CHEMISTRY AND BIOACTIVITY OF CONSTITUENTS OF *Murraya koenigii* (Linn.) Spreng. LEAVES

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CHEMISTRY AND BIOACTIVITY OF CONSTITUENTS OF *Murraya koenigii* (Linn.) Spreng. LEAVES

By

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CERTIFICATE

This is to certify that the thesis entitled "Chemistry and Bioactivity of Constituents of ***Murraya koenigii*** (Linn.) Spreng. Leaves", submitted to the Post Graduate School, Indian Agricultural Research Institute, New Delhi, in partial fulfilment of the requirements for the award of the degree of **Doctor of Philosophy in Agricultural Chemicals,** embodies the result of bona fide research work carried out by **Shri SANJAY SRIVASTAVA** under my guidance and supervision, and that no part of the thesis has been submitted for any other degree or diploma.

Place : New Delhi

Date : December 7th, 1999

(R.P. Singh)
Chairman
Advisory Committee

Dedicated to

my wife

and

children

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(SANJAY SRIVASTAVA)

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ABBREVIATIONS

Greek Alphabets (used as prefixes) :

α	-	alpha
β	-	beta
γ	-	gamma
Δ, δ	-	delta

Units :

cm	-	Centimetre
cm^{-1}	-	per centimetre
h	-	hour
l	-	litre
mg	-	milligram
min	-	minute
ml	-	millilitre
mm	-	millimetre
ppm	-	part per million
psi	-	pounds per square inch

General

Ar	-	Aromatic
br	-	broad
CPI	-	Corrected per cent inhibition
C.P.N.	-	Corrected per cent non-motility
^0C	-	degree celcius
d	-	doublet
Et	-	Ethyl
ED_{50}	-	Effective dosage for 50 per cent inhibition
GC-MS	-	Gas chromatography - Mass spectrometry

GLC	-	Gas liquid chromatography
IR	-	Infra-red spectroscopy
i.d.	-	internal diameter
LT_{50}	-	Lethal time causing 50% non-motility
m	-	multiplet
Me	-	methyl
MeOH	-	Methanol
m.p.	-	melting point
MHz	-	Megahertz
o	-	ortho
p	-	para
Ph	-	Phenyl
PDA	-	potato-dextrose agar medium
PMR	-	Proton magnetic resonance spectroscopy
TLC	-	thin-layer chromatography
υ	-	wave number
λ	-	wave length

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CHAPTER 1

INTRODUCTION

Synthetic pesticides are applied for the effective, efficient and economic pest control. However, their indiscriminate use has resulted in several problems such as pest resistance to pesticides, resurgence of pests and toxic residues in the environment. The continued use may further result in elimination of natural enemies of insects and disruption of eco-system.

In view of the recent tirade against the use of the synthetic insecticides, the botanicals are gaining prominence for the control of pest in agriculture, forestry and health programme. Botanical pesticides are natural products that belong to the group of so called secondary metabolites which includes thousands of alkaloids, terpenoids, phenolics and minor secondary chemicals.

Plants virtually are "natures" chemical factories, providing unlimited natural sources of botanical pesticides. So, more emphasis is laid to obtain pesticides which are

* Specific
* non-toxic to man and beneficial organisms
* biodegradable
* less prone to pest resistance
* cost effective

* serve as prototype for synthesis of new chemicals of high biological activity.

Renewed search for secondary metabolites imparting resistance against insects pest and pathogens to the parent plant is on the increase. There are several plants having resistance to insect pests and other diseases. One such plant is *Murraya koenigii* (Linn.) spreng. There is no report on the biological activity of the chemical constituents of the leaves of curry leaf plant, even though phytochemical investigations are available.

An attempt therefore has been made to utilize the leaves of this plant (*M. koenigii*) in pest control measures not only on the basis of essential oil and pure carbazole alkaloids present in the leaves alone but also by derivatization of carbazoles.

Apart from some medicinal properties like antiinflammatory, anti-convulsant and diuretic etc. shown by this plant, it offers also an opportunity to explore the efficacy of essential oil as a source of insect antifeedant, antinemic, antifungal, larvicidal, insect repellant and insect growth regulatory whereas carbazole alkaloids remain as a source of antifungal antinemic and insect antifeedant. With the above rationale in mind, the present work sets out the following objectives :

(I) Detailed chemical examination of leaves of *Murraya koenigii* (Linn) spreng.

(II) Biological activity evaluation of chemical constituents obtained from the above plant.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Introduction

2.1.1 Classification of Murraya Linn.

Order	-	Rutales
Family	-	Rutaceae; Subtribe-clauseneae
Subfamily	-	Aurantoidae
Genus	-	Murraya
Species	-	*Murraya koenigii* (Linn.) spreng.

Common name in different Indian languages

Assam	:	Nara Singha, Bishahari
Bengali	:	Barsanga, Kariaphulli
English	:	Curry leaf tree
Gujarati	:	Goranimb, poospala, gandla
Hindi	:	Kathnim, methanim, kurry patta
Kannada	:	Karibau
Malayalam	:	Kariveppu
Oriya	:	Barsan, besango
Sanskrit	:	Surabhinimba
Tamil	:	Karivempu, Karuveppilei
Telgu	:	Karepaku

2.1.2 Distribution

The plant is largely cultivated for its fragrant leaves and is common in most parts of India. Also found in Srilanka, Burma, Indo-China, S. china and Australia. Two species occur in India are

* Murraya koenigii (Linn.) Spreng. (Curry leaf tree)
* *Murraya paniculata* (Linn.) Jack Syn. *Murraya exotica* (Linn) (orange Jessamine)

The other important species of genus Murraya found abroad are

* *Murraya elongata* DC - Native to Burma and the richest source of monomeric coumarin
* Murraya euchrestifolia Hyata - native to Taiwan and the richest source of Carbazole alkaloids

2.1.3 The plant

Murraya koenigii (L.) Spreng. is an unarmed small aromatic tree with dark grey bark and closely crowded spreading dark green foliage; leaves imparipinnate, leaflets alternate, obliquely ovate or somewhat rhomboid, gland dotted and strongly aromatic; Flowers white, in much branched terminal corymbose cymes, fragrant; fruits subglobose or ellipsoid berries, purplish black when ripe, 2-seeded.

The leaves of this plant are extensively employed as flavouring in curries and chutneys. Analysis of leaves gave the following value

Moisture	-	66.3%
Protein	-	6.1%
Fat (ether extract)	-	1.0%

Carbohydrate	-	16.0%
Fibre	-	6.4%
Mineral matter	-	810 mg
Iron	-	3.1 mg
Carotene (Vit. A)	-	12,600 i.u.
Nicotinic acid	-	2.3 mg
Vit. C	-	4 mg/100 gm.

The fruit of this plant is edible. It yields 0.76% of a yellow volatile oil with neroli like odour and pepper like taste accompanied by an agreeble sensation of coolness on the tongue.

Parts used : Leaves, roots and bark

2.1.4 Therapeutic uses

Whole plant, leaves and root bark are considered antiemetic carminative, stomachic and tonic

Green leaves - Infusion used as antidiarrhoel antidysenteric and antiemetic: bruised and applied locally to eruptions and poisonous bites:

Root-bark - relieves renal pain

2.2 Chemical Constituents of *Murraya koenigii* (Linn) Spreng.

Murraya koenigii (L.) spreng (curry leaf tree) is a store house of chemically diverse and structurally complex bioactive molecules. It has been a subject of several reviews (Kapil, 1971; D.P. Chakraborty, 1977).

Leaves are the most important source of the ingredients of curry patta that affect insect in various ways. Besides the leaves; fruits, bark,

roots also contain a vaiety of bioactive principles. Chemical constituents isolated from various parts of the curry leaf tree are described as follows:

2.2.1 Seeds

2.2.2 Stem-bark

2.2.3 Root bark/root

2.2.4 Fruits

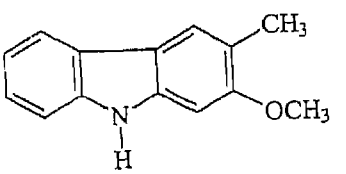
2.2.5 Leaves - Non-volatile compound

2.2.5.1 Leaves - Volatile compounds

2.2.1 Seeds

Members of the C_{13} skeleton Group

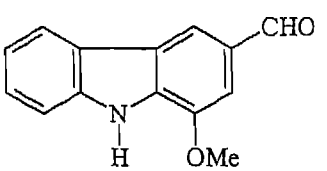
Bhattacharya and Chowdhury (1985) isolated 2-methoxy-3-methyl Carbazole $C_{14}H_{13}NO$, m.p. 245°C from the petroleum ether (60-80°C) extract of the seeds of *Murraya koenigii*

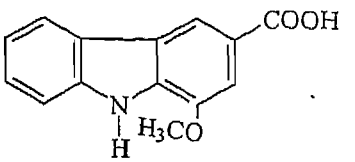


The IR spectrum (υ_{max} 3425, 1640, 1600, 1208, 820, 750 cm^{-1}) and the UV spectrum (λ_{max} 235, 255, 300 and 328 nm) indicated it to be a carbazole alkaloid. The nmr spectrum showed signals at δ8.0 (bs, IH, NH) 7.5 (s, IH, H-4) 7.4-7.2 (complex multiplet 4H, aromatic protons) 6.95 (s, IH, H-1) 3.77 (s, 3H, OCH_3) 2.35 (s, 3H, aromatic C-Me). All these evidences led to the formulation of the alkaloid as 2-methoxy-3-methyl carbazole.

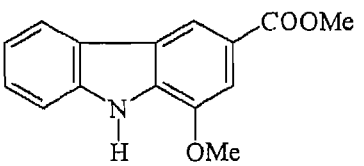
2.2.2 Stem Bark

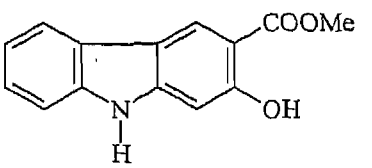
Members of the C_{13} - Skeleton Group - This group consist of the carbazole alkaloids those were mostly isolated from stem bark of *Murraya koenigii* spreng e.g. Murrayanine ($C_{14}H_{11}NO_2$) was isolated from petroleum ether (40-60°) extract by chakraborty *et al.* (1965), Mukoeic acid $C_{14}H_{11}NO_3$ isolated from the acidic fraction of the alcoholic extract by Chowdhury *et al.* (1969).



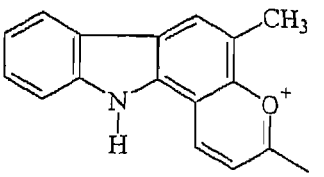


Chakraborty *et al.* (1976) isolated Mukonine ($C_{15}H_{13}NO_3$) and Mukonidine ($C_{13}H_{11}NO_3$) from the stem bark of *M. koenigii*.

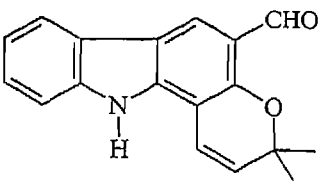




Members of the C_{18} skeleton Group - Girinimbine $C_{18}H_{17}NO$ (M^+ 263) the first member of the group of Carbazole alkaloids with a C_{18}-skeleton was isolated from the stem bark of *M. koenigii* spreng by Chakraborty *et al.* (1964). The mass spectrum of girinimbine showed a molecular ion peak at m/e 263 and high intensity peak at m/z 248 (M-15) which supports the presence of a 2,2 dimethyl - Δ^3 - pyran system in girinimbine and could be represented by the carbazolopyrylium ion (1).



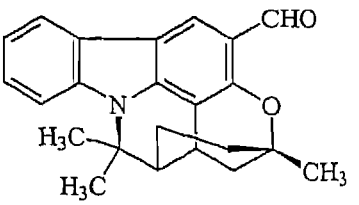
Chakraborty *et al.* (1968) isolated Murrayacine $C_{18}H_{15}NO_2$ (M^+ 277) mp 244-45^0 from the stem bark of *M. koenigii.*



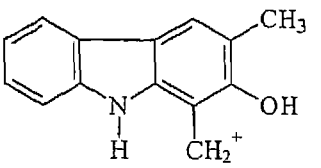
Members of the C_{23} - skeleton group

Chakraborty *et al.* (1966) isolated the first member of this group namely Mahanimbine $C_{23}H_{25}NO$ (M^+ 331) mp 94^0 from the stem bark of *M koenigii* spreng. Later on it was isolated from the leaves and fruit of the plant by Narasimhan *et al.* (1968) and Narasimhan *et al.* (1975) respectively.

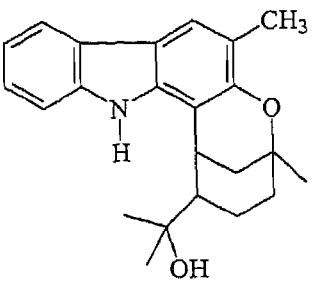
Chakraborty and Chowdhury (1966) isolated Murrayazoline from the alcoholic extract of the stem bark of this plant.



The mass spectrum of murrayazoline showed the base peak at m/z 331 and lacked the high intensity peak at m/z 248 represented by carbazolopyrylium ion. Instead it had a prominent peak at m/z 210 characteristic of the 2,2 dimethyl pyranocarbazole system (2).

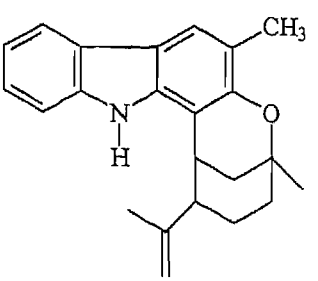


Murrayazolinine $C_{23}H_{27}NO_2$ mp 184° was isolated from the stem bark of this plant by Chakraborty *et al.* (1973).

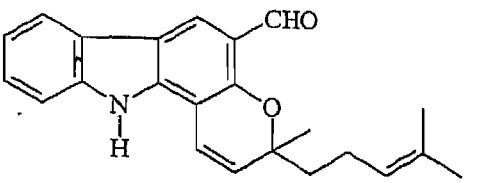


The evidence suggested that murrayazolinine is a pentacyclic alkaloid built on a 2-hydroxy-3-methyl carbazole skeleton to which a monoterpene unit is attached through an ether linkage.

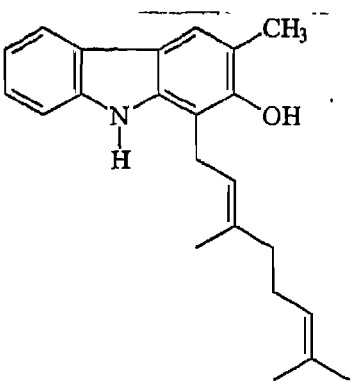
Murrayazolidine $C_{23}H_{25}NO$ (M^+ 331) mp 143°, the first pentacyclic carbazole was isolated from the stem bark by Chakraborty *et al.* (1970). The nmr spectrum of murrayazolidine showed it to have an aromatic substitution pattern similar to those of mahanimbine, murrayazoline etc. It had signals for two vinylic potons (δ 4.8) and for a vinyl methyl group on a double bond at (δ 2.35) besides a benzylic methine protons at δ3.4.



Chakraborty *et al.* (1974) isolated Murrayacinine. $C_{23}H_{23}NO_2$ (M^+ 345) mp 105^0 a new carbazole alkaloid from the stem bark of *Murraya koenigii* spreng.



Rama rao *et al.* (1980) isolated Mahanimbinol $C_{23}H_{27}NO$ (M^+ 333) as an amorphous powder from the hexane extract of Stem wood of *Murraya koenigii* spreng.

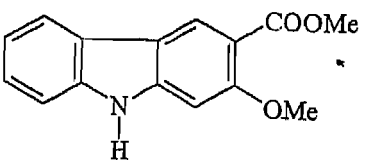


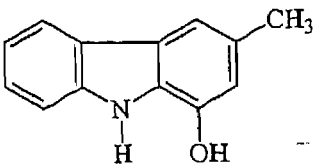
The mass spectrum showed a base peak at m/z 210 formed by the loss of a C_9H_{15} residue from C_{10} side chain. A strong peak at m/z 209 also indicated the presence of OH adjacent to the geranyl group.

Isomurrayazoline $C_{23}H_{25}NO$ (M^+ 331) mp 269-70^0 was isolated from the benzene extract of the stem bark of *Murraya koenigii* spreng by Bhattacharyya *et al.* (1982).

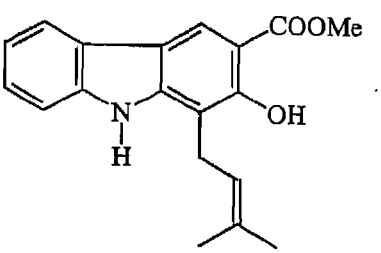
From the stem bark extract of *Murraya koenigii* Bhattacharyya *et al.* (1994) isolated two carbazole alkaloids which have been shown to be

2-methoxy carbazol-3-methyl carboxylate and 1-hydroxy-3-methyl carbazole using spectroscopic and chemical evidence.



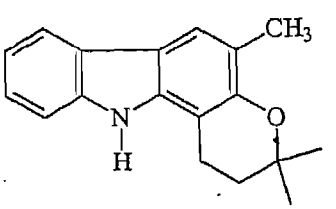


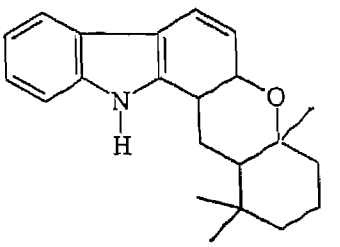
Reisch *et al.* (1994) isolated two non-cyclized possible biogenetic precursors of girinimbine and mahanimbine, 2-hydroxy-3-methyl-1- (3-methyl-2-butenyl) carbazole (girinimbilol) and 2-hydroxy-3-methyl-(3.7-dimethyl-2, 6-octadienyl) carbazole (mahanimbilol) from the stem bark of *Murraya koenigii.*



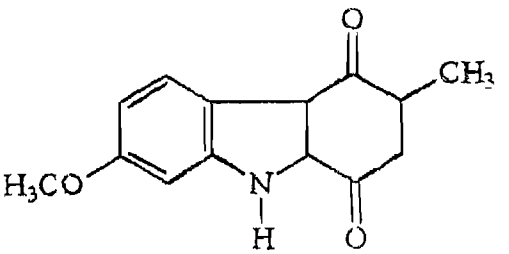
Compounds girinimbilol and Mahanimbilol gave a blue-purple colour on spraying with 10% H_2SO_4 and their UV spectra suggested that they were carbazole alkaloids. The molecular formulae of girinimbilol and Mahanimbilol were shown by high resolution mass spectral mass determination to be $C_{18}H_{19}NO$ and $C_{23}H_{27}NO$ respectively. Their IR spectra showed absorptions at υ_{max} 3450-3550 and ~ 3430 cm^{-1} suggesting that an OH group was present in addition to the NH group.

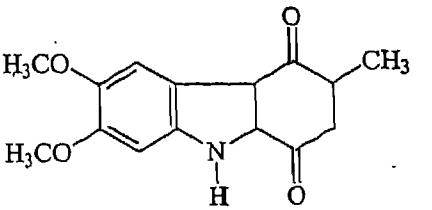
The structures were established by cyclization to dihydrogrinimbine and bicyclomahanimbiline respectively.





Saha and Chowdhury (1998) isolated two new carbazole alkaloids designated as koenigine-quinone A (5) and koenigine-quinone B (6), from the alcoholic extract of the stem bark of Murraya koenigii and their structures were established as 7-methoxy-3-methyl carbazole-1, 4 quinone and 6, 7-dimethoxy-3-methyl carbazole-1, 4 quinone respectively.

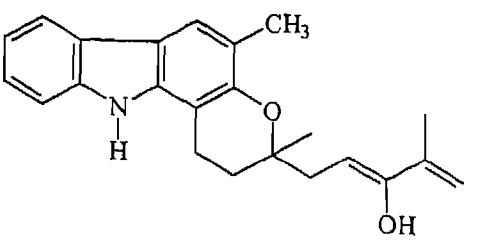




2.2.3 Roots/root bark

Members of the C_{23}-skleton group

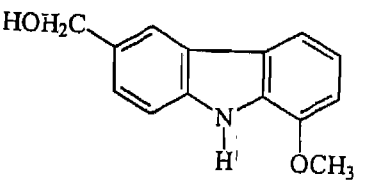
Roy *et al.* (1979) isolated Mahanimboline $C_{23}H_{25}NO_2$ (M^+ 347) mp 170-72^0 from the petroleum ether (60-80^0) extract of the root bark of *Murraya koenigii*.

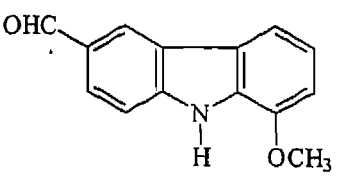


The mass spectrum of mahanimboline showed a molecular ion peak at m/z 347 and a peak at m/z 329 due to loss of H_2O. A high intensity

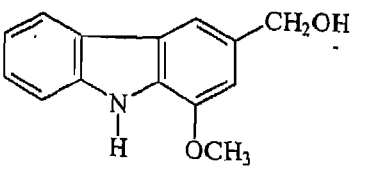
peak at m/z 248 could represent the same carbazolopyrilium ion as that formed from girinimbine and mahanimbine. The uv absorption spectrum (λ_{max} 237, 280, 288 and 330 nm; log ε 4.42, 3.69, 4.25 and 2.75) was very similar to pyranocarbazole alkaloids like girinimbine and mahanimbine and thus indicated the presence of the same pyranocarbazole chromophore in mahanimboline.

Members of the C_{13}-skleton Group - Roy *et al.* (1982) isolated two optically inactive carbazole alkaloids namely Mukoline $C_{14}H_{13}NO_2$ (M^+ 227) mp 115-20^0 and Mukolidine $C_{14}H_{11}NO_2$ (M^+ 225) mp 152-55^0 from the benzene extract of the roots of *Murraya koenigii*.





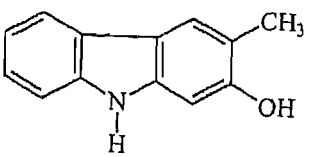
Fiebig *et al.* (1985) isolated Koenoline $C_{14}H_{13}NO_2$ (M^+ 227) mp 213^0 a cytotoxic carbazole alkaloid from the chloroform extract of the root bark of *M. koenigii*.



The IR spectrum of koenoline showed it to be an aromatic compound with - NH- (3445 cm^{-1}) and -OH (3235 cm^{-1}) while the UV spectrum (λ_{max} 335, 323, 289, 279, 238, 251, 241 and 225 nm; log ε 3.58, 3.61, 4.04, 3.86, 4.38, 4.22, 4.71 and 4.56) was strikingly similar

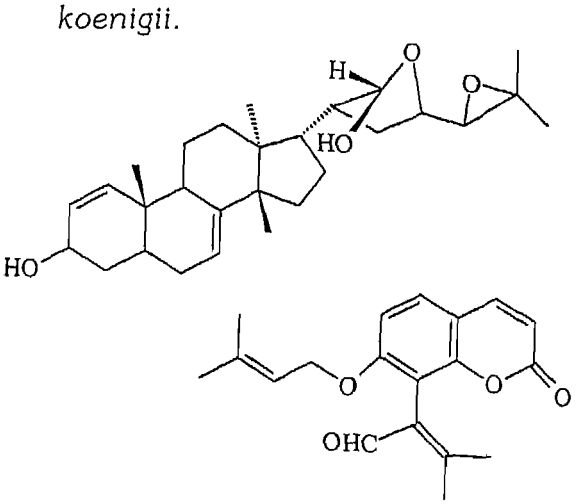
to that of a 1-methoxy carbazole derivative. The non-phenolic nature of the hydroxyl group was suggested from its negative colour raction with $FeCl_3$.

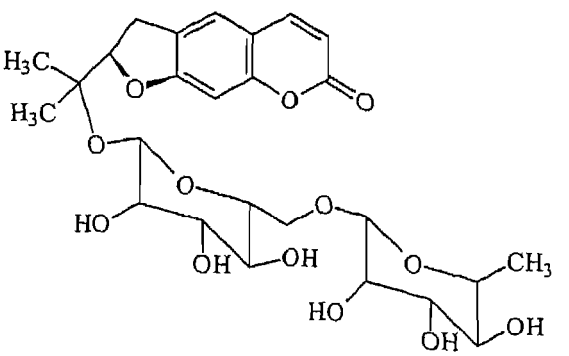
Bhattacharyya *et al.* (1986) isolated 2-hydroxy-3-methyl carbazole $C_{13}H_{11}NO$ (M^+ 197) mp 245^0 from the roots of *Murraya koenigii*.



Its IR spectrum showed bands at 3520 (Phenolic OH) 3400 (-NH) 1635 and 1600 (aromatic system). It has a UV spectrum (λ_{max} 235, 254, 258, 304 and 332 nm; log ε 4.65, 4.25, 4.26, 4.19 and 3.66) suggestive of a carbazole skeleton. The nmr spectrum showed signals at δ 8.2 (bs, 1H, NH) δ 8.1 (s, 1H, OH) δ 8.0-7.1 (complex multiplet, 4H, aromatic proton) δ 7.68 (s, 1H, H-4) δ 7.0 (s, 1H, H-1) δ 2.33 (s, 3H, aromatic methyl).

Srivastava *et al.* (1993) isolated three new compounds, marrayenol, murrayagetin and marmesin-1-0-rutinoside from the root extractof *M. koenigii*.

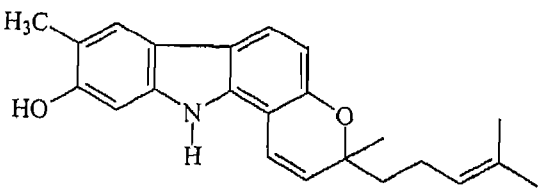


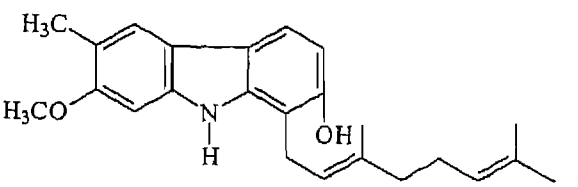


2.2.4 Fruits

Two new carbazole alkaloids Isomahanine and murrayanol were isolated from the fruits of *M. koenigii* by Johannes *et al.* (1992).

These two were isolated along with the five previously reported carbazole alkaloids mahanimbine, murrayazolidine, girinimbine, koenimbine and mahanine. The structure of Isomaharine and murrayanol were confirmed by the formation of 9-methoxymahanimbicine on their methylation and cyclization respectively.





2.2.5 Leaves

Naturally occurring carbazole alkaloids were reported from the plants belonging to the genera Murraya, Glycosmis and clausena of the family Rutaceae. So, far the genus Murraya has been found to elaborate simple carbazoles with a

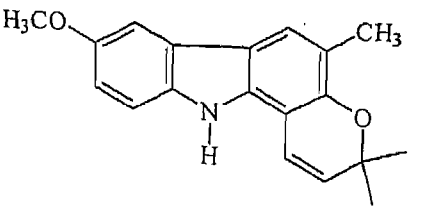
* C_{13} skeleton
* C_{18} skleton
* C_{23} skeleton

Members of C_{13} skeleton group

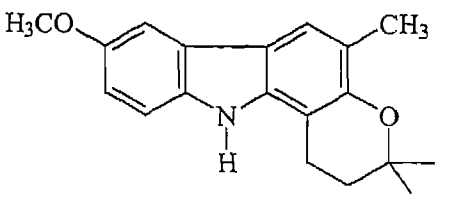
This group of carbazole alkaloids are not so far found in the leaves. However, they were mostly isolated from the stem bark of *M. koenigii* (L.) spreng.

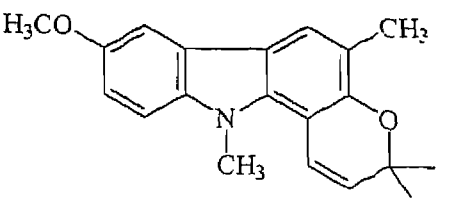
Members of C_{18} skleton group

Two groups of workers [Narasimhan *et al.* (1968) & Kureel *et al.* (1969)] isolated this base $C_{19}H_{19}NO_2$, mp. 194-195° (Mol wt. 293 by mass spectrum) from leaves was optically inactive and shown to be identical with koenimbine.

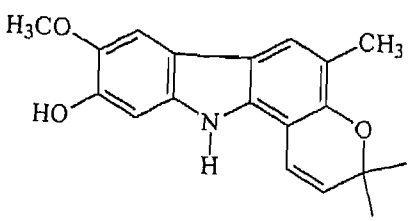


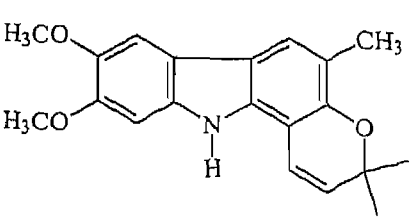
The following derivatives were prepared : Dihydrokoeminbine, m.p. 249°C N-methyl koenimbine, mp. 148°C.





Other carbazole alkaloids e.g. koenigine $C_{19}H_{19}NO_3$ (M^+ 309) m.p. 183-5°C and koenidine $C_{20}H_{21}NO_3$ (M^+ 323) m.p. 224-25°C were isolated from the leaves by three group of workers [Kureel *et al.* (1969); Joshi *et al.* (1970); Narasimhan *et al.* (1970)]



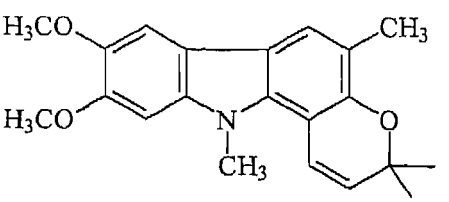


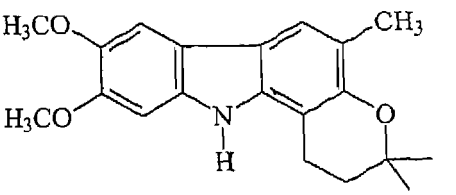
Derivatives

Koenidine on methylation with methyl iodide and sodium hydride in dry benzene gave N-methyl koenidine $C_{21}H_{23}NO_3$, m.p. 189°C. Other derivatives of koenidine prepared by catalytic hydrogenation were :

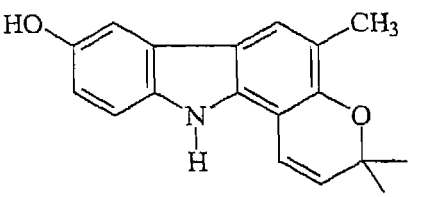
Dihydrokoenidine, m.p. 232°C

N-methyl dihydrokoenidine, m.p. 193-194°C

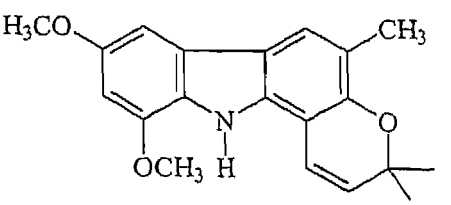




Another member of C_{18} carbazole alkaloid group namely Koenine $C_{18}H_{17}NO_2$ (M^+ 279) m.p. 250-252°C was isolated by Narasimhan *et al.* (1970) from the leaves. This compound gave a positive $FeCl_3$ test for phenols. The assignment of the structure was mainly based on nmr data.



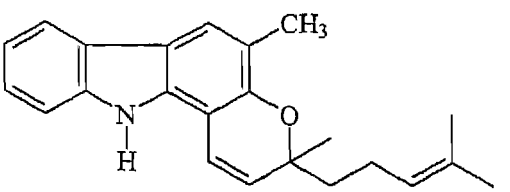
Mukonicine $C_{20}H_{21}NO_3$ (M^+ 323) was isolated from the alcoholic extract of the leaves of *M. koenigii* spreng (Mukerjee *et al.*, 1983).



Members of the C_{23} skleton group

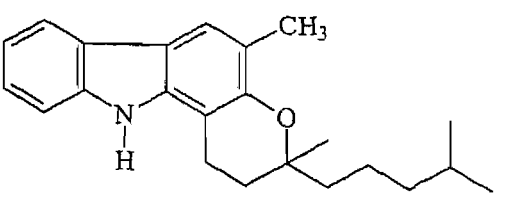
Mahanimbine $C_{23}H_{25}NO(M^+ 331)$ m.p. 94-95°C, the first member of the C_{23} carbazole alkaloid group was isolated from the stem bark by Chakraborty *et al.* (1966). Later on it was isolated from the leaves of *M. koenigii* [Narasimhan *et al.* (1968)]

IR spectrum of mahanimbine showed band for an NH-function, an aromatic C-Me group, double bond and an aromatic residue. UV absorption spectrum showed the presence of pyranocarbazole chromophore in the alkaloid.

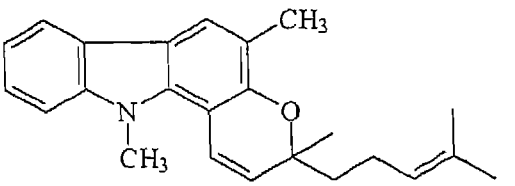


Derivatives

On hydrogenation at 29°C using 5% Pd/C (0.4 gm) as catalyst, mahanimbine (dissolved in ethanol) gave tetrahydro derivative $C_{23}H_{29}NO$ m.p. 108°C.

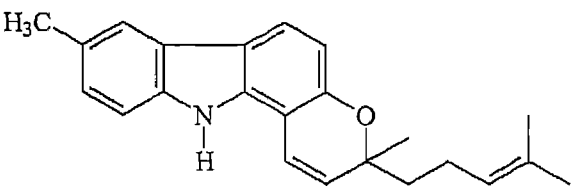


On methylation with dimethyl sulphate in the presence of NaOH, mahanimbine gave N-methyl derivative ($C_{24}H_{27}NO$) m.p. 137°C which was obtained after filteration, washed, dried and crystallized from ethanol.



Structure of mahanimbine have been proposed mainly on the basis of interpretation of nmr and UV spectra of tetrahydro mahanimbine $C_{23}H_{29}NO$ m.p. 108°C [Narasimhan *et al.* (1968)].

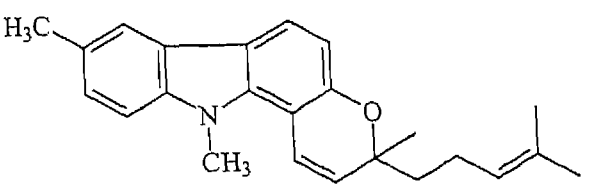
Isomahanimbine was isolated by fractionation of the mother liquor of the leaf extract after the removal of mahanimbine [Joshi *et al.* (1970) and Kureel *et al.* (1970)]



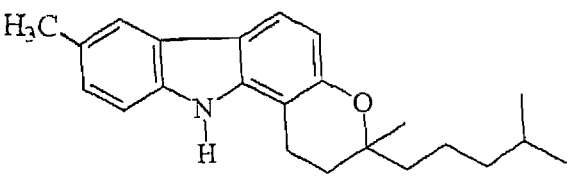
Structure of isomahanimbine was confirmed by synthesis [Kureel *et al.* (1970)].

Derivatives

N-methyl isomahanimbine $C_{24}H_{27}NO$ m.p. 94°C was prepared by dissolving isomahanimbine in acetone and shaken for 15 minutes with NaOH (0.5 gm in 1 ml H_2O) and then for a further 15 minutes after the addition of dimethyl sulphate (1 ml) and worked up as in mahanimbine.

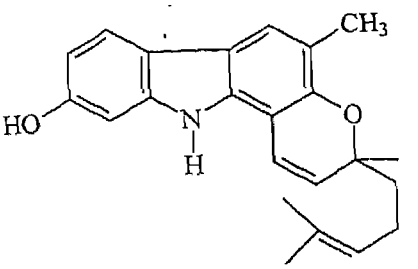


Tetrahydro isomahanimbine ($C_{23}H_{29}NO$) m.p. 166-167°C was obtained by hydrogenating the isomahaninbine (in ethanol)at 29°C using 5% Pd/C (0.2 gm) as catalyst.



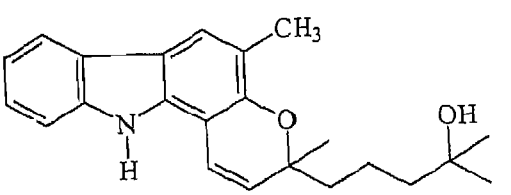
Narasimhan *et al.* (1970) isolated mahanine $C_{23}H_{23}NO_2$ (M^+ 247) m.p. 100°C from the leaves. The UV and IR spectra suggested the presence of the pyranocarbazole system.

The nmr spectrum showed that like mahanimbine it had three high field methyl singlets indicating the presence of a monoterpene unit substituted on the pyran ring.



The structure have been confirned by synthesis of (±) 0-methyl mahanine (Popli, 1972).

Mahanimbinine $C_{23}H_{27}NO_2$ (M^+ 349) m.p. 179°C another carbazole alkaloid isolated from the leaves (Kureel *et al.*, 1970) of *M. koenigii*.

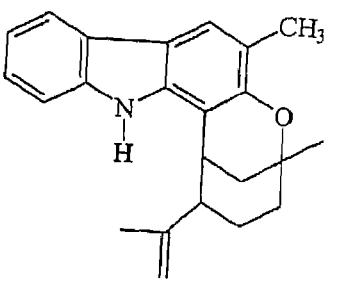


(43)

The IR spectra of this compound showed the presence of an alcoholic hydroxyl (3580 cm^{-1}) function. The structure of mahanimbinine was confirmed by partial synthesis.

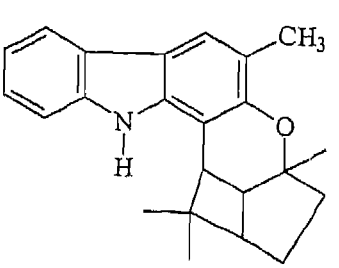
Mahanimbine on treatment with m-chloroperbenzoic acid furnished the expoxide which on reduction with $LiAlH_4$ furnished mahanimbinine, mp 148°C.

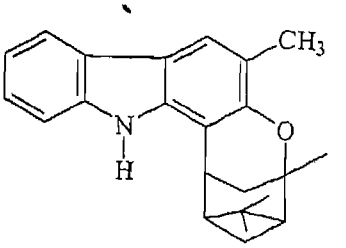
Two groups of workers [Kureel *et al.* (1969) and Dutta *et al.* (1969)] isolated the racemate of murrayazolidine [$C_{23}H_{25}NO$ (M^+ 331) m.p. 143°C stem bark] called cyclomahanimbine or curryanine from the leaves of *M. koenigii* spreng.



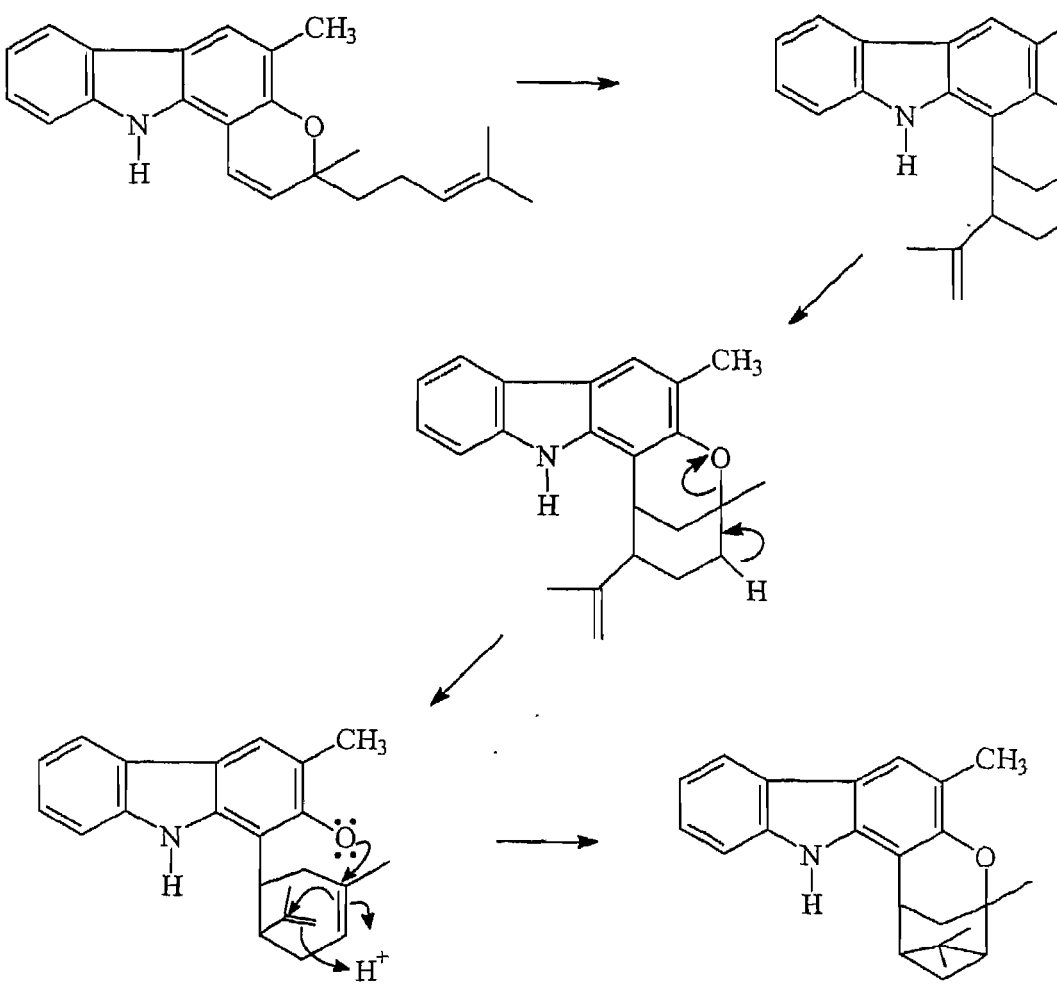
Cyclomahanimbine $C_{23}H_{25}NO$ (M^+ 331) m.p. 146°C was shown to have UV spectrum (λ_{max} 251, 257, 307 & 241 nm) very similar to that of 2-methoxy carbazole indicating the ether linkage at the 2-position of the carbazole nucleus.

Bicyclomahanimbine $C_{23}H_{25}NO$ (M^+ 331) m.p. 145°C has been isolated by Kureel *et al.* (1969) from the petroleum ether extract of *M. Koenigii* leaves and proposed either of two structures.





Structure (A) was preferred because of the conversion of mahanimbine to bicyclomahanimbine on silica gel.



2.2.5.1 Volatiles

The chief components of the essential oil obtained from the leaves by hydrodistillation were isolated by Dutt (1958). They were as follows

dl-phellandrene	(4.6%)
d-Sabinene	(9.2%)
d-α-pinene	(5.5%)
β-Caryophyllene	(26.3%)
dipentene	(6.8%)

Essential oils from curryleaf have also been studied by Nigam & Purohit (1961) and Macleod & Pieris (1982).

Macleod and Pieris (1982) reported that the oil comprised of monoterpene hydrocarbons (16%) and sesquiterpene hydrocarbon (80%) with β-caryophyllene (28.7%), β-gurjunene (21.4%), β-elemene (6.8%) and β-phellandrene (6.1%) as the major components among the 27 compounds positively identified. Leaves of *M. koenigii* from china have been shown by Liquan *et al.* (1988) to contain α-pinene (38.4%), β-pinene (6.3%), β-caryophyllene (12.9%), and γ-elemene (10.1%) as the main constituents with monoterpenoids constituting slightly in excess of 50% of the oil.

However, the plant materials obtained from trees grown in village garden in penang Malaysia by Wong and Tie (1993) identified 62 compounds constituting 96.9% of oil with β-phellandrene (24.4%), α-pinene (17.5%) and β-caryophyllene (7.2%) and Terpinen 4-ol (6.1%) as the major constituents.

The polar components present in the essential oil have not been investigated until now. For this reason Wagner (1995) subjected the

essential oil obtained by steam distillation from the leaves of *M. koenigii* to adsorption chromatographic isolation [MPLC, Kieselgel 60, 460 x 26 mm, gradient elution : 500ml $CHCl_2$ - $CH_3COC_2H_5$-$CH_3COOC_2H_5$ 97:3, 500 ml $CHCl_2$-$CH_3COOC_2H_5$ 90:10, 500 ml $CHCl_2$-$CH_2COOC_2H_5$ 50:50] of the alcohol fraction which was separated into the terpene and sesquiterpene alcohol groups by partition chromatography. Medium pressure chromatography (MPLC) yielded two substances [0.89% (1) and 0.33% (2)] structural elucidations revealed that they were *Kongol*(1) (11-selinen 4α, 5α, 7β, 10β 4-ol or eudesmen 4α ol) and the azulene derivative globulol (2) (10-aromadendranol)

Hiremath *et al.* (1996) revealed 47 components in the essential oil of wild curryleaf. The major components of oil identified were β-caryophyllene (30.1%) and β-pinene (28.57%) followed by β-gurjunene (6.3%) sabinene (3.67%), limonene (2.67%), linalool (0.82%) and β-phellandrene (0.18%).

Singh and Maheshwari (1997) showed that Super Critical Fluid extraction using liquid carbondioxide is convenient and superior in terms of both quantity as well as qualitative properties of the leaves extract as compared to traditional hydrodistillation process.

The variation in the composition of essential oil constituents as we have seen in different experiments may be due to botanical ambiguity or climatic conditions of grown area.

2.3 Identification of carbazole alkaloids

There is a considerable interest in the chemistry of carbazole akaloids and thin-layer chromatography has been found to be suitable for their separation and identification

Carbazoles could be detected on the developed chromatogram by their fluorescence or quenching under UV light as well as from the colours developed by spraying with picric acid, DDQ or HCl.

It has been shown by Roy and Chakraborty (1974) that dichlorodicyano benzoquinone (DDQ) could form a charge transfer complex with carbazoles giving rise to characteristic absorption in the visible region of these substances. DDQ has been used with success as a spray reagent for carbazoles and their detection on developed TLC plates.

In search for a better spray reagent for carbazole alkaloids, Roy *et al.* (1981) examined the behaviour of $FeCl_3$, $SbCl_3$ and conc. HCl as a spray reagents for detecting carbazole alkaloids. In all cases, the results were found to be similar i.e. the coloured spots were detected (blue, violet, green).

Bhattacharyya *et al.* (1984) used benzoyl peroxide as an effective spray reagent for the detection of carbazoles. Carbazoles can be detected at level down to 0.05 μ gm with this reagent, whereas the minimum concentration varies from 10 μ gm to 0.1 μ gm in the case of previously used reagents.

2.4 Bioactivity

2.4.1 Biological properties of carbazole alkaloids and related compounds (Non-volatiles)

Carbazole alkaloids are biologically active displaying antibacterial, antifungal, insecticidal, anticancer antiyeast activity. Of the carbazole alkaloids girinimbine, mahanimbine and murrayanine have antifungal activities (Das *et al.*, (1965)]. The antibiotic activities of some carbazole alkaloids e.g. glycozoline and related compounds were also evaluated (Chakraborty *et al.* (1975).

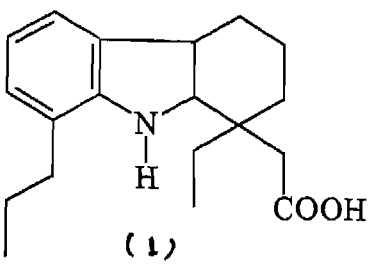
According to the study made by Chowdhury *et al.* (1978) revealed that tetrahydro carbazoles were more toxic to houseflies than the corresponding carbazoles.

Chowdhury and Das (1979) further studied the toxicity of tetrahydro carbazole, carbazole, glycozoline and glycozolidine along with extract of root-bark of *Glycosmis pentaphylla* (Retz) DC (which contain two carbazole alkaloids - glycozoline and glycozolidine) on mosquito larvae (Culex sp). The percentage of mortality of houseflies (*Musca domestica*) at different concentrations of tetrahydrocarbazole and 2-methyl tetrahydro carbazole were also presented in their study.

Antifungal and antibacterial properties of some carbazoles isomeric with glycozoline and girinimbine were studied by Randelia and Patel (1982) but the compounds showed little activity. The authors suggested that symmetrical disposition of the methyl and hydroxy groups on the carbazole ring is essential for maximum antifungal activity.

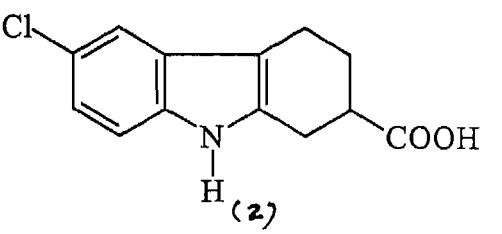
Some tetrahydro carbazoles had low insecticidal properties whereas some substituted 1,2,3,4-tetrahydro carbazoles were found to be active against *Trypanosoma cruzi* (Chowdhury *et al.* (1978)].

Some acidic tetrahydro carbazoles have been shown to have anti-inflamatory activity [Pecca and Albonico (1970)]. Thus 1- ethyl-8 n. propyl 1,2,3,4-tetrahydrocarbazole-1-acetic acid (1) is a novel antiinflammatory agents.



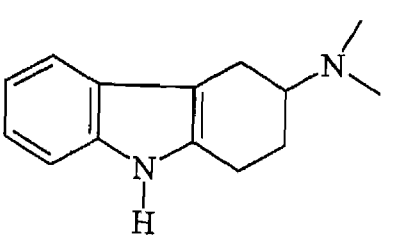
(1)

6-Chloro 1,2,3,4-tetrahydro carbazole -2-carboxylic acid (2)was found to be clinically active in the treatment of acute gout.



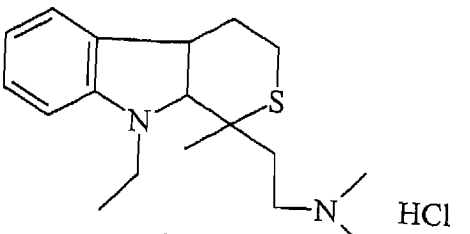
(2)

3-Dimethyl amino- 1,2,3,4-tetrahydro carbazole (3) which has a modified tryptamine structure prevented amphetamine induced stereotyped behaviour in rats and prevented reserpine-induced ptosis in mice (Mooradian *et al.*, 1977)



(3)

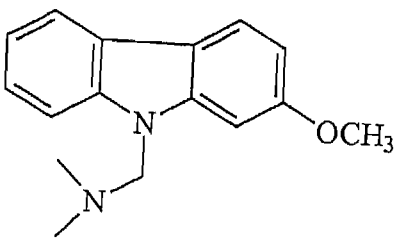
Another potent analogue was 9-ethyl-N,N,1-trimethyl-1,2,3,4 tetrahydrocarbazole-1- ethanamine (4).



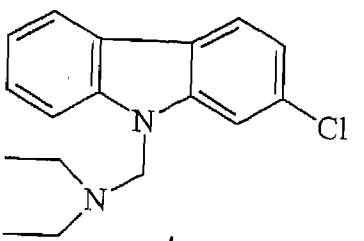
(4)

N-alkylamino carbazoles (5,6) possess significant anticonvulsant and diuretic activity [Shoeb *et al.* (1973)].

Introduction of aminopropyl chain at the N-atom seems to enhance the anticonvulsant activity in combination with CH_3O- at positions 2, 3 and 4.

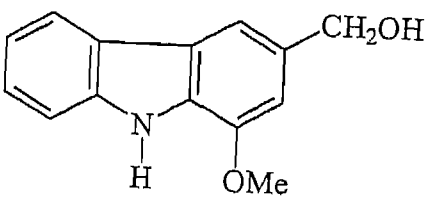


(5)



(6)

Koenoline (7) exhibited cytotoxic activity against the KB cell culture system (Fiebig *et al.*, 1985).

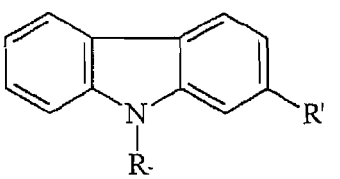


(7)

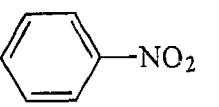
Another study on the toxicity of some N-substituted carbazoles with piperine and piperic acid on mosquito larvae (culex sp.) showed that

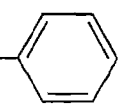
N-methyl, N-acetyl and 2, 9-diacetyl carbazoles and also piperine were highly toxic to mosquito larvae but piperic acid, N-benzoyl carbazole, N-p-nitrobenzoyl carbazole and carbazole were not so.

Hence replacement of NH proton of carbazole by suitable group makes it toxic.



(i) R = H, R' = H (Carbazole)

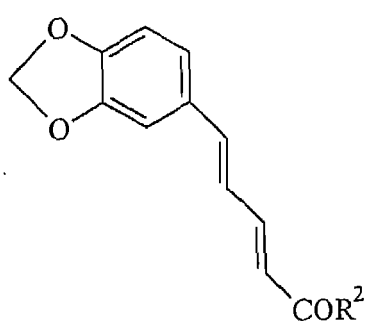
(ii) R =  , R' H [N-p-nitrobenzoyl carbazole]

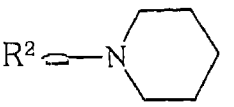
(iii) R =  , R'=H (N-benzoyl carbazole)

(iv) R = $COCH_3$, R' = $COCH_3$ (2,9, diacetyl carbazole)

(v) R = $COCH_3$, R'= H (N-acetyl carbazole)

(vi) R = CH_3, R'=H (N-methyl carbazole)



(vii)  (Piperine)

(viii) R^2 = OH (Piperic acid)

Further investigation of the toxicity of N-substituted carbazoles to mosquito larvae (culex sp.) revealed that replacement of the NH-proton by simple groups e.g. methyl, acetyl enhanced the larvicidal properties but the presence of a long chain like the cinnamoyl group did not enhance the toxicity of carbazoles (Das, 1989).

Antibacterial, anti-inflammatory and antifeedant activity of root extract of *M. koenigii* spreng were evaluated (Srivastava and Srivastava, 1993). The study revealed that antibacterial activity of ethanolic extract of root was found to show positive effect on all the tested organism e.g. *Vibrio cholerae* (Vc), *Salmonella typhimurium* (ST) and *Staphyllococcus aureus* (SA). Streptomycin was used as a standard. The anti-inflammatory activity of root extract was evaluated in albino rats (100-120 g, fasted overnight). The percentage inhibition of inflammation after 5 hours was calculated, oxyphenbutazone was used as a standard. And the antifeedant activity of the extract was determined against 3rd instar grub stages of brinjal bettle. *Henosepilachna vigitioctopunctata* Fabricus.

2.4.2 Biological properties of essential oil (Volatiles)

The terpenes and in particular the lower volatile members of the class have been known from antiquity, having been isolated from plants and used for a variety of human purposes (although seldom as pure compounds) since pre-christian times.

Their volatility which made them easily discoverable in fragrant plant material and at the sametime readily obtainable bysimple distillation of leaves lent to them the term essential oil. Various properties of the oil, such as the fungicidal nature, broad fungitoxic spectrum and absence of phytotoxicity to crop plant suggest that it may be useful as an effective fungitoxicant (seed dresser) against many fungi e.g. *Rhizoctonia solani*, *Alternaria alternata*, *Aspergillus niger*, *R. bataticola* etc.

Kishore *et al.* (1983) extracted the leaves of 22 taxa of Gorakhpur (U.P.) with distilled water (1:1 w/v). The extracts were screened for their volatile fungitoxicity by inverted petri plate method. Only the leaf extract of *Lippia alba* caused complete inhibition of mycelial growth of *Rhizoctonia solani*. However, extracts of 15 other plant species viz. *Annona squamosa*, *Cannabis sativa*, *Citrus aurantifolia*, *Erigeron bonariensis*, *Eupatorium* sp., *Euphorbia geniculata*, *Heliotropium indicum*, *Lantana camara*, *Tagetes errecta*, *Ocimum santum*, *Parthenium* sp. *Sonchus oberaceous*, *Solanum nigrum*, *Tectona grandis* and *Murraya koenigii* spreng also exhibited very strong fungitoxicity showing 82-95% inhibition of mycelial growth.

Tripathi *et al.* (1982) tested the pollens of 54 plants species belonging to 26 families for their fungitoxicity against *Helminthosporium oryzae* Breda de Haan causing leaf blight of paddy.

Out of 54 plants tested only the pollen suspension of *Xanthium strumarium* inhibited the spore germination completely. The pollen suspension of *Acacia arabica*, *A. auriculiformis*, *Antiganon leptopus*, *C. modosa*, *Lantana camara var.* aceleata, *Murraya koenigii spreng* etc. showed marked stimulatory action on the fungal spore germination.

%age inhibition of fungal spore germination was recorded after 24 hrs of incubation at 28 ± 1°C and calculated as per formula

$$\% \text{ inhibition of fungal spore germination} = \frac{Nc - Nt}{Nc} \times 100$$

Nc = Average number of fungal spore germinated in control

Nt = Average number of fungal spore germination in treatment

Pandey and Dubey (1991) showed volatile fungitoxicity of the leaves of *H. suaveolens*, *M. koenigii* and *O. canum* against *Rhizoctonia solani* during a screening of 27 angiospermic taxa

The volatile fungitoxic factors were isolated in the form of essential oils from the leaves of the most active plants viz. *H. suaveolens*, *M. koenigii* and *O. canum* by subjecting the leaves to hydrodistillation through clevenger's appratus.

The inhibition of the test fungus at different concentrations of essential oils was determined by the poisoned food technique of Grover and Moore (1962) using potato dextrose agar medium.

In another set of experiment four concentrations viz. *H. suaveolens* + M. koenigii (Hyp + Mu) *H. suaveolens* + *O. canum* (Hyp + Oci), *M. koenigii*+ *O. canum* (Mu + Oci)and *H. suaveolens* + *M. koenigii* + O. canum (Hyp + Mu + Oci) from the three oils of *H. suaveolens*, *M. koenigii* and *O. canum* were prepared by mixing the individual oils in equal amounts. These mixtures were similarly tested at different concentrations against the test fungus.

During fungitoxicity studies the minimum inhibitory concentration (MIC) of essential oil of *H. suaveolens* and *O. canum was* 3000 ppm

and 500 ppm respectively. it was 5000 ppm and 4000 ppm for Agrosan GN and captan respectively. Thus these oil were superior to the synthetic fungicides.

However, *M. koenigii* oil (MIC-5000 ppm) was as effective as agrosan GN but less efficaceous than captan.

All four combinations showed higher potency than the individual oils. In case HyP + Mu combination. the MIC was 500 ppm while it was 300 ppm for Mu + Oci and Hyp + Mu + Oci combination.

The Hyp + Oci combination showed the strongest efficacy since it was absolutely effective even at 100 ppm.

These oil combinations were then several times more efficaceous than the synthetic fungicides tested.

Murraya koenigii (Linn.) Spreng. is a Rutaceae plant which is ubiquitous in Southern India. The bark, roots and leaves contain alkaloids and essential oil and are successfully used for febrile disorders. dysentery and diarrhoea in Ayurvedic and Yunani medicines. The essential oil is considered to be responsible for antibacterial and fungicidal activity. Since the report of the first carbazole alkaloid. murrayanine from *Murraya koenigii* and the antibiotic properties of these alkaloids in 1975 by Chakraborty *et al.* there has been wider interest on the structure and biochemical properties of these compounds.

The present review relates the advances in the field of chemistry of carbazoles and essential oil from *Murraya koenigii* and the investigation of their various medicinal and biochemical properties as well. The present

work is taken up as there is no report on biological activities e.g. antinemic, antifeedant, insect growth regulatory, antifungal, repellant and larvicidal of the chemical constituents extracted and isolated from the leaves of *M. koenigii*. The chemical constituents namely corbazole alkaloids are derivatized and further studied for antinemic, antifeedant and fungitoxic properties.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and reagents

Carbazole alkaloids and essential oil the test chemicals were isolated and extracted respectively from *Murraya koenigii* spreng. Extraction and isolation procedures are given in the methodology section.

Other reagents and chemicals required for the study were of analytical reagent grade and purchased from the local market. The organic solvents used in chromatography, crystallisation and derivatization were of commercial grade and distilled before use.

3.1.2 Chromatography

3.1.2.1 Thin layer chromatography (TLC)

TLC plates were prepared by spreading a slurry of silica gel G containing 10% gypsum as a binder in water on 20 cm x 5 cm and 20 cm x 20 cm glass plates uniformly using TLC applicator. The thickness of silica gel layer on the plates was maintained at 0.25 mm for identification and 1.0 mm for preparative work. The plates were activated in an oven for two hours at 110^0C. The sample solutions were spotted on the TLC plates using capillary tubes. The plates whenever needed were developed in the following solvent system :

Hexane (100%), Hexane - Benzene (1:1, v/v), Benzene (100%), Benzene-chloroform (9:1, v/v), Benzene chloroform (1:1 v/v) and chloroform (100%).

The developed plates were air dried for five minutes and then visualized by iodine vapour or spraying with 5 per cent vanillin-sulphuric acid mixture. The plates after spraying with vanillin sulphuric acid (5 %) were heated on a hot plate or in an oven at 70-80^{0}C for 10 minutes. When spots appeared the spots were marked on the plates and Rf values were determined by using following formula :

$$Rf = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent}}$$

3.1.2.2 Column chromatography

Carbazole alkaloids were separated and purified by column chromatography using glass column of size (ranging from 1.0 cm to 1.5 cm i.d. and 100 cm to 150 cm in length) containing 60-120 mesh pre activated silica gel. The column was successively eluted with hexane, hexane-benzene, benzene, benzene-chloroform, and chloroform. The fractions were concentrated under reduced pressure in a rotatory vacuum evaporator and monitored by TLC and GC. The fractions of similar Rf values were mixed together.

3.1.2.3 Gas liquid chromatography

The qualitative and quantitative analysis of carbazole alkaloids and essential oil were carried out by Hewlett Packard 5890 A series-II gas liquid chromatograph fitted with flame-ionization detector (FID) using following conditions :

Glass column (2 m x 2 mm i.d.) was packed with OV-17 (3%) on chromosorb W (used as stationary phase) with temperature programming 80^0C - 260^0C @ 10^0 / min. Injector and detector were maintained at a temperature of 250^0C. Nitrogen (IOLAR) was used as carrier gas with flow rate of 30 ml/min.

3.1.3 Spectroscopy

3.1.3.1 Ultra-violet spectra were recorded on HITACHI U 2000 spectrophotometer

3.1.3.2 Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectra were recorded on Nicolet impact ` 700 FT-IR spectrophotometer either as solution in carbontetrachloride or chloroform or using nujol mull or KBr disc.

3.1.3.3 Proton magnetic resonance spectroscopy (PMR)

The proton magnetic resonance (PMR) spectra were recorded on a varian EM-360 L, 60 MHz instrument using duteriochloroform ($CDCl_3$) as solvent containing tetramethyl silane (TMS) as internal standard. The chemical shifts are expressed in δ values and coupling constant (J) are given in hertz (Hz) Notation used for splitting pattern are :

s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and bs = broad singlet.

3.1.3.4 Gas-chromatography-Mass spectroscopy (GC-MS)

A HRGC-MEGA 2 series gas chromatography coupled to a FISON TRIO 1000 ion trap mass spectrophotometer and connected with a panasonic KX-P 1150 multi-mode printer was used. The ionization potential was 70 eV. Gas chromatography was equipped with a SE-54

capillary column (15 m x 0.25 m i.d., film thickness 0.1-0.15 μ). The GC conditions were as follows :

Initial temperature 70°C for 1 minute and then heated upto 250°C (15 minutes) @ 10°C/min Helium was used as carrier gas with a flow rate 2 ml/min.

3.1.4 Hydrodistillation and supercritical fluid extraction technique (SCF)

Essential oil was obtained by hydrodistillation of fresh leaves of *Murraya koenigii* in clevenger's apparatus (Guenther 1949) and by supercritical fluid extraction process . SCF extraction using liquid CO_2 is convenient and superior in terms of both quality and quantity.

3.1.5 Melting points

All melting points were recorded with electrical melting point appratus in an open capillary method and are uncorrected and reported in degree celsius.

3.1.6 Elemental analysis

C, H and N analysis were carried out on Perkin-Elmer model 2400 CHN element analyzer by Dumas total combustion process using acetonitrile as standard. The observed values were within ± 0.5% of the theoretical values. The elemental analysis was done at the division of National facility for Blue Green Algae (NFBGA), IARI, New Delhi-12.

3.1.7 Material required for derivatization of pure compounds

Methyl iodide, Sodium hydride, dimethyl sulphate, 5% palladised charcoal.

3.2 Methods

3.2.1 Extraction of essential oil

Method Ia : The fresh leaves (1 kg) of wild curryleaf plant (*Murraya koenigii*) were collected from IARI campus in 1996 and hydrodistilled in clevenger's appratus for 6 hrs. The moisture was removed by keeping the oil in a dessication over fused $CaCl_2$ overnight. The yield of oil obtained was 0.2% (w/w). The moisture free oil was analysed by GC-MS.

Method Ib : For supercritical extraction process air dried leaves (1 kg) of *Murraya koenigii* were crushed to powder using mixer which were then sieved and particle size of 0.5 mm was packed in extractor thimble using glass wool on both the ends. The extractor was then connected to the rest of assembly and liquid CO_2 was passed through the sample present in extractor thimble. The desired supercritical temperature (20^0C) was maintained with the help of water thermostat and air-driven pump which was used to push liquid carbondioxide to maintain pressure (100 bar). The oil from sample was collected and weighed (0.89% w/w). The oil was analysed on GC-MS and the major components of the oil were identified and enlisted in next chapter.

3.2.2 Extraction and isolation of carbazole alkaloids

Fresh leaves collected from a single plant in Indian Agricultural Research Institute (IARI) campus were dried in shade, powdered (2.0 kg) and extracted twice in cold with petroleum ether (40-60^0C, one litre) to remove fatty subsances. The leaves were then dried and re-extracted with 95% ethanol (2.5 litres) to get a dark coloured viscous residue (21 gm) on removal of solvent under vacuum.

The viscous residue was then re-extracted with hexane, benzene, ethyl acetate and methanol. The *hexane* and *benzene* extracts containing non-volatile constituents were subjected separately to column chromatography over a silica gel (column height 150 cm, i.d. 1.5 cm).

Each column was eluted with hexane, hexane-benzene, benzene, benzene-chloroform and chloroform with increasing proportions.

The ethyl acetate and methanol extracts were obtained in insignificantly low quantities therefore could not be used for further isolation work.

Hexane extract (5.25 gm)

The column fractions (100 ml each) obtained from the hexane extract were subjected to TLC and fractions having similar Rf value were combined.

1. Fractions 9-25 (Eluent - 1:1 hexane - benzene) were combined and the solvent distilled off. The oily residue on crystallization from hexane gave mahanimbine (176 mg) m.p. 93^{0}C.

UV (EtOH) ≡ 223, 239, 288, 330 and 343 mm

IR (Nujol) ≡ 3320, 1640, 1325, 1250, 1140, 900, 840, 745 cm^{-1}

Found C 83.9%; H 7.7% mol wt. by mass spectrum 331

Calc. for $C_{23}H_{25}NO$: C 83.3%, H 7.6%, mol wt. 331

2. Fractions 28-36 (Eluent:benzene) were combined and the solvent distilled off. This gave residue which on crystallization from methylene chloride-hexane (1:9) gave isomahanimbine (143 mg), m.p. 142^{0}C.

UV (EtOH) (λ_{max}) ≡ 225, 238, 281, 290, 335 and 354 nm

IR (Nujol) (υ_{max}) ≡ 3440, 1638, 1600, 1338, 1230, 1170, 910, 805, 745 cm^{-1}

Found : C 83.4%; H 7.8%; N 4.5% mol wt. by mass spectrum 331

$C_{23}H_{25}NO$ requires : C 83.3%; H 7.6%; N 4.2% mol wt. 331

3. Fractions 39-42 and 43-50 [Eluent : Benzene-chloroform (1:1) and chloroform] gave a residue on removal of solvent from the above fractions. The obtained residue on crystallization from ethanol gave cyclomahanimbine (39 mg).

UV (EtOH) (λ_{max}) ≡ 246, 251, 257, 307 and 341 nm

IR (Nujol) (υ_{max}) ≡ 3425(-NH), 1615 and 1602 cm^{-1} (aromatic system)

Found : C 83.5%; H 7.7%; N 4.5% mol wt. by mass spectrum 331.

$C_{23}H_{25}NO$ requires : C 83.3; H 7.6%; N 4.2%, mol wt. 331

Benzene extract (12.4 gm)

1. Fractions 8-15) [Eluent : Hexane - benzene (1:1)]

Fractions were combined and the solvent distilled off. It gave yellowish oily residue which was treated with n-hexane (10 ml). A white solid (62 mg) that separated was filtered and washed with cold n-hexane (2 x 5 ml) and dried in vacuum. This on re-crystallization from n-hexane-ethyl alcohol (98:2) gave koenigine as white needles, m.p. 183-85^{0}C, M^+ 309.

UV (EtOH) (λ_{max}) ≡ 243, 260, 290, 344 and 357 nm

IR (nujol) (υ_{max}) ≡ 3530, 3440, 1650, 1300, 1210, 1120, 850, 770 cm^{-1}

NMR (δ value) ($CDCl_3$) ≡ 2.2 (s, 3H, Ar-CH_3)

3.8 (s, 3H, Ar-OCH_3)

8.78 and 10.64 (bs and s 1H each, NH and Ar-OH)

MS ≡ m/z : 309 (M^+) M.F. - $C_{19}H_{19}NO_3$

294 (m-15) aromatization of chromene ring by the loss of methyl group;

269 (M-30) further the loss of -OMe group.

Found : C 73.76%; H 6.36; N 4.08;

$C_{19}H_{19}NO_3$ requires : C 73.36%, H 6.19%; N 4.53%

2. Fractions 18-24 (Eluent:Benzene)

On removing the solvent of combined similar fraction under vacuum yielded a white solid (69 mg) which crystallized from hexane to give white shining crystals of koenine, m.p. 250-52^{0}C M^+ 279.

UV (λ_{max}) ≡ 236, 296, 335 and 356 nm

IR (υ_{max}) ≡ 3350, 32330, 1650, 1215, 1110, 890, 790, 720 cm^{-1}

NMR (δ-value) ≡ 1.42 (s, 6H, -0-C $(CH_3)_2$]

2.22 (s, 3H, Ar-CH_3)

5.73 (d, J=10Hz, 1H, C-3)

7.55 (s, 1H, C-4)

8.76 and 10.72 (s, 1H each NH & Ar-OH)

MS ≡ m/z : 279 (M^+) MF-$C_{18}H_{17}NO_2$

264 (M-15) aromatization of chromene ring by the loss of methyl group.

Found : C 77.80%; H 6.12%; N 4.80%,

$C_{18}H_{17}NO_2$ requires : C 77.39%, H 6.13%, N 5.01%

3. Fractions 28-48 (Eluent:Benzene-chloroform (1:1)

The similar fractions (each 100 ml) were combined and the solvent distilled off. The obtained residue which on crystallization from $CHCl_3$-hexane (2:8) gave koenimbine (623 mg), m.p. 193°C

UV (EtOH) (λ_{max}) ≡ 237 nm and 296 nm

IR (KBr) (υ_{max}) ≡ 3406, 2980, 2920, 1643, 1643, 1610, 1548, 1490, 1457, 1380, 1295, 1209, 1140, 1110, 1024, 975, 748 cm^{-1}

Found : C 77.79%; H 6.84%; N 4.63;

$C_{19}H_{19}NO_2$ requires : C 77.79%; H 6.53%; N 4.77%

Mass ≡ m/z $C_{19}H_{19}NO_2$ (M^+ 293)

4. Fractions 48-62 (Eluent : Chloroform)

Gave a residue which on crystallization from ethanol gave koenidine (361 mg), m.p 224-25°C.

UV (ethanol) (λ_{max})	-	223, 237, 299 nm
IR (KBr) (υ_{max})	-	3421, 2927, 1650, 1630, 1485, 1216, 1163, 1110, 1064, 998, 874, 770, 720 cm^{-1}
NMR (δ-value)	-	1.42 (s, 6H, -0-C$(CH_3)_2$]
		2.4 (s, 3H, Ar-CH_3)
		5.75 (d, J=10 Hz, 1H, C-3'
		7.5 (s, 1H, C-5)
MS	-	m/z : 323 (M^+) M.F. = $C_{20}H_{21}NO_3$
		308 (M-15) aromatizationof chromene ring by the loss of methyl group.
		293 (M-30) further loss of another methyl group
Found	:	C 74.64%; H 6.81%; N 4.29%
$C_{20}H_{21}NO_3$ requires	:	C 74.28%, H 6.55%, N 4.33%

Fractions 64-86 (Eluent : Chloroform)

Gave a mixture of two compounds showing two spots with different Rf value on TLC plate (In solvent system - Benzene-chloroform (3:2)

This mixture (1.2 gm) was rechromatographed over silica gel (100 g, column height 50 cm, i.d. 1.5 cm) in chloroform. Fractions (each 100 ml) were monitored by TLC.

1. Fractions 5-12, on evaporation of solvent in vacuum and treatment with hexane (10 ml) yielded a solid (52 mg) m.p. 222-24°C, this on sublimation at 210°C (oil both) gave the crystals of koenidine, m.p. 224-25°C.

2. Fractions 15-20 from the rechromatography in chloroform were complex mixtures, solvent was removed to get gummy mass. Hexane was added to the gummy mass and the solution cooled overnight to give white lumps. These were filtered and recrystallized from petroleum ether to get white fluffy crystals of mahanine (46 mg), m.p. 98-100°C.

UV (Ethanol) (λ_{max}) ≡ 240, 295, 340, 360 nm

IR (KBr) (υ_{max}) ≡ 3440, 3325, 1630, 1600, 1210, 1165, 1080, 980, 825, 720 cm^{-1}

NMR (δ-value) ≡ 1.4 (s, 3H, -0-C-(CH_3)

1.5 & 1.6 (s, 3H each -C=C-(CH_3)$_2$]

2.3 (s, 3H, Ar-CH_3)

5.7 (d, J=10Hz 1H, C-3')

9.16 and 10.8 (bs and s 1H each NH & Ar-OH)

MS ≡ m/z : 347 (M^+) M.F.-$C_{23}H_{25}NO_2$ 332 (M-15) aromatization of chromene ring

264 (M-83) loss of C_6H_{11} side chain.

Found : C' 79.86%; H 7.60%

$C_{23}H_{23}NO_2$ requires : C 79.50%; H 7.25%

3.2.3 Identification of some carbazoles by TLC studies

A thin layer spread on glass plate of dimension 10 cm x 20 cm using silica-gel G as the absorbent was prepared. The chromatogram was developed in a solvent system of benzene and chloroform (1:1) by usual technique.

The carbazole alkaloids examined were isolated from *Murraya koenigii* spreng. After the development of chromotogram, the spots were detected by spraying first with conc. HCl. On heating the plate at 90^0C formation of colour on each of the spots was observed.

The coloured spots of different compounds along with Rf are given in table 1.

The same experiment was repeated successively using $FeCl_3$ and $SbCl_3$ as spray reagent and the result are also shown in the same table 1. The coloured spots could be detected at a minimum concentration of 1 µg of carbazoles.

Table 1. Colour developed after spraying the chromatograms with conc. HCl, with 5% $FeCl_3$ and 10% $SbCl_3$ soln. respectively

Compounds	Colour developed with			
	Conc. HCl	5% $FeCl_3$	10% $SbCl_3$	Rf value
Koenimbine	Pinkish violet	Ash blue	Pinkish violet	0.65
Koenidine	Pinkish violet	Greenish blue	Pinkish violet	0.25
Mahanimbine	Pink (dull)	Greenish blue	Bluish violet	0.93
Mahanine	Blue on prolonged heating	Greenish blue	Blue	0.79
Cyclomahanimbine	Bluish	Deep blue	Blue	0.89

3.2.4 Derivatization of carbazoles

3.2.4.1 N-methylation of C_{18}-carbozole

1. N-methyl koenimbine : Koenimbine (250 mg) on methylation with methyl iodide (1 ml) and sodium hydride (0.2 gm) in dry benzene gave N-methyl koenimbine (190 mg), m.p. 148°C

UV (EtoH) (λ_{max}) ≡ 242, 301, 339, 387, 371 nm

IR (KBr) (υ_{max}) ≡ 1640, 1570, 1460 cm^{-1}

2. N-methyl koenidine : Koenidine (210 mg) on methylation with methyl iodide (1 ml) and sodium hydride (0.2 gm) in dry benzene gave N-methyl koenidine (162 mg), m.p. 189°C.

UV (EtoH) (λ_{max}) ≡ 241, 303, 346, 361 nm

IR (KBr) (υ_{max}) ≡ 1640, 1610, 1590 cm^{-1}

3.2.4.2 Hydrogenation of C_{18}-carbazole

1. Dihydrokoenidine : Koenidine (160 mg) was dissolved in ethanol (40 ml) and hydrogenated at 29°C using 5% Pd/C (0.2 g) till no more hydrogen was absorbed. The catalyst was filtered off, the filterate concentrated and the residue crystallized from hexane (70 mg).

Dihydrokoenidine : m.p. 237°C

UV (EtoH) (λ_{max}) ≡ 215, 235, 267, 315, 323 nm

IR (KBr) (υ_{max}) ≡ 3410, 1610, 1305, 1260, 780, 900, 665 cm^{-1}

2. *Dihydrokoenimbine* : Prepared in the similar way as dihydrokoenidine

Dihydrokoenimbine : m.p. 249°C, yield - 65 mg

UV (EtOH) (λ_{max}) ≡ 243, 258, 312, 332, and 346 nm

IR (KBr) (υ_{max}) ≡ 3400, 1620, 1310, 1200 cm^{-1}

3.2.5 Bioassay techniques

3.2.5.1 Antifeedant bioassay

Experiments were conducted during September 1998. A stock culture of *spodoptera litura* (Fab.) was maintained in the laboratory

Rearing of test insect

The tabacco armyworm, *spodoptera litura* (Fab.) was reared in glass jar 20 cm x 10 cm size on castor (*Ricinus communis* Linn.) leaves. The jars were cleaned by removing the remnant leaves and excreta and were supplied with fresh and clean castor leaves. Only about one hundred larvae were kept in each jar so as to avoid overcrowding and unsanitary conditions which may result in fungal growth or development of disease. In this way a clean and disease free culture was maintained.

The fourth instar larvae were transferred into a wooden cage of 60 x 36 cm size having layer (10 cm) of sterilized soil at the bottom. The soil not only helped in the absorption of moisture from the excreta but also served as favourable site for pupation of larvae. Care was taken to clean the cages everyday and the larvae were supplied with fresh and clean castor leaves till pupation. The larvae pupated in earthen coccons after 12-14 days. The moths emerging after a week were collected and transferred to a clean jar (20 x 15 cm) containing a cotton wad soaked

with honey solution and pieces of folded paper for oviposition. The eggs laid on these papers were collected.

Those eggs on the same day were kept together in separate clean jars with castor leaves and were observed daily for hatching. Approximately twelve hour old neonate first instar larvae were removed and used as test material.

The cultures were maintained at 27 ± 1°C, 70-75% relative humidity and 16:8 light-dark and reared on castor leaves until transferred to experimental diets for bioassay studies.

Antifeedancy

A choice bioassay by the leaf disc method [Koul and Isman (1990)] was conducted. Two cm^2 discs were punched out from the castor, *Ricinus communis*, leaves and treated on each side by dipping in acetone solutions of the test compound. The control discs were treated with acetone alone. After drying at room temperature, two discs one treated and the other untreated (control) were placed in petridish (9 cm. dia.) and 5th instar larvae (X 5 replicates) prestarved for 1 hr was released into it. After 4 hr of larval release, leaf consumption was recorded by a digital leaf area meter (model 3100 Lincoin, Nebraska, USA). Percent feeding inhibition for each treatment was calculated.

$$\% \text{ feeding inhibition} = 1 - \frac{T}{C} \times 100$$

Where C = consumption of control disc

T = consumption of treated disc.

From % feeding inhibition data ED_{50} (effective dose which causes 50% antifeedance) values of few effective derivatives were calculated by probit analysis.

3.2.5.2 Antifungal bioassay

Essential oil (SCF & Clevenger), carbazoles (pure & crude) and derivatives of carbazoles were assessed for the fungitoxicity against *Rhizoctonia bataticola*, *Helminthosporium oryzae* and *Rhizoctonia solani*. Fungicidal activity of the test compound was assayed by poisoned food technique (Nene and Thapliyal, 1979) which involves studying the growth inhibition caused by the test compound with respect to a control chemical. The fungal strains of *R bataticola*, *H. oryzae*, and *R. solani* were obtained from Division of Mycology and plant pathology, Indian Agricultural Research Institute, New Delhi and spores were multiplied on potato dextrose-Agar medium (PDA)

Preparation of medium

Ingredients

The medium used for multipliction of fungal species, potato-dextrose-Agar (PDA) was prepared from the following ingredients

Potato	250 g	
Dextrose	20 g	
Agar agar	16 g	(Tuite, 1969)

The potatoes were peeled and chopped into small pieces and boiled in water (1 litre) till they became soft. The potato extract was stained. Glucose (20 g) and agar agar (16 g) were added to the extract. The

mixture was boiled till the agar formed a homogeneous translucent emulsion.

Sterilization

Adequate number of conical flasks (100 ml) were taken (one concentration for each compound) and to each 65 ml of the medium added. The mouths of the conical flasks were plugged tightly with cotton wool. Petridishes and conical flasks containing the medium were placed in an autoclave and sterilized under 15-17 psi pressure for 25-30 minutes. Streptomycin (2-3 drops of a 1000 ppm solution) was added to each flask after cooling to avoid contamination by bacterial growth.

Preparation of concentration of the chemicals

Required quantity (260 mg) of the test compound was weighed and dissolved in acetone. Requisite concentration of this solution was added to PDA medium in a conical flask and mixed well so as to get 1000 ppm, 2000 ppm, 3000 ppm and 5000 ppm. The entire process was carried out aseptically in a laminar flow chamber previously irradiated with UV light for 50 minutes.

Fungitoxicity evaluation

Inoculation

The contents of each conical flask were poured equaly into two petridishes. The medium was allowed to set under UV light in the laminar flow chamber. 5 mm discs of the fungi were transferred aseptically to the petridishes.

Incubation

Once the inoculation was complete, the petridishes were kept in a B.O.D. incubator at 25°C. The incubation period for the three fungii was 7 days each.

Recording of observations

Within 5-7 days, when the growth of the fungal colony in the control treatment had covered almost the entire petridish, the diameter of the fungal colony in each petridish was measured. The fungicidal activity was expressed as per cent inhibition over control by Abbot's formula

$$\text{Per cent inhibition} = \frac{C-T}{C} \times 100$$

Where C =diameter of control treatment

T =diameter of test compound

3.2.5.3 Antinemic bioassay

a) Isolation of juvenile stage II (J_2) of root knot nematode (Meloidogyne incognita)

Egg masses of root knot nematode were obtained from cultures maintained on egg plant (*Solanum melongena* L.) after two months of infection. These were kept on a layer of tissue paper supported on wire gauze for screening according to the method as detailed by Cobb (1920). Freshly hatched J_2 were collected from the suspension in water.

Tests *in vitro* were conducted against the freshly hatched second stage juveniles (J_2) of root knot nematode *Meloidogyne incognita*. Observations were taken after 24 h, 48 h and 72 h on mortality of J_2.

Mortality was confirmed first by transferring J_2 to water for revival test. If the J_2 were alive then transferred them to the new seedlings of soybean to check whether they were able to penetrate or not.

In vitro test

For *in vitro* screening, stock solution of 2000 ppm concentration of each compound (essential oil and carbazole alkaloids) was prepared in acetone, 2-3 drops of Tween-80 were added as emulsifier and the volume made upto 25 ml with distilled water. From this stock solution, double strength (DS) test concentrations of 1000, 500, 250, 125 and 62.5 ppm. were obtained (Sethi and Prasad, 1982).

One ml of nematode suspension containing about 50 freshly hatched J_2 were placed in 12 ml capacity glass vial and one ml solution of test compound containing double the desired concentration was added to each vial. Three replications were maintained. The loosely capped vials were incubated at 30 ± 1^{0}C for 72 hours. A control containing only acetone and tween-80 was similarly maintained.

Triazophos (Hostathion^R 40 EC) (0, 0-diethyl 0-1-phenyl-1H-1, 2, 4 trizole-3yl) phosphorothioate was used as standard. After the desired period of incubation, the number of live and dead larvae were counted with the aid of a steroscopic binocular microscope and mortality was confirmed as per Prasad (1989).

Percentage mortality was calculated from the average of three replications for each concentration and converted to natural mortality according to Abbott's formula. Corrected percentage mortality data were

fed to a BASIC LC_{50} programme version 1.1 (Trevors, 1981) to obtain LC_{50}.

3.2.5.4 Growth inhibition bioassay

The study was performed on 24 h old neonate (1st instar) of *spodoptera litura* were obtained from a laboratory running culture maintained at 27 ± 2°C, 70-75% relative humidity and 16:8 light:dark.

Preparation of diet

Artificial diet used in this study was based on a commercial diet (No. 9763, Bioserv Inc. French Town, New Jersey USA). Composition of diet is reported in Table 2.

Table 2 Composition of the artificial diet used in the bioassay studies

A.	Mixture		
	1.	Yeast powder	40 g
	2.	Ascorbic acid	13 g
	3.	Methyl p-benzoate	8 g
	4.	Sorbic acid	4 g
	5.	Streptomycin sulphate	1g
	6.	Vitamin mixture	5 g
		Total	71 g
B.	Agar agar		0.32 g
C.	Besan		2.63 g

Diet = A (0.45 g) + B + C + 19.0 ml water

Experimental diet were prepared by dissolving 5, 10, 20, 25 and 30 µl of essential oil (in five conc. 500, 1000, 2000, 2500, 3000 ppm) in a small volume of acetone and coating on to the dry constituents of the diet (A + C) and mixing thoroughly. When the carrier had completely evaporated, hot solution of agar-agar was poured into constituents of diet and stirred vigorously for uniform distribution of the active ingredient in the diet. The diet so formed was allowed to cake, maintaining optimum moisture. Control diet was similarly treated with carrier alone acetone.

Different concentrations of essential oil were mixed individually in respective diet in a similar fashion and given to first instar neonate *spodoptera litura* (Fab.) larvae.

For feeding various concentrations of the oil used were 500 ppm, 1000 ppm, 2000 ppm, 2500 ppm, 3000 ppm and 4000 ppm.

After cooling, the diet was cut into 5 pieces and were placed individually into 30 ml plastic cups to each of which were added 2 larvae. The cups were placed in plastic boxes lined with water soaker paper towels to maintain high humidity and boxes were kept in an environment chamber at 27 ± 2 ^{0}C and 16:8 LD. After 7 days, all larvae were weighed and mean weights for each treatment group were expressed as a percentage of control (% growth inhibition).

ED_{50} for growth inhibition

The experiment was done on 24 h old neonate larvae (Koule, Smirle and Isman, 1990). Required volume of acetone stock soln. of each test compound was incorporated into artificial diet to obtain 500, 1000, 2000,

2500, 3000 ppm for SCF essential oil and 500, 1000, 2000, 3000, 4000 ppm for clevenger oil concentrations.

Two larvae were released per cup in an untreated control was simultaneously run after 7d of feeding and larval weights were recorded and growth inhibition determined.

5.3.5.5 Larvicidal Bioassay

Experiment was conducted during May, 1998. A stock culture of mosquito larvae of *Anopheles stephensi* used for bioassay test was obtained from Dr. J.S. Shankar, Pool Officer in the Division of Agricultural Chemicals, IARI, New Delhi-12.

Larvicidal activity

Bioassay tests were carried out to determine the percent mortality of mosquito larvae when exposed to different concentrations of essential oil of *Murraya koenigii* spreng.

100 mg of essential oil extracted from the leaves of *M. koenigii* was dissolved in acetone to make up 10 ml volume. From the 10 ml lot, aliquots 0.5, 2, 3 and 5 ml were withdrawn and further diluted each with one litre of water to obtain different concentrations viz. 5 ppm, 20 ppm, 30 ppm and 50 ppm suspensions respectively. All the above suspensions obtained were kept in different jars (size 4 inchs dia x 6 inches height).

Batches of 25 (3rd/4th instar) larvae in 3 replicates were exposed to different concentrations of essential oil, alongwith appropriate control.

For evaluating the percent mortality of mosquito larvae, the observations were made after 24 hr exposure. At the end of 24 hr observation period, the larvicidal activity expressed in terms of larvae died was calculated as follows :

$$\text{\% mortality of mosquito larvae} = \frac{\text{No. of larvae died}}{\text{No. of larvae kept}} \times 100$$

The percent mortality of mosquito larvae was corrected for control by using Abbot's Formula. From these observations, LD_{50} was determined.

3.2.5.6 Repellant activity

For testing the repellant effect of the essential oil, 10 gm of wheat flour was treated with 1 ml of each 0.1, 0.2, 0.3 & 0.5% of essential oil solutions and thoroughly mixed with food material and was transferred into the chambers of an olfactometer (consisting of 5 chambers). The treated food was placed in four chambers while in the fifth solvent-treated food was placed, which served as a control. At each trial one oil with four doses was tested.

Fifty unsexed, one day starved, 8-10 day old *Tribolium casteneum* obtained from the Division of Entomology, IARI, New Delhi were released in the central arena of the olfactometer. All the treatment were replicated 3 times. The number of insects found in the various chambers were recorded after 1, 4 and 24 hours of the treatment. The per cent repellancy was calculated by the following formula by Hasan Ali and Lwandi (1989).

$$\text{\% Repellancy} = \frac{N_c - N_t}{N_c + N_t} \times 100$$

N_c = no. of insects in control chamber

N_t = no. of insects in treated chamber

3.2.6 Statistical Analysis

1. Probit Analysis

Probit analysis was carried out using a computer software 'LD_{50}' to compute ED_{50} for crude hexane and benzene extracts, pure carbazoles isolated from these extracts, the essential oil extracted from the leaves of *M. koenigii* and derivatives of pure carbazoles.

2. Student t-test

t-test was used for the comparison of ED_{50} value. The ED_{50} value of two replicates were estimated by probit analysis as per Finney's linear regression programme. The ED_{50} were estimated with standard error (SE) as : $ED_{50} \pm SE$. The standard error deviation (SEd) was estimated as

$$SEd = \sqrt{SE_1^2 + SE_2^2}$$

then t-value was computed as

$$t = \frac{ED_{50}(1) - ED_{50}(2)}{SEd}$$

If the computed t-value is greater with $(n_1 + n_2 - 2)$ degree of freedom than tabulated t value at prescribed level of significance, the two ED_{50} are considered significantly different.

CHAPTER 4

RESULTS AND DISCUSSION

4.1.1 Extraction and Identification of essential oil constituents from the leaves of *Murraya koenigii*

Essential oil from the fresh leaves of *Murraya koenigii* Spreng was obtained by following two methods

* Hydrodistillation in clevenger apparatus
* Super Critical Fluid extraction (SCF)

The essential oil so obtained was subjected to GC-MS for identification of its chemical constituents.

Hydrodistilled oil : The GC spectrum of oil showed it to be in mixture of twelve components (2 major and 10 minor). Of these eight compounds were identified on the basis of their mass fragmentation pattern and their comparison with those reported in literature. The per cent composition of these compounds is shown in Table 3. The compounds identified from hydrodistilled essential oil of *Murraya koenigii* (L.) Spreng. leaves are depicted in Fig. 1.

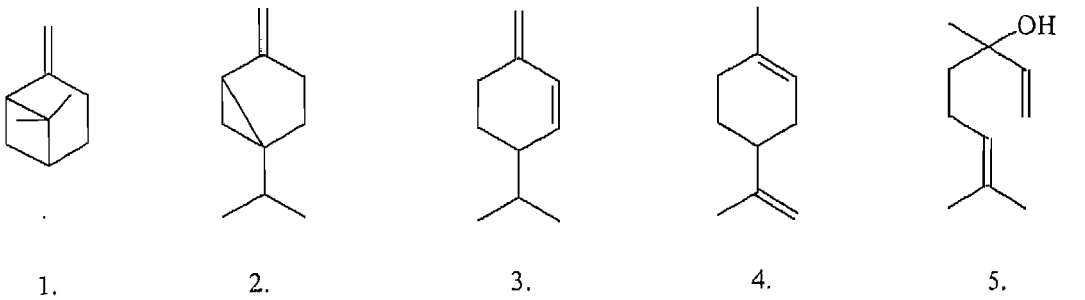
The first two major compounds (M^+, 204) identified as sesquiterpene hydrocarbon were accounted for 50% of the total volatiles detected. The sesquiterpene were tentatively identified on the basis of their mass fregmentation pattern as β-caryophyllene and β-gurjunene.

The first major compound β-caryophyllene showed molecular ion peak at m/z 204 (M^+, %) along with other characteristic ion peaks at m/z 189, 175, 161, 147, 133, 105, 93, 79, 69 [Fig. 2].

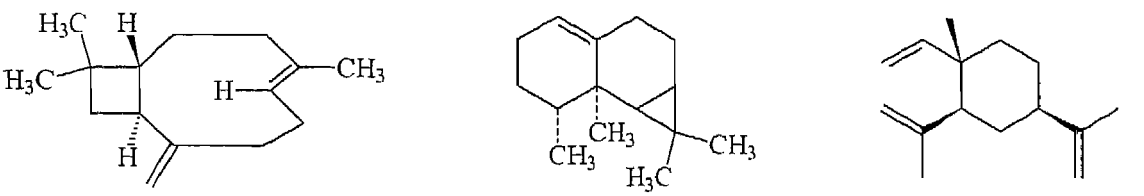
Table 3. Composition of Hydrodistilled oil

S.No.	Compounds	Peak area (%)
1.	β-pinene	5.84
2.	Sabinene	3.67
3.	β-phellandrene	6.1
4.	Limonene	2.67
5.	Linaloc	0.82
6.	β-caryo···llene	29.0
7.	β-gurjunene	21.0
8.	β-elemene	6.9
	Total identified	76.00
	Total unidentified	24.00

The second major compound in the essential oil, β-gurjunene showed molecular ion peak at m/z 204 (M^+) alongwith other fragment ion peak at m/z 189, 174, 159, 147, 133, 119, 105, 93, 91. Their fragmentation pattern differ from one another and the base ion peak for these compounds were 69 (100%) and 93(100%) respectively. Followed by these two, the third compound in the essential oil showed molecular ion peak at M^+ 204 besides other characteristics ion peak at m/z 189 (M^+ - CH_3) 161 (M^+ - 43) indicating loss of 15 mass unit of -CH_3 and 43 mass unit of -C_3H_7 from the parent molecule. Other characteristics ion peak at m/z 147, 133, 119, 93, 81, 79, 77 and 69 and 81 (100%) as base peak was typical of compound proposed as β-elemene (Fig. 3). The fourth compound in the essential oil showed molecular ion peak (M^+) at m/z 136 along with other characteristic ion peak at m/z 119, 93, 91, 77 and 93 (100%) as base peak was typical of compound proposed as β-phellandrene.



1. 2. 3. 4. 5.



6. 7. 8.

Fig. 1 Compounds identified from hydrodistilled extracted essential oil

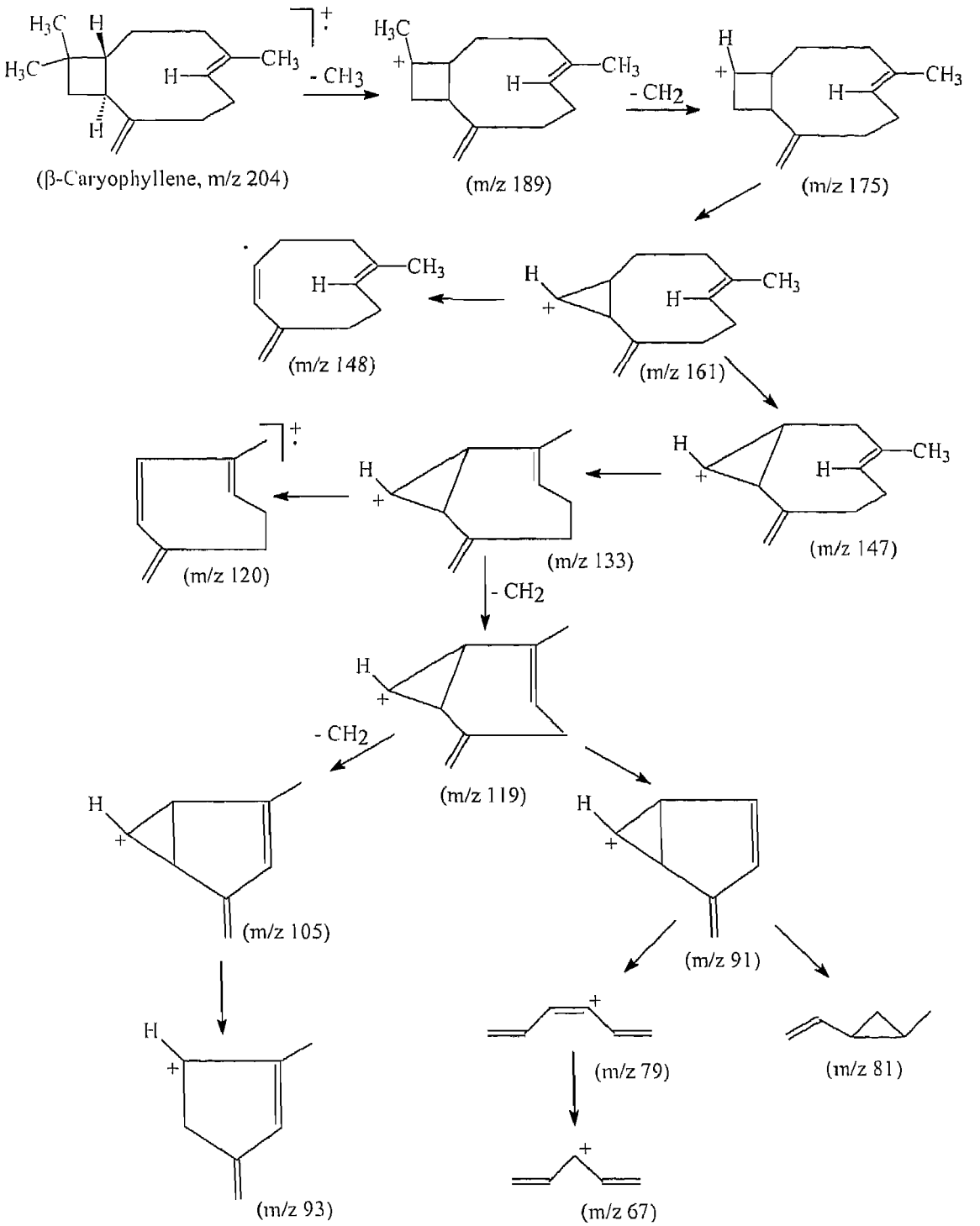


Fig. 2 Mass Fragmentation Pattern ofβ-Caryophyllene

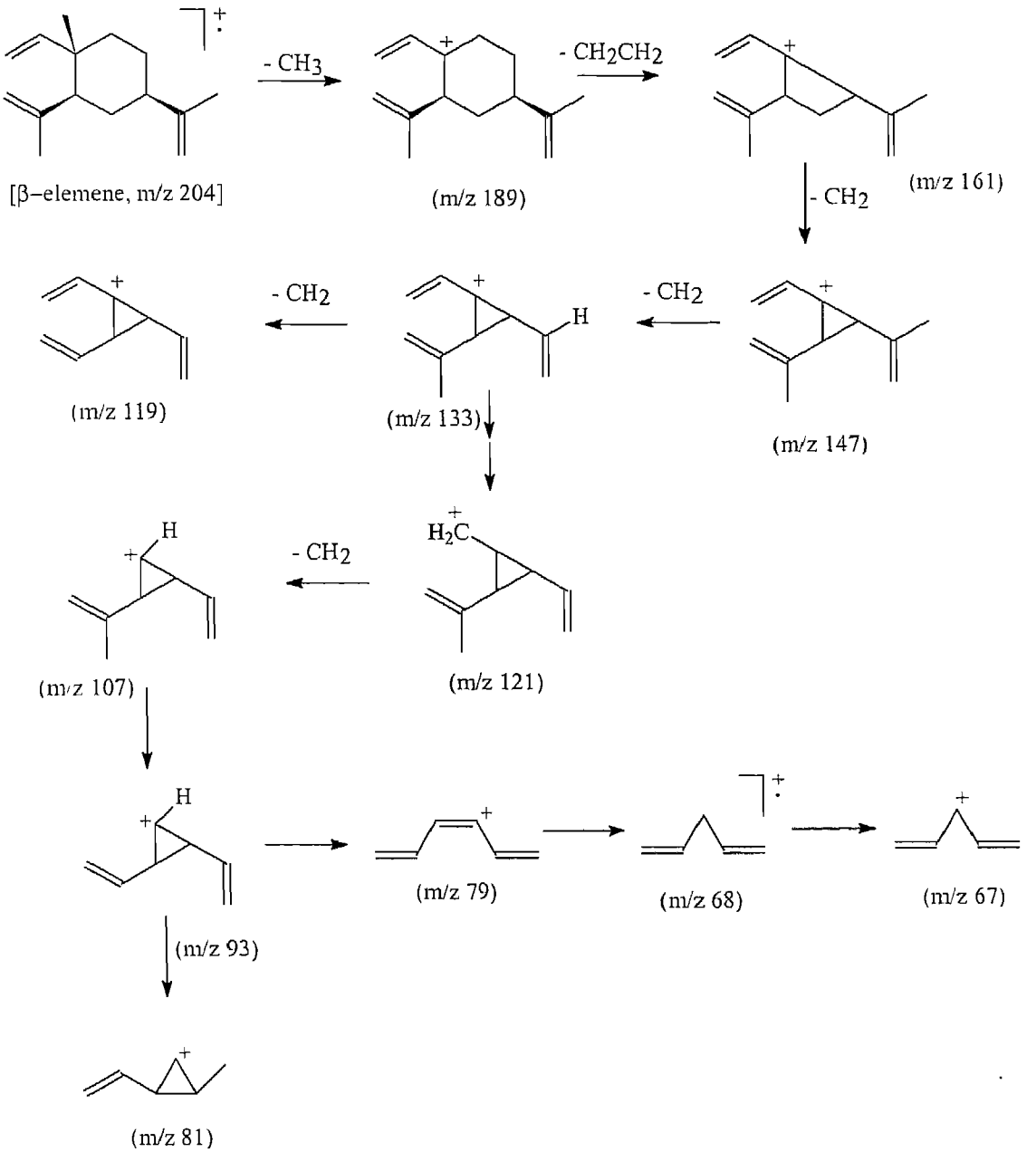


Fig. 3 Mass Fragmentation Pattern of β–Elemene

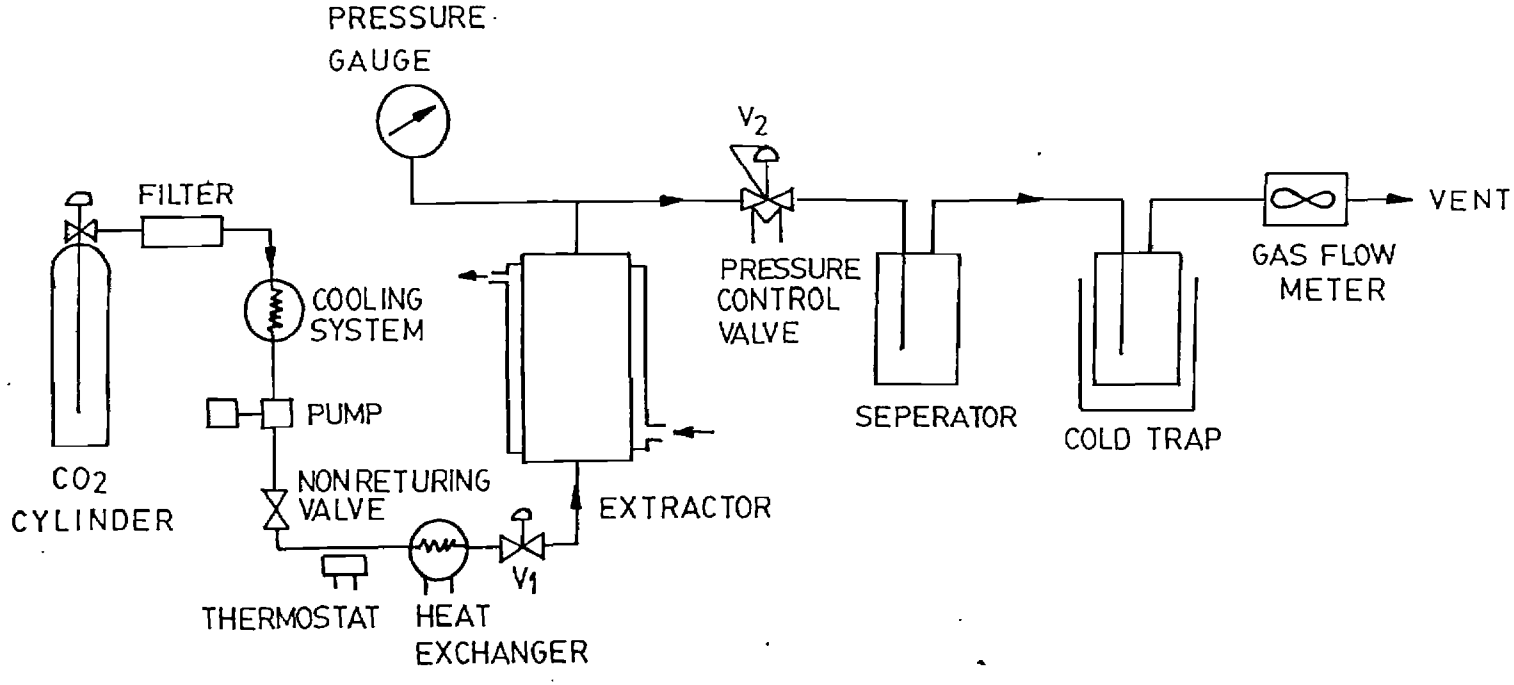


FIG. LIQUID AND SUPERCRITICAL FLUID EXTRACTION APPARATUS.

Rest of the four compounds in the essential oil were present in minor quantities and showed molecular ion peak (M^+) at m/z 154, 136, 136, 136 and these were tentatively identified on the basis of their mass fragmentation pattern and their resemblance with reported literature as linalool, sabinene, limonene and β-pinene respectively.

Super Critical Fluid extracted (SCF) oil

The essential oil was obtained from the fresh leaves collected from the experimental Fields of Indian Agricultural Research Institute. Essential oil was obtained by supercritical fluid extraction technique using liquid CO_2 [Critical temp. and pressure conditions are mentioned in Chapter 3] at Indian Institute of Technology, Hauz Khas, New Delhi.

The essential oil so obtained was subjected to GC-MS for identification of its chemical constituents. The GC spectrum of SCF-oil showed it to be similar to that of hydrodistilled oil. SCF-extracted oil was a mixture of sixteen compounds. Of these eleven compounds were identified on the basis of their mass fragmentation pattern and their comparison with those reported in literature. They are as follows :

β--Caryophyllene, β-gurjunene, β-elemene, β-phellandrene, β-pinene, linalool, elemol, p-Cymene, palmitic acid, lauric acid, mahanimbine (Table 4).

So the most of major and minor compounds in the essential oil extracted by SCF technique were found similar to those extracted with hydrodistillation process.

Besides monoterpenoids and sesquiterpenoids, SCF extracted oil showed the presence of some fatty acids and carbazole alkaloid in it

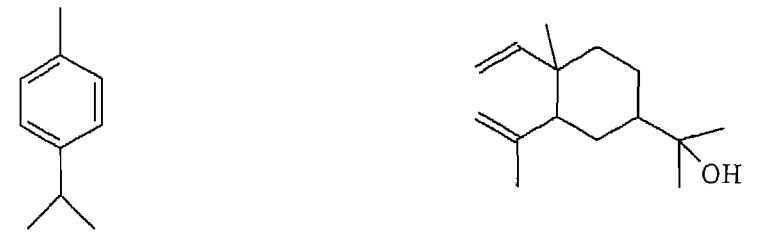
Table 4. Composition of supercritical fluid extracted oil

S.No.	Compounds	Peak area (%)
1.	β-pinene	5.84
2.	β-phellandrene	6.1
3.	Linalool	0.82
4A.	p-Cymene	2.13
5A.	Elemol	4.21
6.	β-caryophyllene	29.0
7.	β-gurjunene	21.0
8.	β-elemene	6.9
9.	Palmitic acid	3.4
10.	Lauric acid	2.7
11.	Mahanimbine	0.98
	Total identified	83.08
	Total unidentified	16.92

indicating the presence of more chemical constituents than hydrodistilled oil (Fig. 4). Later on these fatty acids and carbazole alkaloid were identified on the basis of their mass fragmentation pattern as palmitic, lauric acids and mahanimbine respectively.

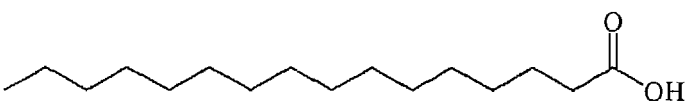
Total eleven compounds identified in SCF-extracted oil and six were already discussed in the hydrodistilled oil and remaining five are to be discussed here.

Seventh compound in the SCF - oil showed the molecular ion peak (M^+) at 222 and was identified as elemol a sesquiterpene alcohol. It showed other characterisic peak at m/z 189, 175, 161, 147, 133, 119, 105,



4A.

5A.



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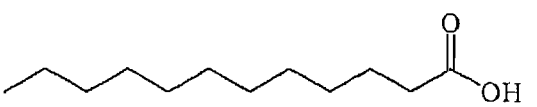

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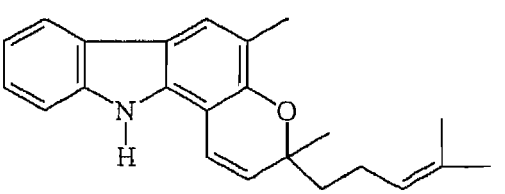
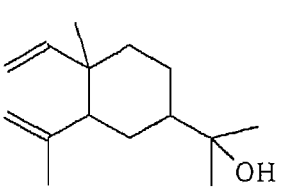
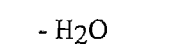


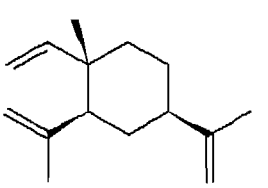
11.

Fig. 4 Compounds identified only from Supercritical Fluid Extraction of essential oil



[Elemol, m/z 222]





[β–elemene, m/z 204]

Rest of the fragmentation pattern is similar to as shown in the Fig. 3

Fig. 5 Mass Fragmentation Pattern of Elemol

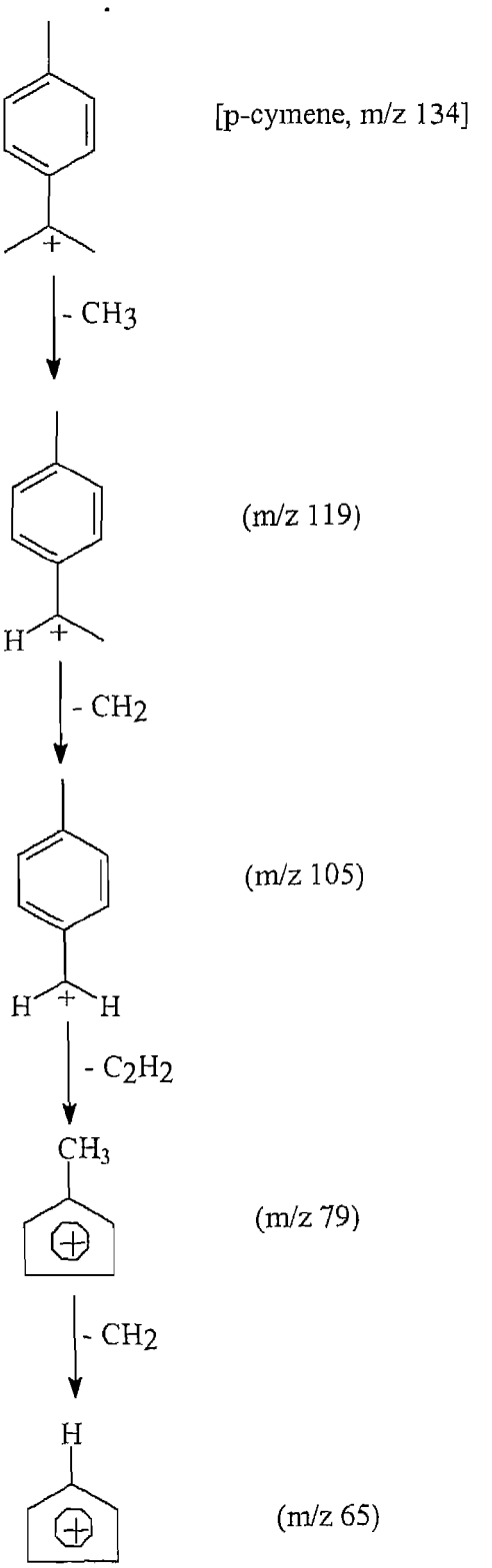


Fig. 6 Mass Fragmentation Pattern of p-Cymene

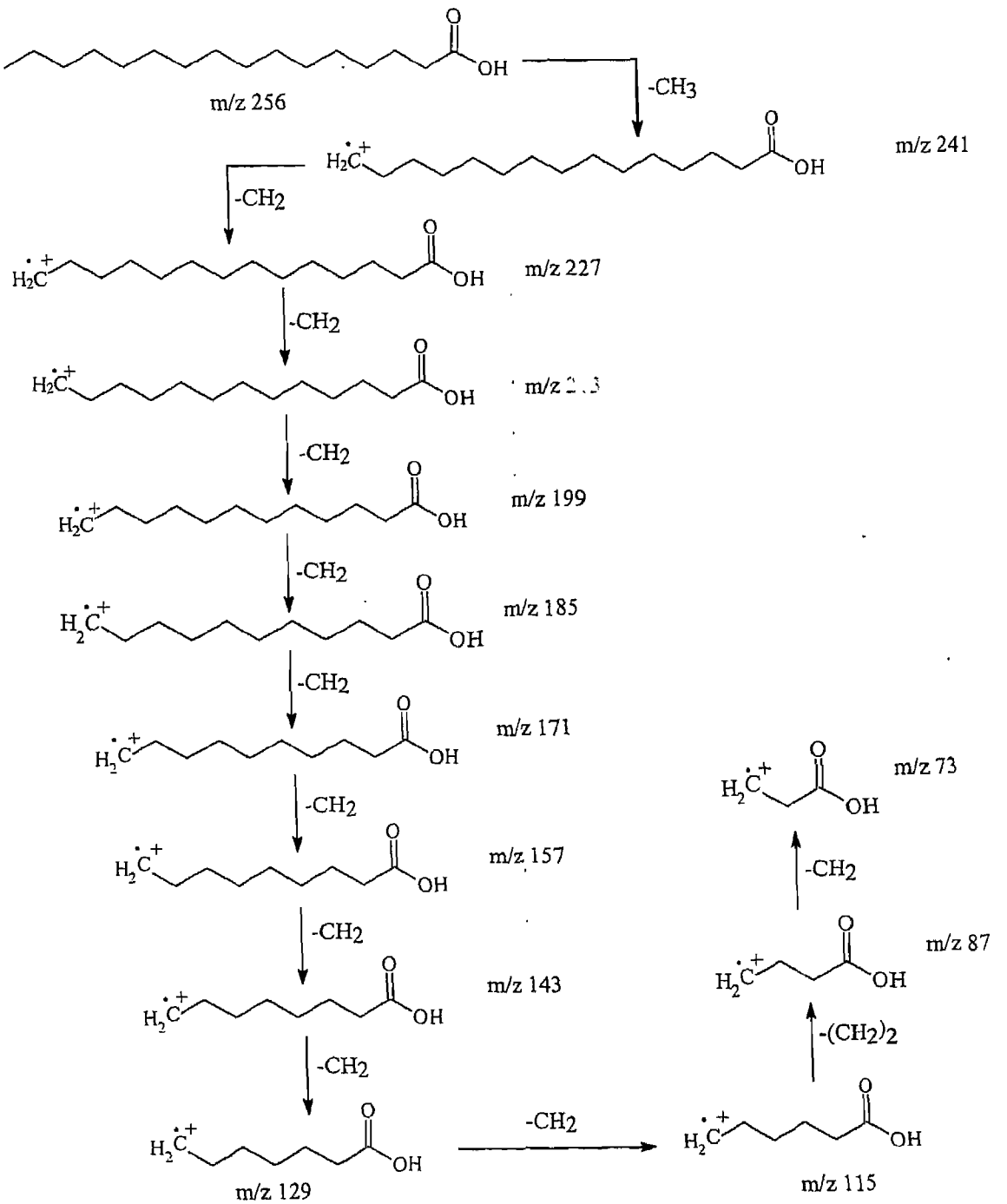


Fig-7 : Mass fragmentation pattern of palmitic acid

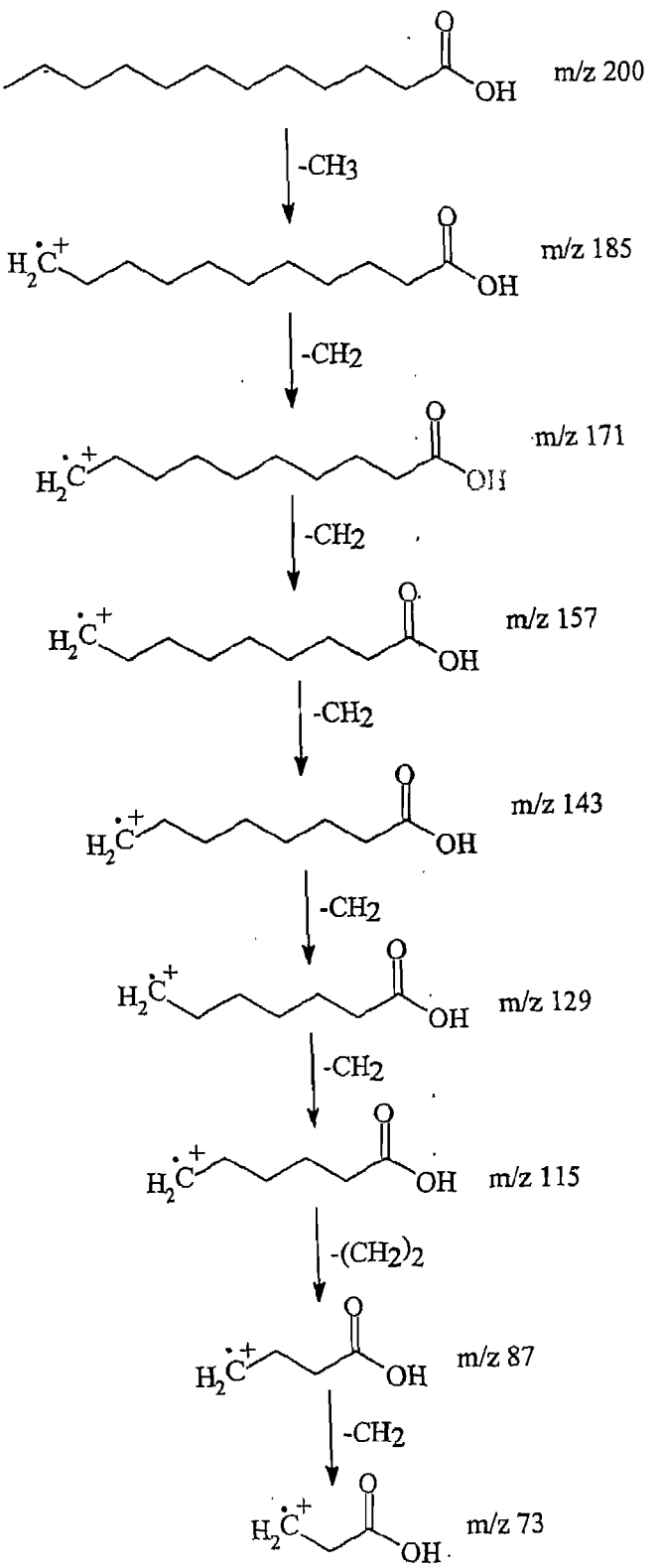
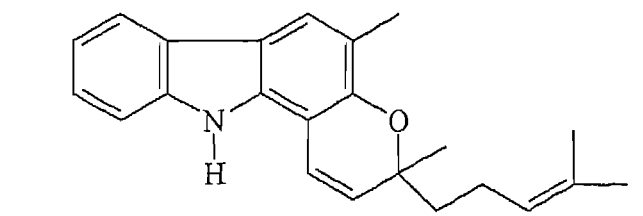
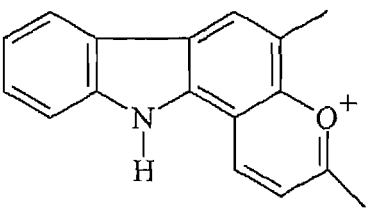


Fig-8 : Mass fragmentation pattern of lauric acid



(m/z 331)

- C_6H_{11} or (M-83)



(m/z 248) [Carbazolopyrylium ion]

Fig. 9 Mass Fragmentation Pattern of Mahanimbine

91, 77 with base peak m/z 71 (100%) was typical of compound proposed elemol (Fig. 5).

Eighth compound in the SCF oil showed molecular ion peak (M^+) at m/z 134 and was identified as p-cymene. Its mass fragmentation pattern resembles with that reported in literature. It showed molecular ion (M^+) peak at m/z 134 and base peak at m/z 119 along with other peak appearing at 91, 69 which was typical of compound proposed as p-cymene (Fig. 6).

Next (ninth) compound showed molecular ion peak (M^+) at m/z 256 and was identified as Palmitic acid. It showed base peak at m/z 73 along with other peak appearing at 227, 213, 199, 185, 171, 157, 143, 129, 115 and 87 which were typical of the sequential loss of CH_2 unit (Fig. 7).

Another (tenth) compound in the SCF oil was identified as lauric acid as it exhibited molecular ion peak at m/z 200 (M^+) along with other characteristic peaks at m/z 171, 157, 143, 129, 115, 87 and 73 (100%). These were formed as a result of sequential loss of 14 mass unit (Fig. 8).

Next (eleventh) compound in the SCF oil gave molecular ion peak (M^+) at m/z 331 and was identified as mahanimbine as C_{23} skeleton carbazole alkaloid. Its mass fragmentation pattern resembles with that reported in literature.

It gave a high intensity base peak at m/z 248 (M-83) which could be represented by the carbazolopyrylium ion (Fig. 9).

4.1.2 Isolation and Characterization of Carbazole alkaloids

Isolation : Dried and finely powdered leaves of *Murraya koenigii* spreng extracted twice with cold petroleum ether. The leaves were then dried and re-extracted with 95% ethanol to get a dark coloured viscous residue after the removal of solvent under vacuum. The viscous residue was then re-extracted with hexane, benzene, ethyl acetate and methanol. The respective solvents were removed under vacuum. The ethyl acetate and methanol extracts were obtained in insignificantly low quantities so could not be used for further phytochemical investigations. The hexane and benzene extracts were subjected to exhaustive column chromatography over a silica gel to obtain pure compounds. Each column eluted with hexane, hexane-benzene, benzene, benzene-chloroform, chloroform with increasing proportions as hexane : benzene 9:1, 8:2, 7:3 and 1:1. On removal of solvents from each fractions the compounds obtained were crystallized at low temperature. From the hexane extract compounds like mahanimbine, isomahanimbine and small amount of Cyclomahanimbine and from the benzene extract compounds like koenigine, koenine, mahanine, koenidine, koenimbine were separated.

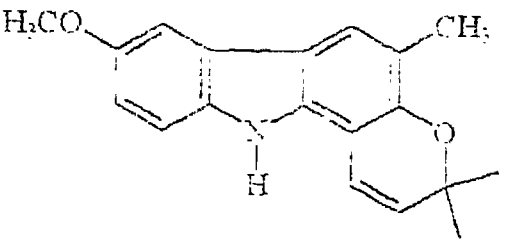
Characterization : Members of C_{18}- and C_{23}- skeleton group carbazoles were characerized on the basis of spectral data and elemental analysis.

Members of C_{18} skeleton Group

1. Koenimbine, m.p. 194°C analyzed for $C_{19}H_{19}NO_2$ (M^+ 293)

The UV spectrum of the alkaloid (λ_{max} 237 and 296 nm) and the IR spectrum (υ_{max} 3406, 2980, 2920, 1643, 1610, 1584, 1490, 1457,

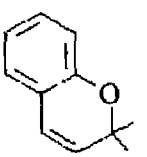
1380, 1295, 1209, 1140, 1110, 1024, 975, 748 cm^{-1}) suggested the presence of a carbazole nucleus.



The nmr spectrum showed the presence of an -NH function between δ 7.75 and 6.85 an aromatic methoxyl and C-methyl group.

The C-4, C-5 protons appeared at δ, 7.6 and 7.5 respectively. Of these the C-4 proton was a singlet suggesting that position 2 and 3 were substituted.

The sharp six proton singlet at δ 1.42 together with doublet for two protons at 5.75 and 6.67 (J = 10 Hz) showed the presence of a 2,2 dimethyl -Δ^3-pyran.



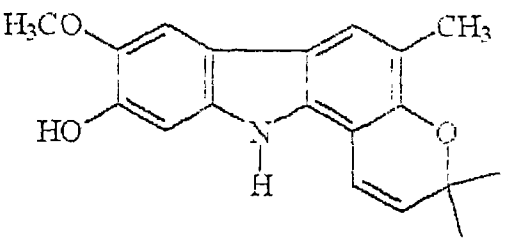
2,2-DimethylΔ^3-pyran

All the above data was in complete agreement with the data given in literature (Kureel *et al.*, 1969 and Narasimhan *et al.*, 1968).

2. Koenigine, m.p. 183°C analysed for $C_{19}H_{19}NO_3$ (M^+309)

The UV spectra of this compound is strikingly similar to that of koenimbine. Koenigine has one hydroxyl group (green coloration with $FeCl_3$). On methylation koenigine is converted to koenidine.

The structure of koenigine rest mainly on the interpretation of the nmr spectrum.



The presence of a proton at 8 position in koenigine was evident from the singlet around δ, 6.81 in its PMR spectrum. The phenolic group was placed at 7-position since the 8-proton appeared at higher field in DMSO than in $CDCl_3$.

The location of the methoxyl group was determined on mass spectral evidence. It was noticed that all the alkaloids of pyranocarbazole series showed an intense peak corresponding to aromatization of chromene ring (Loss of Methyl in Koenimbine, Koenigine etc.).

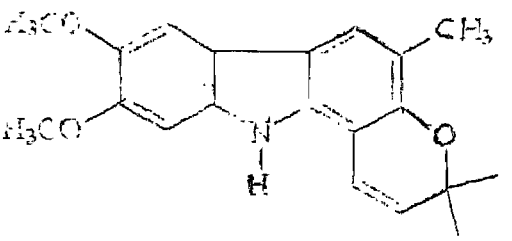
However, a further intense peak at M-30 corresponding to the loss of another methyl group was observed in 'koenigine.

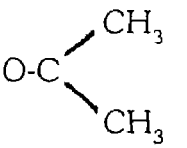
3. Koenidine, m.p. 224°C analysed for $C_{20}H_{23}NO_3$ (M^+ 323)

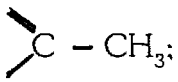
Its UV spectrum λ_{max}^{etoH} 223, 237 and 299 nm indicated the presence of a carbazole nucleus. This was supported by its IR spectrum which showed peaks at 3421 (-NH), 2927, 1650, 1630, 1600 cm^{-1} (unsaturation and aromatic system).

The nmr signals (δ multiplicity, no. of protons under peak and assignment given) : 3.86 and 3.96 both singlet, 3 each, 2 methoxyl

attached to an aromatic ring; 5.75 and 6.65 both doublet (J=10 Hz) one each olefinic protons.



1.42, Singlet, 6H,  ;

2-3, Singlet, 3H,  ;

In addition the spectrum showed three aromatic protons all singlet at δ, 6.91 7.55 and 7.65 respectively as well as a broad signal for a single proton, exchangeable with D_2O at δ 7.90 (> N-H).

The mass spectrum showed apart from the M^+ peak at 323 abundant ions at m/z 308, 293, 292, 264, 250 and 154.

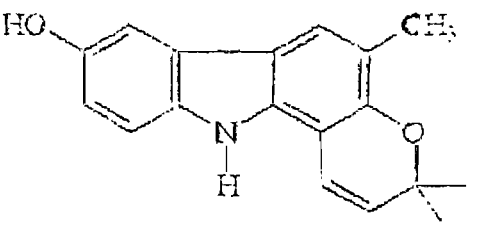
4. Koenine, m.p. 250-52°C analyzed for $C_{18}H_{17}NO_2$ (M^+ 279)

The compound gave a positive ferric chloride test for phenol.

The structure was determined by nmr data :

The C-7 proton (δ 6.74 J=8 Hz & 2 Hz) is ortho coupled with H-8 (δ, 7.18, J=8 Hz) and meta coupled with H-5 (δ 7.20, J = 2 hz) H-4 (δ, 7.55) was singlet.

The presence of an aromatic C-methyl group is also deduced from the nmr spectrum.



From these data and conversion of Koenine to koenimbine by methylation proposed structure for koenine.

Members of the C_{23} - Skeleton Group

5. **Mahanimbine, m.p. 94°C analysed for $C_{23}H_{25}$ NO (M^+ 331)**

It gave bluish green colour with conc. H_2SO_4 and gave a picrate, m.p. 142°C.

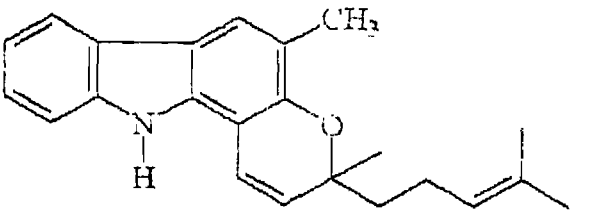
IR Spectrum : Showed bands for an - NH - function, an aromatic C. Me group, a double bond and an aromatic residue (υ_{max}^{CHCl3} 3450, 1650, 1620, 1490, 1379, 1365; $\upsilon_{max}^{K,Br}$ 3350, 1647, 1613, 1575, 1493 Cm^{-1}).

UV spectrum : (λ_{max} 223, 239, 288, 330, 343 nm) was suggestive of the presence of a carbazole system.

Mass spectrum showed a molecular ion peak at m/z 331. The other peaks were at m/z 316, 248. The peak at m/z 248 was more intense than the molecular ion peak. This is suggestive of the presence of a methyl pyranocarbazole system.

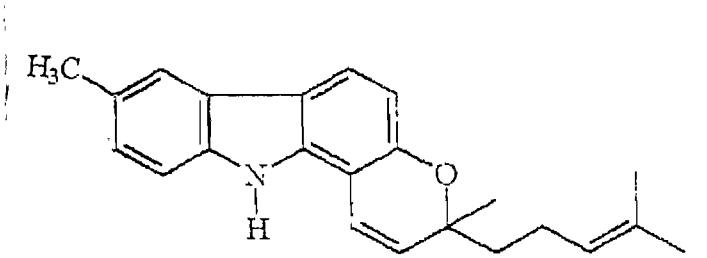
Mass spectral data showed that besides a methyl pyranocarbazole skeleton, it has a C_5H_8 fregment.

NMR spectrum revealed five aromatic protons (besides the NH - proton at 11.08). Two signals (in $CDCl_3$) appeared at considerably low field at 7.67 and 7.83 and were identified with the mutually deshielded C-4 and C-5 protons of carbazole ring. One of the signal was essentially a singlet showing that the proton had no ortho or meta neighbour while the other was a broad multiplet indicating that this proton had an ortho as well as a meta neighbour.



6. Isomahanimbine m.p. 148°C analyzed for $C_{23}H_{25}$ NO (M^+ 331)

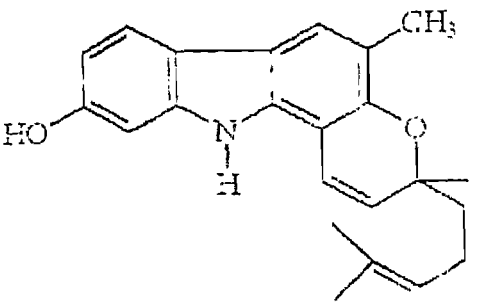
UV spectrum gave the value (λ_{max} 238, 281, 290, 335 and 354) was very similar to that of mahanimbine. IR spectrum showed an - NH - function (3440 cm^{-1}) and unsaturation.



nmr spectrum : The C-3 and C-4 protons appeared as an AB quartet at δ 6.7 and δ 7.7 (J=8.5 Hz) while the C-5 proton appeared as a broad singlet at δ 7.68; the NH-proton at δ 7.65 was masked by other protons. The AB system at δ 7.05 (J=8 Hz) and δ 7.15 (J =8 Hz) was assigned to the C-7 and C-8 protons respectively. The aromatic methyl signal appeared at δ 2.46; the gem-dimethyl group on a double bond at δ 1.55 and 1.65 and the methyl adjacent to the ether oxygen at δ 1.44. Four protons due to the methylene groups appeared as a complex multiplet between δ 1.7-2.4 and the olefinic proton was a triplet at δ 5.1.

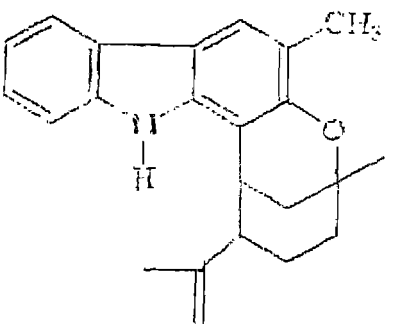
7. Mahanine, m.p. 100°C analyzed for $C_{23}H_{23}NO_2$ (M^+ 247)

UV and IR spectra suggested the presence of the pyranocarbazole system. The nmr spectrum showed that like mahanimbine it had three high field methyl signal indicating the presence of monoterpene unit substituted on the pyran ring. The further appearance of a C-6 proton quartet at δ 6.54 (J=7.8 Hz) a C-8 proton doublet at δ 6.74 (J=2 Hz) a C-4 proton singlet at δ 7.58 and a C-5 proton doublet at δ 7.54 (J=8 Hz) clearly established the structure of mahanine as shown below.



8. Cyclomahanimbine, m.p. 146°C analysed for $C_{23}H_{25}NO$ (M^+ 331)

UV data (λ_{max} 251, 257, 307, 341 nm) was shown to have very similar to that of 2-methoxy carbozole indicating the ether linkage at the 2-position of the carbazole nucleus.



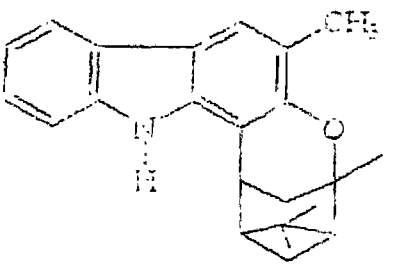
The nmr spectrum of cyclomahanimbine showed it to have an aromatic substitution pattern similar to those of mahanimbine. It had

signals for two vinylic protons (δ 4.8) and for a vinyl methyl group on a double bond at (δ 2.35) besides a benzylic methine proton at δ 3.4.

It has been supported by conversion of mahanimbine to cyclomahanimbine by shaking with hydrochloric acid.

9. Bicyclomahanimbine, m.p. 145°C analysed for $C_{23}H_{25}NO$ (M^+ 331)

UV spectrum (λ_{max} 242 255, 260, 305, 331 nm) indicative of the presence of a 2-methoxycarbazole. The nmr spectrum showed an aromatic substitution pattern similar to that of mahanimbine. The high field methyl signal at δ -0.71 was attributed to attachment to a cyclobutane system.



The given structure of bicyclomahanimbine was further supported by the facts on silica gel mahanimbine gets converted to bicyclomahanimbine.

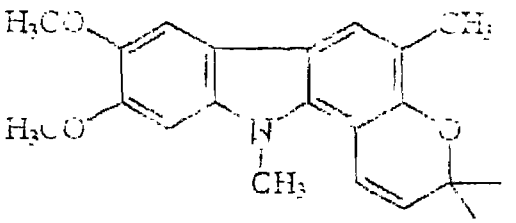
Derivatization of Carbazoles

N-methylation of members of C_{18} skeleton Group

N-methyl koenidine - Methylation of koenidine (210 mg) with methyl iodide (1 ml) and sodium hydride (0.2 gm) in dry benzene gave N-methyl koenidine ($C_{23}H_{23}NO_3$) (Kureel *et al.*, 1969).

Yield of N-methyl koenidine - 162 mg

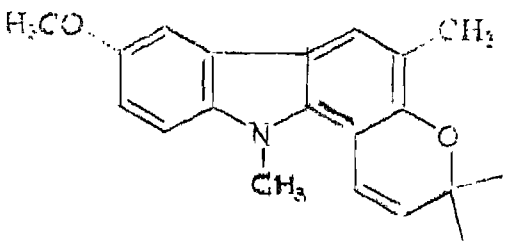
Melting point - 189°C.



UV data showed (λ_{max} 241, 303, 346, 361 nm) the presence of pyranocarbazole system.

IR data : 1640, 1610 and 1590 cm^{-1}.

N-methyl koenimbine - Methylation of koenimbine (250 mg) with methyl iodide (1 ml) and sodium hydride (0.2 gm) in dry benzene as per Kureel *et al.* (1969) method, gave N-meythyl koenimbine (190 mg).



It analyzed for $C_{20}H_{20}NO_2$.

Melting point - 148°C.

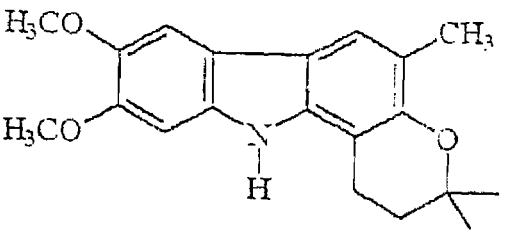
IR data : 1640, 1570, 1460 cm^{-1}.

UV data (λ_{max}^{EtOH} 242, 301, 339, 357 and 371 nm) suggested the presence of carbazole nucleus

Hydrogenation of members of C_{18} - Skeleton group

Dihydrokoenidine : Koenidine (150 mg) was dissolved in ethanol (40 ml) and hydrogenated at 29°C using 5% Pd/C (0.2 g) till no more H_2 was absorbed. The catalyst was filtered off, the filterate concentrated and the

residue crystallized from hexane (70 mg) (Joshi *et al.*, 1970).



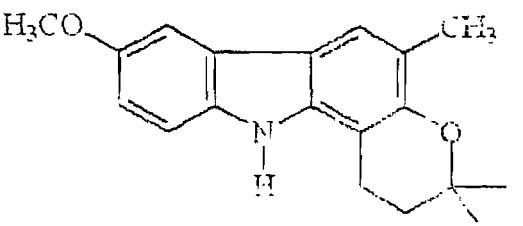
Melting point - 237°C

Analyzed for $C_{20}H_{23}NO_3$

IR data (nujol) : 3410, 1610, 1305, 1260 cm^{-1}

UV (EtOH) : λ_{max} 215, 235, 267, 315, 323 nm found C 74.0%; H 7.2% mol wt. by mass spectrum 325. $C_{20}H_{23}NO_3$ requires C 73.8%, H 7.1%.

Dihydrokoenimbine : Prepared as per the method described earlier.



Melting point - 249°C, yield of dihydrokoenimbine - 69 mg

UV (EtOH) : λ_{max} 243, 258, 312, 332 and 346 nm

IR (Nujol) υ_{max} 3400, 1620, 1310, 1200 cm^{-1}.

4.1.3 Physical methods of structure elucidation of carbazole alkaloids

UV spectra

The ultraviolet absorption spectrum of carbazole (λ_{max} 233, 257, 293, 322, 336 nm; log ε 4.51, 4.18, 4.10, 3.46, 3.39) differs from its carbocyclic analogue fluorene due to the contribution of the lone pair

of electrons of the heterocyclic nitrogen to the chromophoric system. This gives rise to the characteristic carbazole spectrum. Substitutions at different positions cause significant changes and in some cases a diagnostic spectral pattern is obtained. The significant and diagnostic spectra of substituted carbazoles have been recorded in the case of formyl and methoxy carbazoles. Formyl, methoxy and pyranocarbazoles give rise to characteristics spectra which have been extensively utilized in the structure determination of carbazole and related alkaloids. UV spectra is suggestive of the presence of *Carbazole nucleus* in the given compound.

IR Spectra

The infrared spectra of the carbazoles show bands characteristics for imino compounds. The peak of the NH-bands shifts on change of phase. Following useful informations may be obtained with the help of IR spectra;

* The unsaturation associated with 2:2 dimethyl Δ^3-pyran shows an absorption band at 1645 cm^{-1}.
* The presence of an alcoholic hydroxyl and imino function is known from the absorption at 3580 cm^{-1} and 3450 cm^{-1}.
* Usually the aromatic aldehyde peak of the carbazole alkaloids appear in the region 1680 cm^{-1}.

NMR Spectra

The NMR spectra of the carbazole alkaloids have been very helpful in structure elucidation. The carbazole - NH-being acidic it absorbs near δ 10.0 but with substitution the signal for the -NH- proton has been found

to be shifted. The signal for the -NH- proton has been registered at δ 7.6 to 9.8 in different carbazole alkaloids.

The signal for the protons on C-4 and C-5 appear at lower field (around δ 7.17 to 7.5) as these two protons are phenanthrenic and are mutually deshielded. The other aromatic protons of carbazole occur at higher frequency as complex multiplets. Resonances are shielded or deshielded according to the environment of the proton in question.

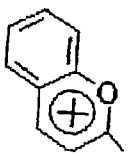
The downfield shift of the ethylenic protons of a chromene ring adjacent to the aromatic ring has been attributed to the deshielding effect of the heterocyclic nitrogen. This has been used to explain the angular fusion of the chromene ring in koenimbine, koenidine and related compounds. The ethylenic proton of the chromene adjacent to the aromatic ring shows a significant shift (δ 0.3 to 0.5) on changing the solvent from $CDCl_3$ to DMSO. This has been utilized in determining the angular fusion of the 2,2 substituted-Δ^3-pyran system in C_{18} and C_{23} carbazole alkaloids. The methyl protons of N-CH_3 carbazoles experience a deshielding effect when an angular 2,2-dimethyl-Δ^3-pyran with an oxygen ether linkage at the 2-position is present.

Mass Spectra

Carbazole being a stable aromatic system, the mass spectral behaviour of the carbazole skeleton provides rather little information for structure elucidation. Substituted carbazoles behave very similar to substituted benzenes.

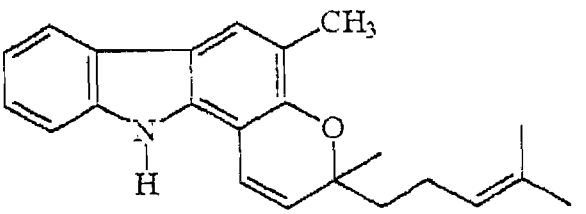
The mass spectral behaviour of carbazole alkaloids containing a 2,2-disubstituted-Δ^3-pyran or 2,2-disubstituted pyran ring has been of much help in their structure elucidation. It is well known that 2,2-dimethyl-

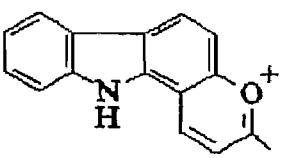
Δ^3-pyran (M^+ 160) gives rise to a high intensity peak at m/z 145 (M-15) due to the formation of the pyrylium ion.



pyrylium ion

Mahanimbine gives a high intensity base peak at m/z 248 (M-15) which could be represented by the carbazolopyrylium ion.





carbazolopyrylium ion

4.2 Bioactivity

Introduction

The main objective of the present investigations is to evaluate the pesticidal properties of the essential oil and carbazole alkaloids obtained from *Murraya koenigii* Spreng and also to boost the environmental protection system by using bioactive principles to lessen the enviromental pollution by destroying plant pathogenic microbes (fungi, nematodes) and mosquito larvae and to inhibit the growth of insects.

Carbazole alkaloids are bioactive on that basis their pesticidal properties such as

* Nematicidal,
* Fungicidal and
* Antifeedancy are discussed in the present investigations.

They are broadly divided into following groups

1. Carbazole alkaloids present in crude hexane and benzene extracts.
2. Pure carbazole alkaloids isolated from the crude extracts
3. N-substituted carbazole alkaloids derivatives (N-methyl derivatives)
4. Partially reduced carbazole alkaloids (dihydro derivatives).

4.2.1 Antifeedant Activity

The essential oil and other important chemical constituent isolated from the leaves of *Murraya koenigii* namely carbazole alkaloids and their derived N-methyl and dihydro derivatives along with crude hexane and benzene extracts were evaluated for their antifeedancy against 5th Instar

larvae of tobacco armywarm (*Spodoptera litura* Fab.) at different concentrations ranging from 500 to 10,000 ppm by "Choice test" method. Castor (*Ricinus communis*) leaves of appropriate size (2 cm^2) were treated with essential oil, carbazoles and their derivatives of different concentrations and were offered as food along with leaves discs treated with acetone which served as control.

After drying at room temperature two leaf discs, one treated and other untreated (control) were placed in petridish (9 cm dia) and 5th instar larva (X 5 replicate) prestarved for 1 h was released into it. After 4 h of larval release, leaf consumption was recorded by a digital leaf area meter (model 3100 lincoin, Nebraska, USA) and the percent. feeding inhibition for each treatment was calculated by per cent feeding inhibition formula mentioned in Chapter 3. The result of feeding inhibition thus calculated are enlisted in Tables 5, 6, 7 and 8.

From the per cent feeding inhibition data, ED_{50} (Effective dose causing 50% feeding inhibition) values were calculated by probit analysis.

Probit analysis was carried out using a computer software 'LD_{50}' to compute ED_{50} for each extracts, pure carbazoles and their derivatives and essential oil.

t-Test was also performed to compare and determine the statistical significance (at 95% level) between ED_{50} values of essential oil and dihydro-derivatives of koenimbine and koenidine.

Benzene extract of the leaves of *Murraya koenigii* showed maximum antifeedancy and was dose dependent. At 1000 and 10,000 ppm dose level it showed over 55% and 80% antifeedancy (Fig. 10).

Table 5. Antifeedant activity of Hexane and Benzene extracts against 5th instar larvae of ***Spodoptera litura***

Dose (ppm)	Per cent feeding inhibition	
	Hexane extract	Benzene extract
500	14.74	46.15
1000	21.65	58.24
2000	32.16	55.49
3000	47.00	73.39
5000	54.67	86.24
10,000	66.45	84.44

PROBIT ANALYSIS

Table value of χ^2 (95%) Result of χ^2	Hexane extract χ^2 (4)=9.456 1.505	Benzene extract χ^2 (4)=9.456 8.156
ED_{50} Value	0.4261%	0.0710%
95% confidence limit	0.3380-0.5373	0.0485-0.1041

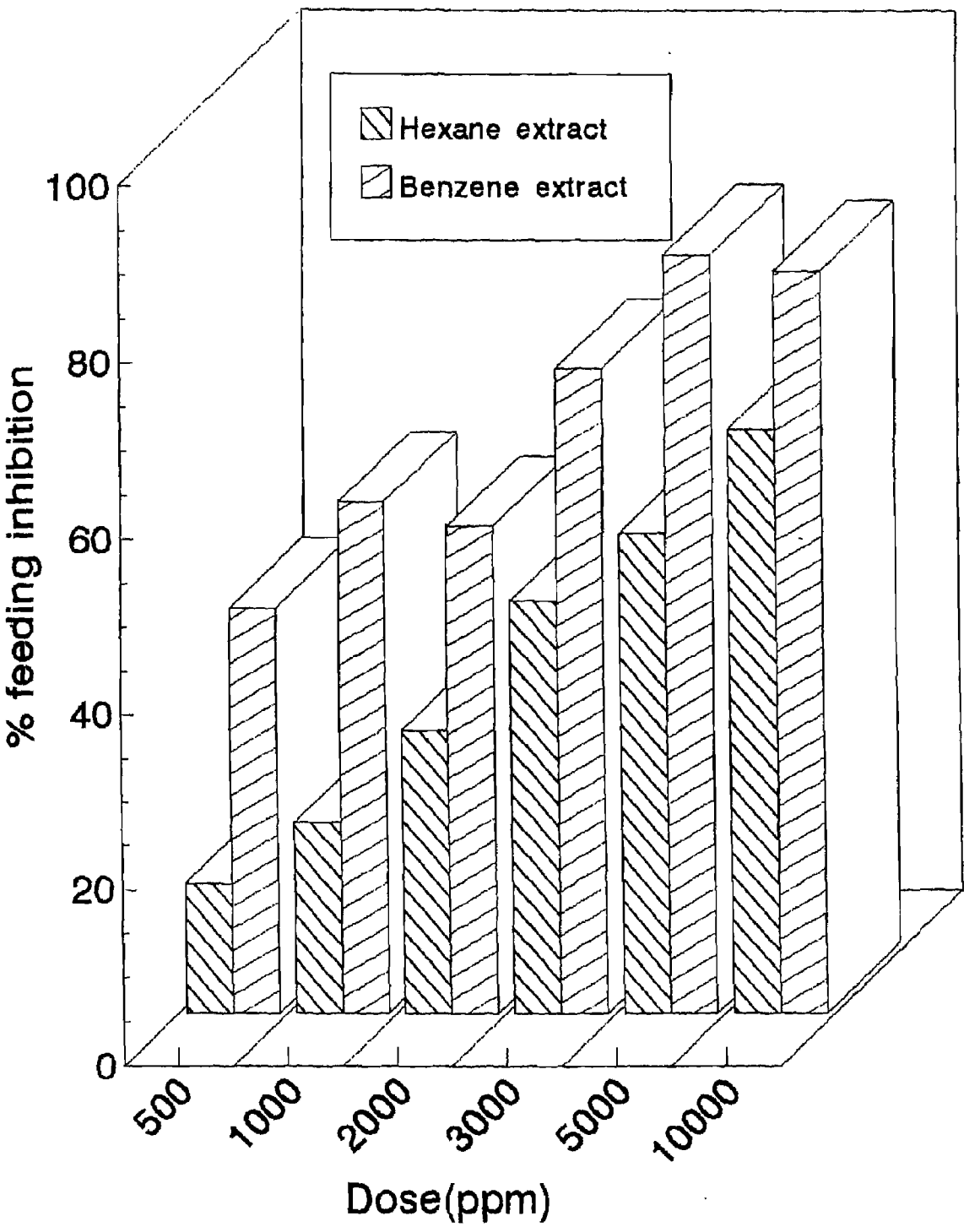


Fig-10 Antifeedent activity of hexane and benzene extractives against *Spodoptera litura*

Table 6. Antifeedant activity of pure carbazole alkaloids isolated from the leaves of ***Murraya koenigii*** against 5th instar larvae of ***Spodoptera litura***

Dose (ppm)	Per cent feeding inhibition in			
	Koenimbine	Koenidine	Mahanimbine	Isomahanimbine
500	35.00	41.74	21.00	19.00
1000	42.15	56.56	37.26	36.00
2000	54.26	47.21	46.15	48.24
3000	74.00	62.56	60.55	58.17
5000	80.33	71.10	74.95	63.00
10000	88.00	73.39	78.45	71.24

PROBIT ANALYSIS

Table value of χ^2 (95%) Result of χ^2	Koenimbine $\chi^2(4)=0.456$ 4.409	Koenidine $\chi^2(4)=9.456$ 6.933	Mahanimbine $\chi^2(4)=9.456$ 2.39	Isomahanimbine $\chi^2(4)=9.456$ 2.888
ED_{50} Value	0.0924%	0.1074%	0.1861%	0.2550%
95% confidence limit	0.0666-0.1284	0.0683 0.1689	0.1573-0.2201	0.2057-0.3172

Table 7. Antifeedant activity of N-methyl and dihydro derivatives of pure compounds isolated from the leaves of ***M. koenigii*** against 5th instar larvae of ***Spodoptera litura***

Dose (ppm)	Per cent feeding inhibition in			
	N-methyl Koenimbine	N-methyl Koenidine	Dihydro Koenimbine	Dihydro Koenidine
500	37.36	29.74	49.21	46.15
1000	46.15	40.86	56.00	55.00
2000	58.24	56.24	74.70	60.45
3000	71.20	68.45	67.26	65.20
5000	76.80	70.83	86.24	76.24
10000	70.00	80.79	91.22	84.24

PROBIT ANALYSIS

Table value of χ^2 (95%) Result of χ^2	N-methyl Koenimbine $\chi^2(4)=9.456$ 8.305	N-methyl Koenidine $\chi^2(4)=9.456$ 1.697	Dihydro Koenimbine $\chi^2(4)=9.456$ 7.151	Dihydro Koenidine $\chi^2(4)=9.456$ 1.652
ED_{50} Value	0.1087%	0.1526%	0.0630%	0.0774%
95% confidence limit	0.0749-0.1571	0.1204-0.1934	0.0438-0.0906	0.0509-0.1175

Table 8. Antifeedant activity of essential oil against 5th instar larvae of ***Spodoptera litura***

Dose (ppm)	Percent feeding inhibition of hydrodistilled oil
50	33.44
100	46.60
250	58.60
500	74.50
1000	82.20
5000	91.60
10,000	96.80

PROBIT ANALYSIS

Table value of χ^2 (95%) Result of χ^2	Hydrodistilled oil χ^2 (4)=9.456 1.505
ED_{50} Value	0.0130
95% confidence limit	0.0094-0.0179

The antifeedant activity of hexane extract was less than benzene extract as at 10,000 ppm it showed Ca. 67.0% feeding inhibition. The antifeedancy result obtained from the extracts indicated that feeding inhibiting active principles were better extracted in benzene than hexane (Table 5).

Some of the carbazole alkaloids obtained from the leaves of *Murraya koenigii* were also evaluated alongwith N-methyl and dihydro derivatives for their antifeedancy against *Spodoptera litura* at different concentrations ranging from 500 to 10,000 ppm.

The data in Table 6 revealed that at lower concentration the data were irregular but at higher concentration (5000 to 10,000 ppm) somewhat stable. At the highest concentration 10,000 ppm (per cent feeding inhibition calculated over solvent control) koenimbine had more than 85% antifeedancy whereas koenidine, isomahanimbine and mahanimbine showed more than 70% antifeedancy. On comparing the ED_{50} value of the pure carbazole isolated from hexane and benzene extracts of the leaves of *M. koenigii*, the order of antifeedant effect was found to be:

Koenimbine	> Koenidine >	Mahanimbine >	Isomahanimbine
(924 ppm)	(1074 ppm)	(1861 ppm)	(2555 ppm)

Investigation of the antifeedant activity of N-substituted carbazoles derivative enlisted in Table 7 revealed that the replacement of -NH- proton of carbazole ring by a methyl group did not have much effect on the antifeedant properties of these compounds but hydrogenation of these compounds to dihydro carbazoles however, enhanced the antifeedant activity quite sharply which could be observed from the comparison of the ED_{50} value of koenimbine, koenidine and their dihydro derivatives

Koenimbine (ED_{50}-924 ppm)	-	Dihydrokoenimbine (ED_{50}-630 ppm)
Koenidine (ED_{50}-1074 ppm)	-	Dihydro koenidine (ED_{50}-756 ppm)

Dihydrokoenimbine and dihydrokoenidine were shown to possess higher antifeedancy than the corrsponding koenimbine and koenidine carbazoles alkaloids. Such a higher antifeedant activity of dihydro carbazole derivatives might be due to the presence of partially reduced carbazole moiety. The results obtained here are in complete agreement with the observation made and studied by Chowdhury and Das (1979).

The essential oil extracted through hydrodistillation in clevenger appratus was active against *Spodoptera litura* larvae. The antifeedancy results of essential oil are listed in Table 8. The ED_{50} value for hydrodistilled oil was 130 ppm.

The antifeedant activity of hydrodistilled oil was quite comparable with dihydroderivative of koenimbine and koenidine (Fig. 11). It was found to be as high as 96.0% at 10,000 ppm from hydrodistilled oil. According to the t-test and confidence limit, the difference between observed ED_{50} values of essential oil and dihydro derivatives of koenimbine and koenidine was found statistically significant at 99% level. GC-MS analysis of the essential oil indicated that oil was a complex mixture of compounds comprising of β-phellandrene, β-gurjunene, β-pinene, limonene, linalool, β-caryophyllene and sabinene.

The antifeedant activity of the essential oil may be due to β-caryophyllene (29.0%) and β-gurjunene (21.0%) which are the major components of the oil.

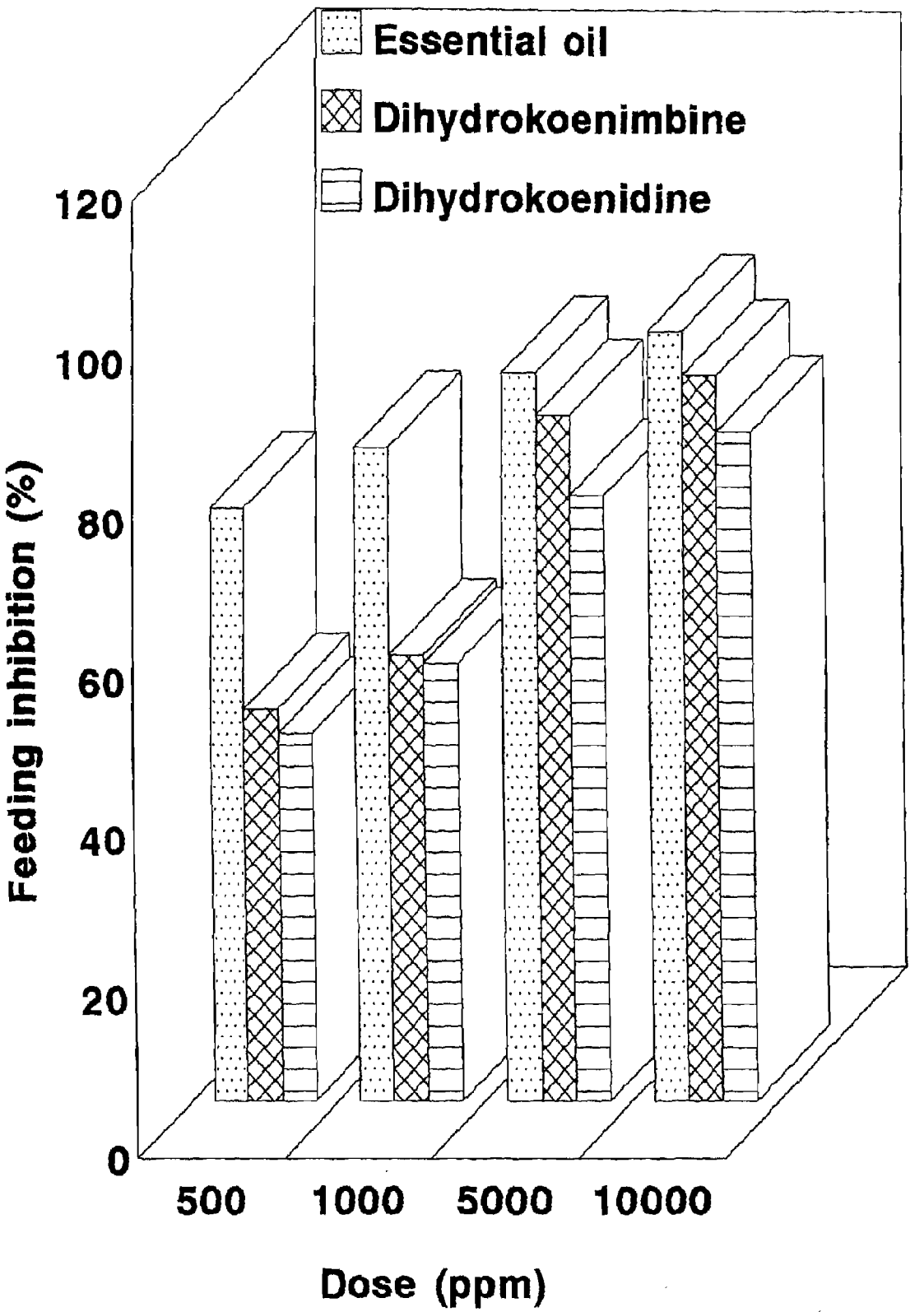


Fig. 11 Comparative study of antifeedant activity between essential oil and dihydroderivatives of koenimbine and koenidine

4.2.2 Antifungal activity

The essential oil and other chemical constituents isolated from the leaves of *Murraya koenigii* namely carbazole alkaloids and their N-methyl and dihydro derivatives along with crude hexane and benzene extracts were evaluated for their antifungal activity against three test fungi viz. *Rhizoctonia bataticola, Rhizoctonia solani* and *Helminthosporium oryzae* at concentrations ranging from 1000 ppm to 5000 ppm by food poisoning technique.

Inhibition in growth of the test fungi was recorded after a period of six days from the date of inoculation. From the per cent growth inhibition data ED_{50} (Effective dose causing 50% growth inhibition) values were calculated by probit analysis.

Probit analysis was carried out using a computer software 'LD_{50}' to compute ED_{50} for essential oil, carbazoles and their derivatives alongwith hexane and benzene extracts.

t-Test was performed to compare and determine the statistical significance of ED_{50} values of two materials.

Essential oil

The observation (Table 9) indicated that the essential oil of *M. koenigii* showed a very good antifungal activity against all the three test organisms.

Perusal of the data of fungitoxicity of all the concentrations of essntial oil against the three test organisms showed a comprehensive outlook of the total essential oil of the plant.

Table 9. Fungitoxicity of essential oil against ***Rhizoctonia solani*** ***Rhizoctonia batalicola*** and ***Helminthosporium oryzae***

Dose (ppm)	Corrected percent growth inhibition of		
	Rhizoctonia solani	***Rhizoctonia batacicola***	***Helminthosporium oryzae***
1000	39.0	47.0	46.0
2000	56.0	64.0	60.0
3000	82.0	89.0	76.0
5000	100	100	96.0

PROBIT ANALYSIS

Table value of χ^2 (95%)	***Rhizoctonia solani*** $\chi^2(1)=3.746$	***Rhizoctonia batacicola*** $\chi^2(1)=3.746$	***Helminthosporium oryzae*** $\chi^2(1)=3.746$
Result of χ^2	3.404	1.303	0.0954
ED_{50}	0.1410	0.1112	0.1214
95% confidence limit	0.1202-0.1654	0.0870-0.1428	0.0929-0.1587

At concentration 1000 ppm of oil the percentage inhibition of mycelial growth of *Rhozoctonia solani* is 39.0 while for *Helminthosporium oryzae* it is 46.0. The oil of *M. koenigii* showed a total inhibition of mycelial growth against *Rhizoctonia solani* and *Rhizoctonia bataticola* at 5000 ppm concentration.

The Chief chemical constituents of essential oil of *M. koenigii* are β-coryophyllene (29.0%) and β-gurjunene (21.0%) (sesquterpenes identified by GC-MS) have bearing on the toxicity of the plant product. The study shows that β-caryophyllene and β-gurjunene, the most abundant chemical constituents of essential oil which contribute towards the highest fungitoxicity of oil. Finally the results suggest that the oil can be tried against *Rhizoctonia solani* which causes damping off and root rot diseases of seedlings and can also be tried well against *Helminthosporium oryzae* mainly responsible for the diseases in wheat, oats and barley.

Carbazole alkaloids

The data in tables 9, 10, 11, 12, 13 and 14 showed that chemical constituents of the leaves of *M. koenigii* namely koeninbine, koenidine, mahanimbine, isomahanimbine and their N-methyl and dihydro derivatives along with hexane and benzene extracts have excellent to good activity against the three test fungi viz. *Rhizoctonia solani, Rhizoctonia bataticola* and *Helminthosporium oryzae* at concentrations 1000, 2000, 3000 and 5000 ppm.

Maximum inhibtion in mycelial growth of all the three test fungi was recorded with benzene extract of the leaves of *M. koenigii* (Table 10) Benzene extract showed complete inhibition of mycelial growth

Table 10. Fungitoxicity of Hexane and Benzene extracts of the leaves of ***M. koenigii*** against ***R. bataticola, R. solani*** and ***H. oryzae***

Dose (ppm)	Per cent growth inhibitionin					
	Hexane extract			Benzene extract		
	Rhizoctonia bataticola	***Rhizoctonia solani***	***Helminthosporium oryzae***	***Rhizoctonia bataticola***	***Rhizoctonia solani***	***Helminthosporium oryzae***
1000	27.0	30.0	32.0	50.0	49.0	52.0
2000	31.0	33.0	31.0	67.0	65.0	69.0
3000	42.0	46.0	43.0	79.0	77.0	81.0
5000	57.0	59.0	54.0	100	100	99.00

PROBIT ANALYSIS

	Hexane extract			Benzene extract		
	Rhizoctonia bataticola	***Rhizoctonia solani***	***Helminthosporium oryzae***	***Rhizoctonia bataticola***	***Rhizoctonia solani***	***Helminthosporium oryzae***
Table value of χ^2 (95%)	$\chi^2(2)=5.937$	$\chi^2(2)=5.937$	$\chi^2(2)=5.937$	$\chi^2(2)=5.937$	$\chi^2(2)=5.937$	$\chi^2(2)=5.937$
Result of χ^2	3.0507	2.2309	2.9263	0.1560	0.0773	0.1752
ED_{50}	0.4161%	0.3626%	0.4750%	0.1023%*	0.1064%*	0.0958%*
95% confidence limit	0.2925-0.5919	0.2623-0.5012	0.2802-0.8051	0.0744-0.1406	0.0771-0.1467	0.0686-0.1337

* From 95% confidence limit and t-test difference in the ED_{50} values of Hexane and benzene extracts was statistically significant at 99 % level.

against *Rhizoctonia solani* and *Rhizoctonia bataticola* at 5000 ppm concentration.

Among chemical constituents, Koenimbine and Koenidine showed a maximum inhibition in mycelial growth against all the three test fungi (Table 11) which was in conformity with the result obtained with benzene extract which had koenimbine and koenidine in major quantities.

Koenidine and Koenimbine showed complete inhibition of mycelial growth against the test fungi *Rhizoctonia solani* and *Rhizoctonia bataticola* whereas Mahanimbine and Isomahanimbine gave 55.0 and 57.0 and 58.0 and 62.0 percent growth inhibition against *Rhizoctonia bataticola* and *Rhizocotonia solani* respectively at 5000 ppm concentration (Table 12).

Maximum antifungal activity shown by Koenidine and Koenimbine at 1000, 2000, 3000 and 5000 ppm might be due to the symmetrical disposition of methyl group and substituted hydroxy group on the carbazole ring which is in complete agreement with the observation made and studied by Randelia and Patel (1982).

Investigation of the fungitoxicity of N-substituted carbazoles (Table 13) revealed that the replacement of the -NH- proton in koenidine and koenimbine by methyl group slightly enhanced the antifungal characters which could be seen by comparing ED_{50} value of Koenidine (898 ppm, 994 ppm, 1047 ppm) koenimbine (986 ppm, 1144 ppm, 949 ppm) and their N-methyl derivatives e.g. N-methyl koenidine (951 ppm, 942 ppm, 1027 ppm). N-methyl koenimbine (850 ppm, 1026 ppm, 938 ppm) against the test fungi *Rhizoctonia bataticola*, *Rhizoctonia solani* and

Table 11. Fungitoxicity of pure compounds isolated from the Benzene extracts of the leaves of ***M. koenigii*** against ***R. bataticola, R. solani*** and ***H. oryzae***

Dose (ppm)	Per cent growth inhibitionin					
	Koenimbine			Koenidine		
	Rhizoctonia bataticola	***Rhizoctonia solani***	***Helminthosporium oryzae***	***Rhizoctonia bataticola***	***Rhizoctonia solani***	***Helminthosporium oryzae***
1000	51.0	48.0	52.0	52.0	51.0	49.0
2000	65.0	60.0	69.0	64.0	65.0	66.0
3000	76.0	74.0	80.0	74.0	77.0	76.0
5000	100	100	100	99.0	100	99.0

PROBIT ANALYSIS

	Koenimbine			Koenidine		
	Rhizoctonia bataticola	***Rhizoctonia solani***	***Helminthosporium oryzae***	***Rhizoctonia bataticola***	***Rhizoctonia solani***	***Helminthosporium oryzae***
Table value of χ^2(95%)	$\chi^2(2)=5.937$	$\chi^2(2)=5.937$	$\chi^2(2)=5.937$	$\chi^2(2)=5.937$	$\chi^2(2)=5.937$	$\chi^2(2)=5.937$
Result of χ^2	0.0748	0,.7222	0.1207	0.0431	0.1654	0.1006
ED_{50}	0.0986%	0.1144%	0.0949%	0.0898%	0.0995%	0.1047%
95% confidence limit	0.0667-0.1460	0.0821-0.1596	0.0669-0.1348	0.0554-0.1457	0.0685-0.1444	0.0746-0.1469

Table 12. Fungitoxicity of pure compounds isolated from Hexane extracts of the leaves of ***M. koenigii*** against ***R. bataticola, R. solani*** and ***H. oryzae***

Dose (ppm)	Per cent growth inhibitionin					
	Mahanimbine			Isomahanimbine		
	Rhizoctonia bataticola	***Rhizoctonia solani***	***Helminthosporium oryzae***	***Rhizoctonia bataticola***	***Rhizoctonia solani***	***Helminthosporium oryzae***
1000	29.0	31.0	3.0	31.0	37.0	34.0
2000	34.0	35.0	37.0	36.0	46.0	38.0
3000	45.0	44.0	49.0	49.0	53.0	46.0
5000	55.0	57.0	61.0	58.0	62.0	53.0

PROBIT ANALYSIS

	Mahanimbine			Isomahanimbine		
	Rhizoctonia bataticola	***Rhizoctonia solani***	***Helminthosporium oryzae***	***Rhizoctonia bataticola***	***Rhizoctonia solani***	***Helminthosporium oryzae***
Table value of χ^2(95%)	$\chi^2(2)=5.937$	$\chi^2(2)=5.937$	$\chi^2(2)=5.937$	$\chi^2(2)=5.937$	X^2 (2)=5.937	X^2 (2)=5.937
Result of χ^2	0.8359	1.3067	1.7256	1.48465	0.0829	0.4176
ED_{50}	0.4089%	0.3907%	0.3110%	0.3563%	0.2418%	0.4255%
95% confidence limit	0.2758-0.6082	0.2636-0.5790	0.2297-0.4212	0.2561-0.4959	0.1766-0.3311	0.2417-0.7491

Table 13. Fungitoxicity of N-methyl derivatives of pure compounds isolated from the leaves of ***M. koenigii*** against ***R. bataticola, R. solani*** and ***H. oryzae***

Dose (ppm)	Per cent growth inhibitionin					
	N-methyl koenimbine			N-methyl koenidine		
	Rhizoctonia bataticola	***Rhizoctonia solani***	***Helminthosporium oryzae***	***Rhizoctonia bataticola***	***Rhizoctonia solani***	***Helminthosporium oryzae***
1000	55.0	50.0	53.0	49.0	52.0	47.0
2000	67.0	68.0	70.0	62.0	64.0	65.0
3000	79.0	81.0	83.0	78.0	78.0	77.0
5000	100	100	100	100	100	99.0

PROBIT ANALYSIS

	N-methyl koenimbine			N-methyl koenidine		
	Rhizoctonia bataticola	***Rhizoctonia solani***	***Helminthosporium oryzae***	***Rhizoctonia bataticola***	***Rhizoctonia solani***	***Helminthosporium oryzae***
Table value of χ^2(95%)	$\chi^2(2)=5.937$	$\chi^2(2)=5.937$	$\chi^2(2)=5.937$	$\chi^2(2)=5.937$	$\chi^2(2)=5.937$	$\chi^2(2)=5.937$
Result of χ^2	0.5173	0.2005	0.4887	0.1601	0.9481	0.0653
ED_{50}	0.0850%	0.1027%	0.0938%	0.0951%	0.0942%	0.1027%
95% confidence limit	0.0534-0.1353	0.0767-0.1374	0.0679-0.1296	0.0735-0.1577	0.0640-0.1387	0.0850-0.1495

Table 14. Fungitoxicity of dihydro-derivatives of pure compounds isolated from the leaves of ***M. koenigii*** against ***R. bataticola, R. solani*** and ***H. oryzae***

Dose (ppm)	Per cent growth inhibitionin					
	Dihydro koenimbine			Dihydro Koenidine		
	Rhizoctonia bataticola	***Rhizoctonia solani***	***Helminthosporium oryzae***	***Rhizoctonia bataticola***	***Rhizoctonia solani***	***Helminthosporium oryzae***
1000	39.0	38.0	40.0	37.0	33.0	38.0
2000	48.0	47.0	46.0	49.0	44.0	44.0
3000	57.0	54.0	54.0	57.0	53.0	50.0
5000	69.0	68.0	60.0	72.0	65.0	61.0

PROBIT ANALYSIS

	Dihydro koenimbine			Dihydro Koenidine		
	Rhizoctonia bataticola	***Rhizoctonia solani***	***Helminthosporium oryzae***	***Rhizoctonia bataticola***	***Rhizoctonia solani***	***Helminthosporium oryzae***
Table value of χ^2(95%)	$\chi^2(2)=5.937$	$\chi^2(2)=5.937$	$\chi^2(2)=5.937$	$\chi^2(2)=5.937$	X^2 (2)=5.937	X^2 (2)=5.937
Result of χ^2	1.0223	0.0516	0.1734	0.5830	0.2154	0.5026
ED_{50}	0.1956%	0.2343%	0.2340%	0.1953%	0.2494%	0.2651%
95% confidence limit	0.1491-0.2565	0.1670-0.3286	0.1596-0.3431	0.1546-0.2469	0.1951-0.3188	0.1858-0.3781

Helminthosporium oryzae respectively at concentrations ranging from 1000 to 5000 ppm. However, hydrogenation of koenimbine and koenidine to their dihydro derivatives which showed reduced antifungal characters against the same three test fungi at the same concentrations (Table 14).

4.2.3 Antinemic activity

Essential oil

In vitro test of essential oil from the leaves of *Murraya koenigii* Spreng was conducted against the freshly hatched second stage juveniles of the root-knot nematode, *Meloidogyne incognita* at the concentrations 1000, 500, 250, 125 and 12.5 ppm after the 24h, 48h and 72h of exposure.

Data on percent non-motility of juveniles of root-knot nematode, *Meloidogyne incognita* tabulated in Table 15 showed that antinemic activity of essential oil was dose and exposure time dependent.

Table 15. Antinemic activity of essential oil against second stage juveniles root knot nematode, *Meloidogyne incognita*

Dose (ppm)	Percent non-motility non-motility (After 24 h)	Per cent non-motility (After 48 h)	Per cent non-motility (After 72 h)
1000	100	100	100
500	88.7	100	100
250	72.0	79.0	87.8
125	60.5	72.7	89.6
62.5	31.6	73.2	73.8
Control	0.0	0.0	0.0

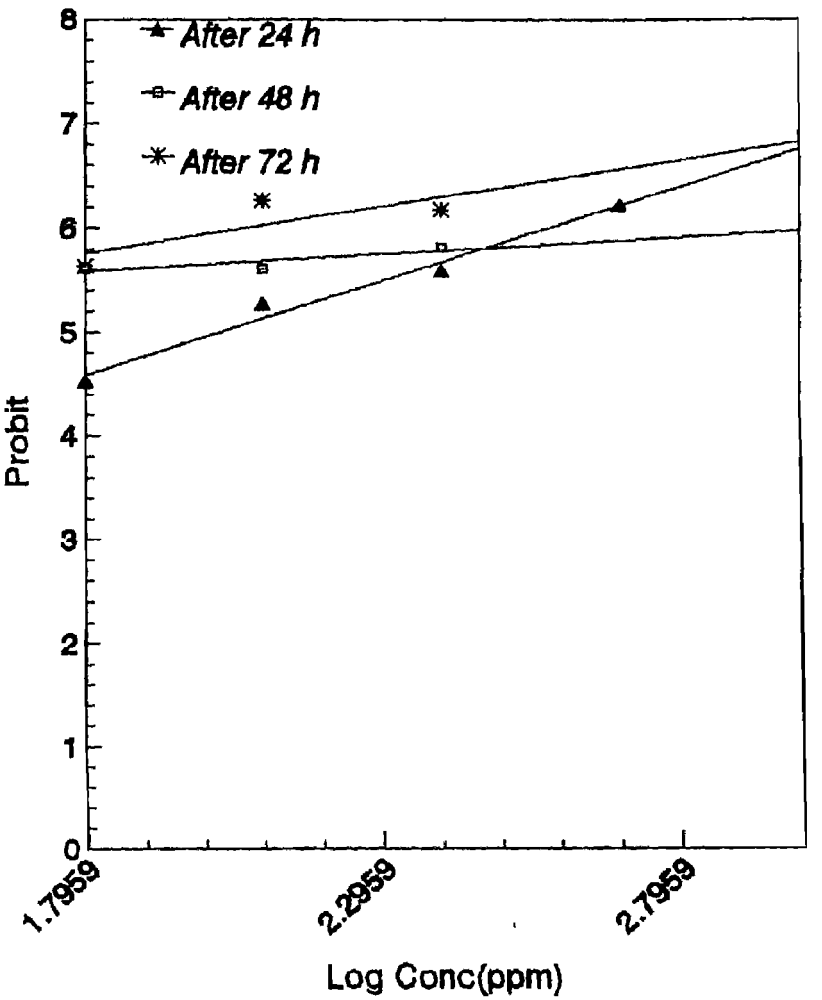


Fig-12 **Nematicidal activity of Murraya oil against root-knot nematode.**

As the concentration of essential oil increased from 62.5 ppm to 1000 ppm, the antinemic activity in terms of non-motility increased from 31.6 to 100% after 24h exposure to the test chemical.

The essential oil showed corrected non-motility more than 50% at 125, 250 and 500 ppm dose level but 31.6% at 62.5 ppm after 24 h exposure. After 48 h of exposure, the essential oil becomes more effective and gave 73.2% non-motility at 62.5 ppm and maximum activity was obained after 72 h of exposure on the nematode population.

Significant differences among the various dose levels of essential oil i.e. log. concentration versus corrected per cent non-motility (probit) of root-knot nematode, *Meloidogyne incognita* after 24h and 48 h and 72 h of exposure illustrated in Fig. 12.

In vitro tests of the chemical constituents isolated from the leaves of *Murraya koenigii* Spreng namely carbazole alkaloids and their methyl derivatives along with crude hexane and benzene extracts were conducted against the freshly hatched second stage juveniles of the root-knot nematode, *Meloidogyne incognita* at the concentrations 1000, 500, 250, 125 and 62.5 ppm after the 24h, 48 hand 72 h. of exposure.

Hexane and benzene extracts

Data on percent non-motility of juveniles of root-knot nematode *Meloidogyne incognita* tabulated in Table 16 and 17 showed that the antinemic activity of different solvent extracts from the leaves of *M. koenigii* was concentration dependent (Prasad and Singh, 1996) also reported the similar results while testing salmeal extracts and three nematicides *in vitro*.

Table 16. Antinemic activity of Hexane extract of the leaves of ***Murraya koenigii*** against second stage juveniles of root knot nematode (***Meloidogyne incognita***)

Dose (ppm)	Corrected mortality (%)			LT_{50} (hrs.)
	After 24 hrs	After 48 hrs	After 72 hrs	
1000	15.4	42.5	74.4	49.8
500	8.4	34.9	58.8	63.08
250	8.0	25.5	29.3	473.0
125	8.2	9.8	21.5	-
62.5	4.7	4.4	17.9	-

Table 17. Antinemic activity of Benzene extract of the leaves of ***Murraya koenigii*** Spreng against second stage juveniles of root knot nematode (***Meloidogyne incognita***)

Dose (ppm)	Corrected mortality (%)			LT_{50} (hrs.)
	After 24 hrs	After 48 hrs	After 72 hrs	
1000	26.0	46.6	74.7	43.67
500	19.0	34.1	60.5	61.82
250	16.5	30.9	31.1	177.4
125	13.2	21.6	26.9	-
62.5	9.0	15.0	19.3	-

The benzene extract at 62.5 ppm after 24 hours exposure showed no activity whereas at 1000 ppm it caused immobility upto 77.7%. ED_{50} value of benzene extract with respect to immobility was calculated to be 352 ppm in 72 hours exposure.

Another important factor which could relate the dependence of antinemic properties of crude, pure carbazole compounds and the corresponding N-methyl derivatives of pure carbazoles with lethal time i.e. LT_{50} was used here.

Benzene extract showed higher antinemic activity than hexane extract against *M. incognita* which could be observed from comparing the LT_{50} (Time taken by extract/pure compounds to cause 50% immobility) value of both the extracts with respect to immobility at 1000 ppm and 500 ppm dose levels. At 1000 ppm, the hexane extract gave LT_{50} value 49.0 hours whereas benzene extract showed it to be 43.0 hours. Similarly at 500 ppm level the benzene extract showed LT_{50} value 61.0 hours whereas hexane extract resulted 63.0 hours.

From the results, it could be inferred that as the concentration of crude extract decreased the LT_{50} value with respect to immobility in the nematode population increased and later on the LT_{50} value became insignificant at low concentration e.g. 62.5 ppm.

Carbazole alkaloids and their N-methyl derivatives

Investigation of antinemic activity of Koenimbine, Koenidine and their N-methyl derivatives revealed that the replacement of -NH- proton of carbazole ring in Koenimbine and Koenidine by a methyl group enhanced the antinemic properties of these carbazole compounds. The maximum

activity of Koenimbine and Koenidine was obtained after 72 hours of exposure on the nematode population (Tables 18 and 19).

Table 18. Antinemic activity of Koenidine isolated from the Benzene extract of the leaves of ***M. koenigii*** against second stage juveniles of root knot nematode (***Meloidogyne incognita***)

Dose (ppm)	Corrected mortality (%)			LT_{50} (hrs.)
	After 24 hrs	After 48 hrs	After 72 hrs	
1000	6.0	42.3	65.5	56.33
500	Nil	38.2	42.3	-
250	Nil	27.0	31.1	-
125	Nil	21.6	23.7	-
62.5	Nil	14.4	21.4	-

Table 19. Antinemic activity of Koenimbine isolated from Benzene extract of the leaves of ***M. koenigii*** spreng against second stage juveniles of root knot nematode (***Meloidogyne incognita***)

Dose (ppm)	Corrected mortality (%)			LT_{50} (hrs.)
	After 24 hrs	After 48 hrs	After 72 hrs	
1000	4.5	40.6	62.6	58.73
500	Nil	37.0	40.6	-
250	Nil	25.6	29.1	-
125	Nil	19.2	21.6	-
62.5	Nil	12.0	19.0	-

The higher antinemic activities of N-methyl koenimbine and N-methyl koenidine could be observed from the comparison of LT_{50} value of N-methyl koenimbine and N-methyl koenidine with those of pure carbazole compounds e.g. Koenimbine and Koenidine at 1000 ppm dose level.

At 1000 ppm, N-methyl koenimbine and N-methyl koenidine gave LT_{50} value 39.0 and 42.0 hours respectively (Tables 20 and 21) whereas pure carbazoles e.g. Koenimbine and koenidine gave LT_{50} value 56.0 and 59.0 hours respectively at the same concentration.

Table 20. Antinemic activity of N-methyul derivative of koenidine isolated from the Benzene extract of the leaves of ***M. koenigii*** against second stage juveniles of root knot nematode (***Meloidogyne incognita***)

Dose (ppm)	Corrected mortality (%)			LT_{50} (hrs.)
	After 24 hrs	After 48 hrs	After 72 hrs	
1000	29.5	51.4	82.3	39.43*
500	21.6	38.2	66.0	54.25*
250	19.1	334.1	37.2	-
125	16.7	25.0	31.1	-
62.5	12.1	19.5	23.4	-

* From 95 % confidence limit limit and t-test, difference in the LT_{50} values of Koenimbine, Koenidine and their corresponding N-methyl derivatives was statistically significant.

Table 21. Antinemic activity of N-methyl derivative of Koenimbine isolated from Benzene extract of the leaves of ***M. koenigii*** Spreng against second stage juveniles of root knot nematode (***Meloidogyne incognita***)

Dose (ppm)	Corrected mortality (%)			LT_{50} (hrs.)
	After 24 hrs	After 48 hrs	After 72 hrs	
1000	26.0	48.7	78.0	42.69
500	19.2	36.2	62.4	48.21
250	16.7	29.8	32.7	60.96
125	14.0	22.1	27.2	72.75
62.5	9.4	16.2	20.1	-

Reason for the enhanced antinemic properties of N-methyl koenimbine and N-methyl koenidine could possibly be due to their greater lipophilicity which might lead to the dissolution of lipid layer of Juveniles.

4.2.4 Insect Growth Inhibition Activity (IGR)

The dose dependent inhibition of growth of twenty four hour old neonate larvae of *Spodoptera litura* by the essential oil extracted from the leaves of *M. koenigii* revealed (Table 22) that the oil extracted with supercritical fluid technique using CO_2 is more effective and having higher IGR activity than the oil extracted by hydrodistillation method in a clevenger's apparatus.

Table 22. Evaluation of essential oil for growth inhibition study against ***Spodoptera litura***

Dose (ppm)	Percent growth inhibition	
	Hydrodistilled oil	Supercritical fluid extracted oil
500	35.00	40.00
1000	45.41	48.00
2000	58.00	59.00
2500	-	66.00
3000	64.00	98.00
4000	96.00	-

PROBIT ANALYSIS

Table value of χ^2 (95%) Result of CHI_2	Hydrodistilled oil $\chi^2(3)=5.937$ 0.1997	Supercritical fluid extracted $\chi^2(3)=5.937$ 0.7699
ED_{50} Value	0.1214%	0.1049%
95% confidence limit	0.0925-0.1594	0.0745-0.1462

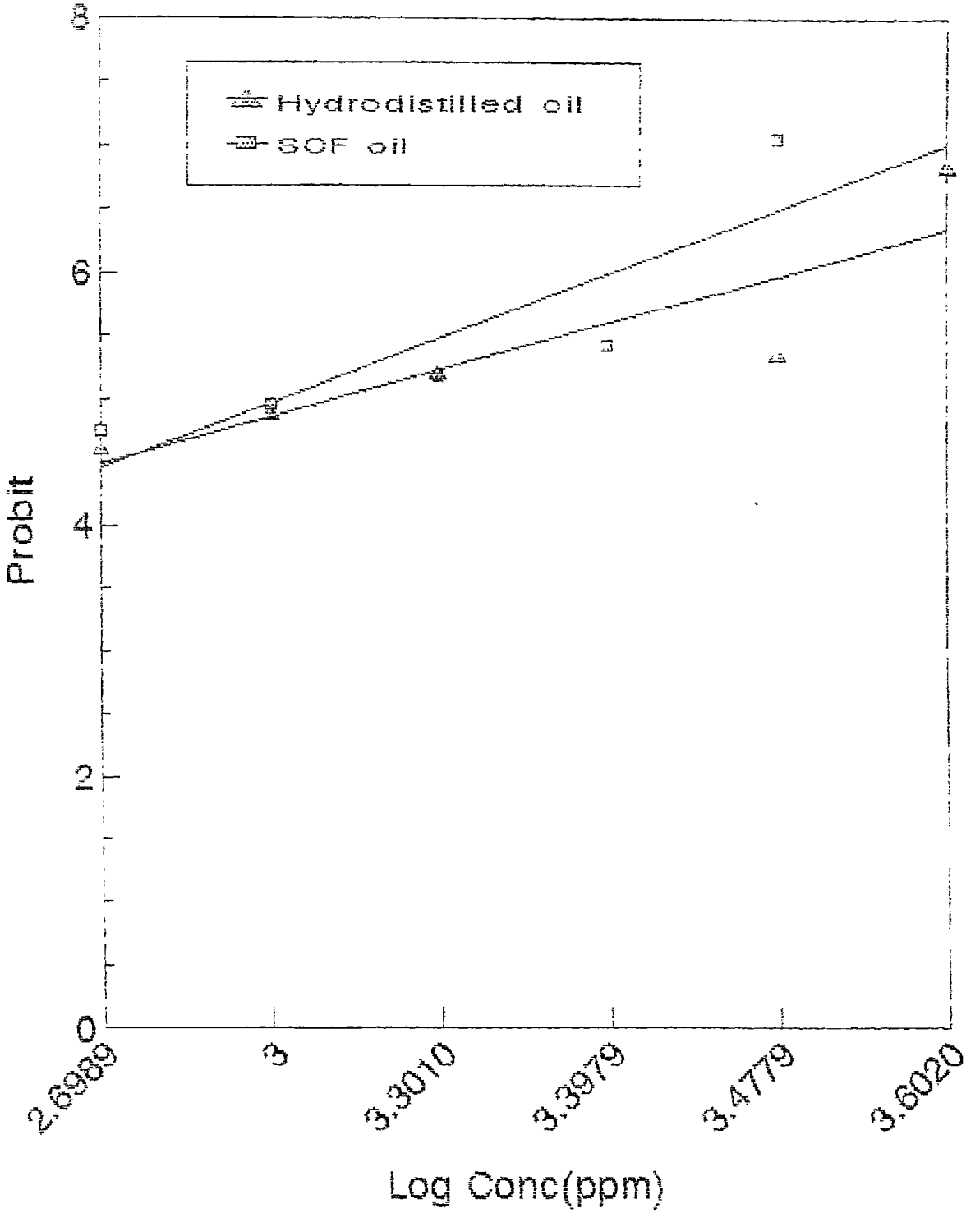


Fig-13 Growth inhibition study of Murraya oil against *Spodoptera litura*

At 3000 ppm level, the supercritical fluid extracted (SCF) oil gave 98% growth inhibition whereas hydrodistilled oil showed only 64% growth inhibition. Hydrodistilled oil reached to 96% growth inhibition at 4000 ppm level.

The presence of more lower boiling fractions (monoterpenoids), fatty acids such as palmitic and lauric and a carbazole alkaloid Mahanimbine in SCF-extracted oil might be responsible for imparting higher IGR activity to this oil than hydrodistilled oil. The difference in the IGR activity between SCF-extracted oil and hydrodistilled oil is illustrated in a best way by line-diagram (Fig. 13).

4.2.5 Larvicidal activity

The essential oil extracted from the leaves of *Murraya koenigii* was evaluated for the larvicidal activity against 3rd or early 4th instar larvae of *Anopheles stephensi* at concentrations 5, 10, 20 and 50 ppm after the 24 hr of exposure.

Data on per cent mortality of mosquito larvae, *Anopheles stephensi* tabulated in Table 23 showed that larvicidal activity of essential oil was dose dependent. As the concentration of essential oil increased from 5 ppm to 50 ppm dose level, the larvicidal activity also increased and gave 10 to 100% mortality after 24 h exposure to the test chemical.

The essential oil showed mortality of mosquito larvae more than 50% at 20 and 50 ppm dose level after 24 h exposure.

LD_{50} value with respect to mortality was calculated to be 15.96 ppm.

Table 23. Larvicidal activity of essential oil against mosquito larvae (***Anopheles stephensi***)

Dose (ppm)	Per cent growth mortality
5	10.0
10	30.0
20	60.0
50	100.0

PROBIT ANALYSIS

Table value of χ^2 (95%) Result of χ^2	$\chi^2(1)=3.746$ 0.0448
ED_{50} Value	15.961 ppm
95% confidence limit	13.474-18.907

The chief chemical constituents of essential oil *M. koenigii* are β-caryophyllene (29.0%) and β-gurjunene (21.0%) and contribute toward the highest larvicidal activity of the oil.

4.2.6 Repellent activity of Murraya koenigii essential oil

Plants are a rich source of renewable bioactive chemicals. A number of plants have been identified for their utility in insect control. The repellent effects of various essential oils on insects are reported by Su *et al.* (1972); Saxena and Kaul (1978); Chander and Ahmed (1986).

In the experiment, the oil extracted from the leaves of *M. koenigii* Spreng by using steam distillation in clevenger technique and supercritical fluid extraction (SCF) technique using liquid CO_2 and both of these obtained oils were assessed for food repellency against storage pest *Tribolium castaneum*. The various test concentations ranging from 1000 to 5000

ppm were used and the result obtained after exposure of 1h, 4h and 24h has enlisted in Table 24.

Table 24. Repellant/Food deterrent effect of hydrodistilled and supercritical fluid extracted essential oil against ***Tribolium castaneum*** (Herbst)

Dose (ppm)	Percent Repellancy/Deterrency in					
	Hydrodistilled oil			Supercritical fluid extracted oil		
	1 hr	4 hr	24 hr	1 hr	4 hr	24 hr
1000	29.47	33.36	35.80	35.80	39.50	42.80
2000	35.60	37.01	39.50	41.26	42.60	45.50
3000	40.50	39.45	42.80	47.71	47.03	50.44
5000	46.03	45.50	47.71	53.48	53.08	54.50
Control	-	-	-	-	-	-

It is observed from the table 24 that steam distilled oil recorded 35.80 to 47.71% food repellency at 1000 to 5000 ppm dose level after the 24 hour of exposure and was superior to control. Whereas SCF extracted oil gave 42.8 to 54.5% food repellency at 1000 to 5000 ppm dose level after 24 hour of exposure and superior to steam distilled oil.

The higher food repellency recorded in supercritical fluid extracted oil at the concentration ranging from 1000 to 5000 ppm against storage pest, *Tribolium castaneum* after the exposure of 1h, 4h and 24 hour may be due to the presence of more low-boiling fractions (monoterpenoids) in SCF-oil than in hydrodistilled oil.

Higher food repellency value of SCF-oil also reflect some of the good features of supercritical fluid extraction process.

* It gives extracts identical to the original fragrance present in the matrix

* Low temperature extraction and good separation is possible for thermally labile compounds.

So supercritical fluid extraction (SCFE) using liquid CO_2 emerged as a very promising technique for extraction of fragrance materials. The essential oil obtained by this process has been identified as the promising agent for IGR activity.

CHAPTER 5

SUMMARY AND CONCLUSION

In view of the growing awareness from the hazards of synthetic pesticides more emphasis is being given for the development of newer pesticides from plants which are expected to be not only free from harmful effects such as environmental pollution, human health hazard, pest resistance and outbreak of minor pests into major ones encountered with the synthetic pesticides but also to serve as lead chemicals which give a new hope for future pest control agents.

The plant which is affected by few insect pest and pathogens could be examined phytochemically to find out the chemicals responsible for the defence mechanism. *Murraya koenigii* (L.) Spreng. is one such plant. Leaves of *M. koenigii* were subjected to phytochemical and bioactivity evaluation.

The essential oil and carbazole alkaloids extracted and isolated from the leaves of *M. koenigii* exhibit wide spectrum of biological activities which include antiinflammatory, anticonvulsant and antidiarrhoeal activity.

Although the plant is well known for its antibiotic and antimicrobial activities but not much work has been done on insect and other pest controlling properties e.g. antifeedancy, antinemic and fungitoxicity of pure constituents namely carbazole alkaloids present in the leaves of *M. koenigii* Spreng.

The present investigation titled "Chemistry and bioactivity of constituents of *Murraya koenigii* (Linn.) Spreng. leaves" with following objectives :

(I) Detailed chemical examination of leaves of *Murraya koenigii*.

(II) Biological activity evaluation of chemical constituents obtained from the above plant has been carried out in the Division of Agricultural chemicals, IARI, New Delhi and its sister Divisions e.g. Entomology, Mycology & Plant pathology.

Super critical fluid extraction technique using liquid CO_2 for obtaining essential oil from leaves of *M. koenigii* was used in Indian Institute of Technology (IIT), Hauz Khas, New Delhi.

Hydrogenation of pure carbazole alkaloids obtained after the rigorous column chromatography of benzene extract of *M. koenigii* was done in the Chemistry Department, Delhi University. Elemental analysis was done at National Facility for Blue Green Algae (NFBGA), IARI, New Delhi.

The major carbazole alkaloids isolated from the leaves of *M. koenigii* were characterized as Mahanimbine, Isomahanimbine, Cyclomahanimbine, Koenimbine, Koenidine, Koenigine, Koenine and Mahanine. Isolation and purification of these carbazole alkaloids is simple and elegant.

Because of the wide range of biological activities, the plant carbazoles have the potentiality to be used either as such or as a lead molecule in pest control by developing some highly active analogue by chemical modification.

An attempt therefore has been made to utilize the leaves of the plant, *M. koenigii* in pest control measures not only on the basis of carbazoles alone but also by transforming carbazoles chemically into their derivatives.

The salient features of the research findings are summarized below

(1) The essential oil comprised of mono- and sesquiterpenoids was extracted from the leaves of *M. koenigii* by two techniques e.g. hydrodistillation by clevenger appratus, super critical fluid extraction technique using liquid CO_2 (SCF). Yield of essential oil was found higher in SCF-extracted technique.

(2) Carbazole alkaloids e.g. Mahanimbine, Isomahanimbine, Cyclomahanimbine, Koenimbine, Koenidine, Koenine, Koenidine, Mahanine of *M. koenigii* were isolated in as much as 97% pure form.

(3) Carbazoles were transformed chemically into several products as described below :

* Reaction of Koenidine with methyl iodide and sodium hydride in dry benzene yielded N-methyl koenidine, m.p. 189^{0}C.

* Reaction of Koenimbine with methyl iodide and sodium hydride in dry benzene gave N-methyl koenimbine, m.p. 148^{0}C.

* Koenidine was converted to dihydrokoenidine by dissolving koenidine in ethanol and hydrogenated at 29^{0}C using 5% Pd/C (0.2 gm).

* Koenimbine was converted to dihydrokoenimbine by dissolving koenimbine in ethanol and hydrogenated at 29°C using 5% Pd/C (0.2 gm).

(4) Antifeedant bioassay of essential oil, crude and pure carbazole alkaloids, dihydro and N-methyl derivatives of pure carbazole alkaloids against 5th instar larvae of tobacco cutworm, *Spodoptera litura* (Fab.) by 'choice test', at concentration from 500 - 10,000 ppm revealed that :

* The essential oil extracted through hydrodistillation showed good activity against *Spodoptera litura* larvae. The ED_{50} value for hydrodistilled oil was 130 ppm.

* Carbazoles are not a strong antifeedant chemical like essential oil. They are moderately antifeedant. However, their dihydro-derivatives showed better antifeedancy.

* Investigation of the antifeedant activity of N-substituted and dihydro carbazoles revealed that the replacement of NH - proton of carbazoles by a methyl group did not have much effect on the antifeedant properties of these compounds but hydrogenation of the carbazoles enhanced the antifeedant activity which could be observed from the comparison of ED_{50} of pure carbazoles and their dihydro derivatives.

(5) Insect growth regulatory activity (IGR). : The dose dependent growth inhibition of 24 hour old neonate larvae of *Spodoptera litura* by the essential oil extracted from the leaves of *M. koenigii* revealed that the oil extracted with supercritical fluid process was more

effective and having higher insect growth regulatory activity than the oil extracted by hydrodistillation method in clevenger apparatus.

(6) Repellant/Food deterrent activity of essential oil extracted from the leaves of *M. koenigii* by using Hydrodistillation in clevenger and supercritical fluid extraction technique using CO_2 against storage pest, *Tribolium castaneum* at concentrations ranging from 1000 to 5000 ppm in olfactometer revealed that the supercritical fluid (SCF) extracted oil showed higher per cent food repellancy than the hydrodistilled oil at concentrations 1000 - 5000 ppm after 24 hours exposure period and was superior to hydrodistilled oil.

(7) Antifungal bioassay of essential oil, crude and pure carbazole alkaloids, dihydro and N-methyl derivatives of pure carbazole alkaloids against three test fungi viz., *Rhizoctonia solani*, *Rhizoctonia bataticola* and *Helminthosporium oryzae* at concentrations ranging from 1000 ppm to 5000 ppm by food poisoning technique revealed that :

* The essential oil showed good antifungal activity against all the three test organisms e.g. *R. solani*, *R. bataticola* and *H. oryzae*. The ED_{50} values of essential oil for *R. bataticola*, *R. solani* and *H. oryzae* were recorded to be 1112 ppm, 1410 ppm and 1214 ppm respectively.

* The hexane extract, benzene extract along with four major constituents viz. Koenidine, Koenimbine, Mahanimbine, Isomahanimbine were tested for their antifungal activity against three fungi *Rhizoctonia bataticola*, *Rhizoctonia*

solani and *Helminthosporium oryzae*. Benzene extract showed maximum inhibition of the growth of the three fungi. Among chemical constituents Koenidine and Koenimbine showed maximum growth inhibition which is in conformity with the results obtained with benzene extracts, which had Koenidine and Koenimbine in major quantities.

* Investigation of the antifungal activity of N-substituted and dihydro carbazoles against three fungi *R. bataticola*, *R. solani* and *H. oryzae* revealed that the replacement of NH-proton of carbazole ring by a methyl group slightly enhanced the antifungal properties of N-methyl carbazole but hydrogenation of the carbazoles however showed significant reduction in the antifungal properties of their corresponding dihydro carbazoles.

(8) Antinemic bioassay of essential oil, crude and pure carbazol alkaloids, and N-methyl derivatives of pure carbazoles against root knot nematode at *Meloidogyne incognita*, concentrations 1000, 500, 250, 125 and 62.5 ppm after 24 h, 48 h and 72 h of exposure revealed that :

* From the data on per cent non-motility of second stage juveniles of root-knot nematode, *Meloidogyne incognita* it could be inferred that the antinemic activity of essential oil was *dose* and *time of exposure* dependent. And a close scrutiny of the data also revealed that the essential oil is the most potent chemical for *M. incognita* control.

* The hexane and benzene extracts were tested for antinemic activity against root knot nematode, *Meloidogyne incognita*.

Benzene extract showed higher antinemic activity than hexane extract which could be observed from comparing LT_{50} value of both the extracts with respect to immobility.

* Investigation of antinemic activity of Koenimbine, Koenidine and their N-methyl derivatives revealed that the replacement of -NH-proton of carbazole ring in Koenimbine and Koenidine by a methyl group enhanced the antinemic properties of these carbzole compounds. Reason for higher antinemic properties of N-methyl koenimbine and N-methyl koenidine presumably be attributed to increased lipophilicity which might lead to the dissolution of lipid layer of juveniles.

(9) Larvicidal activity

The essential oil extracted from the leaves of *Murraya koenigii* Spreng. was evaluated for the larvicidal activity against 3rd or early 4th instar larvae of *Anopheles stephensi* at concentrations 5, 10, 20 and 50 ppm after the 24 hours of exposure revealed that :

* From the data on per cent mortality of 3rd or early 4th instar larvae *Anopheles stephensi* it could be concluded that the larvicidal activity of essential oil was dose dependent. And a close scrutiny of the data also revealed that the essential oil is the most potent chemical for the control of mosquito larvae, *A. stephensi*.

CONCLUSION

Essential Oil

- Essential oil was uniformly found to be active in all the bioassay screenings.
- It showed better antifeedant activity against 5th instar larvae of *Spodoptera litura*, antinemic activity against second stage juveniles of root knot nematode *Meloidogyne incognita*, repellent activity against *Tribolium castaneum* and antifungal activity against *Rhizoctonia solani*, *Rhizoctonia bataticola* and *Helminthosporium oryzae* than that of its non-volatile (Carbazole alkaloids) counterparts.
- The close scrutiny of data also revealed that the essential oil was most potent chemical for the control of mosquito larvae, *Anopheles stephensi* and could better utilized for pest control activities.

Carbazole alkaloids : They are divided into following groups :

(1) Carbazole alkaloids present in hexane and benzene extracts.

(2) Carbazole alkaloids isolated from hexane and benzene extracts.

(3) Structurally modified carbazole alkaloids.

- Between crude extracts, benzene extract was found to be far most antifungal against *Rhizoctonia solani* (ED_{50} - 1060 ppm), *Rhizoctonia bataticola* (ED_{50} - 1020 ppm) and *Helminthosporium oryzae* (ED_{50} - 950 ppm), antifeedant against 5th instar larvae of *Spodoptera litura* (ED_{50} 710 ppm), antinemic against root knot nematode, *Meloidogyne incognita* (ED_{50} - 350 ppm) than hexane extract.

- Among pure carbazole alkaloids - Mahanimbine, Isomahanimbine (Hextane extract), Koenimbine, Koenidine (Benzene extract).

  Antifeedant activity against *Spodoptera litura* followed the order : Koenimbine (ED_{50} 924 ppm) > Koenidine (ED_{50} 1070 ppm) > Mahanimbine (ED_{50} 1860 ppm) > Isomahanimbine (ED_{50} 2550 ppm).

  Antifungal activity against *Rhizoctonia solani*, *Rhizoctonia bataticola* and *Helminthosporium oryzae* showed the order :

  Rhizoctonia solani — Koenidine (ED_{50} 990 ppm) > Koenimbine (ED_{50} 1140 ppm) > Isomahanimbine (ED_{50} 2410 ppm) > Mahanimbine (ED_{50} 3900 ppm).

  Rhizoctonia bataticola — Koenidine (ED_{50} 890 ppm) > Koenimbine (ED_{50} 980 ppm) > Isomahanimbine (ED_{50} 3560 ppm) > Mahanimbine (ED_{50} 4080 ppm).

  Helminthosporium oryzae — Koenimbine (ED_{50} 940 ppm) > Koenidine (ED_{50} 1040 ppm) > Mahanimbine (ED_{50} 3100 ppm) > Isomahanimbine (ED_{50} 4250 ppm).

  Whereas Koenidine (ED_{50} 604 ppm) and Koenimbine (ED_{50} 706 ppm) were found to be nematoxic against *Meloidogyne incognita*.

- Structurally modified carbazole alkaloids : It becomes evident that carbazoles causing bioactivity could be enhanced by making structural modifications e.g. :

(i) Hydrogenation — On hydrogenating the pure carbazole alkaloids to their corresponding dihydro-derivatives, there was a significant increase in the antifeedant properties of these dihydro-derivatives against 5th

instar larvae of *Spodoptera litura* e.g., Dihydro koenidine (ED_{50} 750 ppm) > Koenidine (ED_{50} 1070 ppm) and Dihydro koenimbine (ED_{50} 630 ppm) > Koenimbine (ED_{50} 920 ppm).

(ii) N-methylation — Antinemic activity of pure carbazole alkaloids against second stage juveniles root knot nematode *Meloidogyne incognita* was enhanced by their derivatization into N-methyl derivatives e.g., At 1000 ppm dose level, N-methyl koenimbine (LT_{50} 43.0 hr) > Koenimbine (LT_{50} 59.0 hr) and N-methyl koenidine (LT_{50} 39.0 hr) > Koenidine (LT_{50} 56.0 hr).

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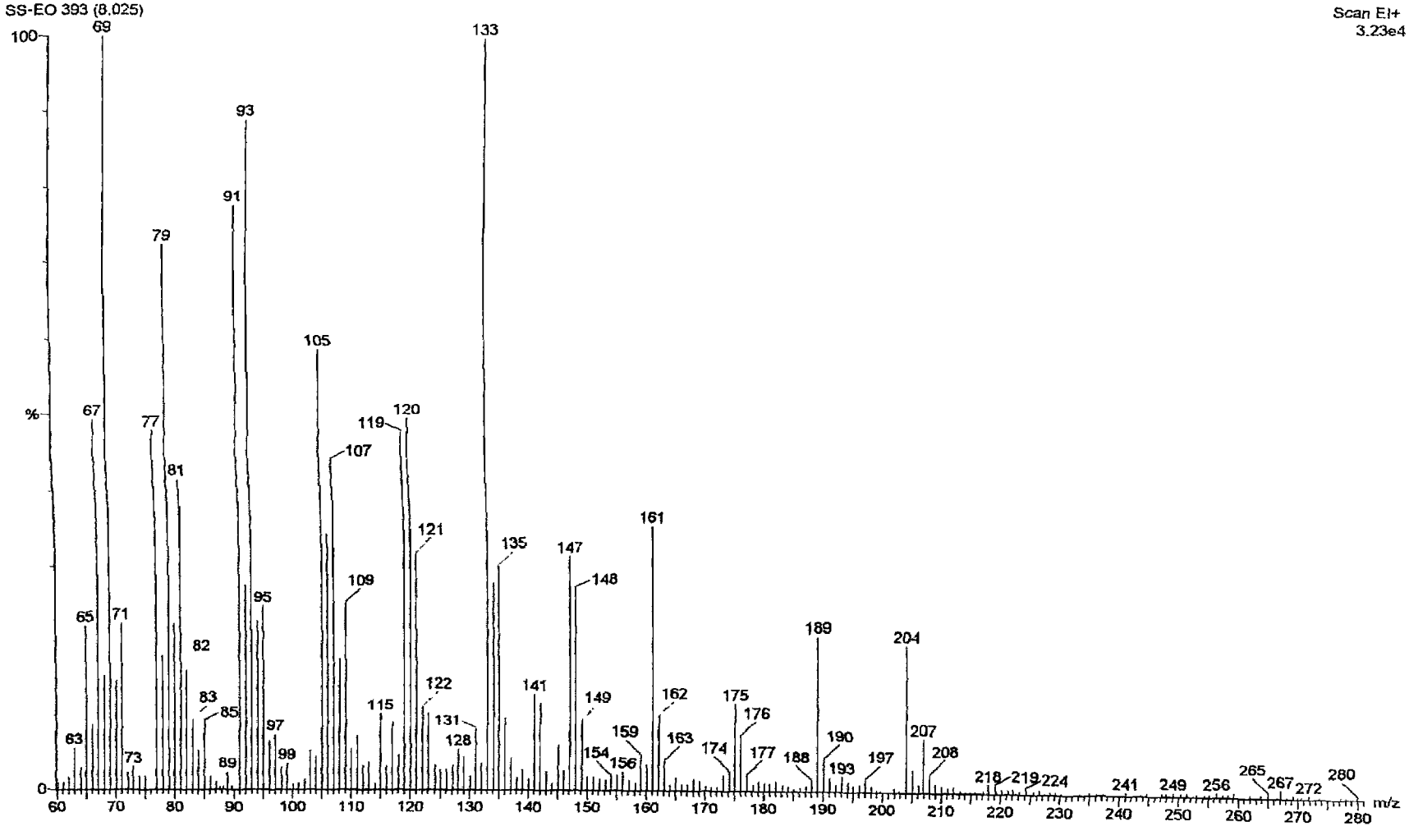

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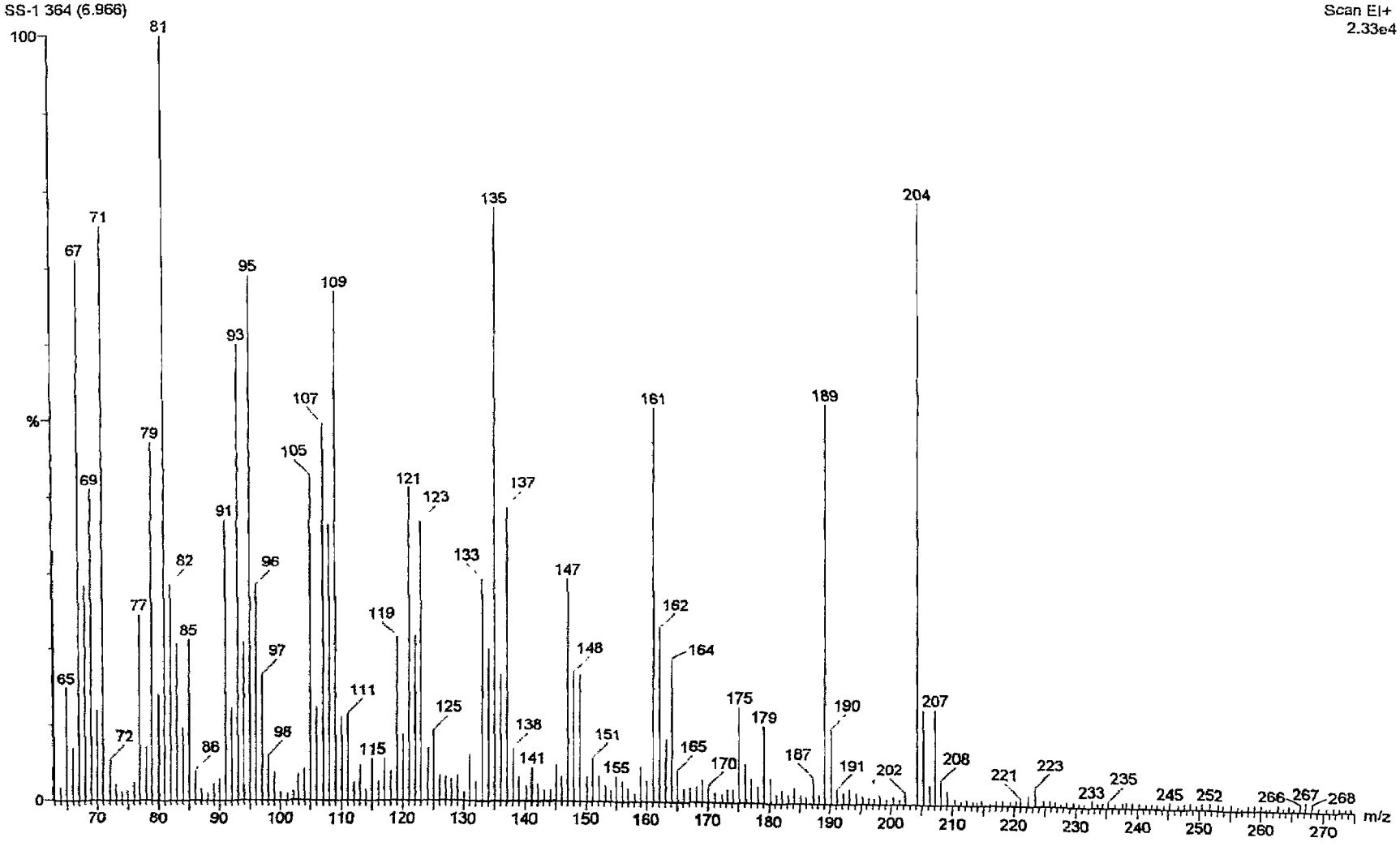

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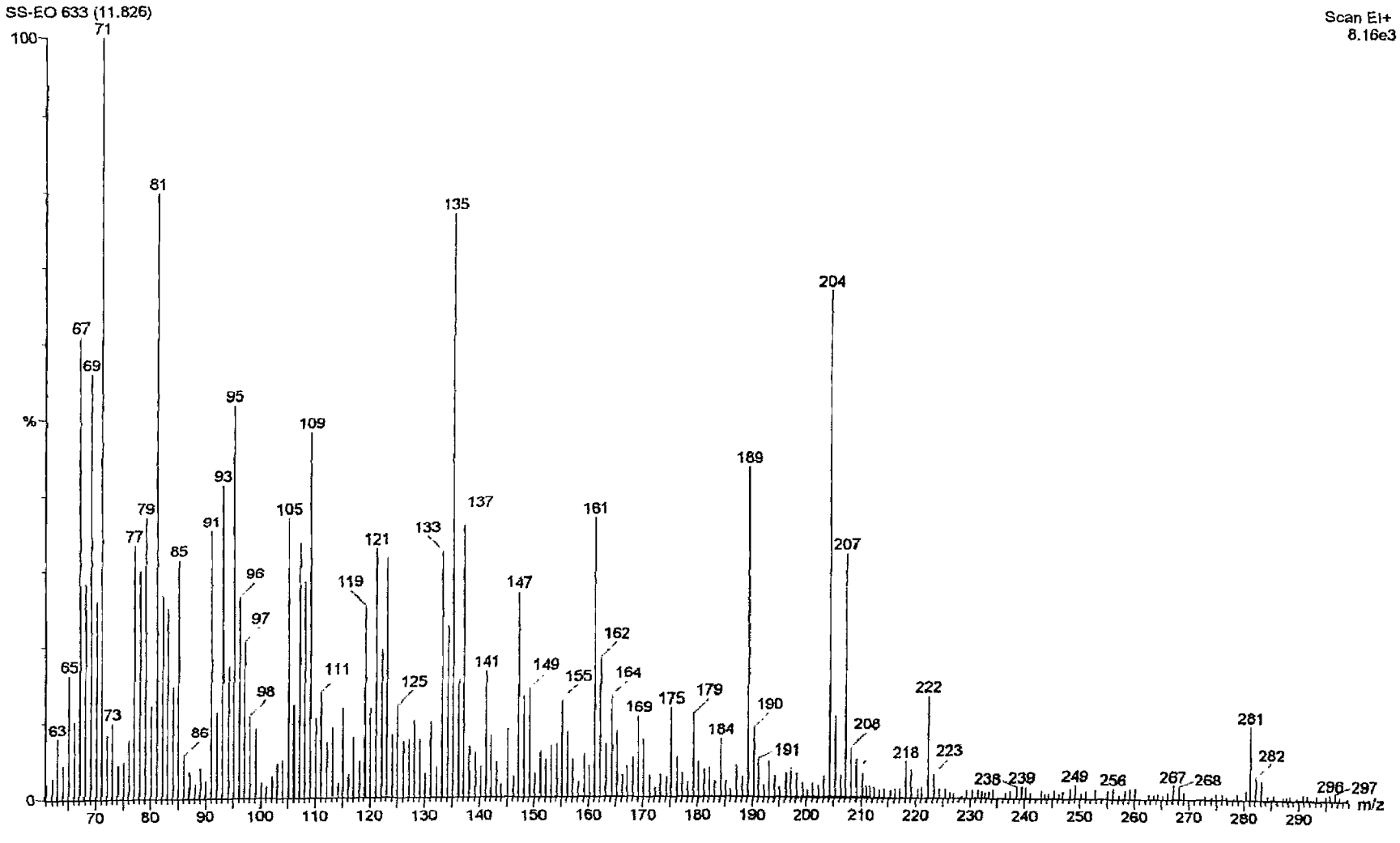

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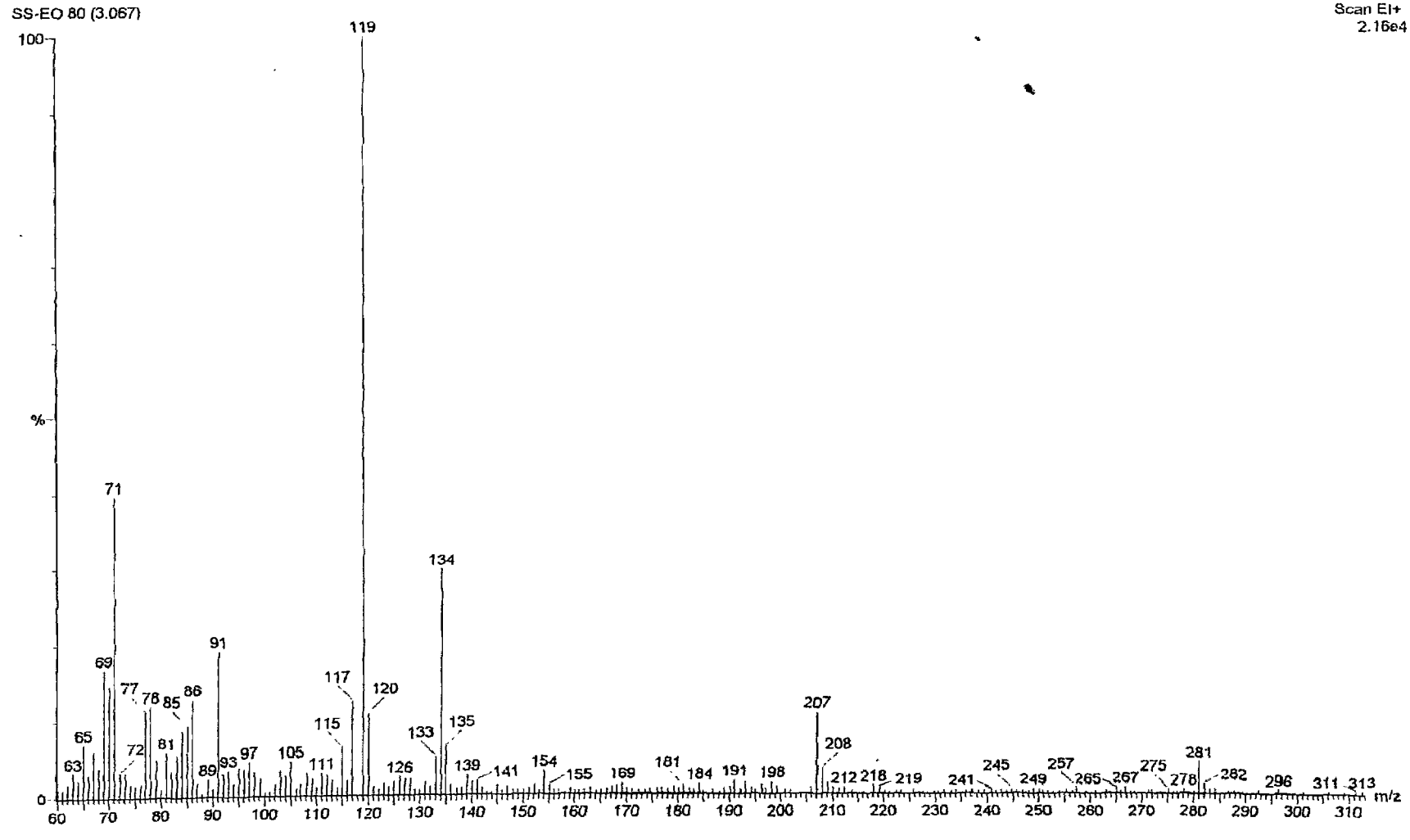

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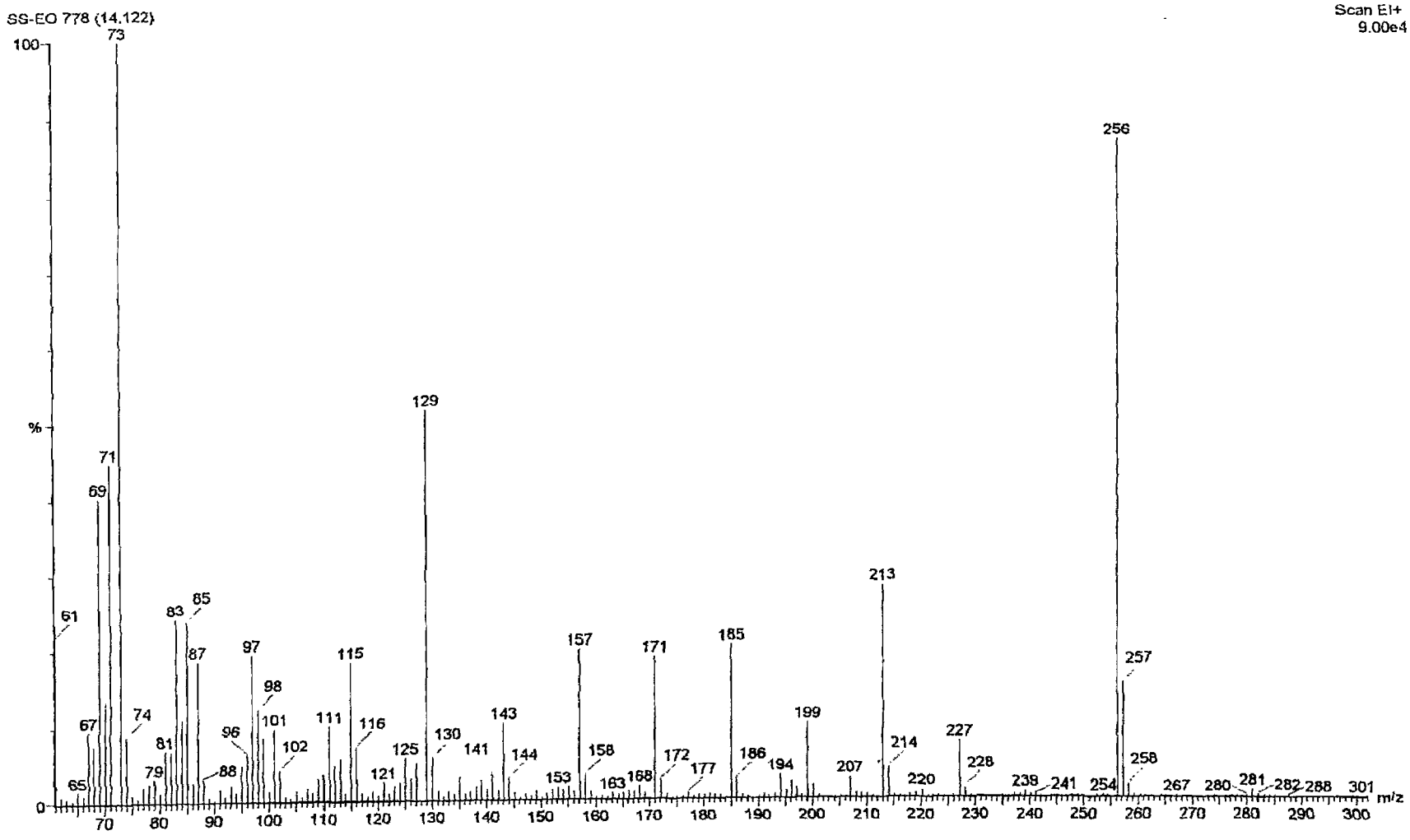

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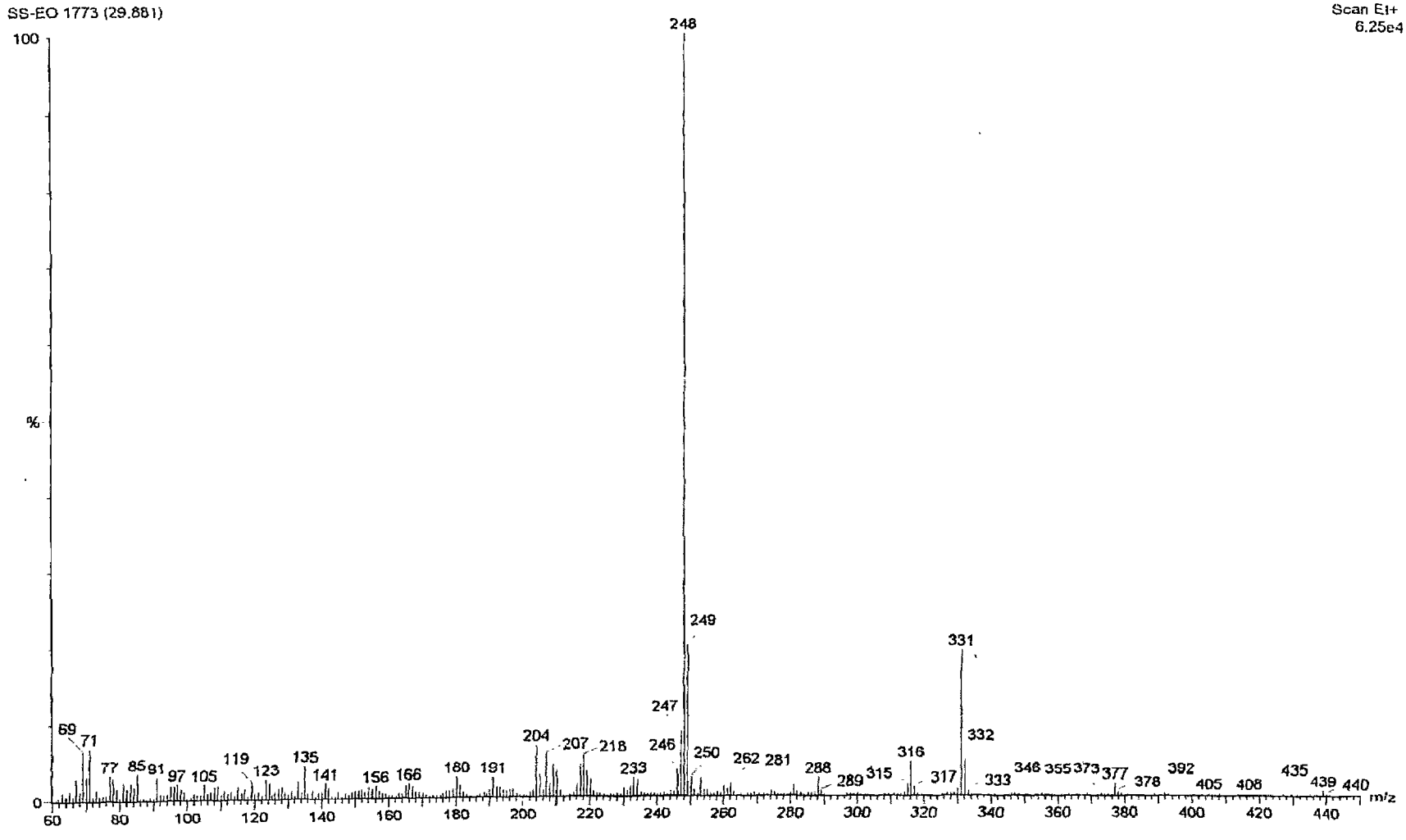

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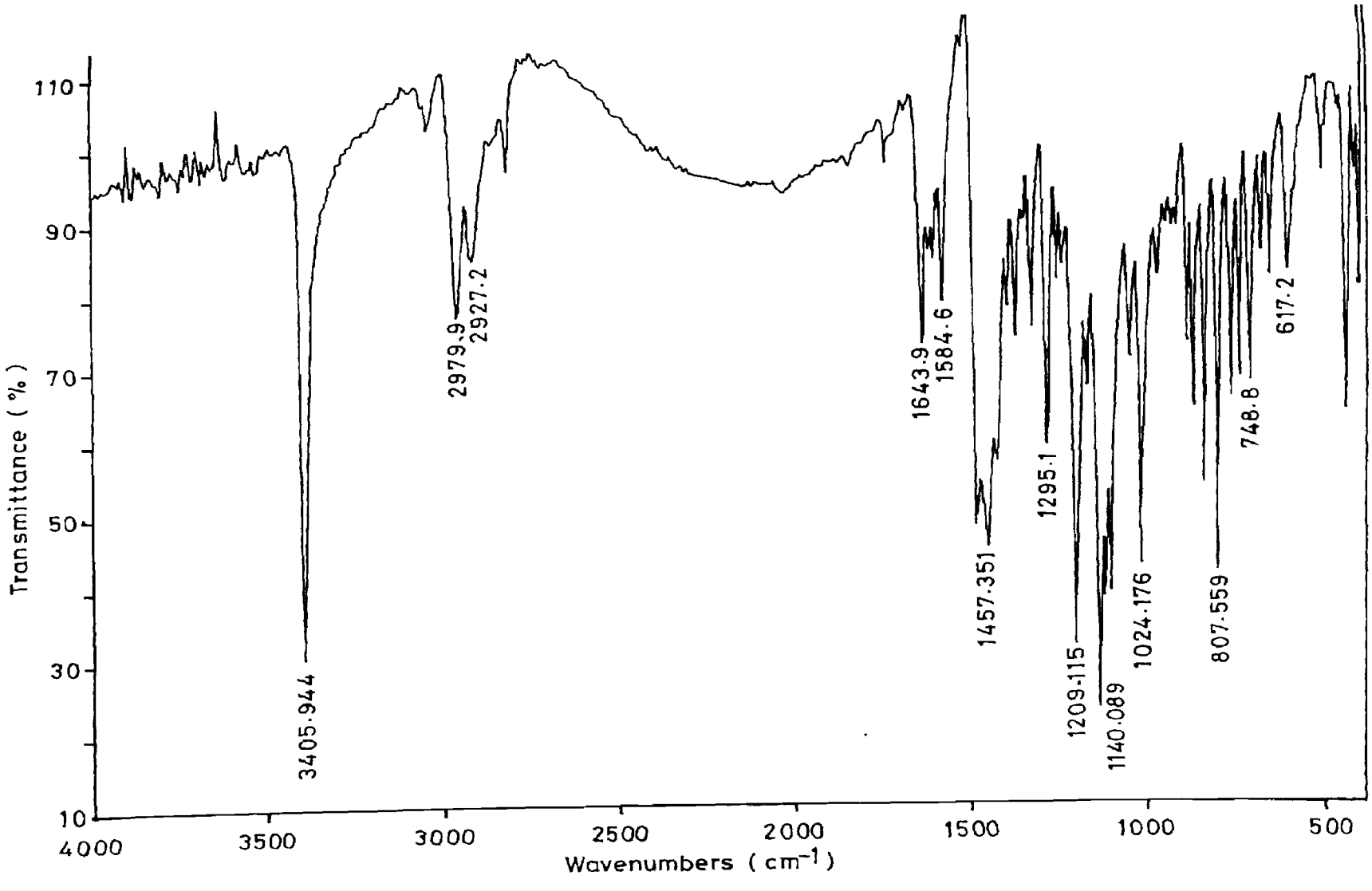

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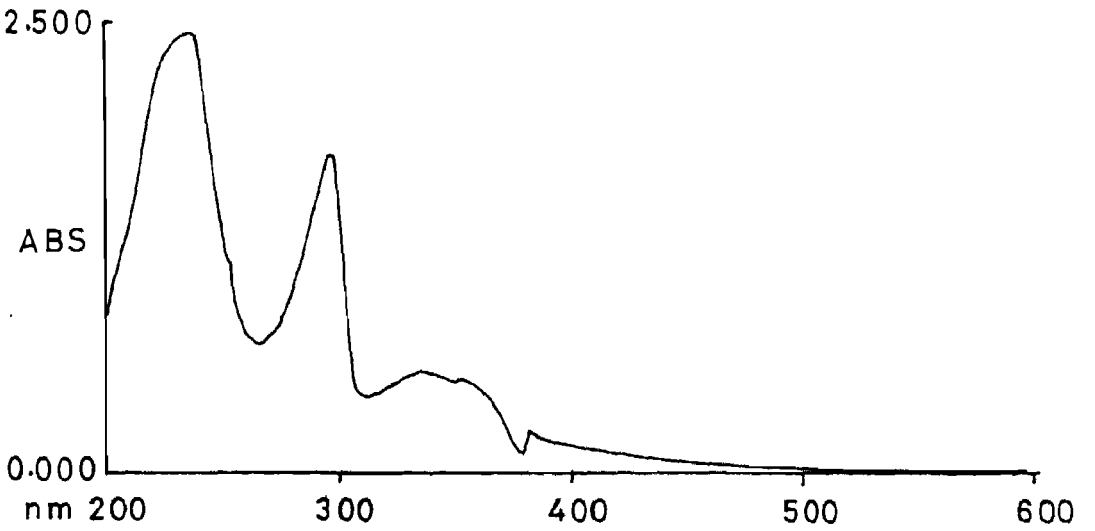

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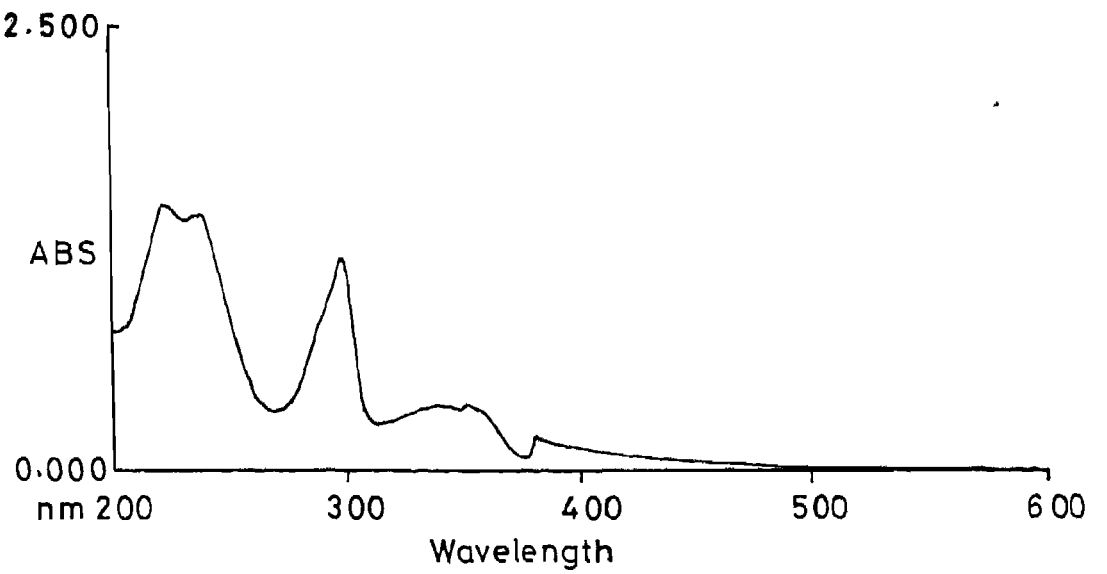

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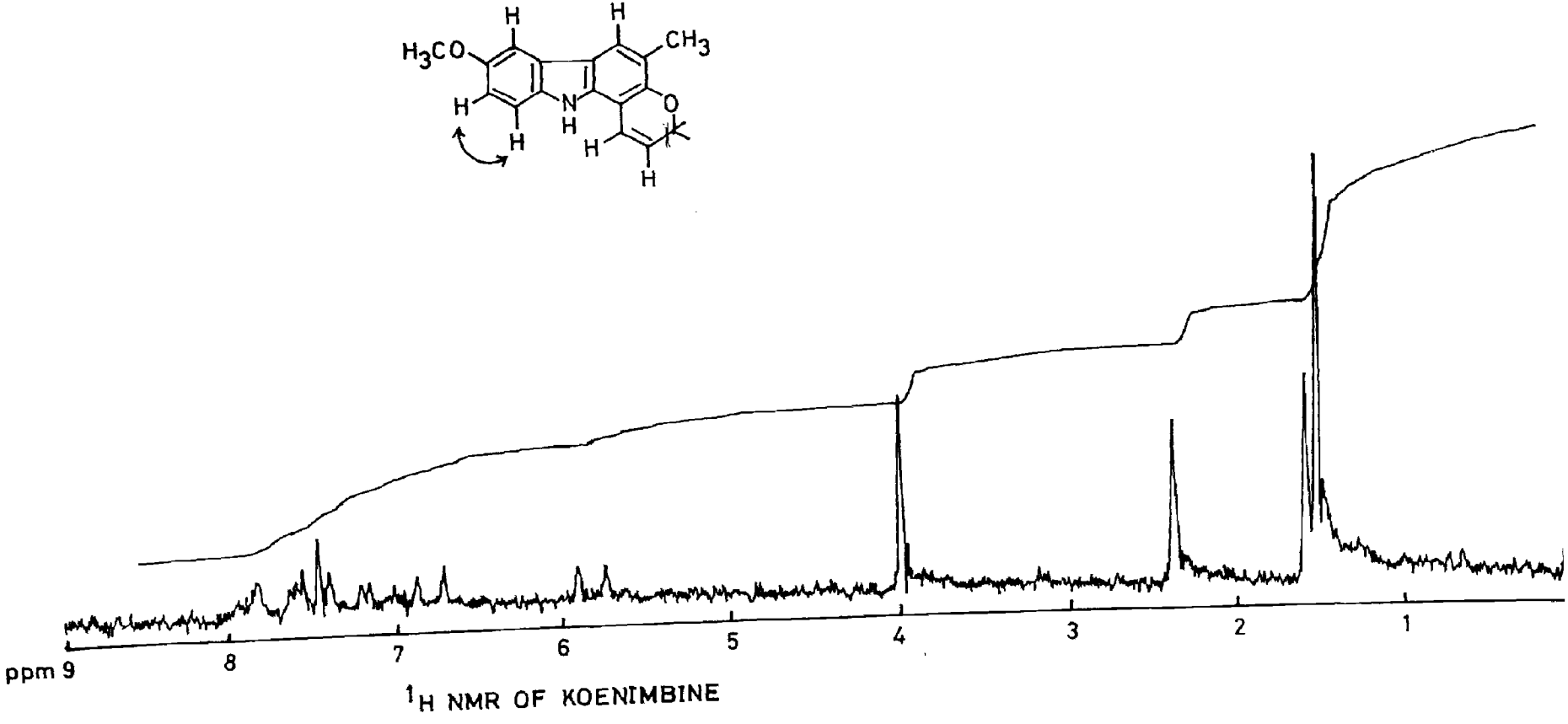
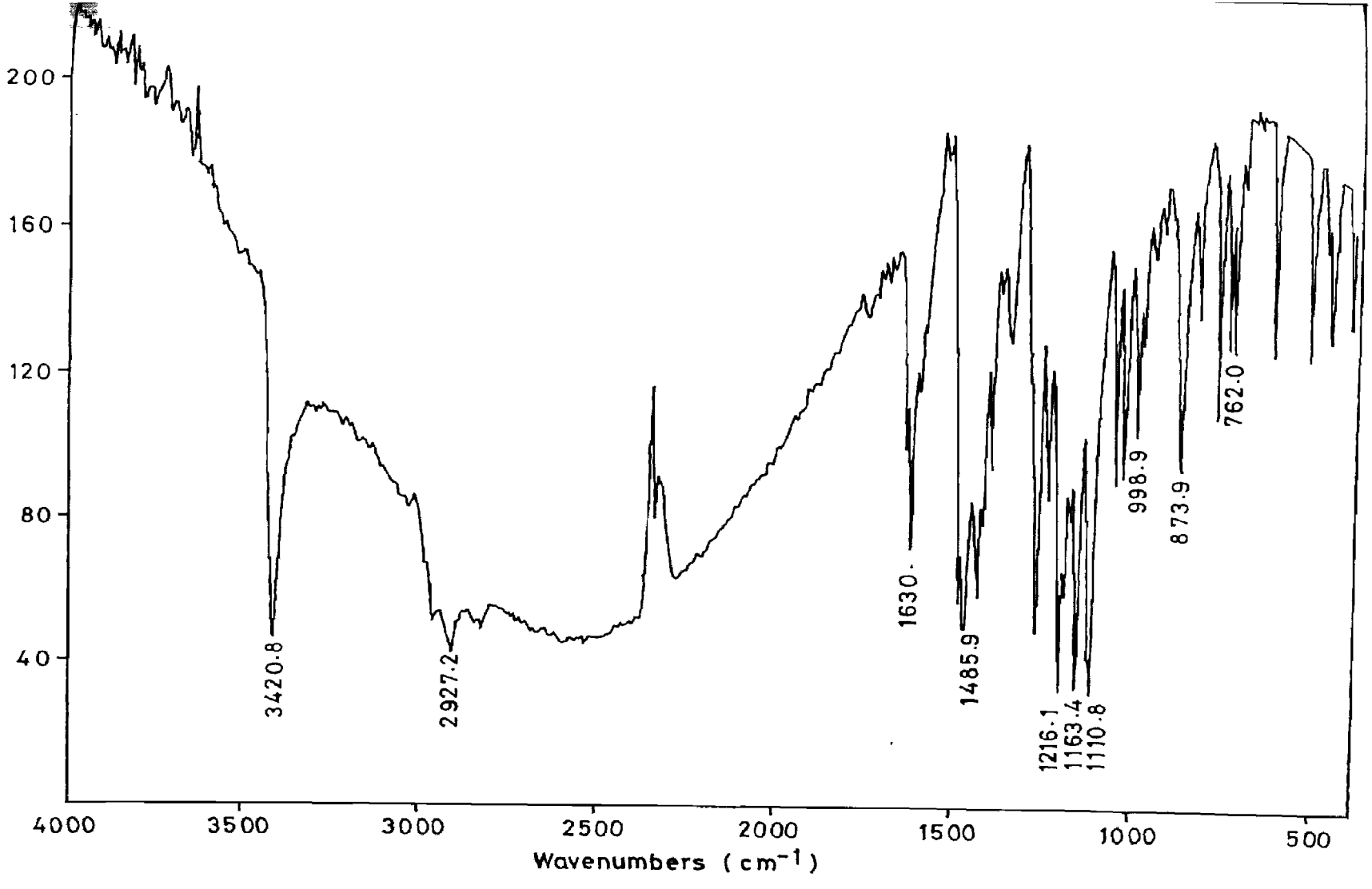


^1H NMR OF KOENIMBINE



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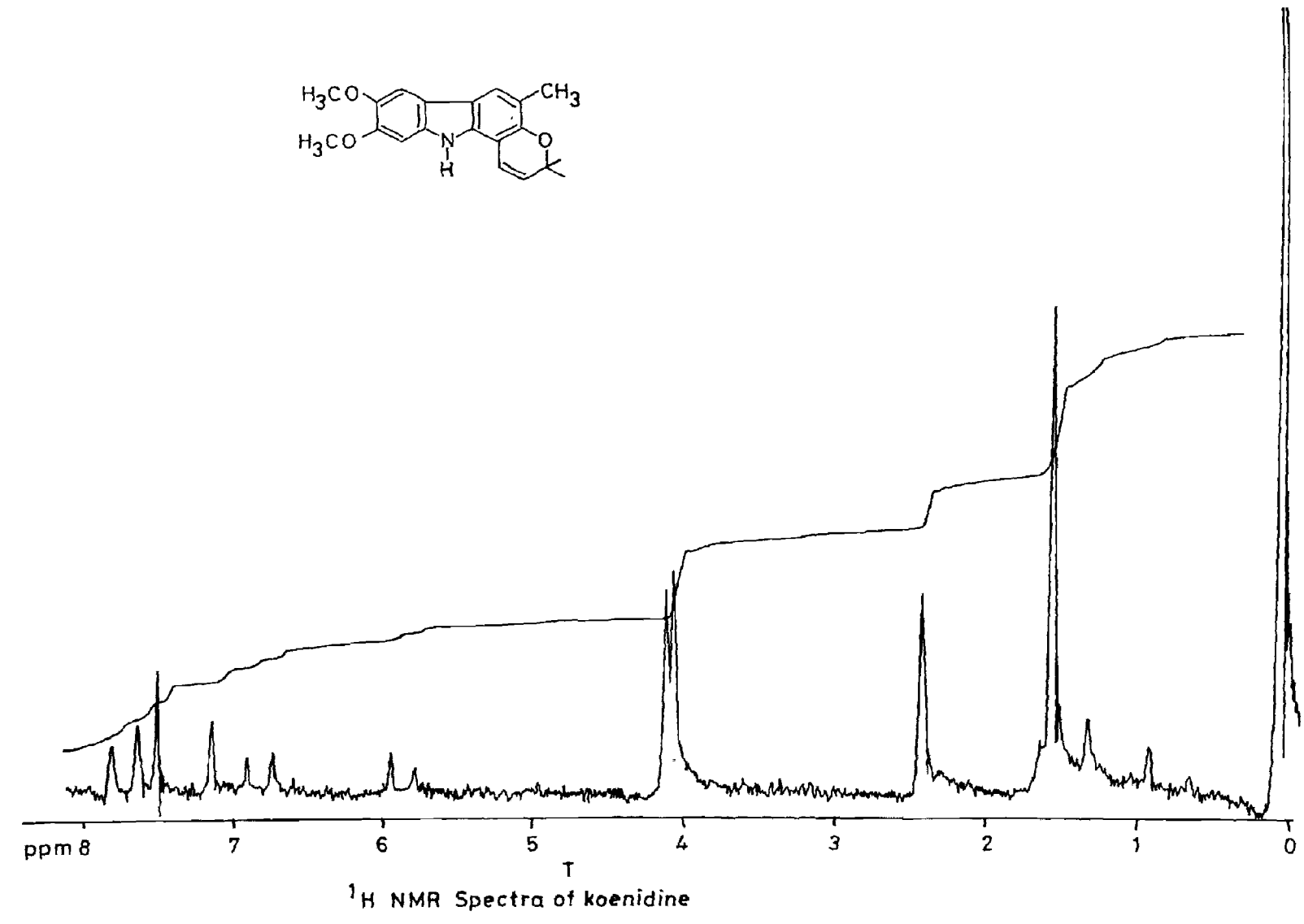

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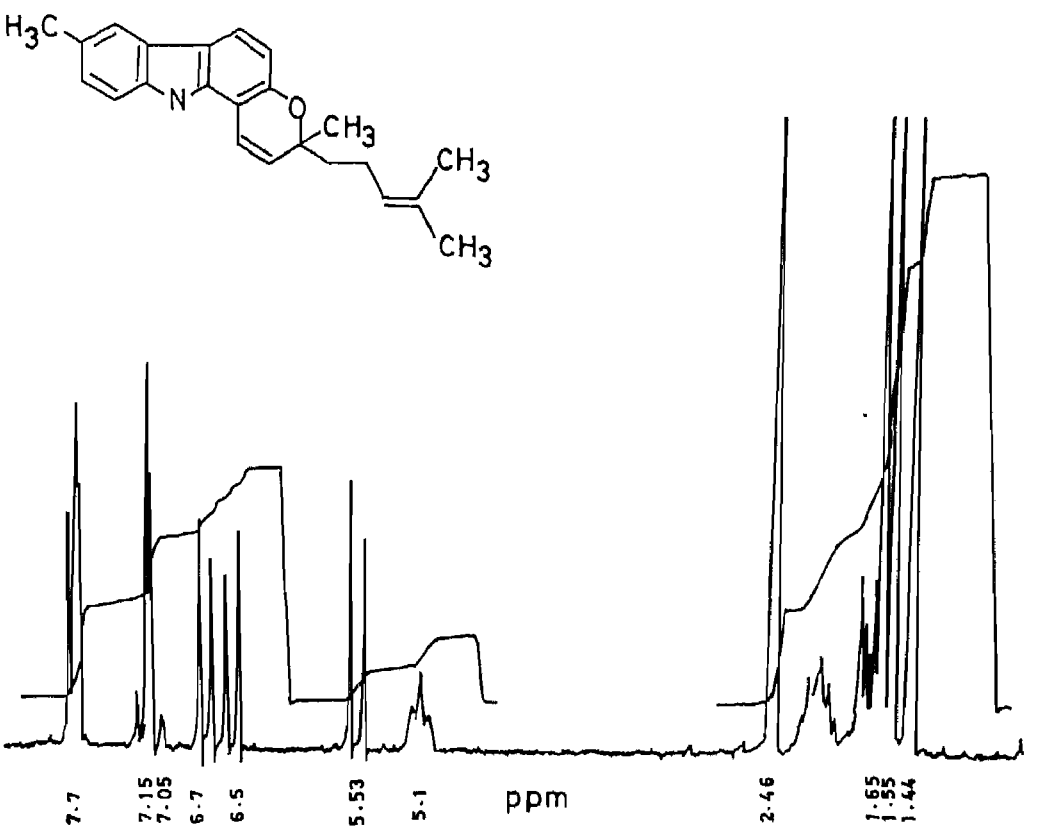
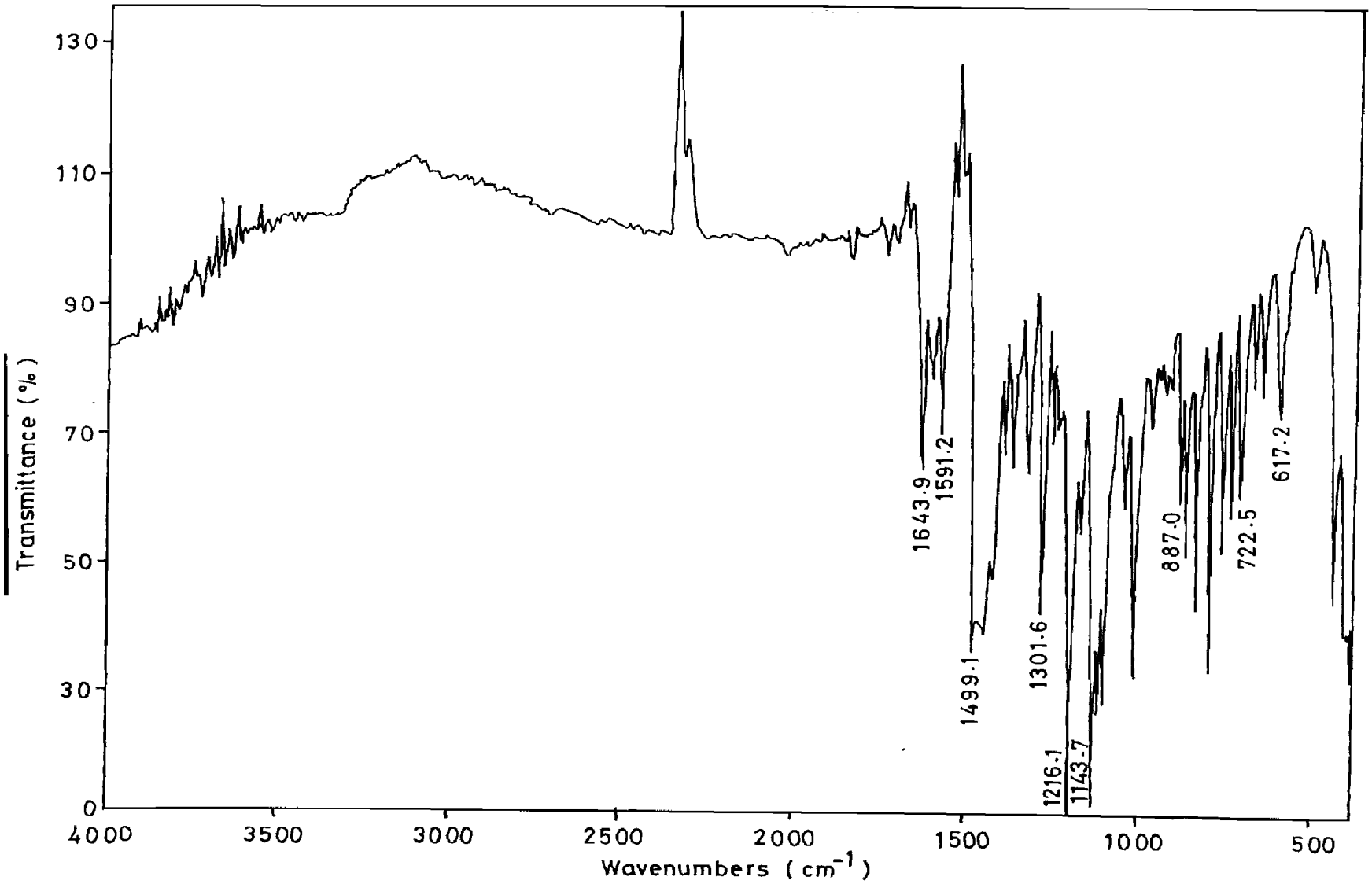
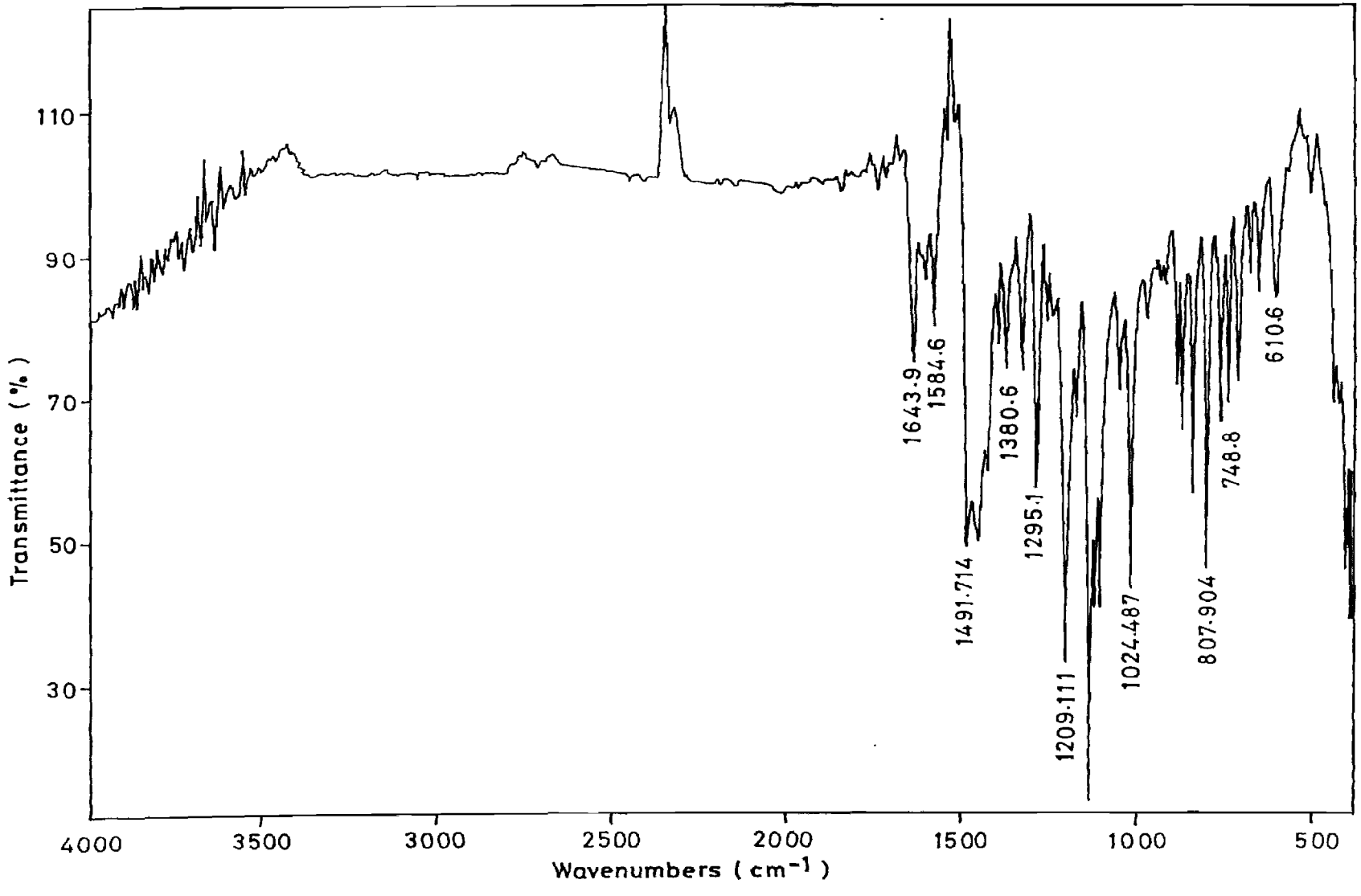


^{1}H NMR OF ISOMAHNIMBINE



IR Spectra of N- methyl koenimbine



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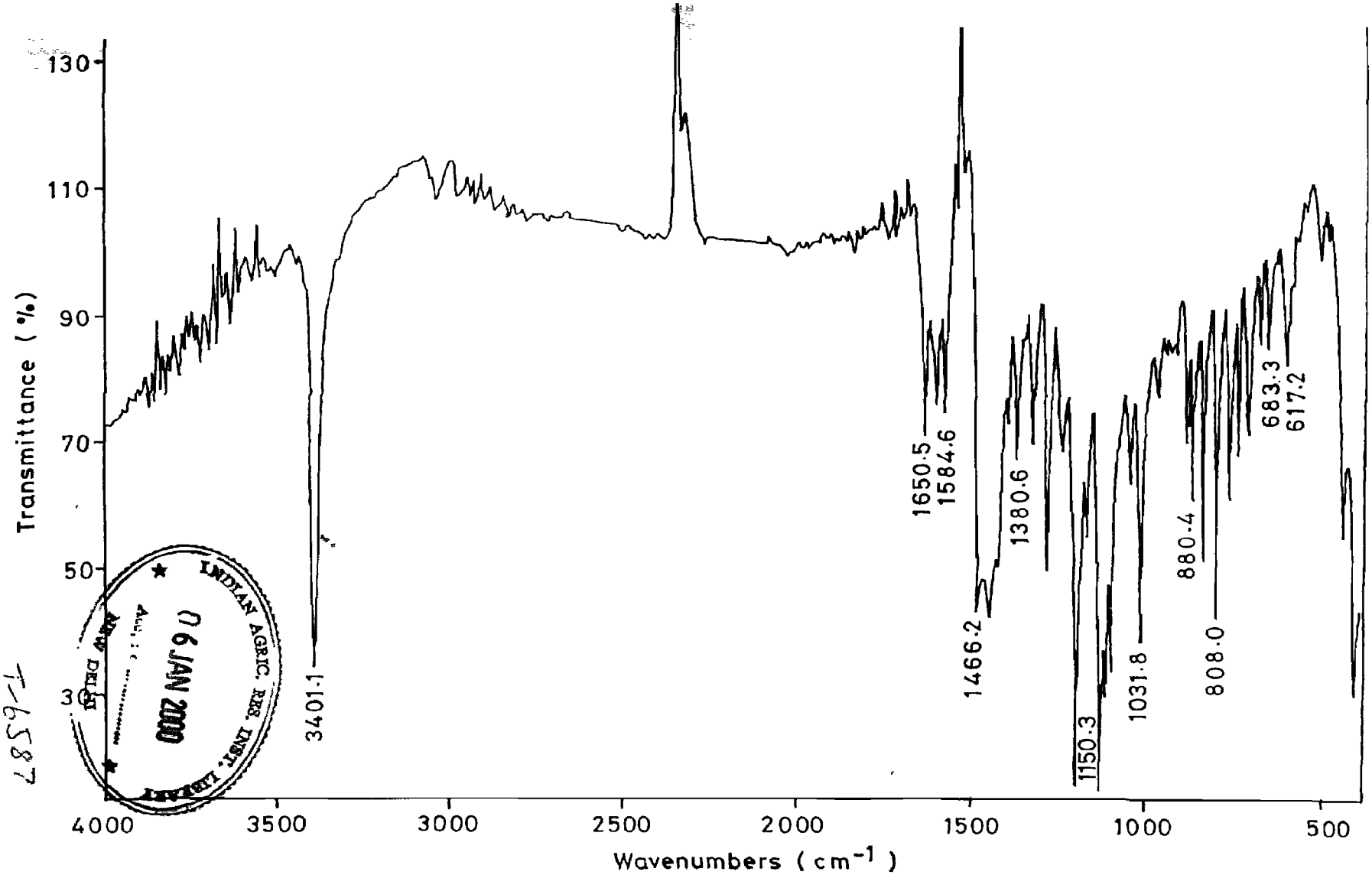

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