STUDIES ON PLANT METABOLITES

THESIS
SUBMITTED TO THE UNIVERSITY OF CALICUT
IN PARTIAL FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN CHEMISTRY

By

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CERTIFICATE

This is to certify that this thesis entitled **"Studies on Plant Metabolites"** is an authentic record of the research work carried out by **Molykutty M. Kaniampady**, in the Department of Chemistry, University of Calicut, under my supervision in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry, under the Faculty of Science of the University of Calicut and that no part thereof has been presented earlier for any other degree.

C.U. Campus,
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Dr. P. Mohamed Shafi
(Supervising Teacher)

DECLARATION

I, Molykutty M. Kaniampady, hereby declare that this thesis is an authentic record of original research work carried out by me under the guidance and supervision of Dr. P. Mohamed Shafi, Professor, Department of Chemistry, University of Calicut. No part of this thesis has previously formed the basis for the award of any degree or diploma as stipulated in the statutes of Calicut University.

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Molykutty

Molykutty M. Kaniampady

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Molykutty M. Kaniampady

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PREFACE

Natural Products Chemistry has originated from mankind's curiosity about colour, taste, odour and the search for cure from human, animal and plant diseases. The term natural product is applied to materials derived from plants, microorganisms, invertebrates and vertebrates which are fine biochemical factories for the biosynthesis of both primary and secondary metabolites. Secondary metabolites play ecologically significant roles in how the living organisms deal with their surroundings and therefore are important for their ultimate survival. During the last few decades natural products' research has advanced tremendously through the fields of chemistry, life sciences, food science and material sciences. Natural products are ubiquitous in our everyday life.

Phytochemistry deals with the study of secondary metabolites isolated from the plant kingdom, their characterisation, reactions, transformations and biological activities. Plants are sophisticated factories where a variety of chemical compounds are manufactured. These derived products are used as medicines, pesticides, perfumes, fragrances and other utility products. The systematic study of medicinal plants and their use in traditional medicine against a particular ailment not only enables to discover the active substances

responsible for that use but also opens interacting avenues for further research.

Plants have fed the world and cured the ailments of its population since time immemorial. The study of naturally occurring substances that have medicinal and other useful properties have been a subject of rapid development and an interesting field of active research. Compounds isolated from natural sources continue to occupy an important place among useful products of modern medicine. The systematic study of medicinal plants used in traditional medicine and the characterisation of compounds isolated using modern scientific tools have increased the economic importance of the plant pharmaceuticals. By and large the pharmacological activity of a medicinal plant resides in the so called secondary metabolites like terpenoids, steroids, alkaloids and the other classes of chemical entities.

With the advent of improved chromatographic separation techniques, the separation of various natural products, including positional and stereoisomers is achieved routinely. Newer spectroscopic techniques such as two-dimensional, high resolution, Nuclear Magnetic Resonance Spectroscopy, Infrared and Raman Spectroscopy, X-ray Crystallography, high resolution Electron Microscopy and Mass Spectroscopy have simplified the structure elucidation of new natural products.

The work presented in this thesis deals with the chemical investigation of the bark of *Macaranga peltata* and the analysis of essential oils from leaves and flowers of *Gliricidia sepium* and *Strychnos nux-vomica* flowers. Chemotaxonomical analyses of the essential oils from four different *Ocimum* species have also been conducted.

The thesis is divided into four chapters and the relevant references are given at the end of each chapter.

The first chapter presents the phytochemical studies on the bark of *Macaranga peltata.* The investigation enabled the identification of two compounds: **3-acetylaleuritolic acid** and **3,4,3'-tri-O-methylellagic acid**. These compounds were characterised using IR, NMR and mass spectral data. This chapter also discusses the chemical transformation of 3-acetylaleuritolic acid to aleuritolic acid, diester of aleuritolic acid and 3-ketoderivative of aleuritolic acid.

The second chapter comprises four sections. Section I is an introduction, highlighting the importance of essential oils and methods of their extraction. Section 2 gives a brief introduction to chemotaxonomy and explains chemotaxonomic significance of secondary metabolites including terpenes, triterpenoids and flavonoids. Section 3 gives an introduction to the four *Ocimum*

species and each of its medicinal properties and uses. Section 4 comprises (a brief overview of GC, GC-MS and SPME techniques) the analytical techniques used for the analysis of essential oils. By means of GC-FID, GC-MS and olfactometry nearly **100 volatiles** were identified as the constituents of the four essential oils and the following chemotypes were attributed to the analysed *Ocimum* samples: *O. americanum,* methyl cinnamate-type; *O. basilicum,* methyl cinnamate - linalool-type; *O. gratissimum,* eugenol type; and *O. sanctum,* methyl eugenol-type. This work has been published as a paper entitled "Chemotaxonomical Analysis of the Essential Oil Aroma Compounds of Four Different *Ocimum* species from Southern India" in the Journal *European Food Research Technology* (Eur Food Res Technol), **217**, 2003, 120-124.

Chapter 3 is divided into two sections. In Section 1, the analysis of the essential oil volatiles of *Gliricidia sepium* leaves is presented. By means of GC and GC-MS **sixteen compounds** were identified in this sample with propyleneglycol, coumarin, (Z)-3-hexenol and β-farnesene as the main constituents. Olfactoric evaluation of the leaf oil was also done. Section II discusses the analysis of the essential oil volatiles of *G. sepium* flowers. **Twenty six compounds** were identified in the flower essential oil with coumarin, hydroquinone and myrtenol as the major constituents.

In consideration of the toxicity of coumarin and hydroquinone, this finding is a warning signal against the use of the leaves as fumigant against mosquitoes and the flower as a food material.

Based on this work an article entitled "Essential Oil Composition of *Gliricidia sepium* (Leguminosae) Leaves and Flowers" has been submitted for publication in *Indian Journal of Chemistry* Section B.

The fourth chapter consists of the analysis of the volatiles of *Strychnos nux-vomica* flowers using GC-FID and GC-MS. **Seventy five compounds** were identified in the flower essential oil with palmitic acid, tricosane, trans and cis epoxylinalool, linalool, α-terpineol, T-cadinol, nerolidol, farnesol, linalool oxide, menthol and linalyl acetate as major constituents.

PHYTOCHEMICAL STUDIES ON MACARANGA PELTATA Muell

Molykutty M. Kaniampady “Studies on plant metabolites” Thesis. Department of Chemistry , University of Calicut, 2006

CHAPTER I

PHYTOCHEMICAL STUDIES ON *MACARANGA PELTATA* Muell.

1.1 INTRODUCTION

Macaranga Thou. belongs to Euphorbiaceae family which consists of 283 genera and 7300 species of almost cosmopolitan distribution, mainly of the tropics but extending also into the temperate regions of northern and southern hemispheres. Two major centres of distribution are tropical America and Africa. *Macaranga* genus contains 240 species[1]. About 12 species are found in India[2]. *Macaranga peltata* Muell. is a small tree commonly found in Indian forests[3]. It is found in Bengal, Bihar, Orissa and the Deccan Peninsula, mostly in the hills[2]. These are small dioecious trees: h-12 m, d-30 cm. Bark 10-15 mm thick, surface pale, greyish brown, mottled with white, smooth, lenticellate; brittle; blaze deep pink-red. Branchlets glaucous. Leaves alternate, simple. Stipules large, ovate-acuminate, petioles 12-35 cm long, blade 12-125 cm x 12-25 cm, orbicular-ovate, tip long acuminate, base peltate, 3 ribbed from base, nerves and ribs prominent beneath, red. Flowers greenish yellow, male in dense panicles, concealed in large bracts and female in smaller panicles, seeds black. It is found throughout Kerala[4].

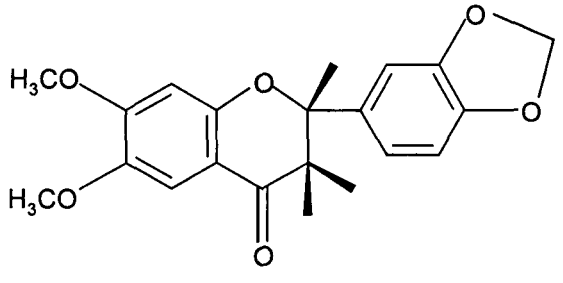
It is called 'Vattakanni' in Tamil, 'Kondatamara' in Telugu, 'Chandakanne' in Kannada and 'Uppila' and 'Vatta' in Malayalam[5]. The tree reproduces freely by seeds which germinate at the commencement of monsoons. It comes up plentifully in old clearings, makes rapid growth and produces large crowded leaves which are not eaten by cattle. It withstands drastic pruning and puts forth flushing growth in 2-3 months. The loppings are applied as green manure to paddy fields in the west coast. It is also useful in coffee plantations for shade. The leaves are rich in nitrogen and potash. They contain water, 60.17; N, 1.3; potassium (K_2O), 0.66; and phosphorus (P_2O_5), 0.18%[6].

A reddish brown gum (kino) exudes from the cut branches, bases of petioles, young shoots and fruits. It is partly soluble in water and is available in the form of hard tears or agglutinated masses with a shiny lustre and little or no taste. A sample of kino from South Malabar contained: moisture, 17.1; tannin 15.0; gum (mostly pararabin), 63.4; and ash, 2.1%. It is used for sizing paper and for taking impressions of leaves, coins, medallions etc; it is used also as a substitute for gum arabic[5]. The gum powder from *Macaranga peltata* has been used in Indian medicine for the treatment of venereal diseases[6]. A decoction of leaves and bark is used as a wash for ulcers. The fruit is eaten in times of scarcity.

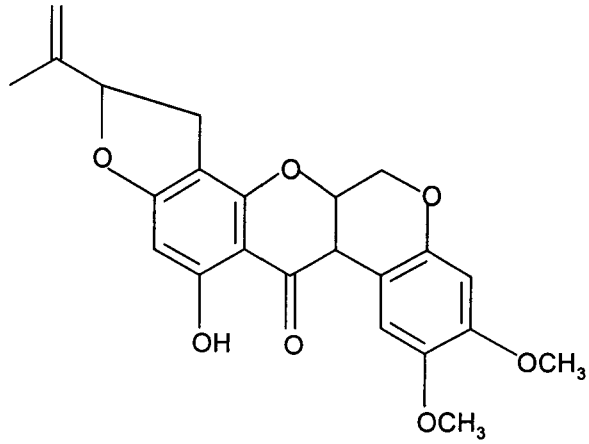
The wood of *M. peltata* is pale brown with a mottled appearance and is reported to be suitable for matches and paper pulp[7].

1.2. WORK SO FAR REPORTED ON *MACARANGA* SPECIES

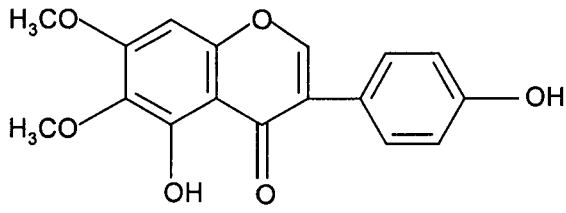
Only three species, apart from *M. peltata* of *Macaranga* genus have been chemically examined so far[8-10]. *Macaranga tenarus* has been previously examined and the isolation of a new diterpene, macaranganol and some other terpenoids were reported[11]. A new flavanone, 6,7-dimethoxy-3'4'-methylenedioxyflavanone, sumatrol and 7-methyltectorigenin have been isolated from the acetone extract of the leaves of *Macaranga indica* Wight[12]. Sultana and Ilyas isolated two chromenoflavones - macaflavone I and macaflavone II from the leaves of *M. indica* Wight[13].



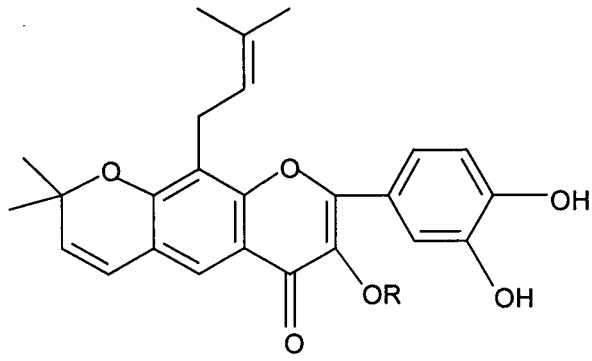
6,7-dimethoxy-3',4'-methylenedioxyflavanone



Sumatrol



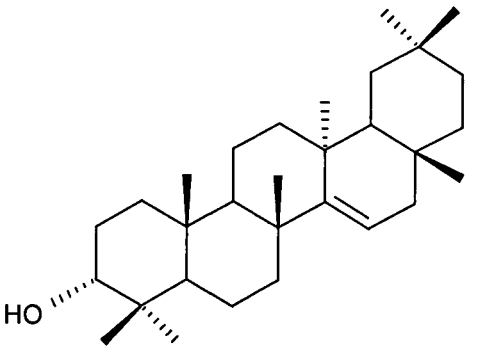
7-methyltectorigenin



R=H Macaflavone I

R = Me Macaflavone II

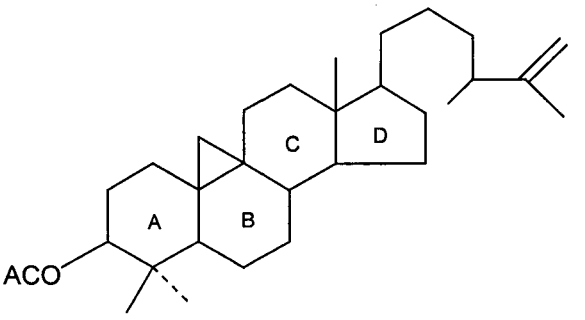
A triterpene 3-epitaraxerol was isolated from *M. denticulata* along with taraxerone and β-sitosterol[9].



3-Epitaraxerol

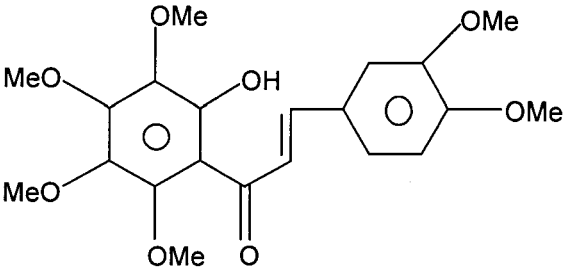
***Macaranga peltata* Muell.**

Anjaneyulu and co-workers[14] isolated a novel tetracyclic cyclotriterpene (cyclopeltenyl acetate) from the heartwood of *Macaranga peltata* Muell. β-sitosterol, n-octacosanol and a few other known triterpenes, like α-and β-amyrins, lupeol and betulin have also been isolated and characterised by them.



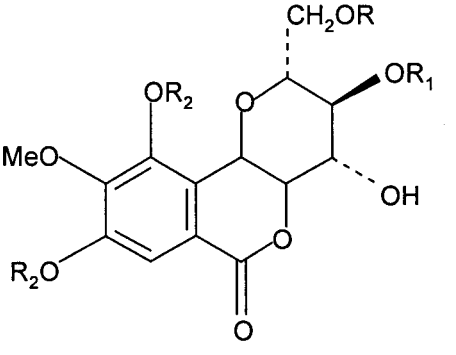
Cyclopeltenyl acetate

Anjaneyulu and Reddy[15] also isolated 6,3',4'-trimethoxyflavanone from the bark of *Macaranga peltata* Muell., along with hexacosanol, friedelin and β-sitosterol. They also isolated and characterised 2'-hydroxy 3',4',5',6',3,4-hexamethoxychalcone for the first time in nature from the bark of *Macaranga peltata*[16].



2'-hydroxy-3',4',5',6' 3,4-hexamethoxychalcone

Ramaiah and co-workers[17] isolated bergenin and three O-methyl ethers of bergenin (I, II and III) from the heartwood of *M. peltata.*



R = R_1 = R_2 = H Bergenin

I R = R_1 = H, R_2 = Me

II R = R_2 = Me, R_1 = H

III R = H, R_1 = R_2 = Me

1.3. PRESENT WORK

Since no previous reports on the chemical composition of *Macaranga peltata* species from Kerala were found, the present investigation was aimed at the isolation and characterisation of compounds present in it.

1.4. MATERIALS AND METHODS

Plant material

The bark of *Macaranga peltata* was collected from Calicut University Campus, Kerala in May 2003 and was authenticated by Dr. A.K. Pradeep, Department of Botany, Calicut University.

Melting point determination

All the melting points of the crystalline isolates were determined using Toshniwal Capillary Melting Point Apparatus.

Optical activity

Optical activity was measured at 20°C using Perkin Elmer Polarimeter 341. The light source used was sodium D-line (589 nm). A 2 ml solution containing 5 mg of the sample was used for the measurement.

Infrared absorption spectroscopy (IR)

The IR spectra of the isolates were recorded as KBr pellets using Shimadzu FTIR-8101A spectrometer.

Nuclear magnetic resonance spectroscopy (NMR)

The proton NMR spectra, ^{13}C-NMR spectra and the HSQC spectra of the isolates were recorded at 400 MHz on an AVANCE 400 NMR spectrometer (Bruker, Karlsruhe, Germany) in DMSO and $CDCl_3$ using tetramethylsilane (TMS) as internal standard. The chemical shifts are reported in ppm (δ).

TOCSY and HMBC spectra of the isolates were recorded at 500 MHz on a DRX 500 NMR spectrometer (Bruker, Karlsruhe, Germany) in DMSO and $CDCl_3$ using tetramethylsilane (TMS) as internal standard.

Mass spectra

ESI-FT-ICR mass spectra were recorded on an apex II FTICR mass spectrometer (4.7 T, Bruker-Daltonics, Bremen, Germany).

The electron impact mass spectra (EIMS) were recorded on an HP 5970 MSD mass spectrometer at ionisation energy 70 eV.

Column chromatography (CC)

Column chromatographic separation of the crude and semipurified extracts were carried out using silica gel (Qualigens, 100-200 mesh).

Thin layer chromatography (TLC)

Thin layer chromatographic plates were prepared using TLC grade silica gel-G (Merck) using Stahl apparatus.

Reagents

1. Leibermann-Burchard reagent (LB reagent)

Acetic anhydride (5 ml) was added carefully to 97% sulphuric acid (5 ml) and this mixture was added to absolute ethanol (50 ml), while cooling in ice. The sprayed plate was heated to 110°C until maximal visualisation of the spots.

With LB reagent triterpenoids were detected as red or pink spots and sterols and its esters were detected as green to blue spots.

2. Vanillin-sulphuric acid reagent

The reagent was prepared by dissolving vanillin (1 g) in ethanol (100 ml) and conc. H_2SO_4 (5 ml) in ethanol (100 ml) separately.

The chromatogram (TLC) was sprayed first with 5% H_2SO_4, followed immediately by 1% ethanolic vanillin. The sprayed plate

was then heated to 110°C for 5-10 minutes until obtaining maximal visualisation of the spots.

With vanillin-sulphuric acid reagent triterpenoids and steroids were detected as various coloured spots (red, yellow, blue or brown).

3. Anisaldehyde-sulphuric acid reagent (AS reagent)

Anisaldehyde (0.5 ml) was mixed with glacial acetic acid (10 ml) and diluted with methanol (85 ml) and conc. H_2SO_4 (5 ml) was added to it and mixed.

The TLC plate was sprayed with AS reagent, heated at 100°C for 5-10 minutes until obtaining maximal visualisation of the spots.

With AS reagent triterpenoids were detected as blue, red-violet, orange or red spots.

4. 20% aqueous sulphuric acid (20% H_2SO_4)

20% aqueous sulphuric acid was prepared. The sprayed plate was heated to 110°C until spots were visualised.

With 20% H_2SO_4, the terpenoids gave brown, pink, purple or yellow colour.

1.5. EXTRACTION, FRACTIONATION AND ISOLATION OF COMPOUND FROM THE PETROLEUM ETHER EXTRACT OF THE BARK OF *M. PELTATA* MUELL.

Dried and finely powdered bark of *Macaranga peltata* Muell. (4 kg) were extracted thrice with petroleum ether (60-80°C, 3 x 7L). The combined extract was then concentrated under reduced pressure to about 500 ml of light yellow coloured solution. Then a cream coloured powdery solid was separated. It was filtered, washed repeatedly with petroleum ether and dried. This on recrystallisation from pyridine yielded 2.0 g of pure substance **MP1**, m.p. 282°C.

TABLE 1.1

Compound isolated from petroleum ether extract

Compound	Extracting solvent	Melting point	Molecular mass
MP1	petroleum ether	282°C	498

1.6. EXTRACTION, FRACTIONATION AND ISOLATION OF COMPOUND FROM THE ALCOHOL EXTRACT OF THE BARK OF *M. PELTATA* MUELL.

Finely powdered bark of *M. peltata* after extraction with petroleum ether (4 kg) was extracted thrice with methanol (3 x 6L). All the alcohol extractions were carried out by refluxing with alcohol for 30 minutes and keeping it for one day. The combined alcohol extract was concentrated under reduced pressure to about 500 ml. About 250 ml of water was added and fractionated thrice with

benzene and then with ethyl acetate (3 x 200 ml each). The benzene fraction on column chromatography did not afford any compound. The ethyl acetate fraction was concentrated, the residue was dissolved in methanol and adsorbed on silica gel (300g, 100-200 mesh) for column chromatography (4 cm x 100 cm, d x l). Elution was carried out using solvents of increasing polarity viz. 2:1 petroleum ether-ethyl acetate (IL) 1:1 petroleum ether-ethyl acetate (3L), 1:2 petroleum ether-ethyl acetate (IL), ethyl acetate (1.5L), 1:1 ethyl acetate-methanol (500 ml) and 1:2 ethyl acetate-methanol (500 ml).

Several 50 ml portions were collected and each fraction was checked by TLC. Fractions were pooled together according to their homogeneity judged from TLC analysis. The fractions 19 to 23 eluted out by 1:1 petroleum ether-ethyl acetate were found to give identical spots on TLC analysis. These fractions on evaporation yielded light yellow coloured substance. This compound was filtered, washed with petroleum ether and recrystallised from pyridine. The yellow needle shaped solid thus obtained was further purified by preparative TLC on silica gel G plates, by dissolving the solid in pyridine and eluting with 3:2 mixture of petroleum ether-ethyl acetate. The yellow base spot was extracted with hot pyridine which upon evaporation yielded a cream fluffy solid **MP2** (10 mg, m.p. 275°C).

TABLE 1.2

Compound isolated from methanol extract

Compound	Eluent composition	Melting point	Molecular mass
MP2	1:1 petroleum ether-ethyl acetate	275°C	344

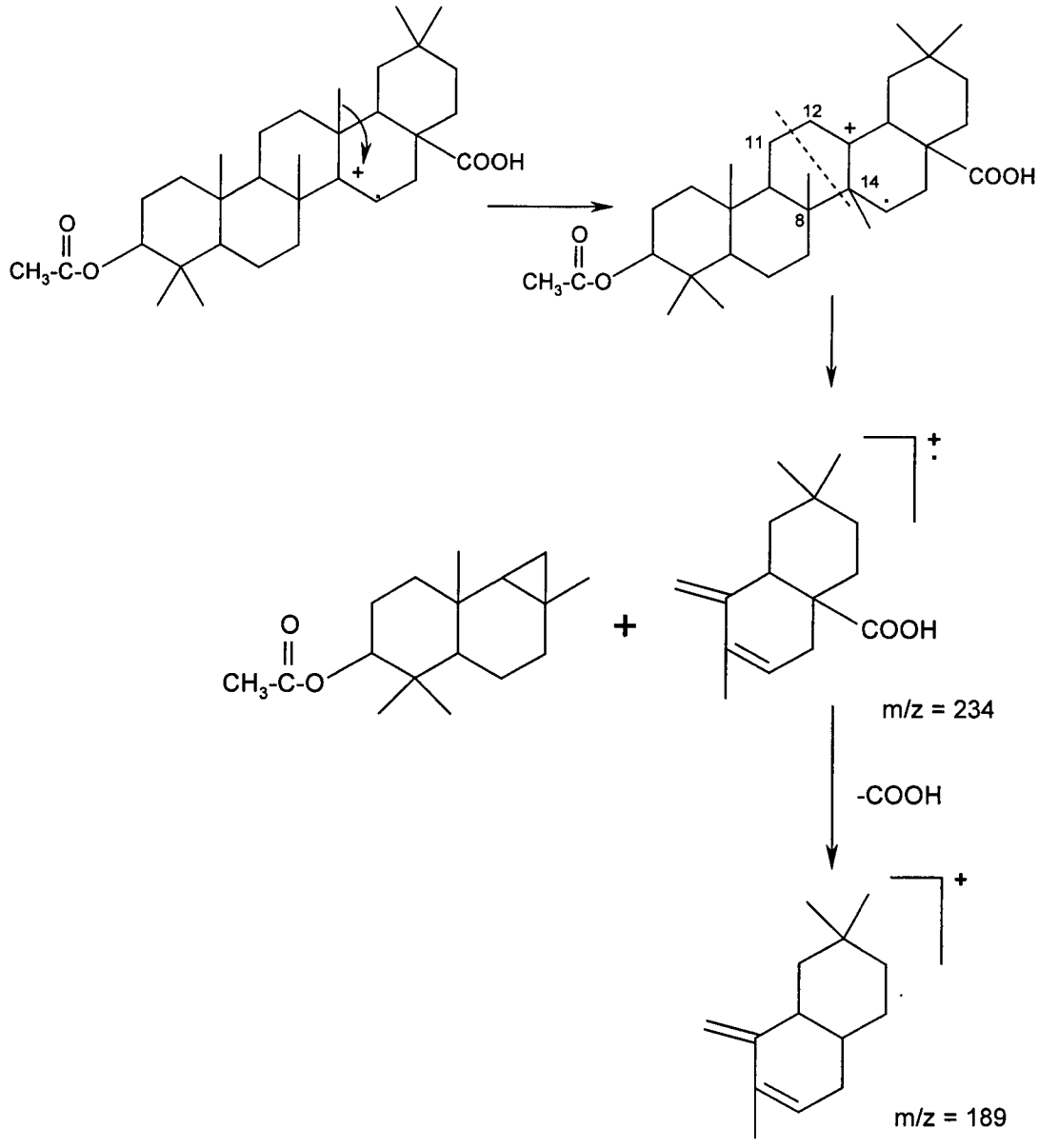
1.7. RESULTS AND DISCUSSION

1.7.1. Characterisation of MP1 (3-acetylaleuritolic acid)

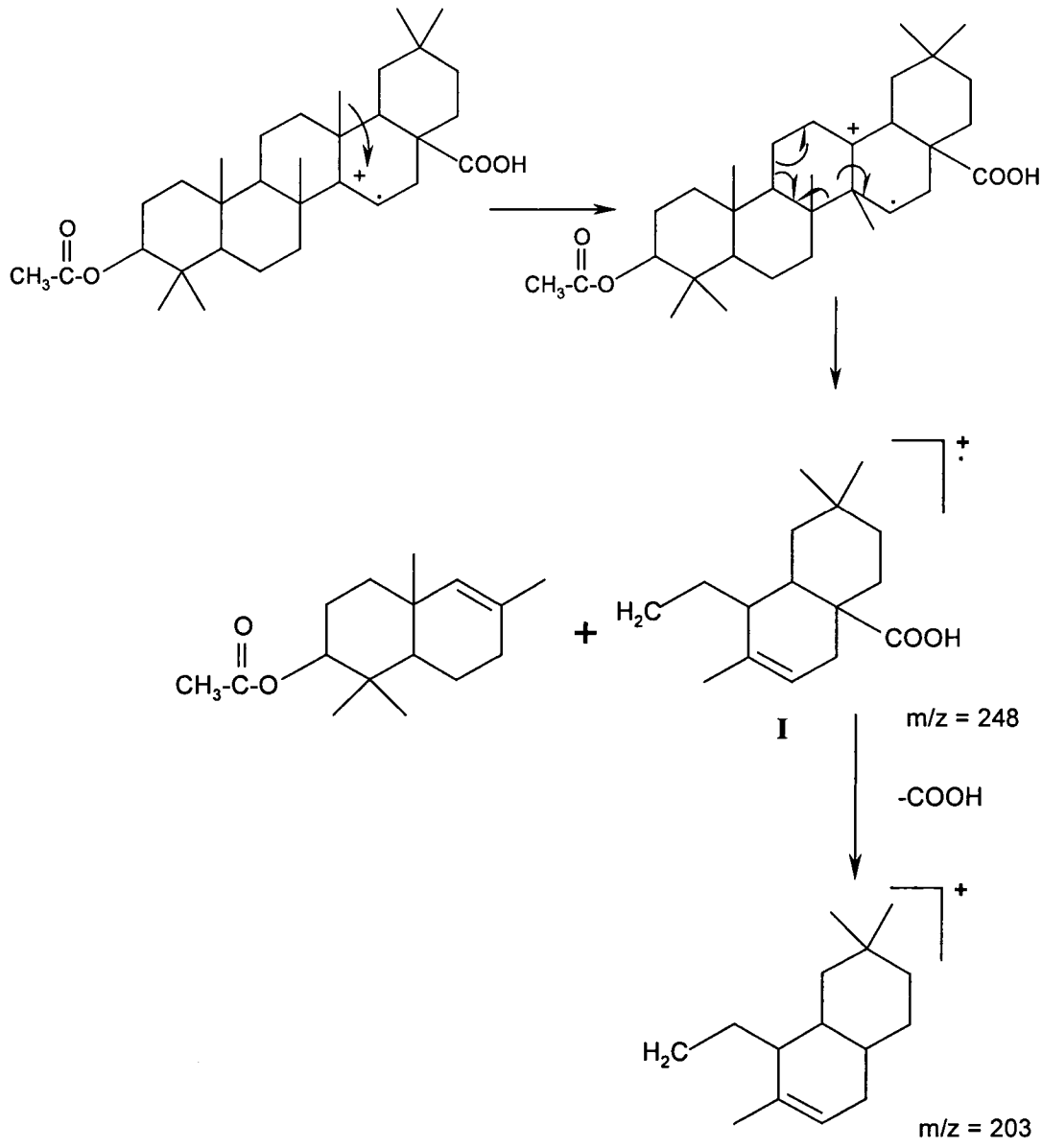
This compound was isolated from the petroleum ether extract as colourless needles (2.0 g). It was recrystallised from pyridine and had a m.p. of 282°C (Lit. 301-302°C)[18]. It answered Liebermann-Burchard reaction showing a persistent pink colour typical for triterpenes; also it gave a blue colour with vanillin - H_2SO_4 and appeared as a pink spot with anisaldehyde – H_2SO_4 reagent. Bayer's reagent was decolourised indicating its unsaturated nature and yielded effervescence with sodium bicarbonate solution. Its IR spectrum [MP1 IR] exhibited absorption bands for carboxylic acid (3050 cm^{-1}, 1688.7 cm^{-1}), ester (1736.9 cm^{-1}) and C=C (1640.5 cm^{-1}) functions and trisubstituted double bond (peak at 830.4 cm^{-1}). The specific rotation in chloroform was found to be $[\alpha]_D$ $^{+}26^{o}$ (Lit. $^{+}23.1^{o}$)[18]. The compound MP1 afforded a corresponding hydroxy acid (MP1a) m.p. 268°c by hydrolysis with alkali and gave a monomethyl ester (MP1b) m.p. 220°C by methylation with CH_2N_2.

The high resolution mass spectrum showed molecular ion peak at m/z 498.3705 corresponding to a molecular formula $C_{32}H_{50}O_4$ (calculated value: 498.3711). It indicated eight degrees of unsaturation, five of them were adjusted in a pentacyclic triterpenic carbon framework and one each in olefinic linkage, ester and carboxylic groups. The EI mass spectrum (MP1 MS) of MP1 was distinctive of pentacyclic triterpenes of Δ^{14}-Taraxerenes in which rings A, B, C and E were saturated[19].

The base peak was at m/z 189. The mechanism for the genesis of this fragment can be proposed by assuming that in the molecular ion, the missing electron is preferentially from the carbon-carbon double bond, migration of C-13 methyl group, then yielding the radical ion. Fission of the 11-12 and 8-14 bonds gave the stable diene[19].

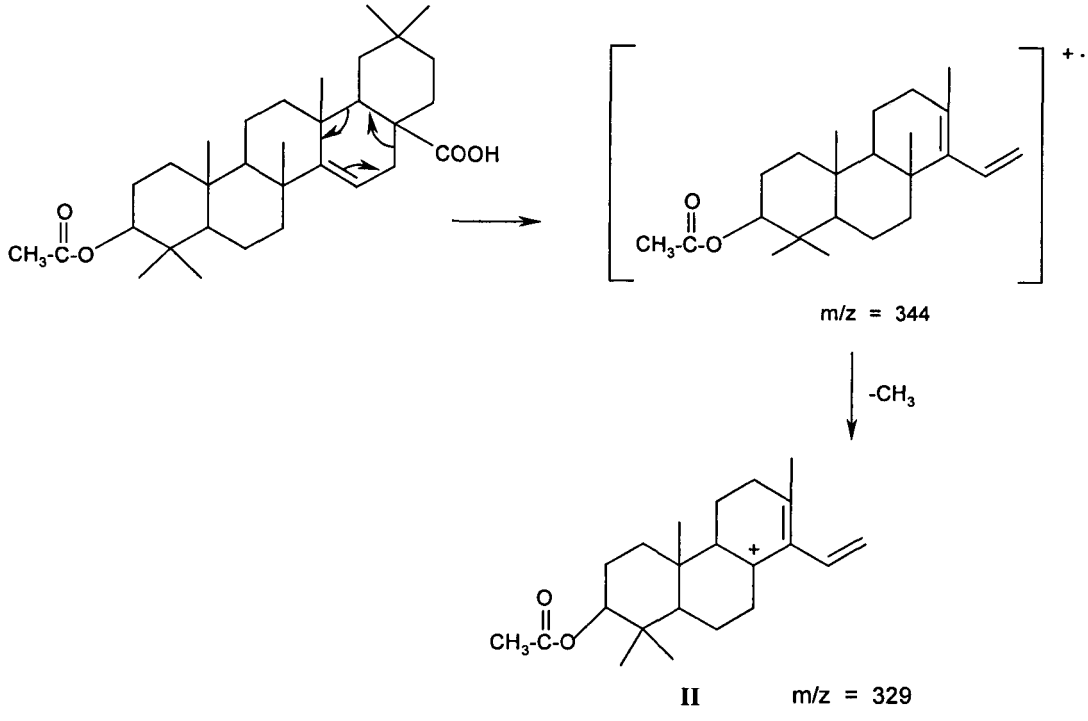


The fragments observed at m/z 248 and 203 in the mass spectrum of MP1 may alternatively be accommodated by the fragmentation given below[20].



The mass spectrum displayed other ion fragments at m/z 483, 452, 438, 423, 409, 395, 344 [retro-Diels-Alder cleavage], 329 and 269.

Retro-Diels-Alder Cleavage



Fragments with m/z = 329 and m/z = 248 (II & I) suggested the existence of the acetoxy group in rings A or B and an angular carboxylic group in rings D or E.

The ^{1}H-NMR spectrum (MP1 ^{1}H-NMR) of MP1 displayed a one-proton downfield broad signal at δ 5.46 assigned to vinylic C-15 proton. A one proton double doublet at δ 4.44 was ascribed to a proton on a carbon atom bearing a secondary acetoxy group. The coupling constants of this proton, 14.3 Hz and 5.7 Hz, confirmed its axial orientation. This observation places the acetoxy group equatorial (β-orientation). A three proton signal at δ 1.97 was

associated with acetyl protons. The remaining seven methyl proton signals between δ 0.78 to 0.88 encountered were corresponding to seven tertiary methyl protons. This, again, is supported by thirteen cross peaks in the HSQC spectrum (MP1 HSQC) of $\rangle$C–H and $-CH_3$ groups (a total of eight methyl groups and five $\rangle$C–H groups). The ^{13}C-NMR spectrum (MP1 ^{13}C-NMR) of MP1 exhibited 32-carbon resonances which compared well with that of acetoxyaleuritolic acid[21] [**Table 1.3**].

TABLE 1.3

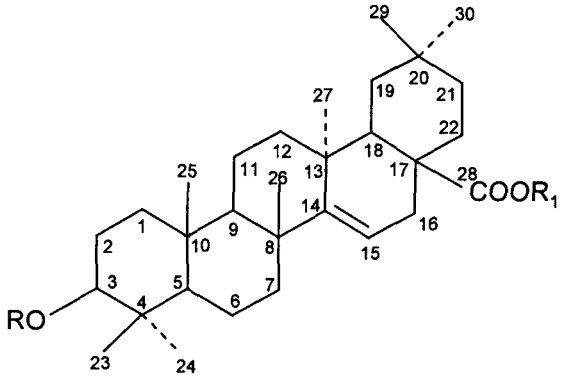
Carbon atom No.	^{13}C-NMR shifts of compound MP1	^{13}C-NMR shifts from literature
1	36.9	37.4
2	26.9	23.4
3	79.83	80.8
4	36.6	37.6
5	54.5	55.6
6	20.2	18.7
7	39.6	35.3
8	38.0	39.0
9	48.03	49.0
10	36.2	37.3
11	17.7	17.3
12	32.6	31.2
13	36.3	37.9

Carbon atom No.	^{13}C-NMR shifts of compound MP1	^{13}C-NMR shifts from literature
14	159.5	160.5
15	115.8	116.8
16	30.2	30.6
17	50.5	51.5
18	40.3	41.3
19	34.2	40.7
20	28.6	29.3
21	32.2	33.6
22	30.8	31.8
23	28.2	27.9
24	16.2	16.6
25	15.5	15.7
26	27.6	28.6
27	25.1	26.2
28	183.5	184.4
29	29.6	33.3
30	22.1	22.4

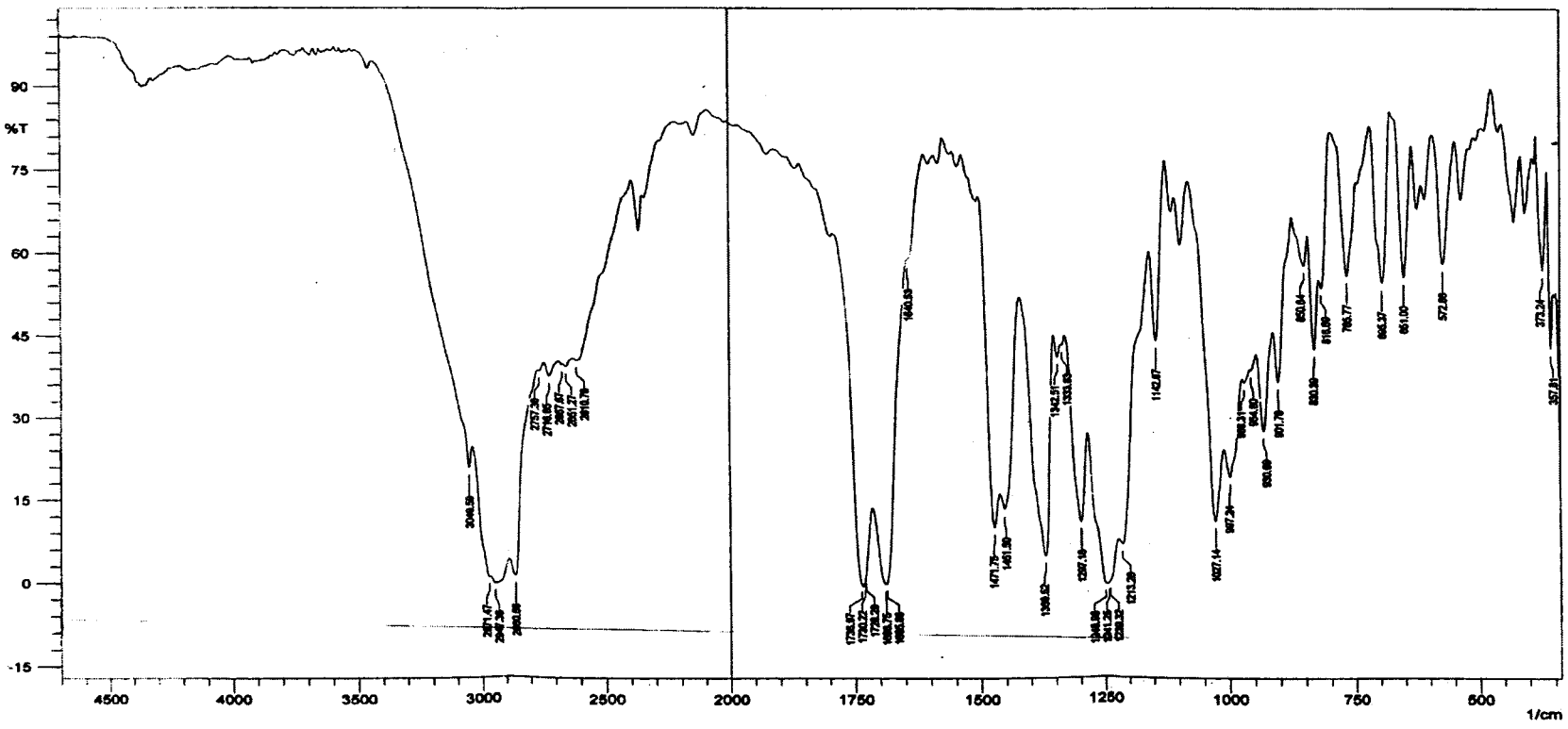
The olefinic proton (δ 5.46) gave cross peaks in the TOCSY spectrum (MP1 TOCSY) with the methylene protons (δ 2.3 & 1.9) on carbon-16. These methylene protons gave cross peaks in the HMBC spectrum (MP1 HMBC) with olefinic carbons (δ 115.86 & 159.49) and also with C-17 (δ 50.52) and C-28 (δ 183.57), the carboxyl carbon.

This observation confirmed the γ-δ unsaturated carboxylic acid structure.

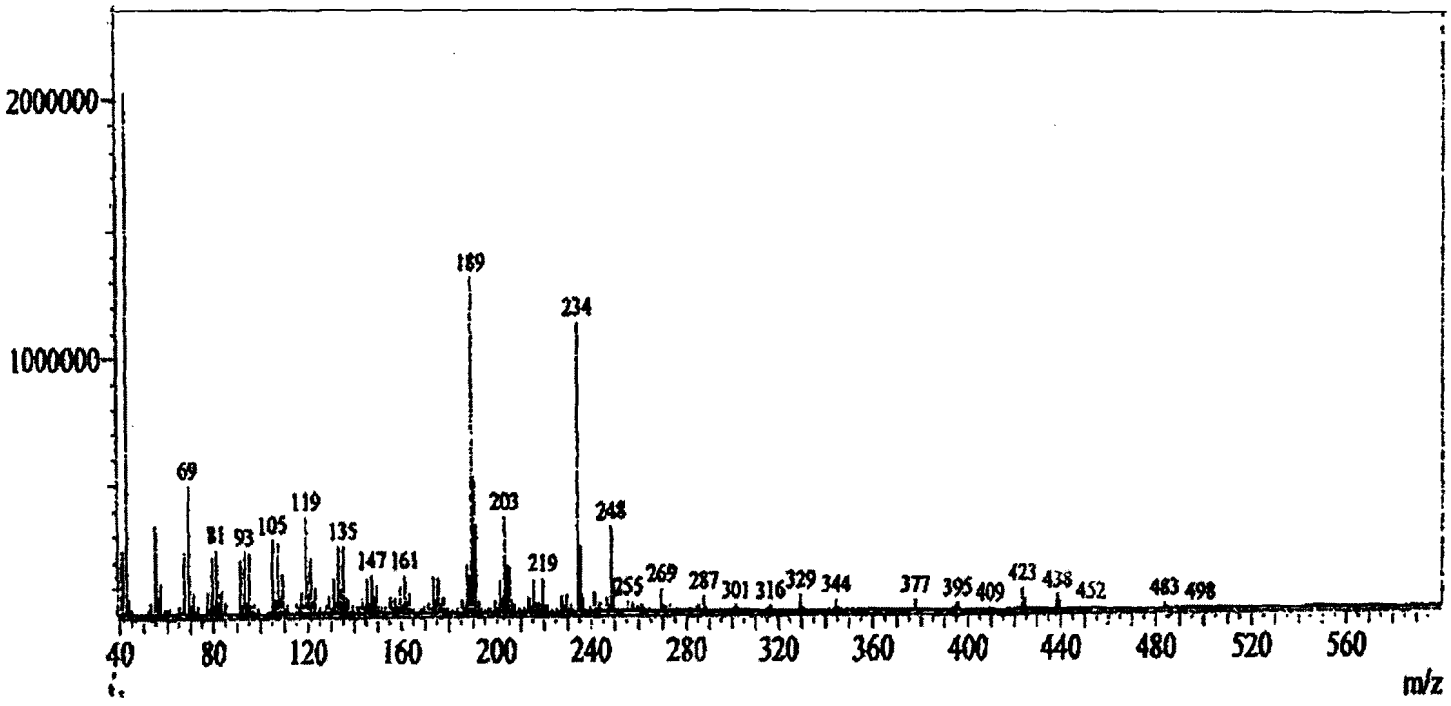
All the spectral data of compound MP1 were found to be quite identical with those of 3-acetylaleuritolic acid reported in literature[18,21-23]. Comparison with the spectral data of 3-acetyl-aleuritolic acid [IR, NMR & Mass], confirmed the compound **MP1** as **3-acetylaleuritolic acid** (3 β-acetoxytaraxer -14-en-28-oic acid).



MP1	R = AC	R_1 = H
MP1a	R = H	R_1 = H
MP1b	R = AC	R_1 = Me

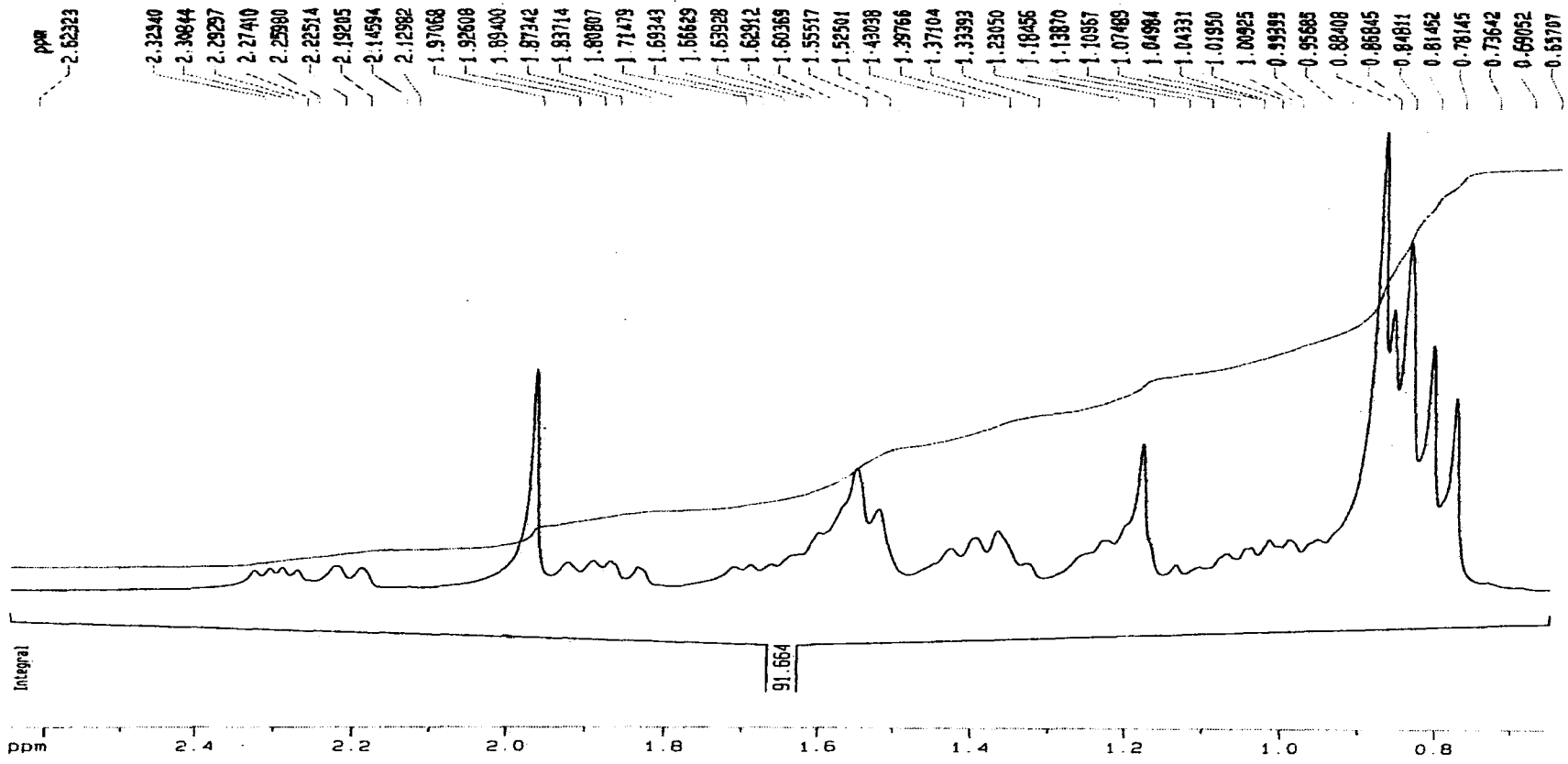


IR spectrum of 3-acetylaleuritolic acid [MP1 IR]

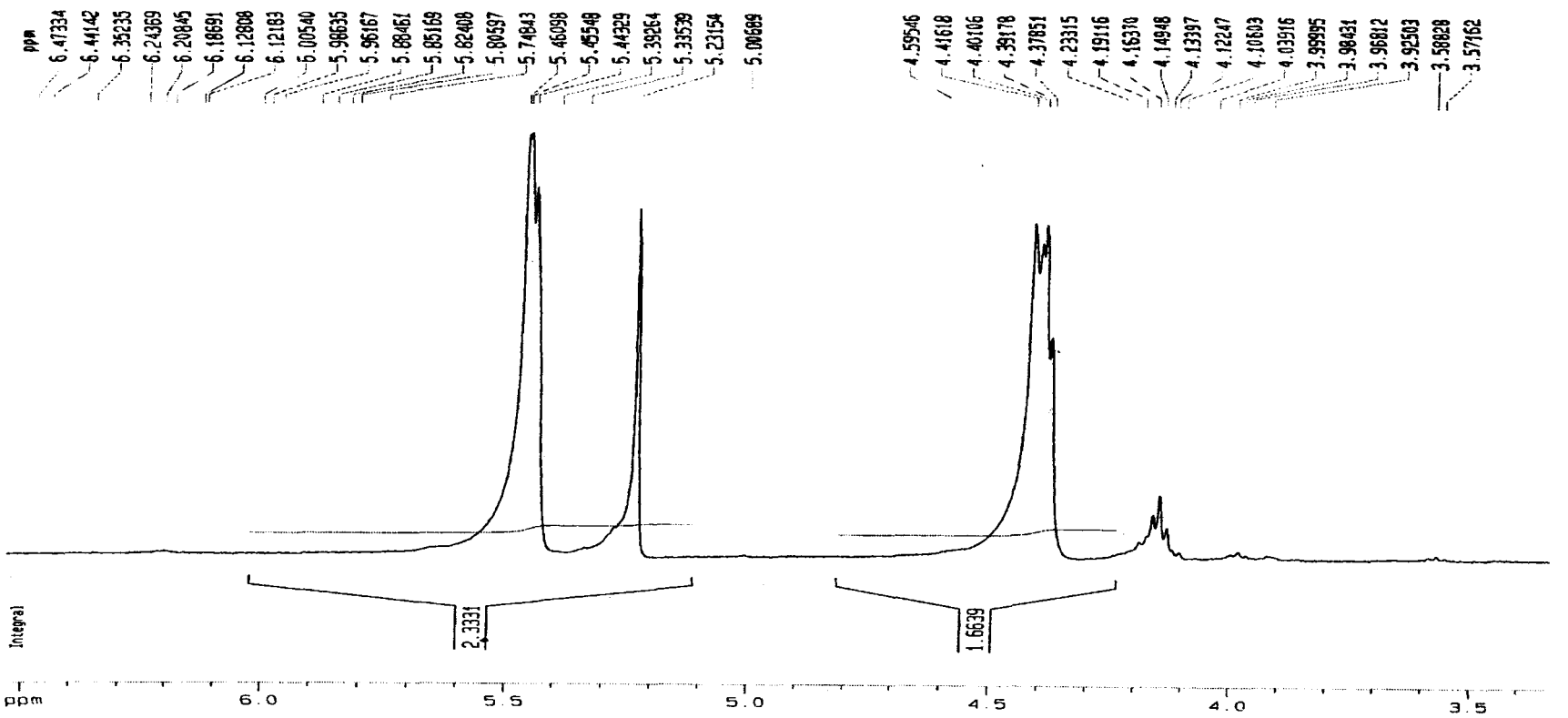


Mass spectrum of 3-acetylaleuritolic acid [MP1 MS]

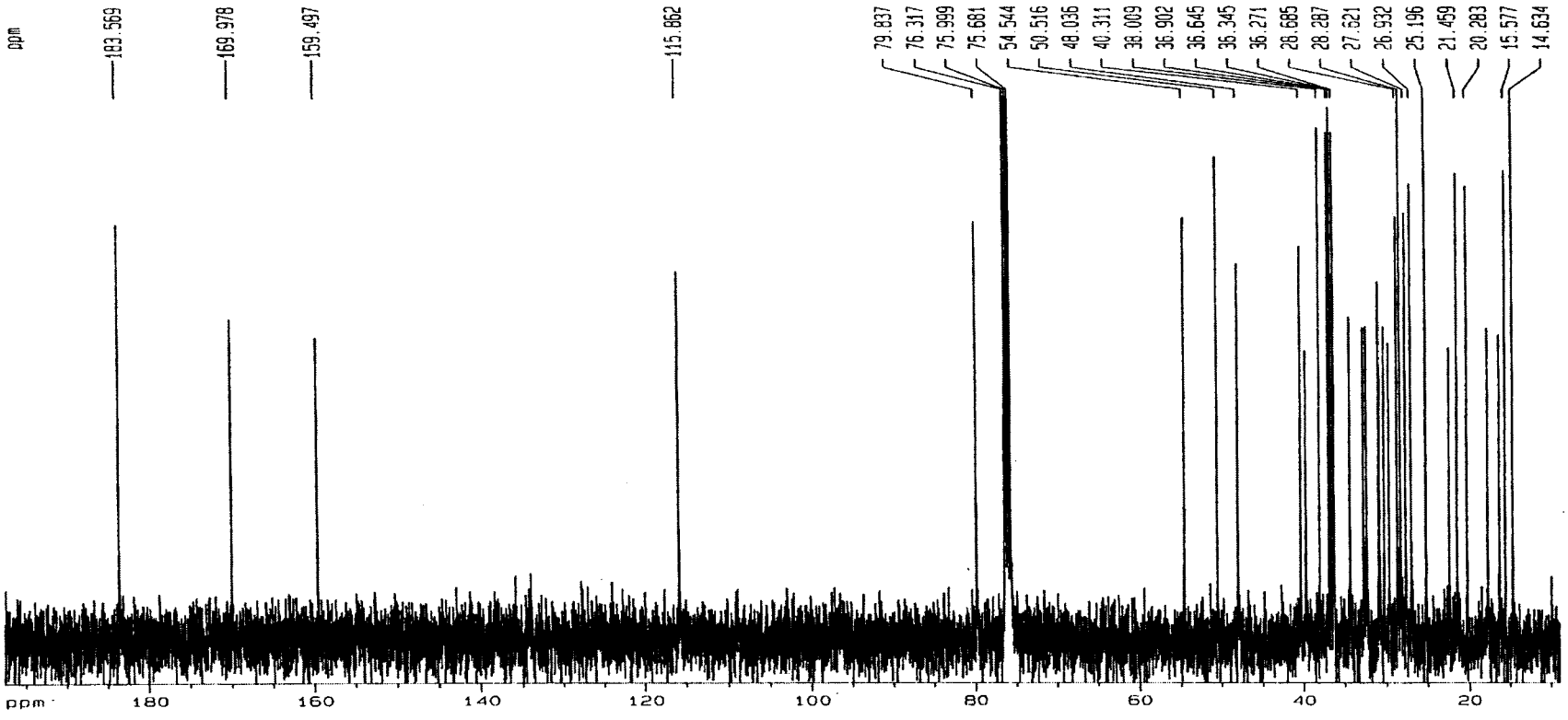
^{1}H-NMR spectrum of 3-acetylaleuritolic acid [MP1 ^{1}H-NMR] (400 MHz, $CDCl_3$, TMS)



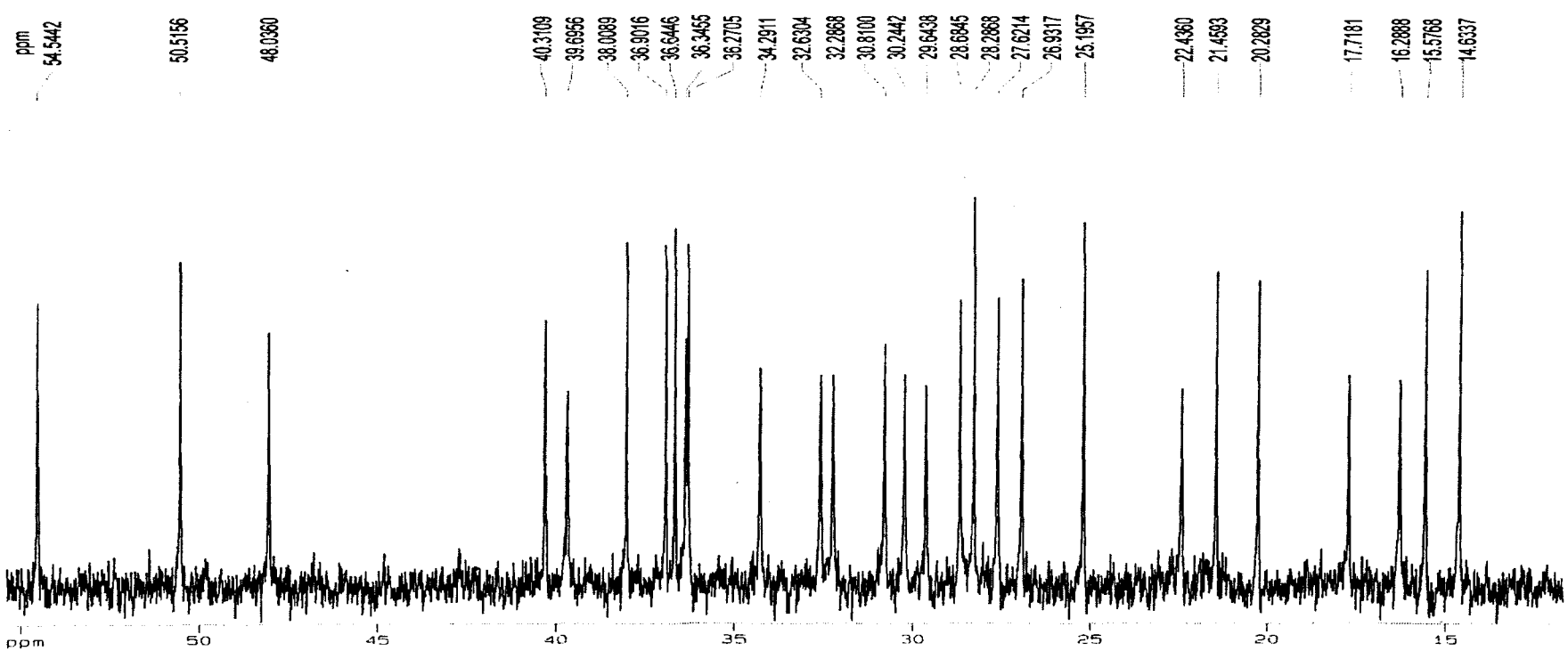
^{1}H-NMR spectrum of 3-acetylaleuritolic acid [MP1 ^{1}H-NMR expansion] (400 MHz, $CDCl_3$, TMS)



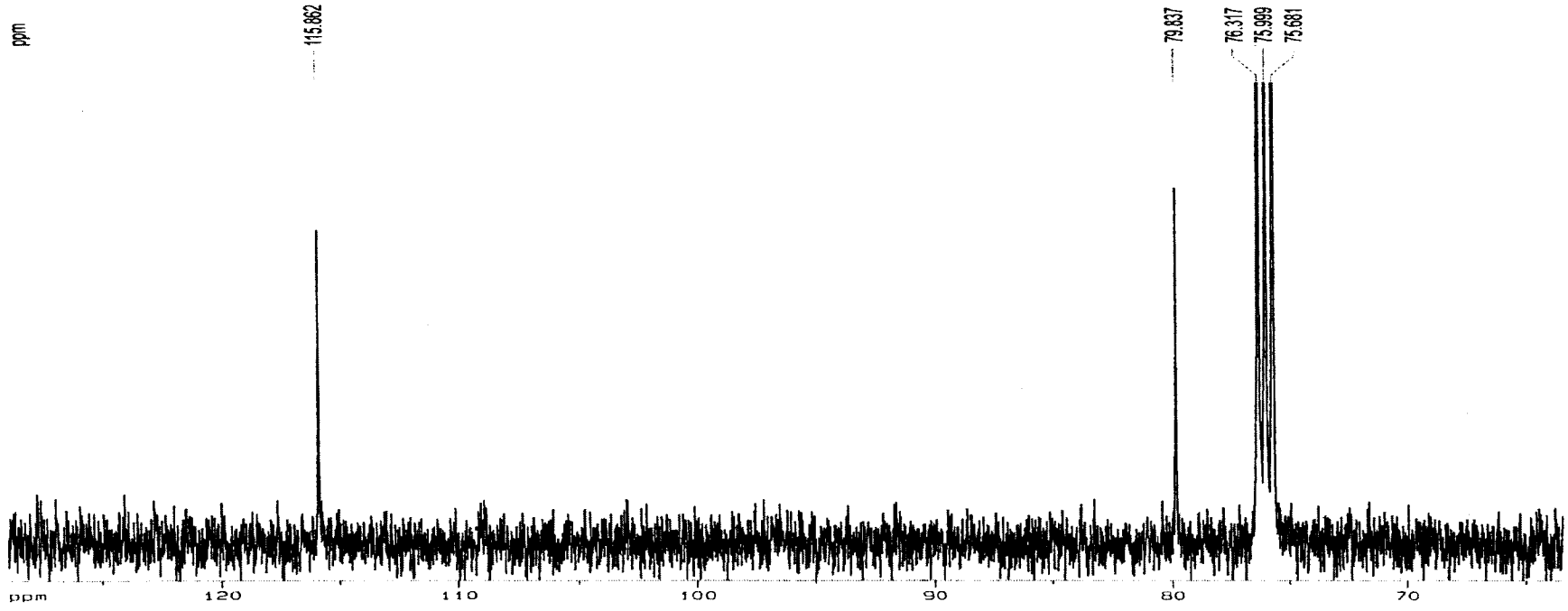
^{1}H-NMR spectrum of 3-acetylaleuritolic acid [MP1 ^{1}H-NMR expansion] (400 MHz, $CDCl_3$, TMS)



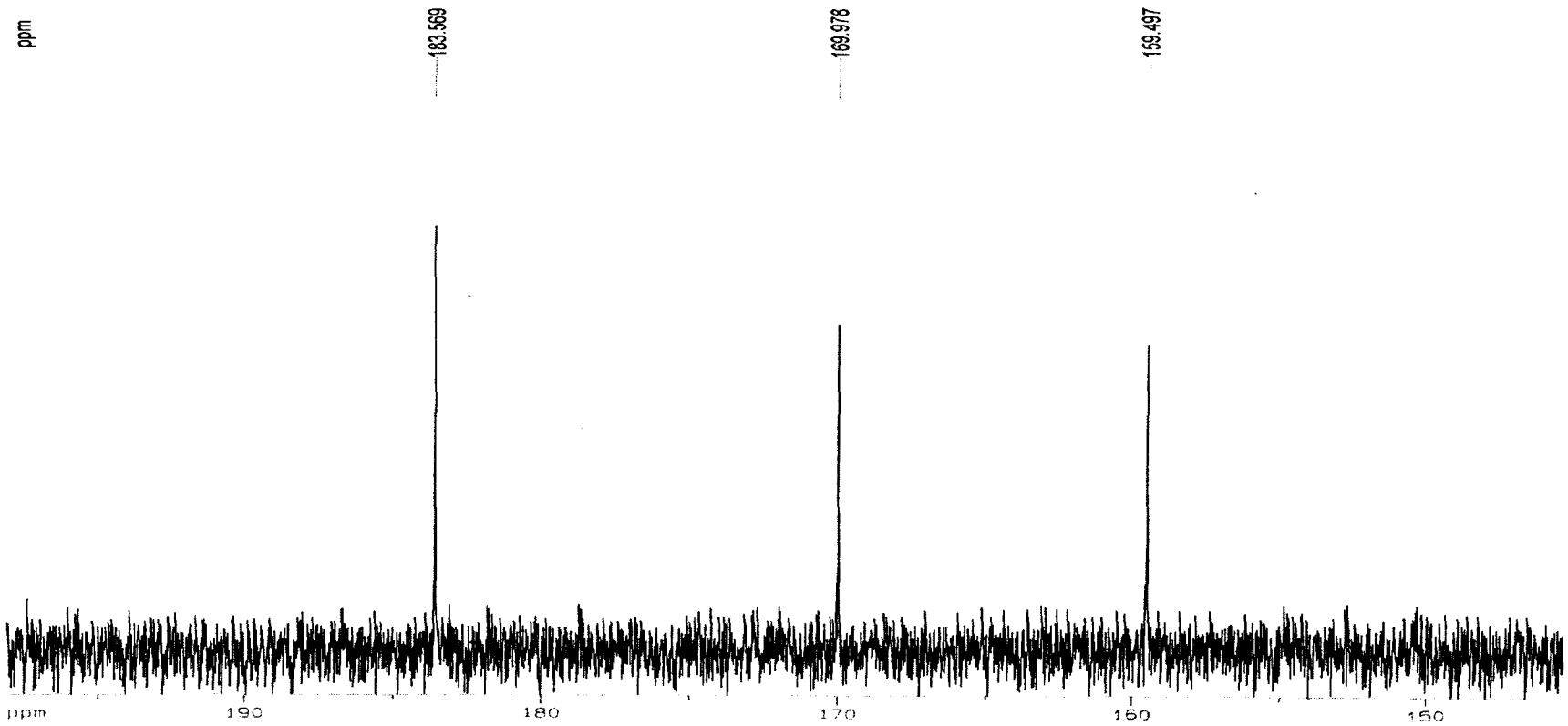
^{13}C-NMR spectrum of 3-acetylaleuritolic acid [MP1 ^{13}C-NMR] (400 MHz, $CDCl_3$, TMS)



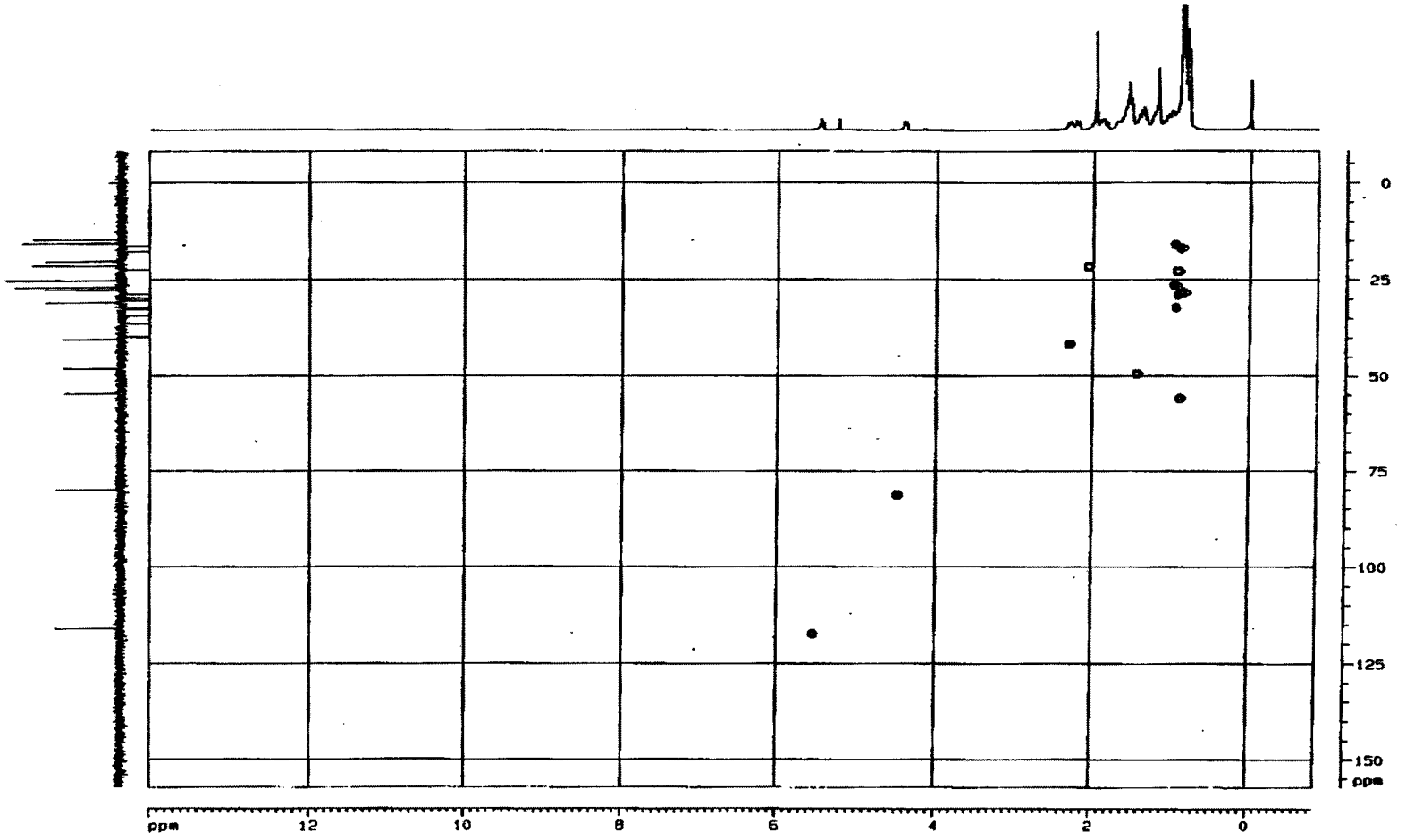
^{13}C-NMR spectrum of 3-acetylaleuritolic acid [MP1 ^{13}C-NMR expansion] (400 MHz, $CDCl_3$, TMS)



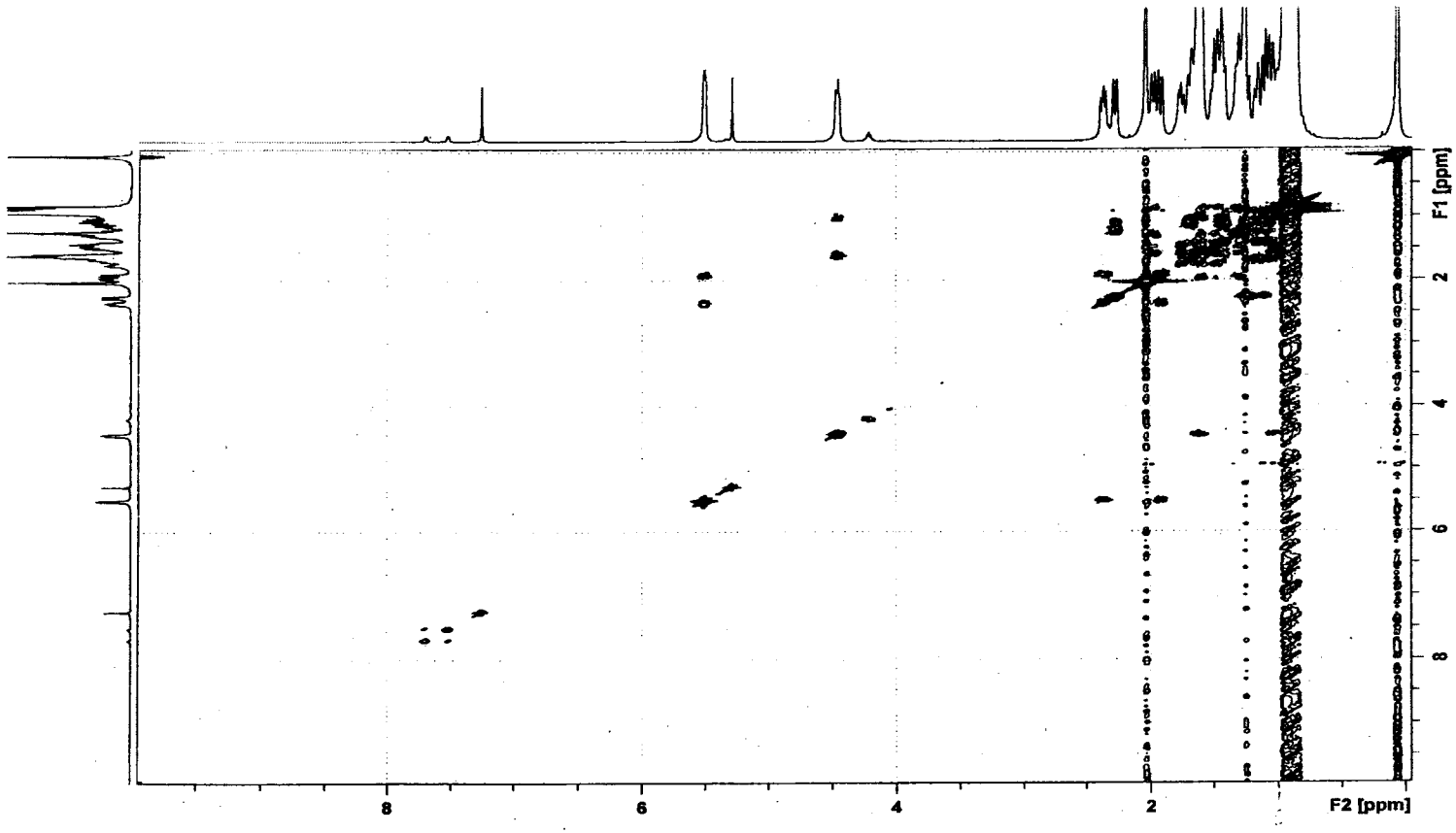
^{13}C-NMR spectrum of 3-acetylaleuritolic acid [MP1 ^{13}C-NMR expansion] (400 MHz, $CDCl_3$, TMS)



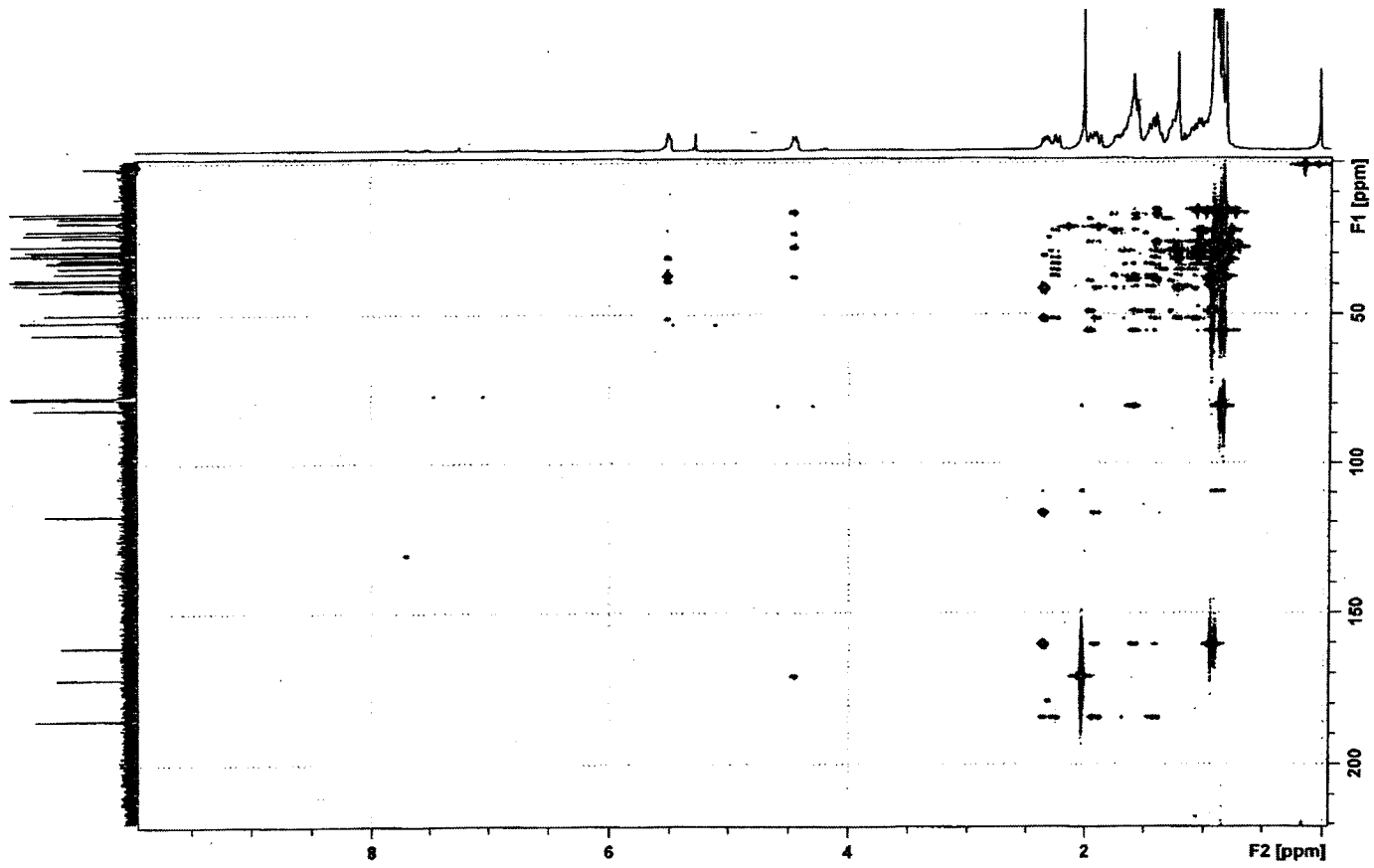
^{13}C-NMR spectrum of 3-acetylaleuritolic acid [MP1 ^{13}C- NMR expansion] (400 MHz, $CDCl_3$, TMS)



HSQC spectrum of 3-acetylaleuritolic acid [MP1 HSQC] (400 MHz, $CDCl_3$, TMS)



TOCSY spectrum of 3-acetylaleuritolic acid [MP1 TOCSY] (500 MHz, $CDCl_3$, TMS)



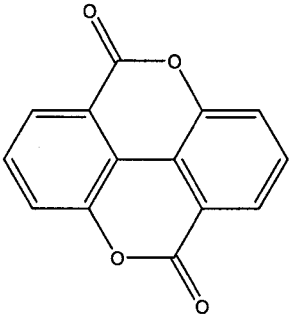
HMBC spectrum of 3-acetylaleuritolic acid [MP1 HMBC] (500 MHz, $CDCl_3$, TMS)

1.7.2 Characterisation of MP2 (3, 4, 3'-tri-O-methylellagic acid)

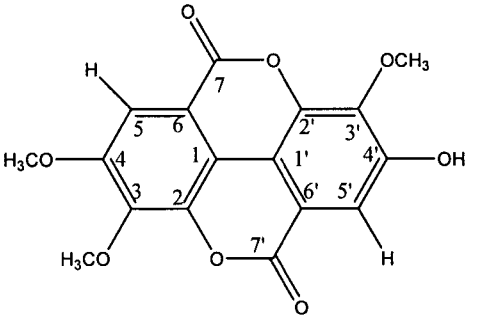
The compound **MP2** isolated as described in section 1.6 melted at 275^{0}C. Its high resolution mass spectrum [MP2 MS] indicated a molecular mass of 344.06077 amu corresponding to a molecular formula $C_{17}H_{12}O_8$ (calculated for $C_{17}H_{12}O_8$ = 344.2656). This formula shows the presence of twelve double bond equivalents.

The IR spectrum [MP2 IR] has characteristic peaks at 3439,1751.4 and 1728.2 cm^{-1}. The sharp nature of the peak at 3439 cm^{-1} indicated the presence of an intra-molecular hydrogen bonded –OH group. The intense absorptions at 1751.4 and 1728.2 cm^{-1} correspond to two carbonyl groups. The ^{1}H-NMR spectrum [MP2 ^{1}H-NMR] showed singlets at δ 3.99, 4.03, 4.04, 7.51, 7.60 and 10.83. In the DEPT 135 spectrum [MP2 DEPT] peaks at δ 57.1, 61.4, 61.7, 107.8 and 112.0 were observed. These peaks with chemical shifts at δ 57.1, 61.4 and 61.7 are quite characteristic of methoxy carbons while the peaks at δ 107.8 and 112.0 are characteristic of aromatic carbon bearing one hydrogen atom. These observations were further confirmed from the cross peaks in the HSQC spectrum [MP2 HSQC] (δH/δC : 3.99/57.1, 4.03/61.4 and 4.04/61.7).The presence of three methoxy groups in the molecule were thus confirmed. The H-H COSY spectrum [MP2 H-H COSY] showed no cross peaks justifying the mutually uncoupled nature of all the different sets of protons.

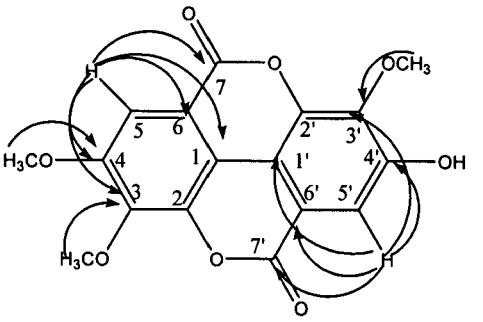
The ^{13}C-NMR spectrum [MP2 ^{13}C-NMR] showed the presence of twelve carbon atoms in the range δ 107-153. This clearly suggested the presence of two benzene rings in the molecule. Two benzene rings plus two carbonyl groups added up to ten double bond equivalents. As the compound has twelve double bond equivalents, the following tetracyclic skeletal structure can be suggested.



Introduction of three methoxy groups and one hydroxy group makes the final structure.



This structure is further confirmed from the cross peaks in the HMBC spectrum [MP2 HMBC] and corresponds exactly to 3,4,3'-tri-O-methylellagic acid reported[24,25].



The reported m.p. 294-295^{0}C agreed fairly well with the isolated compound[24]. Also this compound fluoresced in UV light with a mauve colour that is characteristic of ellagic acid derivatives[24].

The IR carbonyl absorption frequencies of 1751.4 and 1728.2 cm^{-1} also were not against the proposed structure. These ester groups showed different frequencies ranging from 1750-1715 cm^{-1} depending upon the degree of methylation of the ellagic acid structure[24].

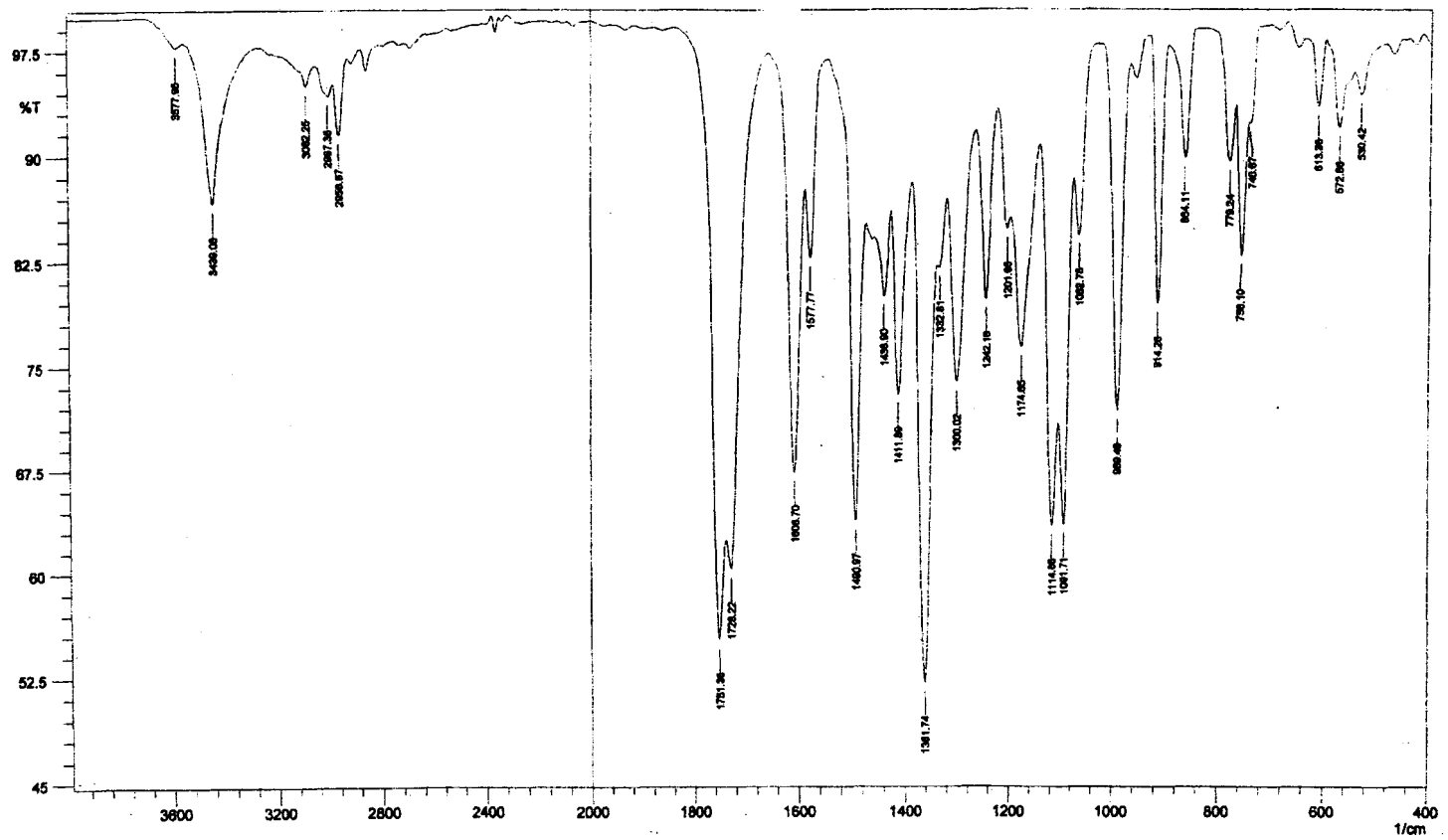
In the EI mass spectrum the base peak and the molecular ion peak were the same. Apart from this, peaks corresponding to ($M-CH_3$), ($M-CH_3CO$) and ($M-2CH_3CO$) were observed along with other peaks. This kind of fragmentation is characteristic of O-methylated ellagic acids[26].

The ^{13}C chemical shift values of compound MP2 and those obtained for 3,4,3'-tri-O-methylellagic acid 4'-O-β-D-glucopyranose are given in the table[27] (**Table 1.4**). They show perfect agreement

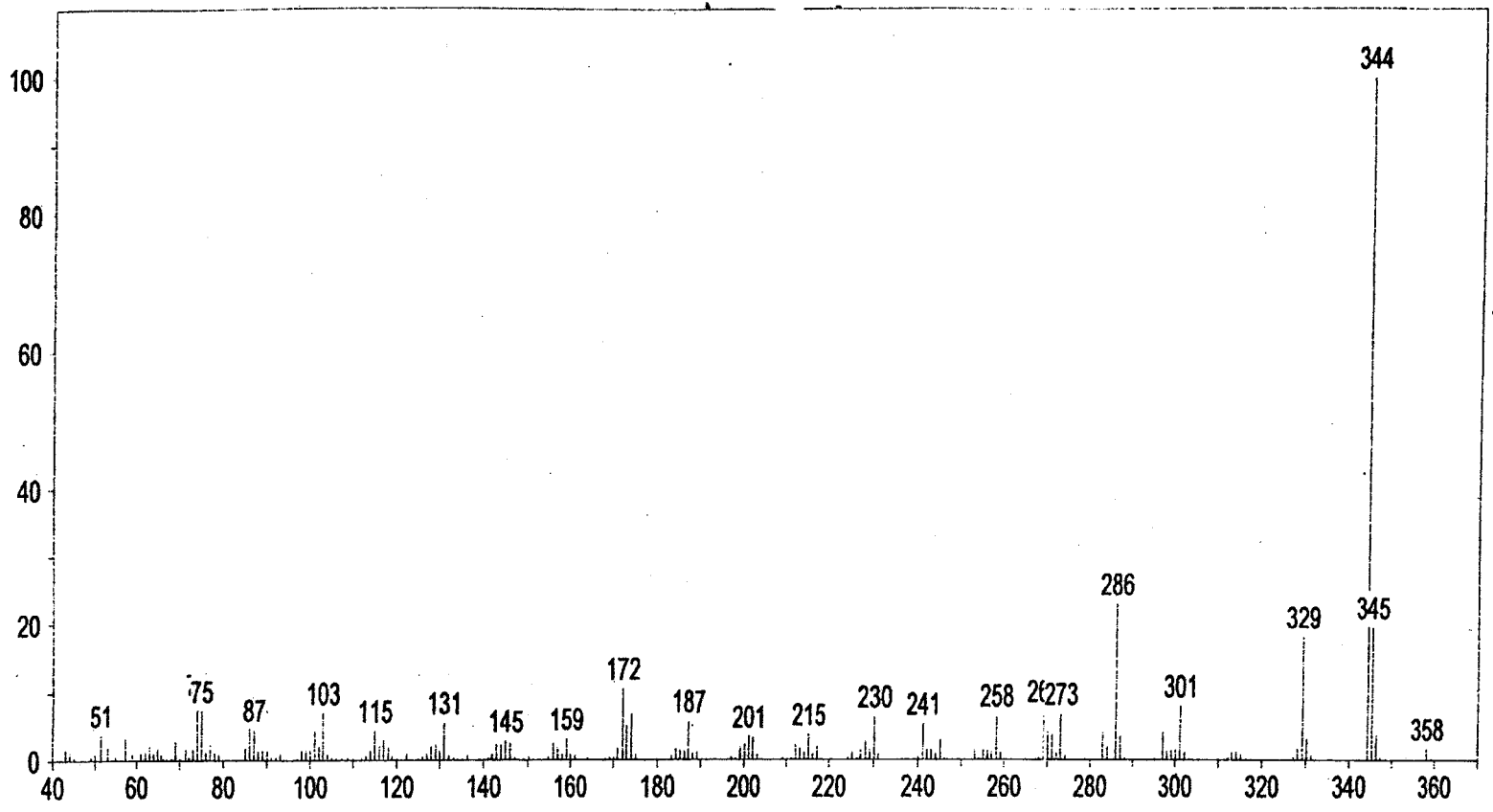
proving the identity of compound **MP2** as **3,4,3'-tri-O-methylellagic acid**.

TABLE I.4

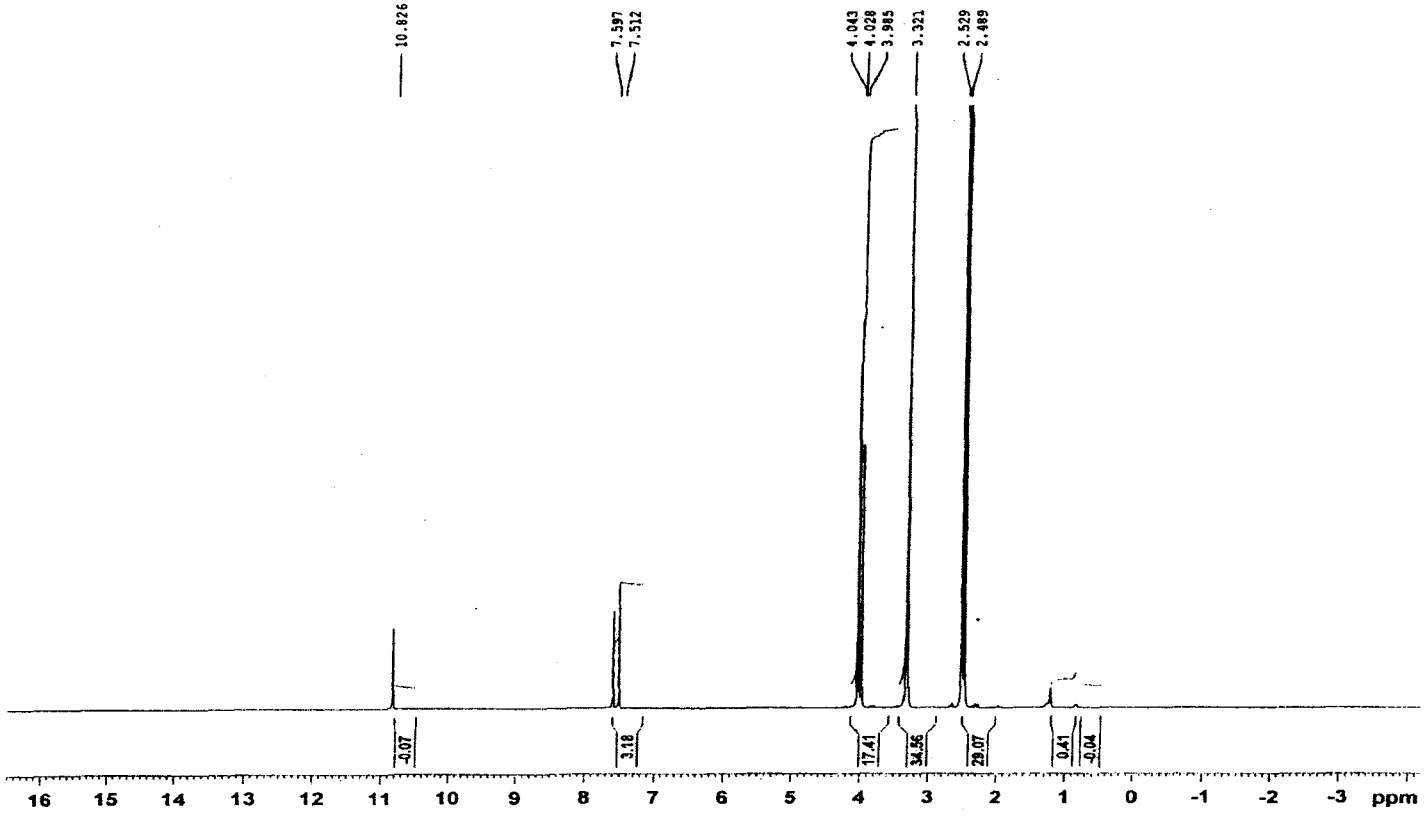
Carbon Atom Number	^{13}C-NMR shifts of Compound MP2	^{13}C-NMR shifts of 3,4,3'-tri-O-methylellagic acid 4'-O-β-D-glucopyranose
1	113.4	112.4
2	141.5	140.8
3	140.84	140.7
4	153.81	154.1
5	107.54	107.3
6	112.52	112.2
7	158.49	158.1
1'	113.42	113.8
2'	140.84	140.9
3'	140.26	141.6
4'	152.70	151.8
5'	111.9	112.0
6'	111.73	112.0
7'	158.31	157.9



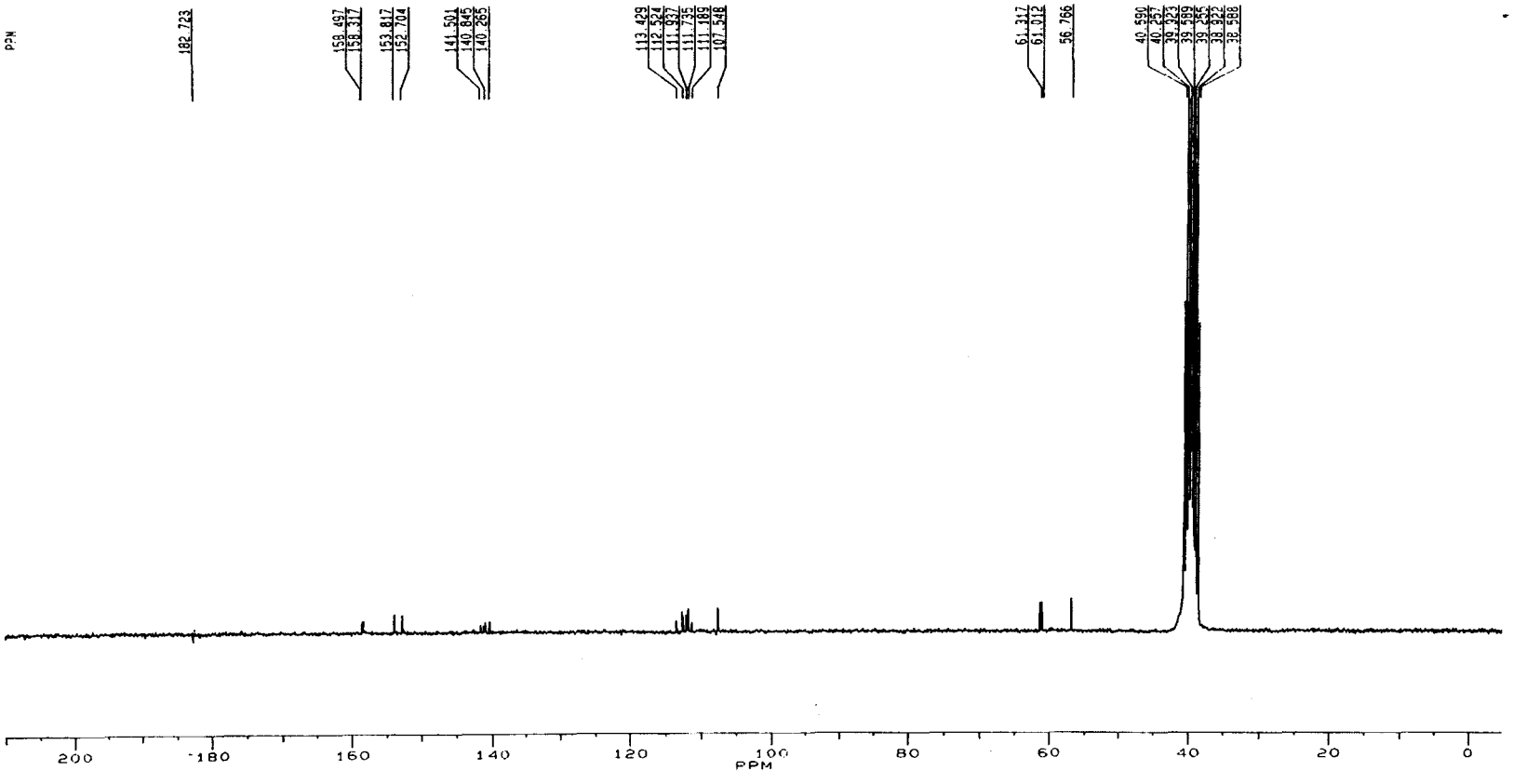
IR spectrum of 3,4,3'-tri-O-methylellagic acid [MP2 IR]



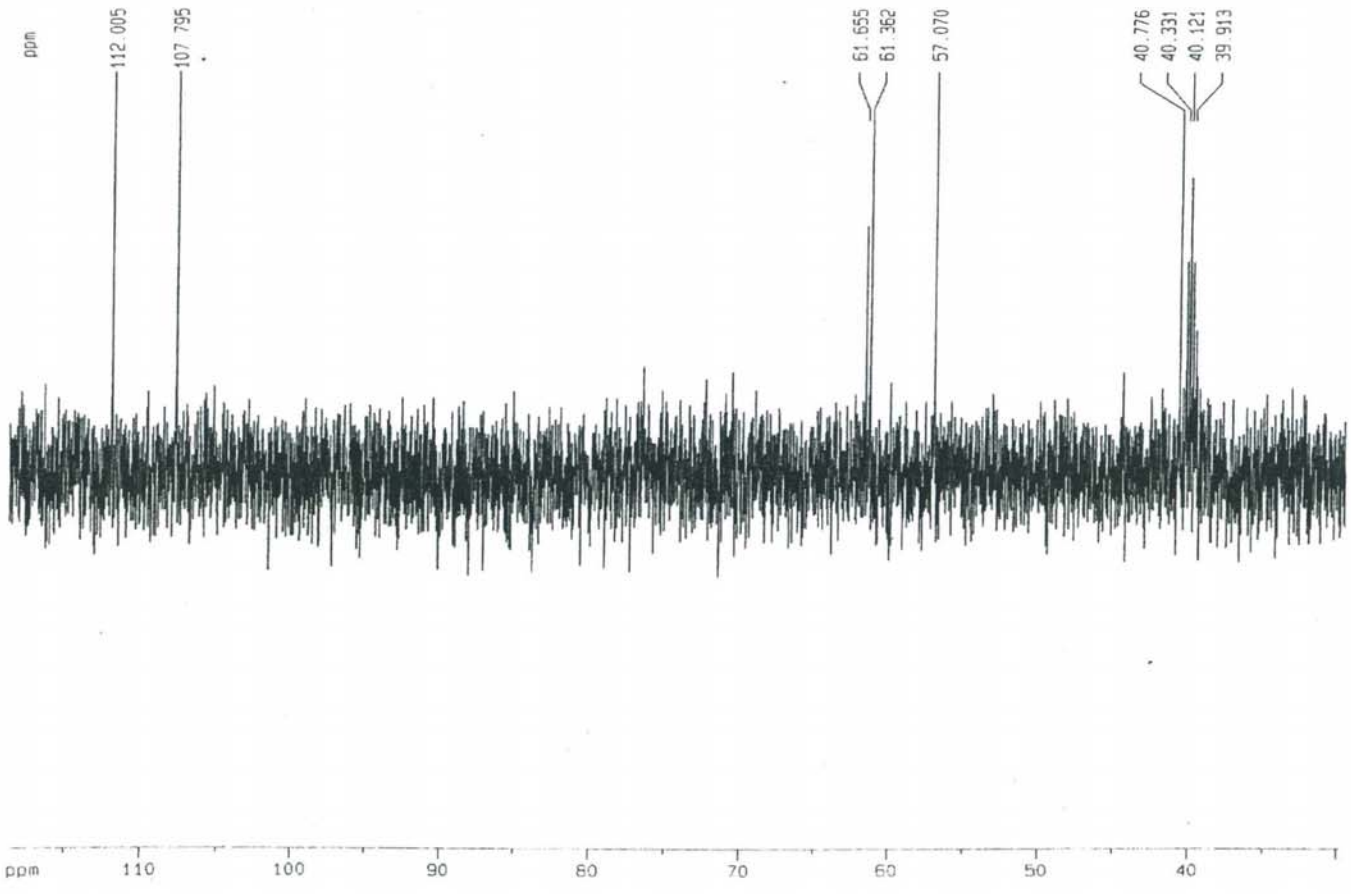
Mass spectrum of 3, 4, 3'-tri-O-methylellagic acid [MP2 MS]



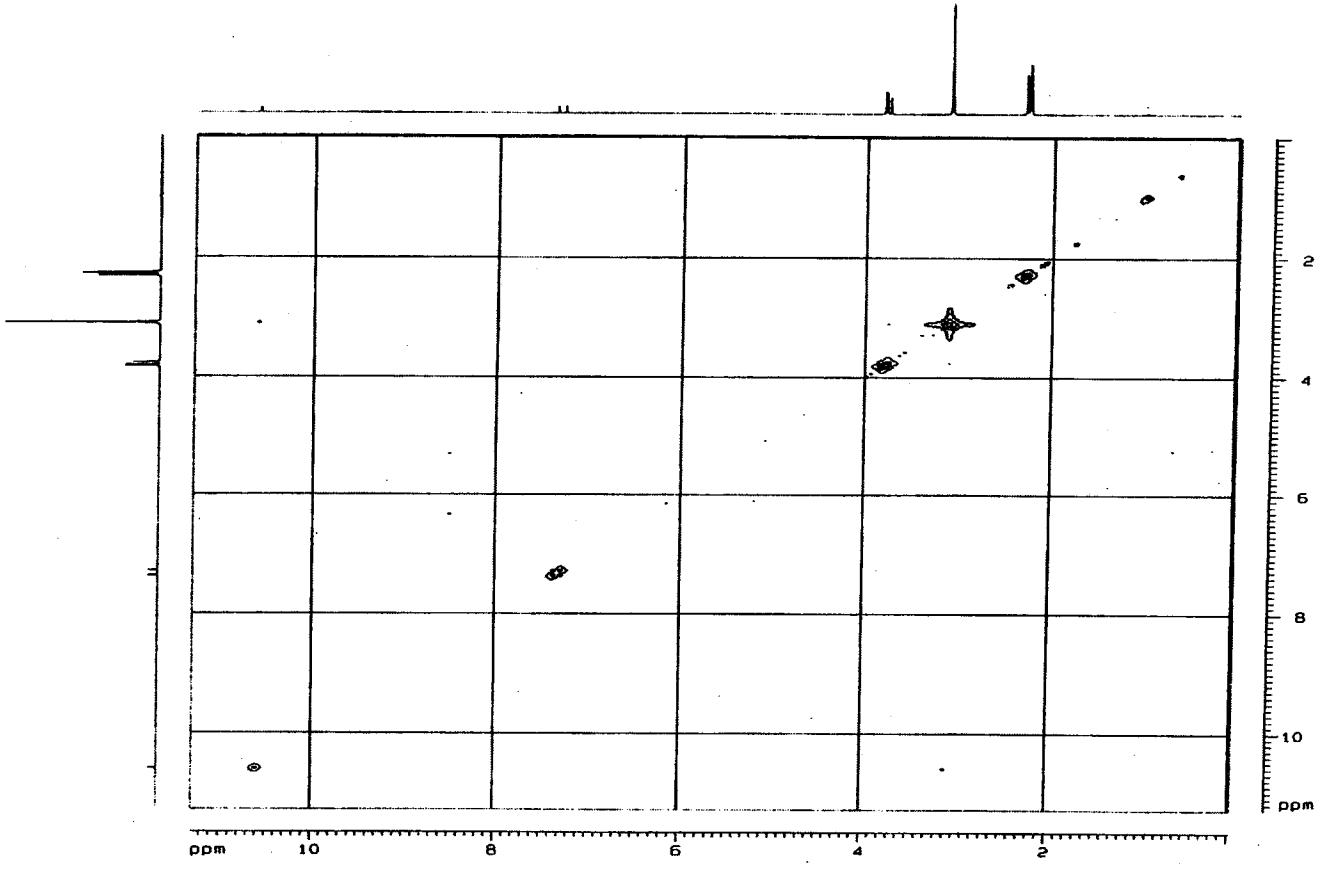
^{1}H-NMR spectrum of 3, 4, 3'-tri-O-methylellagic acid [MP2 ^{1}H-NMR] (400 MHz, DMSO, TMS)



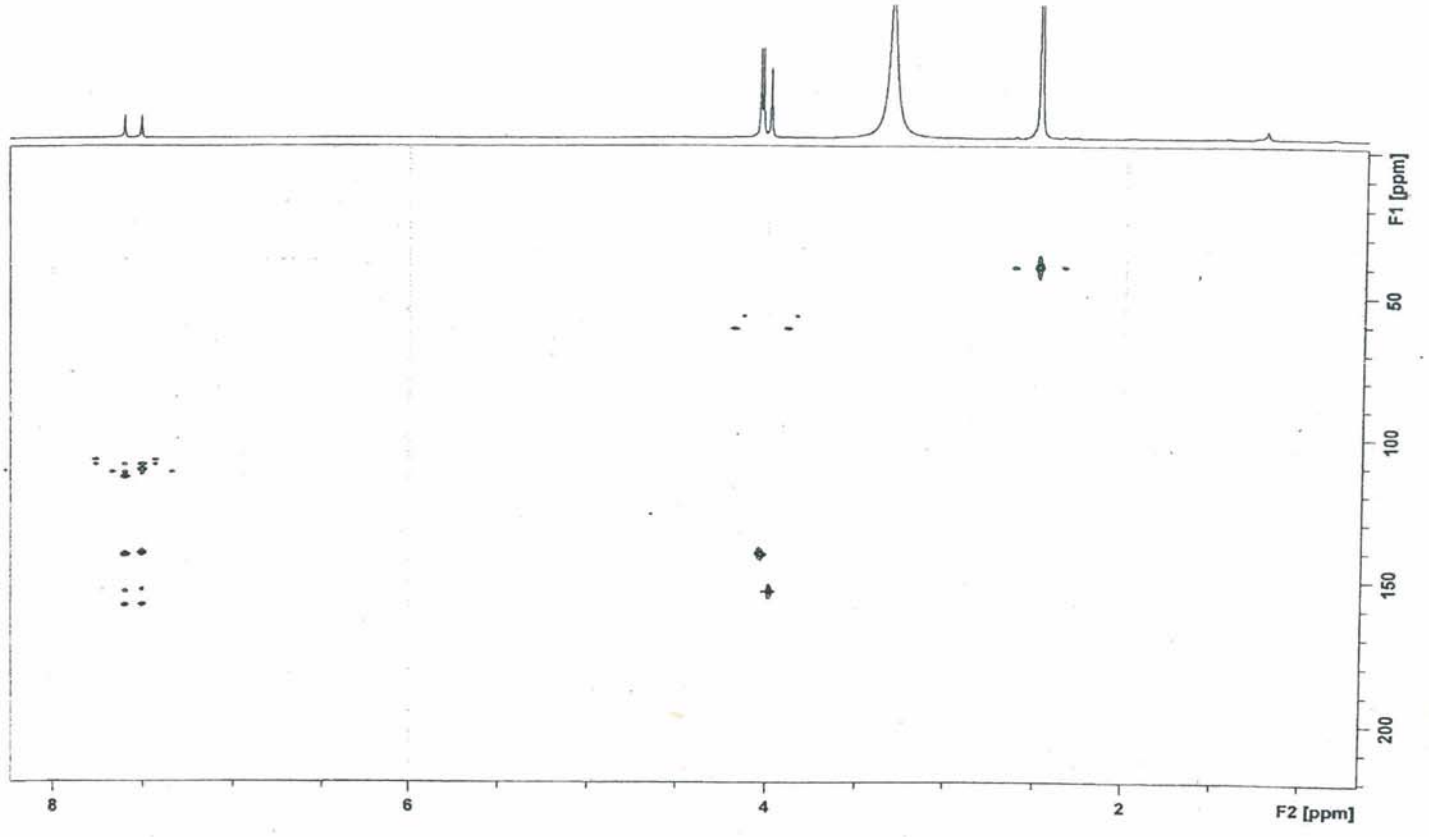
^{13}C-NMR spectrum of 3, 4, 3'-tri-O-methylellagic acid [MP2 ^{13}C -NMR] (400 MHz, DMSO, TMS)



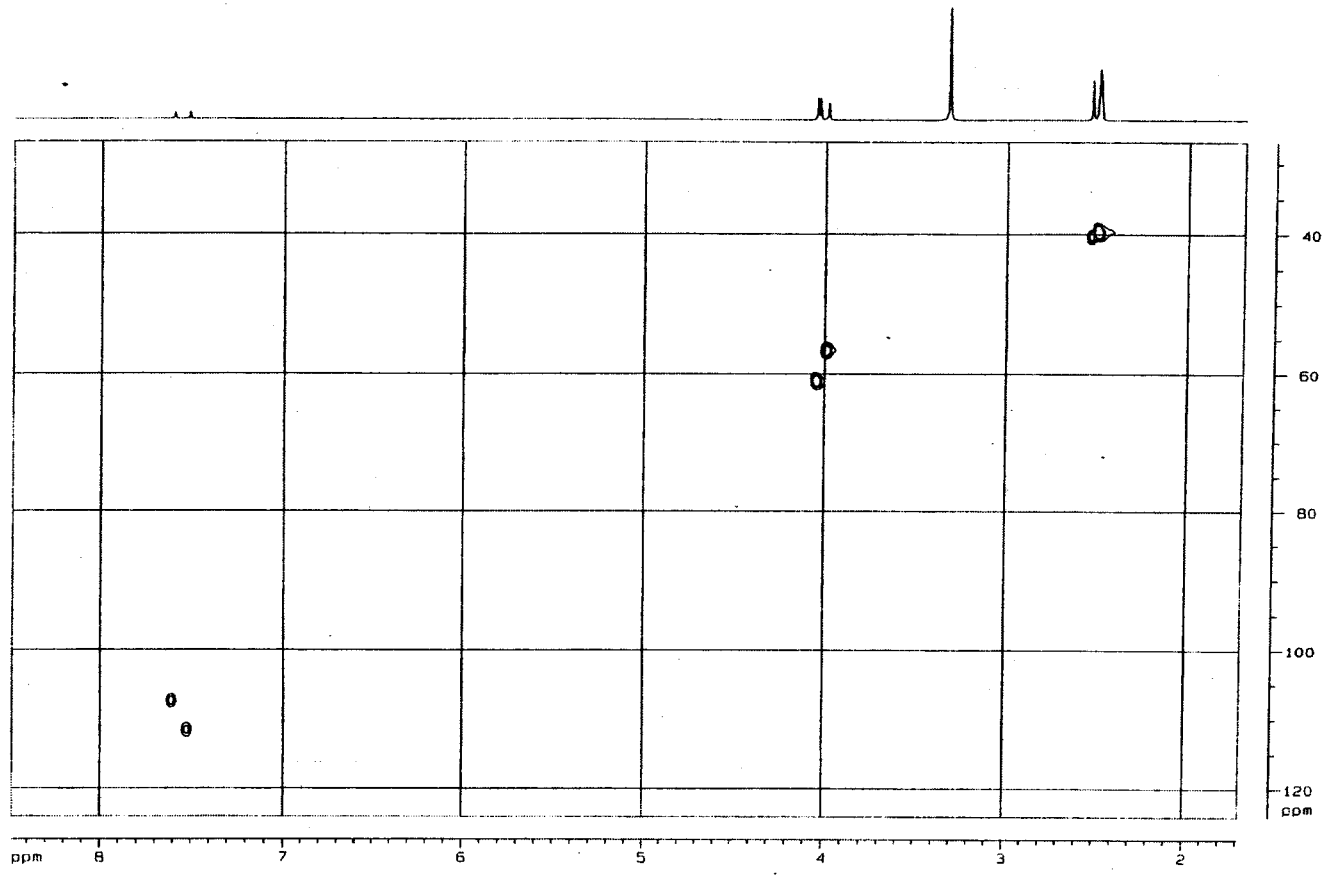
DEPT spectrum of 3, 4, 3'-tri-O-methylellagic acid [MP2 DEPT] (400 MHz, DMSO, TMS)



H-H COSY spectrum of 3, 4, 3'-tri-O-methylellagic acid [MP2 H-H COSY] (400 MHz, DMSO, TMS)



HMBC spectrum of 3, 4, 3'-tri-O-methylellagic acid [MP2 HMBC] (500 MHz, DMSO, TMS)



HSQC spectrum of 3, 4, 3'-tri-O-methylellagic acid [MP2 HSQC] (400 MHz, DMSO, TMS)

1.8. HYDROLYSIS OF 3-ACETYLALEURITOLIC ACID

3-Acetylaleuritolic acid (200 mg) was refluxed with 2 M ethanolic KOH solution (40 ml) for 2 hrs. The solution was acidified, extracted with $CHCl_3$ (3 x 40 ml), the organic phase washed with H_2O (3x20 ml), dried using anhydrous sodium sulphate and evaporated to yield **3-hydroxy derivative (Aleuritolic acid – MP1a)**. It was recrystallised from pyridine m.p. 268°C (Lit. 300-302°C).[22] Its IR spectrum [MP1a IR] showed absorption at 3298.28, 3053.32, 1689.64 and 1467.83 cm^{-1}. Prominent peaks in the mass spectrum [MP1a MS] were at m/z : 456 $[M]^+$, 441 $[M-CH_3]^+$, 423 $[441-H_2O]^+$, 411 $[M-COOH]^+$, 287, 248, 234, 203, 189, 119, 105, 91, 81, 57 and 43. The base peak was at m/z 189 as in the case of 3-acetylaleuritolic acid[19].

1.9. METHYLATION OF 3-ACETYLALEURITOLIC ACID

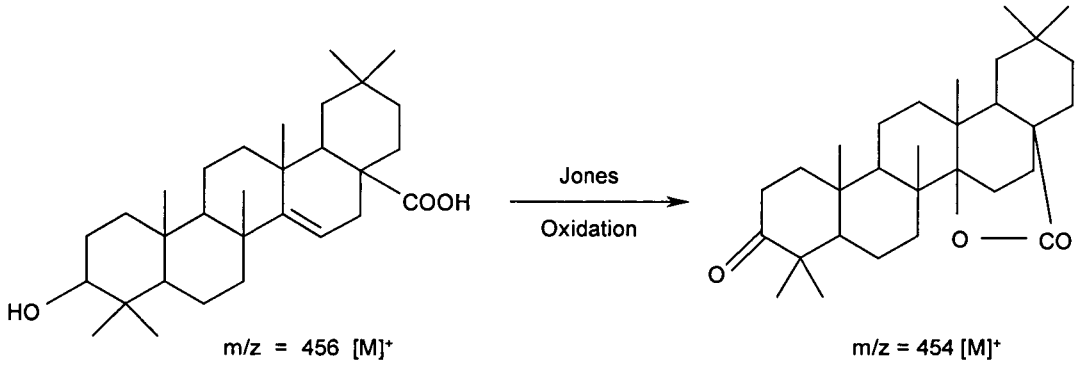
Nitrosomethyl urea was prepared as per reported methods[28] and converted to diazomethane[29]. 3-Acetylaleuritolic acid (100 mg) was shaken with CH_2N_2 solution for 30 minutes. It was filtered and evaporated to get the **methyl ester of 3-acetylaleuritolic acid (acetoxy methyl aleuritolate MP1b)**. Thereafter it was purified by preparative TLC, m.p. 220°C (Lit. 235-238°C)[22]. Its IR spectrum [MP1b IR] showed absorptions at 1728 and 1471.69 cm^{-1}. This compound showed only one absorption for the >C=O group with a

maximum at 1728 cm^{-1}. This can be due to the overlap of the two carbonyl groups of the diester. The mass spectrum [MP1b MS] had M^+ ion peak at m/z 512 which corresponded to the expected diester. Other prominent peaks in the mass spectrum were at m/z: 497, 452, 437, 377, 344, 329, 262, 248, 203, 189, 173, 159, 133, 119, 105, 93, 81, 69 and 55.

1.10. JONES OXIDATION OF ALEURITOLIC ACID

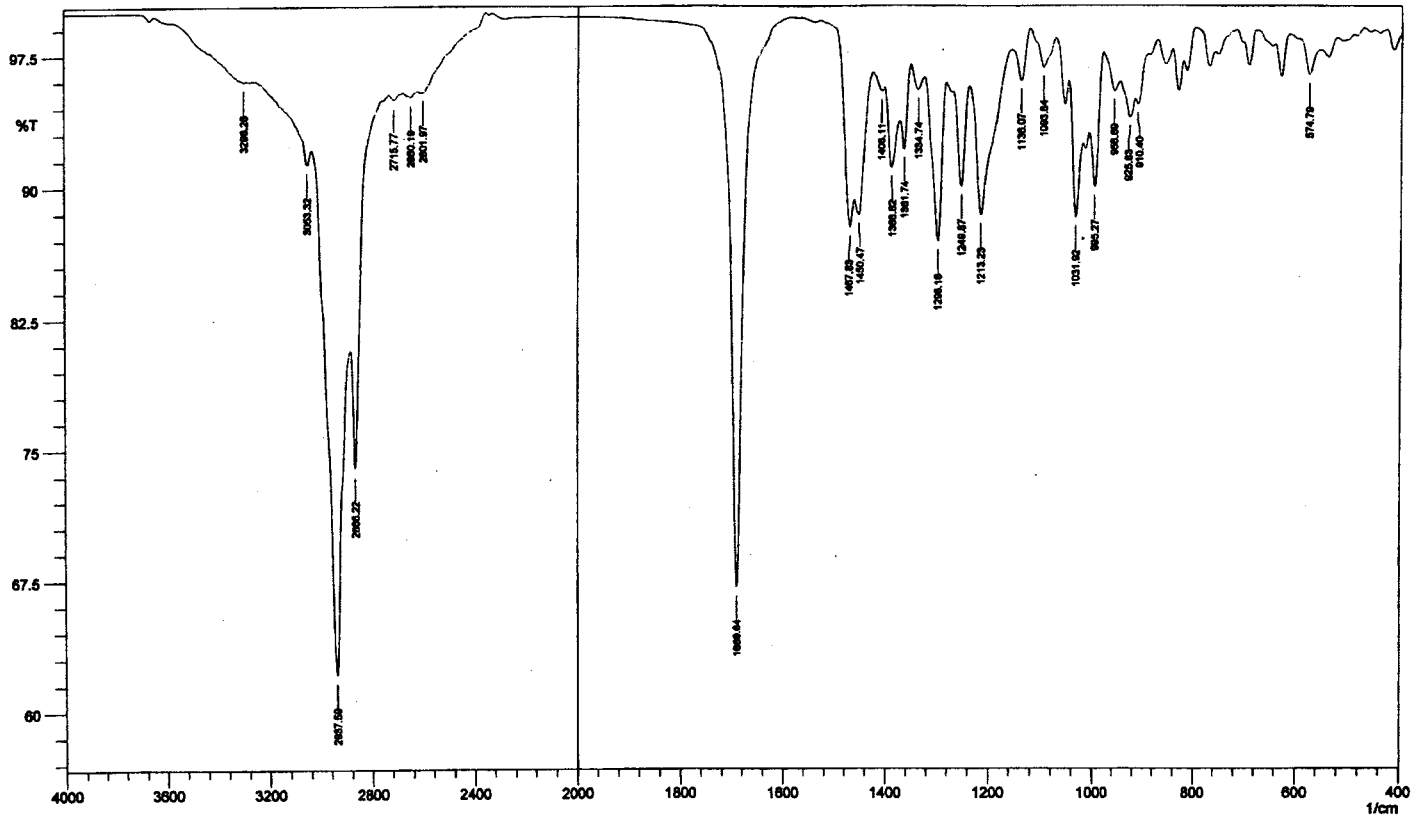
Jones reagent[30] (4 ml) was added to a solution of aleuritolic acid (100 mg) in Me_2CO (50 ml) at 4°C and stirred for 2 hrs using magnetic stirrer. Water (20 ml) was added and the mixture extracted with $CHCl_3$ (3 x 25 ml), washed with water (3 x 20 ml), dried using anhydrous sodium sulphate and evaporated to yield **3-keto derivative (MP1c)**. It was recrystallised from pyridine, m.p. 205°C. Its IR spectrum [MP1c IR] showed absorption at 1761.01, 1703.14 and 1458.18 cm^{-1}.

The IR spectrum showed the presence of the new keto group formed (1703.14 cm^{-1}). However the >C=O absorption at 1688.7 cm^{-1} in aleuritolic acid was absent and a new >C=O absorption appeared at 1761.01 cm^{-1}. This was due to the lactonisation of the γ-δ unsaturated carboxylic acid under the acidic conditions of oxidation. This structure was confirmed from the corresponding molecular mass of the compound [m/z 454].

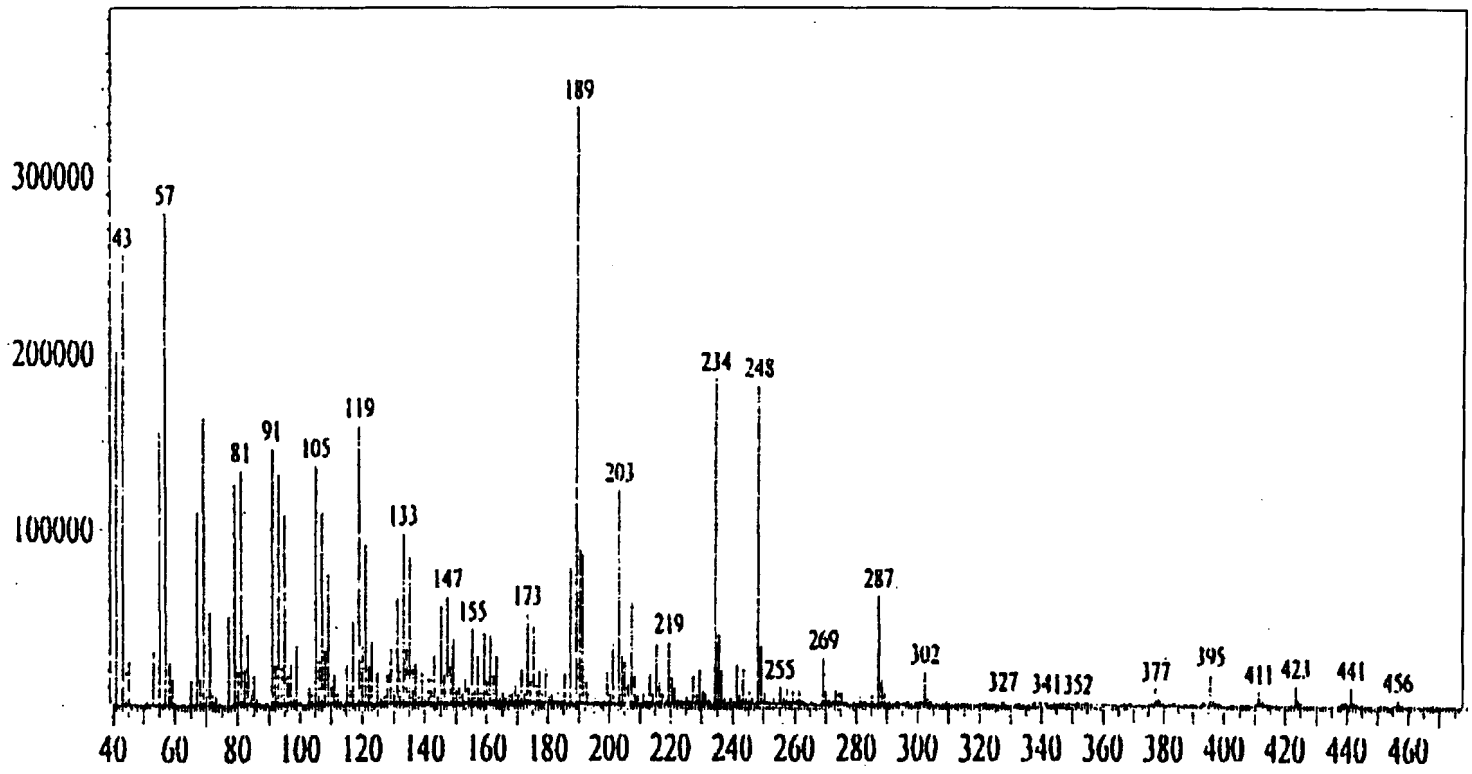


The mass spectrum [MP1c MS] of the compound showed the prominent peaks at m/z : 454 $[M]^+$, 439, 424, 285, 263, 250, 234, 219, 205, 189, 173, 161, 145, 133, 119, 107, 91, 81, 67 and 55.

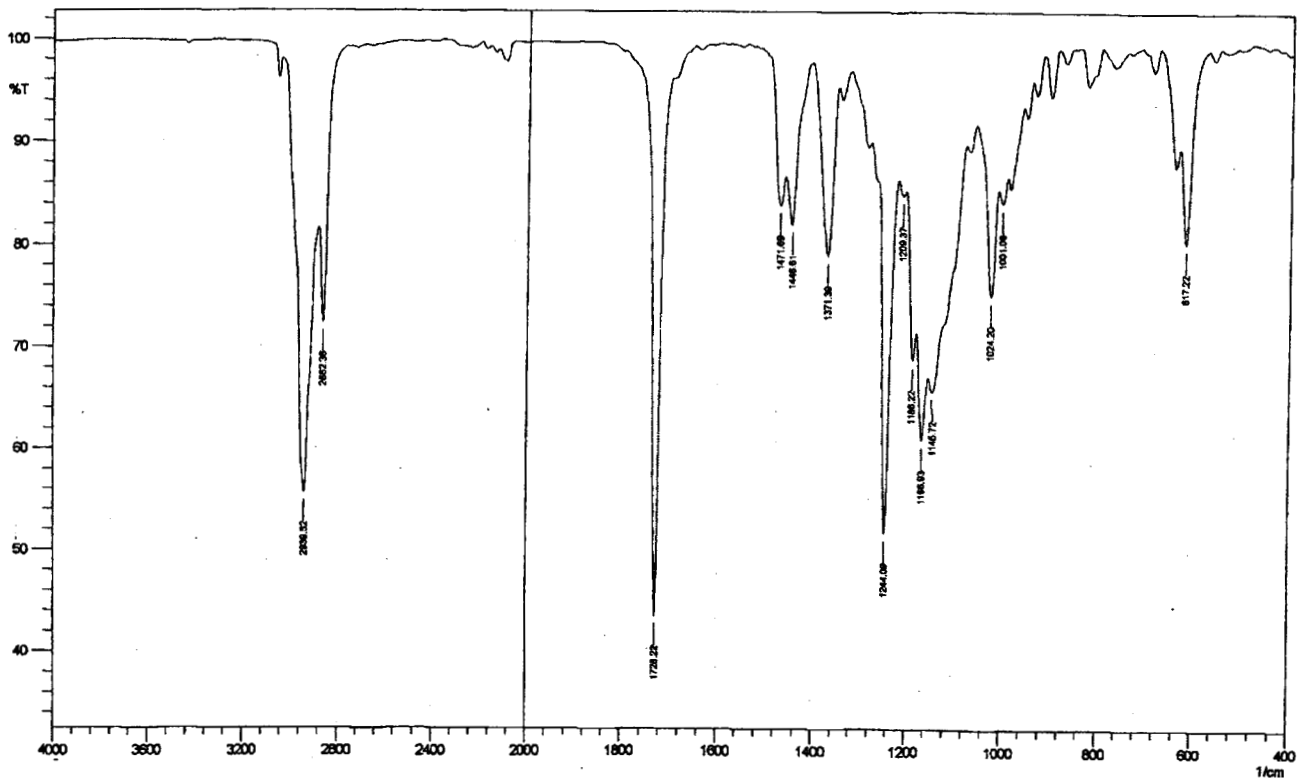
This study is the first to report this compound.



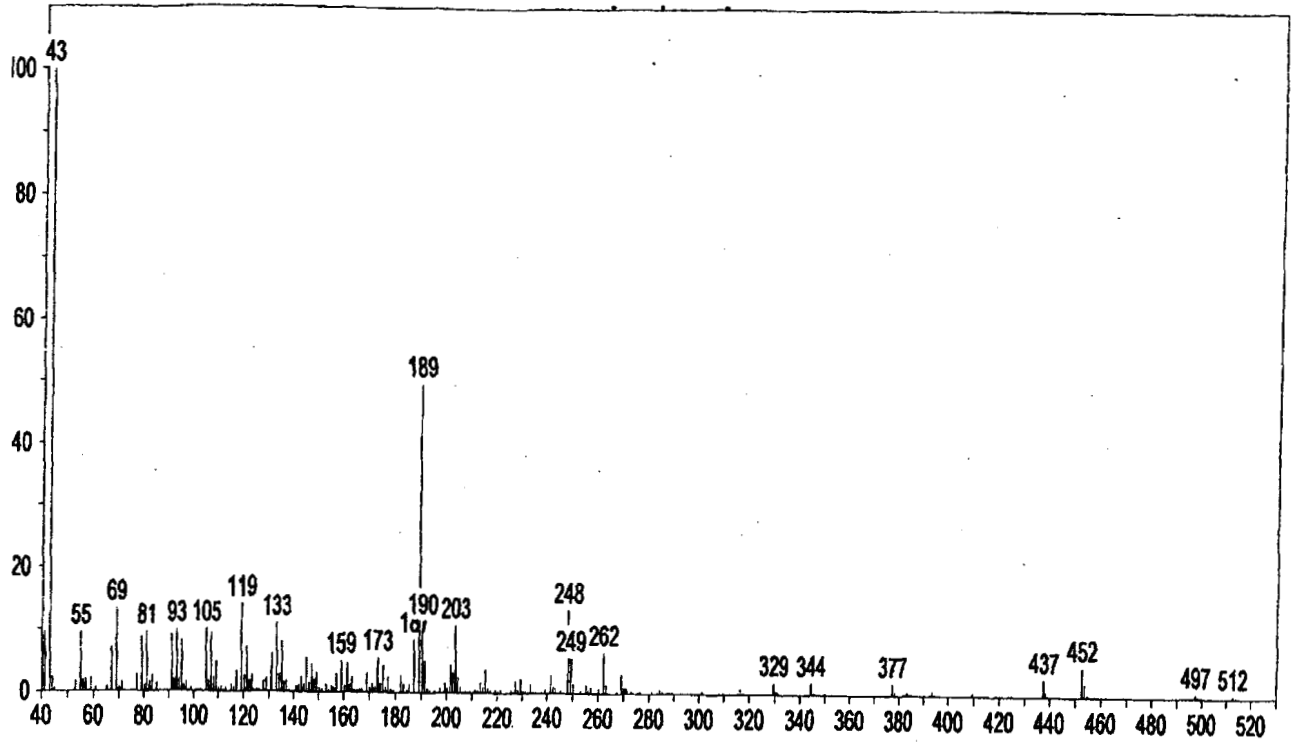
IR spectrum of aleuritolic acid [MP1a IR]



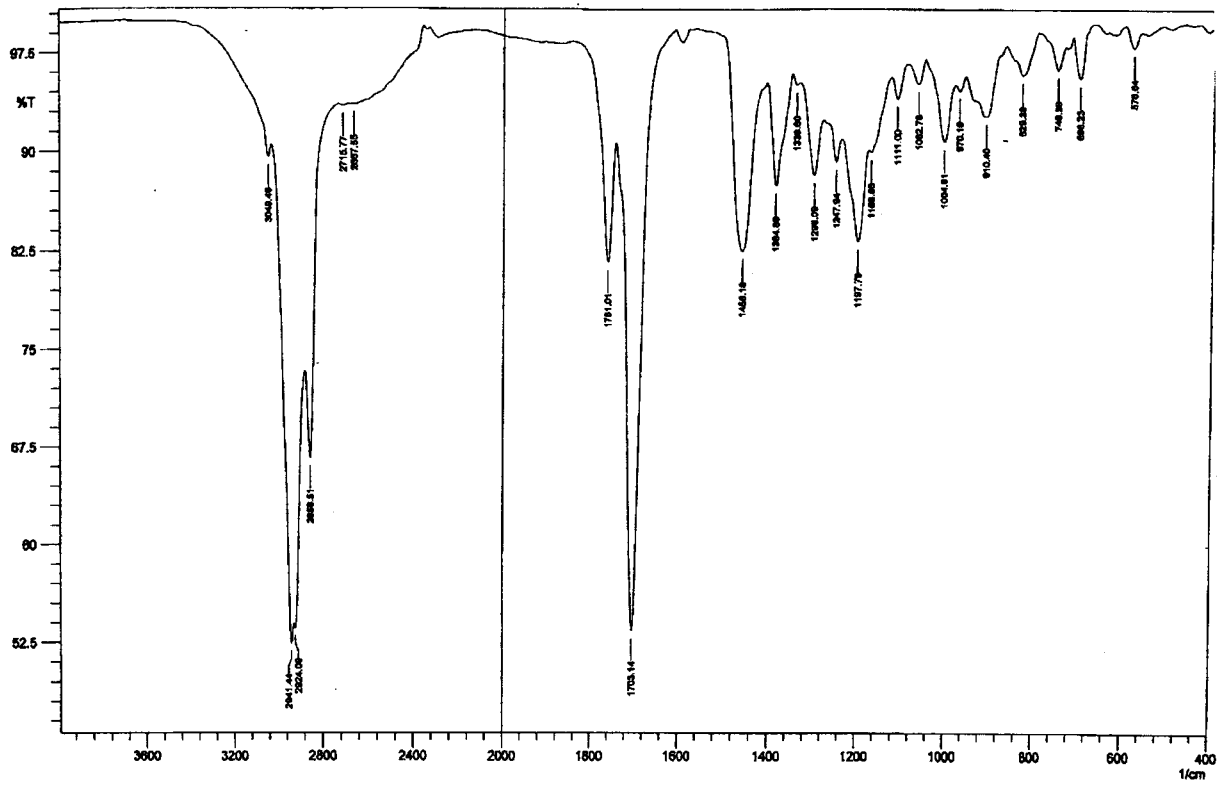
Mass spectrum of aleuritolic acid [MP1a MS]



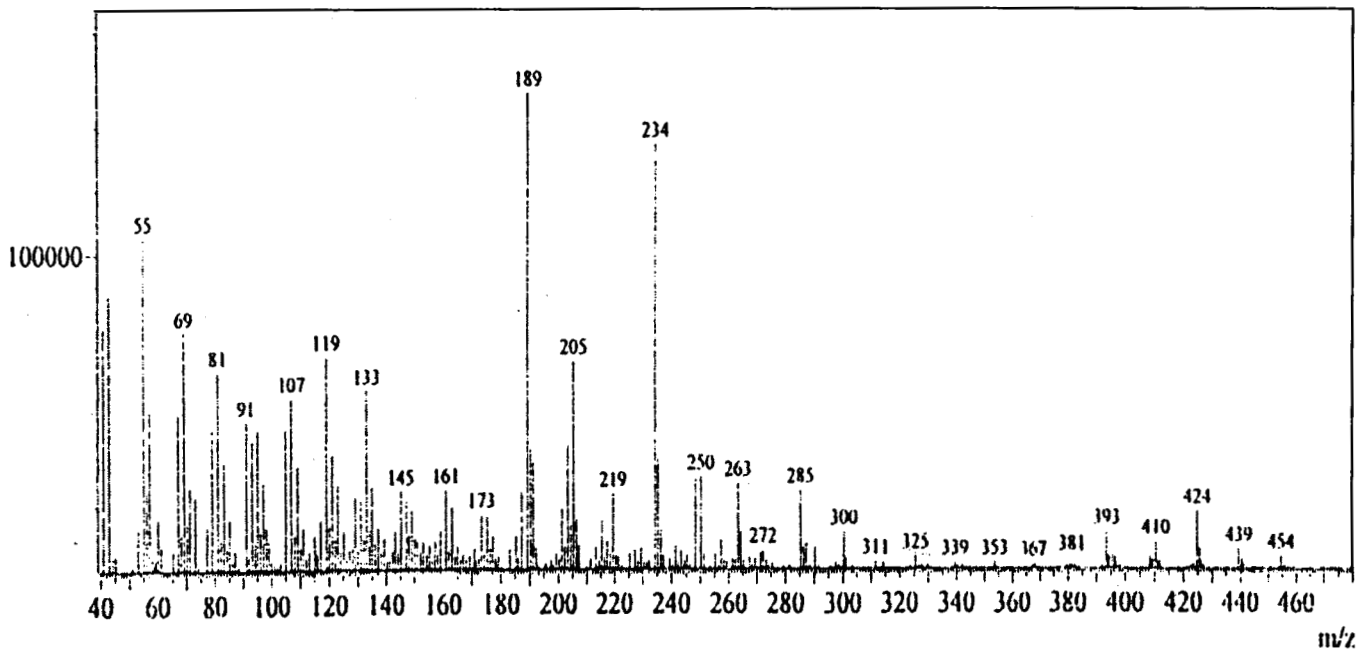
IR spectrum of 3-acetoxy methyl aleuritolate [MP1b IR]



Mass spectrum of 3-acetoxy methyl aleuritolate [MP1b MS]



IR spectrum of 3-keto derivative of aleuritolic acid [MP1c IR)



Mass spectrum of 3-keto derivative of aleuritolic acid [MP1c MS]

1.11 CONCLUSION

3-acetylaleuritolic acid had been earlier isolated and identified from the seeds of *Phytolacca americana* L. growing in Korea which belongs to a separate family Phytolaccaceae.[18] Aleuritolic acid had also been isolated from the bark of *Aleurites montana* (Euphorbiaceae)[22] in West Bengal.

A group of scientists from Andhra University[14-17] isolated a number of compounds from the heartwood and bark of *M. peltata* grown in Andhra Pradesh. The compounds include bergenin, its partial methyl ethers, β-sitosterol, cyclopeltenyl acetate, α- and β-amyrins, lupeol, betulin, n-octacosanol, 6,3',4'-trimethoxyflavanone, hexacosanol, friedelin and 2'-hydroxy, 3',4',5',6',3,4-hexamethoxy chalcone.

Neither **3-acetylaleuritolic acid** nor **3,4,3'-tri-O-methylellagic acid** could be detected in any part of *M. peltata* grown in Andhra Pradesh. The researcher of the present study could not isolate the compounds identified by them. This may be due to regional variation of secondary metabolites present in *M. peltata.*

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CHEMOTAXONOMICAL ANALYSIS OF THE ESSENTIAL OIL AROMA COMPOUNDS OF FOUR DIFFERENT OCIMUM SPECIES

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CHAPTER II

SECTION 1 : CHEMOTAXONOMICAL ANALYSIS OF THE ESSENTIAL OIL AROMA COMPOUNDS OF FOUR DIFFERENT *OCIMUM* SPECIES

II.1. GENERAL INTRODUCTION

In our daily life we come across variety of fruits, flowers, leaves, stems, barks and roots of most of the plants having some pleasant smell. It has been observed that this pleasant smell is actually due to the presence of certain highly volatile oils known as essential oils. They are so named because of their odour or essence. The natural essential oils and their aroma are perhaps the most remarkable products of plant metabolism. Younger plants produce more oils than older ones, but old plants are richer in more resinous and darker oil because of continuing evaporation of lighter fractions of the oil. The oils are stored as micro droplets in the glands of plants. After diffusing through the walls of the glands, the droplets spread over the surface of the plant before evaporating and filling the air with perfume. The most odoriferous plants are found in the tropics, where solar energy is the greatest. Climatic and topographical conditions affect plant chemistry and alter the essential oil content qualitatively and quantitatively. India is considered to be the ancient home of perfumes and aromatic plants,

because it is blessed with a wide variety of soil and climatic conditions that support the enormous plant wealth.

Essential oils are complex mixtures of hydrocarbons and their oxygenated derivatives. The main constituents of essential oils are the terpenoids (i.e., mono and sesquiterpenes) and their oxygenated derivatives such as alcohols, aldehydes, ketones, etc. Essential oils due to their pleasant smell are commercially important especially in perfumery. Moreover, various essential oils show biological activity such as insecticidal, anthelmintic and antiseptic action, so they are also useful in pharmacy[1].

II.2. EXTRACTION OF ESSENTIAL OILS

Essential oils frequently occur as a very small percentage by weight of the original plant material, so the processing of large quantities is often required to obtain considerable amount of oil[2]. In general, four methods are used for the isolation of essential oils. They are steam distillation, expression (pressing), solvent extraction and enfluerage (extraction by using fat). Of these steam distillation is the most commonly used method. At first the plant material is crushed or ground to reduce the particle size and to rupture some of the cell walls of oil bearing glands. The crushed or ground mass is then steam distilled when the essential oils go into distillate from which they are extracted using pure organic solvents like diethyl

ether. Steam distillation may change or modify the components of essential oils. Liquid carbon dioxide extraction[3] retains the true nature characteristic of essential oils and extracts all the desirable components, without drastic treatments, because of its strong solvating power and low viscosity.

The function of the essential oil in the plant is not fully understood. The odours of flowers are said to act as attractants for insects involved in pollination and thus may aid in preservation and natural selection. Leaf oils, wood oils and root oils may serve to protect against plant parasites or depredations by animals. Exudates, which contain essential oils, act as protective seals against disease or parasites, prevent loss of sap, and are formed readily when the tree trunks are damaged. Many components of essential oils are chemically active and thus could participate readily in metabolic reactions.

II.3. ECONOMIC IMPORTANCE

Essential oils have extensive applications in the field of flavour and fragrance industry. For example, mint and cinnamon are used in tooth paste, mouth wash or lozenges. Some combination of essential oils can be found in soaps, detergents, room freshners, papers, printing ink, paint, candles, condiments, floor polishes, etc.

Flavour essential oils are used in baked foods, snacks, soft drinks, liquors, sauces, gravies, salad dressings and other food products[4].

II.4. THERAPEUTIC APPLICATIONS OF ESSENTIAL OILS

The knowledge of therapeutic properties of essential oils is as old as mankind's use of plants as medicaments. Essential oils, being complex mixtures can act in different complicated ways. Therapeutic property of each essential oil is specific. Therefore individual chemicals isolated from essential oils are more often used than the oils themselves for the treatment. There are however, more and more research works on the therapeutic properties of essential oils that in many way are better than antibiotics, due to their wider spectrum of activity. There is also a very interesting phenomenon of synergic activity of two or more essential oils against bacteria in which the addition of one oil to another will increase the original bactericidal activity of individual oils. Even more interesting is the synergic activity of essential oils with antibiotics. It was found that the addition of a small quantity of oil to some antibiotic would increase the activity of the antibiotic several times[5]. Bactericidal activity of essential oils can also be applied in food preservation. Apart from the above few examples of uses of essential oils, there are so many preparations using essential oils as their constituents. "Analgetica" the pain killers contain, cinnamon, clove, eucalyptus, jasmine, mint,

sage and pepper oils. Cold remedies "Antigrippe" include basil, cinnamon, pine, rosemary and thyme.

SECTION 2 : CHEMOTAXONOMY

II.5. INTRODUCTION

Taxonomy is one of the oldest fields of biological science. Organisms, and their relationships to other organisms, have occupied man's thinking for hundreds, if not thousands of years. In order to classify, even at the most elementary level, man had to recognise organisms. To do this he has to observe, make comparisons and to some extent, integrate data, and develop generalisations therefrom[6]. Taxonomy has no data of its own. It is a synthetic science drawing upon data from such diverse fields as morphology, anatomy, cytology, genetics, cytogenetics, and chemistry[7]. Plant chemotaxonomy is one of the more fashionable and rapidly expanding areas of plant taxonomy and seeks to utilise chemical information to improve the classification of plants. In fact chemotaxonomy has many diverse and indeed ancient origins. Perhaps foremost comes the search by herbalists, and latterly by pharmacists, for drugs, which has involved the accumulation of information on the chemical content of a very wide range of plants. The statement that similar sorts of plants have, in general, similar

medicinal properties, i.e., contain similar chemicals, is at least 3000 years old, and the concept probably dates back thousands of years[8].

Although the concept of employing chemical data in systematic investigations is an old one, a genuine interest in an understanding of the possible correlations between plant constituents and classification has been relatively recent. The interest in this type of investigation has increased with the expanded data coming from biochemical, immunochemical and organic chemical research, and the development of relatively quick and simple analytical techniques. With the development of natural product chemistry, botanists and chemists have expressed the opinion that it should be possible to employ chemical constituents in helping to characterise, describe and classify taxa[9].

There are probably three main reasons for the recent rapid growth of chemotaxonomy: the development of many new techniques (notably various forms of chromatography and electrophoresis) which have made the analysis of plant products so much quicker and simpler, and which require much less plant material than formerly; the realisation that behind the universal occurrence in plants of many vital biochemical pathways there is an enormous variation between taxa in many other less vital pathways; and the current belief that evidence from as many sources as possible should be used in plant classification[8].

The contribution of phytochemistry to plant classification is enormous. Although in theory all the chemical constituents of a plant are potentially valuable to a taxonomist, in practice some sorts of molecules are far more valuable than others. Apart from inorganic compounds, which are of relatively little use, three very broad categories of compounds can be recognised: primary metabolites, secondary metabolites and semantides. Primary metabolites are parts of vital metabolic pathways, and most of them are of universal occurrence, or at least occur in a wide range of plants. The presence or absence of such compounds is therefore not of much systematic value. Secondary metabolites perform non-vital functions, and are therefore less widespread in plants. It is of course this restricted occurrence among plants which renders them valuable as taxonomic information. The most well-known groups of compound which have been utilised in this way include alkaloids, phenolics, glucosinolates, amino acids, terpenoids, oils and waxes, and carbohydrates. Semantides are the information carrying molecules – DNA, RNA and proteins. In theory, the sequence of nucleotides and amino acids in these substances should provide all the taxonomic information necessary for classification, and offer an alternative to the study of secondary metabolites, cytology, morphology, anatomy, etc., for the latter are merely manifestations of the former[8].

Most chemical substances which have been found very useful in taxonomy are therefore secondary metabolites or semantides. In the former case they are usually rather large molecules with many side groups which can be variously substituted, thus allowing a wide range of possible types of molecule, a good proportion of which is likely to be of limited occurrence. Many of them are, for this reason, the end products of metabolic pathways, or parts of short side-chains from widely distributed metabolic pathways. The greater the complexity of a molecule the greater the number of steps required for its formation and therefore the narrower its distribution – hence the greater its taxonomic value. Semantides, on the other hand, provide taxonomic data not on the basis of presence or absence, but in terms of sequences, ratios or percentages[8].

Chemosystematic classification of plants would exclude desirability of well preserved or complete specimens for study but even crushed and fragmentary plant parts could be assayed for their chemical constituents and placed accordingly in a system of classification. Such a system would be of far more utility in study of fragmentary fossil specimens which do not exhibit enough morphological and anatomical features for their placement in a system of classification[10].

II.6. CHEMOTAXONOMIC SIGNIFICANCE OF SECONDARY METABOLITES

Abbot pointed out that albuminous compounds and chlorophyll were not likely to be of much use in classification because they were necessary for the maintenance of life and presumably occurred in all species[11]. A similar idea has been expressed, in substance, more recently by Erdtman and others who noted that secondary compounds are probably more useful in systematics than basic metabolites[12].

The choice of secondary metabolites as taxonomic markers is mainly due to the fact that they are more amenable to experimentation than primary metabolites from the taxonomic point of view. The distribution of secondary metabolites in different parts of a plant may not be uniform. The secondary metabolite profile of a plant may change as the plant parts mature with age. Seasonal effects cannot also be ignored. The only way to level out such differences is to examine different batches of material collected during different seasons and to take a statistical average of the total profile[13].

Differences in the secondary metabolites of a plant may also arise due to geographic and environmental factors. For example, the leaves of *Eclipta alba* (Asteraceae) growing in and around Delhi and

other parts of Northern India contain wedelolactone as the major component; desmethyl wedelolactone is present only in small quantities. On the other hand, *E. alba* growing in South India (Bangalore, Calicut and adjoining areas) contains desmethyl wedelolactone as the major compound. This difference can be traced to the difference in the amino acid compositions of the two geographic strains; the North Indian variety is rich in methionine whereas the South Indian variety is poor in this amino acid which plays a vital role in biochemical methylation reactions[13].

Many secondary metabolites have key ecological roles to play. If, in a particular ecosystem, all the indigenous plants need protection against, for example, the insects in the environment, all of them may elaborate the same type of defensive chemical. In that sense that particular secondary metabolite having a pronounced biological action resembles the essential metabolites. This could be one of the possible reasons for the occurrence of certain secondary metabolites in different plants which are otherwise widely divergent. Several plant families specialise in producing a variety of alkaloids, and there is often a significant degree of correlation between the type of alkaloid and the plant family. Thus for example plants of the family Papaveraceae elaborate a wide range of benzylisoquinoline alkaloids. Extensive studies have shown that these compounds have

considerable taxonomic value in the delineation of relationships within this family[13].

One of the early attempts in modern times to apply knowledge of the chemistry of volatile constituents to plant taxonomy was that of Baker and Smith in Australia[14]. These two chemists surveyed essential oils of many *Eucalyptus* species and reported the fact that some, but not all, species could be readily distinguished by a particular terpene make-up. The major contribution of these authors, in historical terms, however, was not the finding that odoriferous constituents could be used to separate species, but rather the recognition that certain species could be further subdivided into 'chemical races' due to both qualitative and quantitative differences in their essential oil constituents.

Volatile patterns have been analysed in relation to taxonomy in such families as the Compositae, Hypericaceae, Labiatae, Orchidaceae and Umbelliferae. The major components of the volatile steam-distillable 'essential oil' fractions responsible in plants for characteristic odours are the terpenoids. These particular chemicals are commercially important as the basis of natural perfumes, of spices and flavourings in the food industry and of certain medicinal preparations. Compounds such as α- and β-pinene, limonene, linalool, Δ^3-carene, β-phellandrene and myrcene are often found

together in leaf oils and it is their quantitative variation which may separate one species from another. These common terpenes are closely related biosynthetically and can be formed by simple modification of the two main C_{10} terpenoid precursors, geranyl and neryl pyrophosphates. Flower and fruit oils tend to have more specialised monoterpenoids such as keto derivatives. Whatever the tissue, complex mixtures are the rule. Although there may be 10 or 15 easily detectable major components in a given essential oil, there may be a hundred or more other terpenoids in trace amounts[15].

Plant volatiles are readily separated and identified by GLC, a chromatographic procedure that produces both qualitative and quantitative data for taxonomic purposes. Although volatile fractions have been analysed in a large number of plants, the substance or substances present in the fraction which are responsible for the actual odour of a given plant are not always known and much further work is needed to relate organic chemistry to odour constitution. Nevertheless, the volatile profile of a particular plant is usually complex and is nearly always characteristic of that plant. Thus it can be employed for chemotaxonomic purposes if similar profiles are available for related taxa. The most widespread group of odour substances are the volatile terpenoids and they have already proved to be of considerable importance as taxonomic markers in gymnosperms[15].

II.7. TAXONOMIC UTILITY OF PLANT TERPENES

From the taxonomic viewpoint, essential oil studies have been mainly useful as an aid in defining the species; for detecting hybridisation in natural populations, in confirming the presence of geographical races, and in confirming generic and tribal limits[15].

Definition of the species

One simple case in *Pinus* may be taken to exemplify the general possibilities of an approach through the terpenoids. There have been difficulties in *Pinus* in determining the circumscription of certain species solely by morphology, and Mirov's investigations of oleoresin terpentines in the group have occasionally provided a key.[16] A case in point is the taxonomy of the Eastern Mediterranean pines: plants referred to as *P. brutia, P. elderica, P. pityusa* and *P. stankewiczii* have either been lumped together or sunk into *P. halepensis*. Mirov *et al.*, however, found that while *P. halepensis* (sensu strictu) contains 95% α-pinene in the oleoresin, terpentines of the other four taxa never have more than 80% α-pinene and contain in addition, 5.25% β-pinene, 10-30% Δ^3-carene and traces of nine other terpenes. The respective terpentines also differ in optical rotation, that of *P. halepensis* being dextrorotatory and those of the other four laevorotatory. These chemical differences clearly establish

the need for maintaining at least two species names for these plants and for separating *P. halepensis* as a distinctive taxon[16].

It may also be possible to use oil patterns for defining species in angiosperms. Leaf oil patterns are complex and highly species-specific. Hybridisation may occur in natural populations of conifers and detection may be difficult by morphology alone. Oil studies have helped to confirm hybrids in natural stands where two related species grow sympatrically[15]. Thus Mirov was able to recgonise hybrids between the jackpine, *Pinus banksiana* and the lodgepole pine *P. contorta* since the oil contains terpenes characteristic of both parents: α- and β-pinene in jackpine and β-phellandrene in lodgepole pine[17].

II.8. TAXONOMIC UTILITY OF TRITERPENOIDS

Triterpenoids are compounds with a carbon skeleton based on six isoprene units and which are derived biosynthetically from the acyclic C_{30} hydrocarbon squalene. They have relatively complex cyclic structures, most being either alcohols, aldehydes or carboxylic acids. They are colourless crystalline, often high melting, optically active substances, which are generally difficult to characterise because of their lack of chemical reactivity. Biologically, however, they are extremely active and some are very poisonous substances. Many triterpenes are known in plants and new ones are regularly

being discovered and characterised. So far only a few are known to be of wide-spread distribution. This is true of the pentacyclic triterpenes α- and β-amyrin and the derived acids ursolic and oleanolic acids. These and related compounds occur especially in the waxy coatings of leaves and on fruits such as apple and pear and they may serve a protective function in repelling insect and microbial attack. Triterpenes are also found in resins and barks of trees and in latex[18].

The comparative biochemistry of triterpenoids has been extensively studied not only in higher plants but also in bryophytes, algae and fungi. In many cases, taxonomic implications have been drawn from their distribution patterns. At the family level, one promising group for future chemotaxonomic exploration is the grasses, or Gramineae, since they contain a series of rare triterpene methyl ethers[18]. Ohmoto and coworkers, in a general survey of 56 species, using both seed and leaf, isolated 28 different triterpenes, many as methyl ethers belonging to the fernane or arborane skeletal types. These authors found some correlations between triterpene methyl ethers and tribal and generic limits, but it is now fairly clear that these markers are most useful at the species level[19]. Such characters have already been exploited for taxonomic purposes within the genera *Saccharum*[20]. In *Saccharum* leaf waxes, there are six triterpene methyl ethers and they have a distribution in different

clones which clearly reflect the chromosome number and geographic origin. *Saccharum officinarum* or sugar-cane is distinctive both qualitatively, in containing arundoin, and quantitatively in having higher concentrations than other species. Triterpenoid markers have also been studied at the generic level in the family Dipterocarpaceae[21].

Of the various nonvolatile terpenoids, the triterpenoids are probably the most widely used as taxonomic markers, largely because of their regular presence in most angiosperm families.

II.9. TAXONOMIC UTILITY OF FLAVONOIDS

Of all the groups of secondary metabolites used by chemotaxonomists probably none has provided more taxonomic data than phenolic compounds. These form a very loose class of compounds having in common only the fact that they are based upon phenol. Most of them are far more complicated in structure than phenol itself, having several aromatic rings and several substitution groups or side chains. The taxonomically most important phenolics are the flavonoids which have a relatively common nucleus with a great variety of types and patterns of side-groups which characterise the individual compounds. There is usually a considerable diversity of flavonoids in any one species; some of these are very widespread, others very rare, and the pattern

and combination of occurrences are on many occasions proved valuable as taxonomic evidence in the flowering plants at all levels from downwards[8].

One of the best examples of the taxonomic value of secondary metabolites concerns flower pigments. Most red, blue and similar colours of flowers and other organs indicate the presence of anthocyanidins, a particular class of flavonoid. Malvidin is a good example. The hydroxyl groups at positions 3, 5 and/or 7 are frequently substituted by a sugar (such as glucose or rhamnose) the resultant compound being known as anthocyanin. The combination of the types of anthocyanidin and types and position of sugars attached to them provides a large number of anthocyanins, which are extremely widely distributed in flowering plants (in almost all families). They are absent, however, from a few families of dicotyledons, where their function is taken over by a quite unrelated class of compounds, the betacyanins. These compounds differ conspicuously from anthocyanidins in that heterocyclic nitrogen-containing aromatic rings are present and their synthesis in the plant is along quite different metabolic pathways. Betanidin, from beet is an example. They resemble anthocyanidins, however, in that they appear to carry out the same functions and they may also be attached to sugar molecules at one of two positions. Closely related to betacyanins are betaxanthins which are yellow pigments

apparently with similar functions to various yellow or cream flavonoids, loosely known as anthoxanthins, which are present in most plants[8].

SECTION 3: CHEMOTAXONOMICAL ANALYSIS OF THE ESSENTIAL OILS FROM *O. AMERICANUM*, *O. BASILICUM*, *O. GRATISSIMUM* AND *O. SANCTUM*

II.10. INTRODUCTION

Ocimum belongs to Lamiaceae (Labiatae) family which consists of about 200 genera and 3200 species. Lamiaceae family is also known as aromatic or mint family. Economically this family is important as a source of volatile aromatic essential oils and garden ornamentals. Some of them are important for essential oils, such as salvia, lavender, rosemary and mint. In addition to many of the above, others serve as important culinary herbs valued for the flavour or aroma imparted to food[22]. The genus *Ocimum* includes at least 60 species and numerous varieties[23]. It is a versatile genus well known for not only medicinal properties but also for economically important essential oils. The essential oils are used in food, perfumery and cosmetic industries. Some *Ocimum* spp. are used in traditional medicine for different applications especially in many Asian and African countries[24]. About 11 species are recorded from India. Numerous references regarding medicinal utility of *Ocimum*

plants are available in ancient reference books like Charak Samhita, Sushruta Samhita and Indian Materia Medica[25]. Most of the species of *Ocimum* grow wild throughout the tropical and subtropical regions of the world. The recurring polymorphism determines a large number of subspecies, different varieties and forms, producing essential oils with varying chemical composition, some present a high camphor content, others are characterised by citral, geraniol, methyl chavicol, euginol, thymol, etc.[26]

The following species of *Ocimum* are reported to occur in India: (1) *Ocimum canum* Sims, Syn. *O. americanum*, (2) *O. basilicum* L., (3) *O. gratissimum* L., (4) *O. adscendens* Willd., (5) *O. sanctum* L., (6) *O. minimum* L., (7) *O. herbaceum* Rasch, (Found in Nepal), (8) *O. viscosum* Roth, (9) *O. kilimandcharicum* Guerke (10) *O. viride* Willd. and (11) *O. grandiflorum* (Orthosiphon Stamineus)[24].

Ocimum basilicum has the widest distribution covering the entire Indian subcontinent. *O. americanum* is confined to the north western part and *O. canum* to only the southern part of the Indian Peninsula. *O. gratissimum* is found mostly in the southern or eastern parts. *O. kilimandscharicum* was introduced into India from Kenya and does not have any natural range of distribution. *O. viscosum* and *O. grandiflorum* are also reported to be exotic.[22] *Ocimum* species are used as pot herbs and find diverse uses in the indigenous system of medicine in many Asian and African countries.

From the industrial point of view *Ocimum* species with oil rich in camphor, citral, geraniol, linalool, linalyl acetate, methyl chavicol, euginol, thymol, etc., are important and can be harnessed for successful utilisation in industry. Out of the chemicals mentioned above, the last four are in great demand in industry. In India the requirement of most of these are met by imports and the demand is on increase[27].

II.11. *OCIMUM AMERICANUM* L.

Ocimum americanum L. var. *americanum* (Syn. *O. canum* Sims, *O. hispidulum* Schum. and Thonn., *O. thymoides* Bak.), is very common, often found in abundance near cultivated fields and on wastelands in the plains and lower hills in most parts of India and also in Sri Lanka, Java, Western Asia and tropical Africa[28]. It is an erect much branched, sweet scented herb upto 90 cm high; stems and branches green or sometimes purplish. Leaves 2.5-5 cm long and 1-2.5 cm wide, ovate, apex acute, margins entire or faintly toothed or lobed. Flowers born in more or less closely set whorls in spicate racemes upto 20 cm long. Fruits (nutlets) 2 mm long, narrowly ellipsoid, black, pitted, mucilaginous when wet[25]. It is called 'Common basil' in English, 'Ramkasturi' in Kannada, 'Tirunitru' in Tamil and 'Kattutulasi' in Malayalam[28].

Medicinal properties and uses of *O. americanum* L.

In Ayurveda, the plant is considered stomachic, anthelmintic, alexipharmic and antipyretic; it is used to treat diseases of the heart and blood, biliousness, leucoderma and itching. In Unani medicine it is considered diuretic and emmenagogue, and is used to treat diseases of the brain, chronic joint pain, inflammation, enlarged spleen and asthma; the juice is considered useful for treating tooth ache, earache and headache, and mixed with camphor it is used to stop nosebleeds. A decoction of the plant is taken internally to relieve coughs and fever, and that of the leaves for treating dysentry, and as a mouthwash for relieving toothache. The leaves widely used for flavouring sauces, soups and salads are considered diuretic and tonic. The leaf paste is used externally for treating parasitic skin diseases. The leaf juice is a popular remedy for ring worm, and is also given to children for treating cold, catarrh and bronchitis. The plant yields a volatile oil that is used as a perfume for soaps and cosmetics[29].

Previous chemical studies on *O. americanum* L.

The essential oil obtained from *O. americanum* at the flowering stage yielded citral, methyl heptanone and methyl nonyl ketone as the major components[29]. The gas liquid chromatography of the oil showed the presence of linalool (80.46%) and caryophyllene (4.16%)

as major components. The other components were α-pinene, camphene, myrcene, 1,8 cineole, camphor, farnesene, terpineol, methyl chavicol, isoborneol acetate, geraniol, methyl euginol, humulene and γ-cadinene[30]. The seed mucilage is composed of d-galactose, d-glucose, d-mannose, l-arabinose, d-xylose, l-rhamnose, d-galacturonic and d-mannuronic acids and three polysaccharides[31].

The chloroform extract of *O. americanum* (whole plant) yielded β-sitosterol, betulinic acid, ursolic acid and two flavonoids viz., pectolinarigenin-7-methyl ether and nevadensin[32]. A new strain of *O. americanum* (methyl chavicol type) yielded on steam distillation, an essential oil which in turn gave a novel neolignan-ocimin, characterised as 1,6-bis (4'-methoxyphenyl)-(E), (E)-hexa-1,5-diene[33].

II.12. *OCIMUM BASILICUM* L.

Ocimum basilicum L. var. *basilicum*, is a small annual herb, indigenous to Persia and Sind, and is cultivated in gardens in India. Basil is native to Southern Asia and the Middle East but it has long been grown in Europe as an ornamental culinary and medicinal herb. It is grown commercially in central and southern Europe. Whole plant is aromatic; leaves and leafy tops have a pungent taste and clove-like odour[34]. It is an erect branching herb, 0.6-0.9 m high, glabrous or more or less hispidly pubescent. Stems and branches are

green or sometimes purplish. Leaves 2.5-5 cm or more long, ovate, acute, entire or more or less toothed or lobed. Flowers born in whorls densely racemose, the terminal raceme usually much longer than the lateral ones. Nutlets about 2 mm long, ellipsoid, black and pitted[35]. It is called 'Sweet basil' in English, 'Kamakasturi' in Kannada, 'Tirunirupachai' in Tamil and 'Ram tulasi' in Malayalam[34].

Medicinal properties and uses of *O. basilicum* L.

Leaves are used for flavouring purposes. Seeds are useful in catarrh, chronic diarrhoea, dysentery, gonorrhoea, nephritis, cystitis, and internal piles; they also relieve the after pains of parturition, they are used as an aphrodisiac in doses of 1 to 3 drachms; a teaspoonful of seeds steeped in a glass of water swell into a mucilaginous jelly and with some sugar forms an excellent drink in the above named diseases. Juice of the leaves is dropped into the ear in earache and dullness of hearing. Mixed with a little ginger and black pepper the leaf juice is given during the cold stages of ague. Leaves dried and powdered and used like snuff dislodge maggots from the nose[34].

Previous chemical studies on *O. basilicum* L.

The principal constituents of the oil obtained from the flowers of *O. basilicum* were methyl cinnamate and linalool. Phenols and aldehydes were not found[36]. The oil obtained from the leaves and

flowers contained α-pinene, ocimene, limonene, cineole (1,8), p-cymene, α-terpinene, methylheptanone, nonylaldehyde, linalool (77.20%), methyl chavicol (3.30%), β-farnesene, borneol, geraniol and methyl cinnamate in varying amounts[37]. In another study, gas liquid chromatography of the *O. basilicum* oil revealed the presence of linalool (59.29%) and methyl chavicol (32.68%) as major components[38].

The seed mucilage comprised d-glucose, d-galactose, d-mannose, l-arabinose, d-xylose and l-rhamnose along with d-galacturonic and d-mannuronic acids[39]. Graded hydrolysis of the mucilage yielded a polysaccharide mainly composed of glucose and mannose[40].

II.13. *OCIMUM GRATISSIMUM* L.

O. gratissimum L. var. *gratissimum* (syn. *O. guineense* Schum. and Thon., *O. suave* Willd., *O. utricifolium* Roth, *O. viride* Willd.) is a widely distributed palaeotropical species of unknown origin, found almost throughout India as a common weed by roadsides and in waste places; often cultivated[28]. It is a tall, much branched perennial shrub, generally 1.2-1.8 m tall, more strongly scented than other species of the genus; stem and branches subquadrangular, woody below. Leaves 6.3-12.5 cm long and 3.8-5.7 cm wide, elliptic-lanceolate, apex acute, base cuneate, margins coarsely crenate-

serrate, gland-dotted, pubescent on both surfaces. Flowers pale greenish yellow, borne in simple or branched racemes, moderately closely whorled. Fruits (nutlets) subglobose, 1.5 mm in diameter, rugose, brown with glandular depressions, not mucilaginous when wetted[26]. It is called 'Lemon basil' in English, 'Nimbe tulasi' in Kannada, 'Peruntulasi' in Tamil and 'Kattutrittavu' in Malayalam[28].

Medicinal Properties and uses of *O. gratissimum* L.

The plant is considered digestive, tonic, stimulant, demulcent, diuretic, anti-emetic, antiseptic and styptic. In Ayurveda the plant is used to treat skin diseases, erysipelas, inflammations and strangury. In Unani practice, the plant is considered carminative and aphrodisiac and used to treat diseases of the brain, heart, liver and spleen, to relieve piles, and to strengthen gums. In Siddha, the whole plant is used as a carminative and diuretic, and to relieve swellings, abscesses, arthritis and coughs. It is used in cough mixtures in combination with other expectorants. Aromatic baths using fumigations prepared from the plant are recommended for the treatment of rheumatism and paralysis. A decoction of the leaves is considered a useful remedy for gonorrhoea and for seminal weakness. The leaf juice is sometimes given to relieve stomach-ache. The seeds are taken to relieve headache, neuralgia and dysentery; their infusion is used to treat urinary disorders. The volatile oil extracted from the plant acts as a local anaesthetic and is used

externally to relieve inflamed joints and other chronic inflammatory conditions. The oil is also used to relieve earache, toothache and abdominal colic in children[28].

Previous chemical studies on *O. gratissimum* L.

The oil from *O. gratissimum* yielded a new sesquiterpene alcohol, gratissimol[41]. The seed mucilage contained pentoses, hexoses, uronic acids and lipids. On complete hydrolysis it yielded d-glucose, d-galactose, d-mannose, d-xylose and l-arabinose besides d-galactouronic and d-mannuronic acids[42].

II.14. *OCIMUM SANCTUM* L.

Ocimum sanctum L. (syn. *O. tenuiflorum* L.) grows all over India, and is commonly planted in front of the house in many states. The plant is considered to be sacred in India, particularly the leaves, which are used in the worship of Vishnu. In many parts of India, the plant is grown in the courtyard and worshipped daily as a necessary ritual for family well-being[34]. It is an erect much branched, strongly aromatic, softly hairy, annual herb generally upto 2 m high; stems and branches usually purplish, subquadrangular. Leaves elliptic, oblong, 2-5 cm long and 1-3 cm wide, apex obtuse or acute, base obtuse or acute, margins entire or serrate, softly pubescent on both sides, minutely gland-dotted. Flowers small, purplish or crimson, borne in close whorled racemes 15-20 cm long. Fruit a minute, sub-

globose or broadly ellipsoid, slightly compressed, dry nutlet enclosed in the enlarged membranous calyx, nearly smooth, pale brown or reddish with small black markings, mucilaginous when wet. It is called 'Holy basil' in English, 'Vishnutulasi' in Kannada, 'Tulasi' in Tamil and 'Krishnatulasi' in Malayalam[28].

Medicinal Properties and uses of *O. sanctum* L.

In Ayurveda the leaves, flowers and occasionally the whole plant are used medicinally in the treatment of heart and blood diseases, leucoderma, strangury, asthma, bronchitis, lumbago and purulant discharges of the ear. The leaf juice possesses diaphoretic, antiperiodic, stimulant and expectorant properties; it is used to treat infantile cough, cold, catarrh, bronchitis, diarrhoea and dysentery, and is applied to the skin to treat ringworm and other skin diseases, and as an ear drop to relieve earache. In southern West Bengal, a paste prepared from the leaves mixed with roots of *Calotropis procera* (Asclepiadaceae) is taken to treat menorrhagia. An infusion of the leaves is used as a stomachic for gastric disorders in children. A decoction of the root is given as a diaphoretic in the treatment of malarial fevers. The seeds are mucilaginous and demulcent and prescribed for genito-urinary disorders. The oil extracted from the leaves by steam distillation is reported to possess antibacterial and insecticidal properties, and is particularly effective as a mosquito repellent[28].

Previous chemical studies on *O. sanctum* L.

Gas liquid chromatography of the essential oil of *O. sanctum* revealed the presence of eugenol (70%) as the major constituent. Other components identified were nerol, eugenol methyl ether, caryophyllene, terpinene-4-ol, decylaldehyde, γ-selinene, α-pinene, β-pinene, camphor and carvacrol[43]. The leaves have also been reported to yield ursolic acid, apigenin, luteolin, apigenin-7-0-glucuronide, luteolin-7-0-glucuronide, orientin and molludistin[44]. In another study, the essential oil from different parts of *O. sanctum* have revealed that the leaf contained the highest percentage of oil followed by inflorescence and stem but the root was devoid of essential oil. The oil yielded eugenol, methyl eugenol, caryophyllene and some identified compounds[45]. *O. sanctum* (old) leaves contained 3.15% calcium and 0.34% phosphorus, along with 4.9% insoluble oxalate[46].

SECTION 4: ANALYTICAL TECHNIQUES

II.15. GAS CHROMATOGRAPHY

Odour or colour comparison was the early method used for the characterisation of essential oils. Specific gravity, refraction index, distillation range and iodine number were then used for characterisation. The modern methods of gas chromatography and allied techniques for identifying components, present in essential oils are highly efficient.

There are two types of gas chromatography: Gas-Solid Chromatography (GSC) and Gas-Liquid Chromatography (GLC). Gas-Liquid Chromatography is based upon the partition of the analyte between a gaseous mobile phase and a liquid phase immobilized on the surface of an inert solid. GLC finds widespread use in all fields of science, where its name is usually shortened as Gas Chromatography (GC). Gas-Solid Chromatography is based upon a solid stationary phase in which retention of analytes is the consequence of physical adsorption[47].

The principle of GC is (like other chromatographic techniques) the differential distribution of components between two phases, one stationary and the other moving. The mobile phase (carrier gas) usually used is Nitrogen. Helium, Argon and Hydrogen are also used depending upon the nature of the mixture. The mobile phase does not interact with molecules of the analyte, its only function is to transport the analyte through the column. The stationary phase may be solid or liquid. Nowadays liquid stationary phase is more in use. Some examples are:

1. Squalene (Non polar)

2. Silicone oils (poly dimethyl siloxane, poly (phenyl methyl) dimethyl siloxane) (Intermediate polarity).

3. Polyethylene glycols or PEG 400 (Highly polar).

4. Polyethylene glycol succinate PEG-S (Highly polar).

In gas chromatography the sample is vapourised and injected into the head of a chromatographic column. Elution is brought about by the flow of an inert gaseous mobile phase. The components will be coming out at different intervals due to the difference in their retention times.

Relative Retention

Retention time of a component is controlled by the distribution ratio of it in the stationary and mobile phases. i.e., the time it spends in the stationary phase.

The adjusted retention time

$$t'_R = t_R - t_M$$

Where t_R is the retention time of the (analyte) component and t_M that of the unretained compound. In the same experiment a reference compound 'S' can also be added so that its retention time.

$$t'_R(s) = t_R(s) - t_M$$

The relative retention

$$r(a,s) = \frac{t'_R(a)}{t'_R(s)} = \frac{t_R(a) - t_M}{t_R(s) - t_M}$$

The relative retention is constant at constant temperature and the same stationary phase.

Retention Indices

Gas chromatography is essentially an analytical technique commonly used for qualitative analysis by comparing the retention data of the analyte with those of the compound which it is thought to be. Simple retention times are not very reproducible and it is better to use relative retentions or retention indices. The most useful system of retention indices is the one due to Kovats[48]. It takes advantage of the linear relation between the logarithms of the adjusted retention times of a homologous series (n-alkanes) and the number of carbon atoms in the molecule. The n-alkanes are used as the reference compounds because of their stability, ready availability, low cost and wide range of boiling points. The retention of any analyte is compared with the two n-alkanes which elute nearest to it. The adjusted retention time of the analyte is measured at the same time as those of n-alkanes which elute in front and behind it (containing 'z' and 'z+1' carbon atoms respectively) and the retention index of the analyte, I is then defined by

$$I = 100\left[\frac{\log t'_R(\text{subst}) - \log t'_R(n - C_Z)}{\log t'_R(n - C_{Z+1}) - \log t'_R\ (n - C_Z)} + Z\right]$$

For n-alkanes, the term log t'_R (subst) - log t'_R (n-C_Z) reduces to zero and they have retention indices equal to the number of carbon atoms in the molecule multiplied by one hundred.

Columns

Two types of columns used in GC are packed and capillary or open tubular columns. Both types of columns are made from strong materials that are non-adsorbent and chemically inert. Stainless steel and glass are the usual materials for packed columns and quartz or fused silica for capillary columns. Capillary columns are of two basic types, namely, wall-coated open tubular (WCOT) and support-coated open tubular (SCOT). WCOT columns are simply capillary tubes coated with a thin layer of stationary phase. In SCOT columns the inner surface of the capillary is lined with a thin film ($\approx$ 30 μm) of a support material, such as diatomaceous earth.

II.16. DETECTORS

A detector, located at the exit of the separation column, senses the presence of the individual components as they leave the column. The detectors are of different types.

1. Thermal conductivity detector (TCD)

In TCD, a heated filament is placed in the emerging gas stream. The amount of heat lost from the filament by conduction to

the detector walls depends on the thermal conductivity of the gas phase.

2. Flame ionisation detector (FID)

The FID detector adds hydrogen to the column effluent. Subsequently the mixture is passed through a jet where it is mixed with external air and burned. This detector is most widely used for gas chromatography. When ionisable material from the column effluent enters the flame and is burned, the current markedly increases. The current flowing through an external resistor is sensed as a voltage drop, amplified and finally sent to a recorder. The FID responds proportionately to the number of $-CH_2$ groups introduced into the flame.

3. Mass spectrometer

Gas chromatography is often coupled with the selective techniques of spectroscopy, thus giving the so-called hyphenated methods that provide the chemist with powerful and pragmatic tools for identifying the components of complex mixtures. Gas Chromatography-Mass spectrometry (GC-MS) and Gas Chromatography-Infrared Spectroscopy-Mass Spectrometry (GC-IR-MS) are the modern analytical methods used for the separation and identification of components of essential oils. When we use GC-MS, the mass spectrometer is a universal detector for gas

chromatographs since any compound that can pass through a gas chromatograph is converted into ions in the mass spectrometer. At the same time, the highly specific nature of a mass spectrum makes the mass spectrometer a very specific gas chromatographic detector. Gas chromatography is an ideal separator whereas mass spectrometry is excellent for identification.

Sometimes a component with critical odour properties may be present in the oil at ultra trace level for which a discrete GLC peak cannot be readily assigned. In this case the use of olfactory detection involving GLC sniff runs is employed to pinpoint the elution time of the trace constituent.

II.17. GAS CHROMATOGRAPHY-OLFACTOMETRY

Aroma chemicals have two sensory odour properties namely intensity and quality[49]. They are usually hydrophobic organic compounds containing a limited number of functional groups. However the presence of functional group is not a pre-requisite for odour. The two odour properties are very difficult to measure objectively with physical instruments. Olfactometers simply generate and deliver an odorous air sample of known concentration to a human subject for assessment. The technique gas chromatography - olfactometry, more commonly referred as GC sniffing is a more useful method to check the olfactoric purity of a sample. Provided that the

GC conditions adequately separate the components of a mixture, each component can be smelt at the exit of the port of a GC column in olfactorily pure state. Repeated analysis of the same sample at successively high dilutions is a methodology commonly used to identify the components that contributed towards the overall odour of the sample. An advantage of the method is its ability to analyse minute quantities of a sample and to assess pure components. Due to the absence of a universal odour perception, sometimes the odour description from two laboratories may be slightly different.

Gel Permeation Chromatography[50] (GPC) is a useful supplementary method for the separation, identification and carbon skeleton determination of mono and sesquiterpenes of essential oils having same molecular formula but different carbon skeletons on the basis of their molecular size.

II.18. SOLID PHASE MICRO EXTRACTION (SPME)

Headspace sampling

Beyond the conventional gas chromatography analysis of gases and low viscosity liquids, some situations are more effectively handled by head-space sampling. This is true only when the vapour above the sample is of interest as with perfumes or food products. In order to obtain the concentration of the volatile components solid

phase micro extraction (SPME) is employed. More volatile components can be analysed by this method.

The SPME device consists of a fused silica fiber of about 1 cm length with a stationary phase coated on the outer surface and bonded to a stainless steel plunger and a holder that looks like a modified microliter syringe. The fused silica fiber can be drawn into a hollow needle by using the plunger on the fiber holder. Organic analytes adsorb on the phase coating the fiber. After sample adsorption, the fused silica fiber is drawn into the needle. The needle is withdrawn from the sample vial and introduced into the gas chromatograph injection, where the adsorbed analytes are thermally desorbed and delivered to a capillary GC column.

A thick phase coat is most effective for adsorbing/desorbing volatile analytes. With SPME we can achieve detection limits in the parts-per-trillion range for many volatile and non volatile compounds. In short, SPME is fast and easy and eliminates the cost and hazards associated with using organic solvents. It can be used for screening sample prior to a detailed analysis. Good precision under consistent sampling conditions also makes the technique viable in quantitative analysis. It reduces time and expense of sample concentration in analysis. Till now, the most successful applications of SPME are for analyses of volatile and semi-volatile organic compounds by coupling SPME with gas chromatography

(GC). The use of SPME in combination with GC-MS offers a very sensitive and rapid method for the determination of odour active compounds in the microgram/litre range.

II.19. PRESENT WORK

The essential oils of *O. americanum, O. basilicum, O. gratissimum* and *O. sanctum* from various geographic origin have been investigated for their composition and chemotaxonomy by many researchers with more than 250 constituents identified. To the best of our knowledge, no comparative analyses of these essential ocimum oils from southern India are reported until now. Therefore, the aim of this work is to identify the aroma compounds of the essential oils of these four *Ocimum* species from Kerala responsible for the characteristic odour of the single sample, to find-out the chemotype of each and to discuss a possible use of them for the flavouring of food products.

II.20. MATERIALS AND METHODS

Plant materials

300g of *Ocimum americanum* (whole fresh plant), 250 g of *Ocimum basilicum* fresh leaves, 270g of *Ocimum gratissimum* fresh leaves and 250g of *Ocimum sanctum* fresh leaves were collected from the area surrounding Calicut University and each species was

identified by a local botanist. The fresh plants and leaves were cut into small pieces of various size and a paste was prepared using an electric grinder.

Isolation of the essential oils

The essential ocimum oils of the homogenised products were obtained by steam distillation for 4 hrs each. The distillates were extracted with diethyl ether (2 x 100 ml) and after drying with anhydrous sodium sulphate the ether was evaporated. The yields of essential oils were as follows: *O. americanum*, 0.5 g; *O. basilicum*, 0.4g; *O. gratissimum*, 0.6g; *O. sanctum*, 0.45 g.

Solid phase microextraction

The essential ocimum oils were placed into brown 2 ml headspace vials (Supleco) and closed using w/PTFE/red rubber-silver septa with caps (Supleco). The solid phase microextraction (SPME)-needle with a 2cm-50/30 μm PVB/Carboxen/PDMS-Stable Flex coated glass fibre (Supleco) was introduced manually through the septa into the headspace of the ocimum oils in the vial. The end of the SPME-fibre was placed 1 cm over the surface of the essential oil and the volatiles trapped at room temperature for 30 min. The SPME-needle with the trapped headspace compounds on the fibre was introduced manually into the gas chromatography (GC)-injector board of the used hyphenated instruments [GC/flame ionization

detection (FID) and GC/mass spectrometry (MS)]. The SPME needle/fibre was located in the GC-injector board during the whole analysis (about 40 min each).

Gas chromatography analysis

GC/FID analysis was carried out using a GC-14A with SPME sleeve adapted to injector, FID and C-R6A-Chromatopac integrator (Shimadzu, Japan) a GC-3700 with FID (Varian, Germany) and C-RIB-Chromatopac integrator (Shimadzu). The carrier gas was hydrogen, the injector temperature 250°C and the detector temperature 320°C. The temperature programme was: 40°C/5 min to 280°C/ 5 min, with a heating rate of 6°C/min. The columns were 30 m x 0.32 mm bonded FSOT-RSL-200 fused silica, with a film thickness of 0.25 μm (Biorad, Germany) and 30 m x 0.32 mm bonded stabilwax, with a film thickness of 0.50 μm (Restek, USA). Quantification was achieved using peak area calculations, and compound identification was partly carried out using correlations between retention times[51-54].

Gas chromatography - Mass spectrometry analysis

For GC/MS measurements a GC-17A with QP 5000 (Shimadzu) a SPME sleeve adapted to injector and Compaq-Prolinea data system (Class 5k-software) a GC-HP5890 with HP 5970-MSD (Hewlett-Packard, USA) and Chemstation software on a Pentium PC

(Bohm, Austria) a GCQ (Finnigan-Spectronex, Germany-USA) and Gateway-2000-PS75 data system (Siemens-Nixdorf, Germany, GCQ-software) were used. The carrier gas was helium, the injector temperature 250°C, interface heating was at 300°C and ion-source-heating at 200°C; EI-mode was 70eV, and the scan range was 41-450 amu. For other parameters, see description of GC/FID, above. Mass spectra conclusions were done using Wiley, NBS, NIST and our own library on-line as well as using published mass spectral data off-line[52,55-57].

II.21. RESULTS AND DISCUSSION

The four essential ocimum oils were olfactorically evaluated by professional perfumers and described with the following aroma impressions. *O. americanum*: herbal-fruity, sweet-balsamic, weak camphor-and pine-notes, green-celery-side-notes; *O. basilicum*; linalool like, sweet-fruity, balsamic-spicy (direction of clover); *O. gratissimum*: intense spicy (direction of clover, eugenol-note), weak peppery; *O. sanctum*; spicy green (methyl eugenol-note with green side-notes in direction of spinach), weak oily-peppery.

Using GC and GC/MS analyses nearly **100 volatiles** were identified as constituents of the four essential oils investigated (**Table II.1**). The main compounds (concentration higher than 3.0%; calculated as percentage peak area of GC analyses using a non-polar

column) of the essential oil of *O. americanum* were (Z)-methyl cinnamate (72.05%), (E)-methyl cinnamate (9.11%), camphor (5.95%) and β-selinene (3.43%). Those of *O. basilicum* were (Z)-methyl cinnamate (34.49%), linalool (28.44%), camphor (13.08%), (E)-methyl cinnamate (6.90%) and geraniol (3.84%): Those of *O. gratissimum* were eugenol (63.36%), (Z)-β-ocimene (9.11%), germacrene D (8.84%) and β-caryophyllene (3.89%) and of *O. sanctum* were methyl eugenol (56.18%), β-caryophyllene (16.60%) and germacrene D (5.10%). In addition 11 more constituents of the 4 essential ocimum oils were identified in concentrations higher than 1.00% as follows: in *Ocimum basilicum*, terpinen-4-ol (1.33%) and T-cadinol (1.32%); in *Ocimum gratissimum*, (E, E)-α-farnesene (2.78%), α-copaene (1.39%) and δ-cadinene (1.06%), and in *Ocimum sanctum*, ledene (1.85%), β-elemene (1.73%), caryophyllene epoxide (1.10%), globulol (1.03%), aromadendrene (1.02%) and α-cadinol (1.02%). In total, 79 further compounds of the four essential oils were found in concentrations between trace (less than 0.01%) and 0.93%.

These analytical results led to the following assignment of chemotypes of the four essential ocimum oils. *Ocimum americanum* : methyl cinnamate type; *Ocimum basilicum* : methyl cinnamate/linalool type; *Ocimum gratissimum* : eugenol type; and *Ocimum sanctum*; methyl eugenol type.

TABLE II.1

Essential oil compounds of *Ocimum americanum* (Oa, whole plant), *O. basilicum* (Ob, leaves), *O. gratissimum* (Og, leaves) and *O. sanctum* (Os, leaves) in order of their Kovats indices (KI) using a non-polar column (concentrations calculated as %-peak area of SPME-GC-FID analysis)

Compounds	*Oa*	*Ob*	*Og*	*Os*	KI
Compounds C_6-C_9					
1-Hexen-3-ol	0.04	0.05	0.02	0.07	767
Hexanal	nd	0.02	0.04	0.08	773
(E)-2-Hexenal	0.01	0.06	tr	0.06	778
Hexan-2-ol	nd	tr	0.05	0.03	784
(Z)-3-Hexenol	0.08	0.35	0.20	0.54	844
(E)-2-Hexenol	0.07	0.09	tr	0.05	853
Hexanol	tr.	0.02	nd	0.07	857
(E,E)-2,4-Hexadienal	0.01	0.02	tr	0.03	876
1-Octen-3-ol	0.44	0.52	0.29	0.17	967
3-Octanol	0.02	tr	0.34	0.09	979
(Z)-3-Hexenyl acetate	0.05	tr	nd	0.04	985
(E)-2-Hexenyl acetate	tr	tr	nd	nd	994
Nonanal	nd	nd	nd	0.04	1085
Nonanol	tr	nd	0.05	0.12	1160
Nonanoic acid	nd	nd	nd	0.21	1283
α-Thujene	0.03	0.03	0.02	nd	927
α-Pinene	tr	nd	nd	nd	932
Camphene	0.04	0.02	tr	0.06	939
Sabinene	0.01	0.02	tr	0.04	967
β-Pinene	nd	tr	nd	0.03	971
Myrcene	0.03	tr	0.17	nd	983
α-Phellandrene	tr	nd	0.11	nd	998

Compounds	***Oa***	***Ob***	***Og***	***Os***	**KI**
δ-3-Carene	nd	tr	tr	nd	1004
α-Terpinene	0.03	tr	tr	nd	1010
p-Cymene	0.08	0.05	0.03	tr	1017
Limonene	0.24	0.09	tr	0.05	1026
β-Phellandrene	tr	nd	tr	tr	1028
(Z)-β-Ocimene	0.03	0.14	9.11	tr	1033
(E)β-Ocimene	tr	0.02	0.37	nd	1042
γ-Terpinene	0.13	tr	nd	tr	1054
Terpinolene	0.05	0.03	tr	nd	1079
Oxygenated monoterpenes					
1,8-Cineole	0.09	0.47	tr	0.01	1025
(E)-Sabinene hydrate	0.07	0.03	tr	nd	1059
(Z)-Linalool oxide	tr	0.08	nd	tr	1064
Fenchone	nd	0.04	0.17	0.19	1078
(E)-Linalool oxide	nd	0.08	tr	nd	1080
Linalool	0.23	28.44	0.72	0.45	1087
Fenchol	0.53	0.15	0.08	0.04	1106
Camphor	5.95	13.08	0.17	0.02	1131
(E)-Verbenol	0.12	tr	nd	tr	1139
Borneol	0.68	0.29	0.03	0.26	1163
(Z)-Verbenol	0.18	0.02	nd	tr	1165
Terpinene-4-ol	0.25	1.33	0.04	tr	1169
Myrtenal	0.02	0.16	tr	0.02	1174
α-Terpineol	0.07	0.34	0.22	0.11	1182
Nerol	0.08	0.21	0.05	tr	1216
Neral	nd	0.09	tr	nd	1227
Geraniol	0.10	3.84	nd	0.13	1241

Compounds	*Oa*	*Ob*	*Og*	*Os*	KI
Geranial	tr	0.27	nd	nd	1254
Myrtenol	0.01	0.76	0.11	0.06	1279
Thymol	0.39	0.06	nd	0.38	1284
Carvacrol	0.04	tr	0.01	0.08	1292
Sesquiterpene hydrocarbons					
δ-Elemene	tr	0.06	0.01	nd	1335
α-Cubebene	0.01	nd	nd	0.03	1351
α-Copaene	0.19	tr	1.39	0.18	1377
β-Bourbonene	nd	0.02	0.09	0.06	1386
β-Cubebene	nd	tr	nd	0.07	1388
β-Elemene	tr	0.20	0.28	1.73	1390
α-Gurjunene	nd	tr	0.11	0.25	1409
β-Caryophyllene	0.94	0.99	3.89	16.60	1422
β-Gurjunene	0.55	0.01	0.14	0.18	1429
α-Bergamotene	0.01	0.05	0.01	0.15	1436
Aromadendrene	tr	0.35	0.48	1.02	1441
Amorphene	0.14	tr	0.27	0.06	1451
(E)-β-Farnesene	0.07	0.15	0.04	0.05	1455
α-Humulene	tr	0.22	0.47	0.65	1457
*Alloa*romadendrene	0.02	0.38	0.57	0.14	1460
α-Guaiene	nd	tr	0.06	0.23	1462
γ-Muurolene	tr	0.12	0.17	0.08	1473
Germacrene D	0.29	0.18	8.84	5.10	1481
β-Selinene	3.43	nd	0.21	0.32	1485
Ledene	0.02	tr	0.08	1.85	1489
α-Selinene	0.05	0.05	0.13	0.07	1492
Bicyclogermacrene	0.06	0.09	0.23	0.01	1495

Compounds	*Oa*	*Ob*	*Og*	*Os*	KI
(E,E)-α-Farnesene	0.08	0.07	2.78	0.09	1499
α-Cadinene	0.01	tr	0.04	0.24	1503
γ-Cadinene	0.05	0.02	0.03	0.72	1511
α-Bisabolene	tr	0.05	0.08	0.13	1515
δ-Cadinene	0.23	0.06	1.06	0.21	1518
Germacrene B	nd	tr	0.36	0.42	1557
Oxygenated Sesquiterpenes					
Nerolidol	0.09	0.07	0.04	0.13	1555
Globulol	0.40	0.06	0.11	1.03	1570
Spathulenol	0.05	0.12	0.10	0.56	1573
Caryophyllene epoxide	0.01	0.11	0.14	1.10	1577
Guaiol	nd	0.02	0.09	0.16	1591
Humulene epoxide	nd	nd	0.03	0.08	1594
β-Bisabolol	tr	0.03	0.06	0.23	1617
T-Muurolol	0.01	0.03	0.09	0.28	1632
T-Cadinol	0.14	1.32	0.26	0.25	1635
Torreyol	tr	0.02	0.04	0.17	1639
α-Cadinol	0.19	0.13	0.26	1.02	1647
α-Eudesmol	tr	0.11	0.03	0.67	1649
α-Bisabolol	0.03	0.08	0.02	0.44	1675
α-Bergamotol	nd	tr	0.01	0.16	1680
Benzenoid compounds					
2-Phenylethyl alcohol	0.09	0.04	0.06	0.89	1104
Methyl chavicol	tr	0.93	nd	0.18	1182
Eugenol	1.12	0.13	63.36	1.66	1336

Compounds	Oa	Ob	Og	Os	KI
(Z)-Methyl cinnamate	72.05	34.49	nd	nd	1348
(E)-Methyl cinnamate	9.11	6.90	nd	nd	1365
Methyl eugenol	0.02	0.29	0.28	56.18	1379

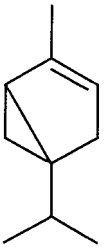
nd : not detected; tr : trace compound (less than 0.01%).

The volatiles of the investigated essential ocimum oils identified attribute to the characteristic aroma impression of the single oil in the following way. In *O. americanum* methyl cinnamates possess herbal-fruity and sweet-balsamic odour notes, camphor and some monoterpenes with pinane-structures' camphor-and pine-notes as well as hexane derivatives' green-notes and selinene derivatives' celery-notes. In *O. basilicum*, linalool and its oxides show linalool notes and eugenol as well as methyl eugenol spicy-notes with direction of clover. In *O. gratissimum* eugenol and methyl eugenol are as described above, whereas peppery-notes can be attributed to (Z)-β-ocimene, germacrene D and β-caryophyllene. In *O. sanctum*, methyl eugenol shows spicy-green-notes as well as β-caryophyllene and its oxide and germacrene D showing spicy-peppery-notes[58-61].

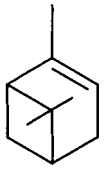
Although phenolic volatiles (e.g. eugenol) and some monoterpenes (e.g. linalool) may show irritation effects especially after application on skin and are therefore evaluated by the fragrance and flavour industry as well as by corresponding departments of the

European Commission as being able to be used only in very low concentrations[62-64], have been well known in medicine and foods for many years without showing any toxic activities on a dramatic scale. Therefore these essential ocimum oils analysed with the compositions discussed above can be used in the same way for the flavouring of foods, such as ready-made meals, soups, potato chips and similar snacks, etc., as with previously investigated essential ocimum oils from other origins without any risk to health after consumption.

Structures of the compounds identified in the essential oils of four *Ocimum* species

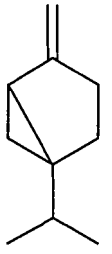


α-Thujene

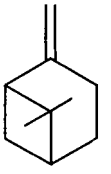


α-Pinene

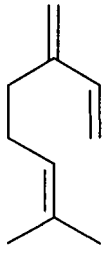
Camphene



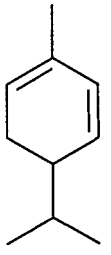
Sabinene



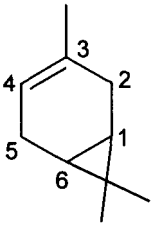
β-Pinene



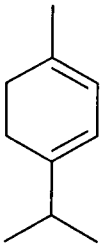
Myrcene



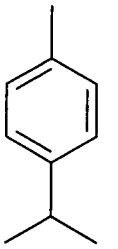
α-Phellandrene



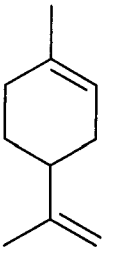
δ-3-Carene



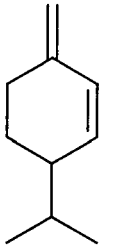
α-Terpinene



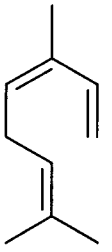
p-Cymene



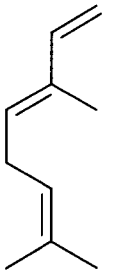
Limonene



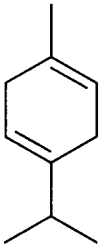
β-Phellandrene



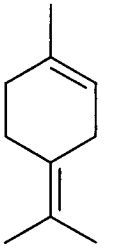
(Z)-β-Ocimene



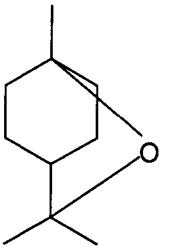
(E)-β-Ocimene



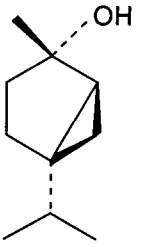
γ-Terpinene



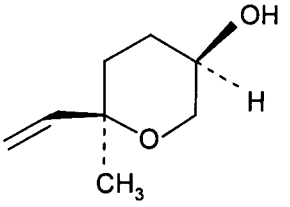
Terpinolene



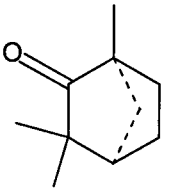
1,8-Cineole



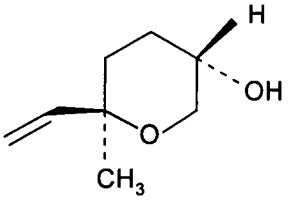
(E)-Sabinene hydrate



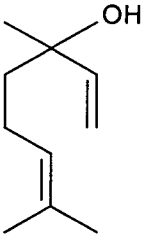
(Z)-Linalool oxide



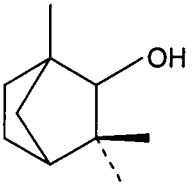
Fenchone



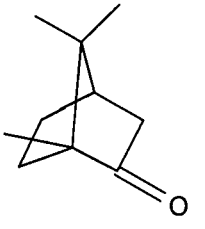
(E)-Linalool oxide



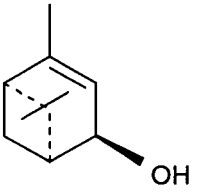
Linalool



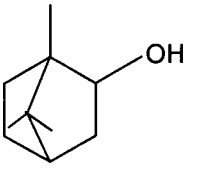
Fenchol



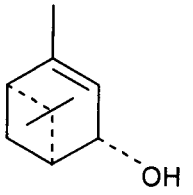
Camphor



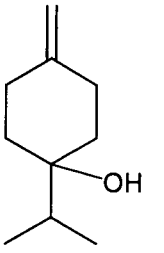
(E)-Verbenol



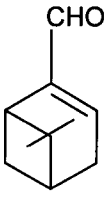
Borneol



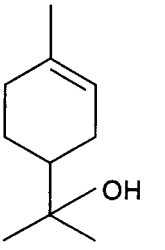
(Z)-Verbenol



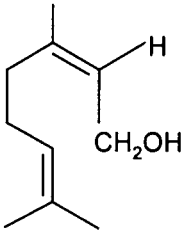
Terpinen-4-ol



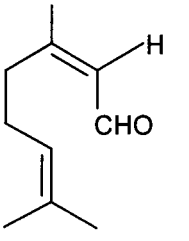
Myrtenal



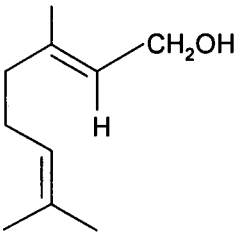
α-Terpineol



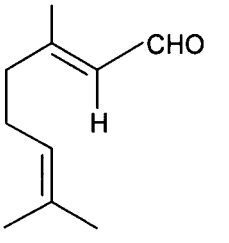
Nerol



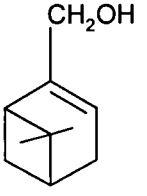
Neral



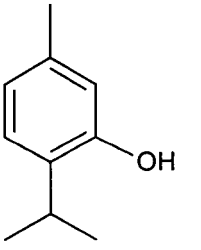
Geraniol



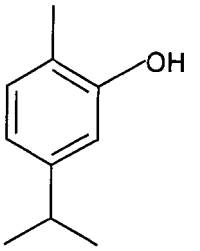
Geranial



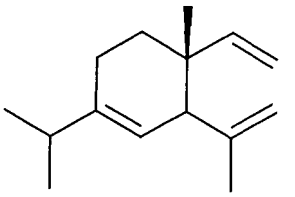
Myrtenol



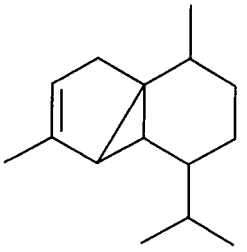
Thymol



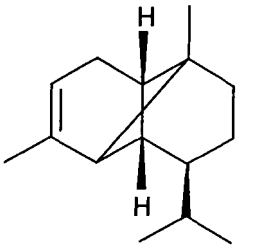
Carvacrol



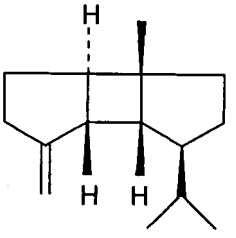
δ-Elemene



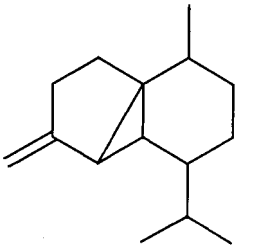
α-Cubebene



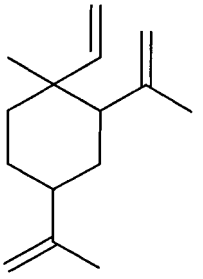
α-Copaene



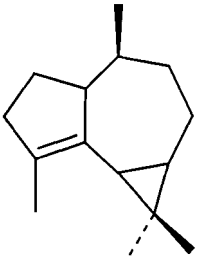
β-Bourbonene



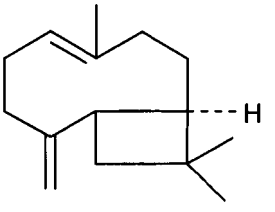
β-Cubebene



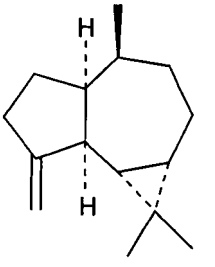
β-Elemene



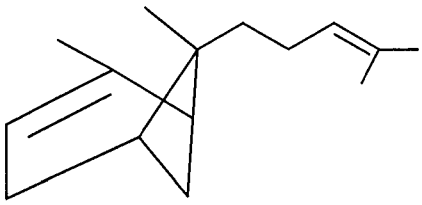
α-Gurjunene



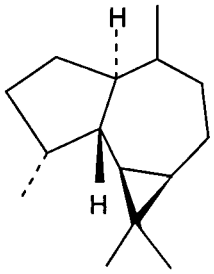
β-Caryophyllene



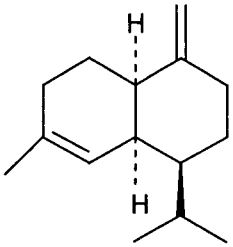
β-Gurjunene



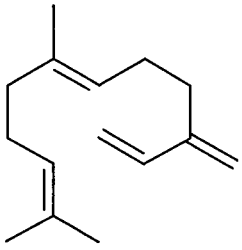
α-Bergamotene



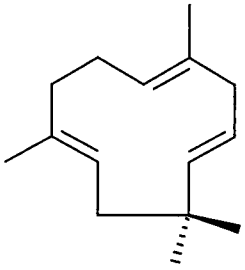
Aromadendrene



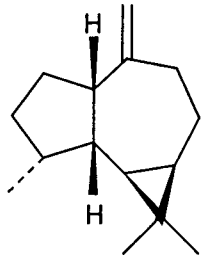
Amorphene



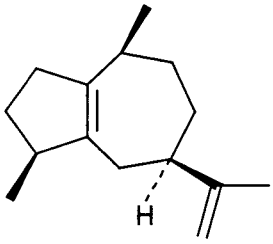
(E)-β-Farnesene



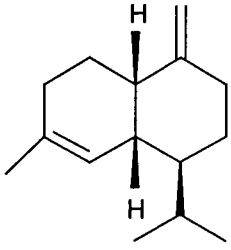
α-Humulene



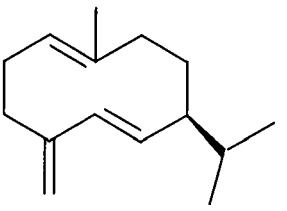
Alloaromadendrene



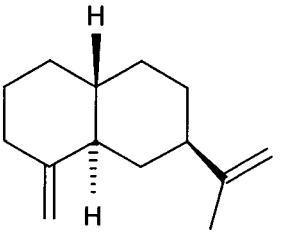
α-Guaiene



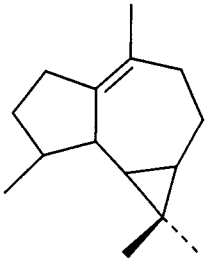
γ-Muurolene



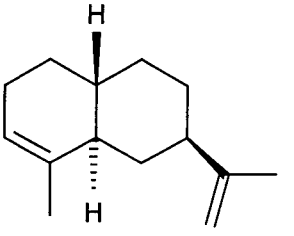
Germacrene D



β-Selinene



Ledene



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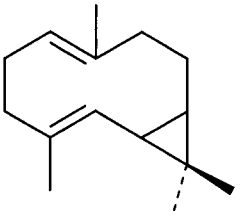
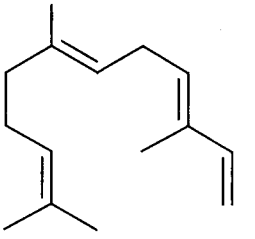
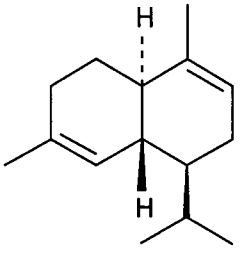


Bicyclogermacrene



(E,E)-α-Farnesene



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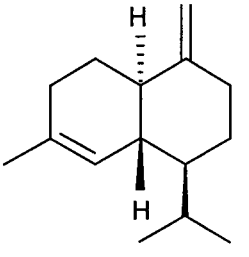

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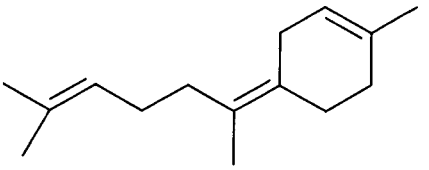

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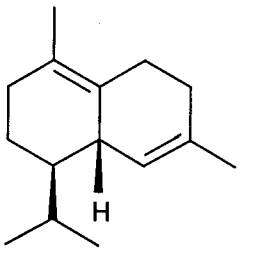
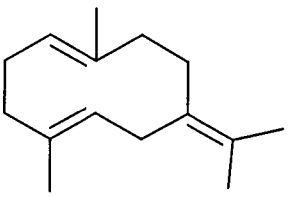
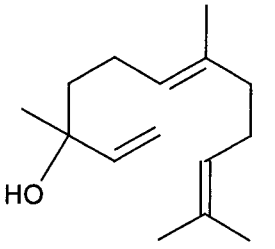


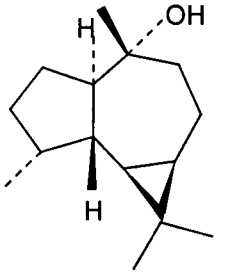
δ-Cadinene



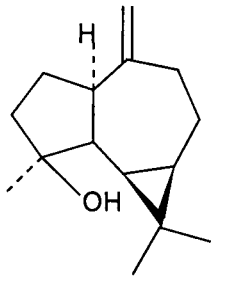
Germacrene B



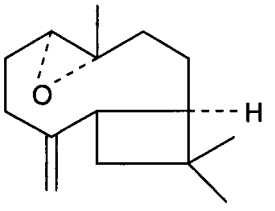
Nerolidol



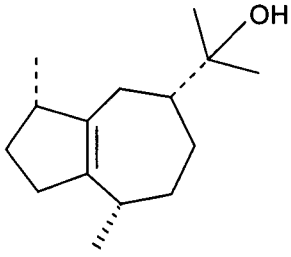
Globulol



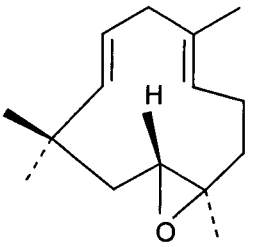
Spathulenol



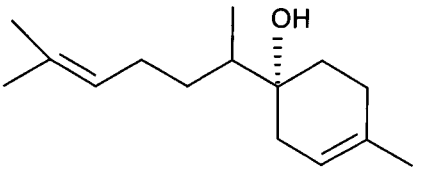
Caryophyllene epoxide



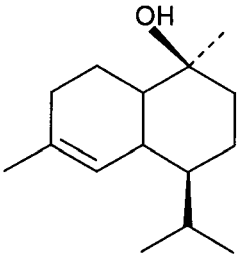
Guaiol



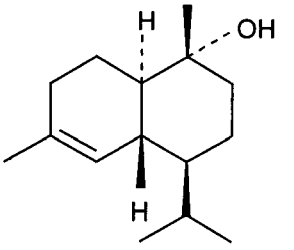
Humulene epoxide



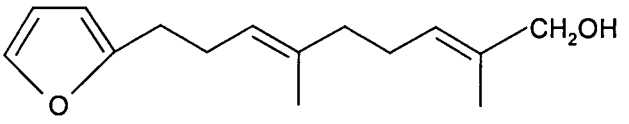
β-Bisabolol



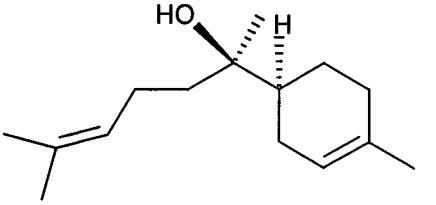
T-Muurolol



α-Cadinol



Torreyol



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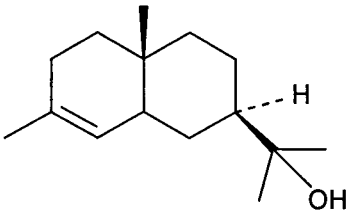
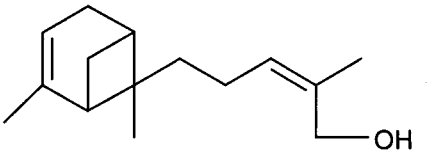
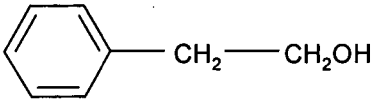


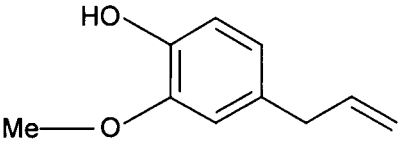
α-Eudesmol



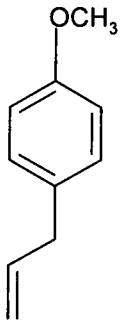
α-Bergamotol



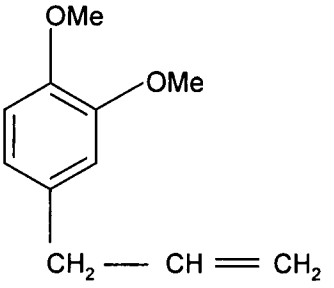
2-Phenylethyl alcohol



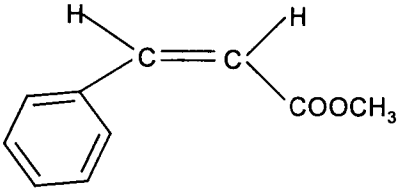
Eugenol



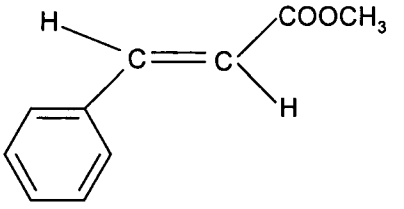
Methyl chavicol



Methyl euginol



(Z)-Methyl cinnamate



(E)-Methyl cinnamate

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ANALYSIS OF THE ESSENTIAL OIL VOLATILES OF GLIRICIDIA SEPIUM LEAVES USING GC-FID, GC-MS AND OLFACTOMETRY

Molykutty M. Kaniampady “Studies on plant metabolites” Thesis. Department of Chemistry , University of Calicut, 2006

CHAPTER III

SECTION I : ANALYSIS OF THE ESSENTIAL OIL VOLATILES OF *GLIRICIDIA SEPIUM* LEAVES USING GC-FID, GC-MS AND OLFACTOMETRY

III.1. INTRODUCTION

Gliricidia sepium (Jacq.) Steud is a tree, 3-10 m high, belongs to the Leguminous family and the subfamily Papilonaceae. The Leguminosae, generally are considered to be one of the 3 largest families of angiosperms, represented by about 550 genera and perhaps 13,000 species. The family is cosmopolitan in distribution, and is represented in India by about 115 genera, of which nearly 35 are naturalised from old world or tropical American sources. The family is divided into 3 subfamilies: Mimosaceae, Caesalpiniaceae and Papilonaceae respectively. They provide many articles of food, fodder, dyes, gums, resins, oils and in addition to this, members of over 140 genera are grown domestically for ornament[1].

Gliricidia is a small genus of trees and shrubs, native of tropical America, of which one species, *G. sepium,* is widely cultivated in the tropics as a shade and ornamental tree. *G. sepium* is a medium-sized tree with short bole, introduced into India primarily as a shade tree in plantations. The tree is grown fairly widely in parts of South India, Bihar and Uttarpradesh upto an

elevation of 3,000 ft. Leaves large, imparipinnate, with 7-15 leaflets, bright green above and pale below; flowers purplish pink or white, borne in great profusion when leaves are shed; pods linear, 4-8 cm long, compressed, containing 10 or more seeds. The tree is fairly free from pests and diseases[2].

Gliricidia sepium is the subject of intensive research because of its potential to enhance the productivity and sustainability of agricultural systems[3], and grows on the east and west coast of Mexico, South America, South India and the Philippines[4]. This legume is a tree used in Mexico as shade for cocoa and coffee plantations and for this reason it is called "Madrecacao" (Mother of cocoa). It is also used as a poison for rodents and in fact the Latin name *Gliricidia* means "rodent poison"[4,5]. It is used also as a hedge plant and the flowers are utilised as food in some places in Mexico. In the Philippines *G. sepium* is one of the best species for reforestation of denuded or grassland areas, and the leaves are used with corn as silage for ruminant feeds[4,6].

It is valued as a source of green manure for paddy in South India and has been recommended for cultivation on bunds of fields. In West Indies, it is often planted as a hedge and trimmed at intervals of 6-8 weeks during the rainy season. The tree is quick-growing and may be propagated by seeds or cuttings. Propagation by cuttings is preferred since seeds are liable to insect attacks and are

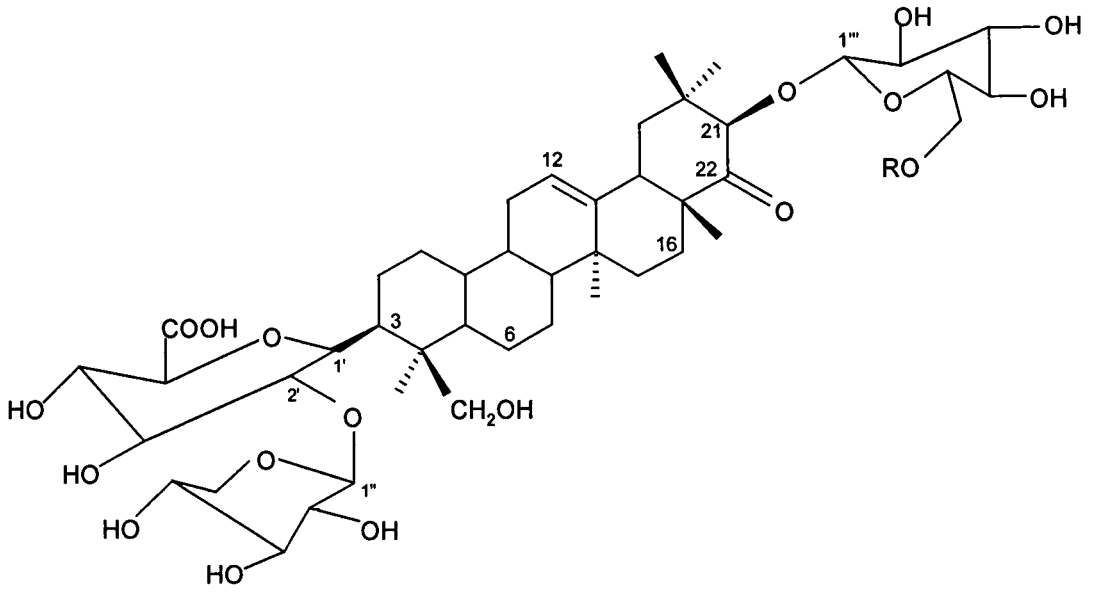
not easy to obtain. The flowers are used as vegetable in Philippines and in Central America[2]. In Panama, decoction of leaves used in utricaria, rash and also in burns and erysepalas[7]. In Guatemala and Costa Rica, the bark decoction is used against protozoan diseases and for the treatment of impetigo and other skin diseases[8]. The wood is durable and is used for house posts, fences, stakes and railway cross-ties[9]. The leaves are used as source of ethylene for advancing the ripening of some fruits[10].

Gliricidia leaf mulch effectively controls the weeds in sorghum fields and increases the crop yield. The leaf extract exhibits lethal effect on the nematode, *Radopholus similis* (Cobb) Thorne. The chopped leaves (10 g/kg soil) when used as green manure, reduce the population of *R. similis* and promote the growth of black pepper under pot conditions[11]. The digested residue, when used as a mulch, increases the level of NPK of the soil and is more suitable to use as fertilizer in comparison to nondigested leaves[12]. Several papers refer to this plant as a nitrogen-fixing tree[13-17].

G. sepium is used both medicinally and for cattle feeding on the Pacific coast of Mexico, Central America, and in tropical regions of South America and Asia[18].

III.2. PREVIOUS WORK

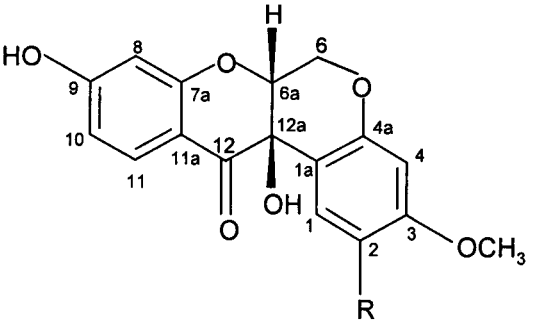
Whetton and co-workers[19] determined the usefulness of this species for feed and feed supplement purposes through the *in vitro* study of degradation products of both crude leaf and soluble protein extracts of the leaf by rumen microbes. Two Oleanene glycosides (1, 2) were isolated[20] from the roots and a series of known aromatic compounds from the leaves. These compounds possess 3β, 21β, 24-trihydroxy-22-oxoolean-12-ene as an aglycon.



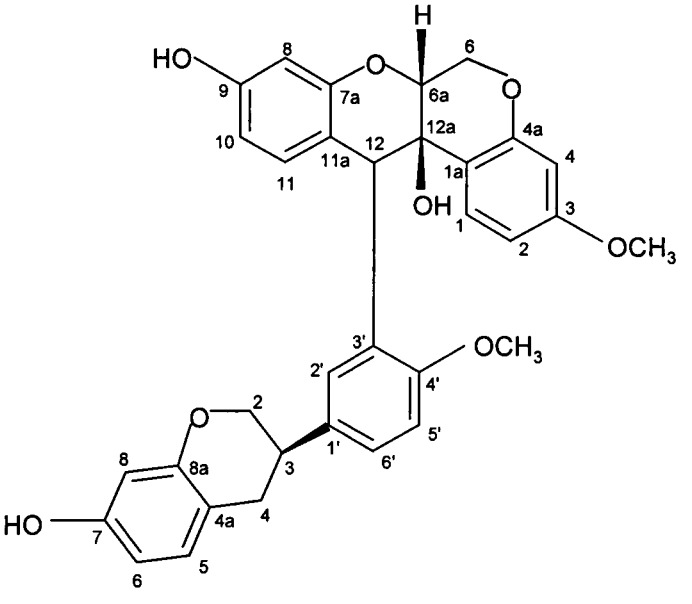
1. R=H
2. R = $COCH_3$

Investigation of methanolic extract of *Gliricidia sepium* bark by Rastrelli and co-workers[21] afforded, in addition to vestitol and 2'-0-methylvestitol, three other 12a-hydroxyrotenoids; gliricidol (1), 2-methoxyglyricidol (2), and gliricidin (3). The structures of 1-3 were

elucidated by analysis of their spectroscopic data. Compounds 1-3 exhibited activity against *Artemia salina* larvae.



1. R = H, gliricidol
2. R = OCH_3, 2-methoxygliricidol



3. Gliricidin

Kojima and co-workers[22] isolated hederagenin-based acetylated saponins from the fruits of *Gliricidia sepium* and were identified by chemical and spectroscopic methods as hederagenin-3-

O-(4-O-acetyl-β-D-xylopyranosyl)-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside, hederagenin-3-O-(3,4-di-O-acetyl-β-D-xylopyranosyl)-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside and hederagenin-3-O-(3,4-di-O-acetyl-α-L-arabinopyranosyl)-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside.

Herath and co-workers[23] isolated an isoflavan 7,4'-dihydroxy-3'-methoxyisoflavan from the insecticidally active hot dichloromethane extract of the heartwood of *Gliricidia sepium*, along with the three other isoflavonoids, isovestitol, formononetin and afrormosin, a pterocarpan, medicarpin and 4-hydroxy-3-methoxy-cinnamaldehyde.

Manners and Jurd[24] isolated three flavonoid constituents: gliricidin, sepinol and gliricidol together with (-)-isomucronulatol from *G. sepium* and their structures were determined from chemical and spectral data.

Rangaswami and Iyer[25] reported the presence of coumarin, o-coumaric acid, melitolic acid and rhamnogalactoside of kaempferol in the leaves of *Gliricidia sepium*. Nair and Subramanian[26] isolated quercetin-3-glucoside from the flowers of *G. sepium*.

Rameshwar Dayal[27] isolated the compounds: astragalin, trifolin, robinin and sucrose from the alcoholic extract of the dried flowers of *G. sepium*. Jurd[28] isolated sepiol together with robinetin

from the heartwood of *G. sepium* and the structure of sepiol determined by spectral and chemical means.

III.3. PRESENT WORK

This work was initiated on the first hand information that the leaves of *G. sepium* are being used as a fumigant to repel mosquitoes in some parts of Kerala, India. The same has also been presented as exhibits in school science fares leading to its popularisation. The objective of the present work was to characterise the volatiles present in the leaves of *G. sepium* and to assess its safety as a fumigant.

III.4. EXPERIMENTAL

The fresh leaves of *G. sepium* were collected from the neighbouring village of Calicut University campus in December 2004. The plant material was identified by Dr. A.K. Pradeep, Department of Botany, Calicut University, Kerala.

Essential oil extraction

The fresh leaves (2 kg) of *G. sepium* were cut into pieces and ground by means of an electric grinder, into paste, which was steam distilled for 2 hrs. The distillate was extracted with diethyl ether (2 x 100 ml) and dried over anhydrous sodium sulphate. After evaporation of the solvent, 0.56 g (0.02% of the fresh weight) of

colourless essential oil was obtained. On keeping the essential oil overnight, a white crystalline solid (GS1) was separated.

Olfactoric evaluations

Olfactometric study enabled the identification of the compounds responsible for different odour exhibited by it. The essential oil was diluted with dichloromethane, 10 μl placed on a commercial odour strip (Dragoco Co.) and its odour characterised by professional perfumers.

Gas chromatography - Mass spectrometry

The GC-MS analysis was carried out by using a Shimadzu GC-17A with QP 5050 and the data system compaq-prolinea (class 5 k-software), Hewlett-Packard GC-HP 5890 with HP-5970 MSD and PC-Pentium (Böhm Co; Chemstation-Software) and Finnigan MAT GCQ with data system Gateway-200-PS75 (Siemens Co., GCQ-software). An apolar 30 m OV-1-type column (0.32 mm i.d. and 0.25 μm film thickness) and helium as carrier-gas was used. Injector temperature: 250°C; interface heating: 300°C; ion source heating: 200°C, EI-mode; scan range: 41-450 amu. For compound identification Wiley - NBS- and NIST- library spectra (on line) as well as reference MS-spectral data were used[29, 30].

GC-FID analyses were carried out using a Shimadzu GC-14A with FID and the integrator C-R6A-Chromatopac and a varian GC-3700 with FID and the integrator C-RIB-Chromatopac (Shimadzu Co.). The same column used for GC-MS was also used for GC-FID. Carrier gas: hydrogen; injector temperature was at 250°C and detector temperature at 320°C; temperature – program: 40°C/5 min to 280°C/5 min with a heating rate of 6°C/min. Quantifications were made by relative % peak-area calculations.

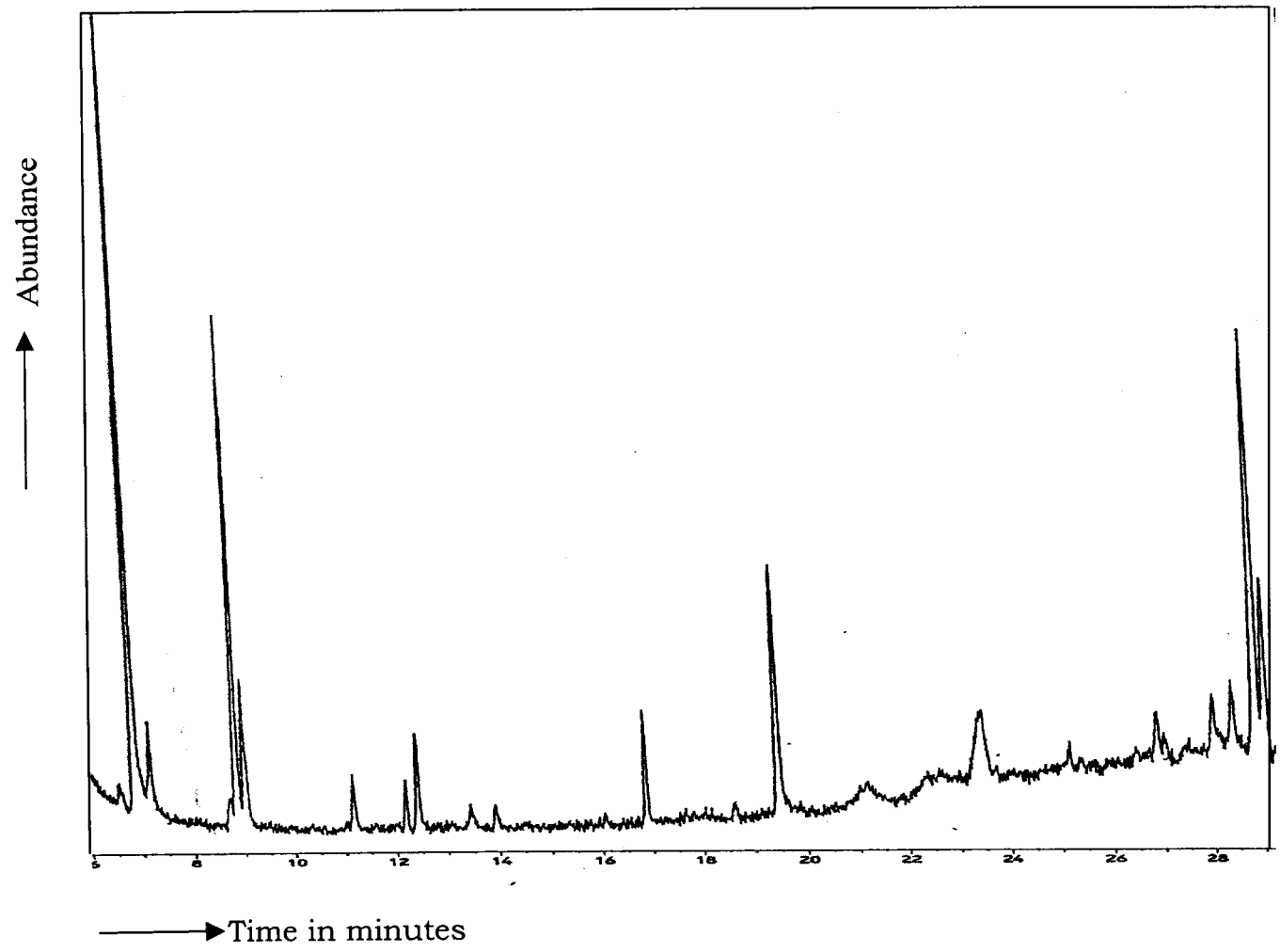
III.5. RESULTS AND DISCUSSION

The essential oil of the leaves of *G. sepium* was olfactorically evaluated as smoky, leather - and tobacco-notes, fatty-herbal, week green- (hay and grass), floral-and spicy-side-notes.

Using Gas Chromatographic spectroscopic systems **16 compounds** were identified in this sample with propyleneglycol (25.1%), coumarin (18.2%), (Z)-3-hexenol (17.7%), β-farnesene (14.2%), (E)-2-hexenol (6.5%), thymol (3.6%) and benzyl alcohol (3.5%) as main compounds (concentrations higher than 3% calculated as percentage peak area using GC-FID with a non polar column).

The leaves are used in some parts of Kerala as a mosquito repellent, by fumigation. This work shows that the volatiles contain coumarin as one of the major components. As coumarin is a known

toxic chemical[31] the fumigation of *G. sepium* leaves as a mosquito repellent pauses serious health risk and should be avoided. The volatiles contain more than 25% of propyleneglycol. This is the first report on the isolation of propyleneglycol from a natural source as per available references. The compounds identified and the corresponding concentrations (%) are given in **Table III.1**.



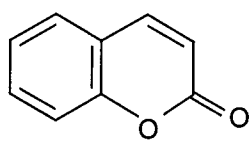
Gas chromatogram of *Gliricidia sepium* leaf essential oil

TABLE III.1

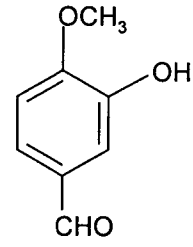
Essential oil composition of fresh leaves of *G. sepium*

Compounds	Percentage
Propyleneglycol	25.1
Coumarin	18.2
(Z)-3-Hexenol	17.7
β-Farnesene	14.2
(E)-2-Hexenol	6.5
Thymol	3.6
Benzyl alcohol	3.5
Caryophyllene	2.3
α-Farnesene	2.0
2-Penten-1-ol	<1
Iso-vanillin	<1
Iso-butyl alcohol	<1
Phenylethyl alcohol	<1
Phenol	<1
Crotonic aldehyde	<1
5,6-dihydro-4H-cyclopenta-(b)-furan	<1

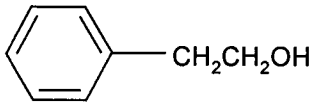
Structures of the identified compounds which are not included in previous chapters are as follows:



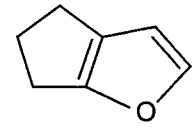
Coumarin



Isovanillin



Phenylethyl alcohol



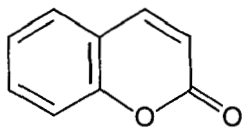
5,6-dihydro-4H-cyclopenta-(b)-furan

III.6. CHARACTERISATION OF GS1

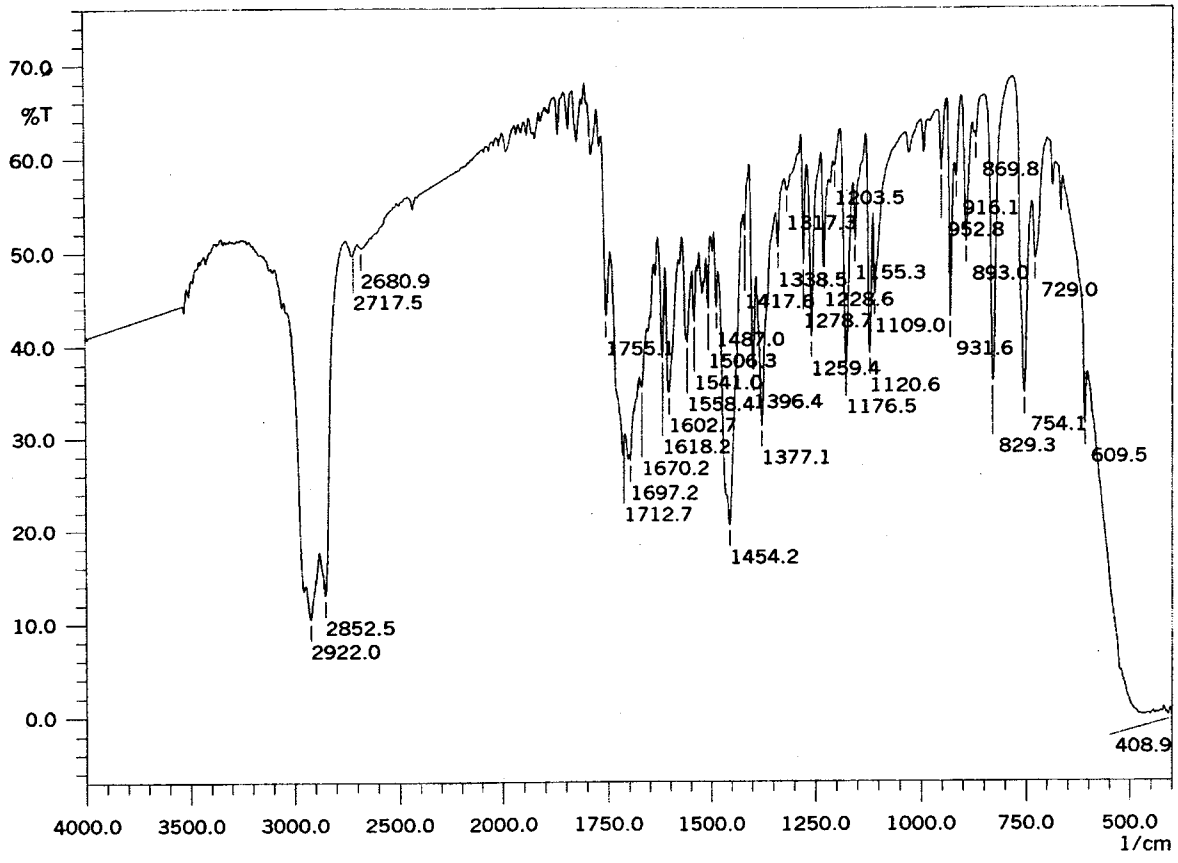
The compound isolated from the leaf essential oil of *G. sepium* by keeping the essential oil overnight was recrystallised from methanol. It was a white crystalline solid having m.p. 69°C (**GS1**). It moved as a single spot on TLC in petroleum ether and ethyl acetate (4:1). IR spectrum (GS1 IR) showed absorption due to >C=O group. A strong absorption at 1712.7 cm^{-1} suggested the presence of >C=O group. The compound GS1, absorbed in the ultraviolet light: λ_{max} 273.9 nm (95% ethanol).

Mass spectrum [GS1 MS] of this compound showed M^+ at m/z 146 and base peak at m/z 118 corresponding to coumarin as already

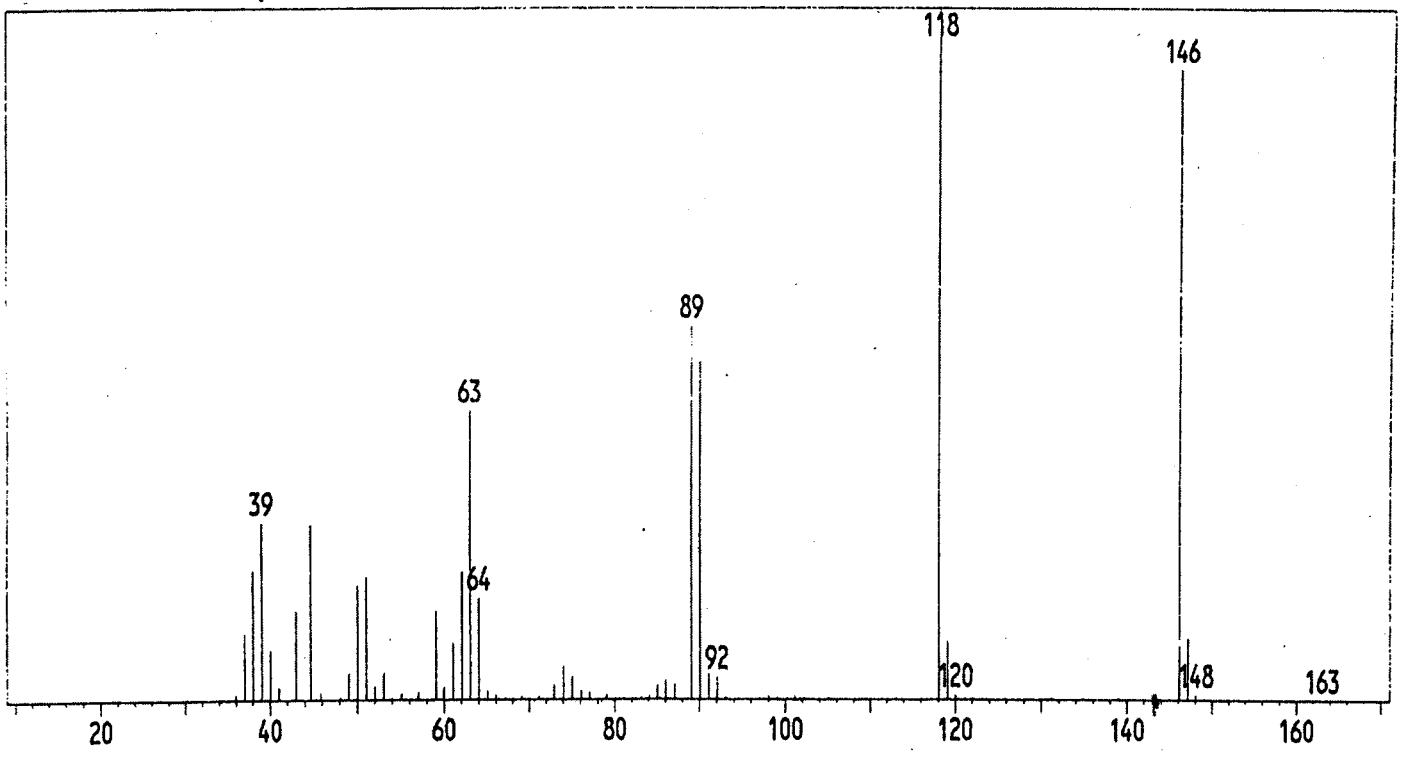
identified by GC-MS. Other fragment ions were at m/z 91, 89, 64, 63, 51 and 39. Its m.p 69°C (Lit. 70°C)[32] and λ_{max} 273.9 nm (Lit. 274.5 nm)[33] were quite comparable to that of **coumarin.**



Coumarin



IR spectrum of Coumarin [GS1 IR]



Mass spectrum of Coumarin [GS1 MS]

SECTION 2 : ANALYSIS OF THE ESSENTIAL OIL VOLATILES OF *G. SEPIUM* FLOWERS USING GC-FID & GC-MS

III.7. PRESENT WORK

Most of the previous chemical investigations have focussed on the isolation of potential allelopathic and toxic compounds from the heartwood, leaves and roots of *G. sepium*. So far no data about the volatiles from *G. sepium* flowers has been published. In the present study, the volatile compounds of the essential oil of fresh flowers are analysed by GC-FID and GC-MS.

III.8. EXPERIMENTAL

Plant material

Fresh flowers of *G. sepium* were collected from Calicut University Campus in December 2004 and the material was identified by Dr. A.K. Pradeep, Department of Botany, Calicut University.

Essential Oil Extraction

The fresh flowers (1.5 kg) of *G. sepium* were cut into small pieces and ground to a paste using an electric grinder. It was then subjected to steam distillation for 3 hrs. About 2L of the distillate were collected and extracted with diethyl ether (3 x 100 ml). The

ether portions were pooled together and dried over anhydrous sodium sulphate. Evaporation of the dry ether extract after the removal of sodium sulphate, over a waterbath yielded 0.41 g (0.02% of fresh weight of the sample) of colourless viscous essential oil. The next day, a white crystalline solid (GS2) was separated from the essential oil.

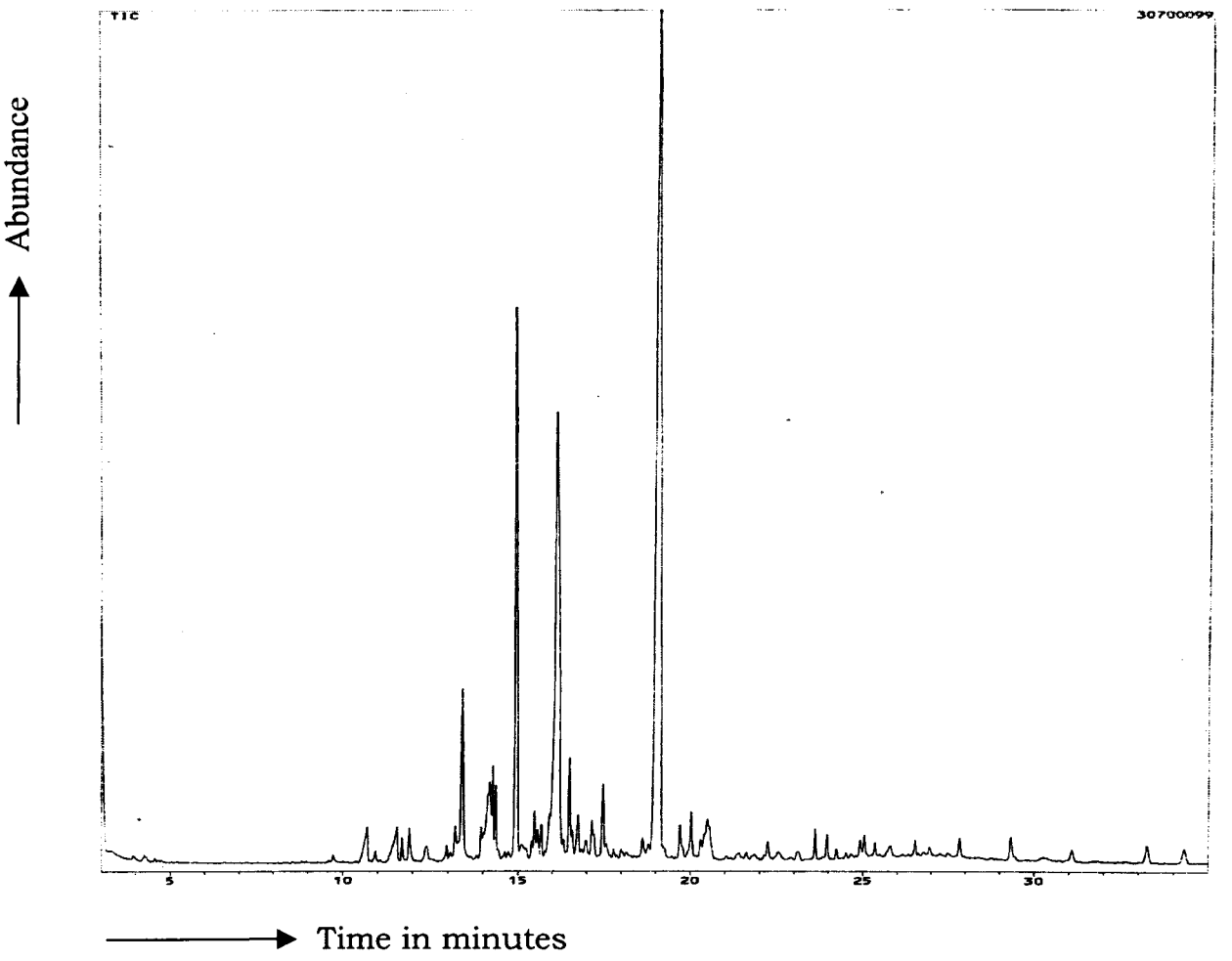
GC-FID and GC-MS

GC-FID and GC-MS conditions were exactly the same as those given in section 1.

III.9. RESULTS AND DISCUSSION

Using Gas Chromatographic spectroscopic systems **26 components** were identified in this sample with coumarin (43.07%), hydroquinone (21.64%), myrtenol (12.73%) and maltol (4.42%) as main compounds (concentrations higher than 3% calculated as % peak area using GC-FID with a non polar column).

It is reported[2] that these flowers are used as food material in Philippines and in Central America. The high percentage of hydroquinone, a topoisomerase II poison[34] and coumarin, a known toxic chemical[31] in the flowers makes it an unhealthy food material. The compounds identified and the corresponding concentrations (%) are given in **Table III.2**.



Gas chromatogram of *Gliricidia sepium* flower essential oil

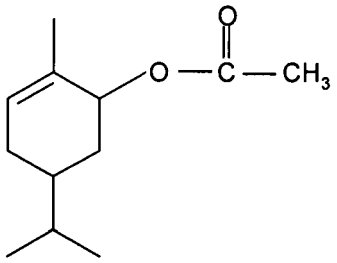
TABLE III.2.

Essential oil composition of fresh flowers of *Gliricidia sepium*

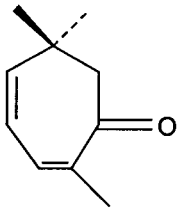
Compounds	Percentage
Benzyl alcohol	0.35
Nonanol	0.62
Maltol	4.42
3-Nonanol*	1.50
2-Octanoic acid*	1.26
2-Butyl-2-hexanol	1.46
Octanoic acid	1.53
2-Butyl-3-hexanol	1.03
Myrtenol	12.73
Dihydrocarveol acetate*	0.30
Eucarvone	0.88
Geraniol	0.72
Nonanoic acid	0.55
Myrtenal	0.78
Hydroquinone	21.64
p-Mentha-1,8-dien-9-ol	1.83
4-Hydroxy-3-methyl acetophenon*	0.37
p-Mentha-1,4-dien-2-ol	0.73
p-Mentha-1, 4-dien-7-ol	0.71
Decanoic acid	0.31
γ-Nonalactone	1.31
Coumarin	43.07
Allyl tiglate*	0.44
Dodecanoic acid	0.64
Tetradecanoic acid	0.46
3-Tetradecanoic acid*	0.36

* Tentative identification

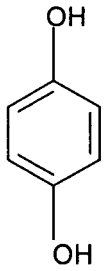
Structures of the identified compounds which are not included in previous chapters are as follows:



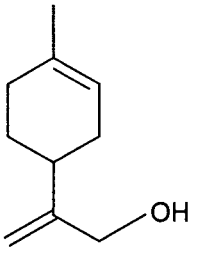
Dihydrocarveol acetate



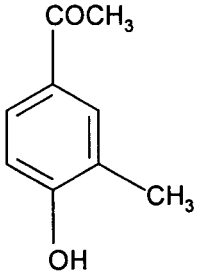
Eucarvone



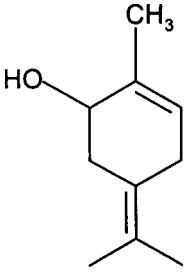
Hydroquinone



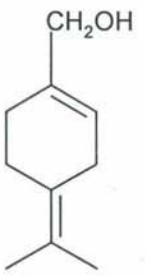
p-Mentha-1,8-dien-9-ol



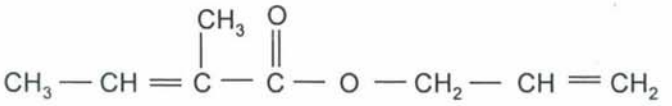
4-Hydroxy-3-methyl acetophenone



p-Mentha-1,4-dien-2-ol



p-Mentha-1,4-dien-7-ol



Allyl tiglate

III.10. CHARACTERISATION Of GS2

The compound isolated as described in section III.8 gave two spots on TLC in petroleum ether and ethyl acetate (4:1). Comparative TLC with coumarin proved the identity of one of them as coumarin. The other compound was separated by using preparative TLC (petroleum ether: ethyl acetate; 4:1). The lower band on extraction with methanol gave a white crystalline solid having m.p. 171°C [**GS2**]. Its IR spectrum [GS2 IR] showed absorption due to hydroxyl group. Two strong absorptions at 3188.1 and 3263.3 cm^{-1} respectively suggested the presence of two hydroxyl groups.

Mass spectrum [GS2 MS] of this compound showed M^+ at m/z 110 and the same is the base peak. This corresponds to

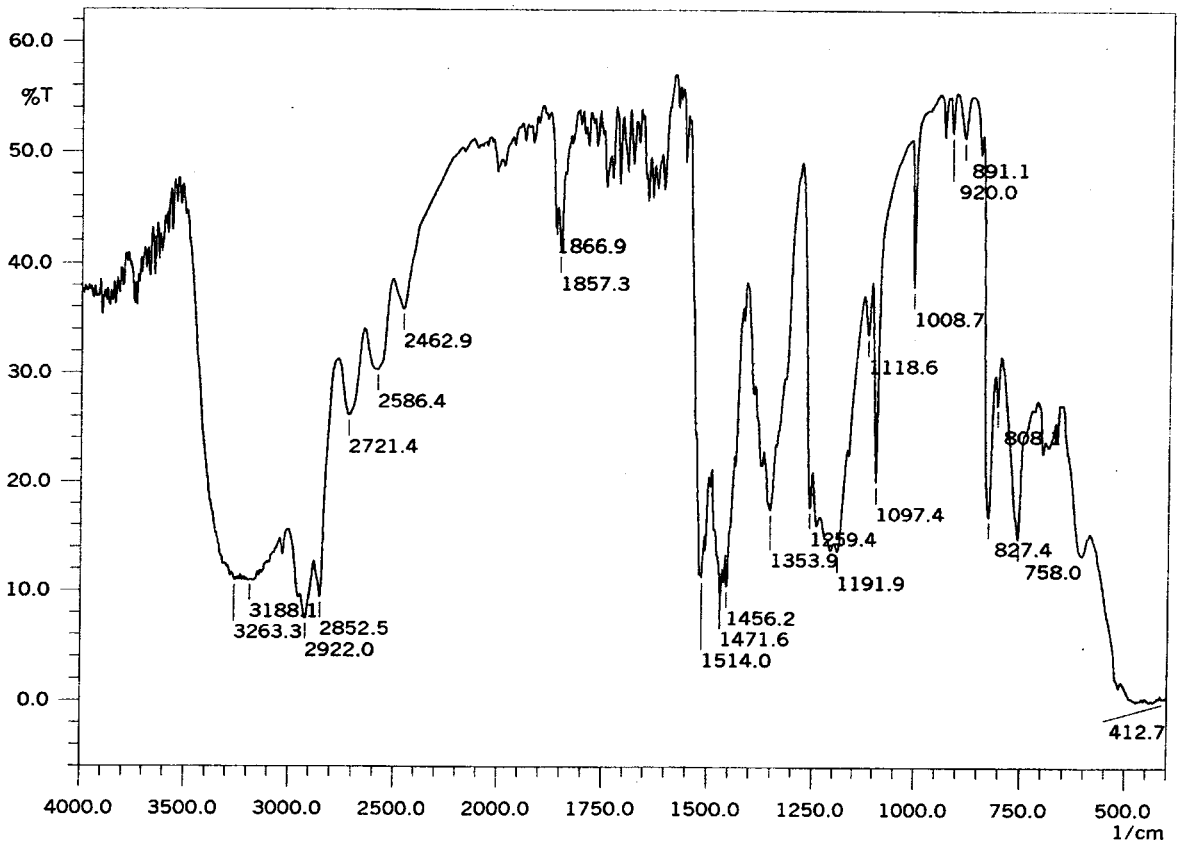
hydroquinone already identified by GC-MS. Other fragment ions were at m/z 82, 81, 64, 53 and 39. Its m.p. (171°C) was comparable to the reported m.p. (172°C) of **hydroquinone**[35].

III.11. ACETYLATION OF GS2 (HYDROQUINONE)

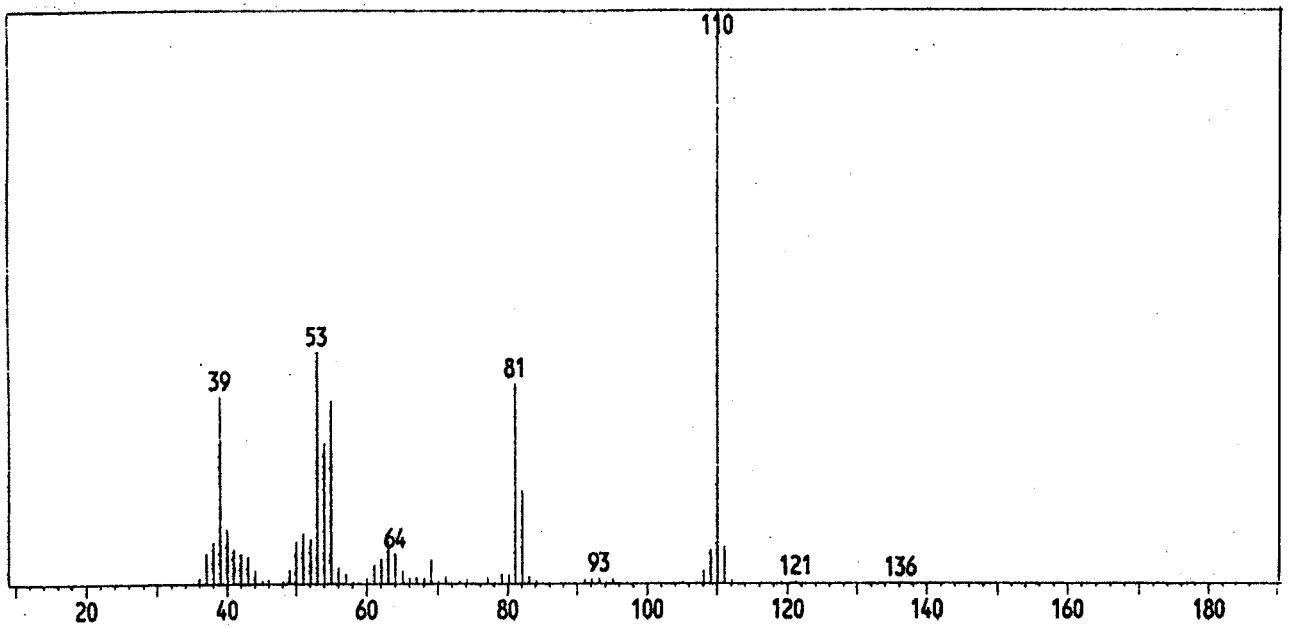
Hydroquinone (100 mg) was dissolved in 3M sodium hydroxide solution (5 ml), added crushed ice (20 g) followed by acetic anhydride (0.5 g). The mixture was shaken vigorously for one minute. On acidification the acetate crystallised out, which was recrystallised from ethanol, m.p 123°C. The m.p was found to be identical to that of **quinol diacetate**[35].

III.12. BENZOYLATION Of GS2 (HYDROQUINONE)

Hydroquinone (100 mg) was dissolved in pyridine (3 ml) and benzoyl chloride (0.5 g) was added to it. After the initial reaction had subsided, the mixture was warmed for 2 minutes over a small flame and poured into 15 ml of water with vigorous stirring. The precipitate was allowed to settle and decanted the supernatant liquid. The residue was stirred thoroughly with 10 ml of one molar sodium carbonate solution, filtered and recrystallised from ethanol, m.p. 199°C. The melting point was found to be identical to that of **quinol dibenzoate**[35].



IR spectrum of Hydroquinone [Gs2 IR)



Mass spectrum of Hydroquinone [GS2 MS]

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ANALYSIS OF THE ESSENTIAL OIL VOLATILES OF STRYCHNOS NUX-VOMICA LINN. FLOWERS USING GC-FID AND GC-MS

Molykutty M. Kaniampady “Studies on plant metabolites” Thesis. Department of Chemistry , University of Calicut, 2006

CHAPTER IV

ANALYSIS OF THE ESSENTIAL OIL VOLATILES OF *STRYCHNOS NUX-VOMICA* LINN. FLOWERS USING GC-FID AND GC-MS

IV.1. INTRODUCTION

Strychnos nux-vomica Linn. belongs to the family Loganiaceae. It is a predominantly pantropical and warm temperate family of 32 genera and nearly 800 species, found in different parts of the world, but well represented in tropical America. Several genera are represented by species indigenous to this country. The genus *Strychnos* is known to contain 200 species[1]. *Strychnos* is a large genus of scandent shrubs or trees, found throughout the tropics and subtropics. This genus exhibits diverse medicinal properties. It is poisonous, the toxic principles acting mainly on the spinal cord[2]. Nearly 20 species occur in India, of which *Strychnos nux-vomica* is renowned for the drug value of its poisonous alkaloids, strychnine and brucine[3]. It is commonly called 'Karaskara' in Sanskrit, 'Mushti' in Telugu, 'Etti' in Tamil and 'Kanjiram' in Malayalam[4]. This tree is wild and plentiful throughout tropical India, commonly in the jungles of southern India, Orissa, Bihar, Uttarpradesh and Srilanka[5].

It is an ever green or deciduous medium sized tree. In favourable situations, trees as high as 30 m with a girth of c. 2.8 m

may be found. Leaves 8-15 cm long, broadly elliptic, obtuse or acute, entire, with prominent central nerves; flowers greenish white, in terminal compound cymes; berries globose, 2.5-5.0 cm in diam., seeds discoid (coin-like), covered with fine and silky hair, embedded in white, bitter pulp[3].

The tree is found growing in regions where the absolute maximum shade temperature varies from 35 to 45°C and minimum from 4 to 18°C, and where rainfall ranges from 75 to 375 cm or more. The tree is a shade bearer, growing under a moderate canopy even in semi-evergreen forests. It produces root-suckers and is free from damage by browsing, as animals avoid it. In moist forests, the tree is evergreen, but in dry areas it sheds the leaves for a short time[3].

IV.2. MEDICINAL PROPERTIES AND USES

Almost all parts of *Strychnos nux-vomica* seem to have been used for one medical purpose or another[6,7]. Alkaloids brucine and strychnine are present not only in the seeds, but also in roots, wood, bark, leaves, fruit pulp and the hard fruit-shells. The fruit is bitter, acrid, pungent; heating, appetiser, tonic, astringent to bowels, antipyretic; cures leucoderma, "vata" and "kapha" diseases of the blood, itching, piles, ulcers, anaemia, jaundice, urinary discharges (Ayurveda)[8]. The fruit is bitter and poisonous; heating, tonic,

aphrodisiac, diuretic, emmenagogue; cures pain in the joints, lumbago, ringworm, piles; useful in paralysis and weakness of the limbs (Yunani)[8].

The leaves when applied as poultice, promote healthy action in sloughing wounds or ulcers, more especially in those cases when maggots have formed. It arrests any further formation of them, and those in the deeper parts perish immediately when the poultice is applied. The root bark is ground up into a fine paste with lime-juice, and made into pills which are said to be affectual in cholera. In the Konkan, small doses of the seeds are given with aromatics in colic, and juice of the fresh wood (obtained by applying heat to the middle of a straight stick to both ends of which a small pot has been tied) is given in doses of a few drops in cholera and acute dysentery. In some districts small quantity of the seeds are taken, apparently as a stimulant, or in lieu of opium[8].

In the Indian archipelago, the wood is used as a popular remedy for dysentery, fevers and dyspepsia. In Srilanka, the roots are ground with water and applied to snake bite. In Cambodia, the seed is used as an emetic. Internally an infusion of the bark is given in epilepsy; externally the bark is used in the treatment of ulcers, atonic and leprotic[8]. A decoction of the leaves is also used as an external application in rheumatism[9].

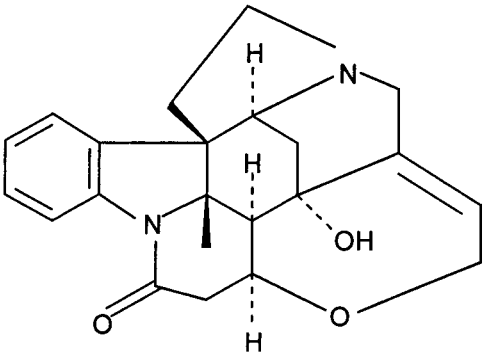
Nux-vomica is a powerful poison in large doses, producing tetanic convulsions and eventually death. In comparatively lesser doses it may result in mental derangement. In the indigenous medicine, it is used as a tonic, stimulant and febrifuge and its preparations are prescribed for nervous disorders. They are also used in the preparation of medicated products for the hair and scalp. Nux-vomica is an effective animal poison, and at present is used more as a poison than as a drug. It is also useful as an insecticide to kill vermin in fields. In South-East Asian Countries, tribals use the seeds in the preparation of arrow- and dart-poisons[3]. Wood is not attacked by termites and used for agricultural implements, tool-handles, ploughs, cart-wheels and fancy cabinet-work[10].

IV.3. PREVIOUS WORK

There are a number of scientific publications on this plant describing the isolation and structure determination of many alkaloids from different parts of the plant. The chemical constituents of the seeds, bark and root have been the subjects of investigation by many workers. The seeds have long been known in pharmacy as the major source of strychnine and brucine, and consequently their alkaloids have been much investigated[11]. Loganin is a bitter glucoside isolated from *Strychnos nux-vomica*[12-14] and other species of *Strychnos*[15]. Inouye and coworkers established the absolute

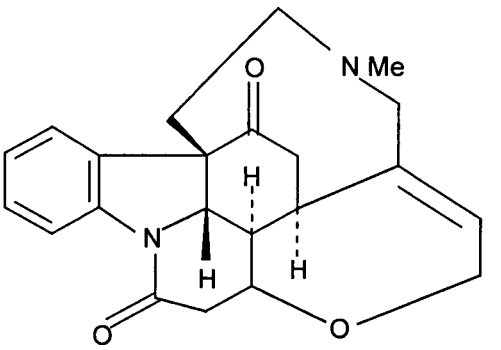
structure of loganin by chemical conversion of asperuloside to loganin penta-acetate[16].

Nicoletti and coworkers determined the structure of 15-hydroxystrychnine, a new alkaloid isolated from the seeds of *Strychnos nux-vomica* along with 12 other substances[17]. By using countercurrent distribution at discontinuously decreasing pH, they made it possible to separate from the raw alkaloid mixture of *S. nux-vomica* seeds nine known susbtances: strychnine, α- and β-colubrine, brucine, pseudostrychnine (=3-hydroxystrychnine), pseudobrucine (= 3-hydroxybrucine), icajine, vomicine and novacine together with four new alkaloids. The structures of three of the latter were established as 3-hydroxy-α-colubrine, 3-hydroxy-β-colubrine[18] and isostrychnine[19].



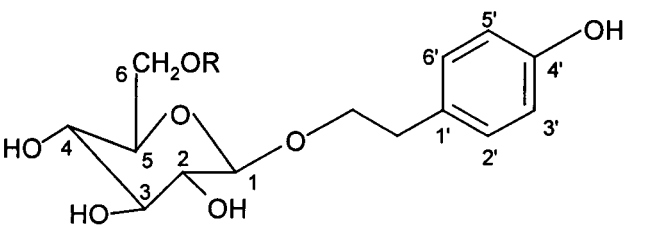
15-hydroxystrychnine

Rodriguez and coworkers isolated 3-methoxyicajine from *S. nux-vomica* and its structure characterised by spectroscopic means[20].

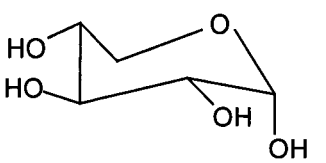


3-methoxyicajine

A phenolic glycoside – cuchiloside – isolated from fruit pulp along with salidroside and their structures were determined by Norman Bisset and coworkers[21].



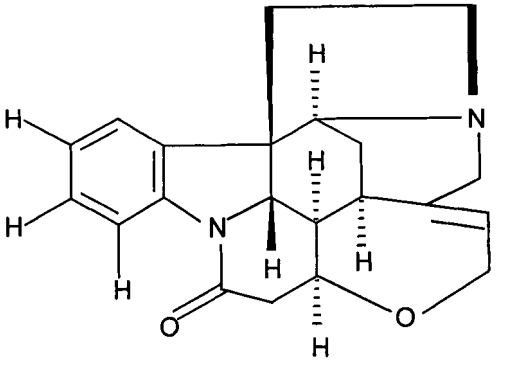
R = H **Salidroside**

R = 

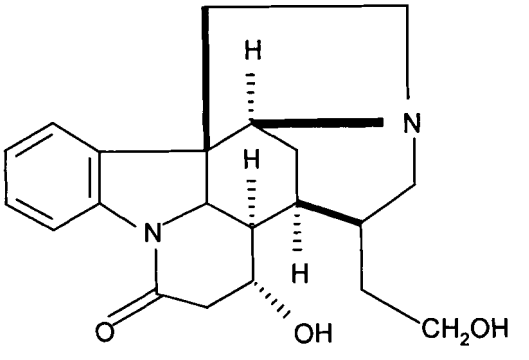
Cuchiloside

Xylose

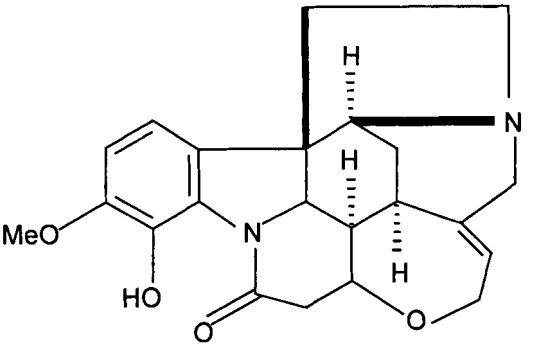
Kemal and coworkers isolated protostrychnine from the bark along with strychnine, normacusine B and 4-hydroxy-3-methoxy-strychnine, and their structures elucidated[22].



Strychnine



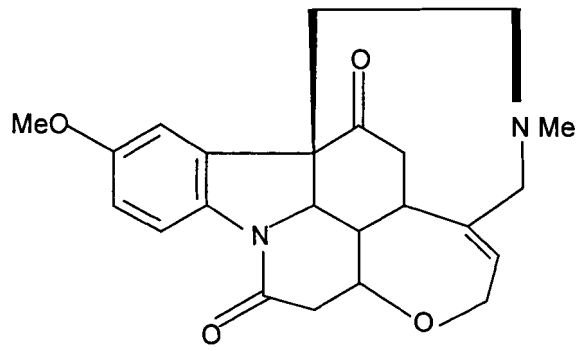
Protostrychnine



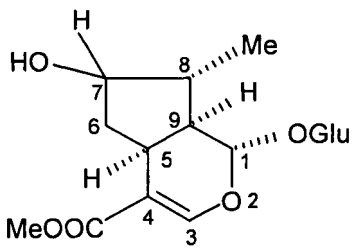
4-hydroxy-3-methoxystrychnine

A new alkaloid N-methyl-sec-pseudo-β-colubrine was isolated from seeds along with 4-hydroxystrychnine by Bisset and Choudhury[23]. They also found that the iridoid mixture in the fruit pulp is

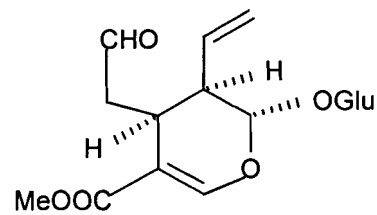
predominantly loganin with small amounts of related compounds, including the biogenetically important secologanin.



N-methyl-sec-pseudo-β-colubrine



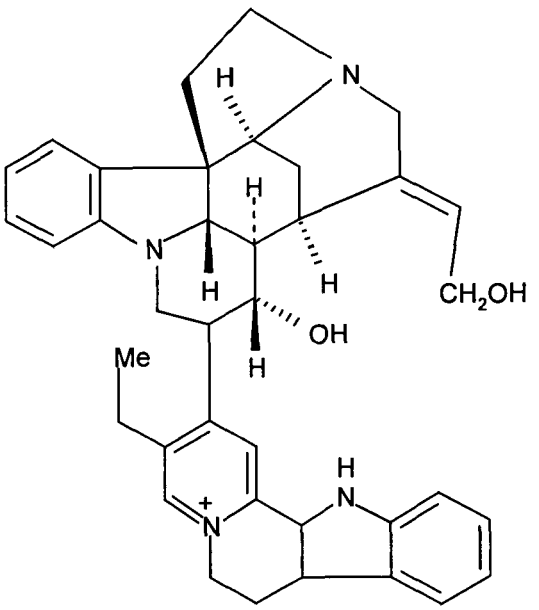
loganin



Secologanin

p-Hydroxybenzoic-, vanillic-, 2-hydroxy-4-methoxy-benzoic-, sinapic- and syringic acids and kaempferol, quercetin and 3'-O-methylquercetin were isolated and identified from *S. nux-vomica.*[24] Corsaro and coworkers[25] reported the chemical composition of polysaccharide fractions from *S. nux-vomica* and *S. innocua* seeds and compared with those from *S. potatorum* seeds. The structural features of the galactomannans from the three *Strychnos* species were also discussed. Rose Gadi Biala and coworkers[26] isolated a

coloured monoquaternary bisindole alkaloid from the roots. The structure of this new orange substance, strychnochrysine was defined by detailed spectroscopic methods.



Strychnochrysine

Baser and Bisset isolated twenty two identified alkaloids from the root bark and leaves of a Sri Lankan *Strychnos* species supplied as *S. nux-vomica*[27]. The following bases have not previously been obtained from this species: 10-hydroxystrychnine, 3,12-dihydroxy-strychnine, 12-hydroxy-11-methoxystrychnine, 3,12-dihydroxy-11-methoxystrychnine, 12-hydroxystrychnine N-oxide, 12-hydroxy-11-methoxystrychnine N-oxide, 19,20-dihydroisostrychnine, 16α-, 17β-dihydro-17α-hydroxyisostrychnine (protostrychnine), O-methyl-macusine B, 16-epi-O-methylmacusine B, and nor-melinonine B.

Zhang and coworkers isolated three iridoids, 6'-O-acetylloganic acid, 4'-O-acetylloganic acid and 3'-O-acetylloganic acid together with two known iridoid glucosides, loganic acid and 7-O-acetylloganic acid from the seeds of *S. nux-vomica*[28].

IV.4. PRESENT WORK

Most of the previous chemical investigations have focussed on the isolation of chemical constituents of the seeds, bark and root of *Strychnos nux-vomica*. So far no data about the volatiles from *S. nux-vomica* flowers has been published. In the present study, the volatile compounds of the essential oil of fresh flowers are analysed by GC-FID and GC-MS.

IV.5. EXPERIMENTAL

The flowers of *Strychnos nux-vomica* were collected from the neighbouring village of Calicut University Campus in March 2005. The plant material was identified by Dr. A.K. Pradeep, Department of Botany, Calicut University, Kerala.

Essential oil extraction

The fresh flowers (1.8 kg) of *Strychnos nux-vomica* were steam distilled for 2 hrs. The distillate was extracted with diethyl ether (3x100 ml) and dried over anhydrous sodium sulphate. After

evaporation of the solvent 0.36 g (0.02% of the fresh weight) of essential oil was obtained.

GC-FID and GC-MS

GC-FID and GC-MS conditions were exactly the same as those given in section 1 of chapter III.

IV.6. RESULTS AND DISCUSSION

Using Gas Chromatographic-spectroscopic systems **seventy-five components** were identified in the flower essential oil of *S. nux-vomica.* The main compounds (concentrations higher than 2% calculated as percentage peak area of GC-FID analysis) were palmitic acid (9.60%), tricosane (5.64%), trans-epoxylinalool (4.79%), cis-epoxylinalool (4.34%), linalool (4.19%), α-terpineol (4.15%), T-cadinol (4.07%), nerolidol (3.84%), farnesol (3.62%), linalool oxide (3.58%), menthol (3.51%), linalyl acetate (3.49%), cis-linalool oxide (2.76%) and myristic acid (2.48%). The compounds identified and their concentrations (%) are given in **Table IV.1**.

TABLE IV.1

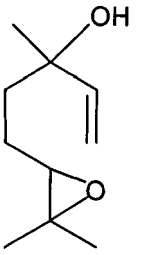
Essential oil composition of fresh flowers of *Strychnos nux-vomica*

Compounds in order of concentration	% peak area
Palmitic acid	9.60
Tricosane	5.64
trans-Epoxylinalool	4.79
cis-Epoxylinalool	4.34
Linalool	4.19
α-Terpineol	4.15
T-Cadinol	4.07
Nerolidol	3.84
Farnesol	3.62
trans-Linalool oxide (furanoid)	3.58
Menthol	3.51
Linalyl acetate	3.49
cis-Linalool oxide (furanoid)	2.76
Myristic acid	2.48
δ-Cadinene	1.95
Oleic acid	1.82
Benzyl benzoate	1.73
β-Caryophyllene	1.67
Benzoic acid	1.56
Nerol	1.41
Pentadecanoic acid	1.36
Benzylalcohol	1.19
Viridiflorol	1.15
Sabinol	0.99
Hydroxylinalool	0.97
Sabinyl acetate	0.89
Cedrol	0.88
Camphor	0.87

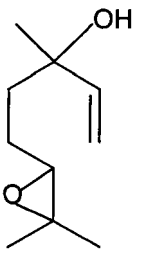
Compounds in order of concentration	% peak area
Cubenol	0.87
Octadeca-9,12-dienoic acid	0.86
Cinnamic alcohol	0.84
α-Terpinyl acetate	0.83
Torreyol	0.79
Docosane	0.77
Pentadecane	0.74
Methylsalicylate	0.69
cis-Carveol	0.62
Widdrenol	0.61
Terpinen-4-ol	0.60
Aromadendrene	0.56
Isoamyl benzoate	0.54
β-Bisabolol	0.54
Tetradecane	0.52
2,3,5,8-Tetramethyldecane	0.51
α-Bisabolol	0.47
trans-para-Mentha-2,8-dienol	0.47
Germacrene D	0.46
Caryophyllene oxide	0.45
α-Copaene	0.43
α-Cadinol	0.42
Geraniol	0.40
β-Bisabolol epoxide	0.38
Cedrene	0.36
Decanoic acid	0.33
Eicosane	0.32
Aromadendrene oxide	0.32
Tridecanoic acid	0.32
α-Guaiene	0.24
Dehydroaromadendrene	0.23

Compounds in order of concentration	% peak area
α-Bisabolol epoxide	0.22
Cinnamic aldehyde	0.21
trans-Carveol	0.21
α-Calacorene	0.19
Verbenol	0.19
Pentadecane	0.18
Coumarin	0.18
Geranyl benzoate	0.17
Neryl acetate	0.16
3,5,9-Trimethyldeca-2,4,8-trien-1-ol	0.15
Viridifluorene	0.14
2,3,4-Trimethyloctane	0.14
Citral	0.13
Carvyl acetate	0.13
Dodecanoic acid	0.13
Geranyl acetate	0.12

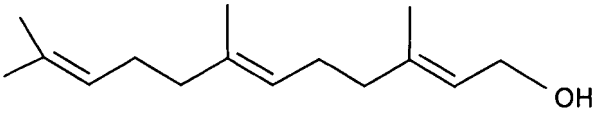
Structures of the identified compounds which are not included in previous chapters are as follows:



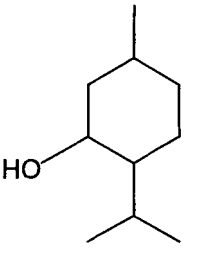
trans-Epoxylinalool



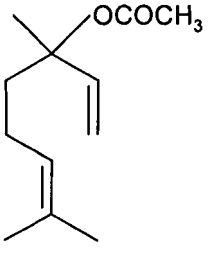
cis-Epoxylinalool



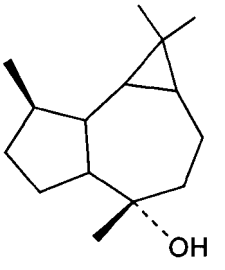
Farnesol



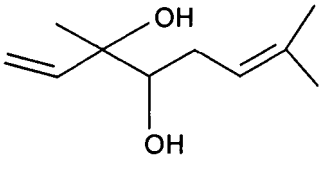
Menthol



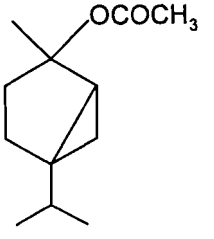
Linalyl acetate



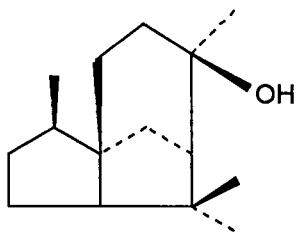
Viridiflorol



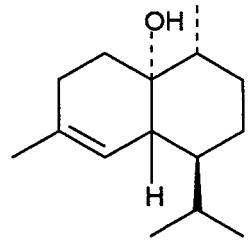
Hydroxylinalool



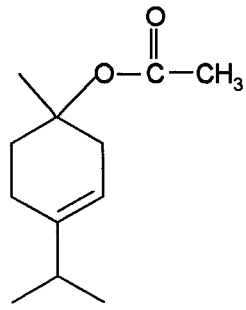
Sabinyl acetate



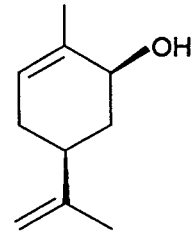
Cedrol



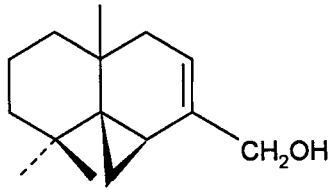
Cubenol



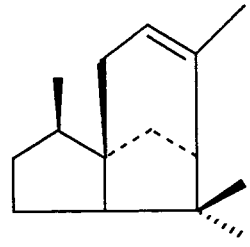
α-Terpinyl acetate



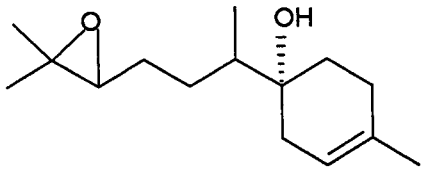
cis-Carveol



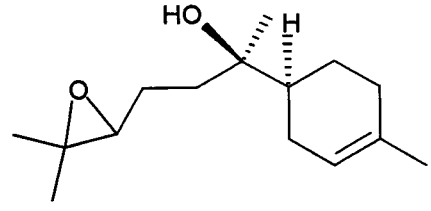
Widdrenol



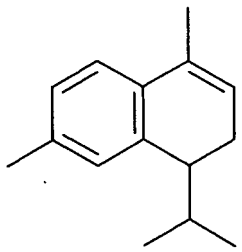
Cedrene



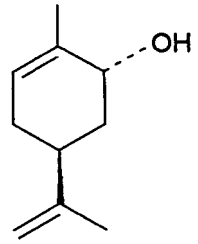
β-Bisabolol epoxide



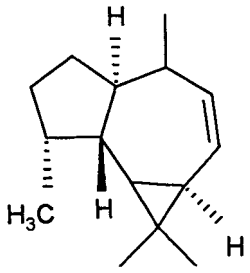
α-Bisabolol epoxide



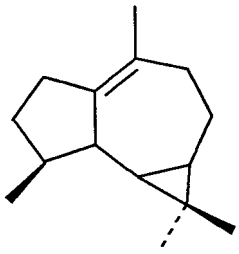
α-Calacorene



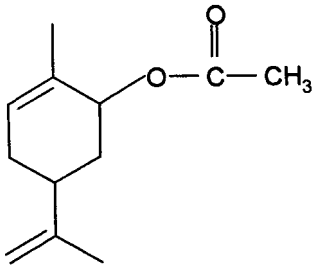
trans-Carveol



Dehydroaromadendrene



Viridifluorene



Carvyl acetate

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