STUDIES ON SECONDARY METABOLITES OF A FEW PLANTS IN MALABAR

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 the thesis entitled "*Studies on secondary metabolites of a few plants in Malabar"* is an authentic record of the research work carried out by **George. T. Abraham.**, in the Department of Chemistry, 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,

Dr. P. Mohamed Shafi
(Supervising Teacher)

DECLARATION

I, **George. T. Abraham.**, 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.

C.U. Campus, **George. T. Abraham.**

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CONTENTS

			Page No.
PREFACE			
CHAPTER I			1-28
	PHYTOCHEMICAL STUDIES ON *CISSUS GLUACA*		
	1.1	Introduction	1
	1.2	Medicinal properties and uses	1
	1.3	Work so far reported on *Cissus* species	2
	1.4	Present work	3
	1.5	Materials and methods	3
	1.6	Extraction, fractionation and isolation of compounds from the petroleum ether extract of *C. gluaca*	7
	1.7	Extraction, fractionation and isolation of compounds from the acetone extract of *C. gluaca*	8
	1.8	Results and discussion	9
	1.8.1	Characterization of MG1 (Mixture of esters)	9
	1.8.2	Characterization of MG2 and SM2 (Friedelin)	15
	1.9	Conclusion	26
CHAPTER II			29-86
	ANALYSIS OF THE ESSENTIAL OIL OF THE ROOTS AND LEAVES OF THE MEDICINAL PLANT *KAEMPFERIA GALANGA* L .		
	SECTION 1 **GENERAL INTRODUCTION**		29-55
	2.1.1	Essential oil	29
	2.1.2	Methods of production of essential oil	35
	2.1.3	Chemical composition	37
	2.1.4	Essential oils in practice	38
	2.1.5	Antimicrobial properties	42
	2.1.6	Analytical methods	44
	SECTION 2		56-68
	ANALYSIS OF THE ROOT ESSENTIAL OIL OF *KAEMPFERIA GALANGA*		
	2. 2. 1	Introduction	56
	2.2.2	Medicinal properties and uses	57
	2.2.3	Previous work	57
	2.2.4	Present work	58
	2.2.5	Materials and methods	58
	2.2.6	Results and discussion	60

SECTION 3 69-81

ANALYSIS OF THE LEAF ESSENTIAL OIL OF *KAEMPFERIA GALANGA*

2.3.1 Present work 69
2.3.2 Materials and methods 69
2.3.3 Results and discussion 69
2.3.4 Experimental 77

CHAPTER III 87-104

SECTION 1 87-96

ANALYSIS OF THE LEAF ESSENTIAL OIL OF *SPILANTHES CILIATA*

3.1.1 Introduction 87
3.1.2 Medicinal properties and uses 88
3.1.3 Previous work 89
3.1.4 Present work 90
3.1.5 Materials and methods 90
3.1.6 Results and discussion 92

SECTION 2 97-101

CHEMICAL TRANSFORMATIONS OF THE ESSENTIAL OIL OF *SPILANTHES CILIATA*

3.2.1 Introduction 97
3.2.2 Esterification of alcohols in *S. ciliata* essential oil 97
3.2.3 Results and discussion 98

CHAPTER IV 105-125

CHEMICAL INVESTIGATION OF *SPILANTHES RADICANS*

4.1 Introduction 105
4.2 Present work 106
4.3 Experimental 107
4.4 Results and discussion 110
4.4.1 Characterization of G_1 (β-Sitosterol) 118

CHAPTER V 126-145

ANALYSIS OF THE ESSENTIAL OILS OF *ARTABOTRYS ODORATISSIMUS* FRUITS AND LEAVES

5.1 Introduction 126
5.2 Medicinal properties and uses 126
5.3 Previous work 127
5.4 Present work 127
5.5 Experimental 128
5.6 Results and discussion 130

PREFACE

Plants are perhaps one of the most interesting and mysterious things of the universe and it is not unreasonable that man, from time immemorial, has devoted much of his time and energy to study this plant kingdom. Plants play a pivotal role in the existence of man kind. They are elixir of life. Ever since the birth of man the plant had provided him with food, shelter and drugs. Plants are the most amazing factories where a variety of chemical compounds are manufactured. Many of the perfumes, medicine, pesticides, etc are derived from plants. The traditional health practices in India, especially Ayurveda make use of plants very effectively and have been validated by practice over thousands of years. The last few decades have witnessed tremendous advancement in natural products' research through the field of Chemistry, life science, food science, material science etc. Natural products are ubiquitous in our day to day life.

Synthetic drugs in the form of drugs, pesticides etc. have been successful in combating diseases of man, his domestic animals and plant crops. However, their side effects are of major concern in the modern world. Besides many of the microbes and insects have become resistant to most of these chemicals. Hence we hopefully turn to nature as a resort and resource in finding new strategies to fight diseases. In this respect plants serve as a vast

reservoir of biodegradable chemicals, many of which may have evolved in the defence of predators.

Phytochemistry deals with the study of secondary metabolites isolated from plant kingdom, their characterization, reactions, transformations and biological activities. Modern spectroscopic techniques and instrumentation have given a new kind of impetus to this branch of science. Plants are sophisticated factories where a variety of chemical compounds are manufactured. These derived products are used as medicine, pesticides, perfumes, fragrances and other utility products. The study of naturally occurring substances that have medicinal and other useful properties have been a subject of rapid developments and an interesting area of active research. Compounds isolated from natural sources continue to occupy an important place among useful products of modern medicine. Hence plants continue to kindle the interest of researchers.

The work presented in this thesis deals with chemical investigation of *Cissus gluaca*, analysis of the essential oil of the roots and leaves of *Kaempferia galanga*, analysis of the essential oil of *Spilanthes ciliata*, chemical investigation of *Spilanthes radicans* and analysis of the essential oil of fruits and leaves of *Artabotrys odoratissimus.*

This thesis is divided into five independent chapters and relevant references are given at the end of each chapter.

The first chapter presents the phytochemical studies on the stem of *Cissus gluaca*. The investigation enabled the identification of friedelin and mixture of long chain esters in the stem. These compounds were characterized using IR, NMR and mass spectral data.

The second chapter comprises of three sections. Section I is an introduction highlighting the importance of essential oil. A brief overview of analytical techniques is also illuminated in this section. Section II deals with the analysis of the root essential oil of *Kaempferia galanga* by GC-MS and GC-FID. The root essential oil of *K. galanga* was olfactorically evaluated and the odour was described as aromatic spicy and pleasant fresh. Seventy constituents were identified and the main compounds were ethyl *trans*-p-methoxycinnamate, ethyl *trans*-cinnamate, pentadecene and 1,8-cineole were found, among further mono and sesquiterpenes, aliphatic hydrocarbons, aliphatic alcohols and cosmetically interesting compound isoamyl *trans*-p-methoxycinnamate. For the first time the *cis* isomar of ethyl cinnamate was identified as minor constituent of this *Kaempferia* species.Two compounds with retention indices close to each other with identical mass spectra were found to be present in this oil. These two compounds were ethyl p-methoxycinnamates (*cis* and *trans*). In order to identify which is which, *trans* isomer was synthesised and co-injected to assert the identity of the isomer. An article based on this work has been published (Analysis of the Essential Oil of the Roots of the Medicinal Plant *Kaempferia galanga* L. *(Zingiberaceae)*

from South-India, *Acta Pharmaceutica Turcica* **XLIII(2),** 2001, 107-110). Section III deals with the analysis of the leaf essential oil of *Kaempferia galanga.* The leaf essential oil of *K. galanga* was olfactorically evaluated and the odour was spicy, pleasant fresh, dry hay like and smoky. Sixty six constituents were identified by GC-MS and GC-FID methods. The main compounds were ethyl *trans*-p-methoxycinnamate, ethyl *cis*-p-methoxy cinnamate, *cis*-hex-3-enol, borneol, ethyl *trans*-cinnamate and caryophyllene oxide.

The third chapter consists of two sections. Analysis of the volatiles of *Spilanthes ciliata,* using GC-MS, GC-FID and olfactometry form the subject matter of first section. Forty eight compounds could be identified in the essential oil, with (E)-2-hexenol, as the main constituent. The constituents of the oil responsible for the characteristic aroma impressions are also discussed. Based on this study an article entitled "Essential Oil Analysis of *Spilanthes acmella* Murr.Fresh Plants from Southern India" has been published in the journal *J. Essent. Oil Res.,* **17**, 2005, 429-431. Section II gives the chemical transformations of the essential oil of *Spilanthes ciliata.* The essential oil of *S. ciliata* contain large number of alcohols and it has a pleasant smell. In this section these alcohols are subjected to esterification by using cinnamoyl chloride, benzoyl chloride and acetyl chloride. The resulting oils were analysed by GC, GC-MS and olfactometry

The fourth chapter consists of the phytochemical studies and essential oil analysis of *Spilanthes radicans.* Phytochemical studies of this plant led to the isolation and identification of -Sitosterol. Using GC-FID and GC-MS the essential oil and two fractions A and B obtained from chromatography of *Spilanthes radicans* was studied and 90 volatiles could be detected. The essential oil and the two root extract of *Spilanthes radicans* were olfactorically evaluated. The main compounds were 2-tridecanone, 1-pentadecene, *trans*- β-Caryophyllene, elemol and guaiol. The sample A also contains sulphur containing compounds which imparted fresh – Allium like top note to it. An article based on this work has been published (Chemical composition and olfactoric characterization of *Acmella radicans (Jacq.)* R.K. Jansen var. *radicans* from Southern India, *Flavour Fragr.J.*, **21**, 2006, 88-91).

The fifth chapter consists of the study on the essential oil composition of fruits and leaves of *Artabotrys odoratissimus*, analysed by GC-MS, GC-FID and olfactometry. Eighty compounds could be identified and the main compounds of the essential oil of the fruits and leaves respectively were the sesquiterpiens β-caryophyllene (14.7%, 17.3%), *trans* nerolidol (8.2%, 1.9%), δ-cadinene (7.3% , 4.2%), α-copaene (6.4% , 9.3%), *trans*, *trans*-α-farnesene (5.8% , 7.4%), τ-cadinol (4.3% , 2.9% and caryophyllene oxide (3.2% , 6.8%). The essential oils of *Artabotrys odoratissimus*, were olfactorically evaluated and the odour was described as floral-fruity, dry-woody, herbal

spicy in the background (fruits) and green-grassy, herbal woody, weak floral, earthy-smoky-fatty in the background (leave). Based on this study an article entitled “Analysis of the Essential Oils of Artabotrys odoratissimus Fruits and Leaves from South-India” has been published in the *Journal of Essential Oil Bearing Plants* **1(2-3)**, 1998, 94-103.

CHAPTER I

PHYTOCHEMICAL STUDIES ON *CISSUS GLAUCA*

1.1 INTRODUCTION

Cissus glauca Roxb. (Syn. *Cissus repens* Lam.) is a handsome trailing herb belongs to *Vitaceae* family[1,2]. It is distributed in eastern India, Maharashtra, Konkan south wards up to Kerala and the Andamans. The plant is commonly known Marigampuli in Malayalam , Elakombullaballi in Kannada, Neelaboddu in Telugu and Diboria in Oriya[3].

It is a slender glabrous climber; branches glaucous; tendrils stout, forked. Leaves up to 15x9cm, broadly ovate, cordate at base, acuminate at apex, dentate-crenate, glabrous; petioles up to seven cm long. Flowers in leaf-opposed compound umbellate cymes, small, greenish yellow; peduncles solitary or fascicled; pedicels slender, reddish. Calyx truncate. Berries ellipsoid-pyriform, 5mm across, 1- seeded, black when ripe. Flowering occurs mainly between August and October[4].

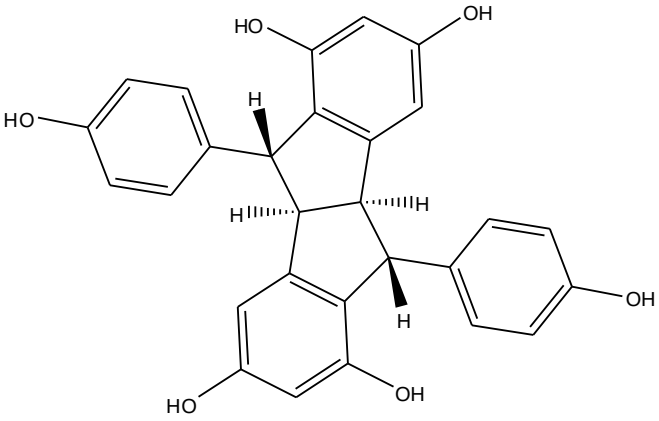
1.2 MEDICINAL PROPERTIES AND USES

The young shoots are succulent pleasantly acidic and edible; often it is substituted for sorrel. The leaves are also eaten raw or in soup. Fruit is acrid but eaten. The stem can be made in to ropes. The paste of the root and also of

the leaf is applied as a suppurate. Leaves are warmed and rubbed on the skin for skin diseases and itch[3]. The fruit is antiscorbutic, astringent, carminative, cardio tonic, cooling, emollient and is used in anorexia, colic, dyspepsia, heart diseases, thirst and ulcers. It overcome loss of appetite, indigestion, flatulence, liver and spleen diseases, cough and other respiratory disorders[5].

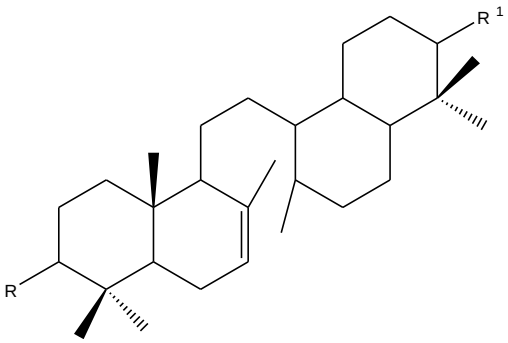
1.3 WORKS SO FAR REPORTED ON *CISSUS* SPECIES

Azif Zaman and co-workers isolated pallidol from *Cissus pallida* [6].



Pallidol

Bhutani and co-workers isolated two new unsymmetric tetracyclic triterpenoid onecer-7-en-3α,21β-diol (I) and onecer-7-en-3β,21α-diol (II) along with β–sitosterol, δ–amyrin and δ-amyrone from *Cissus qaudrangularis*[7]



I—R=□—OH, R^1=□—OH

II—R=β—OH,R^1=α—OH

Remiah and co-workers isolated β–Sitosterol and leucopelargonidin from *Cissus glauca*[8].

1.4 PRESENT WORK

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

MATERIALS AND METHODS

Plant material

The stem of *Cissus glauca* was collected from Calicut University Campus, Kerala in April 2006 and was authenticated by Dr. A.K. Pradeep, Department of Botany, Calicut University. A voucher specimen of the plant has been deposited in the specially maintained Herbarium of Chemistry Department, Calicut University.

Melting point determination

The melting points of all the crystalline isolates were determined using Toshniwal capillary melting point apparatus.

Infrared absorption spectroscopy (IR)

The IR spectra of the isolates were recorded as KBr pellets using JASCO FTIR-4100 spectrometer.

Nuclear magnetic resonance spectroscopy (NMR)

The ^{1}H NMR spectra and HSQC spectra of the isolates were recorded at 500 MHz on a Bruker spectrometer / Varian UNITY plus-300 spectrometer, using $CDCl_3$ as solvent with TMS as internal standard. The chemical shifts are reported in ppm (δ).

^{13}C NMR spectra of the isolates were recorded at 125 MHz on a Bruker spectrometer in $CDCl_3$.

Mass spectroscopy (MS)

Mass spectra of the isolates were recorded on a apex II FTICR mass spectrometer (Bruker).

Column chromatography (CC)

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

Thin layer chromatography (TLC)

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

Reagents

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

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 are detected as red or pink spots and sterols and their esters are detected as green to blue spots.

Vanillin-sulphuric acid reagent

The reagent was prepared by dissolving 1g. vanillin in 100 mL ethanol and 5 mL conc. H_2SO_4 in 100 mL ethanol 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 maximal visualisation of the spots.

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

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 then onc. 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 maximal visualisation of the spots were obtained.

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

2,4-Dinitrophenylhydrazine reagent.

Suspended 2,4-Dinitrophenylhydrazine (2 g) in methanol (100 mL), and then conc. H_2SO_4 (4 mL) added slowly and filtered.

With 2,4-Dinitrophenylhydrazine reagent ketones and aldehydes are detected as orange colour.

1.6 EXTRACTION, FRACTIONATION AND ISOLATION OF COMPOUND FROM THE PETROLEUM ETHER EXTRACT OF *C. GLAUCA*

Dried and finely powdered stem of *Cissus glauca* (3.5kg) was extracted with petroleum ether (60-80c,3x5L). The combined extract was then concentrated under reduced pressure to about 500ml of light yellow coloured solution when a white powdery solid separated out. It was filtered, washed repeatedly with petroleum ether and dissolved in petroleum ether- ethyl acetate mixture (8:1) and absorbed on silica gel (150g,60-120 mesh). After drying, it was taken in a chromatographic column (3cm x 60 cm; d x l) and eluted with solvents of increasing polarity viz. petroleum ether (1L), 8:1 petroleum ether – ethyl acetate (600 ml), 5:1 petroleum ether – ethyl acetate (500 mL), 1:1 petroleum ether – ethyl acetate (500 mL), ethyl acetate (500 mL) and 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. Fractions 2 to 7 obtained by petroleum ether elution on evaporation gave a white powdery substance MG1 (0.2 g) and it melted at 69^0C.

The crude petroleum ether extract, after the removal of white powdery solid, was absorbed on 400 g silica gel (60 to 120 mesh)and packed in a column (4 cm x 100 cm;d x l). The column was then eluted with petroleum ether, different combinations of petroleum ether – ethyl acetate, ethyl acetate and methanol in that order. Homogeneity of different fractions were analysed by TLC. Fractions 1 to 4 obtained by 8:1 petroleum ether – ethyl acetate elution on evaporation gave a white substance, which on recrystalisation from petroleum ether – acetone (1:1) yielded 0.05g of pure crystals MG2, m.p 245^0C

TABLE 1.1

Compound	Eluent composition	Melting point
MG1	Petroleum ether	69^0C
MG2	8:1 Petroleum ether - ethyl acetate	245^0C

1.7 EXTRACTION, FRACTIONATION AND ISOLATION OF COMPOUND FROM THE ACETONE EXTRACT OF *C. GLAUCA*

Finely powdered stem of *Cissus glauca* after extraction with petroleum ether (3.5 kg) was extracted thrice with acetone (3 x 5 L). The combined extract was concentrated under reduced pressure to about 500 ml of light green coloured solution. Then a white powdery substance separated out. It was filtered, washed repeatedly with acetone and dissolved in hot benzene and adsorbed on silica gel (100 g, 60 -120 mesh) for column chromatography

(3 cm x 75 cm; d x l). Elution was carried out using solvents of increasing polarity viz. petroleum ether (750 mL), 10:1 petroleum ether – ethyl acetate (600mL), 5:1 petroleum ether – ethyl acetate (600mL), 1:1 petroleum ether – ethyl acetate (600mL), and ethyl acetate (600mL). Several 50 mL fractions were collected and fractions were pooled together according to their homogeneity judged by TLC. The fractions 4 to 9 obtained by 5:1 petroleum ether - ethyl acetate elution on evaporation gave a white substance. It was repeatedly washed with petroleum ether and dried. This on recrystalisation from 1:1 petroleum ether – acetone yielded 0.4 g of pure crystals, SM2, m.p. 245^0C.

TABLE 1.2

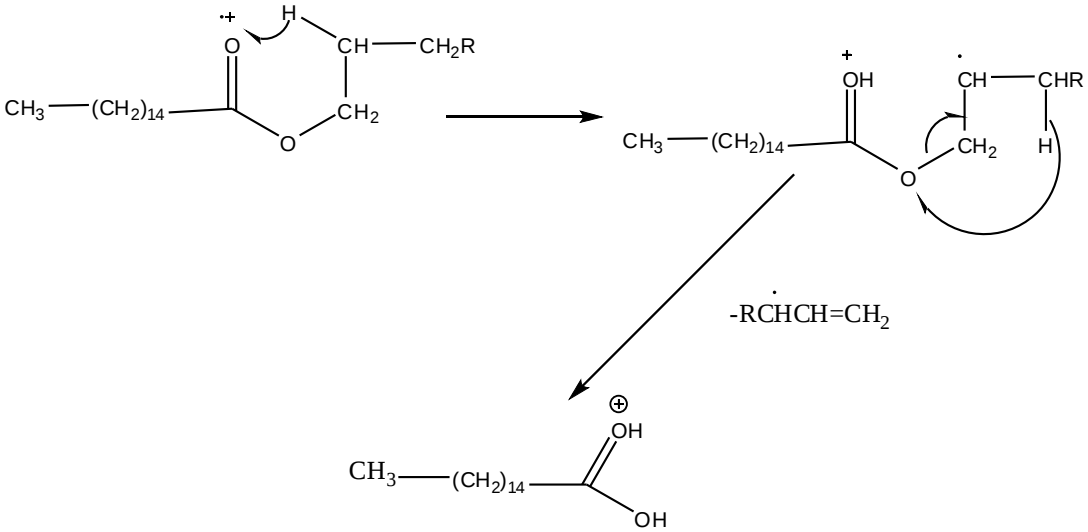
Compound	Eluent composition	Melting point
SM2	5:1 petroleum ether - ethyl acetate	245^0C

1.8 RESULTS AND DISCUSSION

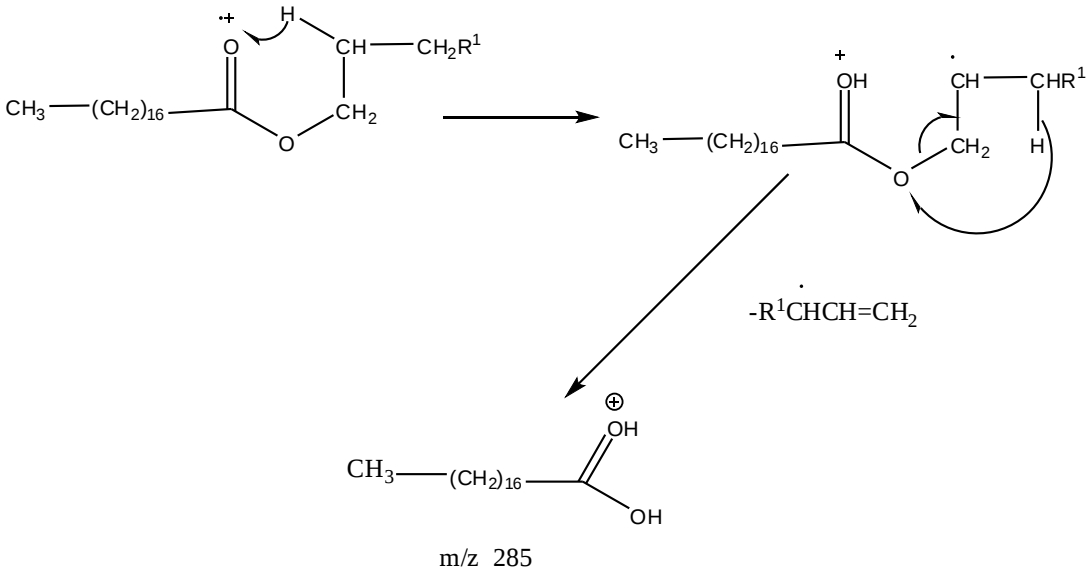
1.8.1 Characterisation of MG1 (Mixture of esters)

The compound MG1 was isolated from petroleum ether extract of the plant and it melted at 69^0C. This compound didn't answer LB reaction, indicating that it was not a triterpenoid or sterol. As it didn't decolourise Bayers reagent, it is a saturated compound. The IR spectrum gave characteristic absorption at1735.62cm^{-1}, indicating the presence of ester

carbonyl group and other frequencies at 719 $^{-1}$cm and 729 $^{-1}$cm are of long chain hydrocarbon part. Fragmentation pattern of the compound in the mass spectrum showed regular difference of 28 and 14 mass units proving again its straight chain hydrocarbon nature. The ^{1}H NMR spectrum showed a two proton triplet at 4.05 (—O—CH_2—CH_2—). From IR, ^{1}H NMR and mass spectral data, it was concluded that MG1 was mixture of long chain esters. In the mass spectrum, peaks at m/z 257 and m/z 285 of these esters are due to the transfer of two hydrogen atom to the fragment containing the oxygen atom, followed by the elimination of alkyl moiety[9]. This type of fragmentation pattern becomes increasingly common as the alkyl chain of the alcohol moiety lengthens. This can be illustrated as follows;

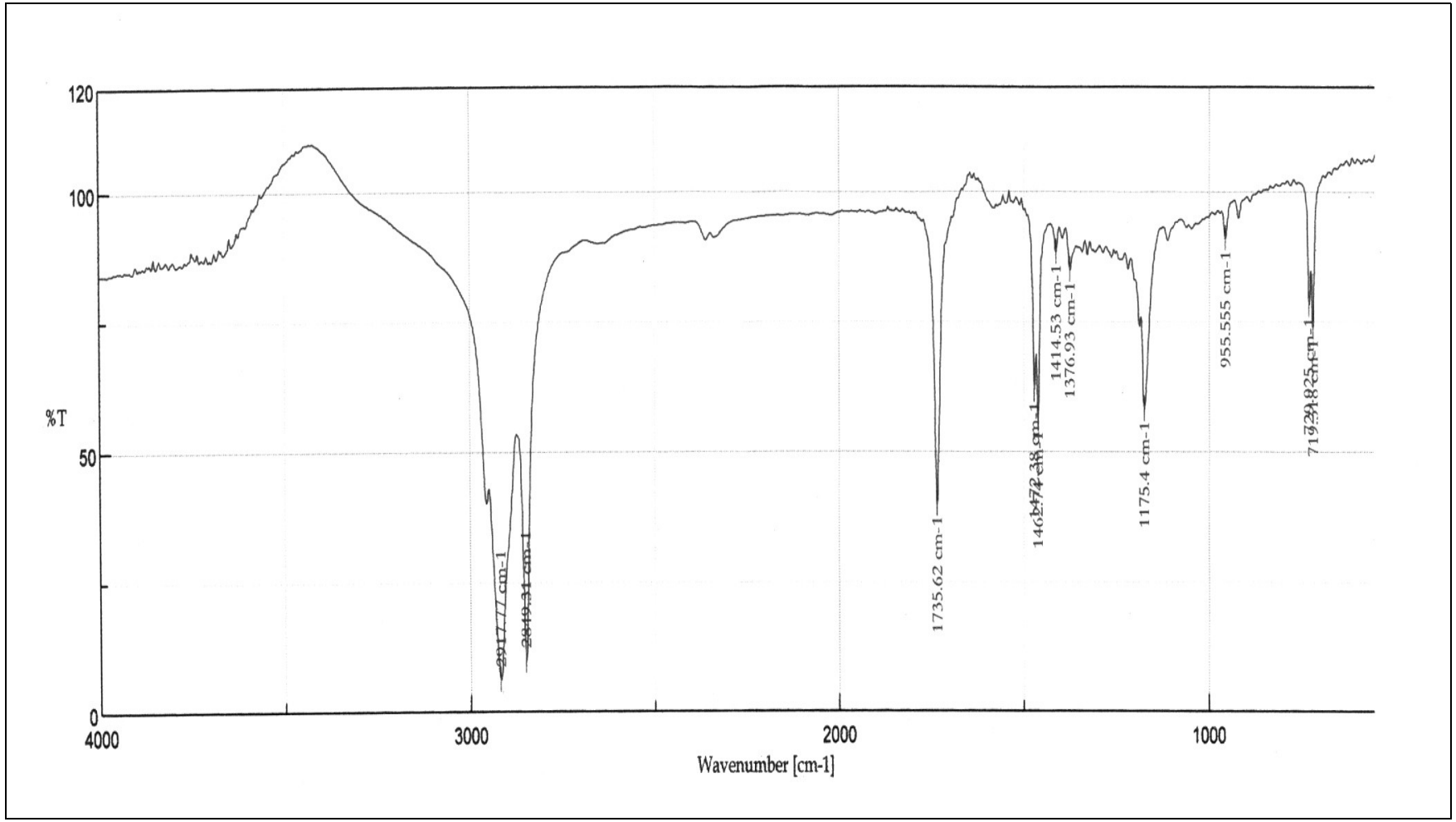


m/z 257

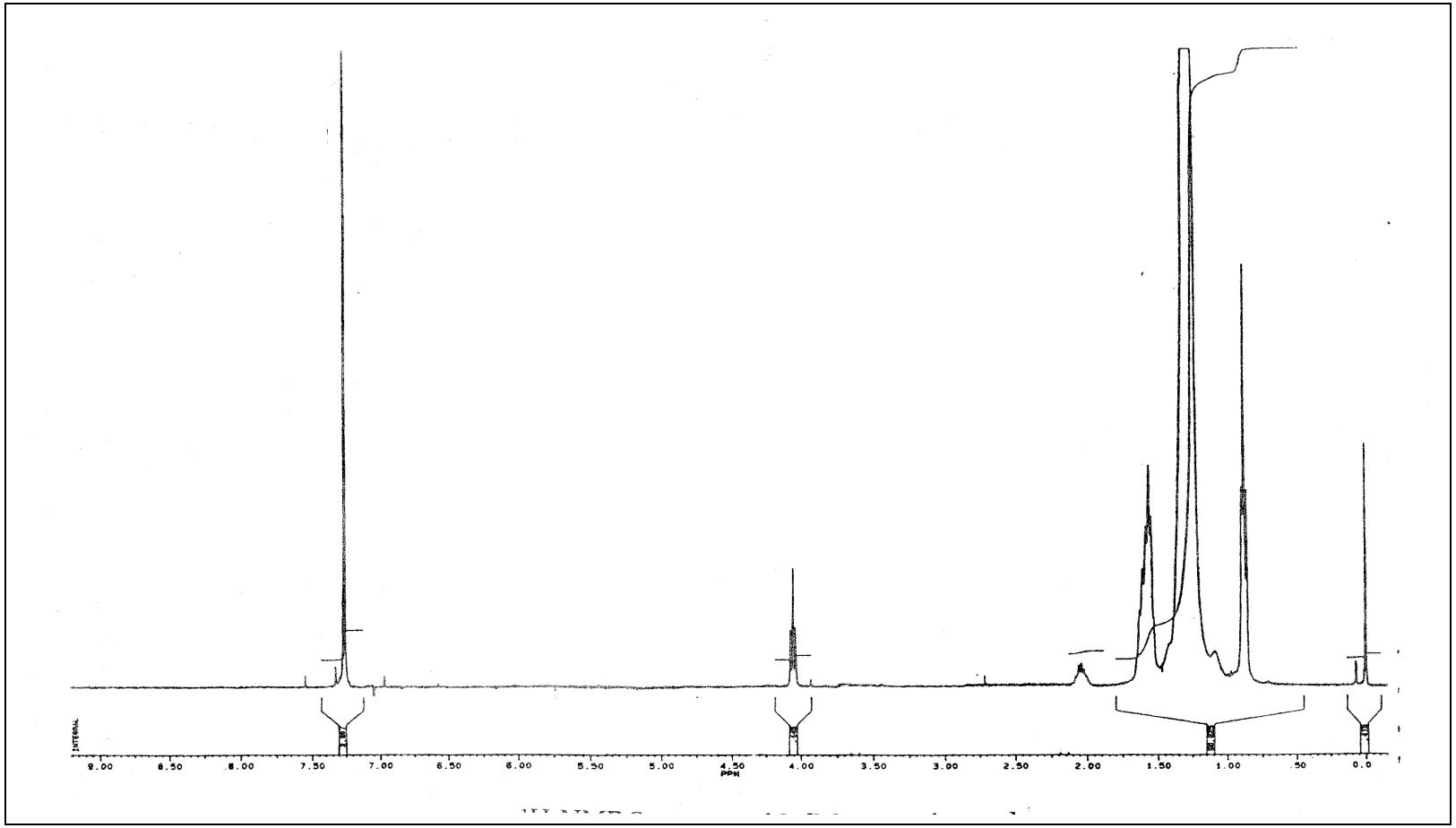


m/z 285

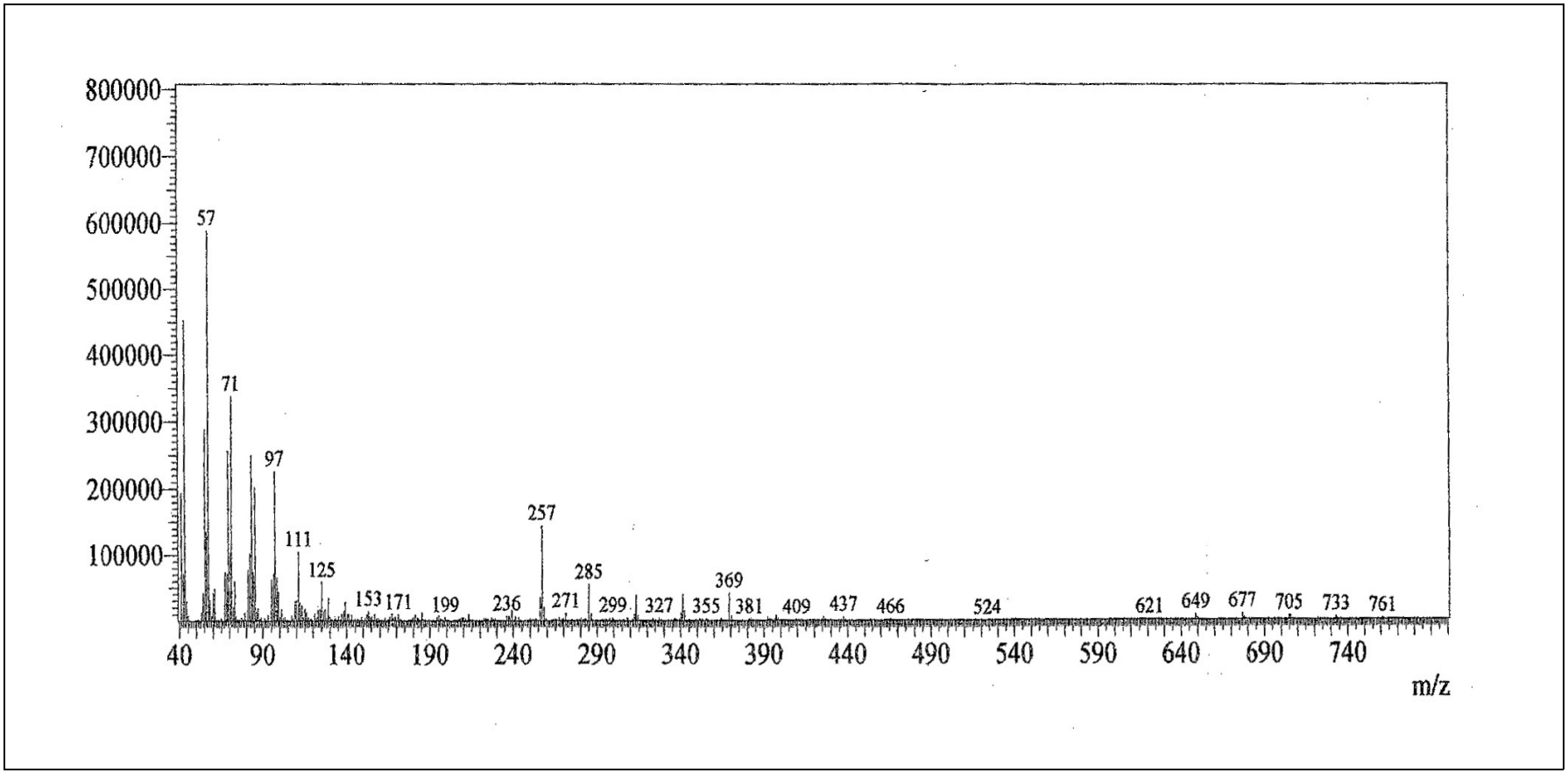
From the above fragmentation it can be concluded that the compound present in MG1 are esters of $CH_3(CH_2)_{14}COOH$ (palmitic acid) and $CH_3(CH_2)_{16}COOH$ (stearic acid)[10].



IR Spectrum of MG1 (Mixture of esters)



^{1}H- NMR Spectrum of MG1 (Mixture of esters)



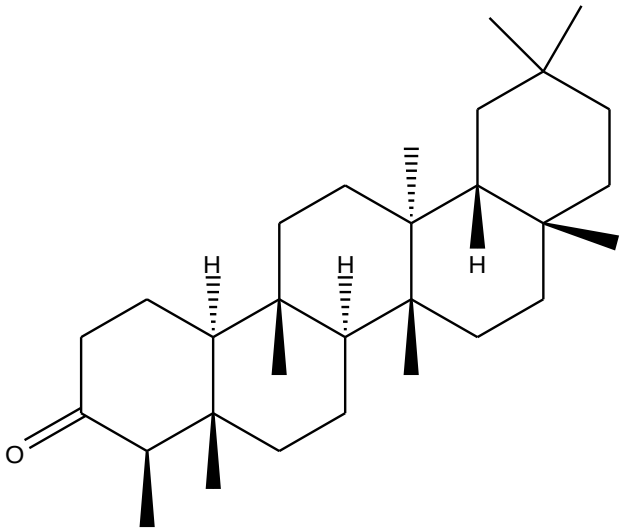
Mass Spectrum of MG1 (Mixture of esters)

1.8.2. Characterisation of MG2 and SM2 (Friedelin)

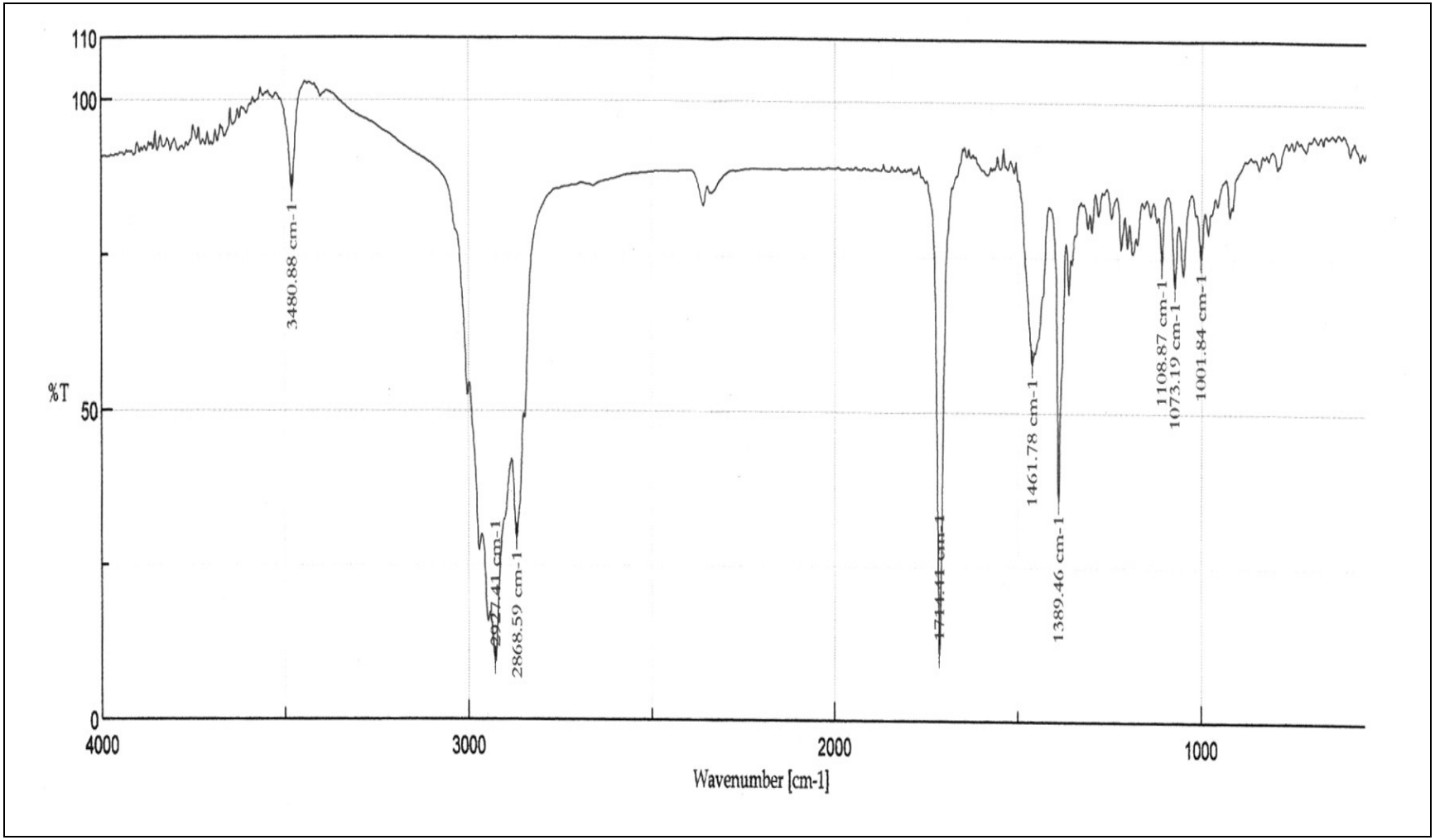
Compound MG2 (m.p. 245^0C) was isolated from petroleum ether extract and was recrystallised from 1:1 petroleum ether – acetone mixture. SM2 (m.p. 245^0C) was isolated from acetone extract of the plant and was recrystalised from 1:1 petroleum ether – acetone mixture. These two compounds answered Liebermann- Burchard reaction showing a persistent pink colour typical for triterpenes[11]; also they gave a blue colour with vanillin – H_2SO_4 and appeared as pink spots with anisaldehyde –H_2SO_4 reagent. They gave orange colour with 2,4-Dinitrophenylhydrazine reagent, indicating the presence of carbonyl group in them. They didn't decolourise Bayers reagent, indicating saturated nature of the compound. TLC comparison revealed that these two compounds (MG2 & SM2) were identical. Their mixed melting point was undepressed and IR spectra were identical.

In the mass spectrum of this compound the molecular ion appeared at m/z 426. Its ^{13}C NMR spectrum indicated the presence of thirty carbon atoms. Presence of a carbonyl oxygen, probably that of a ketone, was evident from the IR spectrum (a strong absorption at 1714.14cm^{-1}). Also the absence of olefinic linkage was evident from its reaction with Bayers reagent (as given above). When these data were put together we arrive at a molecular formula $C_{30}H_{50}O$. This formula suggested six equivalents of hydrogen deficiency. One of them is represented by carbonyl oxygen and the remaining five by a

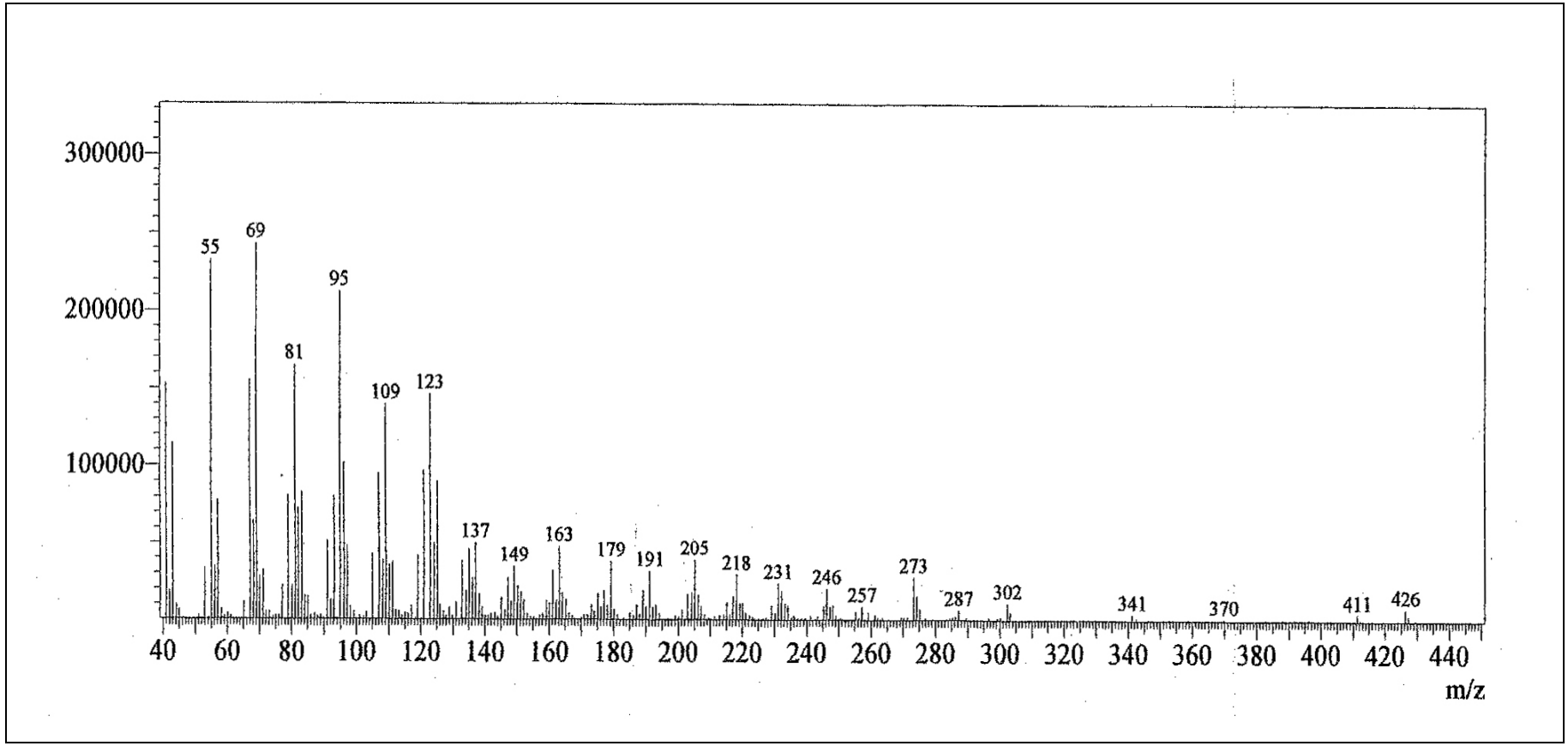
pentacyclic ring structure. The ^{1}H NMR spectrum showed the presence of seven tertiary methyl groups and one secondary methyl group. Test with LB reagent was positive for a triterpenoid. So it can be concluded that the compound under investigation was a pentacyclic triterpenoid containing a keto group. From its melting point (245^{0} C) and molecular formula ($C_{30}H_{50}O$), one possible structure is that of friedelin.



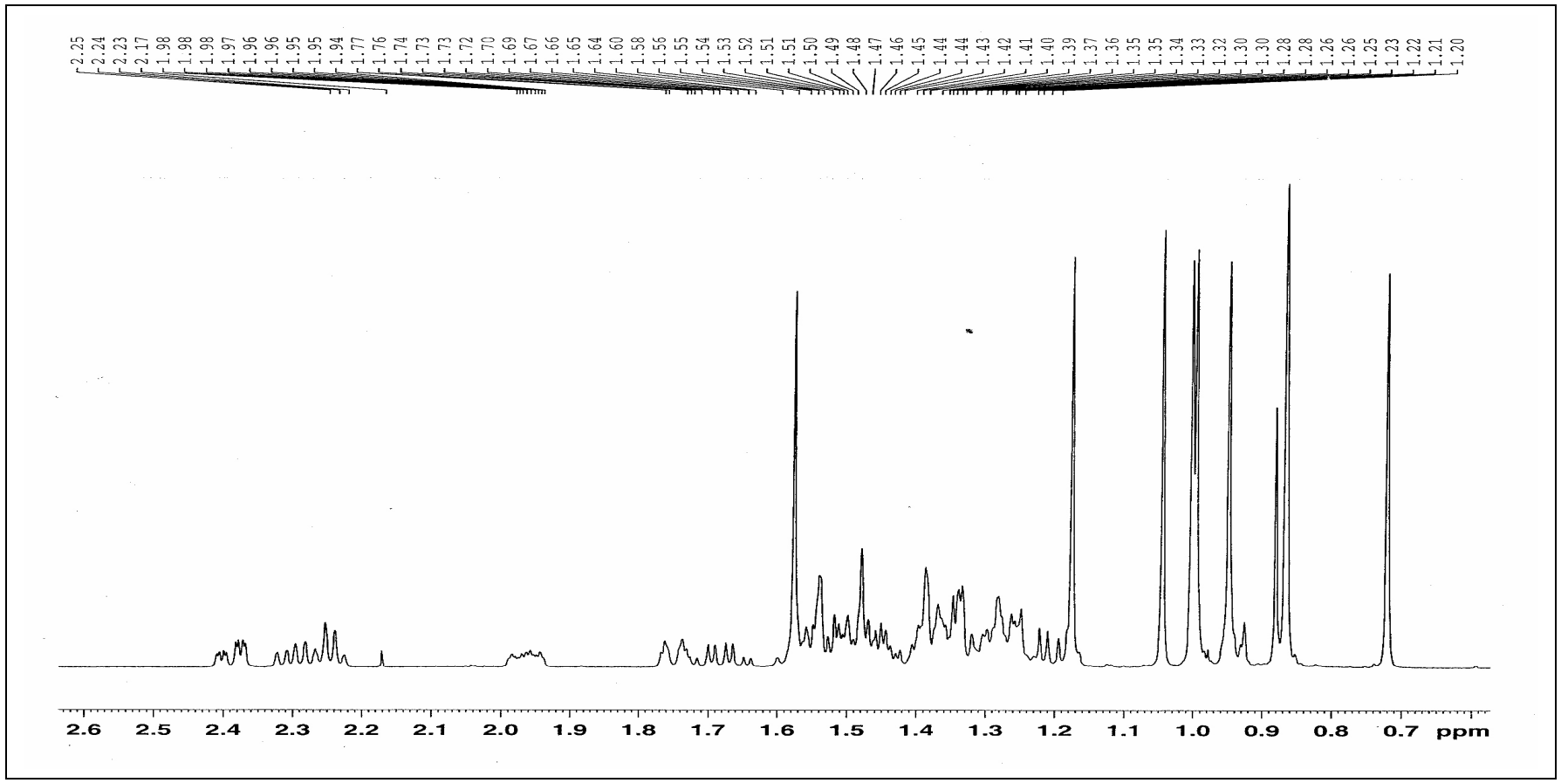
Friedelin



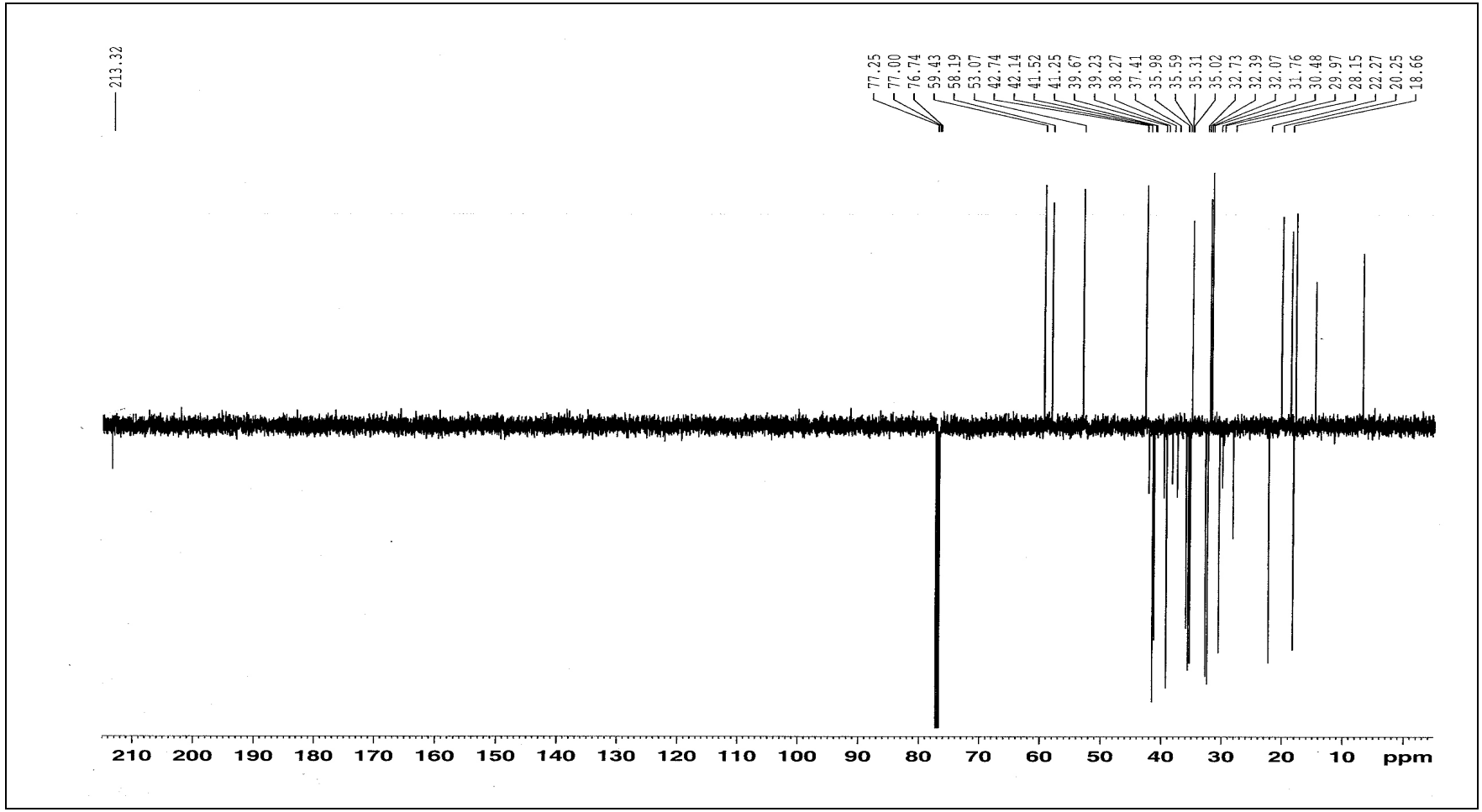
IR spectrum of MG2/SM2 (Friedelin)



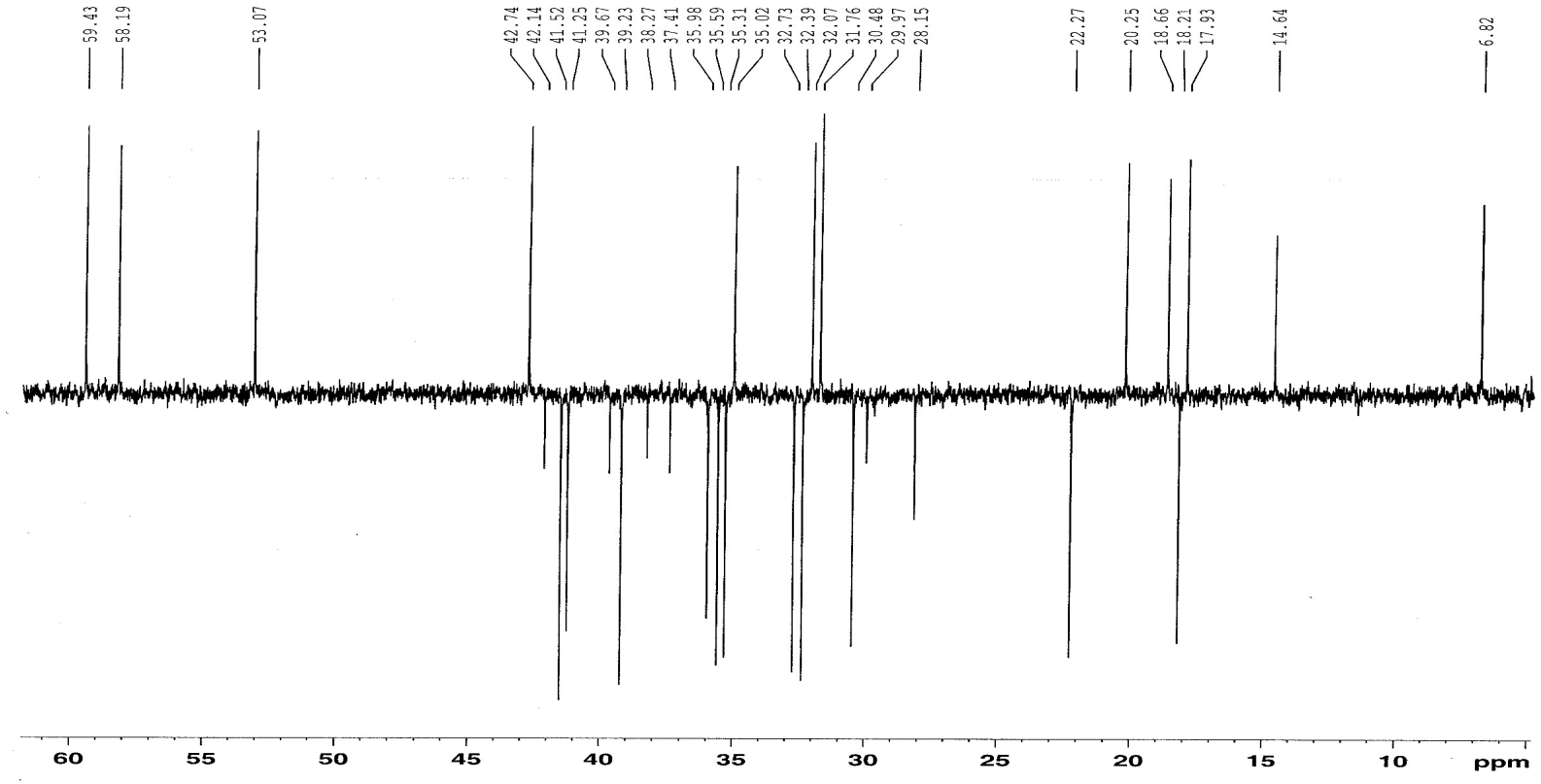
Mass spectrum of MG2/SM2 (Friedelin)



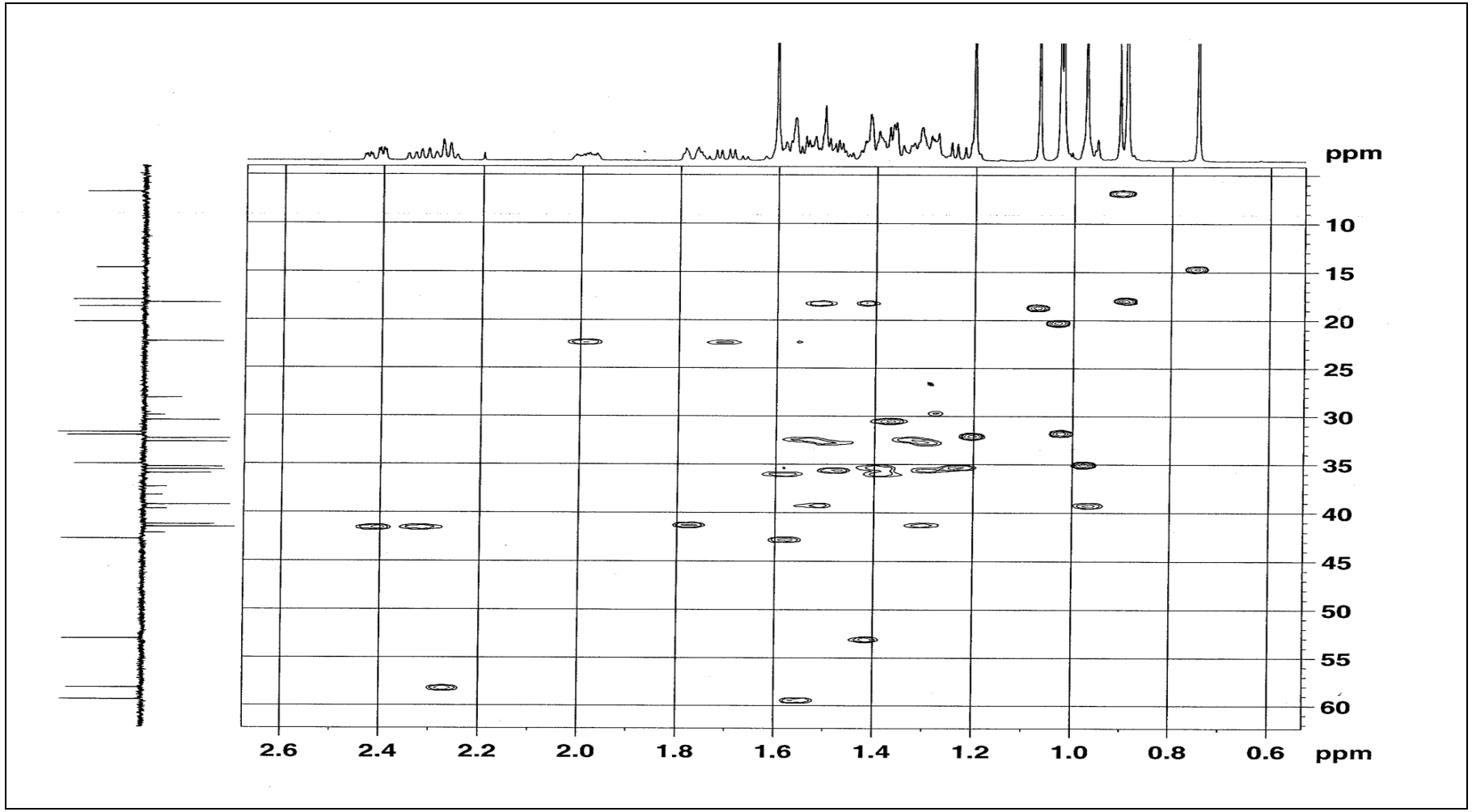
^{1}H-NMR spectrum of MG2/SM2 (Friedelin)



^{13}C-NMR APT spectrum of MG2/SM2 (Friedelin)

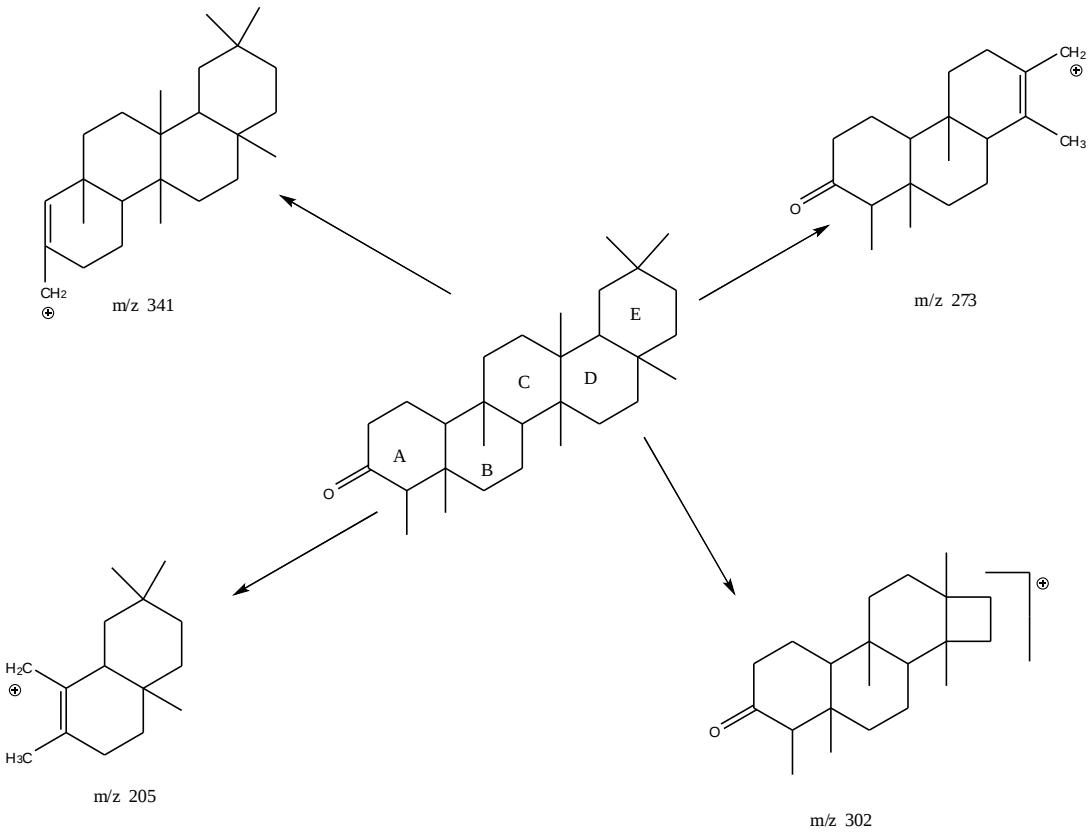


^{13}C-NMR APT (expanded) spectrum of MG2/SM2 (Friedelin)



HSQC spectrum of MG2/SM2 (Friedelin)

Mass spectral fragmentation pattern was quiet typical of pentacyclic ring structure, containing seven methyl groups in rings other than A (m/z 341). The mass peak at m/z 273 indicated that the carbonyl oxygen is present in ring A, B or C. Another peak at m/z 302 was proof for the presence of three methyl groups in ring E[11]. In the mass spectrum peak at m/z 341 originated by the loss of ring A, the peak at m/z 302 by the loss of ring E, and the peak at m/z 273 by the loss of ring D and E. Possible structures for the selected fragment ions in the mass spectrum of compound MG2/SM2 (friedelin)[12,13] is given below;



The above structure is supported by the APT spectrum which contains twelve positive peaks (eight CH_3 and four CH groups) and eighteen negative peaks (seventeen CH_2 groups and one quaternary carbon). HSQC spectrum showed cross peaks between the methyl proton at δ 0.72 and carbon at δ

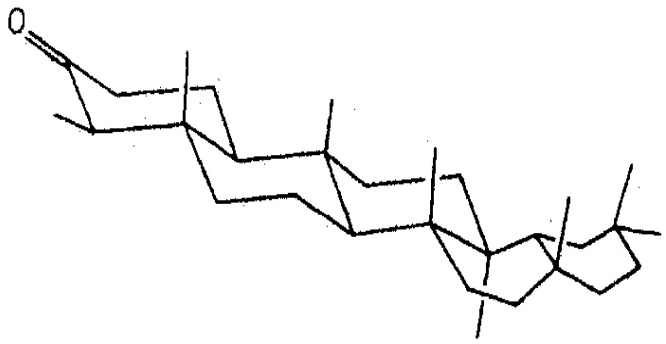
14.63 (C-24), methyl proton at δ 0.87 and carbon at δ 17.93 (C-25), methyl proton at δ 0.95 and carbon at δ 35.02 (C-30), methyl proton at δ 1.00 and carbon at δ 20.25 (C-26), methyl proton at δ 1.00 and carbon at δ 31.76 (C-29), methyl proton at δ 1.05 and carbon at δ 18.66 (C-27), methyl proton at δ 1.18 and carbon at δ 33.07 (C-28). The secondary methyl group (C-23, δ 6.82) appeared as a doublet at δ 0.875. One peak of the doublet overlapped with tertiary methyl proton at δ 0.87.The ^{13}C NMR spectrum of the compound exhibited 30-carbon resonances which compared well with the reported values of friedelin[14] [Table 1.3]

Table 1.3

Carbon atom No	**^{13}C NMR shifts of the compound**	**^{13}C NMR shifts from literature[14]**
1	22.27	22.3
2	41.52	41.5
3	213.35	213.2
4	58.19	58.2
5	42.14	42.1
6	41.25	41.3
7	18.21	18.2
8	53.07	53.1
9	37.41	37.4
10	59.43	59.4
11	35.59	35.6
12	30.48	30.5

13	39.67	39.7
14	38.27	38.3
15	32.39	32.4
16	35.98	36
17	29.97	30
18	42.74	42.8
19	35.31	35.3
20	28.15	28.1
21	32.73	32.7
22	39.23	39.2
23	6.82	6.8
24	14.64	14.6
25	17.93	17.9
26	20.25	20.2
27	18.66	18.6
28	32.07	32.1
29	31.76	31.8
30	35.02	35

From methyl to methyl nuclear overhauser enhancements Francisco Radler and coworkers arrived at the conformation (I) for friedelin, which contain boat conformation for ring D and E[15].



(I)

1.9 CONCLUSION

The triterpenoid compound friedelin was isolated and identified along with long chain esters from *Cissus gluaca*. Friedelin is found to be present in many plants[16-20]. *Cissus gluaca* grown in Andrapradesh had been subjected to chemical investigation by Remiah and co- workers[8]. However they could not isolate friedelin from this plant. Presence of friedelin in *Cissus gluaca* grown in Kerala can be due to regional and climatic variations.

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CHAPTER II

ANALYSIS OF THE ESSENTIAL OIL OF THE ROOTS AND LEAVES OF THE MEDICINAL PLANT *KAEMPFERIA GALENGA* L. (*ZINGIBERACEAE*)

SECTION 1

GENERAL INTRODUCTION

ESSENTIAL OIL

In our daily life we come across the fruits, flowers, leaves, stems, barks and roots of nearly all 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. Essential oils are highly volatile substances isolated by a physical process from an odoriferous plant. The oil bears the name of the plant from which it is derived; for example, rose oil or peppermint oil. Such oils were called essential because they were thought to represent the very essence of odour and flavour. They have been known and traded since ancient times. Many essential oils contain isoprenoids. Some, such as oil of winter green (methyl salicylate) and orange oil (Limonene), have one predominant component, but most have dozens or hundreds. Trace components impart characteristic odour, which synthetic or blended oils can rarely duplicate. The essential oils are volatile liquids, mostly insoluble in water, but freely soluble in alcohol, ether, and vegetable and mineral oils. They are usually not oily to the touch[1].

Essential oils have three primary commercial uses; as odorants in perfumes, soaps, detergents and other products; as flavours in baked foods, candies, soft drinks and many other foods; and as pharmaceuticals, in dental products and many medicines.

Occurrence

Essential oils come from the flowers, fruits, leaves, roots, seeds, and bark of many plants. Oil of lavender, for example, is derived from flowers, oil of patchouli from leaves, and oil of orange from fruits. The oils are formed in the green (chlorophyll-bearing) parts of the plant, and with plant maturity are transported to other tissues, particularly to the flowering shoots. Essential oils are found in the vegetable structures to which they give their characteristic odour and are intimately connected with the vital processes that take place in plants. In plants, they may be formed by the hydrolysis of certain glycosides or directly by protoplasm or by decomposition of the resinogenous layer of the cell wall. Inside the vegetable cells, the essential oils are contained in the "vacuoles", cavities of roundish form bound by a single membrane, the *tonoplast*, and containing an aqueous solution full of a juice, the "vacuolar juice". The vacuole is a cellular organelle, probably originating from the endoplasmic reticulum, into which the "secondary products" or the products of refusal of the metabolism are poured.

Depending on the plant family, the essential oil may be elaborated in different specialized secretory structures: in the *Apiaceae*, the oils are secreted in vittae, in the *Lamiaceae* they are elaborated in the glandular hairs, in the *Hyperycaceae*, in the *Myrtaceae* and in the *Rutaceae* they are poured again into lisigenous or schizogenous pockets, in the *Pinaceae* and in the *Asteraceae* they are found again in the secretive tissues and so on.[2]

The function of the essential oil in a plant is not well understood. Odours of flowers probably aid in natural selection by acting as attractants for certain insects. Leaf oils, wood oils, and root oils may serve to protect against plant parasites or depredations by animals. Oleoresinous exudations that appear when the trunk of a tree is injured prevent loss of sap and act as a protective seal against parasites and disease organisms. Few essential oils are involved in plant metabolism, and some investigators maintain that many of these materials are simply waste products of plant biosynthesis.[3]

History of essential oils

The first records of essential oils come from ancient India, Persia, and Egypt; and both Greece and Rome conducted extensive trade in odoriferous oils and ointments with the countries of the Orient. Most probably these products were extracts prepared by placing flowers, roots and leaves in fatty oils. They have been found in three thousand years old tombs in the pyramids and early Greek physicians, including Hippocrates, mentioned aromatic plant

essences and oil massages for their healing and mood-enhancing qualities. The ancient civilizations of Mesopotamia, more than 5,000 years ago, had machines for obtaining essential oils from plants. The Romans associated essential oils and their fine aroma with wealth and success. Ayurveda, the world's oldest healing system, has long recommended essential oil massage as a health treatment for many conditions.

In most ancient cultures, odorous plants or their resinous products were used directly. Only with the coming of the golden age of Arab culture, was a technique developed for the distillation of essential oils. The Arabs were the first to distill ethyl alcohol from fermented sugar, thus providing a new solvent for the extraction of essential oils in place of the fatty oils that had probably been used for several millennia[3]. The knowledge of distillation spread to Europe during the Middle Ages, and isolation of essential oils by distillation was described during the 11th to 13th centuries. These distilled products became a speciality of the European medieval pharmacies, and by about 1500, the following products had been introduced: oils of cedarwood, calamus, costus, rose, rosemary, spike, incense, turpentine, sage, cinnamon, benzoin, and myrrh[4]. The alchemical theories of the Swiss physician and alchemist Paracelsus played a role in stimulating physicians and pharmacists to seek essential oils from aromatic leaves, woods, and roots.

The development of the modern perfume industry started in France, the world centre of perfumery[3]. ‘Perfumes are fragrant substances, generally of complex composition, which gratify the sense of smell’. ‘Perfumery is the art of production of perfumes by compounding fragrant substances and in association with cosmetics’.[6] A few decades ago perfumes were composed primarily from natural materials. Later, isolates obtained from them were also used for compounding perfumes. Today the variety of available natural perfume and flavouring materials is small compared to several thousands of synthetic materials. This apparent competition between natural and synthetic products has been the major cause of development of a large number of perfume and flavouring materials.

In many cases isolation of naturally occurring perfumery materials from essential oils has not been economical either because of the high cost of essential oils from which they are isolated or because of their occurrence in insignificant proportions in the oils. Hence attempts have been made to prepare them synthetically and in many cases they have been successful and gradually it extends to produce new odorous compounds which are not found in nature.[8]

But as far as flavours are concerned they are often created exclusively from synthetic raw materials; however, certain flavour types cannot be satisfactorily reproduced without the use of an overwhelming proportion of

natural oils and extracts with minute additions of safe and harmless, powerful, synthetic flavouring materials.[8]

Essential oils production in India

The art of perfumery is of great antiquity in India and many travellers of bygone days have referred to the exquisite perfumes produced in India from aromatic flowers, fruits, woods, roots, resins and grasses. Indian perfumes entered Europe in medieval times under the name of Arabian perfumes. India lost its dominant position as a supplier of high grade perfumes and aromatics towards the latter half of the nineteenth century[10]. They failed to keep pace with technological development in western countries and it gradually languished. It was only the early years of the present century that interest in aromatic chemicals and perfumes was revived and researches carried out in many institutes to bring the great aromatic wealth of the country. These researches paved the way for the establishment of gum rosin and turpentine industry and also the commercial production of many aromatic oils. The essential oils research committee set up by the Council of Scientific and Industrial Research in 1941 stimulated further research on the cultural as well as chemical aspects of aromatic plants.[7]

Commercial importance

Essential oils are generally expensive, with prices ranging from several U.S. dollars per kilogram on the low side to several thousand dollars per

kilogram. The high price of the natural oils coupled with their limited availability has encouraged a search for substitutes. Great progress has been made in the synthesis of individual components such as geraniol, citral, linalyl acetate, and the like. These synthetics have been combined with natural oils to extend supplies, and they have also been blended together in an attempt to duplicate the oils themselves. Such reconstituted oils usually lack certain of the odour notes of the natural products, because of the absence of trace ingredients, often unidentified, that may be present in the natural oils. They also tend to have a more “chemical” odour, because of trace impurities in the synthetics that are different from the components of natural oils[3].

2.1.2 METHODS OF PRODUCTION OF ESSENTIAL OIL

The first step in the isolation of essential oils is crushing or grinding the plant material to reduce the particle size and to rupture some of the cell walls of oil-bearing glands. Steam distillation is by far the most common and important method of production, and extraction with cold fat (enfleurage) or hot fat (maceration) is chiefly of historical importance.

Three different methods of steam distillation are practiced. In the oldest and simplest method a vessel containing water and the chopped or crushed plant material is heated by a direct flame, and the water vapour and volatile oil are recovered by a water-cooled condenser. This original method is being replaced by a process in which the plant material is suspended on a

grid above the water level, and steam from a second vessel is introduced under the grid. The volatiles are condensed and the oil is separated. In the third process, the vessel containing the plant material on a grid is heated to prevent condensation of steam, so that dry distillation is attained[4].

In southern France essential oils were extracted with cold fat long before the introduction of extraction with volatile solvents. This process is applied to flowers that do not yield an appreciable quantity of oil by steam distillation or whose odour is changed by contact with boiling water and steam. In this process, flowers are spread over a highly purified mixture of tallow and lard and are left for a period varying from 24 hours to 72 hours. During this time most of the flower oil is absorbed by the fat. The petals are then removed (defleurage), and the process is repeated until the fat is saturated with oil. The final product is called pomade[4] (*e.g.*, pomade de jasmine).

In most cases, it is possible to shorten the long enfleurage process by extracting the essential oils using molten fat for one to two hours at a temperature ranging from about 45° to 80° C (110° to 175° F). The fat is filtered after each immersion, and after 10 to 20 extraction cycles the pomade is sold as such, or it may be extracted with alcohol to yield the oil residue.

Since both enfleurage and maceration are rather expensive processes, some essential-oil specialists have shifted almost completely to using volatile

solvents for the recovery of essential oils from plant materials that could not be processed by steam distillation. Petroleum naphthas, benzene, and alcohol are the primary solvents.

The procedure called expression is applied only to citrus oils. The outer coloured peel is squeezed in presses, and the oil is decanted or centrifuged to separate water and cell debris. The method is used for oil of sweet and bitter orange, lemon, lime, mandarin, tangerine, bergamot, and grapefruit. Much oil is produced as a by-product of the concentrated-citrus-juice industry[4].

The use of super critical carbon dioxide for the extraction of essential oils from plant materials has emerged out recently. The method has several advantages, highly volatile components can be preserved, and the process does not involve heating for the removal of solvent[5].

2.1.3 CHEMICAL COMPOSITION

Terpenes, organic compounds consisting of multiples of isoprene units (containing five carbon atoms), are by far the most dominant constituents of essential oils. Individual oils, however, may contain appreciable quantities of straight chain, aromatic, or heterocyclic compounds. Thus allyl sulfides are characteristics of oil of garlic, traces of indole and anthranilic acid esters are found in orange oil, straight chain alcohols and aldehydes are recognized in oil of violets, and phenols and other aromatic compounds are common to

many oils. Both hydrocarbons and oxygenated compounds such as alcohols, aldehydes, ketones, acids, esters, oxides, lactones, acetals, and phenols are responsible for the characteristic odours and flavours[5].

In some oils one or only a few components predominate: thus oil of wintergreen contains about 98 percent of methyl salicylate; orange oil, about 90 percent of *d*-limonene; bois de rose, 90 percent of linalool; and cassia, up to 95 percent of cinnamaldehyde. Trace components are very important, since they give the oil its characteristic natural odour.

2.1.4 ESSENTIAL OILS IN PRACTICE

Essential oils extracted from vegetable materials offer a great range of applications.

The action of essential oils is carried out particularly:

a) *On the skin*: Some products are able to cause reactions of the skin such as erythema, a light local anaesthetic action, or an increase in micro circulation. Notable application possibilities exist; for instance the use for the care of some rheumatic forms, for example, the arrest of hair loss due to infections and for the treatment of itching and cephalea.

b) *On the digestive apparatus*: Some aromatic substances when in contact with the mucous of the mouth induce an increase in salivation as a simple reflex action and, subsequently, an activation of peristaltic movements.

Diuretic, tonic, and sometimes antitoxic, functions are associated with this.

c) *On the blood circulation*: The tonic action of the camphor and vaso-constricting of the borneol are examples of specific activity.

d) *On the respiratory apparatus*: The high volatility of the essential oils facilitates the possibility of absorption into the body through the bronchitis and the lungs where they have sedative, balsamic and decongestant functions. For example, menthol is suitable in the infection of the primary respiratory tract acting as an antiseptic and local analgesic.

e) *On the nerve centers*: This activity is underlined by the sense of comfort and relaxation that derives from the olfactory perception of a Eau De Cologne or a perfume. The aromatic principles opportunely selected, if inhaled, can have cardio tonic, tonic, antispasmodic, exciting and narcotic effects on the central nervous system: eugenol has, for instance, a practical use in dentistry as local analgesic. Research into rosemary oil, that is used in popular medicine to treat states of exhaustion, have shown that the locomotive activity of rats significantly increases when they have been in environment in which rosemary oil or 1, 8-cineole (the main constituent of this oil) has been vaporized[11]. In popular medicine too lavender sap oil is used for its relaxing properties (due to the presence of the linalool and its esters) and tests on guinea–pigs have shown that motor activity is also

reduced by 80% when the guinea–pigs are kept in environments in which lavender oil or one of its major constituents has been vaporized.

Evidence suggests that plant essential oils possess strong antioxidant properties. Antioxidants could help the body in dealing with the destructive free radical propagation and the complex phenomenon of skin ageing. Antioxidative properties of essential oils and their skin penetrating characteristics should be of interest to the cosmetic industry particularly. For instance, a single essential oil could serve at the same time as fragrance, antimicrobial and antioxidant agent for particular cosmetic formulations could add great marketing value to new products.

In therapeutic use, pleasant aromatics can raise our spirits and address specific clinical symptoms. A few drops of lavender can aid insomnia and bergamot, chamomile, and sandal wood to be relaxing. The psychologically stimulating effect of jasmine, lemon, lemongrass, peppermint, and basil had also been reported. Other aromas found to be relaxing were rose and lavender. Sweet orange essential oil was found to be effective in both induction of anesthesia and recovery time in children.[12]

The germicidal properties are well known that they can either have ‘cidal’ effect if they kill micro organisms or have ‘static’ effect if they inhibit the growth of them.[13]

The essential oils are incorporated in products for external use such as creams, lotions, ointments for different purposes and they have different functions:

a) Superficial action, such as perfuming agents or as co-adjuvant of biologically active products; this is the most important aspect from the cosmetology point of view.

b) With deep penetration through the epidermis a therapeutic action on some organs. The essential oils are used as pulmonary disinfectants: their absorption by cutaneous applications introduces more advantages in comparison to that achieved through administration of inhalations. In the latter case, the product only reaches the bronchuses whereas the former case allows the attainment of hematic transport of the product to the pulmonary tissue where they can carry out its therapeutic action. Probably, the therapeutic activity of the oils is due to their lipophilic properties that allow them to interact with the lipids of cell membranes modifying the activity of the Ca^{++} channels.

Aromatherapy is the ancient art and science which has been used for over 3000 years for its healing, rejuvenating, relaxing and invigorating benefits on the mind, body and spirit. The sense of smell is the fastest route to the brain to create effects; aromas we like lift our mood and promote a sense of well being. It is a healing art that aims to rejuvenate body, mind and

spirit.[10] The different smells (aromas), and the chemical constituents of the oils, are said to produce different emotional and physiological reactions. They can be massaged into skin, added to bath water or vaporized in an oil burner. Through inhalation and transdermal absorption, essential oils produce physiological changes in all the complex systems of the human body.

The most important suggested area of essential oils in therapeutic applications are in urology, dermatology, sleep and nervous disorder, laxatives, erosive gastritis, cardiac, and vascular systems, colds and coughs as well as antiparasitic. Apart from their use as therapeutics, antibiotics, germicides, insecticides, flavour and fragrance industry, essential oils are used to mask odours or to scent insecticides, spray and all products used to clean environments.

2.1.5 ANTIMICROBIAL PROPERTIES

The main features of all essential oils besides the fragrance are their antimicrobial properties. All essential oils have been proved to possess both antibacterial and antifungal activity, although some are more active than others. The effectiveness differs depending on the oil and is strictly linked with its chemical composition, but it is always dose dependent. Terpenoids are considered to be the main group of essential oil constituents responsible for their antimicrobial activity. Terpenoids may serve as an example of lipid soluble agents which affect the activities of membrane-catalyzed enzymes, for

example their action on respiratory pathways. Specific terpenoids with functional groups, such as phenolics, alcohols or aldehydes, also interfere with membrane-integrated or associated enzyme proteins, stopping their production or activity[12]. All these chemical reactions at the metabolic level lead to the growth inhibition of the sensitive microorganisms.

When essential oils are used as flavouring agents in food and beverages as well as in perfumery and cosmetics, their antimicrobial activity is displayed twice. Firstly, essential oils protect the products against microbial contamination by delaying the onset of spoilage or by inhibition of the growth of pathogens. Secondly, when consumed as food additives or applied as cosmetic ingredients essential oils can beneficially act on human health.

The food and cosmetic industries have tended to reduce chemical preservatives in their products due to increasing pressure from consumers and replace chemicals by natural products. Essential oils are to be an excellent alternative for synthetic preparations and that is the reason for an extensive assessment of their antimicrobial activity. The antimicrobial and antifungal activities of essential oils have greatest importance in food and perfumery industries as well as in medicine.

Essential oils used in combination with synthetic antibiotics could be a great benefit because it could reduce environmental pollution and prevent from antibiotic– resistant strain formation.[9]

2.1.6 ANALYTICAL METHODS

The efficient analytical methods applied to the analysis of essential oils can be classified into two different groups.

First the Chromatographic methods like gas Chromatography (GC), high–performance liquid chromatography (HPLC) and supercritical fluid chromatography (SFC), including multidimensional and chromatographic coupling techniques resulting in the separation of individual components.

The second is the hyphenated techniques, that means instrumental on-line coupling of chromatographic separation devices to spectrometers, like coupling of GC with Mass spectrometry (MS), GC-quadrupole mass spectrometry with electron ionization (EI) or with Chemical ionization (CI), GC-ion trap tandem MS, Gas chromatography with Fourier transform infrared spectrometry (GC-FT-IR), Linked GC-FT-IR-MS, GC-UV or Gas chromatography with atomic emission spectroscopy (GC-AES), GC-NMR as well as coupling of HPLC with MS. The advantages of these techniques is that more information about the structure of the separated components and its identification. The hyphenated methods are the powerful and pragmatic tools for identifying components of complex mixtures.

Odour or colour comparison was the early methods used for the characterisation of essential oils. Specific gravity, refractive index, distillation range, iodine number were then used for characterisation. Now the modern methods of gas chromatography and allied techniques have been employed for the determination of the volatile components present in the essential oils.

Gas chromatography (GC)

Chemical analysis of essential oils is generally done using GC (quantitative analysis) and GC-MS (qualitative analysis). Identification of the main component is carried out by the comparison of both the GC retention times and MS data against those of the reference standards with known source. Sometimes, identification by GC-MS must be confirmed by retention indices (Kovats Indices) on two columns of different polarity; and claims for the identification of new constituents should be supported by co-injection with authentic compounds. Recently some 900 Kovats' indices of 400 individual compounds were summarized from the general literature.[11]

The principle of GC is the differential distribution of the components between two phases (one stationary and the other mobile). The mobile phase (carrier gas) usually is nitrogen. Depending on the nature of the mixture Ar, He, H_2 are also used. The stationary phase may be solid or liquid. Nowadays liquid stationary phase is more in use.

According to the nature of the stationary phase, Gas Chromatography can be divided into two classes. If the stationary phase consists of silica, alumina or carbon, the chromatography is termed as gas solid chromatography (GSC) and if the stationary phase is a non volatile liquid held as a thin layer on a solid support, then the technique is known as gas liquid chromatography (GLC). The most common support used in GLC is diatomaceous earth or kieselguhr. Because of tailing caused by nonlinear adsorption isotherms in GSC, GLC has now become the most important and widely used technique. The availability of versatile and specific detectors and the possibility of coupling the gas chromatograph to a mass spectrometer or an infrared spectrophotometer further enhance the usefulness of gas chromatography.

The main advantages of gas chromatography in analysis are, the technique has strong separation power and even quite complex mixtures can be resolved into constituents. The sensitivity of the method is quite high. It is a micro-method and only a few milligrams of sample are enough for analysis. The speed of analysis is quite fast, it gives good precision and accuracy. It involves relatively simple instrumentation, operation of a gas chromatograph and related calculations do not require highly skilled personnel and thus the technique is very suitable for routine analysis. The cost of equipment is relatively low and its life is generally long.

a. Instrumentation

Basically all gas chromatographs, whether GSC or GLC consist of four basic components

i) Carrier Gas

ii) Sample injection system

iii) The column

iv) One or More Detectors with appropriate read out.

i) Carrier gas

The carrier gas is allowed to flow through the system, carrying the sample in the vapor state through the column. For selecting a carrier gas the following considerations should be taken into account; a) it should be chemically inert b) it should be suitable for the detector employed and the type of sample analysed c) it should be available at low cost d) it should be readily available in high purity e) it should not cause the risk of fire and explosion hazard. f) it should give best column performance consistent with required speed of the analysis.

ii) Sample injection system

The carrier gas is connected from the gas reservoir to the sample port injector. The sample must be converted into vapour state. The injection port is heated to a temperature which will ensure rapid vapourisation but not thermal

degradation of the solute. A solid sample may be dissolved in a suitable solvent and injected as solution. The solute vapour mixes instantaneously with the flowing carrier gas and is swept into the column.

iii) Columns

In the column, the different components in the vapourised samples are separated from each other by virtue of their different interaction with the column packing. Two type of columns are commonly employed in GLC, the capillary column and packed column.

Capillary column is fabricated from capillary tubing, the bore of which is coated with a very thin film of the liquid phase. Since packing provides the basis for separation process, column packing is very important. Mostly capillary columns with dimethylpolysiloxane (Methylsilicone: non-polar) and carbowax 20M polar phases are used. Carbowax 20M phases include DB-Wax, BP-20, PEG 20M and HP 20, while methylsilicone phases include SE-30, SF-96, OV-1, Ovlytical101, BP1, CPSIL-5CB, SP 2100, DB 1, DB 5 and HP1. Among these fused-silica capillary GC columns DB 1 or DB 5 and CPSIL 5 are mostly preferred for essential oils.[12]

The column container for packed column is usually stainless steel, copper, nickel or glass. Inner diameters may range from 1.6 to 9.5 mm. These columns are packed with either a solid substrate (GSC) or a liquid coating on an inert solid support (GLC).

The solid inert support:

The main role of the solid phase support is to provide support to the thin uniform film of liquid phase. The most important requirements of a solid support are

a) It should be porous and hence have a large surface area.
b) It should be capable of providing good mechanical strength.
c) It should consist of small, uniform and spherical particles.
d) It should be chemically inert at elevated temperatures.
e) It should be readily wetted by the liquid phase to give a uniform coating.

Various supports have been fashioned from powdered Teflon, alumina, carborundum and micro glass beads, but the most common one is kieselguhr. Kieselguhr or diatomaceous earth is a form of hydrated silica containing many hydroxyl groups on its surface. This can serve as sites to which solute molecules can be absorbed.

The stationary liquid phase:

For a satisfactory separation, the most important requirements of a liquid phase are

i) It should be a good solvent for the components of the sample.

ii) It should be thermally stable.

iii) The solvent power of the liquid phases should be different for each components of the sample.

iv) It should be chemically inert towards the sample

v) It should be of low volatility, its boiling point should be at least 200 ^{0}C higher than the maximum operating temperature for the column.

No single liquid meets all these requirements. Among solutes of similar polarity the elution order usually follows the order of boiling points. When there is sufficient difference in boiling point, a very clean separation is achieved. Solutes having almost identical boiling points, but different polarities require a liquid phase that will selectively retain one or more of the components by dipole interaction or adduct formation.

iv) Detectors

Any physical property, which varies widely from one gas to another and which can be easily monitored form the basis of the detector. Based on these physical properties, detectors are of various types. These include thermal conductivity detector (TCD), flame ionisation detector (FID), electron capture detector (ECD), thermionic emission cross section detector,argon ionisation detector, gas density balance, microwave excited discharge detector etc. Some other main detectors offered by commercial manufactures are helium ionisation, flame thermo couple, piezoelectric absorption detector,

argon triode detector, micro detector, and photo-ionisation detectors, sulphur chemiluminescence detector etc.[17].

Thermal conductivity detector (TCD) or Katharometer (KCD)

The thermal conductivity detector responds to all types of organic and inorganic compounds including those not detected by FID. Further, it does not destroy the eluted components and therefore it is suitable for preparative work. It is however less sensitive than the FID with a minimum detection limit of 10^{-5}g.

The principle is based on the rate of heat loss from a heated wire placed in a gas stream (made of Pt or W), which depends on the thermal conductivity of the gas, so the temperature of the wire changes, consequently the resistance.

Two similar filaments are placed in a balanced Wheatstone bridge with pure carrier gas flowing over one of them and the effluent gas from the chromatographic column through the other. The change in the composition of the effluent gases disturbs the balance of the Wheatstone bridge.

Flame ionistion Detector(FID)

The ionization detectors are based on the electrical conductivity of gases. So the flame ionization detector is the most widely used and generally applicable detector for gas chromatography. With a burner (H_2-air flame), the

effluent from the column is mixed with H_2 and air and then ignited electrically. Most organic compounds, when pyrolysed at the temperature of a H_2/air flame, produce ions and electrons that can conduct electricity through the flame. The resulting current ($\simeq 10^{-12}$A) is then directed into a high impedance operational amplifier for measurement.

FID exhibits a high sensitivity, low noise and is easy to use. The ionization of carbon compounds in a flame is a poorly understood process, although it is observed that the number of ions produced is roughly proportional to the number of reduced carbon atoms in the flame. The FID responds to the number of carbon atoms entering the detector per unit time. It is a mass sensitive, rather than a concentration sensitive device.

b. Basic parameters

b.i. Retention time (t_R)

The time required for the maximum for solute peak to reach the detector in a gas chromatographic column is called retention time. Or the time lapsed between the sample introduction and appearance of peak maxima.

b.ii. Adjusted retention time (t'_R)

It is the difference between retention time and gas hold up time.

$t'_R = (t_R - t_M)$

b.iii. Gas hold up time (t_M)

It is the retention time of a solute (usually air) that has no affinity for the stationary phase.

b.iv. Retention volume (V_R)

It is the volume of gas required to carry a component through the column.

Retention volume $V_R = t_R F_c$

where t_R is the retention time and F_c is the volume flow rate of the gas to carry a component through the column.

Experimentally, the retention volume is calculated from the product of retention time, t_R, and gas flow rate at the column outlet adjusted to column temperature 'F_c'. An additional correlation factor 'J' is applied in order to correct the gas volume for co-compressibility in the column.

b.v. Adjusted retention volume (V'_R)

It is the difference between retention volume and gas hold up volume.

$$V'_R = V_R - V_M$$

But,

$$V_M = t_M.F_C \quad \text{and} \quad V_R = t_R.F_C$$

$$\therefore \quad V'_R = (t_R - t_M) F_C$$

Subscripts R and M refer to species that are retained and moved in the column.

b.2. Retention indices

In gas chromatography the component is identified by comparing the retention data of the analyte with standard data. Usually the relative retention or retention index (I) is taken as comparison. The most useful system of retention indices is the one due to Kovats. It takes advantage of the linear relation between the logarithms of the adjusted retention times of a homologue series of n-alkanes and the number of carbon atoms in the molecules. 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 time 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

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

Where Z is the number of carbon atoms of the n-alkane.

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 atom in the molecule multiplied by one hundred. Retention data are generally expressed in terms of Kovats retention indices RI.[18] The indices indicate where compounds will appear on a chromatogram, with respect to straight chain alkanes injected with the sample. By definition the retention index for a normal paraffin is 100 times the number of carbon atoms in the compound regardless of the columns used or the chromatographic conditions.

Gas chromatography - mass spectrometry (GC-MS)

GC-MS is an established technique for the analysis of complex mixtures, holding a prime position in analytical chemistry because of its combination of sensitivity, wide range of applicability and versatility. The technique is capable of obtaining mass spectra of a few picograms or nanograms of each component. Mass spectrometry is a powerful method for identifying pure substances, but the mass spectra of mixtures are too complicated to be useful. Therefore combined gas chromatography with mass spectrometry provides a very effective tool for the qualitative characterization of complex mixtures by exploiting first the resolving power of GC to obtain the pure component in a flowing stream and then the strength of mass spectrometry to identify those separated compounds. In a mass spectrum the m/z values are plotted against relative abundances, arbitrarily assigning the

most abundant ion in the spectrum as 100 percent. This is the base peak in the spectrum.

SECTION 2

ANALYSIS OF THE ROOT ESSENTIAL OIL OF *KAEMPFERIA GALENGA* L.

2.2.1 INTRODUCTION

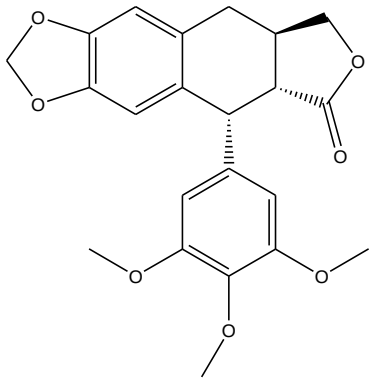
Kaempferia galenga L. (Syn. *Alpinia sessilis*) belongs to *Zingiberaceae* family. It is native to India and cultivated throughout India, Malesia, Africa, Java, China and Sri Lanka.[19-26] The plant is commonly known as Kacholam in Malayalam and Kechule kelangu in Tamil[27]. It is a handsome glabrous perennial aromatic herb with very fragrant under ground part. The under ground rhizome 2-3 x 1-2 cm crowded, strongly aromatic, root numerous, spherical white tubers. Leafy shoot stemless almost horizontal, near the ground. Leaves few, 2 or 3 lamina, 10-15 x 6-10 cm broadly ovate to orbicular, base rounded to sub cerdate, tip broadly pointed, upper surface dark green, glabrous, lower surface pale green white with violetish tinge towards the tip densely hairy. Inflorescence sessile, 6-12 or more flowered, enclosed within 1.5 – 3.5 cm long imbricating leaf sheaths white with light green tip. One flower opens at a time, flowering season June to July. Corolla tube 2.5 cm long. Connective of anther produced into a quardrate 2-lobed appendage, fruits oblong-3-celled and 3-valved capsules, seed arillate.[27-28]

2.2.2 MEDICINAL PROPERTIES AND USES

Kaempferia galanga is a reputed remedy for respiratory aliments like cough, bronchitis and asthma. It is also good for skin diseases, rheumatism, leprosy, ulcer, helminthiasis and fever.[20, 22, 26, 27] The powder prepared from the rhizome is mixed with honey and given for cough and pectoral affections.[26] The tuber is boiled in oil and applied externally for blocking of nasal tract. *Kaempferia* species is also used as a food additive.[24] The rhizome of *K. galanga* is widely used in the ayurvedic system of medicine in the treatment of various inflammatory diseases, diabetes mellitus, and obesity[29]. The alcoholic extract of *K galanga* show wound healing activity in Wister rats[30].

2.2.3 PREVIOUS WORK

Deoxypodophyllotoxin[23], ethyl *trans*-cinnamate and ethyl *trans*-p-methoxycinnamate[31] were isolated from rhizome[22]. The known compounds of rhizome (ethyl *trans*-p-methoxycinnamate and ethyl *trans*-cinnamate), show monoamine oxidase inhibiting[32] and larvicidal effects.[22-24] Deoxypodophyllotoxin ($C_{22}H_{22}O_7$) showed cytotoxic activity by inhibiting HeLa cells.[23]



Deoxypodophyllotoxin

2.2.4 PRESENT WORK

There is only one reported work on the compounds of the essential oil of *K. galanga* from Malaysia with the aim to detect constitutents responsible for the medicinal effects.[25] The work so far reported on the essential oil composition of *K. galanga* was few while essential oil studies on *Alpinia galanga* and *A. officinalis* are available.[31-35] Until now no investigation report on the volatiles of *K. galanga* root and leaf essential oil from South India was available. The present work was aimed to examine the essential oil composition of roots and leaves of *K. galanga* using GC-FID and GC-MS.

2.2.5 MATERIALS AND METHODS

Kaempferia glanga roots were collected from Calicut University surroundings in spring 2000 and identified by Dr. A.K. Pradeep, Department of Botany, Calicut University, Kerala. The fresh 300g rhizomes were ground into a paste using an electric grinder. The essential oil was obtained by steam distillation of the paste for 3 hours. The distillate was extracted with diethyl

ether and dried using anhydrous sodium sulphate. After evaporation of the ether under vacuum a yield of 0.6 g (0.2%) light yellow coloured essential root oil was obtained.

The GC-FID analyses, were carried out using a GC-14 A with FID and the integrator C-R6A-Chromatopac (Shimadzu Co. Japan). GC 3700 with FID (Varian Co., Germany) and the integrator C-RIB-Chromatopac (Shimadzu Co., Japan); carrier gas: Hydrogen; injector temperature: 250°C; detector-temperature: 320°C; temperature-programme: 40°C/5 min. to 280°C/5 min. with a heating rate of 6°C/min.; Column: 30 m x 0.32 mm bonded FSOT-RSL-200 fused silica (film thickness: 0.25 μm, Biorad Co., Germany) and 30 m x 0.32 mm bonded stabil wax (film thickness 0.50 μm, Restek Co., USA); quantification by percentage peak area calculations and compound identification partly by retention time correlations according to refernces.[36-40]

Gas chromatography-Mass spectrometry analysis

For GC-MS analysis GC-17A with a QP 5000 (Shimadzu Co., Japan) and the data system Compaq-ProLinea (USA, Class 5k software), then a GC HP5890 with a HP5970-MSD (Hewlett-Packard Co., USA) and the PC Pentium, a GCQ (Finnigan – Spektronex Co., Germany) with the Gate way–2000-PS75 data system were used. Carrier gas: helium; injector- temperature: 290°C; interface-heating: 300°C; ion source-heating: 200°C; EImode: 70 eV; Scan range: 41-450 amu; other parameters are same as

GC-FID part. Compound identification was achieved by mass spectra correlation with Wiley – NBS, NIST and library data as well as published spectral data.[37, 41, 42]

2.2.6 RESULTS AND DISCUSSION

The root essential oil of *K. galanga* was olfactorically evaluated and the odour was described as aromatic spicy (direction of fresh *Zingiberis officinalis* roots) and pleasant fresh (direction of *Eucalyptus* and *Peppermint*). Using GC-FID and GC-MS, 70 constituents were identified in this essential oil (Table 2.1).

Table 2.1

Volatiles of the root essential oils of *Kaempferia galanga*

Compound	Concentration (%)*
Ethyl *trans*-p-methoxycinnamate	52.5
Ethyl *trans*-cinnamate	26.3
Pentadecane	4.9
1,8-Cineole	2.4
Borneol	1.2
para-Cymene	0.8
δ-3-Carene	3.5
α-Gurjunene	0.5
α-Terpineol	0.5
Carvone	0.4
Artemisia alcohol	0.4

Ethyl *cis*-p-methoxycinnamate	0.4
para-Cymen-8-ol	0.4
Germacrene D	0.3
trans-Isomyrcenol	0.3
Ethyl *cis*-cinnamate	0.3
Carvone oxide	0.3
δ-Cadinene	0.3
Camphene	0.2
Verbenol	0.2
Limonene	0.2
Myrtenal	0.2
β-Caryophyllene	0.2
Bornyl acetate	0.2
α-Pinene	0.2
Terpinene-4-ol	0.2
β-Pinene	0.1
Hexadecanol	0.1
Verbenone	0.1
Linalool	0.1
Germacrene B	0.1
Methyl *trans*-methoxycinnamate	0.1
β-Phellandrene	0.1
Octanol	0.1
Isoamyl *trans*-p-methoxycinnamate	0.1
Muurolol	0.1
2-Hydroxy-1,8-cineole	0.1
δ-Cadinol	0.1
Caryophyllene oxide	0.1
trans-β-Ocimene	0.1
β-Myrcene	0.1

γ-Terpinene	0.1
Terpinolene	0.1
Pentadecanol	0.1
α-Humulene	0.1
Piperitenone	0.1
Isobornyl acetate	0.1
Tridecane	0.1
Decanol	0.1
β-Elemene	0.1
Hexadecane	0.1
α-Terpinene	tr
Octanal	tr
Heptadecane	tr
Limonene oxide	tr
Dihydrocarveol	tr
Carvacrol	tr
Tetradecane	tr
χ-Elemene	tr
Germacrene A	tr
Benzaldehyde	tr
Sabinene	tr
Alloaromadendrene	tr
α-Copaene	tr
cis-β-Ocimene	tr
para-Methoxybenzaldehyde	tr
Indole	tr
Cinnamic acid	tr

* calculated as %-peak area of GC-FID analyses
tr = trace compound (less than 0.1%)

As main compounds (Concentration higher than 2%), ethyl *trans*-p-methoxycinnamate (52.5%), ethyl *trans*-cinnamate (26.3%), pentadecene (4.9%), and 1,8-cineole (2.4%) were found, among further mono and sesquiterpenes, aliphatic hydrocarbons and aliphatic alcohols. For the first time the *cis* isomar of ethyl cinnamate was identified as minor constituent of this *Kaempferia* species as well as the cosmetically interesting compound isoamyl *trans*-p-methoxycinnamate.

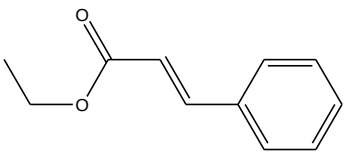
Two compounds with retention indices close to each other with identical mass spectra were obtained. These two compounds were ethyl p-methoxycinnamates (*cis* and *trans*). In order to identify which is which, *trans* isomer was synthesised and co-injected to assert the identity of the isomer.

The analytical data can be correlated to the olfactoric data very well. The cinnamate derivatives are responsible for the aromatic-spicy odour impression, where as especially the monoterpenes, like 1,8-cineole, borneol, δ-3-carene, carvone and carvone oxide generally possess pleasant fresh odour notes.

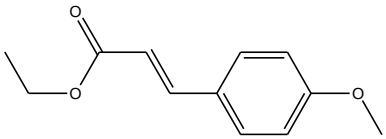
The correlation of this composition of the root essential oil of *K. galanga* with published biological data[19-26] allows for the statement that this sample can be used in medicinal application as well. Also for the food (additive) and cosmetic (UV-B-light-protection filter) industry as isoamyl *trans*-p-methoxycinnamate is an efficient UV protector to skin.

In conclusion we can report that the essential oil of *K. galanga* roots are rich in ethyl *trans*-p-methoxycinnamate, ethyl *trans*-cinnamate and 1,8-cineole and for the first time the *cis* isomer of ethyl cinnamate was found in this *Kaempferia* species. The characteristic aromatic-spicy and pleasant fresh odour of this oil can be attributed to the main cinnamate derivatives as well as to some monoterpenes. The correlation of the composition of this sample with published biological, cosmetic and nutritional data of this *Kaempferia* species shows the useful application of this oil in medicinal treatment, cosmetic products and foodstuff.

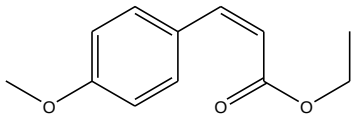
The structure of the identified compounds are given below (structures of structurally simple compound are not included).



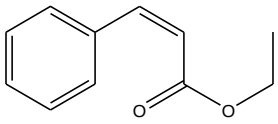
Ethyl *trans*-cinnamate



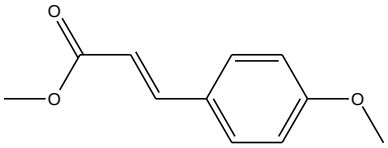
Ethyl *trans*-p-methoxy cinnamate



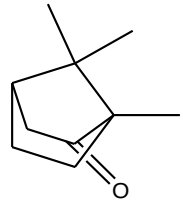
Ethyl *cis*-p-methoxy cinnamate



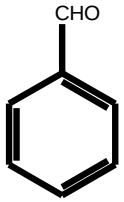
Ethyl *cis* –cinnamate



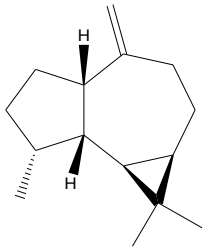
Methyl *trans*-p-methoxy cinnamate



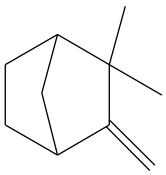
Camphor



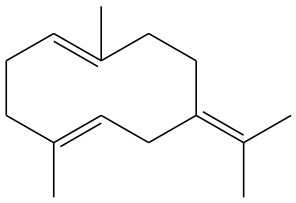
Benzaldehyde

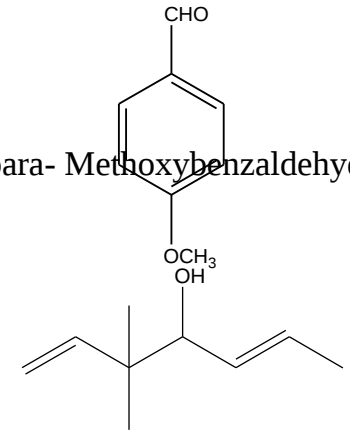


Alloaromadendrene



Camphene

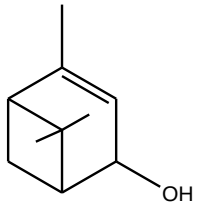




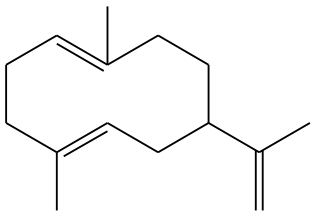
para- Methoxybenzaldehyde

Artemisia alcohol

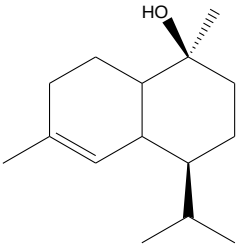
Germacrene B



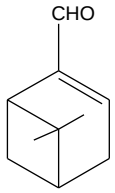
Verbenol



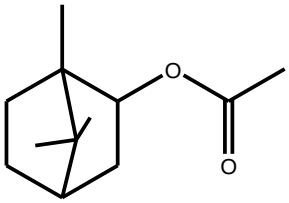
Germacrene A



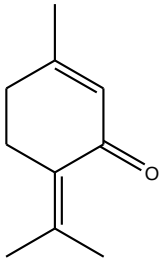
Muurolol



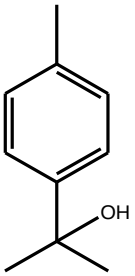
Myrtenal

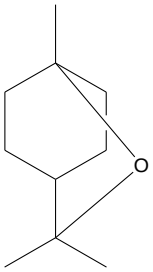


Bornylacetate

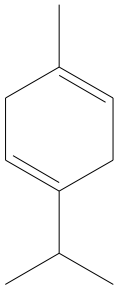


Piperitenone

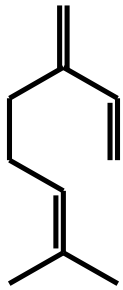




p-Cymen-8-ol

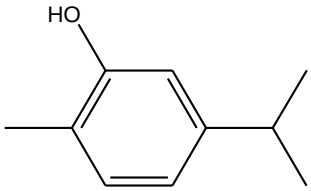


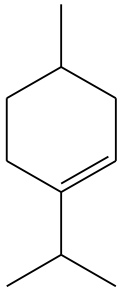
1,8-Cineole



γ-Terpinene

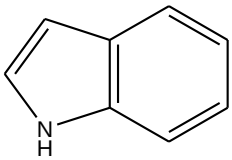
β-Myrcene

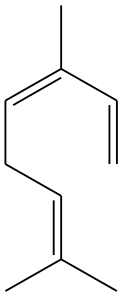




Carvacrol

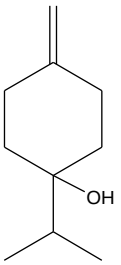
α- Terpinene

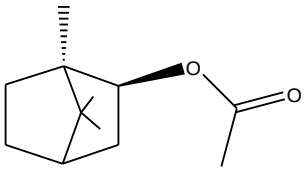




Indol

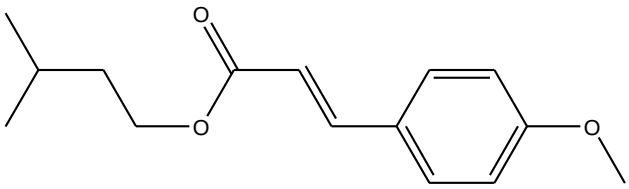
cis-β-Ocimene





Terpinen-4-ol

Isobornyl acetate



Isoamyl *trans*-p-methoxycinnamate

SECTION 3

ANALYSIS OF THE LEAF ESSENTIAL OIL OF *K. GALANGA*

2.3.1 PRESENT WORK

So far no work on the leaf essential oil of *K. galanga* has been reported. A physical examination of the odour of leaf and root shows that there is considerable difference. The root essential oil contains low quantity of isoamyl *trans*-p-methoxycinnamate which has UV-B-light-protection property and hence important in skin cream. Therefore it was thought worth while to analyse the leaf essential oil composition of *K. galanga*.

2.3.2 MATERIALS AND METHODS

The fresh plants of *K. galanga* were collected from Kannur district in September 2000 and the plant material was identified by Dr. A.K. Pradeep Department of Botany, Calicut Univesity. One kg leaves of the fresh plants were cut into pieces and ground by means of an electric grinder into a paste, which was steam distilled for 3 hour. The distillate was extracted with diethyl ether and dried over anhydrous sodium sulphate. After evaporation of the solvent 0.05 g (0.005%) essential oil was obtained.

The essential oil of *K. galanga* was analysed by GC-FID and GC-MS as described in the first section.

2.3.3 RESULTS AND DISCUSSION

The leaf essential oil of *K. galanga* was olfactorically evaluated and the odour was spicy, pleasant fresh, dry hay like and smoky. Sixty six

constituents were identified by GC-MS and GC-FID methods from the essential oil of *K. galanga* and are given in Table 2.2. The main compounds (conc. greater than 2%) were ethyl *trans*-p-methoxycinnamate (35.63%), ethyl *cis*-p-methoxy cinnamate (21.51%), *cis*-hex-3-enol (6.27%), borneol (5.37%), ethyl *trans*-cinnamate (3.81%) and caryophyllene oxide (3.37%).

Table 2.2

Volatile of the leaf essential oil of *Kaempferia galanga*

Compound Name	Concentration %	RI	Odour by GC-O
trans-Hex-3-enol	0.06	849	
cis-Hex-3-enol	6.27	852	
trans-Hex-2-enol	tr	863	
Hexanol	0.31	865	
α-Pinene	tr	938	
Benzaldehyde	tr	964	
Heptanol	0.08	969	
Sabinene	0.03	977	
β-Pinene	0.03	982	
Dehydro-1,8-cineole	0.02	996	
Octanal	0.03	1003	
3-Carene	0.01	1015	
para-Cymene	0.01	1029	
Limonene	tr	1033	
1,8-cineole	2.49	1037	Cineol-eucalyptus
trans- 2-Octenol	0.32	1067	
Octanol	0.57	1069	
cis-Linalool oxide (furanoid)	tr	1076	

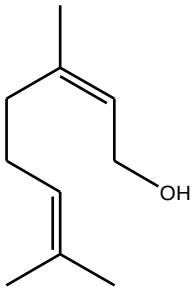
trans-Linalool oxide (furanoid)	tr	1092	
Linalool	0.38	1033	Linalool, bergamot
Fenchol	0.07	1067	
cis -2,8-p-Menthadienol	0.12	1069	
Nopinone	0.10	1076	
Pinocarveol	1.03	1092	
Camphor	0.20	1101	
4-Vinyl anisole	tr	1106	
Isoborneol	tr	1121	
α– Phellandrene-8-ol	0.29	1123	
Pinocarvone	0.30	1165	
Borneol	5.37	1169	Dry-hay-like borneol
Isopinocamphone	0.10	1176	
Terpinen-4-ol	0.45	1184	
meta-Cymen-8-ol	0.33	1184	
para-Cymen-8-ol	0.31	1186	
1(7),8-Menthadien-2-ol	0.14	1190	
α–Terpineol	0.44	1193	
Myrtenol	1.00	1197	
Myrtenal	0.56	1203	
Verbenone	0.17	1206	
trans-Carveol	0.37	1218	
Carvone	0.11	1226	
Geraniol	0.05	1250	
Anisic aldehyde	0.45	1256	
Hydroquinone	0.84	1263	
Thymol	tr	1273	
Carvacrol	0.05	1295	

Vinylguaiacol	0.05	1305	Smoky cresolic
cis -Carveyl acetate	0.60	1321	
Methylanthranilate	0.03	1354	
cis-Ethyl cinnamate	0.30	1383	
trans -Methyl cinnamate	tr	1393	
Tetradecane	tr	1400	
Methyl eugenol	tr	1409	
Caryophyllene	0.07	1431	
α-Ionone	tr	1441	
trans Ethyl cinnamate	3.81	1477	Soft sweet confectionary
β-Ionone	0.06	1500	Sweet violet ionone
Tridecan-2-one	tr	1497	
Pentadecane	0.22	1500	
γ-Cadinene	0.05	1516	
Caryophyllene oxide	3.37	1610	
Anisyl butyrate	0.15	1609	
Humulene epoxide	0.20	1636	
Epi-cubenol	0.46	1640	
Ethyl *cis*-p-methoxycinnamate	21.51	1671	
Methyl *trans*- p-methoxycinnamate	0.29	1682	
Ethyl *trans*- p-methoxycinnamate	35.63	1773	weak sweet slightly spicy

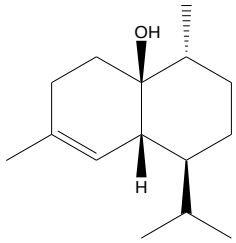
tr = trace compound (<0.1%)

The analytical data can be correlated to the olfactoric data very well. The cinnamate derivatives are responsible for spicy odour impression. Pleasant fresh odour due to 1,8-cineole and other monoterpenes. Dry hay like odour note was due to borneol and smoky odour attributed to vinyl guaicol. Cosmetically important isoamyl *trans*-p-methoxycinnamate was not present in the leaf essential oil of *K. galanga*.

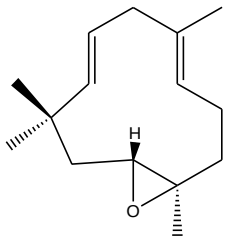
The structures of the identified compounds which are not included in other chapters are given below.



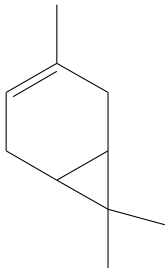
Geraniol



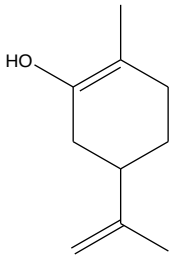
Epi-cubenol



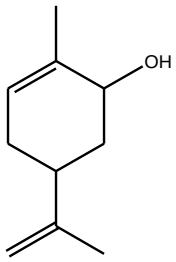
Humulene epoxide



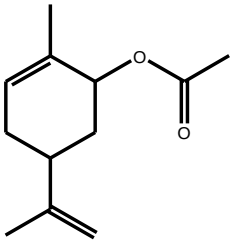
3-Carene



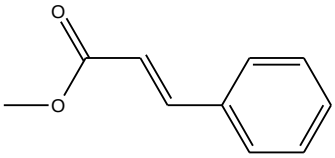
1,8- methadienene-2-ol



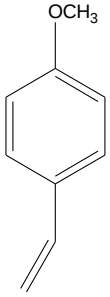
trans-Carveol



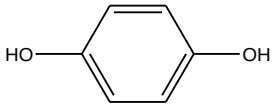
cis-Carveyl acetate



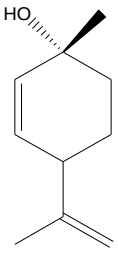
Methyl *trans*-cinnamate



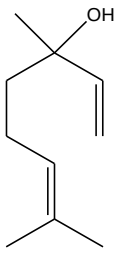
4-Vinylanisole



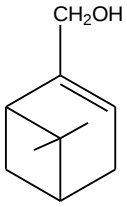
Hydroquinone



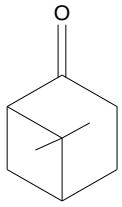
2,8-p-Menthadienol



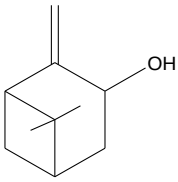
Linalool



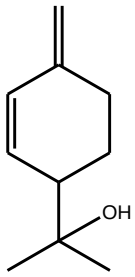
Myrtenol



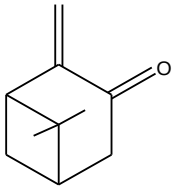
Nopinone



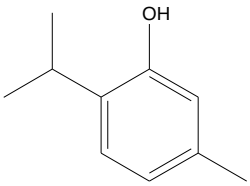
Pinocarveol



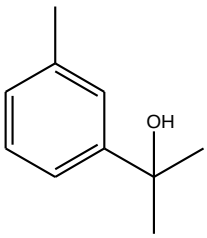
α-Phellandrene–8-ol



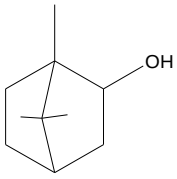
Pinocarvone



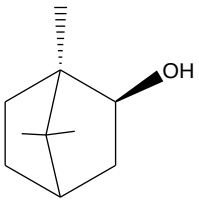
Thymol



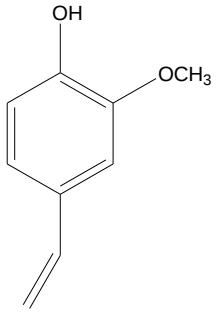
meta-Cymen-8-ol



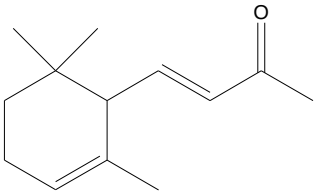
Borneol



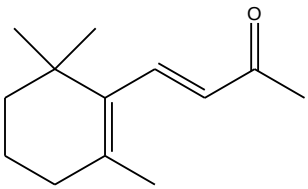
Isoborneol



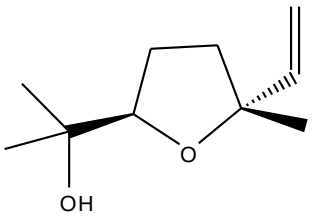
Vinylguaiacol



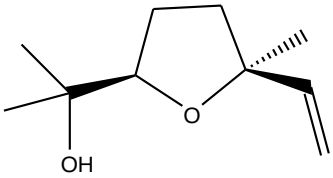
α–Ionone



β–Ionone



trans-Linalool oxide (Furanoid)

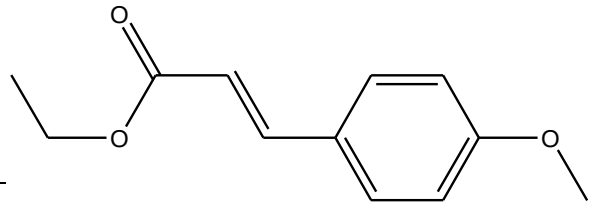


cis-Linalool oxide (Furanoid)

2.3.4 EXPERIMENTAL

Preparation of Ethyl *trans*-p-methoxycinnamate

2g of *trans* p-methoxycinnamoyl chloride (Zigma-Aldrich Co.) was refluxed with 5 ml absolute ethyl alcohol in pyridine using 100 ml R. B. flask and water condenser for 2 hours. After two hour the mixture was poured into cold water and taken to a separating funnel in ether medium. Then the mixture was washed with dil. HCl to remove pyridine. The mixture was then washed with sodium carbonate solution to remove *trans*-p-methoxycinnamoic acid (may be formed from its chloride). The mixture was then washed with distilled water and dried over anhydrous sodium sulphate. After evaporation of ether and recrystallisation from ethanol, pure ethyl *trans*-p-methoxycinnamate (m.p 49^{o}C) was obtained. All the spectral data confirmed the structure and stereochemistry of the compound.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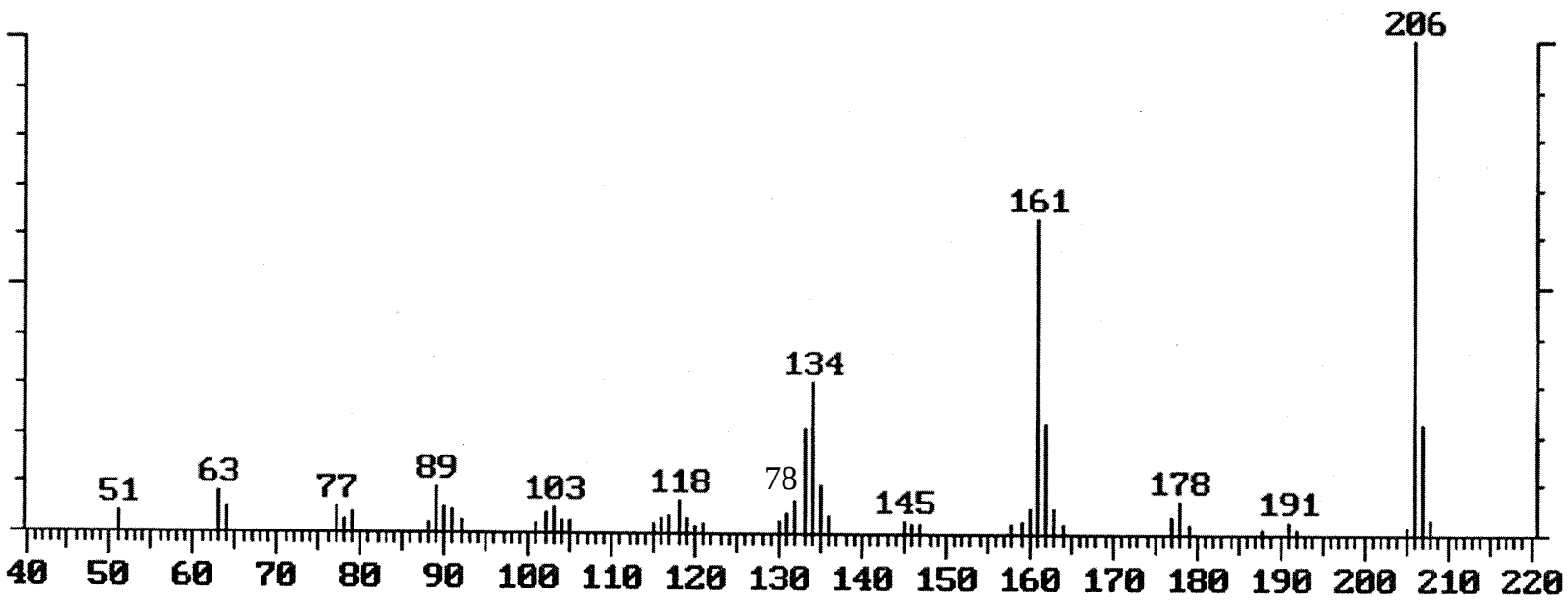
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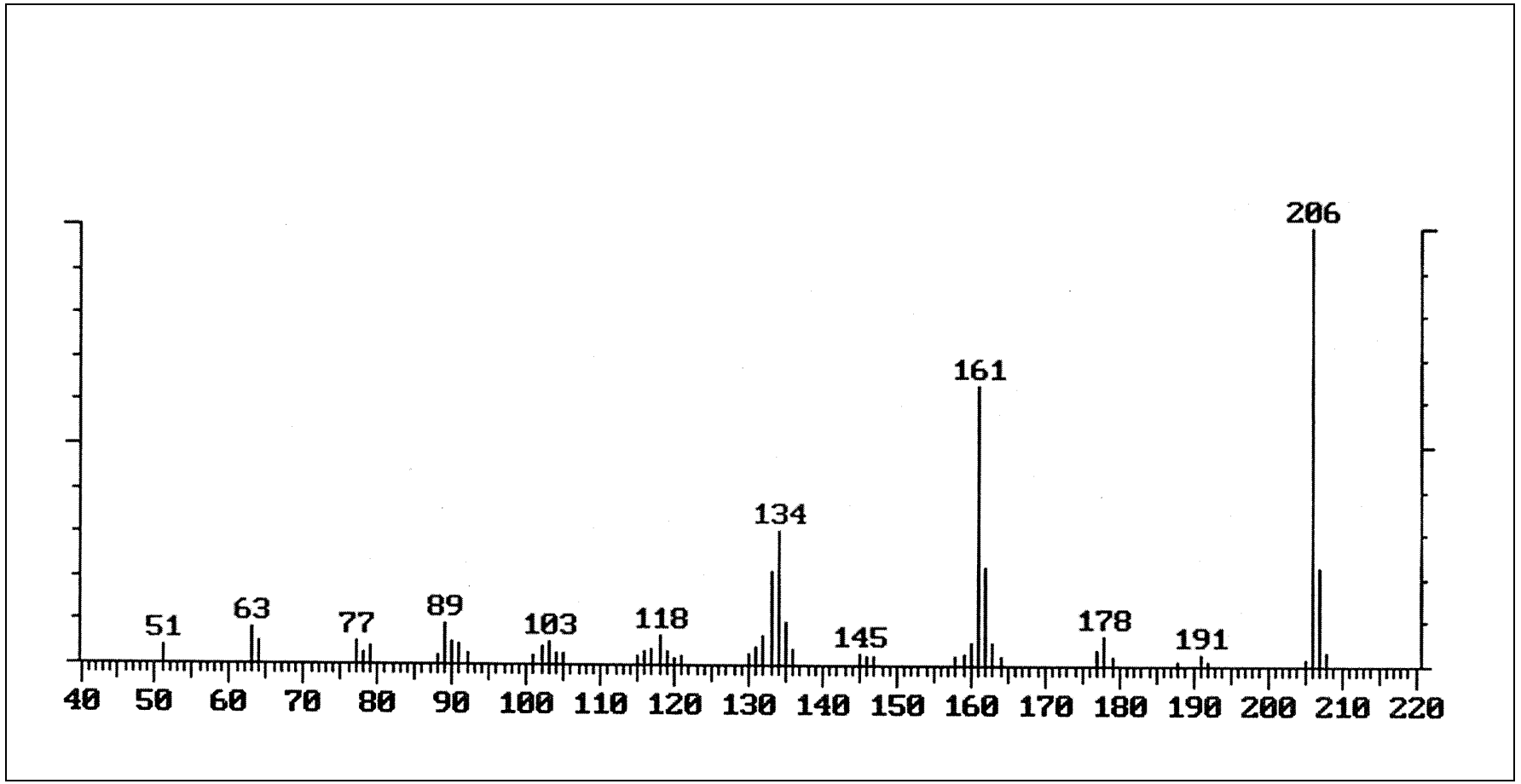

Fig. 2.1 Mass spectrum of ethyl *trans* -p-methoxycinnamate

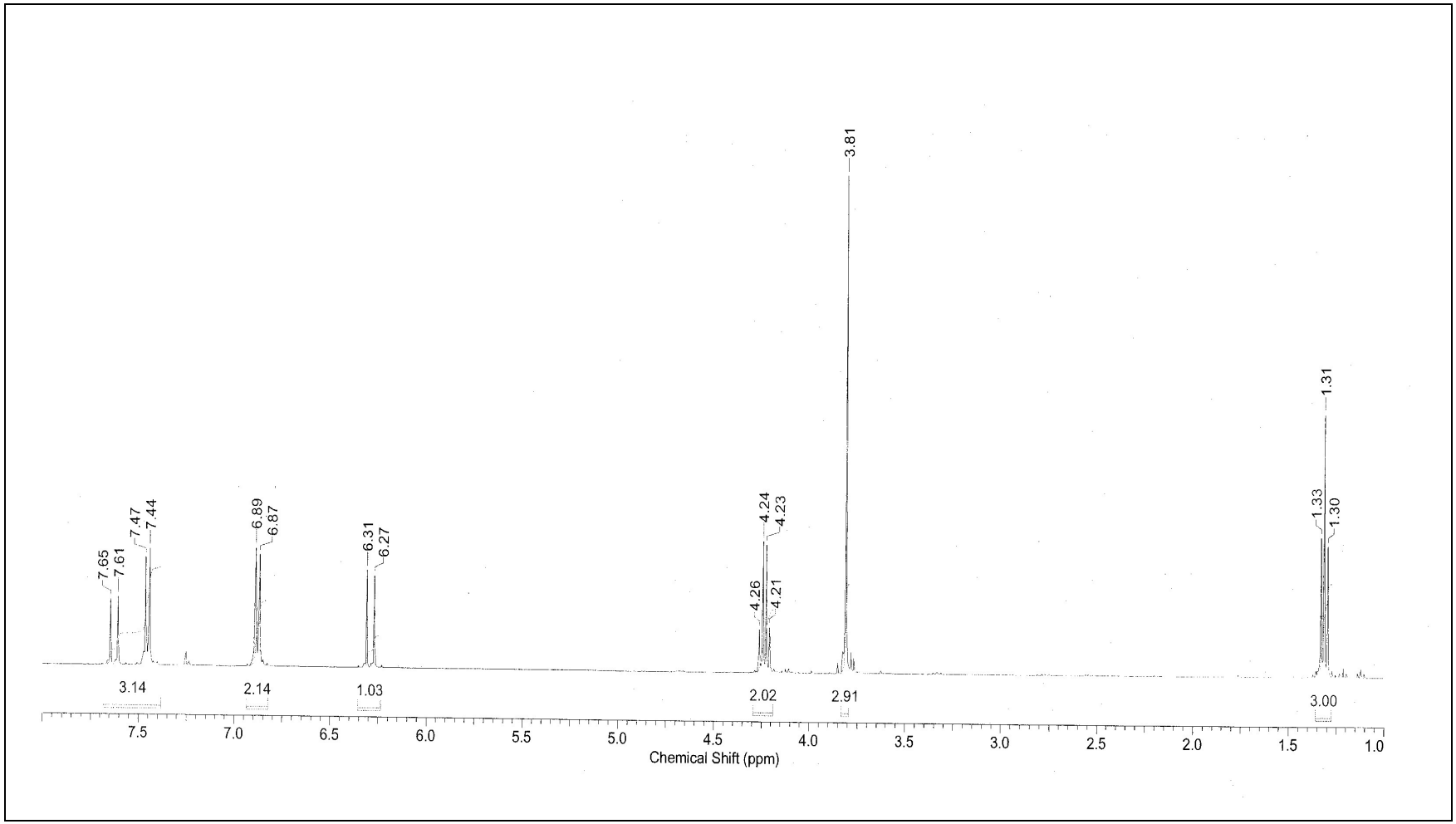

Fig. 2.2 ^{1}H-NMR spectrum of ethyl *trans* -p-methoxycinnamate

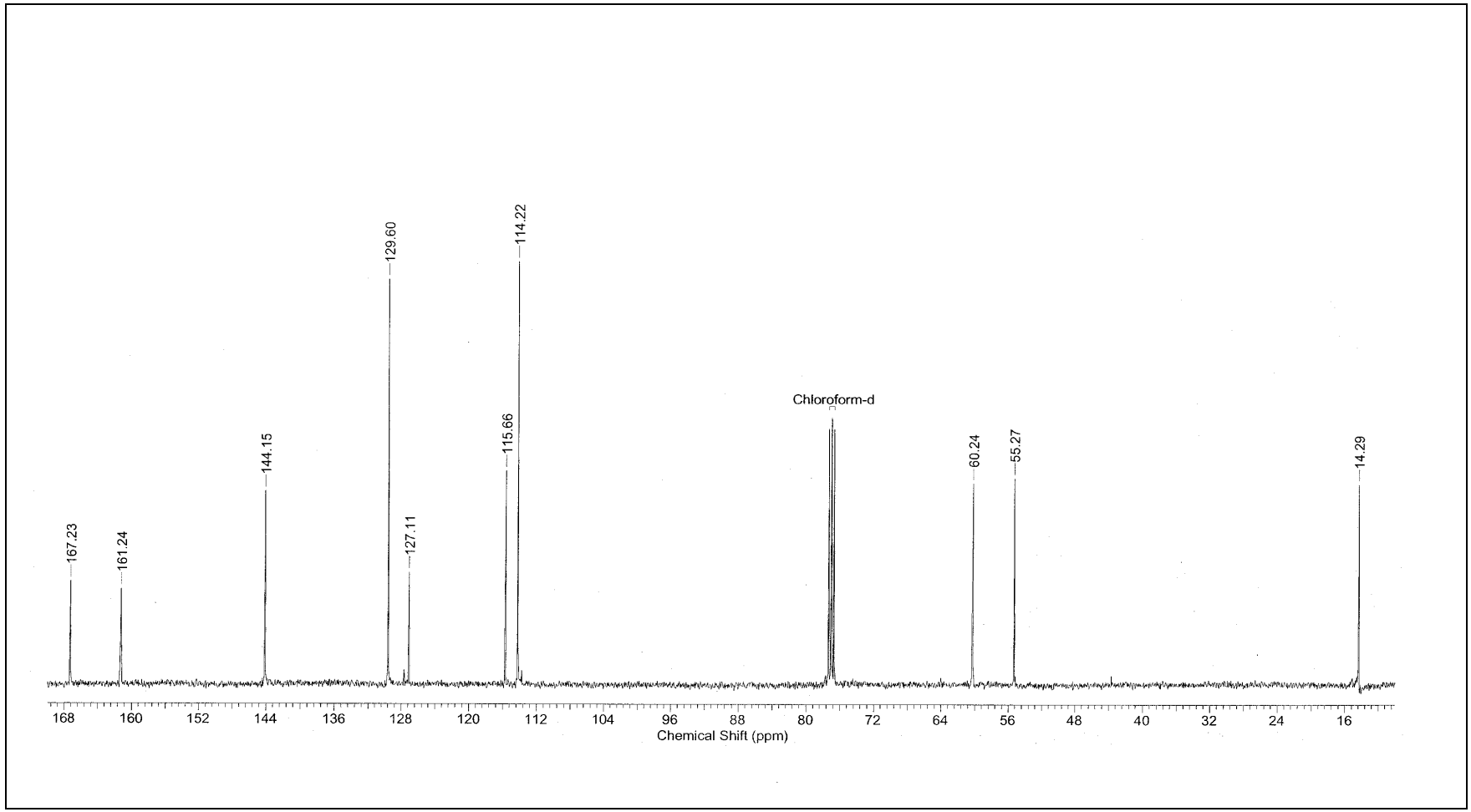

Fig. 2.3 ^{13}C -NMR spectrum of ethyl *trans* -p-methoxycinnamate

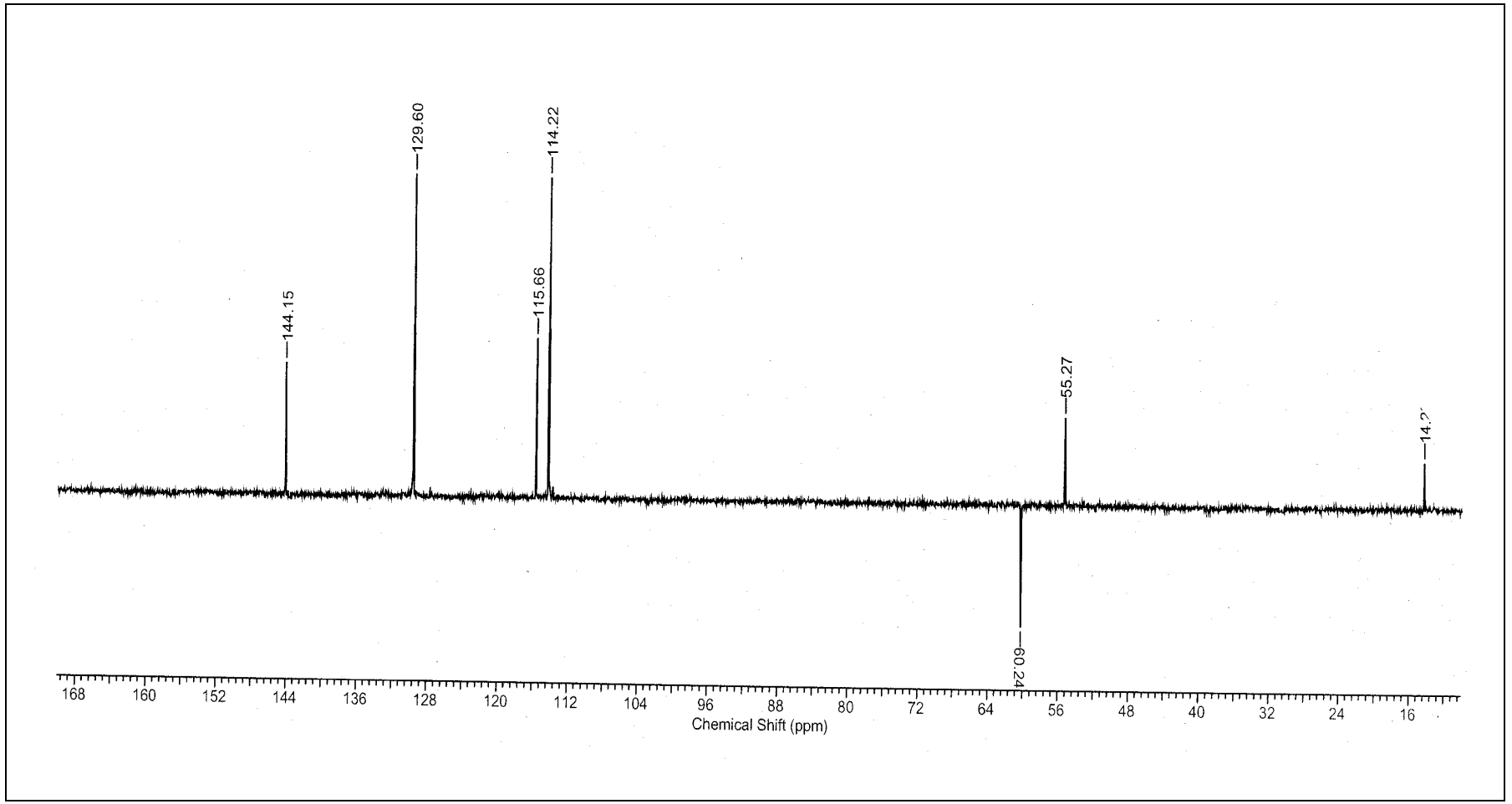

Fig. 2.4 DEPT spectrum of ethyl *trans* -p-methoxycinnamate

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CHAPTER III

ANALYSIS OF THE ESSENTIAL OIL OF *SPILANTHES CILIATA*

SECTION 1

ANALYSIS OF THE ESSENTIAL OIL OF *SPILANTHES CILIATA*

3.1.1 INTRODUCTION

Spilanthes ciliata syn. *Spilanthes acmella* belongs to *Asteraceae* family. This species of plants are distributed along the new and old world tropics. In India, six species are known; *S. paniculata*, *S. calva*, *S.radicans*, *S. ciliata*, *S.uliginosa* and *S.oleracea*.[1] This species commonly known as tooth-ache plant is accredited with local anaesthetic action due to spilanthol content in the flower heads. The flower heads are pungent and are chewed to relieve tooth-ache. In Kerala *S. ciliata*, the common species, is also used for this purpose[1]. *S. ciliata* is common in India, known under various common names such as Kuppa mangal (Malayalam), Vanamugali, Hemmugulur (Kannada), Akkalkara, Pipulka (Marathi), Maratimogga (Telungu), Akarkarha, Pokarmul (Punjabi), and Pirazha (Assami).[2]

Spilanthes ciliata is a diffuse herb, rooting at lower nodes stem terete, glabrous; leaves 7 x 4 cm, ovate-acute base rounded, margin serrate, petioled, heads rayed; 1 cm across, axillary, usually solitary, rarely 2 to 3 in each axial, yellow, subglobose turning conical; peduncle, 5-8 cm; involucral bracts 2-seriate shorter than ray florets, inner series narrower and smaller 6 x 2 mm,

elliptic-subacute, 3-nerved from base, margins narrowly winged, minutely pubescent, palea 3 mm long concave; boat shaped, obtuse or retuse at the expanded tip, keeled along the back, usually glabrous, ray florets 8-12, female ligulate, corolla tube short, 1 mm long limb 2.5 x 2 mm 3-lobed, lobes short, middle one slightly smaller than the laterals; decieluous; achenes trigonous, black glabrous, strongly margined and ciliate along the margins; surface lenticular. Disc florets many, bisexual, pappus of 2 unequal bristles, almost half as long as corolla tube, corolla tube abruptly expanded from middle, lobes, much shorter than the tube erect or spreading densely papillose with in, anther tips exerted from the tube achenes 2 x 0.5 mm, oblong, truncate at apex 2 angled and laterally compressed, black, glabrous strongly margined, ciliate along the margin.[1]

Mainly distributed in the neotropics, this species is now fairly common in wet or marshy places in Kerala.

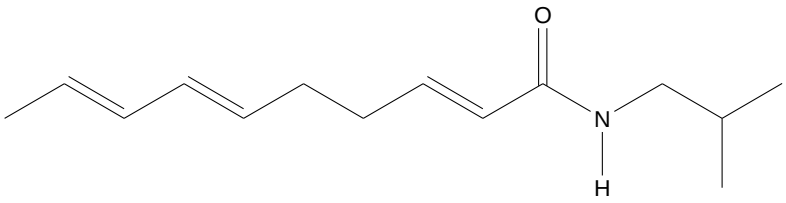
3.1. 2 MEDICINAL PROPERTIES AND USES

The flower heads are pungent, chewed to relieve tooth-ache and affections of throat and gums, and paralysis of tongue. It is also used as a remedy for stammering in children. A tincture made from the flower heads is used as a substitute for the tincture of pyrethrum to treat inflammation of jaw-bones and caries. It is regarded as a stimulant and sialogogue.[2] The plant is boiled in water and the liquid as well as the solid are administrated against

dysentery. The decoction is also given as a diuretic and lithotriptist agent and is employed as a bath for rheumatism and as a lotion in scabies and psoriasis. The root is used as a purgative.[4] The plant is employed as a fish poison in several parts of India. Ether extract of the fresh flower-tops of the plant is effective against anopheles mosquito larvae (as a soap suspension) even in great dilutions with water. Ethanolic extracts of the herb were found to affect the blood pressure of dogs and cats.[2] The ethanol extract of the whole plant of *S. ciliata* show antihepatotoxic activity.[5]

3.1.3 PREVIOUS WORK

The plant, plant parts and extracts of *S. ciliata* are used in local medicine for various treatments. The extracts are rich in sesquiterpenes and alkamides with the amide spilanthol as target compound.[6-14] Spilanthol is found to be identical with affinin.[2,3]



Spilanthol (N-isobutyldeca-2, 6,8-trienoamide)

Spilanthol is highly toxic to adult house flies and anopheles larvae. It is pungent and possesses strong sialogogic action and act as a local anaesthetic and a powerful insecticide[2].

Only few papers discuss the composition of essential oils of *Spilanthes*.[15-17] To the best of our knowledge no information is available on the essential oil composition of fresh plants of *S. cilicta* from South India.

3.1.4 PRESENT WORK

The present work was aimed at examining the essential oil composition of *Spilanthes ciliata* using GC-FID and GC-MS. Also olfactoric properties of the essential oil were studied and the odour-compound relation were correlated.

3.1.5 MATERIALS AND METHODS

Whole fresh plant of *S. ciliata* were collected from Calicut University Campus Kerala, India during March 2003. The plant was identified by Dr. A.K. Pradeep (Department of Botany, Calicut University) and a voucher specimen deposited in the specially maintained Herbarium of Calicut University, Chemistry Department.

1.2 kg of the fresh plants were sliced into small pieces and ground to paste using an electric grinder. The homogenized paste was hydrodistilled for three hour and the collected distillate extracted with diethyl ether. The ether

extract was dried over anhydrous sodium sulphate. After evaporation of the ether a colourless oil in a yield of 5g (0.41%) was obtained.

The oil composition was analysed by a combination of GC and GC-MS. GC analysis of the oil was carried out on a Shimadzu GC-14A (FID) and a Varian GC-3700 (FID) gas chromatograph fitted (with a 30 m x 0.32 mm (film thickness 0.25 μm) chemically bonded apolar FSOT-RSL-200 (Biorad) and with a 30m × 0.32mm (film thickness 0.50μm) Stabilwax (Restek) fused silica column, respectively. The sample was injected by splitter using hydrogen as carrier gas. The column temperature was programmed from 40°C (5 min) to 280°C (20 min) at 6°C/min. The compound identification was partly possible by coinjection of pure compound and correlation with published retention time data[18-22].

GC-MS analysis was carried out in a Shimadzu GC-17A/QP5000, on a HP-5890 GC/HP-5970 MSD and on a Finnigen MAT GCQ (Carrier gas Helium, Elmode, 70eV, Scan range 40-450 amu and ion-source temperature 200°C each, column see GC-part) equipped with Wiley/NBS and NIST libraries. For additional mass spectral correlation, published data[18,21-23] were used.

3.1.6 RESULTS AND DISCUSSION

Forty eight constituents were identified from *S. ciliata* oil. The significant main compounds (concentration calculated as relative percentage peak area of GC-FID analysis, apolar column) were (E)-2-hexenol (25.7%), 2-tridecanone (13.1%), germacrene D (11.1%), hexanol (11%), β-caryophyllene (10.8%), and (Z)-3-hexenol (5.1%) and further compounds found in medium concentration were (Z)-3-hexadecene (4.5%), 1-octen-3-ol (2.6%), and spilanthol (2.5%). The compounds identified in the essential oil of *S. ciliata* are listed in order of elution (Table 3.1). Retention indices (RI); were calculated using a mixture of a homologous alkane series from n-hexane up to n-hexadecane from an apolar FSOT-RSL column (DB-5 like) and the percentage calculated by relative percentage peak area are also provided.

Table 3.1

Percentage composition of the essential oil of *Spilanthes ciliata*

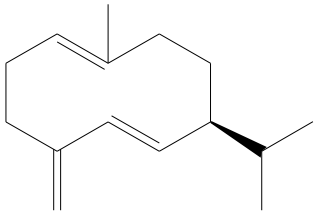
Compound	Concentration (%)	RI
Isoamyl alcohol	0.1%	737
(E)-3-Hexenol	0.8%	847
(Z)-2-Hexenal	0.1%	852
(Z)-3-Hexenol	5.1%	855
(E)-2-Hexenal	0.2%	857
(Z)-2-Hexenol	0.9%	860
(E)-2-Hexenol	25.7%	862

Hexanol	11.0%	865
Heptanol	0.2%	967
Sabinene	0.1%	974
1-Octen-3-ol	2.6%	978
para-Cymene	0.1%	1024
Limonene	0.1%	1028
Benzyl alcohol	0.1%	1031
(E)-β-Ocimene	0.1%	1048
Linalool	1.1%	1096
2-Phenylethyl alcohol	0.1%	1106
(Z)-3-Nonenol	0.1%	1155
Nonanol	0.1%	1167
(E,Z)-2, 6-Nonadienal	0.1%	1179
Terpinen-4-ol	0.1%	1182
Borneol	0.1%	1192
Verbenone	0.1%	1206
(E)-2-Decenol	0.1%	1269
(Z)-3-Tridecene	1.9%	1285
2-Undecanone	0.9%	1294
(E,Z)-2, 6-Decadienal	0.1%	1321
Eugenol	0.7%	1358
α-Copaene	0.8%	1377
β-Elemene	0.1%	1390
β-Caryophyllene	10.8%	1435
α-Humulene	0.5%	1454
Germacrene D	11.1%	1483
α-Amorphene	0.1%	1485
2-Tridecanone	13.1%	1495
Bicyclogermacrene	0.4%	1499
Elemol	0.1%	1547

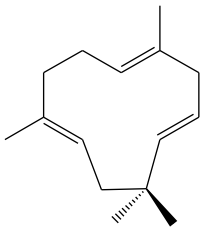
Spathulenol	0.1%	1575
(Z)-3-Hexadecene	4.5%	1578
Caryophyllene oxide	0.7%	1582
(E)-2-Dodecenyl acetate	0.3%	1606
β-Oplopenone	0.5%	1650
α-Muurolol	0.1%	1653
α-Cadinol	0.5%	1661
(E)-Nerolidol	0.5%	1673
α-Bisabolol	0.1%	1724
(E,E)-Farnesol	0.6%	1724
Spilanthol	2.5%	1852

Comparing the analytical result of this investigation of this fresh plant oil of this *Spilanthes* species with sensory data published[24-28], it is to assume that the green herbal aroma can be correlated to the hexane derivatives, 2-tridecanone and some sesquiterpenes as well as the warm-earthy-woody one especially to germacrene D, β-caryophyllene and 1-octen-3-ol. Medicinal spicy odour notes are known from hexanol, germacrene D and 2-tridecanone. The weak floral-fruity aroma can be attributed to linalool and some mono and sesquiterpenes in lower concentrations. Fatty-waxy aroma impressions is exhibited by nonane derivatives, (Z)-3-tridecene and (Z)-3-hexadecene, while moody-smoky odour notes in this background are known from some hexane and octane derivatives.

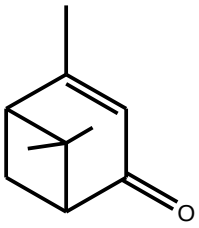
In summary, the essential oil of fresh plants of *S. ciliata* is rich in (E)-2-hexenol, 2-tridecanone, germacrene D, hexanol, β-caryophyllene and (Z)-3-hexenol. These compounds are also of high importance for the characteristic aroma of this oil, which has been investigated for the first time. Structures of identified compounds are as follows:



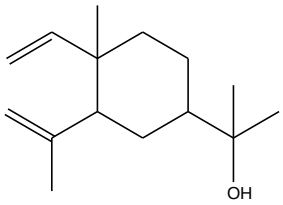
Germacrene D



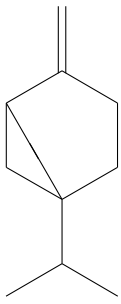
α-Humulene



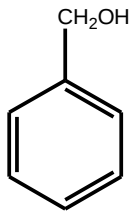
Verbenone



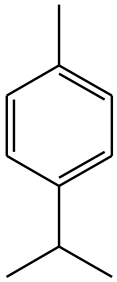
Elemol



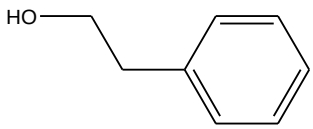
Sabinene



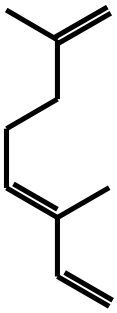
Benzyl alcohol



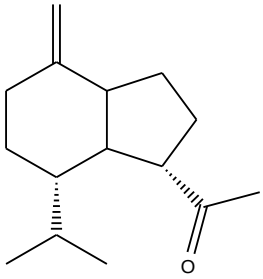
p-Cymene



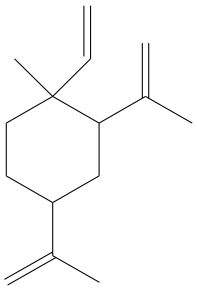
2-Phenylethyl alcohol



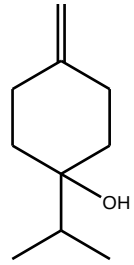
(E)-β-Ocimene



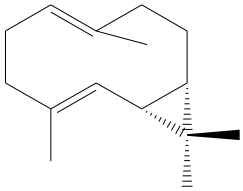
β-Oplopenone



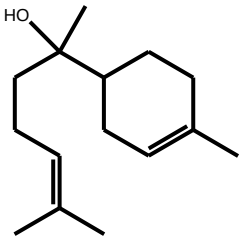
β-Elemene



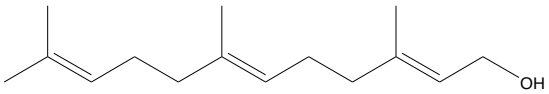
Terpinen-4-ol



Bicyclogermacrene



α-Bisabolol



(E,E)- Farnesol

SECTION 2

CHEMICAL TRANSFORMATIONS OF THE ESSENTIAL OIL OF *SPILANTHES CILIATA*

3.2.1 INTRODUCTION

The essential oil obtained by steam distillation of *S. ciliata* contain large number of alcohols and it has a pleasant smell. It is well known that acetates (eg. ethyl acetate, amyl acetate), benzoates (eg. ethyl benzoates) and cinnamates (eg. ethyl cinnamate) have very characteristic and commercially important odour. Hence it was proposed to convert the alcohols present in the essential oil of *S.ciliata* into their corresponding acetates, benzoates and cinnamates. The resultant oil was analysed by GC, GC-MS and olfactometry

3.2.2 ESTERIFICATION OF ALCOHOLS IN *S. CILIATA* ESSENTIAL OIL

1. Cinnamoylation of alcohols in *S. ciliata* essential oil

The essential oil (2g) of *S. ciliata* was refluxed with cinnamoyl chloride in pyridine using a 100 ml R.B. flask and water condenser for one hour. After one hour the mixture was taken to a separating funnel in ether medium and washed with dil. HCl to remove pyridine. The mixture was then washed with sodium carbonate solution to remove cinnamic acid (may be formed from cinnamoyl chloride). The mixture was then washed with

distilled water, the ether extract was dried over anhydrous sodium sulphate. After the evaporation of solvent ether, 1.5g oil was obtained.

2. Acetylation of Alcohols in *S. ciliata* essential oil

Method adopted for the acetylation was same as that of cinnamoylation.

3. Benzoylation of Alcohols in *S. ciliata* essential oil

Here also the method adopted was exactly same as in the case of cinnamoylation.

GC and GC-MS methods used were exactly as in the case of *S. ciliata* essential oil analysis.

3.2.3. RESULTS AND DISCUSSION

GC-MS analysis of the reacted oils show the presence of esterified alcohols. However, the products did’nt have any major change in the odour. Hence, detailed study was not pursued to correlate the esters to their respective odour.

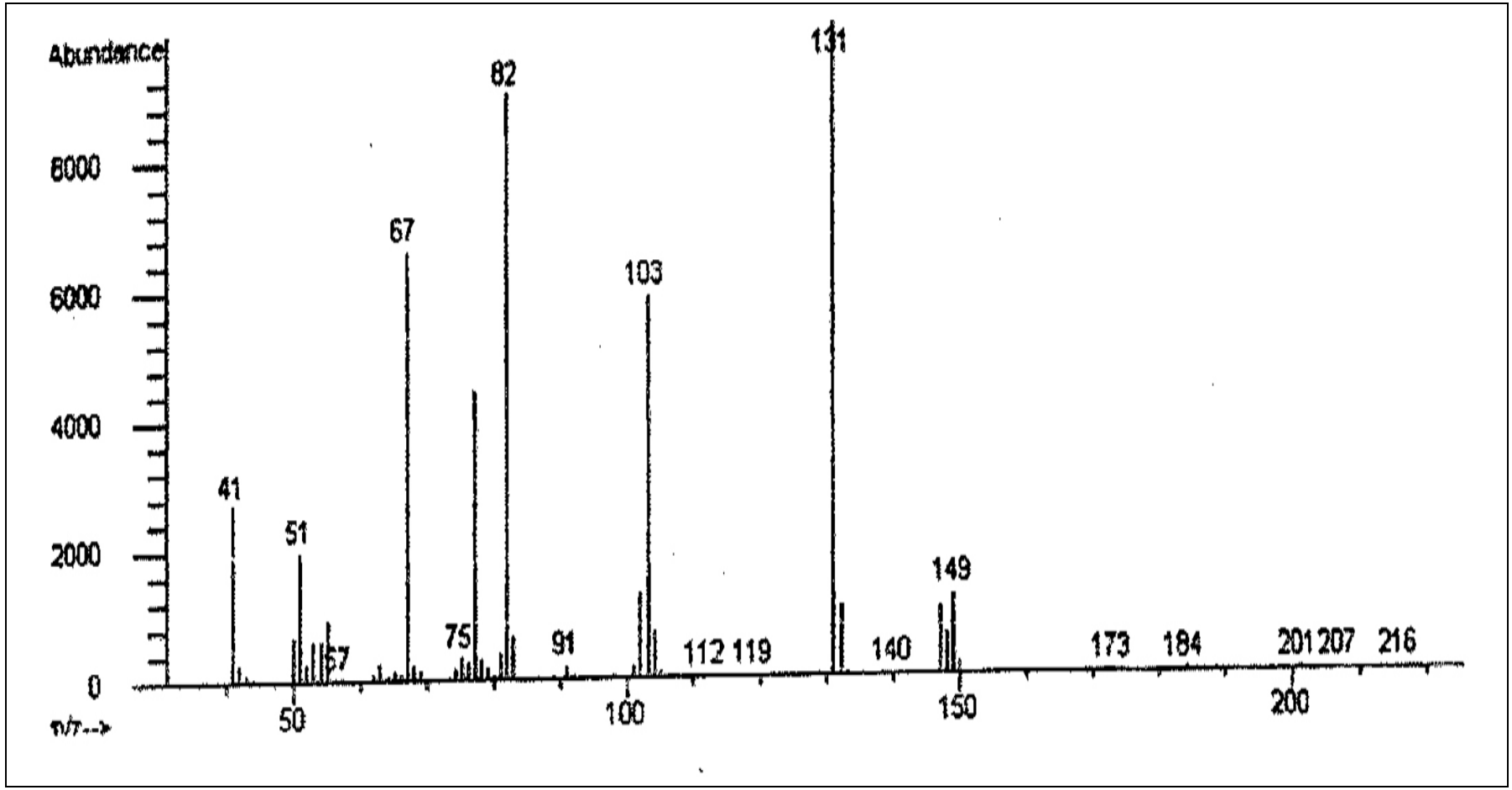


Fig. 3.1 Mass spectrum of (Z)-3-Hexenyl cinnamate

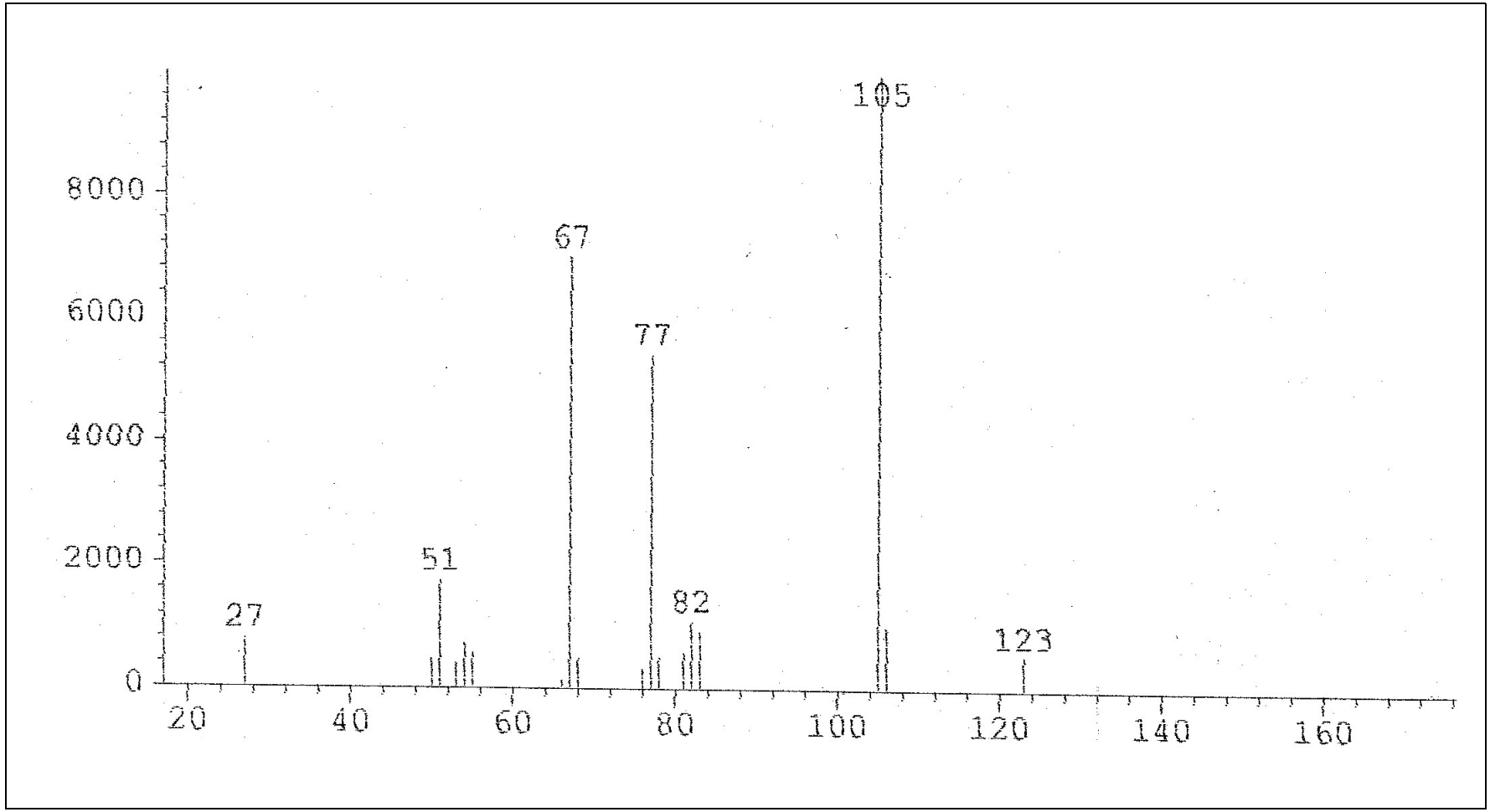


Fig. 3.2 Mass spectrum of (Z)-3-Hexenyl acetate

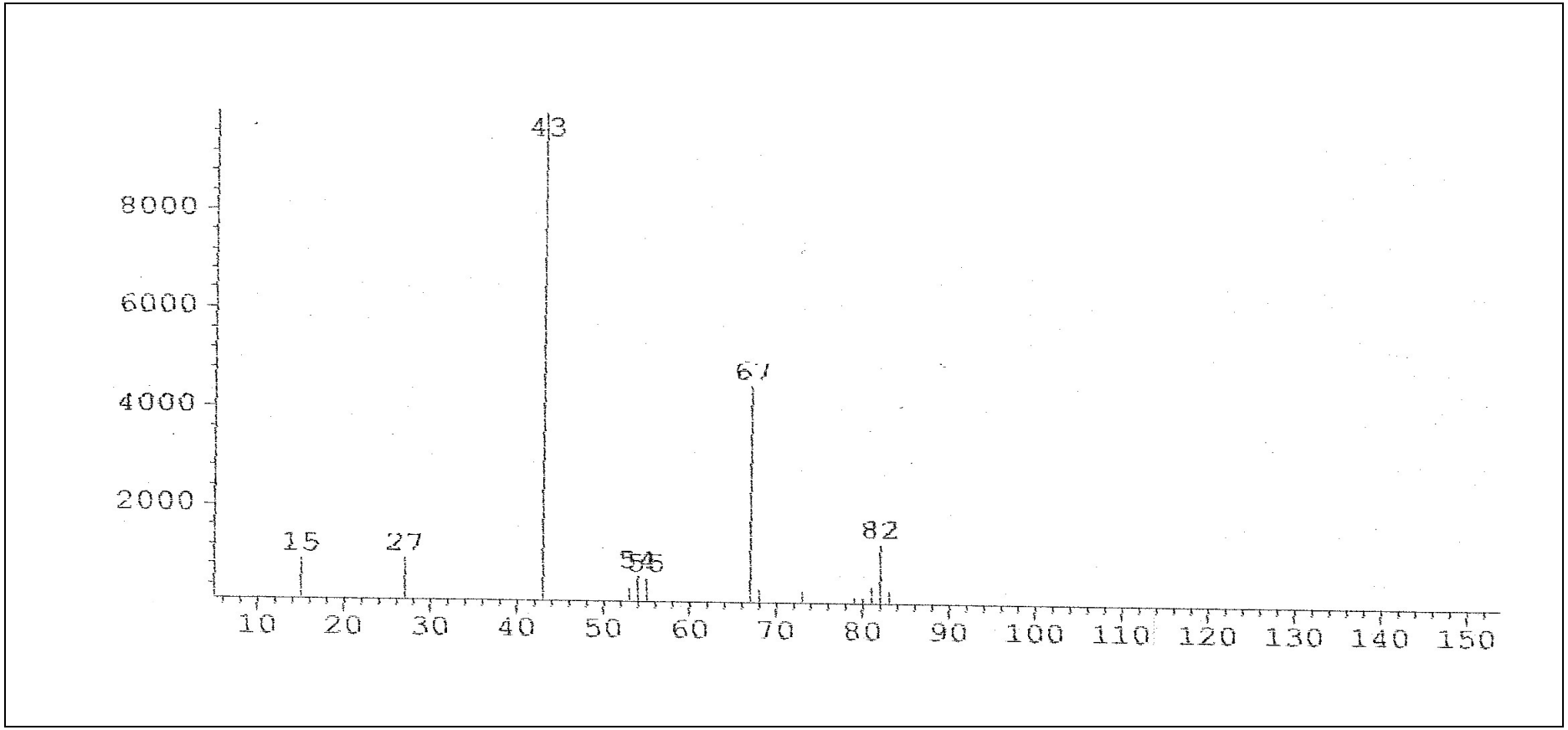


Fig. 3.3 Mass spectrum of (Z)-3-Hexenyl benzoate

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CHAPTER IV

CHEMICAL IVESTGATION OF *SPILANTHES RADICANS*

4.1 INTRODUCTION

Spilanthes radicans (Jacq) R.K. Jansen var. *radicans* (syn. *Acmella radicans* Jacq., syn. *Spilanthes ocymifolia* var. *acutuserrata* A.H. Moore) is an annual, erect and ascendent weed (30-60 cm tall), native to central (Nicaragua, Costa Rica & Mexico) and South America (Colombia) as well as being widespread in warm climate areas around the world, such as Cuba, Cuvacao, India and Tanzania[1], especially at an altitude of 40-130 m. It belongs to *Asteraceae* family and six species of this family are reported in India.[2] [*S. paniculata*, *S. calva*, *S. ciliata*, *S. ulginosa S. radicans,* and *S. oleracea*). The leaves of *S. radicans* are oval, 1-8 cm long and 1-5 cm wide with additional pale-yellow spreading leaves 4.5 cm long 0.5-1 mm wide, sometimes with purple coloured shading. The yellow, green white or white flowers are characterized by their four lobed corolla with a flowering time between August and May. The seeds are fringed with two or three bristles (0.4-1.6 mm long)[2].

All parts of the plant are acrid, but the flower heads are by far the most pungent. They are chewed to relieve tooth ache and affections of throat and

gums as well as to paralyse the tongue. In western India, *Spilanthes* flower heads are known as a popular remedy for stammering children. A tincture made from the flower heads is also substituted for a tincture of pyrethrum for the treatment of inflammation in the jaw-bone. Plant parts, especially chewed seeds, are regarded as a stimulant and sialagogue. The whole plant is boiled in water and the liquid as well as the solid are administered against dysentery. A decoction is used as a diuretic and lithotriptist agent and employed as a bath against rheumatism, but also as a lotion against scabies and psoriasis. The juice of the plant is effective as a vulnerary and the pounded herb as a poultice to dress wounds. *Spilanthes radicans* roots are used in several parts of India as a purgative, while the whole plant is a fish poison.[3] This *Spilanthes* species is also known for its insecticidal, fungicidal and antibacterial activities[4], with an ether extract of fresh flower tops as a very effective agent against Anopheles mosquito larvae.[3]

4.2 PRESENT WORK

Investigation on compounds of *Spilanthes radicans* are very rare and only one paper was found with alkamides identified to have plant growth regulator functions.[4] So far no studies on the compositions of essential oils or extracts of *Spilanthes radicans* fresh whole plants and roots have been published. Therefore the aim of this work was to isolate and identify the compounds of this member of the *Astroeraceae* family. It was also proposed

to identify the essential oil components and characterize the compounds responsible for the pleasant scent, by means of GC-FID, GC-MS and olfactometry.

4.3 EXPERIMENTAL

Essential oil extraction

Fresh plants of *Spilanthes radicans* were collected from Kannur district of Kerala State (South India) in January 2003. The sample was identified by Dr. A.K. Pradeep, Department of Botany, Calicut University Kerala. A voucher specimens (No.42) has been deposited in the specially maintained herbarium of the Department of Chemistry at Calicut University. The fresh plant (800 g) was sliced into small pieces and ground to a paste using an electric grinder. The paste was hydrodistilled for three hours and the distillates extracted with diethyl ether (2 x 100 ml). The ether extracts were collected and dried using anhydrous sodium sulphate. After ether evaporation a colourless oil with a yield of 0.5g (0.62%) was obtained.

Compound isolation

Two kg of dry roots were powdered and extracted with petroleum ether (3x4L) at room temperature and the combined extracts evaporated under reduced pressure. A white powdery substance separated out which on recrystallisation from methanol yielded 1 g of pure substance G_1, M.P 139^{o}C.

Table 4.1

Compound isolated from petroleum ether extract

Compound	Extracting Solvent	M.P
G_1	Petroleum ether	139°C

The crude petroleum ether extract, after the removal of white powdery solid was adsorbed on 500 g of silicagel (Merck Co., 60-120 mesh) and packed in a column (4 cm x 100 cm d x l). The column was then eluted with petroleum ether, different concentration of petroleum ether-ethyl acetate, ethyl acetate and methanol in that order. Several 50 ml portions were collected and each fractions was checked by TLC. Fractions were pooled together according to their homogeneity judged from TLC analysis. Fraction 1 to 6 obtained by petroleum ether elution combined together and on evaporation of the solvent yield 1.3 g (sample A). The residual plant material (after the petroleum ether extraction) was extracted three times with 3L ethyl acetate. After concentration of this extract under vaccum was absorbed on silica gel for column chromatography. The column was first eluted with 1:1 mixture of ethyl acetate – petroleum ether, the first two 50 ml fraction were taken as sample B with a yield of 3.8 g upon evaporation of the solvent.

Sample A and B were syrupy and on TLC examination showed the presence of large number of compounds. Hence, they were analysed by GC-FID and GC-MS in order to identify the compounds present.

Olfactoric evaluation

The essential oil of the fresh whole *Spilanthes radicans* plant was placed in a commercial odour-strip olfactorily evaluated by aroma-trained chemists with a characteristic aroma for each sample.

Gas chromatography

GC-FID analyses were carried out using a GC-14A with SPME glass liner in the injector, FID and C-R6A-Chromatopac integrator (Shimadzu, Japan) a GC-3700 with FID (Varian, Germany) and a C-RIB-Chromatopac integrator (Shimadzu). The carrier gas was hydrogen, injector temperature 250^0C; detector temperature: 320^0C; the temperature for 5 min increasing to 280°C for 5 min with a heating rate of 6°C/min. The columns were a 30 m x 0.32 mm bonded FSOT-RSL-200 fused silica, with a film thickness of 0.25μm (Biorad, Germany) and a 30 m x 0.32 mm bonded stable wax, with a film thickness of 0.50 μm (Restek USA). Quantification was achieved using peak area calculations and compound identification was carried out partially using correlations between retention times [5-9].

Gas chromatography - Mass spectrometry analysis

For GC-MS measurements a GC-17A with QP 5000 (Shimadzu) SPME glass linear in the injector and Compaq-ProLinea data system (Class 5k. Software), a GC-HP 5970 with HP5970 MSD (Hewlett-Packard), USA and Chemstation software on a Pentium PC (Bohm, Austria), a GCQ (Fennigen – Spectronex, Germany–USA) and Gateway–2000–PS75 data system (Siemens – Nixdorf, Germany, GCQ Software) were used. The carrier gas was Helium, injector temperature, 250^{o}C interfase heating at 300^{o}C, ion-source heating at 200^{o}C. EI-mode was 70 eV; and the scan range was 41-450 amu. Other parameters were same as the description of GC-FID above. Mass spectra correlations were done using Wiley, NBS, NIST and Published data. [5,7,10].

4.4 RESULTS AND DISCUSSION

The essential oil and the two root extract of *Spilanthes radicans* were olfactorically evaluated as follows; pleasant herbal – woody – spicy, green earthy with floral fatty side-notes. For sample B the scent was similar to the essential oil odour but more intense with a warm-herbal spicy base note, additionally possessing a moody side – note and fatty waxy background–note, while for sample A fresh vegetable and Allium-like top note, a herbal woody and a week floral fruity fatty aroma were found.

Using GC-FID and GC-MS (both with two columns of different polarity) about 90 volatiles could be detected and more than 70 of them

identified in all three samples (Table 4.2). The main compounds (concentration higher than 3%, calculated as percentage peak area of GC-FID analysis using a polar carbo wax column) were;

Essential oil

2-tridecanone (30.1%), 1-pentadecene (25.2%), *trans*-β-Caryophyllene (12.8%), elemol (4.6%), and guaiol (3.1%).

Sample A

1-pentadecene (54.1%), 2- tridecanone (10.3%), 2-pentadecanone (6.7%), 2-pentadecanol (4.1%), *trans*-β-caryophyllene (3.6%), and 1-tridecene (3%).

Sample B

2-tridecanone (58.2%), 1-pentadecene (15%), and palmitic acid (16.6%).

Table 4.2

Compounds of three different samples of *S.radicans*

Compound	RI	EO	A	B	Aroma impressions
Ethanol	922	tr	nd	nd	Etheral, alcohol-like
Hexanal	1063	tr	tr	nd	Fatty, grassy, green, penetrating
Myrcene	1143	tr	tr	tr	Sweet-balsamic, plastic-side-note
δ-3-Carene	1149	tr	tr	nd	Sweet, refined limonene-note, penetrating
α-Phellandrene	1168	tr	tr	nd	Minty, herbal, spicy
Heptanal	1172	0.1	0.3	nd	Fatty, sweet, woody, nutty, fruity
α-Terpinene	1187	tr	tr	nd	Terpene-like
β-Phellandrene	1212	tr	tr	nd	Herbal, spicy
(Z)-β-Ocimene	1224	tr	tr	nd	Spicy (estragon and basil-notes)
p-Cymene	1243	0.1	0.4	0.2	Weak citrus-note
(Z)-3-Hexen-1-ol	1249	tr	tr	nd	Green ('leaf alcohol'), fresh-grass-like
(E)-β-Ocimene	1252	tr	tr	nd	Spicy
2-Octanone	1278	0.2	0.2	0.1	Mushroom-like, moody, earthy
Terpinolene	1283	tr	nd	nd	Sweet-piney, slightly sweet-anisic
(E)-1-Hexen-3-ol	1299	tr	tr	nd	Intense green with bitter- and fatty-notes
Hexanol	1327	0.2	0.3	tr	Alcoholic, ethereal, medicinal
1-Tridecene	1347	tr	3.0	0.1	Weak fatty-herbal
Diisopropyl disulphide	1352	tr	0.5	nd	Sulphur, *Allium*-like
1-Methylpropyl propyl disulphide	1369	nd	0.4	nd	Sulphur, *Allium*-like

Di-(1-methylpropyl)-disulphide	1391	tr	1.3	tr	Sulphur, *Allium*-like
Heptanol	1419	tr	0.4	nd	Woody, heavy, oily
1-Octen-3-ol	1423	0.3	0.2	1.9	Mushroom-like, moody-earthy
δ-Elemene	1468	tr	tr	0.2	Weak woody, herbaceous
Fenchyl acetate	1473	tr	tr	nd	Fresh, herbal
α-Cubebene	1481	0.1	tr	tr	Herbal-woody
Decanal	1485	0.1	tr	nd	Sweet-waxy, floral, citrus-note
Octanol	1516	0.3	0.4	0.2	Mushroom-like, moody, earthy
Linalool	1522	tr	nd	nd	Floral, citrus-lemon-orange notes
1-Pentadecene	1540	25.2	54.1	15.0	Weak fatty-floral
α-Copaene	1547	0.1	1.2	0.4	Woody, spicy
α-Gurjunene	1562	0.3	2.1	0.8	Herbal, woody
Fenchol	1573	tr	0.1	nd	Fresh, herbal, minty
Pinocarvone	1577	tr	tr	nd	Fresh, camphoraceous
Aromadendrene	1587	1.2	0.3	tr	Woody, spicy
β-Elemene	1590	tr	nd	nd	Herbal, woody
Terpinene-4-ol	1599	0.1	0.1	tr	Fresh, herbal, minty
trans-β-Caryophyllene	1601	12.8	3.6	0.3	Terpene-odour, woody, spicy

α-Humulene	1657	2.1	0.1	0.2	Weak woody
(E)-β-Farnesene	1666	tr	tr	nd	Mild, sweet, warm
Germacrene D	1684	0.3	2.1	0.3	Dry-woody
Carvone	1715	tr	tr	nd	Spicy, fresh, herbal
Decenol	1720	0.1	0.1	tr	Floral, fruity, waxy
α-Terpineol	1732	tr	0.1	nd	Liliac odour, floral, fruity
Verbenone	1735	tr	tr	nd	Minty, spicy
2-Phenylethyl acetate	1786	tr	tr	tr	Floral, rose-like, honey-notes
α-Cadinene	1788	0.3	tr	0.2	Dry-woody, weak medicinal
(E)-Carveol	1790	tr	tr	tr	Spicy, herbal
2-Tridecanone	1793	30.1	10.3	58.2	Warm, herbaceous, slightly spicy
(Z)-Carveol	1819	tr	tr	tr	Herbal, spicy
Benzyl alcohol	1830	0.7	0.3	0.1	Faint aromatic
(E)-2-Decenal	1841	tr	tr	tr	Floral, waxy, orange-note
2-Phenylethyl alcohol	1857	2.2	1.0	nd	Floral, weak rose-like
2-Tridecanol	1898	2.1	2.7	1.6	Sweet-fruity
Caryophyllene oxide	1979	1.1	0.3	0.6	Woody, spicy
2-Pentadecanone	1996	0.2	6.7	0.9	Spicy, herbal
Nerolidol	2003	0.5	0.3	0.2	Rose-, apple-, green-, citrus-like, woody

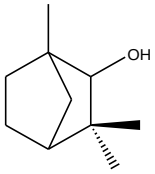
epi-Cubenol	2037	0.9	0.1	tr	Sweet-woody
Elemol	2077	4.6	tr	0.2	Weak woody, green-herbal
2-Pentadecanol	2095	0.3	4.1	0.5	Floral
Eugenol	2117	0.6	nd	nd	Strong spicy, cinnamon and clove-like
γ-Eudesmol	2135	1.3	0.4	0.2	Sweet-woody
Spathulenol	2151	2.2	0.5	tr	Spicy-woody
δ-Cadinol	2165	0.8	tr	tr	Spicy
α-Eudesmol	2186	1.3	0.2	tr	Sweet-woody
β-Eudesmol	2193	1.3	0.2	tr	Sweet-woody
α-Cadinol	2197	0.4	0.1	tr	Spicy
Guaiol	2214	3.1	tr	0.1	Woody, weak spicy
Farnesol	2259	tr	tr	nd	Floral-oily
Caryophyllenol	2296	0.6	0.1	tr	Spicy
Phytol	2569	0.9	0.1	0.2	Herbal-balsamic
Palmitic acid	2862	tr	0.2	16.6	Waxy

tr = trace compound (less than 0.5%)
nd = not detected
EO = Essential oil

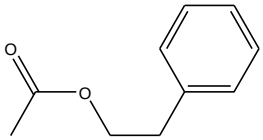
Using correlations of analytical data with odour notes of identified essential oil compounds published else where[11-17], we can state that the pleasant warm-herbal woody-spicy odour notes can be especially attributed to

2-tridecanone, *trans*-β-caryophyllene, 2-pentadecanone, elemol, guaiol, 1-tridecene, α-humulene, α-gurjunene and further caryophyllene derivatives, floral and floral-fatty notes are characteristic for 1-pentadecene, 2-pentadecanol, 2-phenylethyl alcohol and nerolidol, 2-tridecanol and low concentrated compounds such as p-cymene, decanol and nerolidol evoke fruity notes, fresh green can be attributed to elemol and some monoterpenes in very low concentrations, while 1-octen-3-ol, octenol, 2-octenone and hexane derivatives, identified only as trace compounds, show green-earthy odour impressions. In addition the woody-mushroom like side-notes of sample B is typical for 1-octen-3-ol (1.9% in the extract) and further octane derivatives (eg. Octanol), and the waxy background note for palmitic acid (16.6%) as well as the significant fresh – Allium like top note of sample A is especially characteristic for identified disulphides.

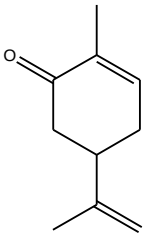
To summarise the investigation of the essential oil and two root extract of *Spilanthes* radicans, we can conclude that the samples were characterized by a significant, pleasant aroma each with more than 70 constituents. The main compounds were found to be 2-tridecanone, 1-pentadecene and *trans* β-caryophyllene as well as tridecene, pentadecane and some sesquiterpenic compounds. Structure of the identified compounds are given below;



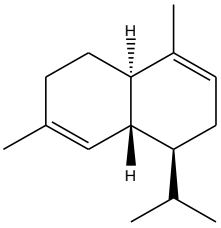
Fenchol



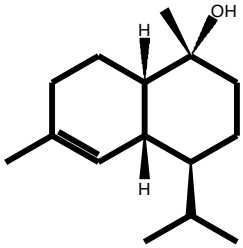
2-Phenylethyl acetate



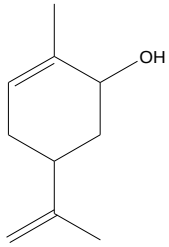
Carvone



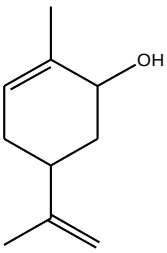
α-Cadinene



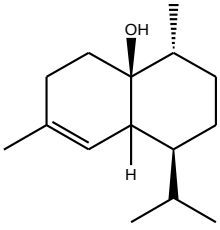
α-Cadinol



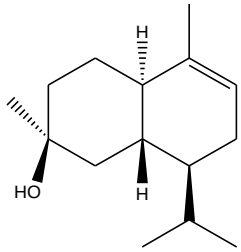
(E)-Carveol



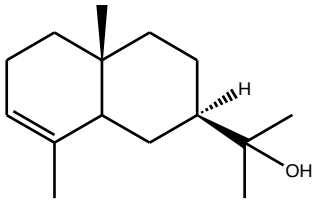
(Z)-Carveol



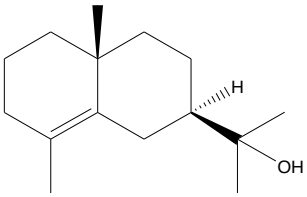
epi-Cubenol



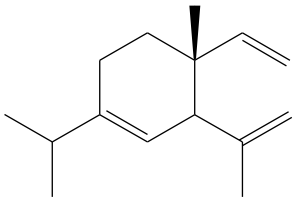
δ-Cadinol



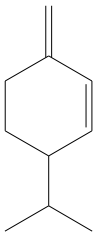
α –Eudesmol



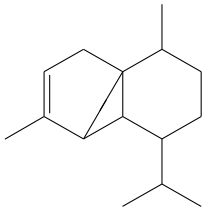
γ- Eudesmol



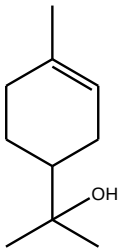
δ–Elemene



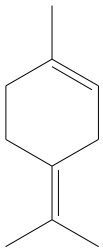
β-Phellandrene



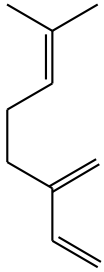
α –Cubebene



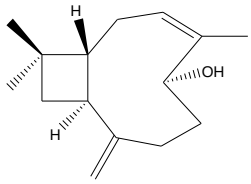
α-Terpineol



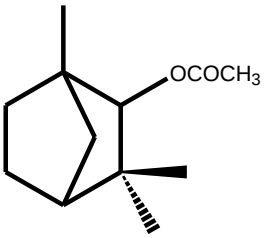
Terpinolene



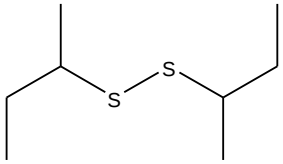
Myrcene



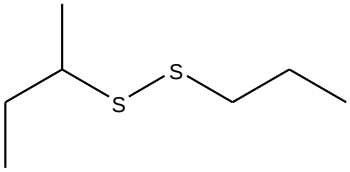
Caryophyllenol



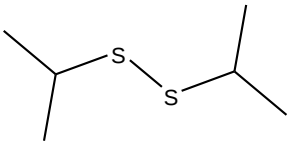
Fenchylacetate



Di-(1-Methylpropyl)-disulphide



1-Methylpropyl propyl disulphide

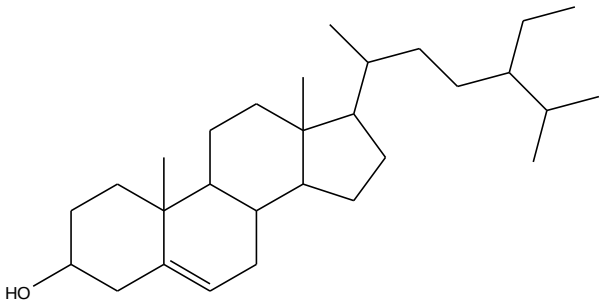


Diisopropyl disulphide

4.4.1 Characterization of G_1 (β-Sitosterol)

This compound was isolated from petroleum ether extract. The plant roots were extracted with petroleum ether three times and the combined extract was concentrated by evaporation. Then a white crystalline solid separated out. It was filtered and washed with petroleum ether, then with

methanol gave a white crystal, on recrystallisation from methanol gave pure crystal melting at 139ºC. It gave bluish-green colour with Liebermann – Burchard reagent, indicating it was a sterol, with vanillin- H_2SO_4 it gave blue spot and with anisaldehyde–H_2SO_4 appeared as red-violet spot.[18] It decolourised Bayer's reagent, indicating unsaturation in the compound. Mass spectrum of this compound showed m+ at m/z 414 and other prominent peaks were at 396 (m^+ - H_2O), 382 (m^+ - CH_3OH), 367 (382 - CH_3), 329 (m^+ -C_6H_{13}), 315 (m^+-C_7H_{15}), 273 (m^+-R) where the side chain R = $C_{10}H_{21}$, 255 (273-H_2O), 231 [m^+-(R+C_3H_6)], 213 (231-H_2O), 159, 145, 119, 107 and 91. IR absorption spectra showed the presence of hydroxyl group (broad absorption with maximum at 3441.4 cm^{-1}). Band due to gem dimethyl groups (doublet at 1383.1 and 1385.1 cm^{-1}) –C-H stretching and bending bands (2981, 2816, 1464, 1470 cm^{-1}) were also observed in the spectrum. Proton NMR spectrum was identical with that of β-sitosterol. A direct comparison of R_f (0.33 in benzene) with an authentic sample established its identity.



β-sitosterol

The derivatives, acetate (m.p. 125^0C) and benzoate (m.p. 144^0C) of the compound were prepared and found to be identical with β-sitosterol acetate (m.p.127^0C) and β-sitosterol benzoate (m.p.145^0C)[19]. Mixed melting point of β-sitosterol with an authentic sample was undepressed.

Experimental

1. Acetylation of G_1 (β-sitosterol**)**

100mg of G_1 was refluxed with 1 ml acetic anhydride in pyridine for two hours. Poured the reaction mixture to cold water, filtered, washed with water, dried and then recrystalised from petroleum spirit. The product melted at 125^0C.

2. Benzoylation of G_1 (β-sitosterol**)**

100 mg of G_1 was refluxed with 2 ml freshly distilled benzoyl chloride and pyridine on a sand bath for two hours. Poured the reaction mixture to cold water, filtered, washed, dried and recrystalised from benzene. The product of benzoylation of G_1 melted at 144^0C.

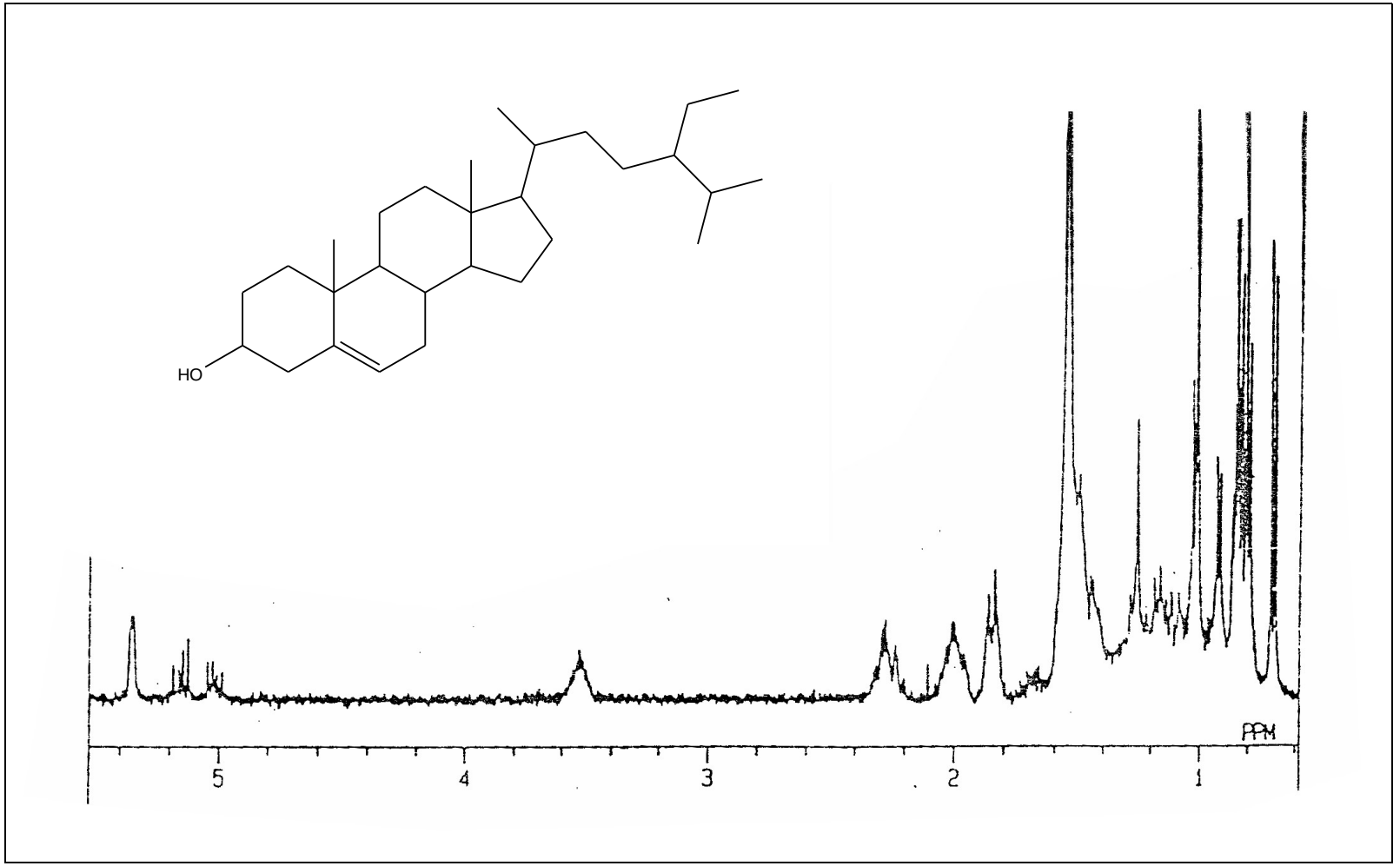


Fig. 4.1 ^{1}H-NMR spectrum of G_1 [β-sitosterol] (400 MHz, $CDCl_3$, TMS)

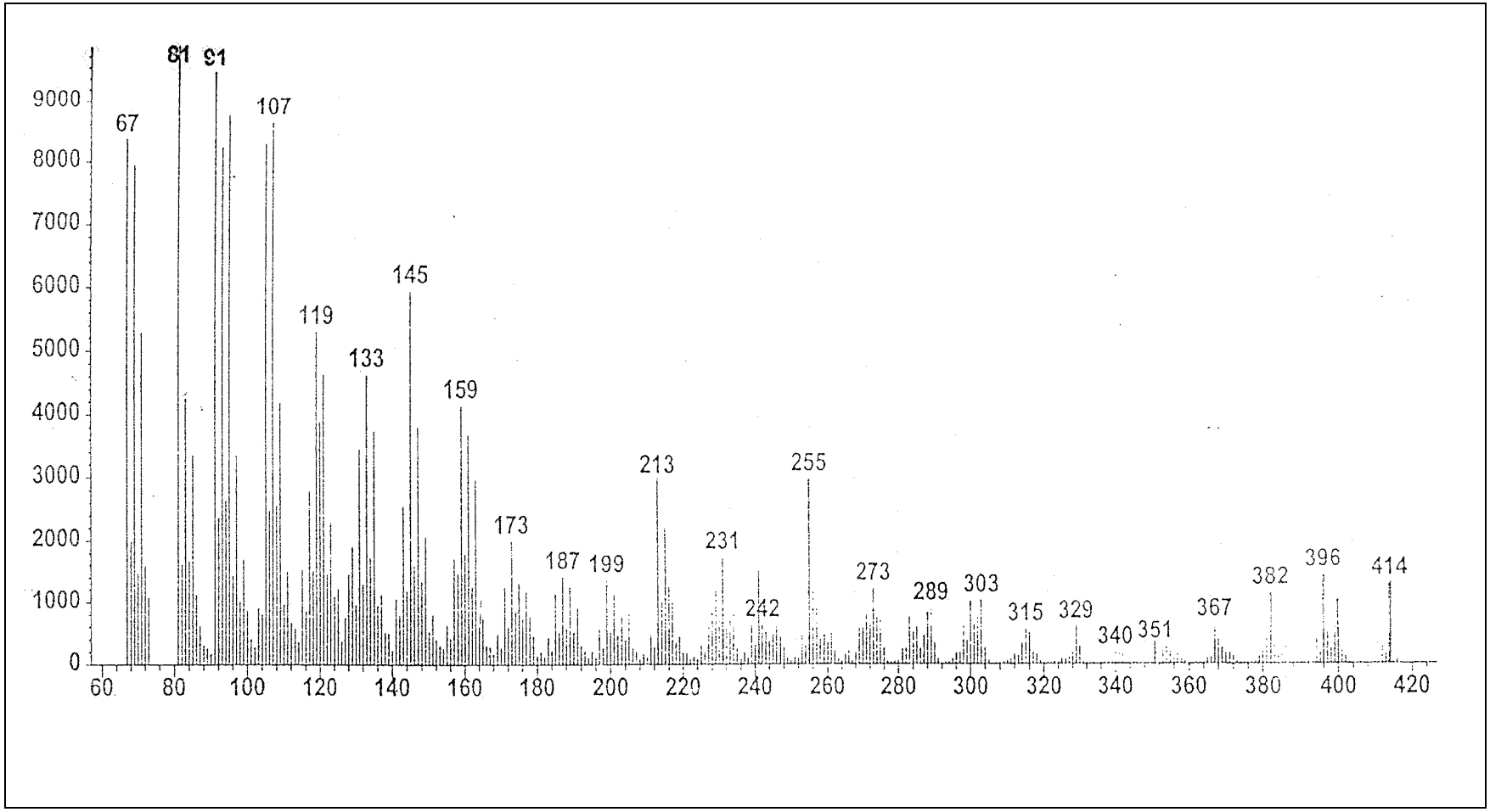


Fig. 4.2 Mass spectrum of G_1 [β-sitosterol]

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CHAPTER V

ANALYSIS OF THE ESSENTIAL OILS OF *ARTABOTRYS ODORATISSIMUS* FRUITS AND LEAVES

5.1 INTRODUCTION

Artabotrys odoratissimus R.Br. is a shrub native to eastern Asia and belongs to *Annonaceae* family.[1] This plant is commonly known as Manoranjini (Malayalam), Manoranjitham (kannada), Harchampa (Hindi), Kalchampa (Beng), Lilochampa (Guj), and Harachampaka (San).[2] It is a straggling shrub, leaves oblong - lanceolate, 6-10 x 2-4 cm long, acuminate, cuneate at base, shining above. Flowers fragrant, solitary or few flowered, peduncle. Sepals 3, recurved. Petals 6 in two whorls of 3 each, clawed at base, green at first turning yellow. Berries yellow 6-10 in a cluster. Flowering and fruiting occur most part of the year. It is indigenous to Indian Peninsula and Srilanka.[2]

5.2 MEDICINAL PROPERTIES AND USES

Artabotrys odoratissimus is an ornamental plant[3]. Fruits of this plant are recorded as containing fixed and volatile oil glycosides and resins, extracts are reported to exhibit hypotensive and spasmogenic as well as cardiac stimulating effects on some animals and cardiac depressant on others.[4]

Decoctions of the leaves are used as a remedy for cholera and have been found to exhibit antifertility effects in rats.[1-5] The essential oil of *A. odoratissimus* has shown excellent to good antihelmintic property against tape worms, earth worms and round worms.[6] Its flowers are used in the treatment of vomiting, biliousness, blood and heart diseases, itching, sweating, foul breath, thirst and headache[6].

5.3 PREVIOUS WORK

Two papers discuss the composition of the essential oil of the root or stem or bark from the African (Gabon & Zaire) and Asian (Malaysia) species of *Artabotrys lastourvillensis*,[7] *A. insignis* Engl. *A. pierreanus* Engl. *A. rufus* De Wild., *A. thomsonis* Oliv. and *A. venustus*.[8] Additional data on effects and less volatile compounds were presented in literature for *A. thomsoni* Oliv., *A. insignis* Engl. A. *pierreanus* Engl et Diels and *A. rufus* De Wild from Congo.[9,10] From the stem bark of *A. odoratissimus* two steroids (β-sitosterol and stigmasterol), an aromatic compound benzyl benzoate and a noraporphine alkaloid asimilabine were isolated[11]. No information has been given for the essential oils of *A. odoratissimus* fruits and leaves from India.

5.4 PRESENT WORK

The objectives of the present work is to identity the volatiles of the essential oil of *Artabotrys odoratissimus* fruits and leaves and to discuss the

constituents which are responsible for the characteristic odour, their biological activities as well as partly for the folk medicinal use of the plant.

5.5 EXPERIMENTAL

Plant material

The ripe fruits and leaves of *A. odoratissimus*, were collected from the Calicut University Campus and plant material was identified by Dr. A.K. Pradeep, Department of Botany, Calicut University of Kerala. A voucher specimen is deposited in the specially maintained Herbarium of the Department of Chemistry at Calicut University.

Essential oil extraction

The fleshy part of ripe fruits was removed from the seeds by means of knife and 400 g of them ground into a paste in a mixer grinder. Water was added to this paste and subjected to steam distillation for three hours. The essential oil was extracted with ether and dried over anhydrous sodium sulphate. A yield of 0.55 g (0.13%) of the essential oil was obtained on removal of the solvent.

450 g of fresh leaves were ground in to a paste and a yield of 0.85g (0.18%) of the essential oil was obtained similarly as in the case of fruits .

Olfactoric evaluations

The essential oils were diluted with dichloromethane, 10 µl placed on a commercial odor-strip (Dragoco Co.) and the odour characterized by professional perfumers.

Gas chromatography

GC analysis 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.). As columns a 30 m x 0.32 mm bonded unpolar FSOT-RSL-200 fused silica (film thickness 0.25µm, Biorad Co.) and a 30 m x 0.32 mm bonded polar stabil wax (film thickness 0.50 µm, Restek Co.) were used. Additional parameters are as follows. Carrier gas: Hydrogen; injector - temp. 250°C, detector temperature: 320°C, temp. program 40°C/5 min to 280°C/5 min with a heating rate of 6°C/min quantification by %-peak-area-calculation (unpolar column).

Some single compounds could be identified by coinjection of pure compounds and correlation of their retention times (using retention indices) with published data.[12-14]

Gas chromatography - Mass spectroscopy

The sample was analysed by the GC-MS system Shimadzu GC-17A with QP 5000 and the data system Compaq-ProLinea (Class 5k-Software) Hewlett-packard GC-HP5890 with HP-5970 MSD and PC-Pentium (Bohm Co., Chemstation-Software) and Finnigan MAT GCQ with data system Gateway-2000-PS75 (Siemens Co., GCQ-Software) Additional parameters are as follows: Carrier gas helium; injector temperature: 250^0C; interface heating: 300^0C; ion-source-heating 200^0C; Elmod: scan range 41-450 amu, other parameters are same as that of GC-FID. For compound identification Wiley-NPS-and NIST Library spectra as well as reference MS spectra[13,15-19] data were used.

5.6 RESULTS AND DISCUSSION

The essential oils were olfactorically evaluated as follows. Floral-fruity (Ylang-Ylang-like and direction of dried plums respectively), dry-woody (pine wood like), herbal spicy in the background (fruits) and green-grassy (top-note), herbal woody (cedar wood-notes), weak floral (passion-flower, like), earthy-smoky-fatty in the background (leave).

Using GC-MS analysis 80 compounds could be identified. As main compounds (concentration higher than 4%, calculated as percentage peak area of GC-FID analysis) of the essential oil of the fruits and leaves respectively of

Artabotrys odoratissimus, the sesquiterpiens β-caryophyllene (14.7%, 17.3%), *trans* nerolidol (8.2% , 1.9%), δ-cadinene (7.3% , 4.2%), α-copaene (6.4% , 9.3%), *trans, trans*-α-farnesene (5.8% , 7.4%), τ-cadinol (4.3% , 2.9% and caryophyllene oxide (3.2% , 6.8%) were found. The identified compounds and the corresponding concentration (%) are given (Table 5.1).

Table 5.1

Composition of the essential oil of the fruits (eof) and leaves (eol) of *Artabotrys odoratissimus*

Compounds	**eof**	**eol**	**RI**
Butyl acetate	0.6	0.1	770
Hexanal	0.1	0.7	778
(E)-2-Hexenal	0.2	1.2	831
(Z)-3-Hexenol	0.4	0.9	845
(E)-2-Hexenol	0.1	0.3	854
Hexanol	0.7	0.1	859
1,3-Butandiol	0.2	nd	941
α-Thujene	0.1	0.4	944
α-Pinene	0.5	0.1	951
β-Pinene	0.2	0.6	957
1-Octen-3-ol	nd	0.2	964
(E)-3-Hexenyl acetate	tr	0.4	982
Myrcene	tr	0.3	985
(E)-2-Hexenyl acetate	tr	0.2	995

Hexanoic acid	nd	0.3	999
Para-cymene	nd	0.1	1012
Limonene	0.4	0.7	1023
cis-β-Ocimene	tr	0.2	1027
trans-β-Ocimene	tr	0.5	1033
cis-Linalool oxide (furanoid)	1.2	tr	0164
1-Nonen-3-ol	0.8	nd	1068
Terpinolene	1.1	0.3	1080
Linalool	2.2	1.4	1085
(Z)-3-Hexenyl ethyl acetal	1.3	0.3	1089
Benzoic acid	1.7	nd	1094
Verbenone	0.4	0.9	1177
α-Terpineol	1.1	1.6	1185
α-Cubebene	1.4	3.2	1362
α-Ylangene	1.6	1.8	1394
α-Copaene	6.4	9.3	1399
β-Cubebene	0.3	0.1	1402
Cyperene	0.1	nd	1405
Longifolene	0.4	1.3	1408
β-Elemene	0.9	0.6	1410
α-Gurjunene	0.1	0.3	1412
β-Bourbonene	tr	0.2	1415
α-Cedrene	nd	0.4	1417
α-Himachalene	0.1	0.3	1420
α-Santalene	nd	0.1	1422

β-Caryophyllene	14.7	17.3	1424
Aromadendrene	0.2	0.3	1427
γ-Elemene	0.1	nd	1430
Sativene	tr	0.2	1432
trans-β-Bergamotene	0.3	0.4	1435
Thujopsene	nd	0.6	1437
α-Bergamotene	1.3	0.4	1440
trans-β-Farnesene	2.9	1.8	1448
γ-Decalactone	0.1	tr	1450
α-Amorphene	3.1	2.7	1452
α-Guaiene	tr	0.2	1455
α-Humulene	0.3	nd	1462
δ-Decalactone	0.4	nd	1464
Germacrene D	0.9	0.6	1468
Alloaromadendrene	0.2	1.2	1474
ar-α-Curcumene	0.1	0.4	1476
trans, *trans*-α-Farnesene	5.8	7.4	1490
α-Muurolene	1.3	2.6	1494
cis-α-Bisabolene	0.2	0.3	1497
Linalyl pentanoate	1.5	nd	1500
Calamene	0.1	0.5	1503
δ-Cadinene	7.3	4.2	1505
β-Selinene	0.2	2.1	1507
γ-Cadinene	2.1	0.3	1510
cis-Nerolidol	2.7	0.5	1536

Elemol	0.2	1.4	1540
trans-Nerolidol	8.2	1.9	1551
Spathulenol	1.4	0.6	1553
Caryophyllene alcohol	0.5	1.7	1557
Longifolene	0.1	1.3	1566
Caryophyllene oxide	3.2	6.8	1574
α-Cedrene epoxide	0.1	0.6	1582
Guaiol	1.2	nd	1584
Cedrol	nd	0.5	1606
β-Eudesmol	0.5	tr	1610
γ-Dodecalactone	0.2	nd	1640
τ-Cadinol	4.3	2.9	1649
α-Cadinol	0.7	0.2	1652
β-Bisabolol	0.5	1.1	1663
trans, *trans*-α-Farnesol	0.9	1.0	1733
Phytol	1.2	1.6	2080
Fatty acids and their esters	0.9	1.7	--
Higher hydrocarbons (higher than 16C)	4.2	0.8	--
Kaurane Derivatives	2.3	0.4	--
Unknown	About 1	2	--

1. nd = not detected
2. tr = trace compound (less than 0.1%)
3. RI = Refractive index

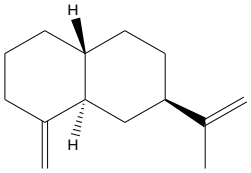
The correlation of olfactoric data with gas chromatographic-spectroscopic ones allows the following statement; lanalool, nerolidol and cadinol derivatives, α-terpineol, α-ylangene and lactones are odorous constituents responsible for the floral fruity scent, pinene and caryophyllene derivatives for the dry woody and para-cymene and ocimene derivatives for the herbal spicynotes (fruits). The green-grassy odour can be attributed to hexane derivatives, the herbal woody (cedarwood) one to cedrene and caryophyllene derivatives, linalool and cadinol derivatives show a weak floral scent and fatty acid and their esters, higher hydrocarbon and some sesquiterpenes (side-notes) possess the earthy smokey-fatty tonality (leaves).[20- 22]

Referring to the biological activities of identified constituents of the essential oils of the fruits and leaves of *A. odoratissimus* the following conclusion can be arrived at. The main compounds caryophyllene, cadenene, nerolidol and farnesene derivatives as well as minor concentrated monoterpenes, such as linalool, pinene and terpinene derivatives are known for their antimicrobial effects.[23,24] In addition linalool in medium concentration (2.2% in this fruits oil and 1.4% in the leaf oil) should be mentioned for its mild sedative and spasmolytic activity. [25-27]

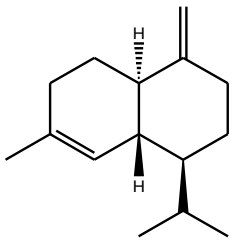
In summary the essential oils of the fruits and leaves respectively of *A. odoratissium* possess a characteristic pleasant odour, with sesquiterpenes, β-

caryophyllene, *trans*-nerolidol, δ-cadinene, α-capaene, *trans*, *trans*-α-faransene and caryophyllene oxide as main compounds. These constituents are partly responsible for the odour impression and for possible biological activities of both samples investigated.

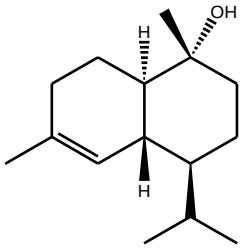
The structure of the identified compounds of *A. odoratissimus* are given below.



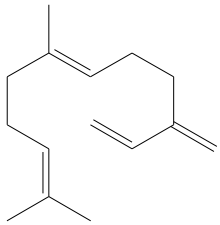
β-Silinene



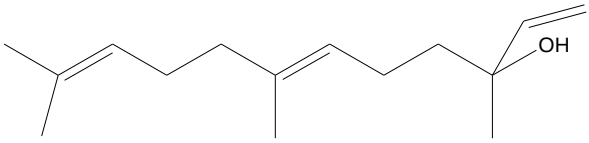
γ-Cadinene



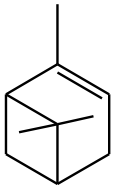
τ-Cadinol



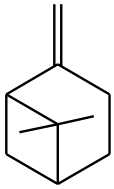
trans-β-Farnesene



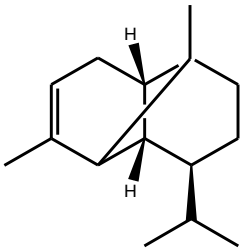
trans-Nerolidol



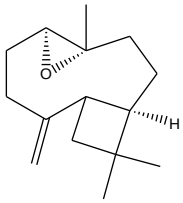
α -Pinene



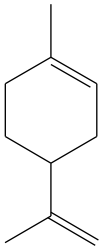
β-Pinene



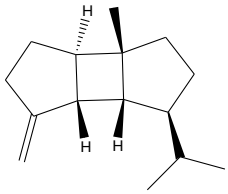
α-copaene



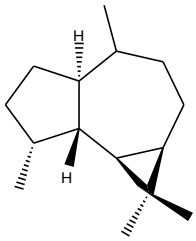
Caryophyllene epoxide



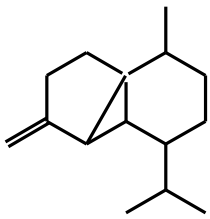
Limonene



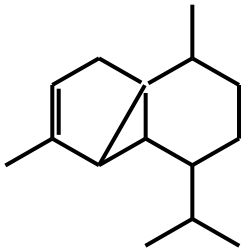
β-Bourbonene



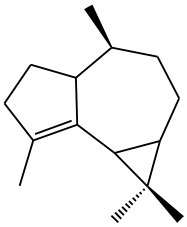
Aromadendrene



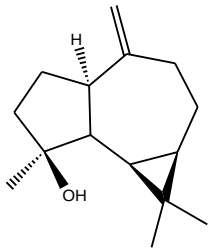
β- Cubebene



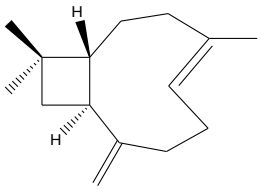
α-Cubebene



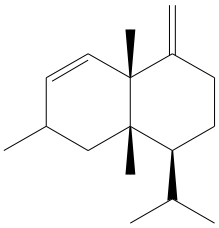
α-Gurjunene



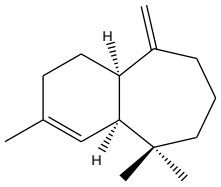
Spathulenol



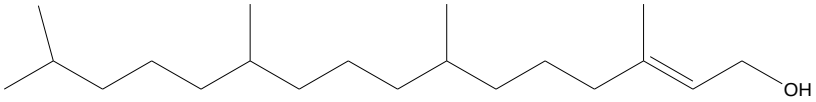
β-Caryophyllene



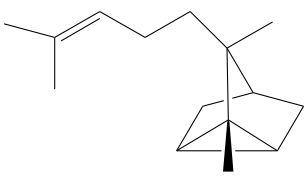
α-Amorphene



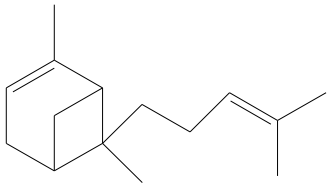
α-Himachalene



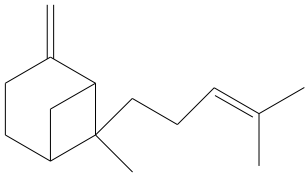
Phytol



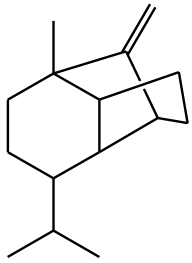
α-Santalene



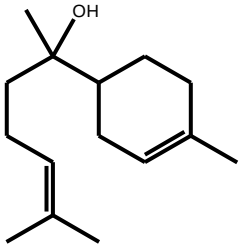
α–Bergamotene



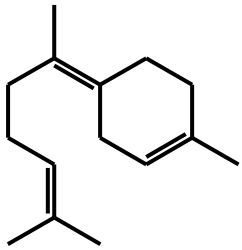
trans-β-Bergamotene



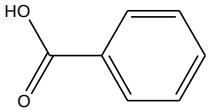
Sativene



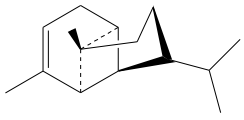
β-Bisabolol



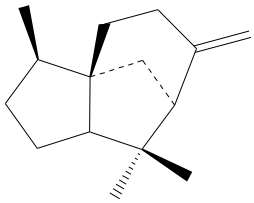
Bisabolene



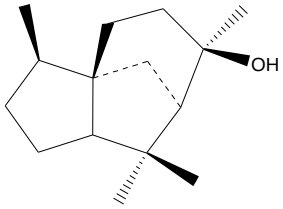
Benzoic acid



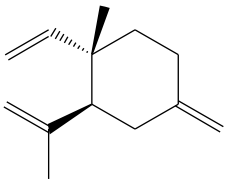
α–Ylangene



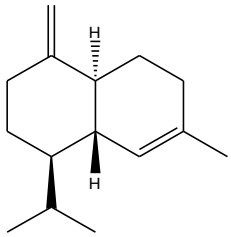
β-Cedrene



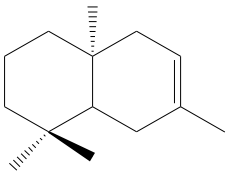
Cedrol



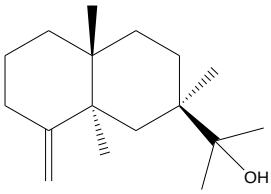
γ-Elemene



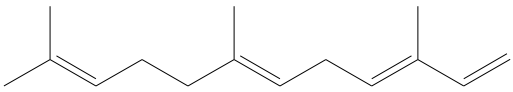
δ-Cadinene



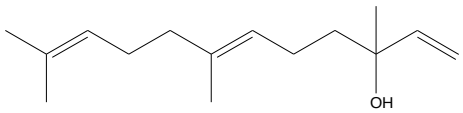
Thujopsene



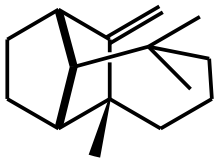
β-Eudesmol



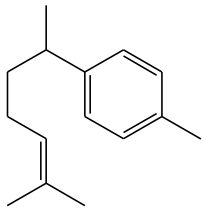
(E, E)- α-Farnesene



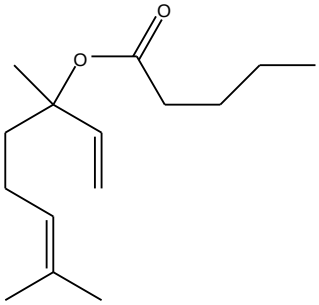
Nerolidol



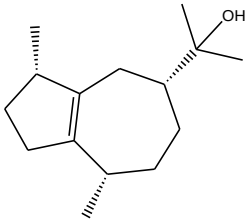
Longifolene



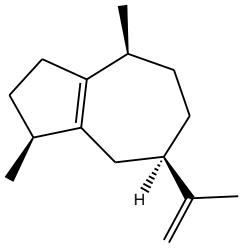
ar-Curcumene



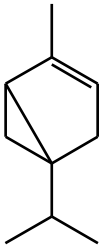
Linalylpentanoate



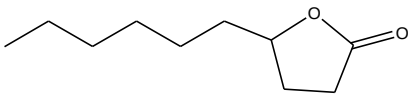
Guaiol



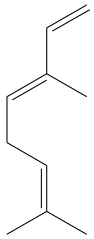
α-Guaiene



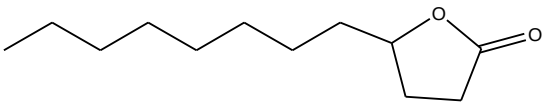
α-Thujene



γ-Decalactone



trans–β-Ocimene



γ-Dodecalactone

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