	cm-1	%T
3151.50	57.22	3434.25	61.05	2854.46	53.66
1738.58	69.72	1598.11	68.19	1453.62	61.20
1384.16	55.68	1197.26	72.60	1113.75	69.62
1034.37	67.43	540.22	77.75		

Fig. 3.9

CHAPTER FOUR

4.0 DISCUSSION

Preliminary phytochemical screening of the bark of *Mitragyna inermis* revealed the presence of steroids and terpenes, alkaloids, tannins, carbohydrates, cardenolides, saponins and flavonoids.

Saponification of the petroleum ether extract gave 0.84 g or 93.97% as the unsaponifiable matter, (P_1), while the saponifiable portion (P_2) gave 84.5 mg or 6.03% of the constituent in the petroleum ether extract, indicating that the unsaponifiable compounds which include steriods and terpenes are more than the saponifiable compounds - usually fatty acids. Methylation of P_2 and subsequent separation of the constituents via gas chromatography showed seven major peaks, each with its retention time, with peak 5 being the most abundant compound present based on its peak height and area. (Fig 3.1 and 3.2).

Mass spectra of the separated peaks and computer based library search for each peak suggested three compounds with similar fragmentation pattern as the parent compound. Peak two with retention time 18.167 mins., has a base peak of m/z 43 (100%) in its mass spectra, which is due to the cation $CH_3CH_2CH_2^+$. (1) Other peaks in the mass spectra which correspond to 2-methyl octanoic acid methyl ester m/z 172 (Fig. 3.3) showed m/z at 129, 97, 87, 69 and 55. M/z 129, is as a result of loss of propyl group from the parent compound. M/z 97 is the fragment obtained as a result of loss of acetoxy group and branched methyl group and subsquent loss of H' to give (3) of M/z = 97. This in turn loses

an alkene (CH_2=CH_2/28) to give the radical cation $^+C_5H_9$ of m/z = 69 (4) (Appendix 1.1). m/z = 87 is due to the cleavage of C-C bond next to the branched carbon to give the fragment (5). M/z 55 is the cation $^+C_4H_7$ formed from the loss of acetoxy group: CH_3COO/59, propyl group: C_3H_7/43 and branched methyl group CH_3/15 from the parent compound.

Peak four showed fragmentation pattern similar to that of 9-oxo decanoic acid methyl ester (Fig. 3.4). Peak of m/z 43 is due to the cation CH_3CO^+/43. M/z = 169, is the ion formed as a result of loss of methoxy group -OCH_3/31 from the parent compound thereby giving the m/z value of 200 which is not stable in the mass spectra. M/z 143 is due to loss of fragment CH_3COCH_2/57, thereby giving the cation (3). Maclafferty rearrangement gives the fragment of m/z 126, which loses a methyl radical CH_3/15 to give the fragment (6): of m/z = 111, which in turn loses the carbonyl group CO/28 to give the cation (7): of m/z 83. This in turn loses an alkene CH_2=CH_2/28 to give the cation $^+CH_2$-CH_2-CH=CH_2 of m/z 55 (Appendix 1.2). 97 is a result of cleavage of bonds next to acetoxy group, and acetyl group leaving the fragment of mass unit 98 (8) which loses a hydrogen ion to give a weak peak observed at m/z 97 10). This in turn eliminate a molecule of alkene (CH_2=CH_2/28) to give a stronger peak observed at m/z = 69 (11).

Peak five, the most abundant peak has a base peak of m/z = 55 (100%) in the mass spectrum. Other peaks in the mass spectra corresponding to azeliac acid ester showed m/z at 185, 125. 97, 83, 74, and 43. M/z of 185 is due to the loss of methoxy group, OCH_3/31, thereby giving a molecular ion of m/z 216 which is similar to that of Azelaic acid dimethyl ester (see Fig. 3.5), m/z 74 which

is due to Maclafferty rearrangement which occurs as a result of β - cleavage and transfer of γ-hydrogen to the carbonic oxygen(2). (appendix 1.3) , and is one of the diagnostic ions in the mass spectra of long chain methyl esters of fatty acids. (Silverstein, *et. al.*, 1983), (Kemp, 1991). m/z (125) results from loss of acetoxy group (CH3C00/59) and methoxy group (OCH_3/31) leaving the fragment(3): which loses a hydrogen radical ($H^{\bullet}$) to give the carbonyl cation(4) of m/z = 125. This on further loss of the carbonyl group (-CO/28) gave a more stable ion at m/z 97 $(CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH{=}CH_2)^+$ which has higher abundance than m/z = 125 (see Fig. 3.5). M/z 111, resulted from the loss of m/z 74, and methoxy group (M^+ - 74 - 31), which on loss of the carbonyl (CO/28), gave a more stable peak m/z 83 (7). This in turn loses a neutral molecule, (ethene ($CH_2{=}CH_2$/28) to give a very stable ion of m/z = 55, which is the base peak(8). The fragment of m/z = 43, resulted from the loss of propyl radical C_3H_7 from the fragment (M^+ - 59).

Mass spectrum of peak seven showed similar fragmentation pattern with that of myristic acid methyl ester (Fig 3.6). and is one of the three compounds from the computer library search.

The base peak at m/z = 74 (2), resulted from the Maclarfferty rearrangement which again is one of the characteristic ions obtained in mass spectra of long chain fatty acid methyl esters. (Kemp, 1991). The base peak of myristic acid ester is also m/z 74. Other diagnostic ions are m/z 227 which is as a result of loss of a methyl group from molecular ion, thereby giving a molecular ion of 242 which is that of methyl myristate. (Fig 3.6). M/z at 199, 143, and 87,

are characteristic fragments of methyl myristate. (Budzikiewicz *et al.*, 1967). Where m/z 199 is as a result of transfer of hydrogen radical ($H^{\bullet}$) from carbon 10 (C -10) of the carbon skeleton to the carbonic oxygen and subsequent fission of C_{11} and C_{12} bond to give a stable ion (4) of m/z 199. M/z 143 is as a result of transfer of hydrogen radical ($H^{\bullet}$) from carbon six (C-6) to the carbonic oxygen and fission of the C_7 - C_8 bond to give the ion (6) of m/z = 143, while m/z 87 resulted from the transfer of a hydrogen radical ($H^{\bullet}$) from carbon six to carbonic oxygen and similar shift of Hydrogen ion ($H^{\bullet}$) from C - 2 to C - 6, and subsequent fission of C_3 - C_4 bon to give the fragment (7) of m/z = 87. Thus the ions m/z 199, 143 and 87 all have a difference of mass unit 56. These are characteristic fragments for methyl ester of formula $[(CH_2)_nCOOCH_3]^+$ where n = 2, 6, 10 etc. (Budzikiewicz *et al*, 1967). M/z 129 is due to migration of hydrogen ion ($H^{\bullet}$) from C - 5 to the carbonic oxygen and fission of C_6 - C_7 bond to give a fragment (9) of m/z 129. Fission of C_2 - C_3 bond gives the radical ion $[CH_2{=}CH\text{-}CH_2\text{-}CH_2]^+$ m/z = 55. M/z 43 is as a result of loss of propyl ion $(C_3H_7)^+$ during fragmentation. Thus the ions m/z 199, 143, 129, 87., 74, 55 and 43 in peak seven correspond to that of myristic acid methyl ester (Fig 3.6 and appendix 1.4).

4.2 SAPARATION AND IDENTIFICATION OF RHYNCHOPHYLLINE (XXIX) FROM ALKALOIDAL EXTRACT (AE)

Thin layer chromatography of AE and its visualization using Dragendorff's spray reagent showed it to contained alkaloidal spots (table 3.5). Column chromatography of AE, and subsequent monitoring of separation by TLC led to

the isolation of F_3 an amorphous solid powder, m.p: 213 - 214 °C. UV spectrum of F_3 in methanol showed λ_{max} at 210 nm, 230 nm and 280 nm. The presence of absorption at 280 nm suggests the presence of aromatic nucleus UV spectrum of rhynchophylline in ethanol showed λ_{max} at 208.3 nm, 243.3 nm and 282 nm Beckitt *et. al.*, (1965).

Infrared spectrum of F_3 neat and in nujol (Fig 3.8 and 3.9) showed stretching frequencies at 3443.32 cm^{-1} and 3434.85 cm^{-1} which is attributed to the free indolic (N - H) stretching. Bindra, (1973) gave the absorption of indolic nitrogen (N - H) stretching in the range of 3500 - 3200 cm^{-1}.

Rhynchophylline has an N - H stretching frequency of 3415 cm^{-1} using KBr disc. Bindra, (1973). Frequencies at 3151 and 3149 cm^{-1} respectively are attributed to bonded (N - H). Frequencies in the range 1750 - 1730 cm^{-1} are characteristic of saturated carbonyl group stretching frequency (C=O str.) Thus frequencies observed for F_3 at 1730 and 1738 cm^{-1} respectively are due to the C=O stretching frequency. This can be confirmed by the frequencies at 1121.9 cm^{-1}, and 1273 cm^{-1} Fig. 3.5), and 1197 cm^{-1} and 113.75 cm^{-1} (Fig. 3.6) which is attributed to C - O stretching frequency which are in the range 1300 - 1100 cm^{-1}. (Kemp, 1991) and is often strong. Frequencies at 2957 cm^{-1} and 2855 cm^{-1} are due to the a symmetric and symmetric (C - H) stretching in alkane. The same frequency range was observed when the sample was run in nujol (Fig 3.9) confirming the presence of alkyl group in F3. Frequency in the range 700 - 850 cm^{-1} is usually an indication of aromatic substitution. Thus frequencies at 742 cm^{-1} and 825 cm^{-1} are due to substitution on the benzene ring. A weak band at

1598.11 cm^{-1} is likely to be the C = C stretching in aromatic nucleus (Fig 3.9), Thus from I. R spectra, the presence of λ_{max} of 280 nm have been confirmed by the frequencies at 742 cm^{-1}, 825 cm^{-1} and 1598.11cm^{-1} in the I. R. spectra of F_3.

F_3 was found to be soluble in chloroform, acelone, slightly soluble in methanol and insoluble in benzene. There was no depression of melting point when mixed melting point was carried out with authentic sample of rhynchophylline.

CONCLUSION

From the phytochemical screening it can be concluded that the bark of *Mitragyna inermis* contain saponifiable compounds which on separation by gas chromatography and mass spectrometry it was found to contain the fatty acids and their derivatives: azelaic acid, myristic acid, 2-methyl octanoic acid and 9-oxo decanoic acid with azelaic acid being the most abundant of saponifiable compounds.

From the alkaloid extract, an alkaloid was isolated, and based on the melting point, mixed melting point, UV and I.R spectra, it was found to be rhynchophylline. Further work needs to be carried out using the pure isolated compounds to further authenticate the basis for the traditional claims of this plant.

HIM IBRAHIM

CHAPTER FIVE

5.0 PHARMACOLOGICAL SCREENING

5.1 Rational for the project

The aim of this work is to assay pharmacologically the effect of the methanolic extract of the bark *M. inermis* on isolated rabbit and guinea pig ileum in order to ascertain the purported uses of the plant in relieving gastro-intestinal disturbances (Dalziel, 1955) which include relieving abdominal colicky pain.

5.2 MATERIALS

5.2.1 Test animals

Adult male rabbit weighing 1.56 kg and Guinea pig 340 g were obtained from the animal house,Faculty of Pharmaceutical Sciences, ABU, Zaria and starved 24 hours before the commencement of the practical work.

5.2.2 Apparatus

(1) Recording microdynanometer and Transducer model No. 7050, (Ugo Basile Italy)

(2) Thermostatically controlled tissue bath (20 ml)

(3) Dissecting kit

5.2.3 Standard Drugs and Sample Used

The standard drugs and crude methanolic extract were prepared by directly weighing and dissolving the known weight of the drug/extract in the

required volume of de-ionized water. Stock concentration prepared were as follow.

(i) Acetyl choline 10 µg/ml

(ii) Atropine sulphate 10 ng/ml

(iii) Histamine 10 µg/ml

(iv) Mepyramine 100 µg/ml

(v) Methanolic extract 100 mg/ml

Physiological solution (See appendix 1.5).

Tyrode solution was used and was freshly prepared.

Isolated tissue Rabbit and guinea pig ileum were used.

5.3 Experimental Methods

The isolated rabbit ileum ,3 cm in length,was placed in a petri dish containing tyrode solution aerated by electric pump. The tissue was transferred to an organ bath and allowed to stabilize for 1 hour after the tissue was washed three time with the physiological solution. The temperature of the bath was maintained at 37 °C. Dose response curve was obtained for Acetyl Choline at bath concentration of 0.025 µg, 0.05 µg, 0.1 µg, 0.2 µg and 0.4 µg, each addition being followed by washing of the tissue three times before the next concentration was added. Dose response was also obtained for the extract from the lowest bath concentration of 0.5 mg to 4 mg each addition followed by washing of the tissue three times before the next concentration is added, after the tissue have rested for 1 minute. Tissue response for acetyl choline at (0.2 µg) was repeated

twice. After washing the tissue, the lowest concentration of the extract (0.5 mg) was incubated in the bath for 30 seconds and challenged with 0.2 μg of acetyl choline, this was repeated with varying concentration of the extract up to a maximum of 4 mg, each being challenged with fixed concentration of acetyl choline (0.2 μg). The effect of atropine at bath concentration 0.025 ng was determined when challenged with 0.2 μg of acetyl choline. The same procedure was repeated using guinea pig ileum, but histamine was used to obtain dose-response contraction before incubating the extract in varying concentrations each concentration being challenged with 0.4 μg of histamine. Lastly mepyramine at concentration 0.25 μg was incubated in the bath and 30 seconds later, 0.4 μg of histamine was introduced. All effects were recorded by the microdynanometer and transducer.

5.4 RESULTS

Table 5.1: **Effect of Ach on Isolated Rabbit Ileum**

Volume (ml)	Response (cm)	Conc. (g/ml)	% Response	Log. Conc.
0.05	3.7	0.025×10^{-6}	69.81	-7.6
0.10	3.9	0.05×10^{-6}	73.50	-7.3
0.20	4.5	0.1×10^{-6}	84.90	-7.0
0.40	4.9	0.2×10^{-6}	92.45	-6.7
0.80	5.3	0.4×10^{-6}	100	-6.4

Table 5.2: **Effect of Increasing Concentration of Methanolic extract on Ach induced contraction**

Volume (ml)	Response (cm)	Conc. (g/ml)	% Response	Log. Conc.
0	4.4	-	75	=
0.1	4.4	0.5×10^{-3}	75	-3.3
0.2	4.2	1.0×10^{-3}	71.58	-3.0
0.4	4.1	2.0×10^{-3}	69.88	-2.7
0.8	4.1	4.0×10^{-3}	69.88	-2.4

Table 5.3 **Effect of Atropine (0.025 ng/ml) on Ach (0.2 μg/ml)**

Volume (ml) of Atropine	Conc. of Atropine (ng/ml)	Volume of Ach (ml)	Conc. Of Ach (μg/ml)	Response (cm)
0.05 ml	0.025	0.4	0.2	-

Table 5.4: **Effect of Histamine on Isolated Guinea Pig ileum**

Volume (ml)	Response (cm)	Conc. (g/ml)	% Response	Log. Conc.
0.1	2.0	0.05×10^{-6}	41.66	-7.3
0.2	3.2	0.1×10^{-6}	66.67	-7.0
0.4	4.2	0.2×10^{-6}	87.5	-6.7
0.8	4.7	0.4×10^{-6}	97.9	-6.4
1.6	4.8	0.8×10^{-6}	100	-6.1

Table 5.5: **Effect of Increasing concentration of methanolic extract on Histamine induced contraction**

Volume (ml)	Response (cm)	Conc. (g/ml)	% Response	Log. Conc.
0.	4.1	-	75	-
0.1	4.1	0.5×10^{-3}	75	-3.3
0.2	4.1	1.0×10^{-3}	75	-3.0
0.4	3.9	2.0×10^{-3}	71.34	-2.7
0.8	3.5	4.0×10^{-3}	64.02	-2.4

Table 5.6: **Effect of Mepyramine (0.25 μg/ml) on Histamine (0.4 μg/ml)**

Volume Mepyramine (ml)	Conc. Mepyramine (g/ml)	Volume of Histamine (ml)	Conc. Of Histamine (g/ml)	% Response (cm)
-	-	0.8	0.4×10^{-6}	4.7
0.05	0.25×10^{-6}		0.4×10^{-6}	0.5

5.5 DISCUSSION

Acetyl choline (Ach) as expected caused a dose-dependent contraction of the isolated rabbit ileum (Fig 5.1A). Similarly a plot of percentage maximum response of acetyl choline against log of concentration, showed an increase in percentage of contraction up to the highest concentration where percentage contraction is 100. (Fig. 5.1). Acetyl choline, an ester of choline, acts on muscarinic receptors present in smooth muscle to bring about contraction via cholinergic transmission, Rang and Dale, (1987). Dale, (1914) noted that various esters of choline elicited responses that were similar to those of either nicotine or

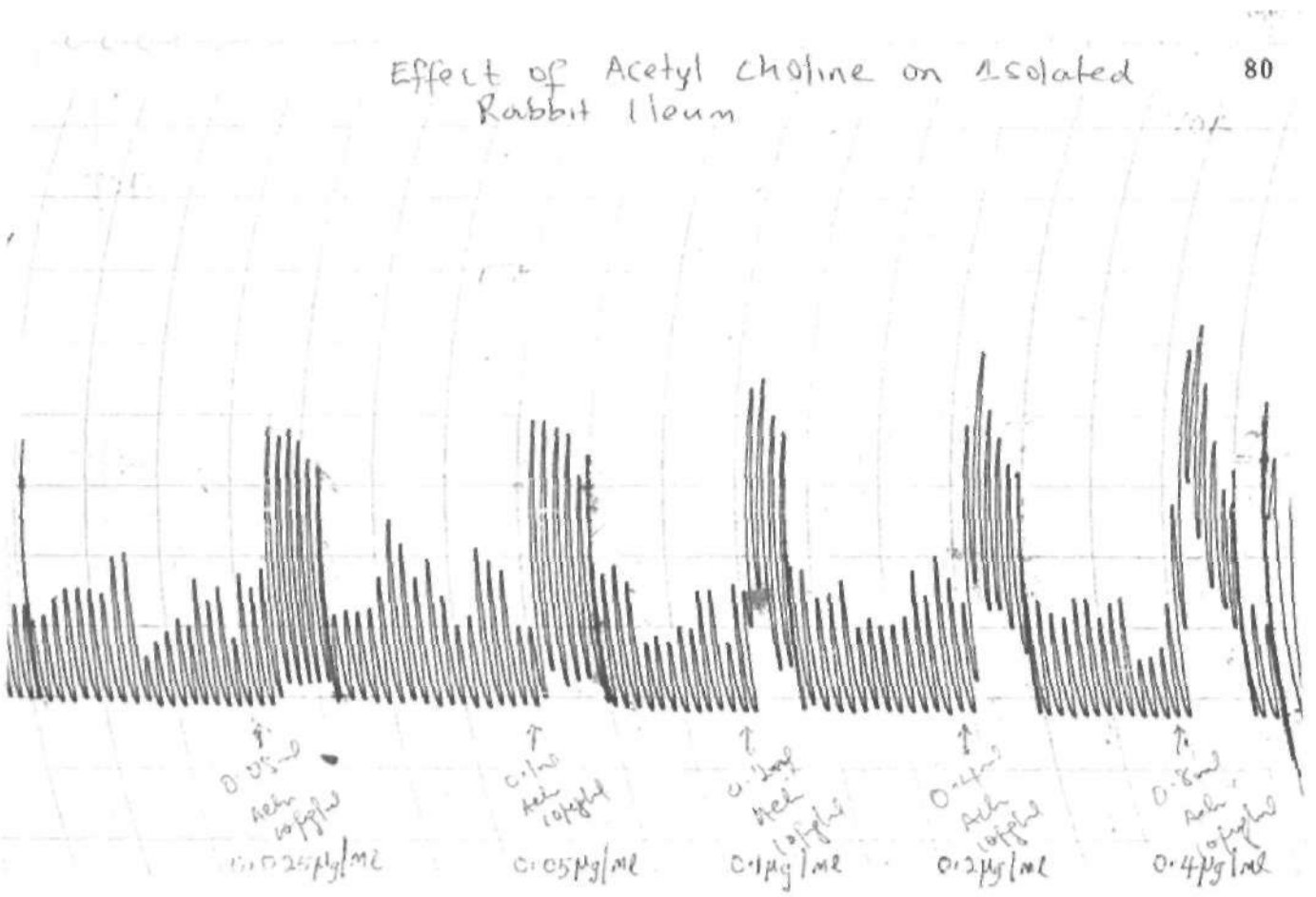


Fig 5.1A

Fig. 5.1A

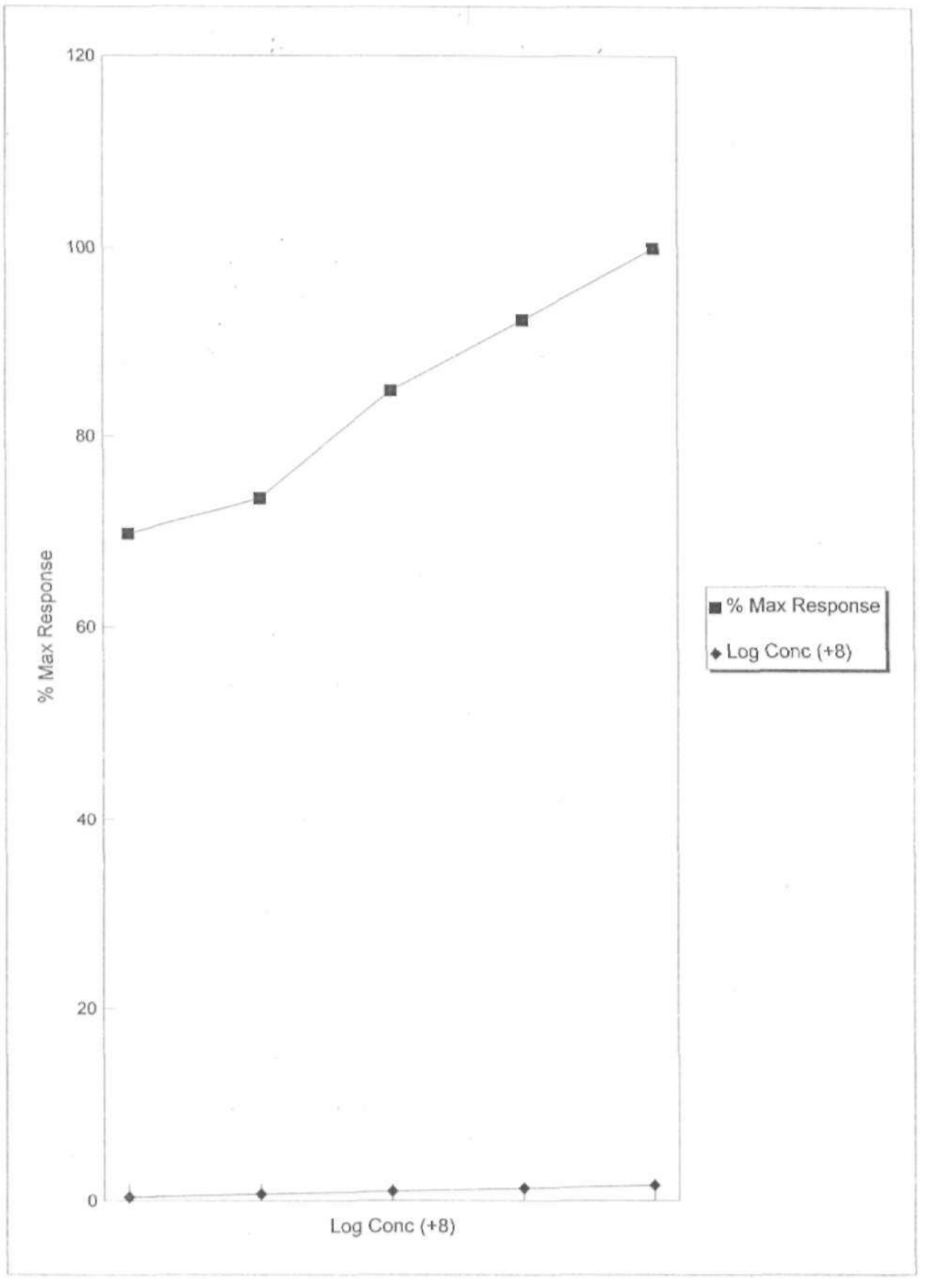


Fig 5.1 Effect of Acetyl Choline on Isolated Rabbit Ileum

muscarine depending on pharmacological preparation and suggested that Ach was a neuro transmitter in the autonomic nervous system and had dual action which he termed nicotine action (nicotinic) and muscarine action (muscarinic). The ability of tubocurarine and atropine to block nicotinic and muscarinic effects of Ach respectively provided support for the type of cholinergic receptors Goodman & Gilman, (1990). Thus the blockade of the response of Ach at concentration of 0.2 μg when incubated with 0.025 ng of atropine confirms that response of Ach on the rabbit ileum is via muscarinic receptors present in the rabbit ileum which is a smooth muscle. The methanolic extract which showed a dose dependant relaxation of the rabbit ileum, (Fig 5.1b) had very little effect on the acetyl choline response when incubated and challenged with Ach, At concentration of 0.5 mg, there was no change in Ach response. At conc. of 1 mg, it showed very little change in the response while at the highest concentration of 4 mg, it did not affect the response of acetyl choline (Fig. 5.2, and table 5.2), thus indicating that the action of the extract on the rabbit ileum in relaxing the smooth muscle of the rabbit ileum in opposition to that of acetyl choline is not atropine like or is not parasympatholytic inclined as compared to atropine which blocked the response of acetyl choline (Table 5.3)

The isolated guinea pig ileum was used to study the effect of histamine in guinea pig ileum because of the high sensitivity of the guinea pig ileum to histamine (Melville, 1973) hence the guinea pig ileum is usually used for initial screening of drugs for antihistaminic activity and involves assessing the activity of the test substance in antagonizing the histamine induced spasms of segments

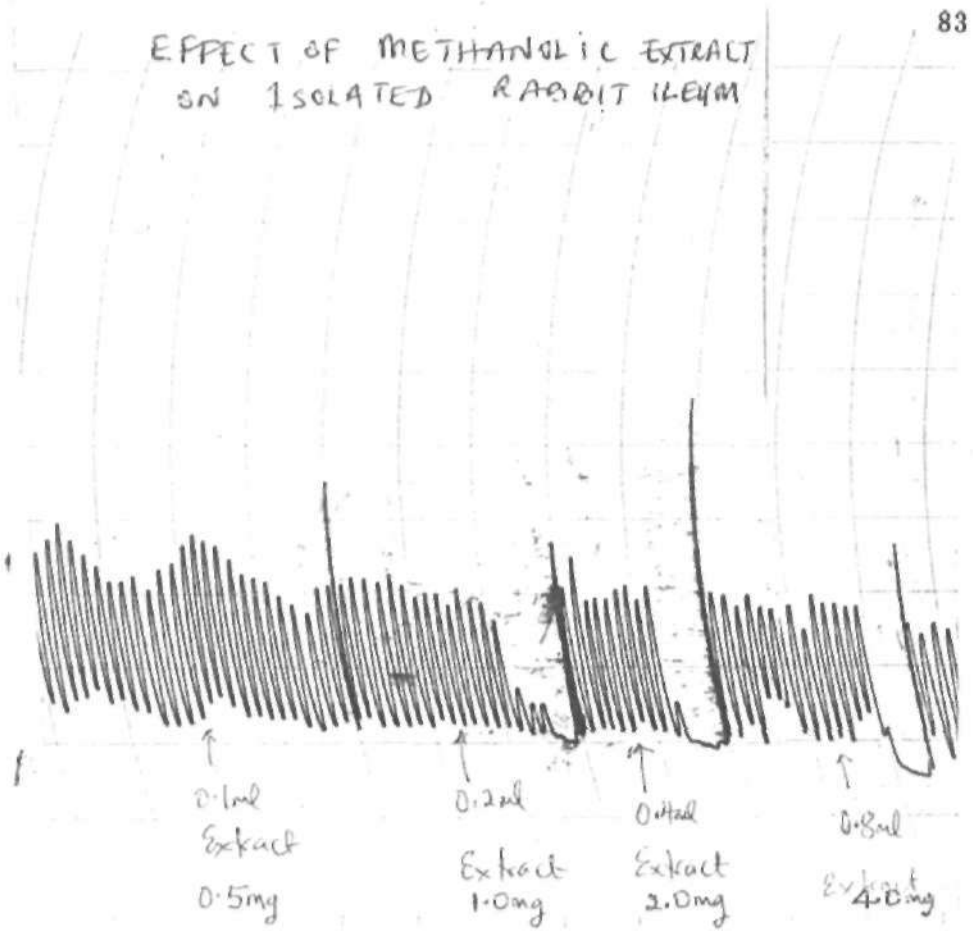

EFFECT OF METHANOLIC EXTRACT
ON ISOLATED RABBIT ILEUM
0·1ml
Extract
0·5mg
0·2ml
Extract
1·0mg
0·4ml
Extract
2·0mg
0·8ml
Extract
4·0mg

Fig. 5.1B

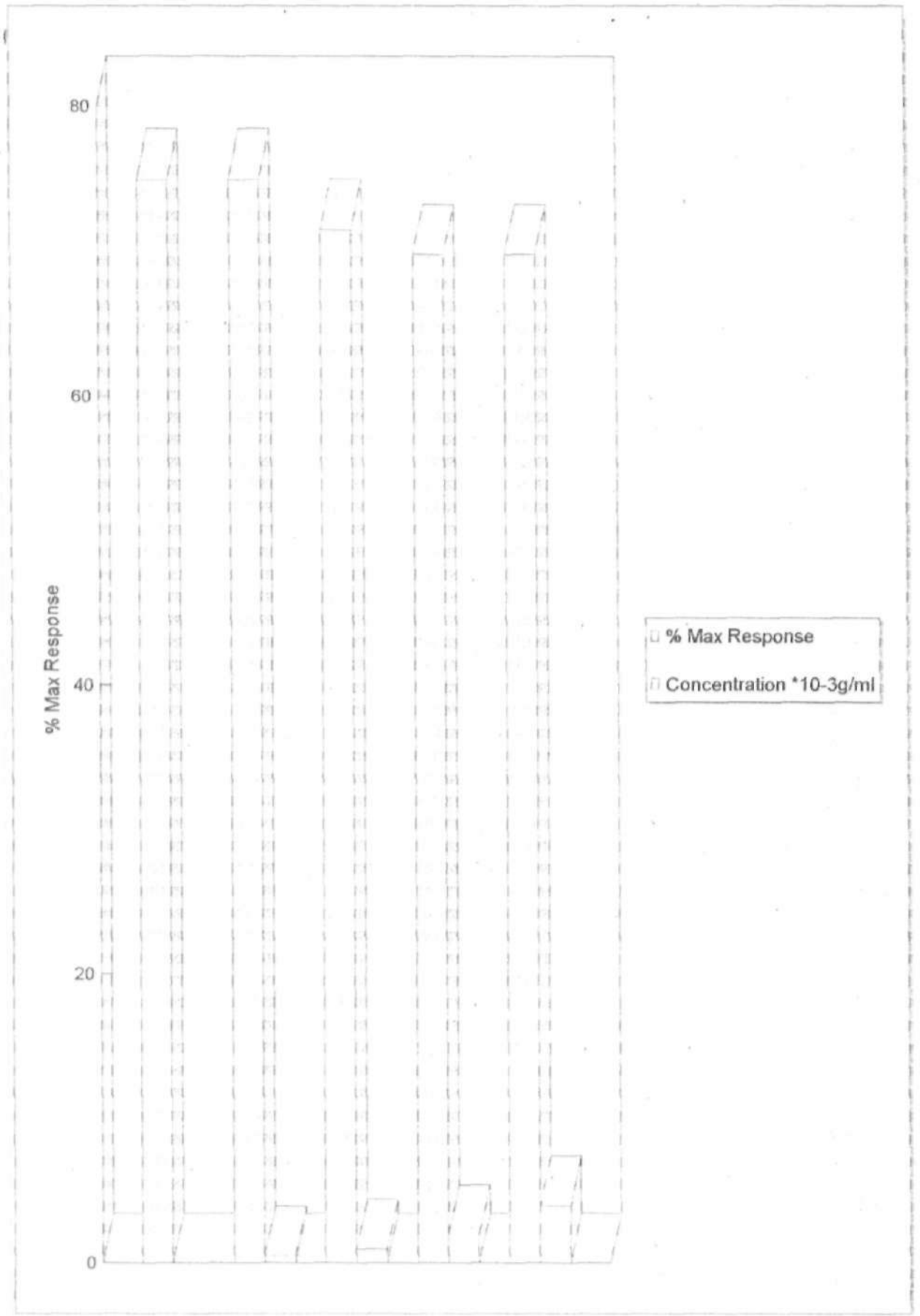


Fig 5.2 Effect of Extract on Acetyl Choline Induced Contraction

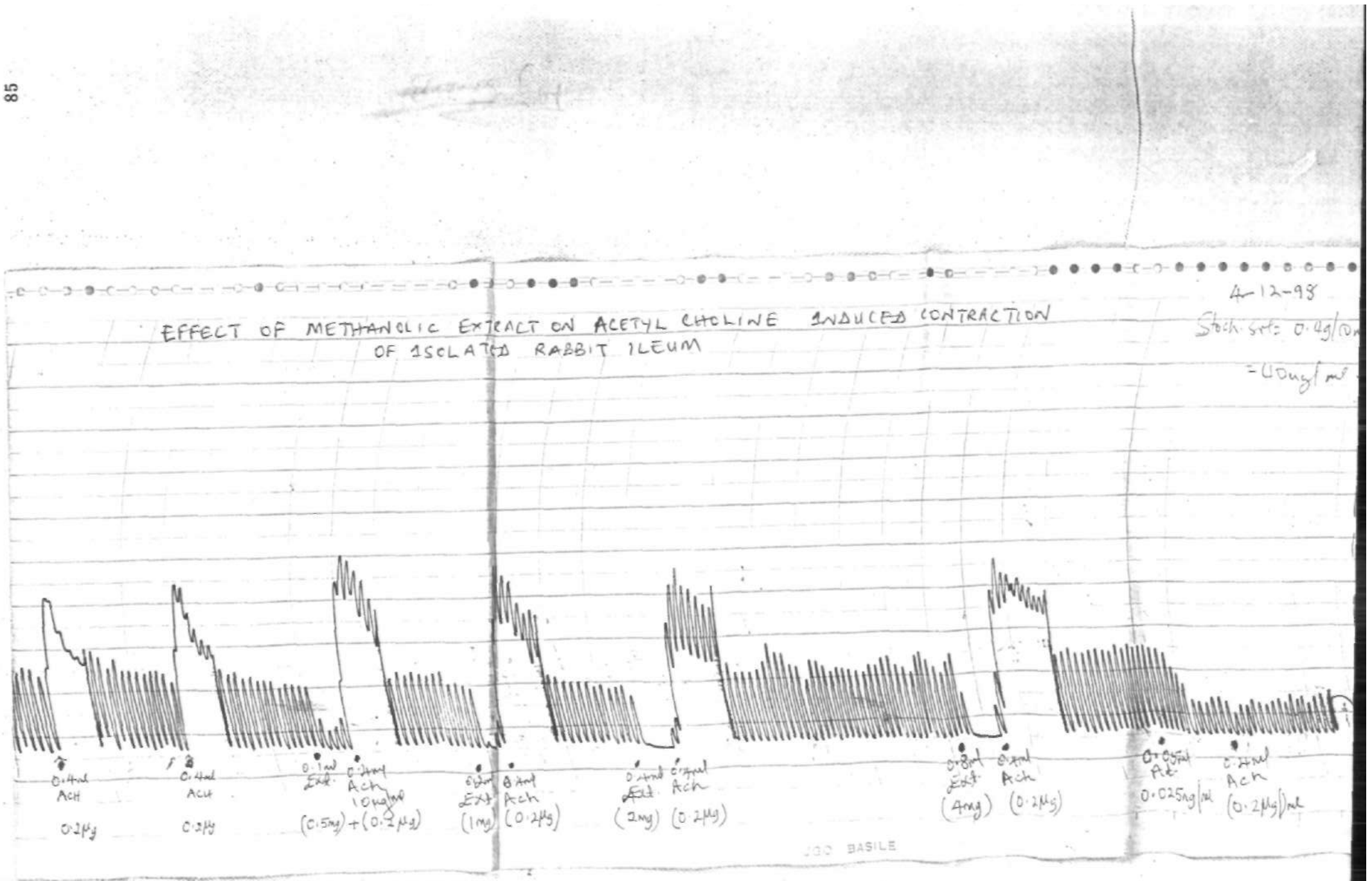

EFFECT OF METHANOLIC EXTRACT ON ACETYL CHOLINE INDUCED CONTRACTION OF ISOLATED RABBIT ILEUM
4-12-98
0·4ml ACH 0·2µg
0·4ml ACH 0·2µg
0·1ml Ext. (0·5mg) 0·2ml Ach 10ng/ml (0·2µg)
0·2ml Ext. (1mg) 0·4ml Ach (0·2µg)
0·4ml Ext. (2mg) 0·2ml Ach (0·2µg)
0·8ml Ext. (4mg) 0·4ml Ach (0·2µg)
0·05ml Ach 0·025ng/ml 0·2ml Ach (0·2µg/ml)
JOG BASILE

Fig. 5.2B

of uterus, ileum or isolated guinea pig ileum Melville, (1973). Histamine obtained via decarboxylation of histidine is found in most tissues of the body particularly in high concentration in the gastro-intestinal tract (G. I. T.). It acts on H_1-receptors present to cause contraction, Rang and Dale (1987). Many of the actions of histamine in the gut is mediated via H_1 receptors, Ash and Schild (1966). H_1 antagonist inhibits most response of smooth muscle to histamine, Garrison and Wall (1990). Thus (Fig. 5.3 & Table 5.4), histamine showed a dose-dependent contraction of the guinea pig ileum, this action is likely to have been mediated via the H_1-receptors which are usually present in the gut,Ash & Schild(1966). The effect of histamine response in the presence of mepyramine was however reduced by mepyramine (table 5.6), thus confirming the antihistaminic activity (H_1 antagonism) of mepyramine. The effect of methanolic extract in the presence of histamine at concentration of 0.5 and 1 mg of extract when challenged with 0.4 µg of histamine did not affect the percentage response of the ileum to histamine (Fig. 5.5 and Table 5.5). However, at concentration of 2 mg, the response of the ileum to histamine was reduced from 4.1 to 3.9 cm that is from 75% to 71.34%. maximum response, and similarly the highest concentration of the extract 4 mg also reduced the response further to 3.5 cm or to 64.02% that is by doubling the concentration of the extract from 2 mg to 4 mg, the response was reduced from 3.9 to 3.5 cm, that is about 7% reduction in the percentage response of histamine to the tissue (table 5.5, and Fig 5.4) thus indicating that the methanolic extract might have a weak antihistaminic activity on isolated guinea pig ileum.

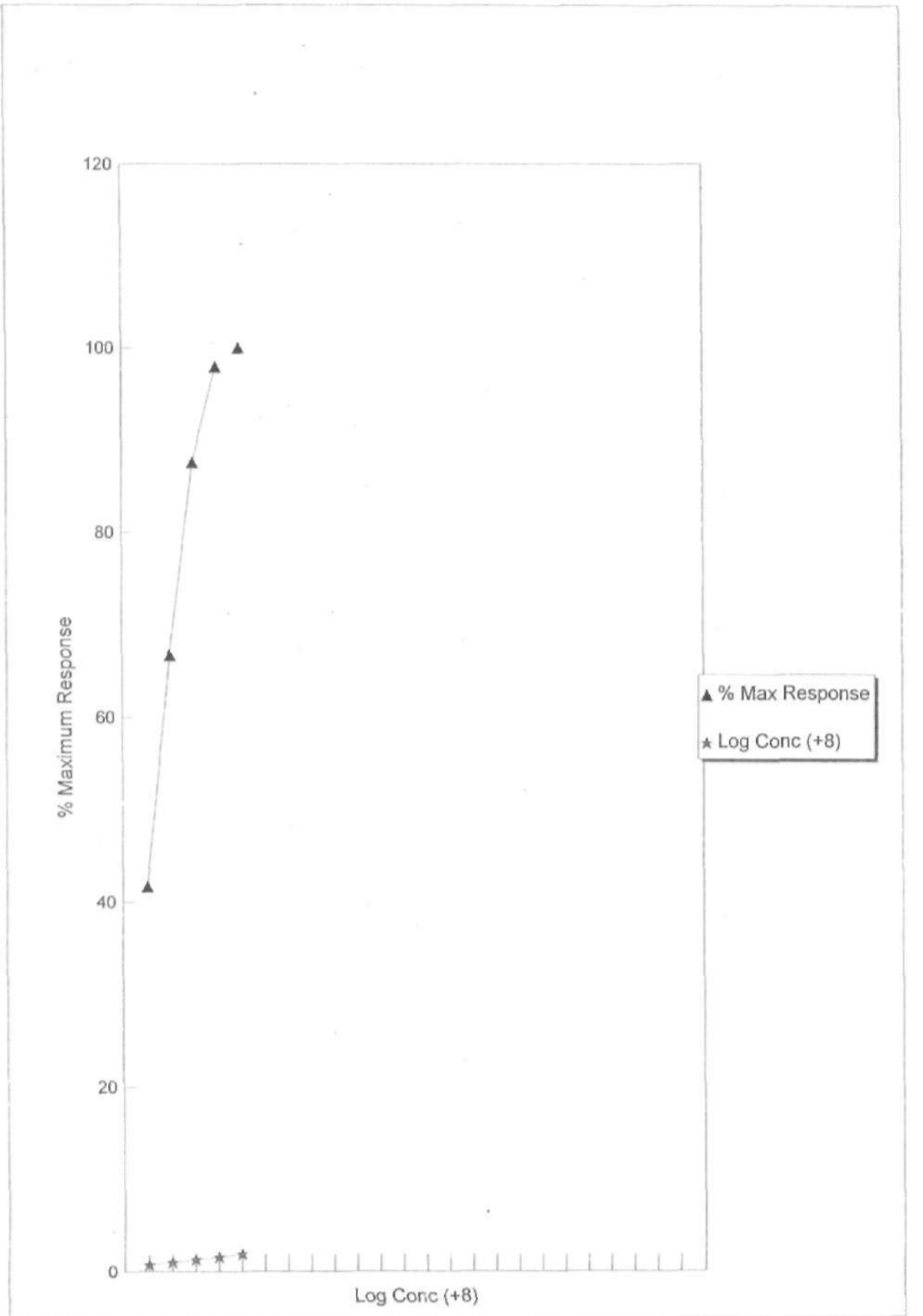


Fig 5.3 Effect of Histamine on Isolated Guinea Pig Ileum

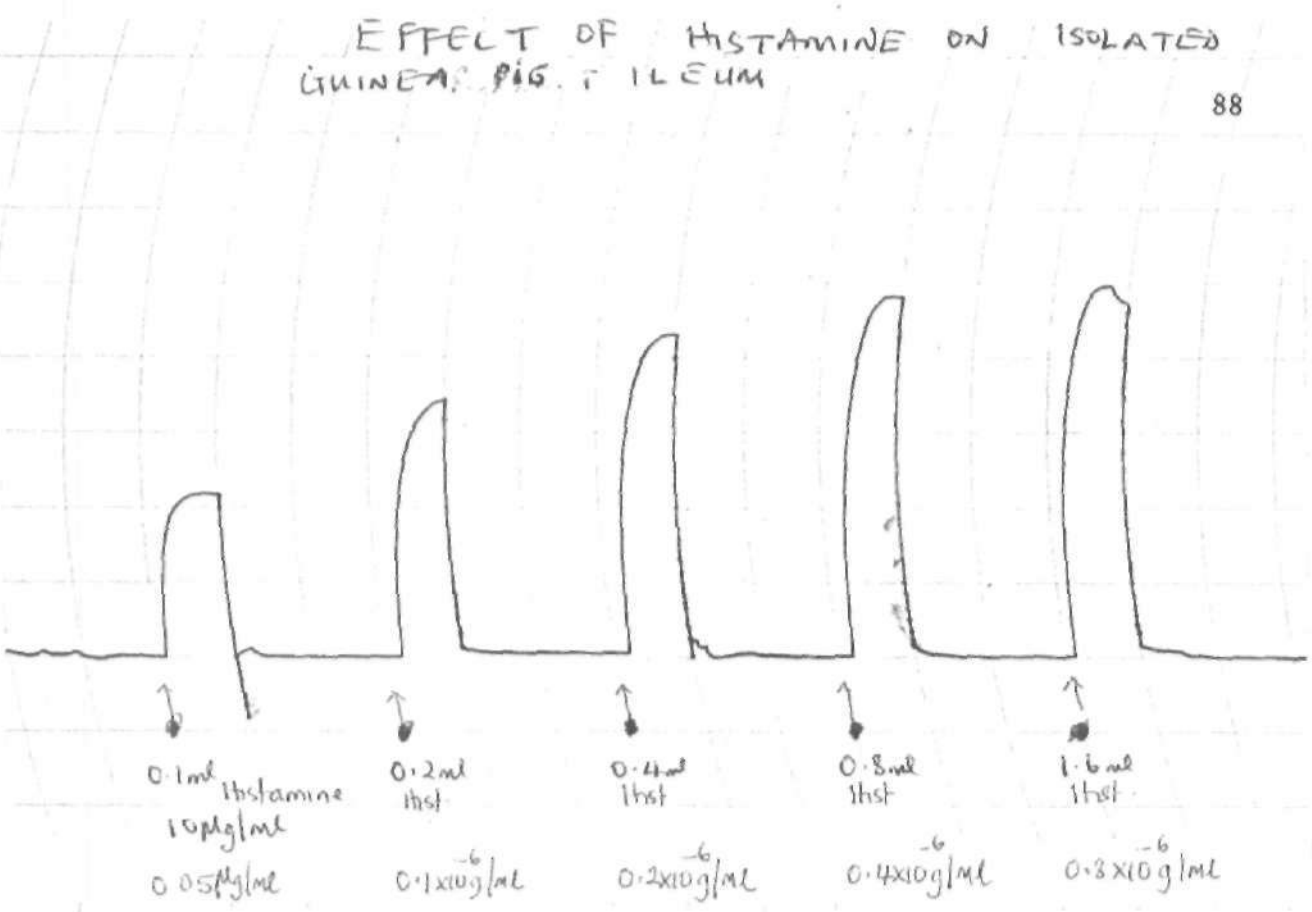

EFFECT OF HISTAMINE ON ISOLATED GUINEA PIG. ILEUM
0·1ml Histamine 10µg/ml
0·05µg/ml
0·2ml Hist
0·1x10⁻⁶g/ml
0·4ml Hist
0·2x10⁻⁶g/ml
0·8ml Hist
0·4x10⁻⁶g/ml
1·6ml Hist
0·8x10⁻⁶g/ml

Fig. 5.3A



Fig 5.4 **Effect of Increasing Concentration of Extract on Histamine Induced Contraction**

EFFECT OF METHANOLIC EXTRACT ON HISTAMINE INDUCED CONTRACTION ON ISOLATED GUINEA PIG ILEUM.

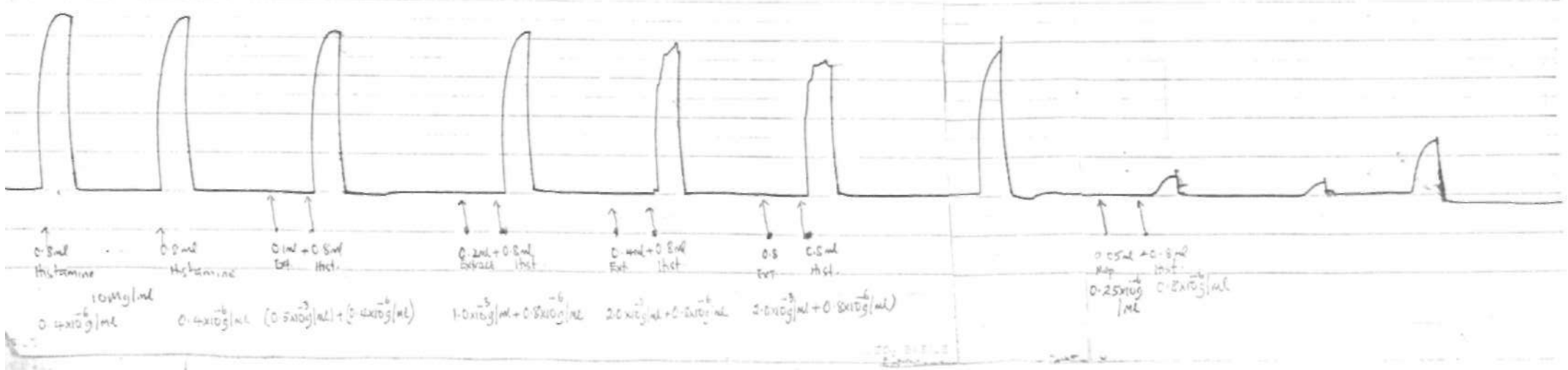


Fig 5·4 A

Fig. 5.4A

5.6 CONCLUSION

Pharmacological screening of the plant further confirms one of the local uses of the plant in relieving abdominal colicky pain. Hence, there is the need to further scientifically validate these claims and other uses, using pure compounds isolated from the plant.

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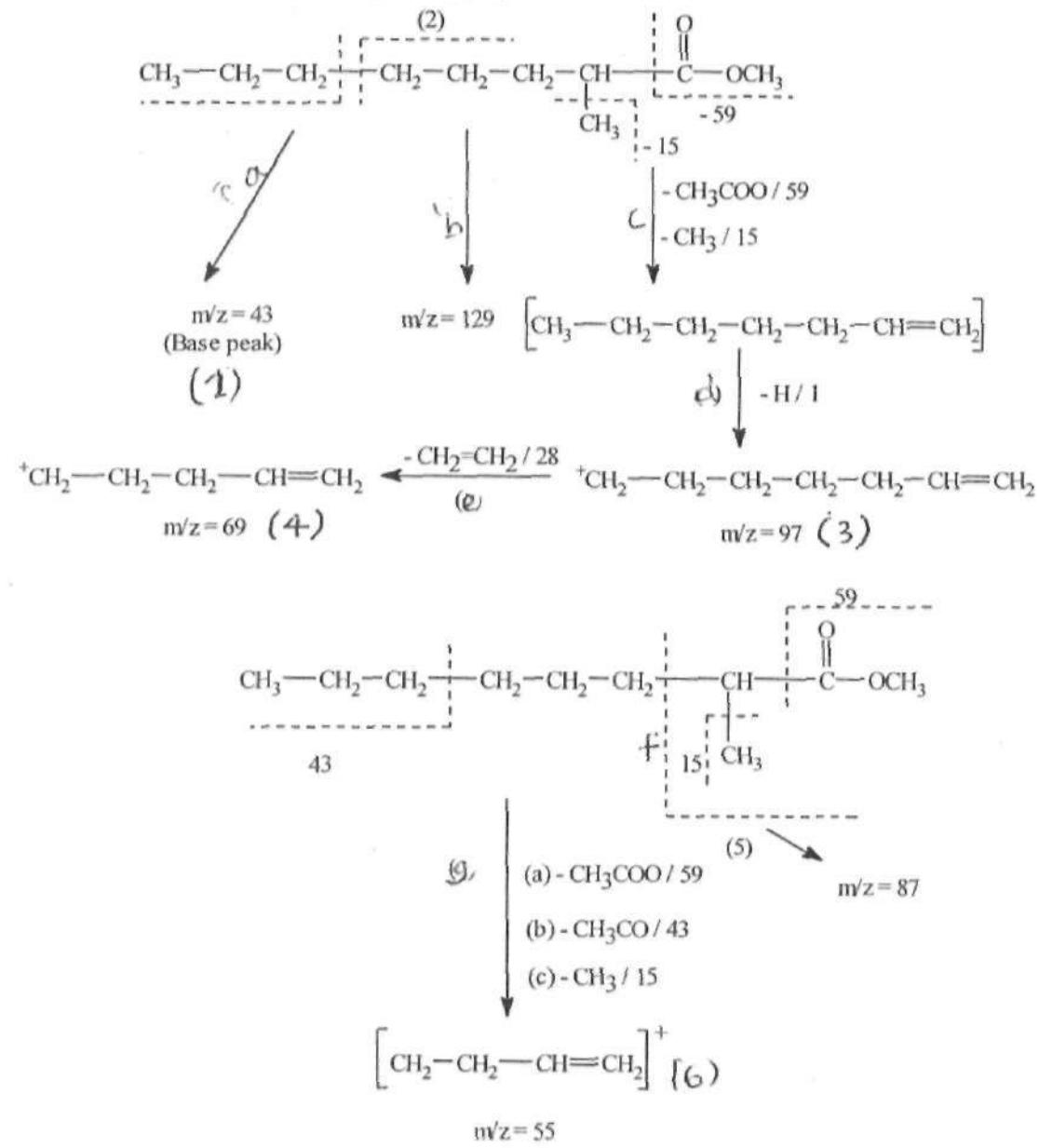
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Appendix 1.1

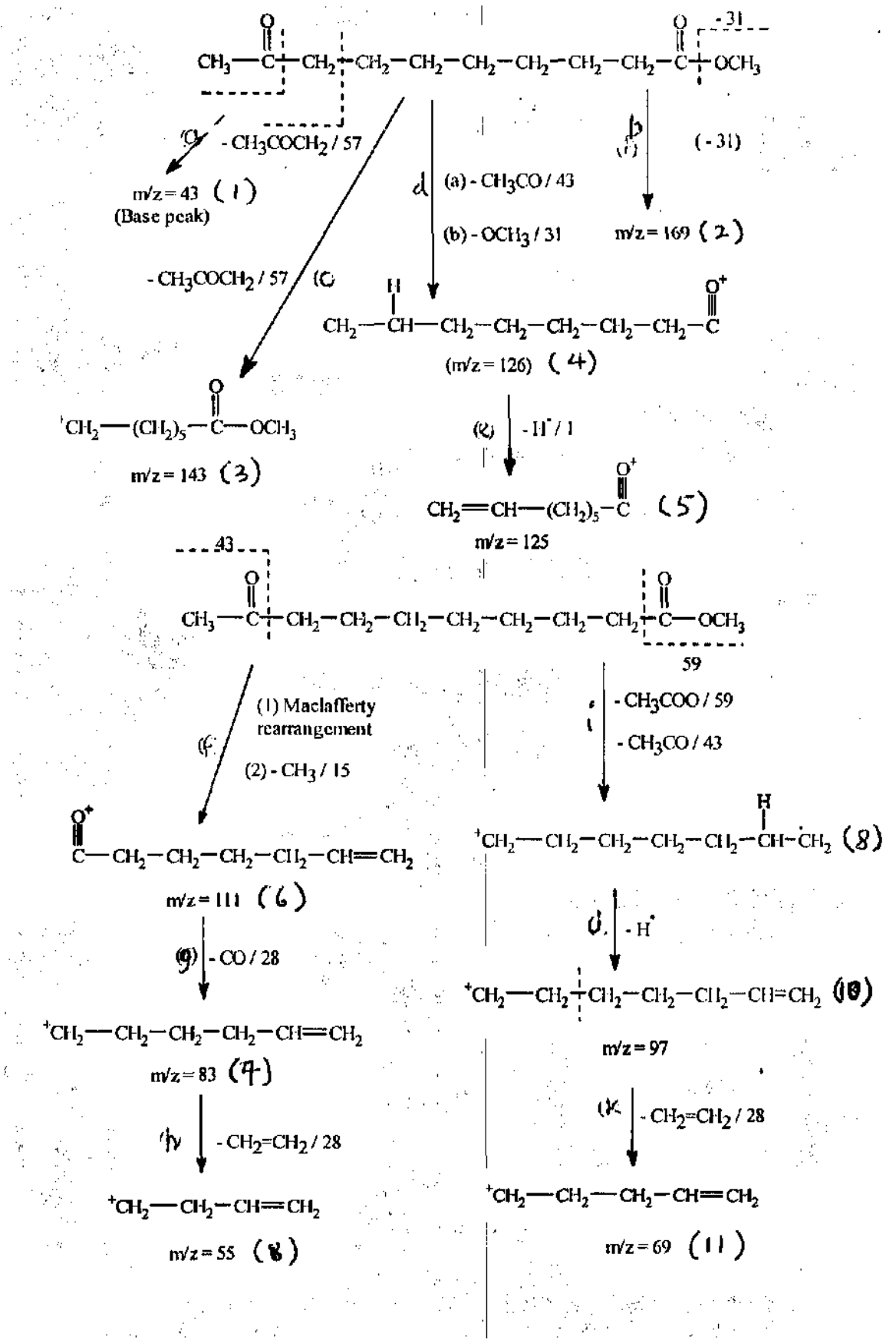
Fragmentation pattern of peak two.

2-methyl heptyl acetate (2-methyl octanoic acid methyl ester).
($C_{10}H_{20}O_2$) M.W. = 172.



Appendix 1:2

Fragmentation Pattern of 9-oxo-decanoic acid methyl ester ($C_{11}H_{20}O_3$)
M. W. = 200.

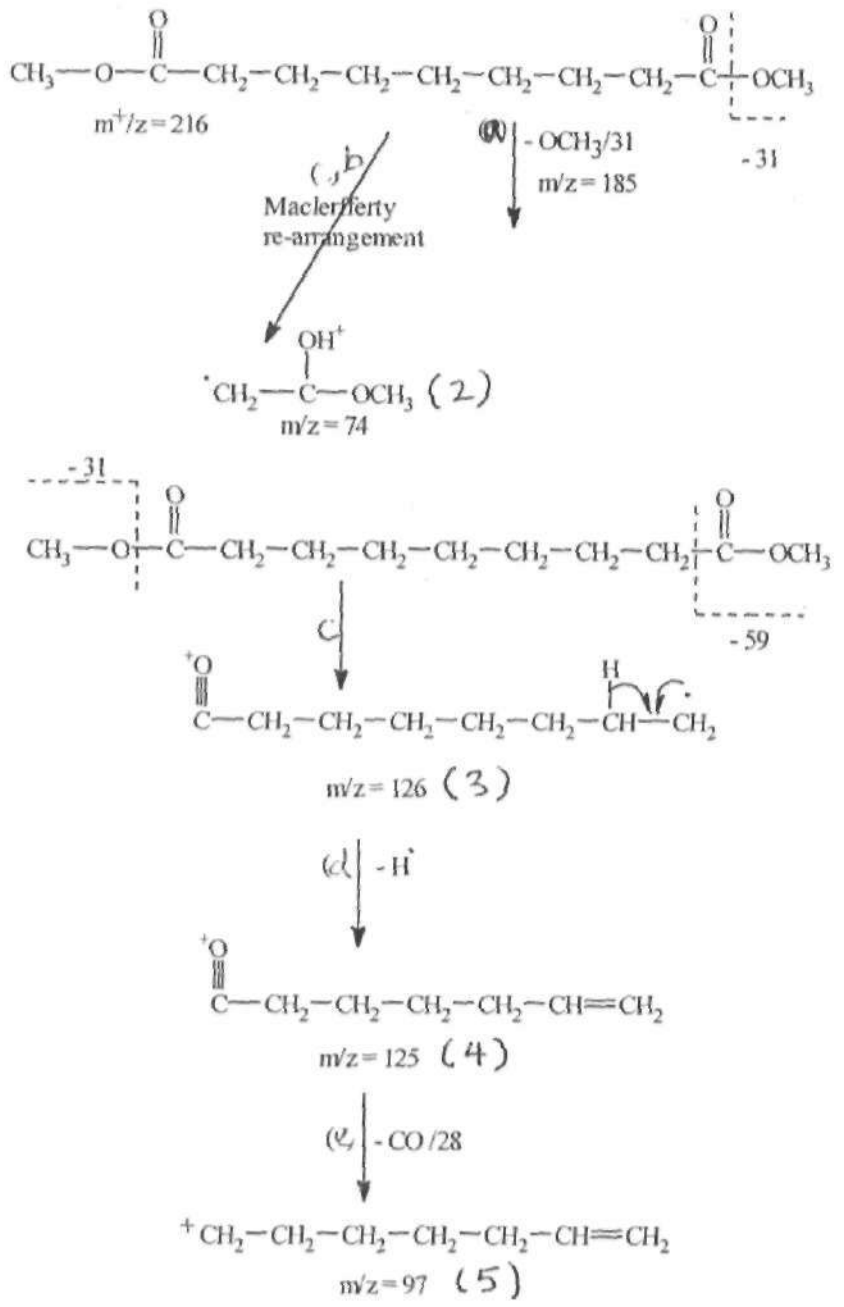


Appendix 1.3

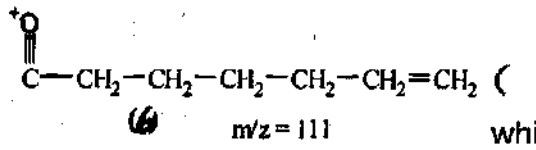
Fragmentation Pattern of Azelaic acid methyl ester.

(Nonanedioc acid dimethyl ester). Peak 5.

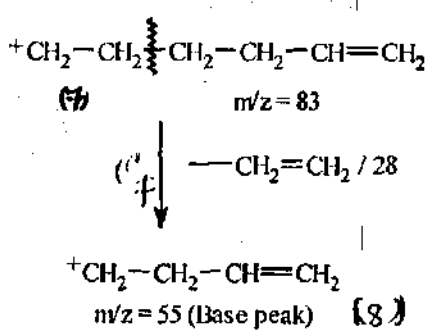
$(C_{11}H_{20}O_4)$ m/z = 216



(M - 74) from Maclerfferty rearrangement gives the fragment m/z = 142, which on loss of methoxy group $-OCH_3/31$, gives the ion:



(6) m/z = 111 which on further loss of - CO / 28 gives another radical ion of m/z 83:



(7) m/z = 83

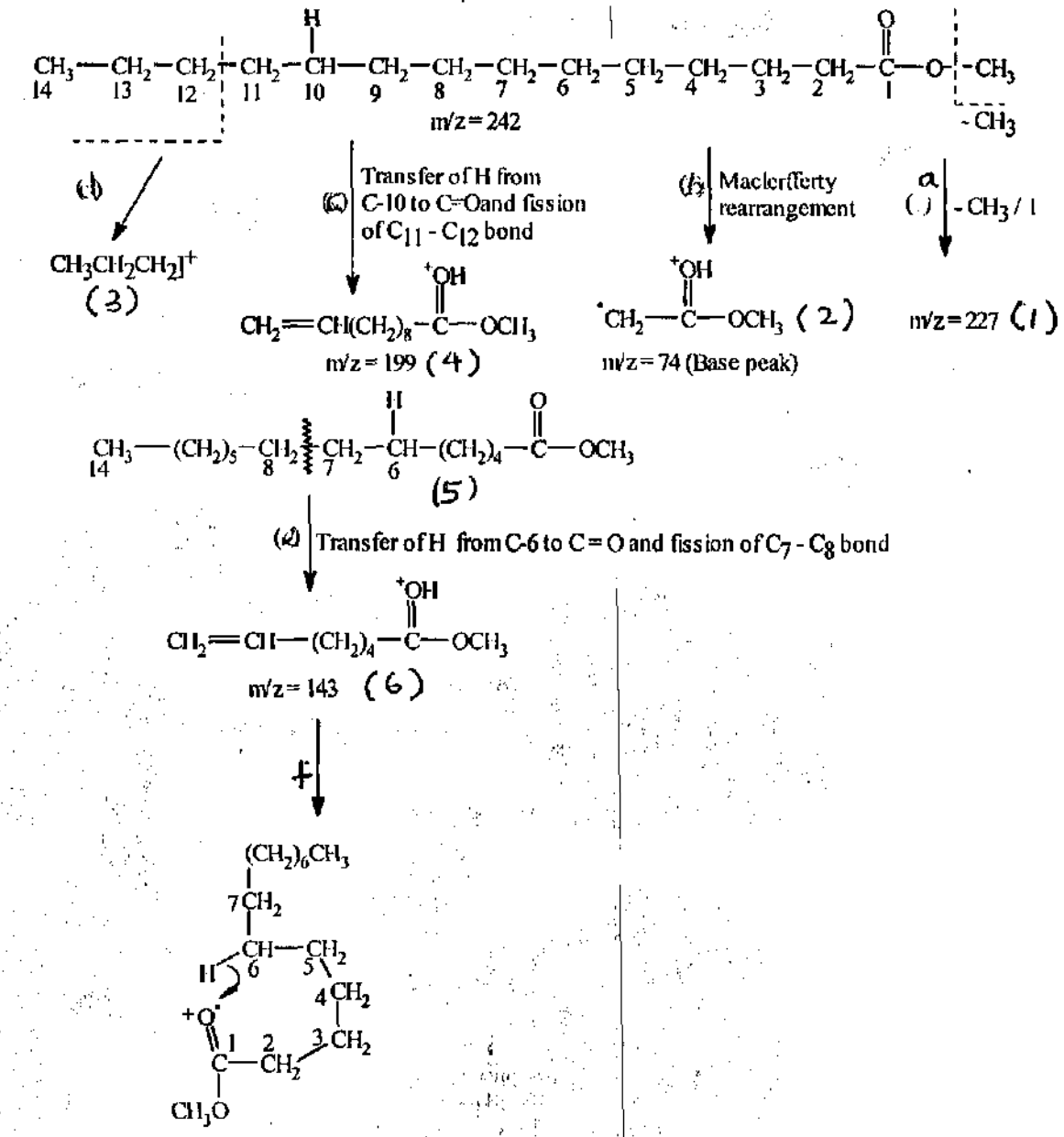
$-CH_2=CH_2$ / 28

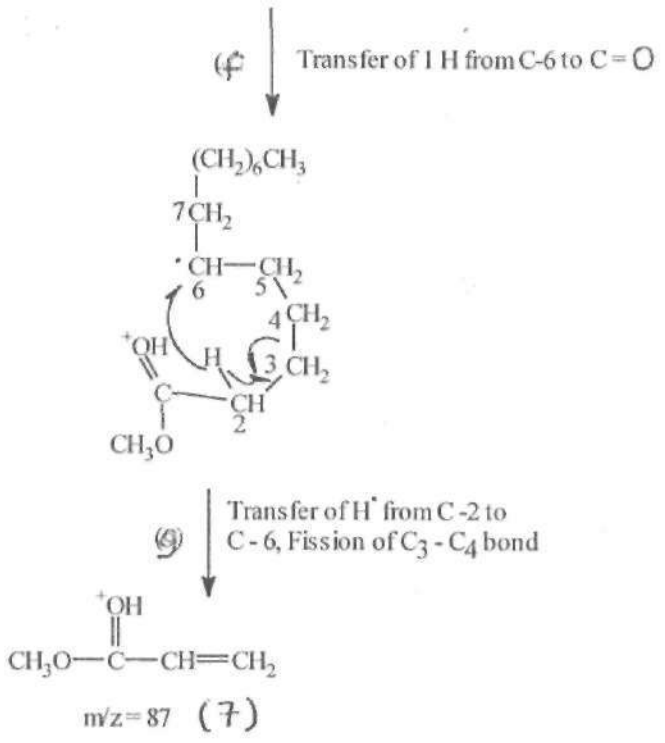
(8) m/z = 55 (Base peak)

which further loses a neutral alkene molecule (ethene) to give a more stable ion of m/z = 55 which is the base peak.

Appendix 1.4

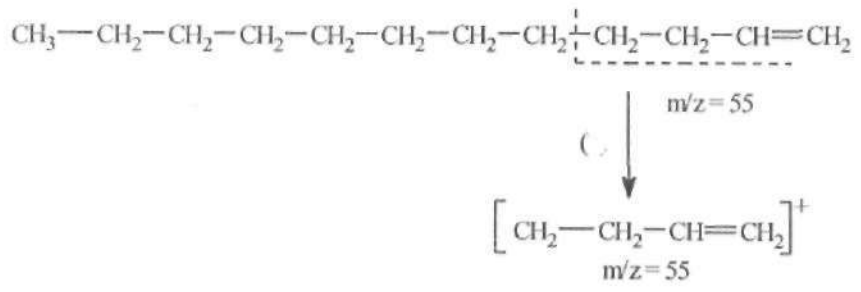
Fragmentation of peak Seven (Tetradecanoic acid methyl ester) (Myristic acid methyl ester), ($C_{17}H_{30}O_2$), m/z = 242



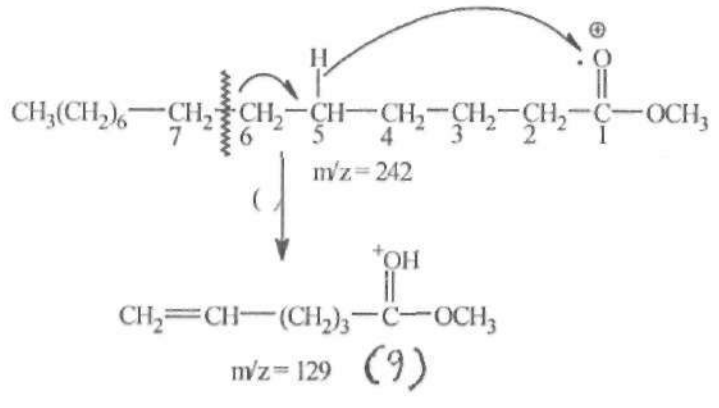


(Budzikiewicz *et. al.*, 1967).

m/z = 55 is as follows: m/z = 227 on undergoing Maclerfferty rearrangement gives the fragment (M - 74) which on further cleavage gives the radical cation of m/z = 55



m/z = 129 is formed as follows:



Appendix 1.5

Composition of 10 L Tyrode's Solution

Sodium chloride	90 g
potassium chloride	20 ml
Sodium dihydrogen phosphate (10%)	5 ml
D-glucose	10 g
Sodium bicarbonate	10 g
Calcium chloride (10%)	20 ml
Magnesium chloride ($MgCl_2.6H_2O$)	1 ml.