PHYTOCHEMICAL INVESTIGATION OF A FEW PLANTS AND SCREENING OF SOME SECONDARY METABOLITES FOR THEIR BIOLOGICAL PROPERTIES

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By

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CERTIFICATE

This is to certify that this thesis entitled "**Phytochemical investigation of a few plants and screening of some secondary metabolites for their biological properties**" is an authentic record of the research work carried out by **Beena Jose**, in the Department of Chemistry, University of Calicut, under my supervision in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry, under the Faculty of Science of the University of Calicut and that no part thereof has been presented earlier for any other degree.

It is also certified that Beena Jose has passed the Ph.D. Preliminary Qualifying Examination in 2003.

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Dr. P. Mohamed Shafi
(Supervising Teacher)

DECLARATION

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

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Sr. Beena Jose

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PREFACE

Plants are an integral part of nature. Ever since the birth of man the plant kingdom has provided him with food, shelter and drugs. Plants are sophisticated factories where a variety of chemical compounds are manufactured. They are now recognized for the production of a large variety of chemicals. These plant-derived products are used as medicines, pesticides, perfumes, fragrances, etc. The Indian system of medicine, Ayurveda, makes use of plant kingdom very effectively and has stood the test of time.

Synthetic chemicals 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. Moreover many of the microbes and insects have become resistant to most of these chemicals. Hence we look to nature as an ally and resource in finding new strategies to combat diseases. In this respect, plants serve as a vast reservoir of biodegradable chemicals, many of which may have evolved in the defence of predators.

Phytochemical studies have become more encouraging with the advent of modern spectroscopic techniques and instrumentation. Hithero impossible separations have become possible with the improved chromatographic techniques. The revolutionary advances in the field of spectroscopy and computers have made structural elucidation more simple and reliable.

The work presented in this thesis deals with the chemical investigation of thorns and leaves of *Canthium parviflorum* and air-dried *Biophytum sensitivum*, the analyses of essential oils from *Zanthoxylum rhetsa* seeds, *Anisochilus carnosus* spikes and roots and air-dried *Biophytum sensitivum*. The chemical transformation and the antimicrobial activities of *Zanthoxylum rhetsa* seed essential oil have also been studied.

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

The first chapter has two sections, section I presents the phytochemical studies on the thorns and section II, on the leaves of *Canthium parviflorum*. The investigation enabled the identification of seven compounds, which include long chain ester, taraxerol, d-mannitol, petunidin, β-sitosterol, mixture of long chain acids and alcohols and sakuranetin-4'-O-glycoside.

The second chapter comprises of three sections. Section I is an introduction highlighting the importance of essential oils and their method of extraction. A brief overview of analytical techniques is also illuminated in this section. Section II deals with the influence of the extraction methods on the essential oil composition of *Zanthoxylum rhetsa* seeds, steam distilled from aqueous, alkaline and acid media. Based on this work an article entitiled 'Influence of pH on essential oil composition of *Zanthoxylum rhetsa* seeds obtained by steam distillation' has been accepted for publication in *Flavour and Fragrance Journal*. The chemical transformation of *Z. rhetsa* seed essential oil, obtained by steam distillation from alkaline medium, containing 72.7% sabinene, has been described in section III of this chapter. The seed oil, upon treatment with peracetic acid got converted into a viscous liquid. GC-MS analysis of this viscous liquid resulted in the identification of 23 compounds of which p-menthane-1, 2, 7-triol was the major component. Earlier reports suggested that, sabinene on reaction with peracetic acid was converted into p-menthane-1, 2, 4-triol. This controversy in the results prompted us to investigate further on this. The viscous liquid obtained by peracetic acid treatment of *Z. rhetsa* seed essential oil was heated with 50% H_3PO_4 for 3 h. The resulting mixture, on GC-MS analysis showed phellandral (p-menth-1-en-7-al) as major component. The formation of this conjugated aldehyde is possible only from p-menthane-1, 2, 7-triol and p-menthane-1, 2, 4-triol cannot yield this product.

Chapter 3 is divided into two sections. Analysis of the essential oil of air-dried *Biophytum sensitivum* is presented in section I. By means of GC and GC-MS sixty-eight compounds were identified in this sample, with 1,4-dimethoxybenzene as major component. 1,4-Dimethoxybenzene is found to be the main component present in the volatiles of lotus flower and contribute to the pollination of the flower as insect attractants. It can be suggested that, the pollination of *B. sensitivum* also is aided by the presence of 1,4-dimethoxybenzene, as it acts as an insect attractant. Based on this work, an article entitled 'Medicinal Used Plants from India: Analysis of the Essential Oil of air-dried *Biophytum sensitivum* (L.) DC.' has been published in the journal *Scientia Pharmaceutica* **72**, 2004, 87-96. The characteristic odour imparted by air-dried *Biophytum sensitivum* essential oil also was a subject of study. Section II discusses the phytochemical studies on *Biophytum sensitivum*. This investigation led to the identification of mixture of alkanols and long chain esters.

The fourth chapter consists of two sections. Analysis of the volatiles of *Anisochilus carnosus* spikes, using GC-FID, GC-MS and olfactometry form the subject matter of the first section. Hundred and eleven compounds could be identified in the spike essential oil, with caryophyllene oxide, 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 'Medicinal Used Plants from India: Analysis of the Essential Oil of *Anisochilus carnosus* (Lamiaceae) Spikes' has been published in the *Journal of Essential Oil Bearing Plants* **6(1)**, 2003, 78-85. Section II gives an account of Solid Phase Micro Extraction (SPME) of *Anisochilus carnosus* root essential oil. The aroma compounds of the essential oil of fresh roots of aldehyde is possible only from p-menthane-1, 2, 7-triol and p-menthane-1, 2, 4-triol cannot yield this product.

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A. carnosus as well as four corresponding SPME samples of the oil (two SPME-headspace samples and the other two SPME-samples obtained by introducing SPME needle directly into the root essential oil) were analysed by GC-FID, GC-MS and olfactoric methods. Sixty-nine volatiles could be identified in this sample with 13-C hydrocarbon megastigma-4, 6(Z), 8(E)-triene as major component. Megastigmatrienes are thought to be derived in nature from β-ionone via the readily dehydrated β-ionol. The correlation of the olfactoric data with gas chromatographic-spectroscopic data showed that, the SPME headspace technique is more effective to analyse the aroma compounds from the essential oil than introducing the needle directly into the oil. Based on this work an article entitled 'Solid Phase Micro Extraction in Essential Oil Analysis on the Example of an *Anisochilus carnosus* (Lamiaceae) Root Oil from South-India' has been accepted for publication in the *Journal of Essential Oil Bearing Plants.*

The fifth chapter has three sections. Section I gives an introduction to antimicrobial studies. Antibacterial activity assay of *Zathoxylum rhetsa* seed essential oil obtained by steam distillation from aqueous, alkaline and acid media, against four bacteria – *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae* and *Proteus vulgaris*, is given in section II. Evaluation of antifungal activity of *Zanthoxylum rhetsa* seed essential oil against *Phytophthora capsici*, the foot rot pathogen of black pepper, is presented in section III of the final chapter. The activity of the oils was checked at all the four different phases of growth of the fungus, namely vegetative growth, sporangial formation, zoospore liberation and zoospore germination. Out of the three essential oils tested, the oil extracted from acid medium was found to be highly active against the tested microorganisms.

Terpinenes and terpinene derivatives are reported to have antimicrobial activities. α- and γ-terpinenes and terpinolene are the major components of acid extract and the antimicrobial activity of the oil can be attributed to the presence of these components. The poor antimicrobial activity of alkali and aqueous extracts revealed that, sabinene, the chief constituent of the extracts, was a poor antimicrobial agent.

CANTHIUM PARVIFLORUM

CHAPTER I

SECTION 1 : PHYTOCHEMICAL STUDIES ON THE THORNS OF *CANTHIUM PARVIFLORUM* LAM.

1.1 Introduction

The genus name *Canthium* is derived from the Malay word 'canti', which was the name given to a tree in Malacca, the first described species of the genus. There are approximately 230 *Canthium* species distributed in the tropical and warm temperate regions of Asia, Africa and Australia. About 16 species are seen in India, Burma (Myanmar), Ceylon (Sri Lanka) and Andamans.[1] Local Canthiums range in size from shrubs to small trees. They have entire, opposite leaves and bear small, sometimes dense axillary clusters. Small rounded or lobed drupes are borne as fruit, ranging in colour from orange/red to shining black. Many Australian species occur in drier inland areas, but quite a few local species occur in rainforest or on its margins.[2]

Canthium parviflorum Lam. Syn. *Plectronia parviflora* is a rigid, thorny, deciduous shrub or small tree with stiff and spreading branches armed with stout, straight, nearly horizontal, paired spines above leaf axils. It is commonly called 'Kara' in Malayalam. It belongs to Rubiaceae family.[3] Leaves are long, flowers small, white or pale greenish yellow, fruit compressed, yellow when ripe, edible and two seeded. This plant occurs naturally on an elevation of 1000 m in western peninsular India from Gujarat and Maharashtra southwards, in Bihar, Orissa, Myanmar and in Sri Lanka.[1] In central India, flowering occurs mainly between May and July and fruiting in November-December.

A decoction of the edible leaves, as well as the roots of this plant, is prescribed in certain stages of flux, and the latter is supposed to have anthelmintic qualities, though neither has much sensible taste or smell. In

Indo-China, the bark and the young branches are given as medication for dysentery.[4,5] The edible leaves and fruit constitute the Ayurvedic drug 'Kari', which is astringent and effective against cough and indigestion. The plant is sometimes used as an antispasmodic.[6] The stem yields a fiber and the wood is hard and suitable for turning.

I.2 Work so far reported on *Canthium* species

I. *Canthium parviflorum* Lam.[7-11]

A rigid shrub, seen in the western part of the Indian peninsula from Konkan southwards to Ceylon, ascending up to 4000 ft. The plant is gregarious and useful for hedges.[1]

Dutta and co-workers[12] isolated d-mannitol from the alcoholic extract of the leaves of *C. parviflorum*. Mannitol, produced by the reduction of mannose, is very common in algae, fungi and lichens as well as in higher plants[13]. The major function of sugar alcohols is in the storage of energy, but mannitol may also be involved in the mechanism of translocation in phloem in higher plants. Other possible functions include osmo-regulation and protection of plants from desiccation and frost damage.[14]

II. *Canthium dicoccum*

This plant is widely distributed in Sikkim, Himalayas, Khasia, Jaintia hills, all plain districts of Madras state on both sides of the peninsula.[4] Wood is used for agricultural implements and for making combs, toys, posts and rafters. Bark is employed as a febrifuge and applied externally to fractures and it is also used in fever.[15] Leaves of this plant contain flavones, tannins and hydrocyanic acid[4].

Mukherjee and co-workers[16] isolated α-amyrin from the benzene extract and oleanolic acid from the ethanolic extract of the bark of *C. dicoccum.*

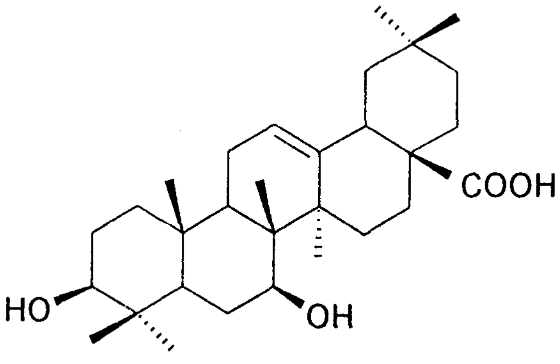
Dan and co-workers[17] found that, the separation of the ethanolic extract of the leaves of *Canthium dicoccum* into acid and neutral fractions followed by column chromatography yielded aesculetin dimethyl ether, scopoletin, lupeol and β-sitosteryl acetate.

Chatterjee and co-workers[18] isolated aesculetin dimethyl ether, scopoletin and a triterpene acid sapogenin, acetyl ursolic acid from the stem bark of *C. dicoccum.* The ethanolic extract of the air-dried, powdered, defatted stem bark of *C. dicoccum* was divided into ether soluble and insoluble parts, the former on preparative tlc over silica gel G gave aesculetin dimethyl ether and scopoletin. The ether insoluble part was hydrolysed with acid and the aglycone part was separated into acid and neutral fractions. The acid part on column chromatography over silica gel gave acetyl ursolic acid.

Herath and co-workers[19] separated sitosterol, quinovaic acid, acetyl quinovaic acid and scopoletin from the bark of *C. dicoccum.*

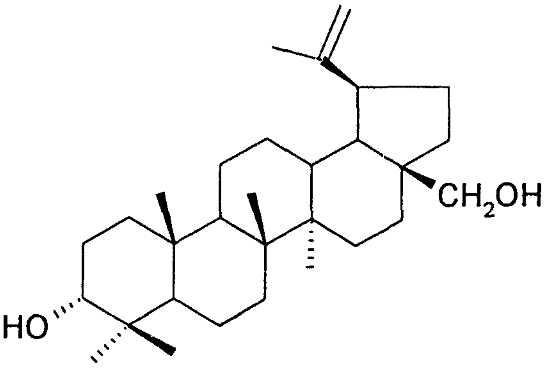
Unnamed alkaloids have been isolated from several species of *Canthium,*[20,21] while the peptide alkaloid canthiumine has been isolated from *C. euryoidesis.* Only traces of the alkaloids were found in the bark, timber and leaves of *C. dicoccum.*

A triterpene acid namely canthic acid isolated from the stem bark of *Canthium dicoccum* has the following structure.



Canthic acid

Another compound 3-epibetulin has also been isolated from this plant[22].



3-Epibetulin

I.3 Present work

Canthium parviflorum Lam. is the least chemically investigated species of the genus *Canthium.* Since no previous reports on the chemical composition of the thorns of *C. parviflorum* Lam. were found, the present investigation is therefore aimed at isolating and identifying the compounds present in it.

I.4 MATERIALS AND METHODS

Plant material

The thorns of *Canthium parviflorum* Lam. was collected from Trichur District of Kerala in May 2002 and was authenticated by Dr. A.K. Pradeep Department of Botany, Calicut University. A voucher specimen of the plant has been deposited in the Herbarium of Chemistry Department, Calicut University.

Melting point determination

All the melting points of 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 Shimadzu FTIR-8101A spectrometer .

Ultraviolet spectroscopy (UV)

UV spectra of the isolates were recorded using Shimadzu 1601 spectrometer.

Nuclear magnetic resonance spectroscopy (NMR)

The proton NMR spectra of the isolates were recorded at 500 MHz in DMSO and $CDCl_3$, using tetramethyl silane (TMS) as internal standard, using Bruker spectrometers. ^{13}C NMR spectra were recorded at 125 MHz in $CDCl_3$, using TMS as internal standard. The chemical shifts are reported in ppm (δ).

Electron impact mass spectra (EIMS)

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

Column chromatography (CC)

Column chromatographic separation of the crude and semipurified extracts were carried out using silica gel (Qualigens, 60-120 mesh).

Thin layer chromatography (tlc)

Thin layer chromatographic plates were prepared using tlc grade silica gel-G (Merck). Layer thickness 0.2mm (prepared using Stahl apparatus).

Paper chromatography (PC)

Paper chromatography was carried out using Whatmann No.1 filter paper.

Reagents

Liebermann-Burchard Reagent (for triterpenes and sterols). The reagent was prepared as follows:

Acetic anhydride (5 ml) and 97% sulphuric acid (5 ml) were mixed carefully with cooling and ethanol (50 ml) was added to that mixture.

Treatment of the plates:

Heated to 110°C until maximal visualization of the spots.

I.5 Extraction, fractionation and isolation of the compounds from the petroleum ether extract of the thorns of *Canthium parviflorum* Lam.

Dried and finely powdered thorns of *Canthium parviflorum* Lam. (2.82kg) were extracted thrice with petroleum ether (60-80°C, 3 x 7 L). The combined extract was then concentrated under reduced pressure to about 500ml of dark brown coloured liquid. Then a yellow powdery solid separated out. It was filtered, washed repeatedly with ethyl acetate and dissolved in hot ethyl acetate and adsorbed on silica gel (50g, 60-120 mesh). After drying, it was taken in a chromatographic column (3cm x 60cm; d x l) and eluted with solvents of increasing polarity viz. petroleum ether (1 L), 7:1 petroleum ether-ethyl acetate (500 ml), 1:1 petroleum ether-ethyl acetate (600 ml), ethyl acetate (700 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 3-10 obtained by petroleum ether elution on evaporation gave a white powdery substance, which on recrystallisation from chloroform yielded 150mg of pure substance $\mathbf{T_1}$, m.p.78°C.

The crude petroleum ether extract, after the removal of yellow powdery solid, was adsorbed on 400g of silica gel (60-120 mesh) and packed in a column (4cm x 100cm; 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. Fractions 1 to 3 obtained by 4:1 petroleum ether-ethyl acetate elution, on evaporation gave white crystalline solid, $\mathbf{T_2}$ (1g, 274°C).

Table I. 1

Compounds isolated from petroleum ether extract of *Canthium parviflorum* Lam. thorns

Compounds	Eluent composition	Melting point	Molecular mass
T_1	Petroleum ether	78°C	522
T_2	4:1 Petroleum ether-ethyl acetate	274°C	426

I.6 Extraction, fractionation and isolation of compounds from the alcohol extract of the thorns of *C. parviflorum* Lam.

Finely powdered thorns of *C. parviflorum* Lam. after extraction with petroleum ether was extracted twice with methanol (2x5L). Final extraction was carried out by refluxing with methanol for 30 minutes and keeping for one day. Both refluxed and unrefluxed methanol extract were syrupy in appearance and gave identical spots on tlc analysis. So they were mixed together and filtered by applying suction.

The combined alcohol extract was concentrated under reduced pressure to about 500 ml. About 250 ml of water was added and extracted thrice with benzene and ethyl acetate (3 x 200 ml each). The benzene extract on column chromatography did not afford any compound. Ethyl acetate extract was concentrated, the residue was dissolved in methanol and adsorbed on 300g of silica gel, packed in a column (4cm x 100cm; d x l). Elution was carried out using solvents of increasing polarity viz. 6:1 petroleum ether-ethyl acetate (1.3L), 3:1 petroleum ether-ethyl acetate (1.5L), 1:1 petroleum ether-ethyl acetate (1L), ethyl acetate (1.5L), 3:1 ethyl acetate-methanol (1.5L) and methanol (500ml). 3:1 ethyl acetate-methanol fraction gave a pale yellow coloured compound T_3, recrystallised from hot ethanol as white powdery solid (50mg, m.p. 158°C).

Table I. 2

Compound isolated from ethyl acetate extract

Compound	Eluent composition	Melting point	Molecular mass
T_3	3:1 ethyl acetate-methanol	158°C	182

Finally the crude methanol extract of *Canthium parviflorum* Lam. thorns were refluxed with MeOH- Conc.HCl (150ml MeOH:40ml Conc.HCl) for 30 minutes. It was then cooled, filtered and the vine red coloured filtrate was twice washed with ethyl acetate to remove flavones.[23] It was then extracted with amyl alcohol and sufficiently concentrated for direct paper chromatography. It was chromatographed one dimensionally on paper in BAW (n-Butanol: Acetic acid: Water, in the ratio 4:1:5) and forestal (HCl: Acetic acid: Water, in the ratio 3:30:10) as eluents. The visible spectra of this compound showed bathochromic shift in the presence of 5% alc. $AlCl_3$. The persistent vine red colour, even after heating with MeOH-Conc.HCl suggested the presence of anthocyanidin, $\mathbf{T_4}$.

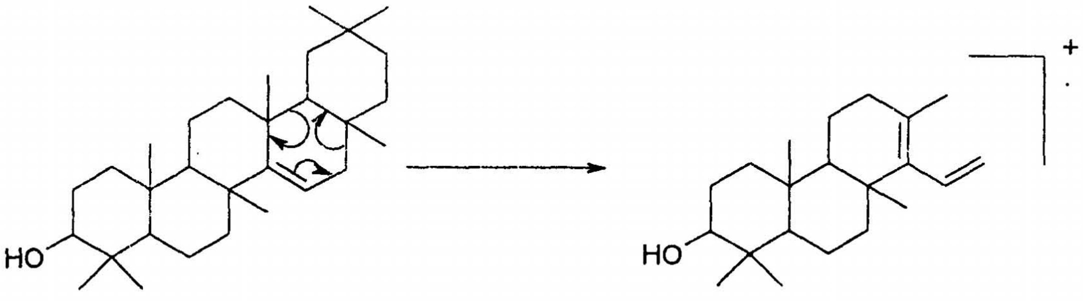
I.7 RESULTS AND DISCUSSION

I.7.1 Characterization of T_1

The compound $\mathbf{T_1}$ melted at 78°C. It gave a brown spot on spraying with H_2SO_4 and strong heating. It did not answer Liebermann Burchard reaction, indicating that it was not a triterpenoid or a sterol. Mass spectrum of this compound showed M^+ at m/z 522. Fragmentation pattern with a regular difference of 14 mass units showed its straight chain hydrocarbon nature. The IR spectrum gave characteristic absorption at 1736cm^{-1} indicating the presence of ester carbonyl group and also other absorption bands at 734cm^{-1} and 719cm^{-1} were indicative of a long hydrocarbon chain. The mass spectral fragmentation pattern and IR data suggested that it was a long chain ester with the molecular formula $C_{35}H_{70}O_2$.

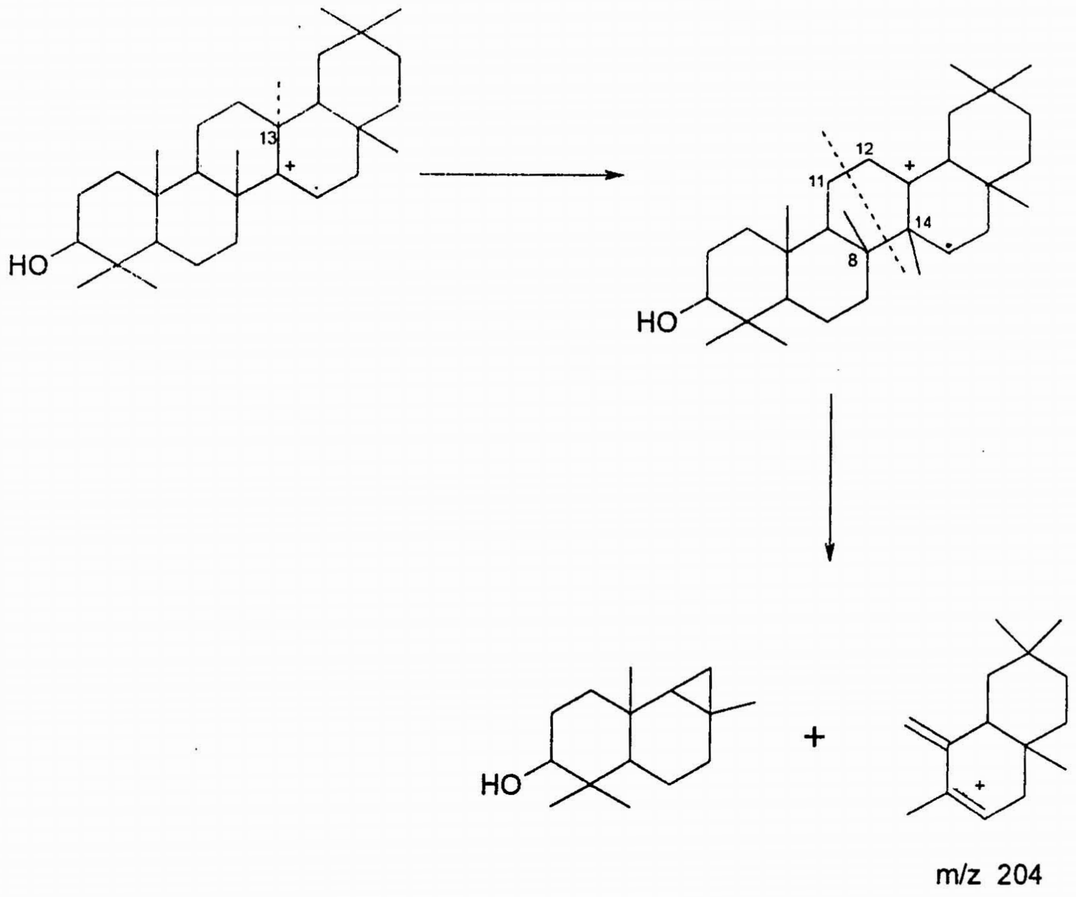
I.7.2 Characterization of T_2 (Taraxerol)

This compound was isolated from the petroleum ether extract on elution with 4:1 petroleum ether-ethyl acetate as colourless needles (1g). It was recrystallised from benzene and had a m.p. of 274°C. It answered Liebermann-Burchard reaction showing a persistent pink colour typical for triterpenes. It gave blue colour with vanillin-H_2SO_4 and appeared as a pink spot with anisaldehyde-H_2SO_4 reagent. It decolourised Baeyer's reagent, indicating its unsaturated nature. Mass spectrum of T_2 showed M^+ ion at m/z 426 corresponding to a molecular formula $C_{30}H_{50}O$. The IR spectrum of this compound showed a broad prominent absorption with the maximum at 3487.7cm^{-1}, indicating the presence of –OH group. The proton NMR spectrum showed eight tertiary methyl singlets at δ 0.80, 0.82, 0.91 (6H; 2 x Me) 0.93, 0.95, 0.98 and 1.09 indicating that it belonged to the pentacyclic group of triterpenes. The ^{1}H NMR and ^{13}C NMR spectra showed the presence of a trisubstituted double bond [^{1}H NMR: δ 5.54 (1H, dd, vinylic proton); ^{13}C NMR: δ 158.17 (unsaturated quarternary carbon), 116.97 (unsaturated CH carbon).[24] A multiplet at 3.2 in the ^{1}H NMR corresponding to one proton is that of a carbinol methine proton. This is supported by the presence of a carbinol methine carbon absorption at δ 79.1 in the ^{13}C NMR spectrum. These two observations along with the IR absorption for –OH group proved the presence of a secondary hydroxyl group. Prominent peaks in the mass spectrum were at m/z 426 (M^+), 411 (M^+-CH_3), 393 ($411-H_2O$), 302 (retro-Diels Alder-cleavage). 287 ($302-CH_3$), 269 ($302-CH_3-H_2O$), 218, 204 and 189 ($204-CH_3$).

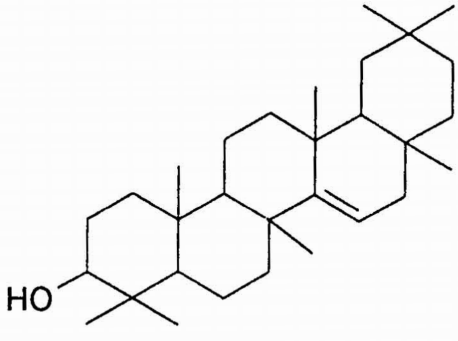


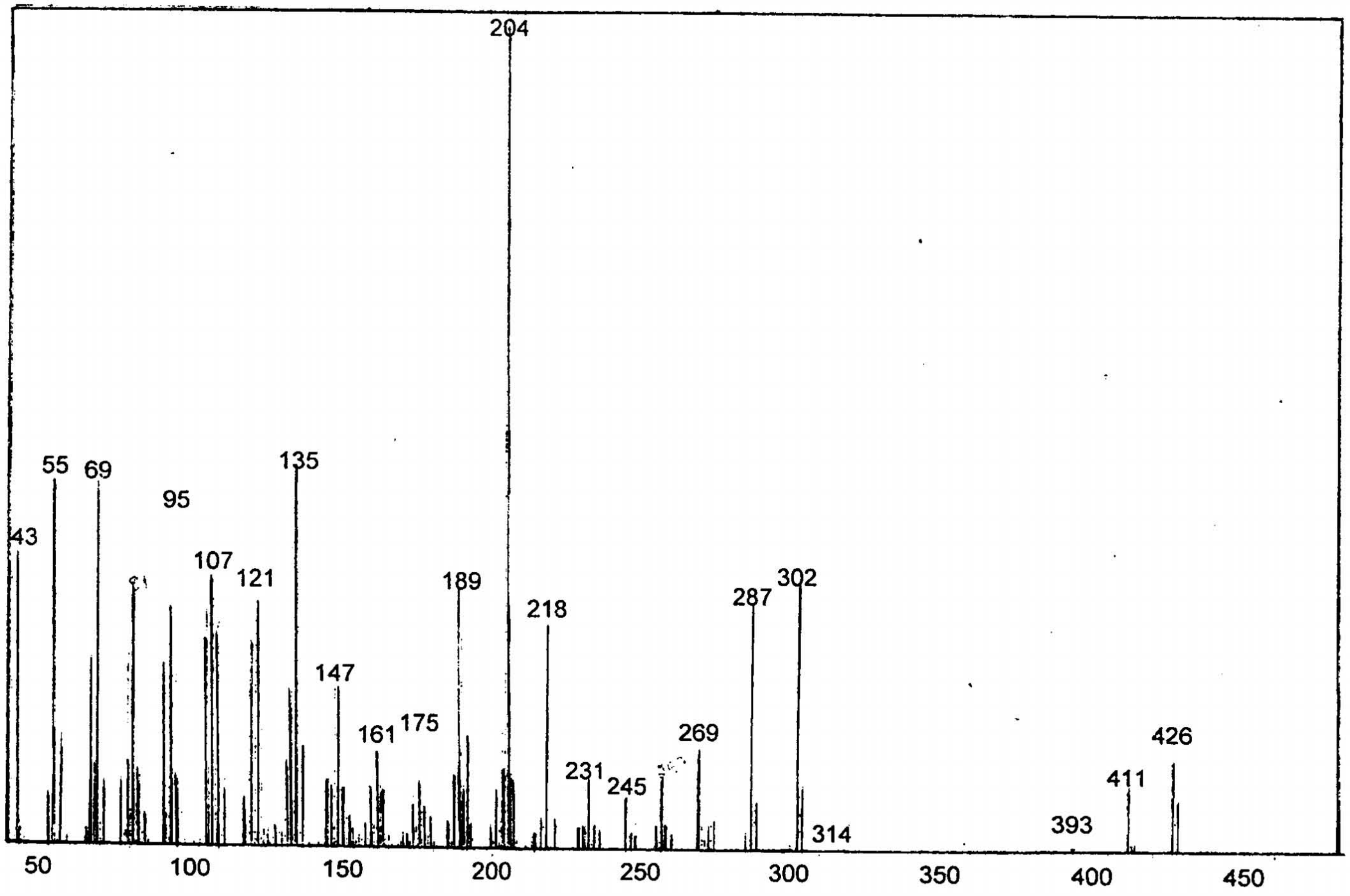
m/z 302

The base peak was at m/z 204. The mechanism for the genesis of this fragment can be proposed by assuming that in the molecular ion, the missing electron is preferentially from the carbon-carbon double bond, migration of C-13 methyl group, then yielding the radical ion. Fission of the 11-12 and 8-14 bonds gave the stable diene.[25]

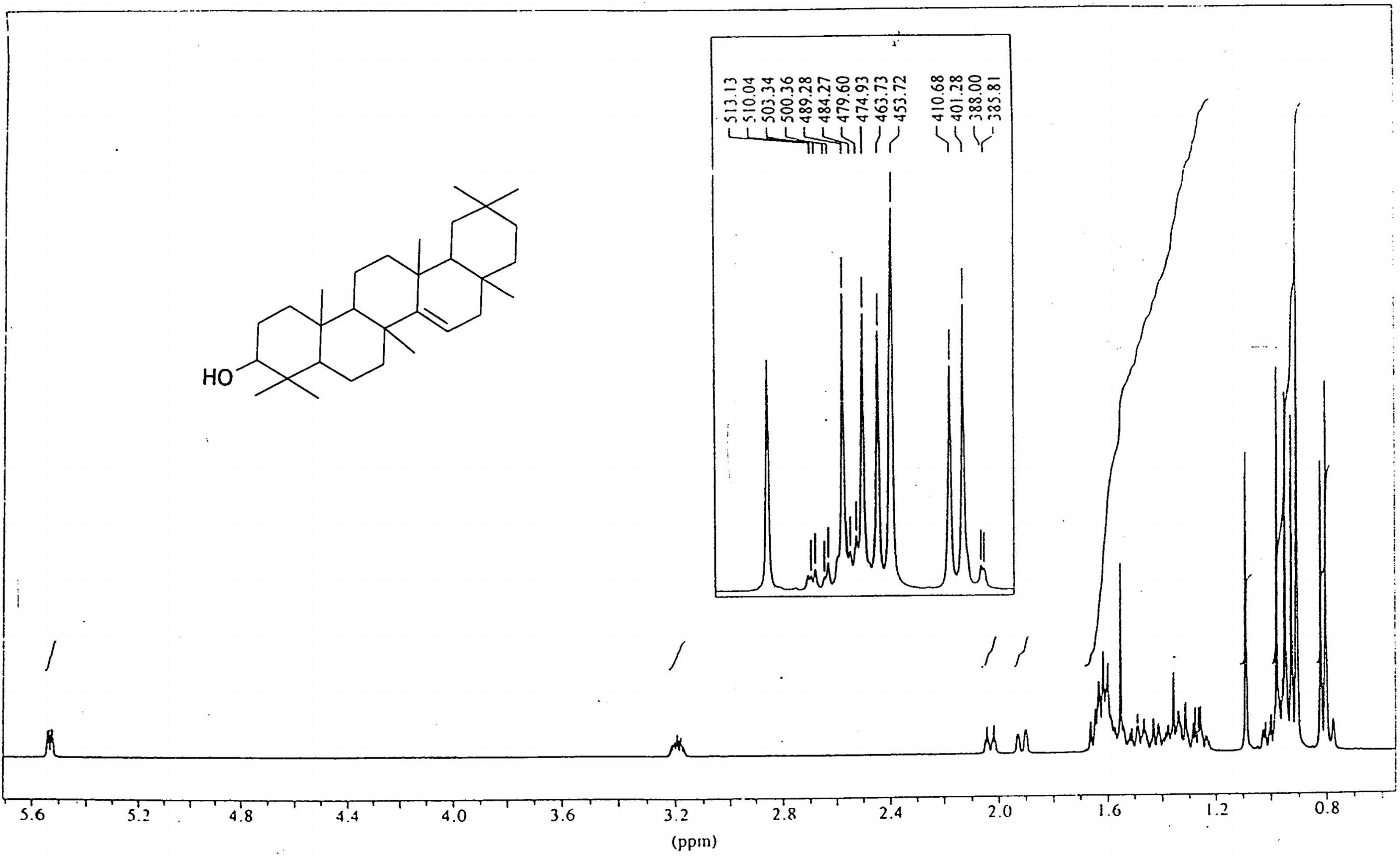


All the spectral data of compound **T$_2$** were found to be quite identical with those of taraxerol reported in literature.[24,26] Direct comparison with authentic sample (mmp, tlc, ^{1}H NMR, ^{13}C NMR) confirmed the compound as taraxerol.

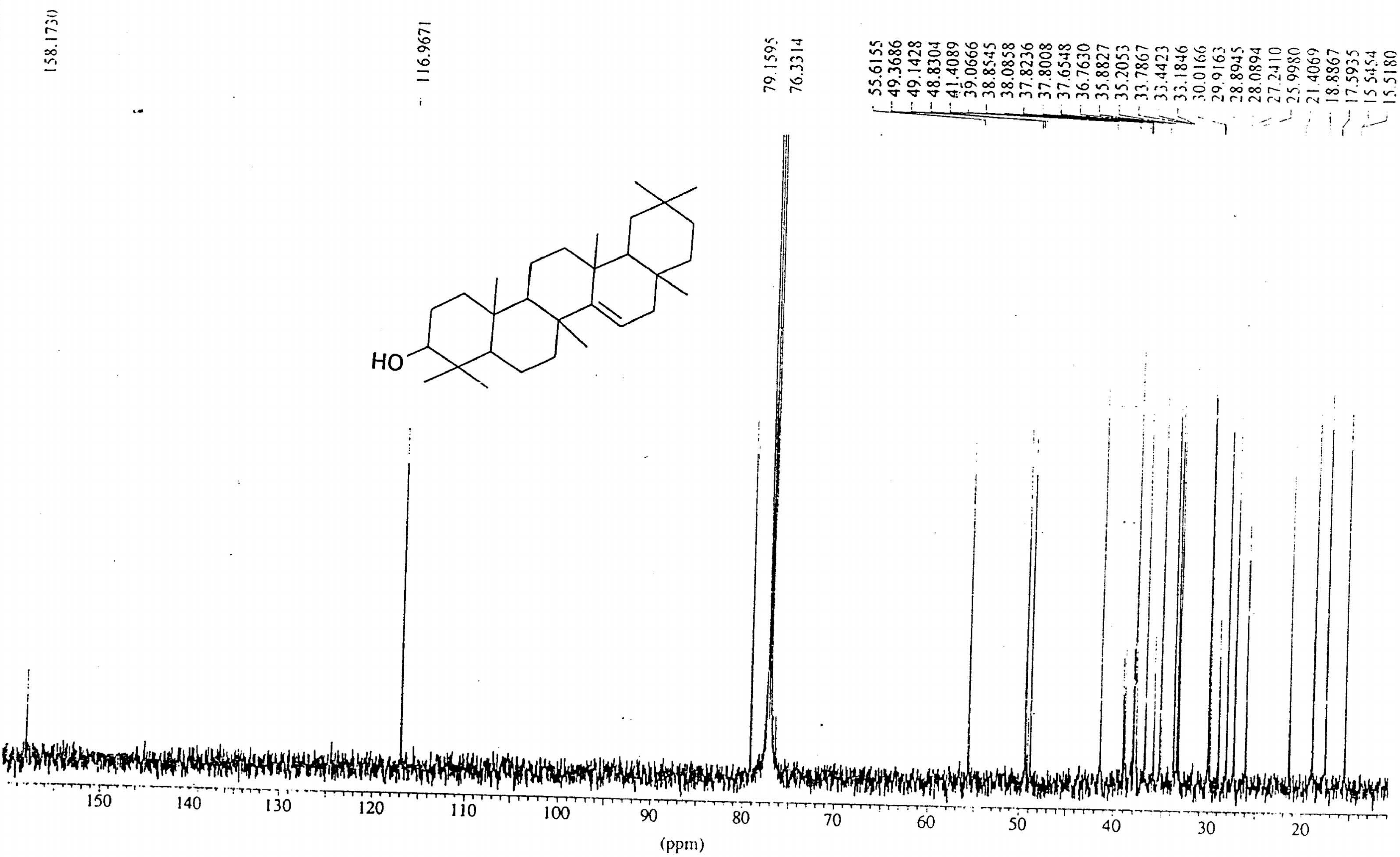




Mass spectrum of T_2 [Taraxerol]



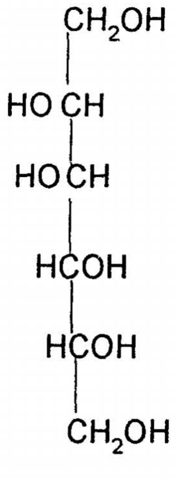
^{1}H-NMR spectrum of T$_2$ [Taraxerol] (500 MHz, CDCl$_3$, TMS)



^{13}C-NMR spectrum of T_2 [Taraxerol] (125 MHz, $CDCl_3$, TMS)

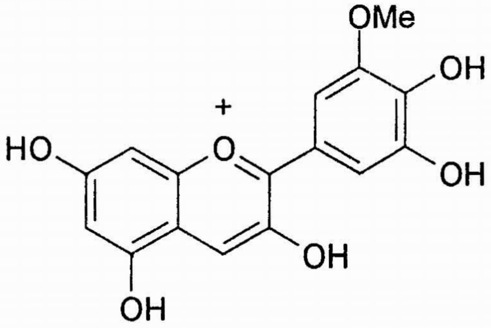
I.7. 3 Characterization of T_3 (d-mannitol)

Compound T_3 was isolated from the ethyl acetate extract on elution with 3:1 ethyl acetate-methanol as a dull yellow coloured fluffy substance (500 mg). It was recrystallised from hot EtOH and had a m.p. of 158°C. It was soluble in water and did not answer Molische's test. It gave brown colour with alkaline $AgNO_3$. Paper chromatography using n-propanol-ethyl acetate-water (7:1:2) as eluent showed the same Rf as that of d-mannitol. Alkaline $AgNO_3$ was used as spray reagent. Mixed m.p. with an authentic sample of d-mannitol was undepressed. Thus the compound T_3 was identified as the sugar alcohol, d-mannitol, a primary metabolite.



I.7.4 Characterization of T_4 (Petunidin)

The compound T_4 was obtained from crude methanol extract of *Canthium parviflorum* thorns. The persistent vine red colour when heated with 2M HCl for 30 minutes at 100°C indicated the presence of anthocyanidin. This on alkalification changed to blue-green and the colour slowly faded. The visible spectrum in MeOH-HCl showed absorption at 544nm and the observed bathochromic shift by the addition of 2 drops of 5% alc. $AlCl_3$ was in good agreement with that of the anthocyanidin, petunidin present in black grapes.[27] Petunidin was extracted from black grapes using MeOH-HCl and it was compared with **T$_4$** by performing paper chromatography in forestal and BAW. The measured Rf values (Rf x 100), 46 in forestal and 52 in BAW were quite comparable with that of petunidin.[28]



Anthocyanins are the most important and widespread group of colouring matters in plants. They are all based chemically on a single aromatic structure, that of cyanidin, and all are derived from this pigment by methylation or by glycosylation.[29] These intensely coloured water soluble pigments are responsible for the colours in the petals, leaves and fruits of higher plants. Numerous shades of colours are due to same skeletal structure, but different substitution. Usually these coloured pigments attract insects and thus facilitate pollination. So it was interesting to note that, anthocyanin was present in the thorns of *C. parviflorum.*

Anthocyanins are reported to have antioxidant activity [30,31] and also they have the power to protect the cells from the adverse effect of UV light.[32] Probably these may be the reason for the occurrence of anthocyanin in the thorns of *C. parviflorum.*

SECTION 2 : PHYTOCHEMICAL STUDIES ON THE LEAVES OF *CANTHIUM PARVIFLORUM* LAM.

I.8 Introduction

The leaves and fruits of *C. parviflorum* Lam. are useful in medicine and only d-mannitol has been so far isolated from the leaves of this plant.[12] So the present work is aimed at isolating and characterizing as many constituents as possible from the leaves of *C. parviflorum* Lam.

I.9 MATERIALS AND METHODS

The leaves of *C. parviflorum* were collected from Trichur District of Kerala in May 2002 and was identified by Dr. A.K. Pradeep, Department of Botany, Calicut University.

Experimental set up for tlc, column chromatography, IR spectra and mass spectra were same as that described in section I of chapter 1. Proton NMR spectra were recorded using Brucker spectrometer at 400 MHz, 500 MHz and ^{13}C NMR at 125MHz, in DMSO and $CDCl_3$ using TMS as internal standard. Melting points of the crystalline isolates were determined using Toshniwal Capillary Melting Point Apparatus.

GC-MS analysis

The mixture of compounds were analysed by GC-MS. GC-MS was performed with Hewlett Packard HP 6890 series GC system with Hewlett Packard 5973 mass selective detector. DB-5 column (U.S.A) of length 30m with 0.32mm internal diameter and film thickness of 0.25μm was used. Helium was used as carrier gas at a flow rate of 2.5ml/min. Temperature programme was 40°C to 290°C with a heating rate of 5°C/min. For compound identifications Wiley 275.MS Library data were used.

I.10 Extraction, fractionation and isolation of compounds from the leaves of *C. parviflorum* Lam.

Extraction: Shade dried finely powdered leaves of *C. parviflorum* (3.1 kg) were extracted thrice with petroleum ether (60-80°C, 3 x 7L). The combined petroleum ether extract was concentrated to get 250 ml of dark green coloured liquid, and no crystalline compound could be isolated from it.

I.10.1 Fractionation of the alcohol extract of the leaves of *Canthium parviflorum* Lam.

The powdered leaves of *C. parviflorum* after extraction with petroleum ether was extracted thrice with methanol (3 x 5L). The combined alcohol extract was concentrated under reduced pressure to about 500 ml of dark green viscous liquid. Then a white crystalline solid separated out. It was filtered, washed with ethyl acetate and methanol to give a white fluffy substance $\mathbf{L_1}$ (1.5 g, m.p. 158°C).

The concentrated methanol extract was syrupy in nature and filtered by applying suction. The residue in the filter paper was extracted with hot methanol and adsorbed on silica gel (300g, 60-120 mesh) for column chromatography (4cm x 100cm; d x l). It was eluted with 8:1 mixture of petroleum ether-ethyl acetate and continued using solvents of increasing polarity viz 6:1, 4:1, 2:1, 1:1 mixtures of petroleum ether-ethyl acetate, combination of ethyl acetate and methanol (2:1) and finally with methanol. Several 100 ml fractions were collected and homogeneity of each fraction was decided by tlc assay. Identical fractions were pooled together and concentrated by evaporation under reduced pressure.

I.10.2 Isolation of compounds from different chromatographic fractions of alcohol extract

The 1 to 6 fractions collected by elution using 6:1 mixture of petroleum ether-ethyl acetate, on evaporation followed by recrystallisation from methanol, furnished long, colourless needles **L_2** (1.6g, m.p. 139°C).

Table I.3

Compound isolated from methanol extract

Compound	Eluent composition	Melting point	Molecular mass
L_2	6:1 petroleum ether-ethyl acetate	139°C	414

The crude methanol extract, after suction filtration was concentrated to 300 ml. Equal volumes of water was added to it and extracted thrice with benzene followed by ethyl acetate (3 x 200 ml each). The benzene extract after evaporation was dissolved in hot methanol and adsorbed on 250g of silica gel for column chromatography (4cm x 100cm, d x l). The column was then eluted with solvents of increasing polarity i.e., petroleum ether, 8:1, 6:1, 4:1, 2:1 mixtures of petroleum ether- ethyl acetate, ethyl acetate, 8:1, 4:1 and 1:1 mixtures of ethyl acetate-methanol and finally with methanol. Several 100 ml fractions were collected and homogeneity of each fraction was checked by tlc analysis. Identical fractions were pooled together and concentrated by evaporation under reduced pressure.

I.10.3 Isolation of compounds from different chromatographic fractions of benzene extract

Fractions 1 to 8 eluted out using 8:1 mixture of petroleum ether-ethyl acetate, were found to give identical spots on tlc analysis. On concentrating these fractions by evaporation, a deep orange red oily mass was obtained. It was chromatographed over a silica gel column (100g, 3cm x 60cm; d x l) prepared in petroleum ether and eluted with petroleum ether followed by 10:1 mixture of petroleum ether-ethyl acetate. The 10th fraction eluted out with 10:1 mixture of petroleum ether-ethyl acetate, on evaporation gave an orange red viscous oily liquid (**L_3**) and was analysed by GC-MS.

Fractions 15 to 20 obtained from benzene extract, on elution with 8:1 mixture of petroleum ether-ethyl acetate, after evaporation yielded white needles and were found to be the same as **L_2**.

I.10.4 Fractionation and isolation of compounds from ethyl acetate extract

Ethyl acetate extract was concentrated, the residue dissolved in methanol and adsorbed on 250g of silica gel, packed in a column (4cm x 100cm; d x l). Elution was started with 3:1 mixture of petroleum ether-ethyl acetate and continued by solvents of increasing polarity, viz. various combinations of petroleum ether-ethyl acetate (1:1), ethyl acetate-petroleum ether (2:1), ethyl acetate and finally with 4:1 mixture of ethyl acetate and methanol.

The fractions 20 to 25 eluted out, by ethyl acetate were found to give identical spots on tlc analysis. These fractions on evaporation yielded greenish yellow flakes. These flakes were filtered, washed with ethyl acetate and recrystallised from hot methanol. The yellow powdery solid thus obtained was further purified by preparative tlc on silica gel G plates, by dissolving the solid in methanol and eluting with 7:1 mixture of ethyl acetate-methanol. The yellow upper spot was extracted with hot MeOH which upon evaporation yielded an yellow powdery solid L_4 (600 mg, m.p. 232°C).

Table I.4

Compound isolated from ethyl acetate extract

Compound	Eluent	Melting point	Molecular mass
L_4	Ethyl acetate	232°C	286

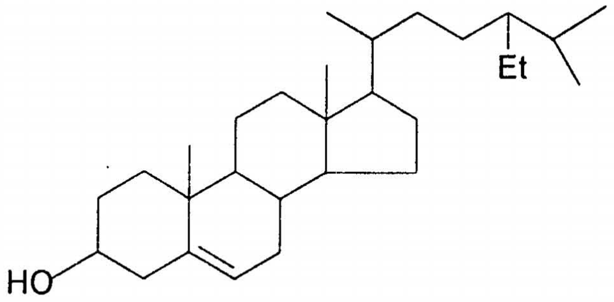
I.11 RESULTS AND DISCUSSION

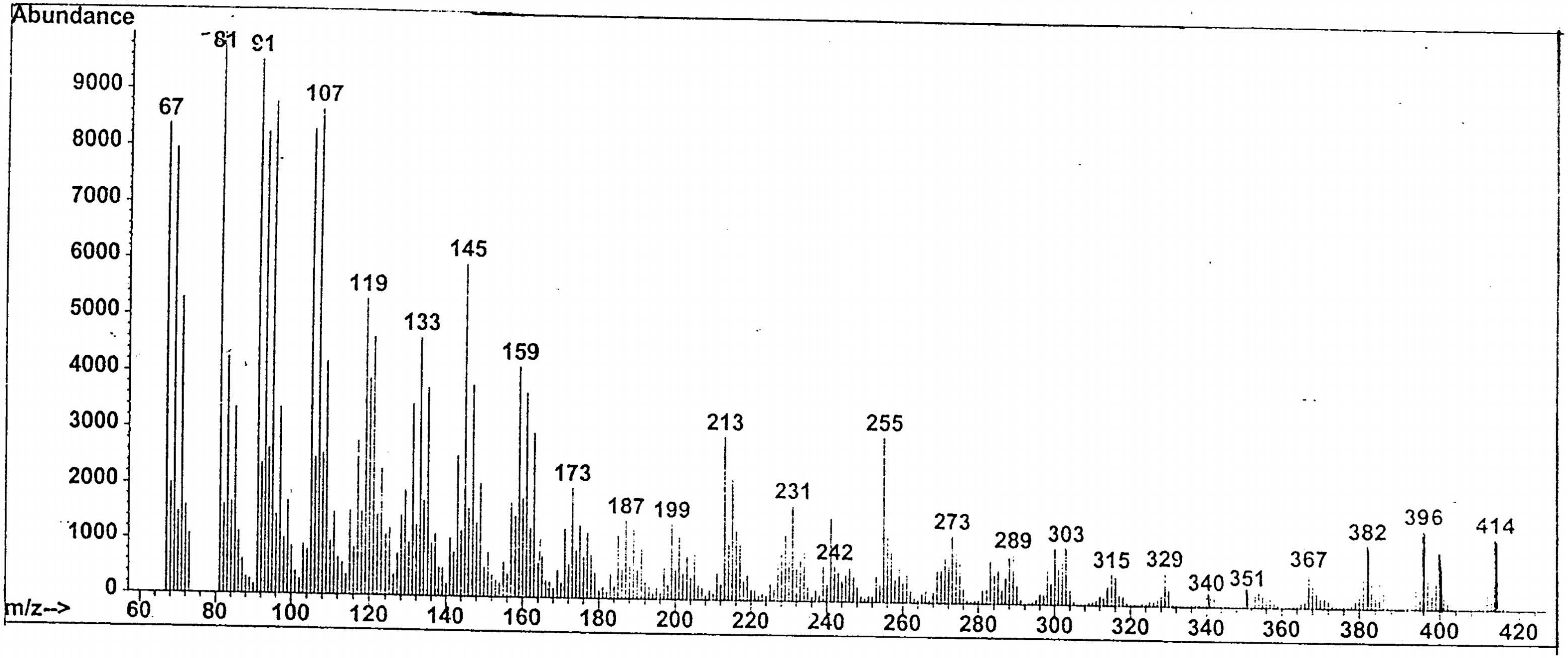
I.11 .1 Characterization of L_1 (d-mannitol)

The compound L_1 crystallises out from crude methanol extract of *C. parviflorum* leaves, on repeated recrystallisation from hot MeOH gave white fluffy substance (1.5g, m.p. 158°C). It was soluble in water and did not answer Molische's test for carbohydrates. It gave brown colour with alkaline $AgNO_3$. Mixed melting point with an authentic sample of d-mannitol was undepressed. Thus the compound L_1 was identified as d-mannitol

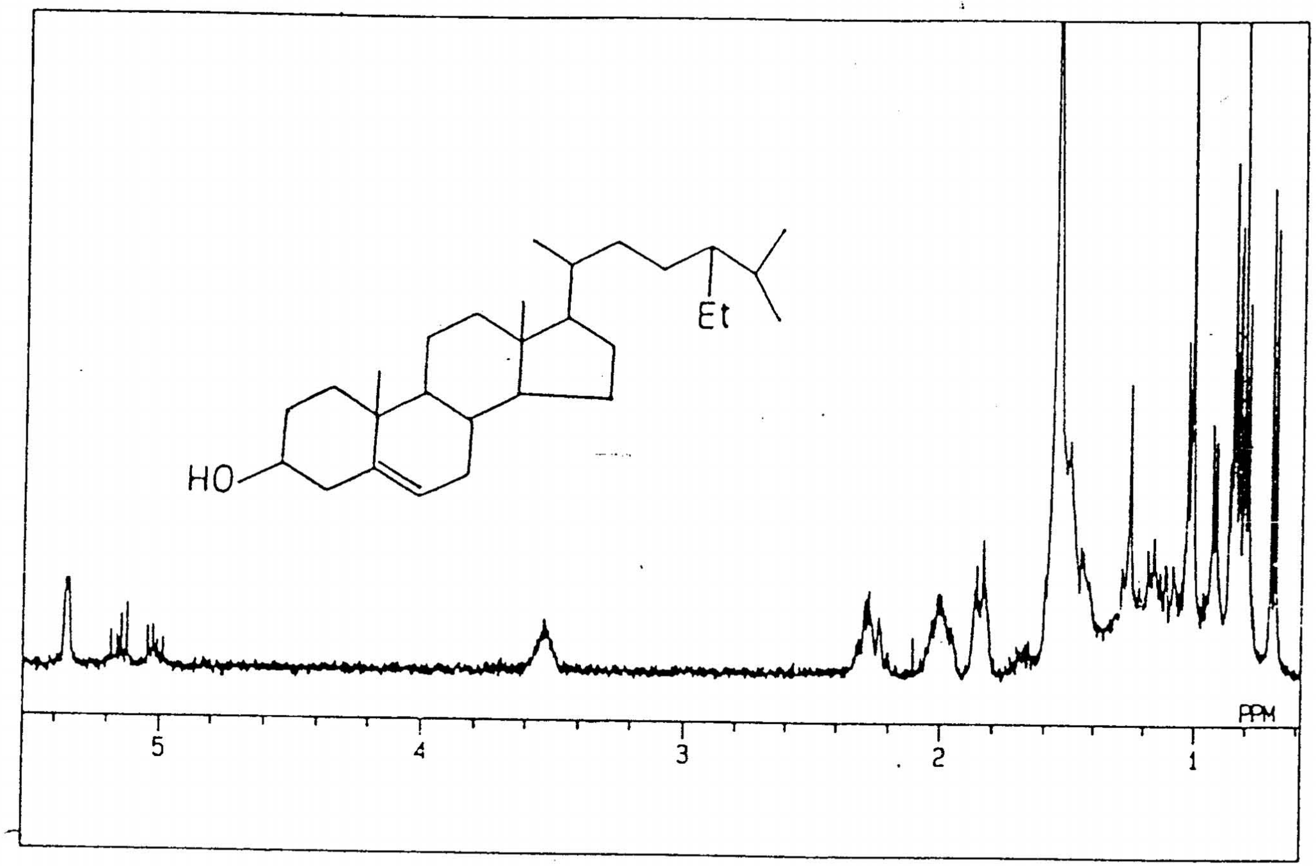
.I.11 .2 Characterization of L_2 (β-Sitosterol)

This compound was isolated from the alcohol extract, on elution with 6:1 petroleum ether-ethyl acetate, which upon recrystallisation from methanol yielded colourless needles (1.6g) melting at 139°C. It gave bluish-green colour with Liebermann-Burchard reagent, indicating that it was a sterol. With vanillin-H_2SO_4, it gave blue spot and with anisaldehyde-H_2SO_4, it appeared as red-violet spot. It decolourised Baeyer's reagent, indicating unsaturation in the compound. Mass spectrum of this compound showed M^+ at m/z 414 and the other prominent peaks were at 396 ($M^+ - H_2O$), 382 ($M^+ - CH_3 - OH$), 367 ($382 - CH_3$), 329 ($M^+ - C_6H_{13}$), 315 ($M^+ - C_7H_{17}$), 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. Infrared absorption spectrum showed the presence of hydroxyl group (broad absorption with maximum at 3441.4cm^{-1}). Bands due to gemdimethyl groups (doublet at 1383.1 and 1385.1cm^{-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 Rf (0.33 in benzene) and m.m.p. with an authentic sample established its identity.





Mass spectrum of L_2 [β-Sitosterol]



^{1}H-NMR spectrum of L_2 [β-Sitosterol](400MHz, $CDCl_3$, TMS)

I.11 .3 Characterization of L_3

The component $\mathbf{L_3}$ was isolated from the benzene extract of *C. parviflorum* leaves as an orange red viscous oily liquid. IR spectrum of $\mathbf{L_3}$ showed strong absorption at 3451cm^{-1}, indicating the presence of –OH group. An intense absorption at 1713cm^{-1} suggested the presence of carbonyl >C =O group and the observed bands at 1641.6cm^{-1} was due to the unsaturation in the compound. GC-MS analysis of the compound $\mathbf{L_3}$, resulted in the identification of 13 compounds, of which 9, 12, 15-octadecatrien-1-ol (43.8%), hexadecanoic acid (25.7%), octadecanoic acid (6.17%) and dodecanoic acid (4.99%) were the major constituents. The different components identified and their percentage compositions are given in table I.6

Table I. 6 : Chemical composition of L_3

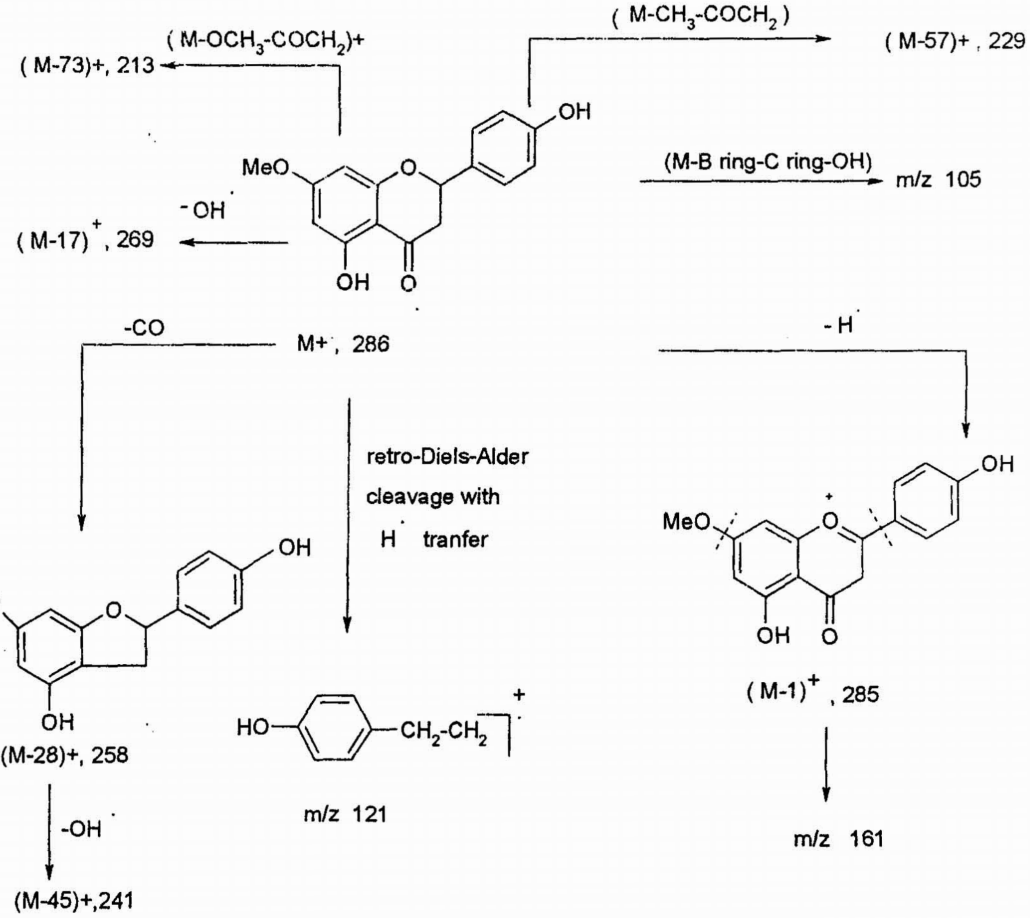
Compounds	Concentration (%)
β-Fenchyl alcohol	0.42
Dodecanoic acid, methyl ester	0.24
Dodecanoic acid	4.99
Tetradecanoic acid	2.13
Hexadecanoic acid, methyl ester	1.74
Hexadecanoic acid	25.7
Heptadecanoic acid	0.27
10, 13- Octadecadienoic acid methyl ester	0.78
9,12,15-Octadecatrienoic acid	1.76
Phytol	4.78
Octadecanoic acid, methyl ester	0.66
9, 12, 15-Octadecatrien-1-ol	43.8
Octadecanoic acid	6.17

I.11 .4 Characterization of L_4 (Sakuranetin-4'-O-glycoside)

Compound L_4 was isolated from the ethyl acetate extract of *C. parviflorum* Lam. leaves, on elution with ethyl acetate and recrystallised from methanol as pale yellow powdery solid (600 mg) which melted at 232°C. This compound gave a yellow spot on tlc and the colour being intensified by fuming with NH_3. With Mg-Conc. HCl (Shinoda test), it gave intense cherry-red colour, which indicated the flavonoid nature of the compound.[33]

The compound exhibited characteristic UV absorption characteristic of flavanones at 229, 267 and 305 nm in EtOH, and showed bathochromic shift with 2N NaOH, indicating the presence of phenolic group.[34] The observed bathochromic shift with 5% alc. $AlCl_3$/HCl suggested the presence of free hydroxyl group at C-5 of flavanone whereas the absence of shift with NaOAc revealed that the 7-OH group is blocked.[35] With NaOAc/Boric acid, the compound gave no appreciable shift in the UV absorption, showing the absence of O-dihydroxyl group.[36]

Infrared spectral data showed carbonyl absorption at 1653.2cm^{-1} (due to H-bonding) and hydroxyl absorption at 3466.5cm^{-1}. The mass spectrum gave an intense molecular ion peak (base peak) at the m/z 286 corresponding to the molecular formula $C_{16}H_{14}O_5$. The NMR spectral studies proved that, the compound is a glycoside and the intense diagnostic peak at m/z 286 is due to the aglycone fragment, formed by the fission of the O-glycosyl group from the aglycone skeleton and replaced by a hydrogen.[37] The molecular fragments at m/z 285 (aglycone–1)$^+$, 269 (aglycone–OH)$^+$, 258 (aglycone–CO)$^+$, 241 (aglycone–CO–OH)$^+$, 229, 213, 161, 121 (retro-Diels-Alder cleavage with H$^\cdot$ transfer), 105, and 93 in the mass spectrum, suggested that the methyl group was in ring A and the hydroxyl group in ring B.



Mass spectral fragmentation of the aglycone skeleton of compound L_4

The proton NMR spectrum showed a sharp one-proton singlet at δ 12.55 ppm for chelated hydroxyl [38-40] (H-5) and a three-proton singlet at δ 3.66 for a methoxyl group.[38,41] A pair of doublets at δ 8.06 (2H, d, J = 15Hz) and δ 6.85 (2H, d, J = 15Hz) were characteristic of H-2', H-6' and H-3', H-5' protons of the 1, 4-disubstituted aromatic ring system.[42,43] The one-proton doublets at δ 6.34 and δ 6.11 with a coupling constant of 2.5Hz, typical of two *meta* coupled protons, were assigned to H-~~6~~8 and H-~~8~~6 respectively.[40,44,45] The H-6 doublet occurs consistently at higher field than the signal for the H-8 and the appearance of H-6 and H-8 doublets at upfield is indicative of the absence of 7-O-glycosylation. [46,47] The one-proton double doublet (actually two doublets merge, J_{trans} = 12Hz, J_{cis} = 3Hz) appeared near 5.2 ppm, as a result of the coupling of the C-2 proton with the two C-3 protons, was assigned to H-2 of flavanone.[48-52] The overlapping quartets (multiplet) at about 2.7 ppm were due to the two C-3 protons [48-51] (2H, J = 17Hz, 3Hz). C-3 protons couple with each other (J = 17 Hz) in addition to their spin-spin interaction with the C-2 proton, thus giving rise to two overlapping quartets.[49]

The HMQC spectrum of the compound exhibited sugar carbon signals grouped at 60.5-76.2 ppm, with C"-1, as usual downfield at 102.2 ppm [53]. These carbons are directly bonded to sugar protons in the region δ 3.23-3.54. It was quite evident from the HMQC spectrum that the C"-1 of sugar is coupled with proton at δ 5.36ppm (H"-1 proton of the sugar moiety). In the ^{1}H NMR spectra, the three-proton signal at δ 1.24 was assigned to rhamnose methyl group.[40,47,50,54] The downfield shift of C"-1 of the sugar moiety clearly indicates the O-glycosylation of the compound.[55] The observed signals at δ 4.82, 4.46 and 4.40 ppm are due to the sugar OH protons, and the nature of the sugar moiety was not fully understood. Some of the sugar proton absorptions was found buried in the peak of water molecule, which is usually found as an impurity in DMSO.[56,57]

In the ^{13}C NMR spectrum of the compound, ^{13}C chemical shift at δ 197 ppm, accounted for the C-4 carbonyl of the flavanone.[58,59] ^{13}C NMR signals produced by DMSO-d_6 obscured the C-3 resonances of flavanone appeared at 41.6 ppm.[60] The carbon resonances in the ^{13}C spectrum of the compound were assigned by comparing it with those data available from literature.[61-63] The values are given in table I.7.

Table I.7

^{13}C NMR Spectral Assignment of Flavonoid aglycone

C. No.	δC (ppm)	C.No.	δC (ppm)
2	76.2	10	102.2
3	41.6	1'	121.1
4	197.0	2'	131.4
5	161.06	3'	115.3
6	99.5	4'	160.2
7	156.3	5'	115.3
8	94.3	6'	131.4
9	156.3	OMe	68.2

The correlation between directly bonded carbon and hydrogen (HMQC) and that of carbon and hydrogen of two or three bond distance (HMBC) are given in the tables I.8 and I.9 respectively.

Table I.8

HMQC Spectrum of L_4

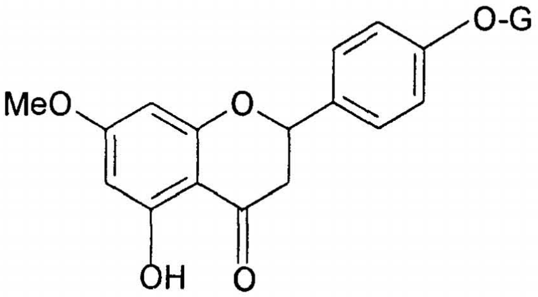
Proton	δ H (ppm)	δ C (ppm)
H-2', H-6'	8.06	131.4
H-3', H-5'	6.85	115.3
H-8	6.34	94.3
H-6	6.11	99.5
Sugar H"-1	5.36	102.2
-OCH_3	3.66	68.2
Sugar Protons	3.54	71.7
	3.46	60.5
	3.38	73.6
	3.34	76.2
	3.23	60.5
Rhamnosyl-CH_3	1.24	29.3

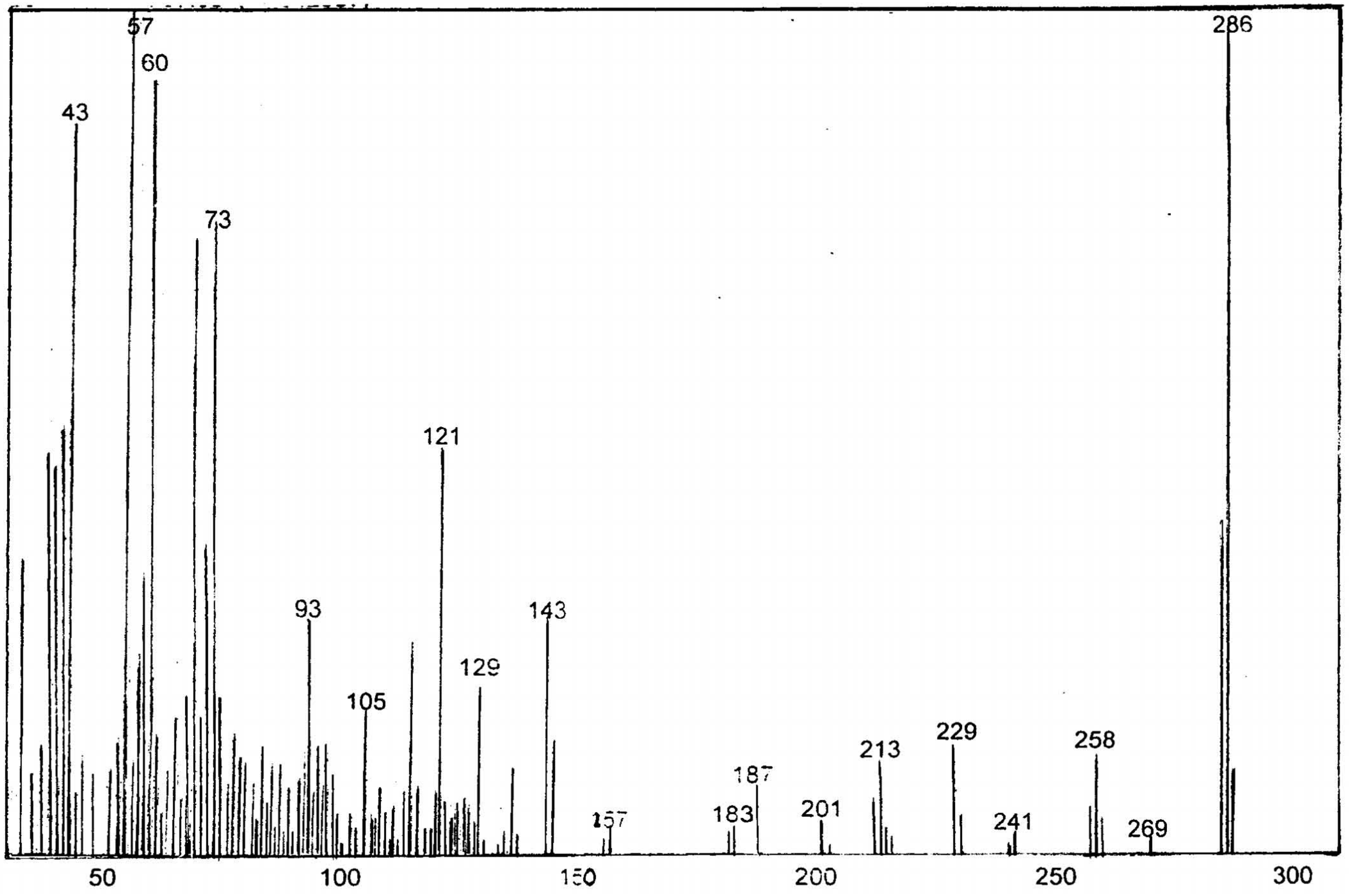
Table I.9

HMBC Spectrum of L_4

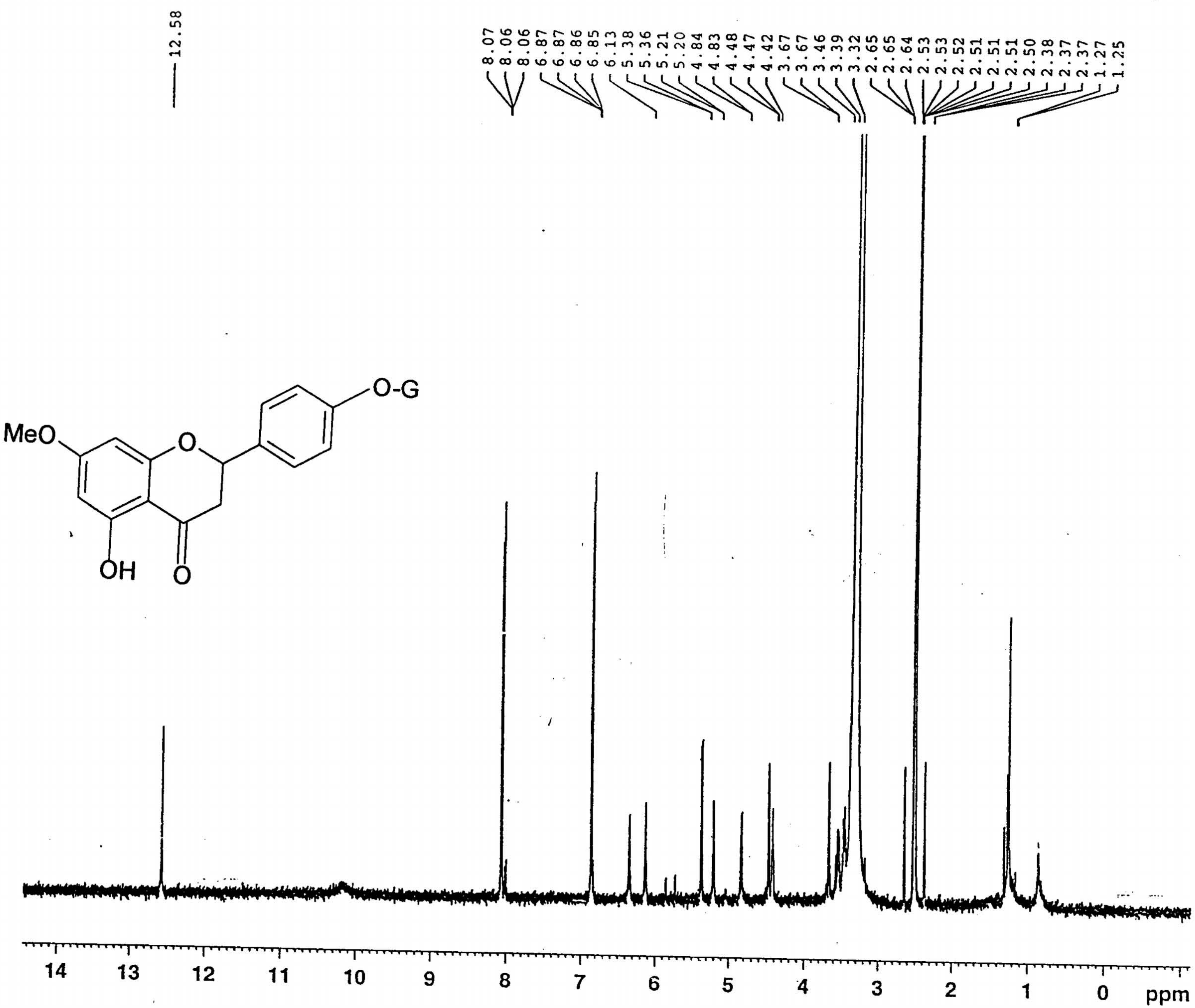
δ H (ppm)	δ C (ppm)
8.06	160.1
8.06	156.3
6.85	160.2
6.85	121.1

All the spectral data are quite comparable with that of sakuranetin-4'-O-glycoside reported [64] earlier, and the structure of L_4 is given below:

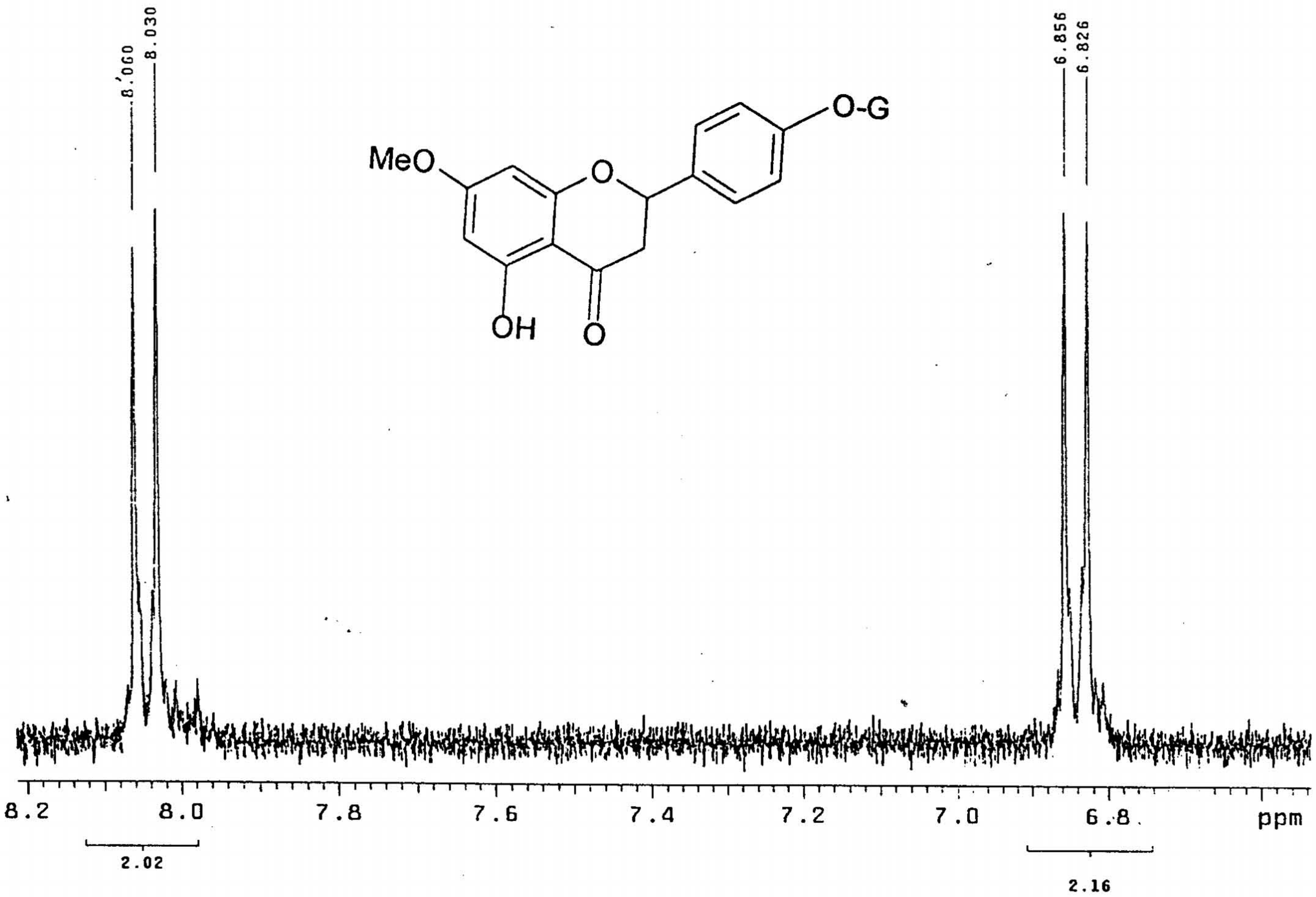




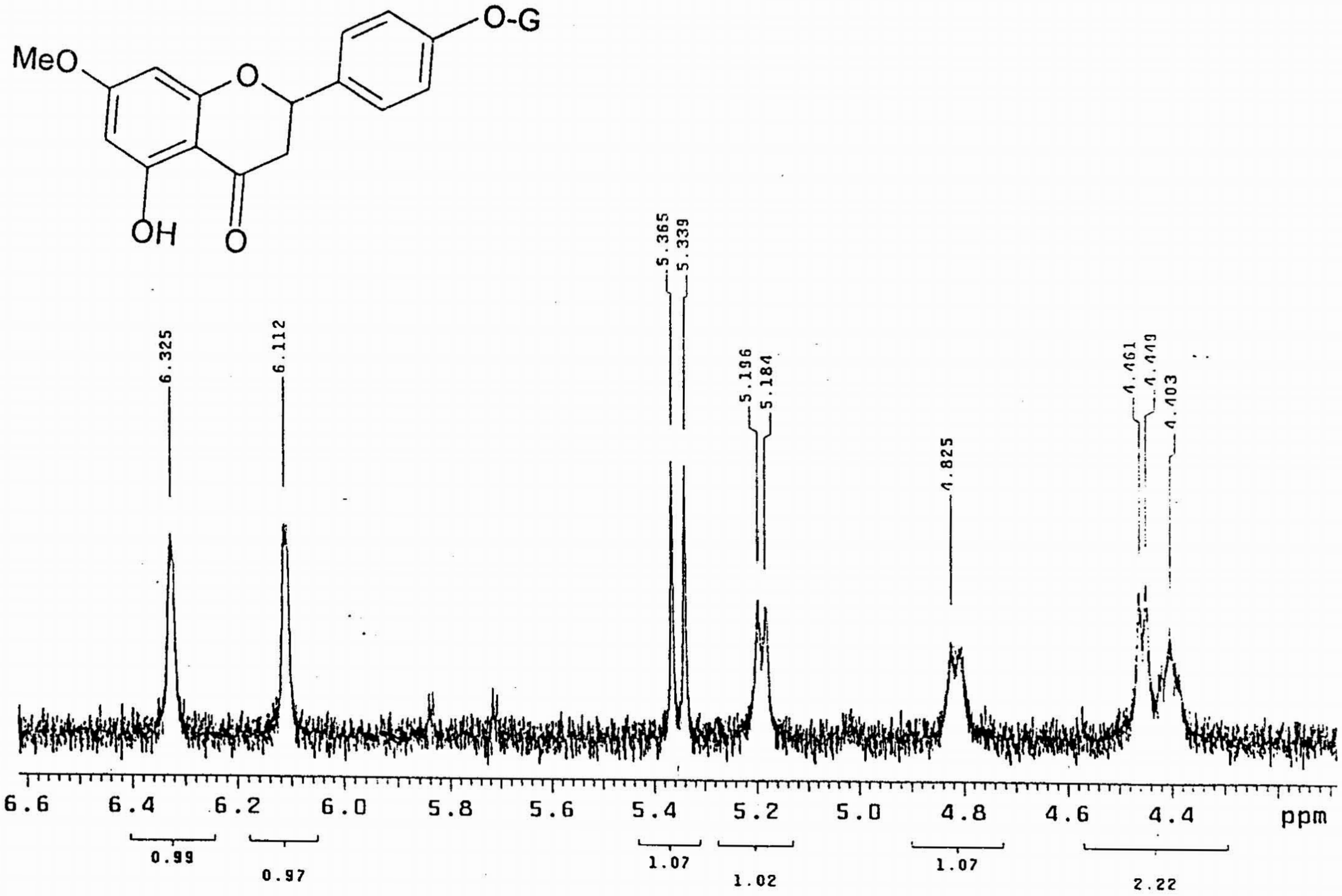
Mass spectrum of flavonoid aglycone [Sakuranetin]



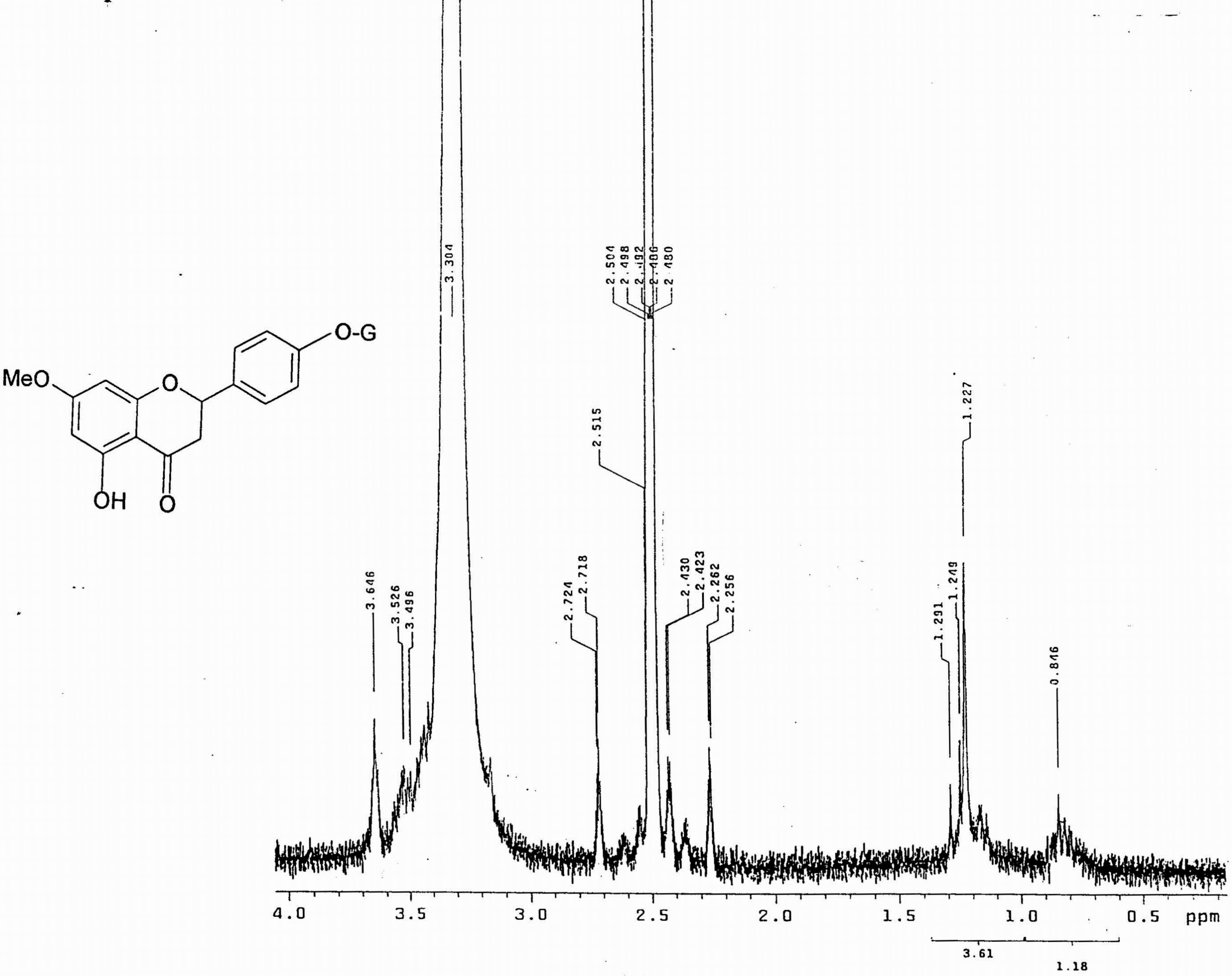
^{1}H-NMR spectrum of L_4 [Sakuranetin-4'-O-glycoside] (500 MHz, DMSO, TMS)



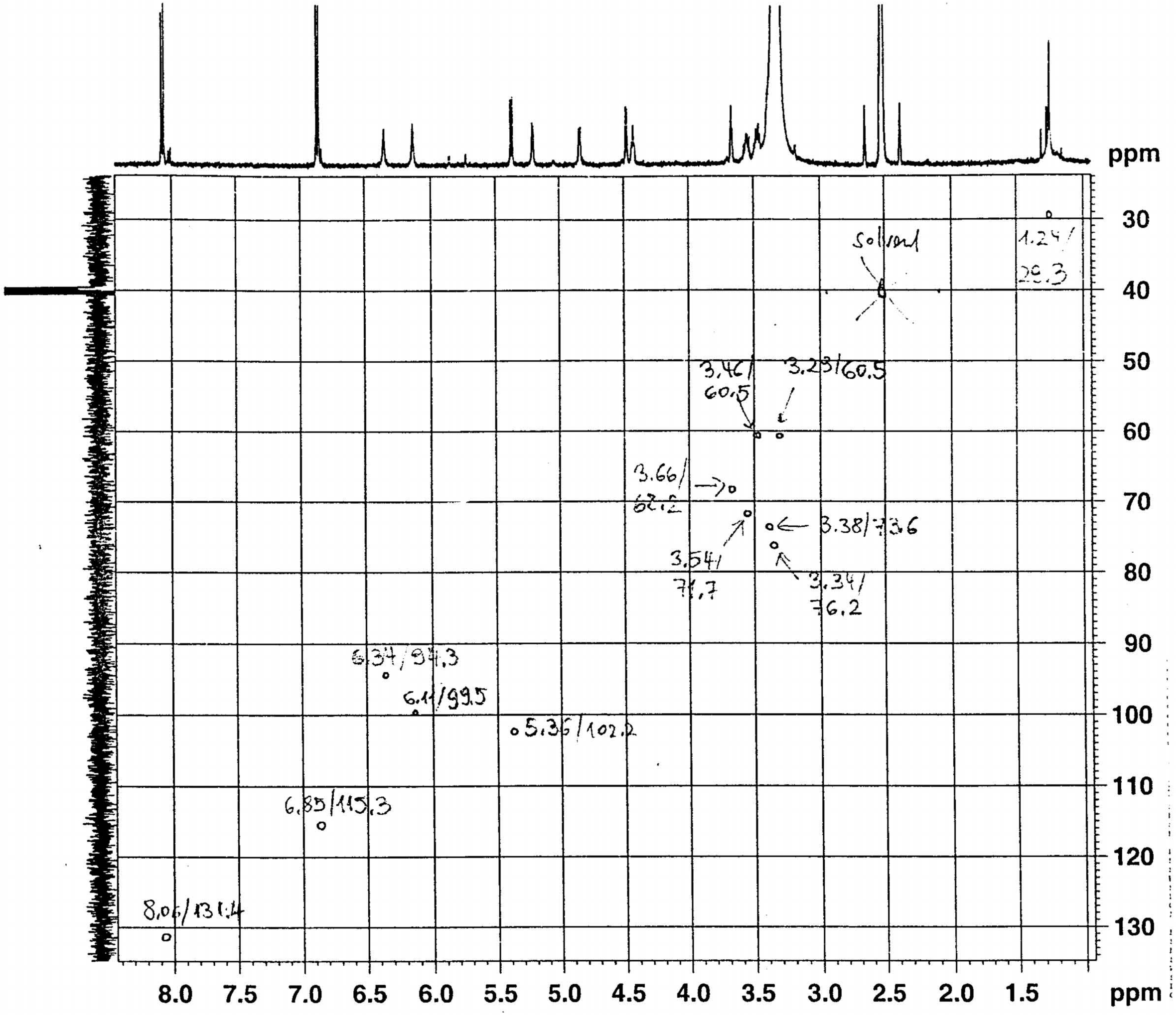
^{1}H-NMR spectrum of L_4 [Sakuranetin-4'-O-glycoside] (500 MHz, DMSO, TMS)



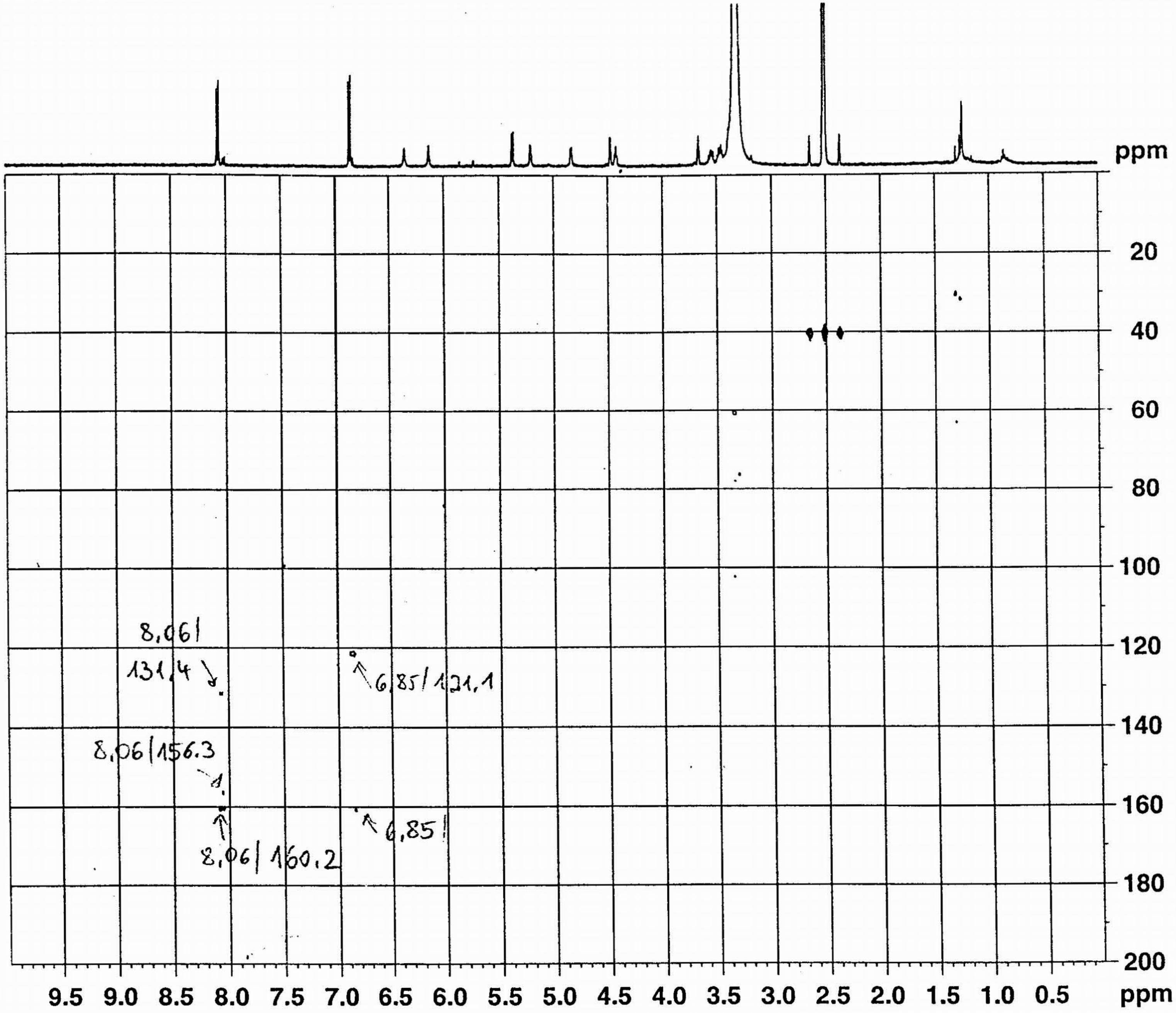
^{1}H-NMR spectrum of L_4 [Sakuranetin-4'-O-glycoside] (500 MHz, DMSO, TMS)



^{1}H-NMR spectrum of L$_4$ [Sakuranetin-4'-O-glycoside] (500 MHz, DMSO, TMS)



HMQC spectrum of L_4 [Sakuranetin-4'-O-glycoside] (500 MHz, DMSO, TMS)



HMBC spectrum of L_4 [Sakuranetin-4'-O-glycoside] (500 MHz, DMSO, TMS)

REFERENCES

1. The Wealth of India, Raw Materials Vol.II. New Delhi: CSIR, 1950, p.66.
2. Floyd, A.G., Rainforest Trees of Mainland South-Eastern Australia. Sydney: Inkata Press, 1989.
3. Ram, P.R., Mehrotra, B.N., Compendium of Indian Medicinal Plants Vol.II. 1993, p.137.
4. Chopra, R.N., Nayar, S.L., Chopra, I.C., Glossary of Indian Medicinal Plants. New Delhi: CSIR, 1956, p.49.
5. Kirtikar, K.R., Basu, B.D., Indian Medicinal Plants. Dehra Dun: Bischensingh Mahendrapalsingh, 1984, p.1284.
6. Parrotta, J.A., Healing Plants of Peninsular India. New York: CABI Publishing, 2001, p.616.
7. Asolkar, L.V., Kakkar, K.K., Chakre, O.J., Second Supplement to Glossary of Indian Medicinal Plants with Active Principles, Part I (A-K). New Delhi: CSIR, 1992, p.414.
8. Chopra, R.N., Nayar, S.L., Chopra, I.C., Glossary of Indian Medicinal Plants. New Delhi: CSIR, 1956, p.330.
9. The Wealth of India, A Dictionary of Indian Raw Materials Vols. I-XI (1948-1976), revised and enlarged Vols. I-III (1985-1992) New Delhi: CSIR, 1993.
10. Yoganarasimhan, S.N., Medicinal Plants of India Vol.I. Karnataka: Interline Publishing, 1996, p.645.

11. Vaidya Ratnam Varrier's Arya Vaidya Sala, Indian Medicinal Plants- A compendium of 500 species Vol.I. Madras: Orient Longmann, 1994, p.366.

12. Dutta, H.K., Ganguly, S.N., Bhattacharya, A.K., *J. Indian Chem. Soc.* **43 (5)**, 1966, 380.

13. Lewis, D.H., Smith, D.C., Sugar Alcohols in Fungi and Green Plants. I. Distribution. *New Phytologist* **66**, 1967a, 143.

14. Harborne, J.B., Phytochemical Methods. London: Chapman and Hall Ltd, 1973, p.226.

15. Ambasta, S.P. (ed). The Useful Plants of India. New Delhi: CSIR, 1992, p.102.

16. Mukherjee, K., Bose, L., *J. Indian Chem. Soc.* **52**, 1975, 1112.

17. Dan, S., Dan, S.S., Mukhopadhayay, P., *J. Indian Chem. Soc.* **59**, 1982, 419.

18. Chatterjee, T.K., Nagar, A., Barua, A.K., Mukherjee, K., *J. Indian Chem. Soc.* **59**, 1982, 418.

19. Herath, W.H.M.W., Sultanbawa, M. U.S., Wannigama, G.P., *Phytochemistry* **18**, 1979, 1385.

20. Willaman, J.J., Li, H.L., *Lloydia* **33**, 1970, 187.

21. Willaman, J.J., Schubert, B.G., *U.S. Dep. Agric. U.S.D.A. Tech. Bull. No.*1234, 1961.

22. Chatterjee, T.K., Basak, A., Barua; A.K., (Mrs.) Mukherjee, K., Roy, L.N., *Trans. Bose Res. Inst.* **42**, 1979. 85.

23. Harborne, J.B., Phytochemical Methods, London: Chapman and Hall Ltd, 1973, p.61.

24. Sakurai, N., Yaguchi, Y., Inoue, T., *Phytochemistry* **26**, 1987, 217.

25. Budzikiewicz, H., Wilson, J.M., Djerassi, C., *J. Am. Chem. Soc.* **85**, 1963, 3692.

26. Agarwal, K.P., Roy, A.C., Dhar, M.L., *Indian J.Chem.* **1**,1963,28.

27. Harborne, J.B., Phytochemical Methods, London: Chapman and Hall Ltd, 1973, p.64.

28 Dey, P.M., Harborne, J.B., Methods in Plant Biochemistry Vol.I. New York: Academic Press, 1989, p.332.

29 Harborne, J.B., Phytochemical Methods, London: Chapman and Hall Ltd, 1973, p.59.

30. Ruberto, G., Renda, A., Piattelli, M., Rapisarda, P., Starrantino, A., *Journal of Agricultural and Food Chemistry* **45(2)**, 1997, 467.

31. Ju, Z.G., Yuan, Y.B., Liu, C.L., Zhan, S.M., Wang, M.X., *Post harvest-Biology and Technology* **8(2)**, 1996, 83.

32 Takahashi, A., Takeda, K., Ohnishi, T., *Plant and Cell Physiology* **32(4)**, 1991, 541.

33 Dey, P.M., Harborne, J.B., Methods in Plant Biochemistry Vol.I. New York: Academic Press, 1989, p.292.

34 Harborne, J.B., Mabry, T.J., Helga, M., The Flavonoids. London: Chapman and Hall, 1975, p.46.

35 Dey, P.M., Harborne, J.B., Methods in Plant Biochemistry Vol.I. New York: Academic Press, 1989, p.304.

36 Harborne, J.B., Mabry, T.J., Helga, M., The Flavonoids. London: Chapman and Hall, 1975, p.58

37 Harborne, J.B., Mabry, T.J., Helga, M., The Flavonoids. London: Chapman and Hall, 1975, p.122.

38 Dey, P.M., Harborne, J.B., Methods in Plant Biochemistry Vol.I, New York: Academic Press, 1989, p.313.

39 Mabry, T.J., Markham, K.R., Thomas, M.B., The Systematic Identification of Flavonoids. New York: Springer-Verlag, 1970, p.262.

40 Markham, K.R., Techniques of Flavonoid Identification. London: Academic Press, 1982, p.73.

41 Mabry, T.J., Markham, K.R., Thomas, M.B., The Systematic Identification of Flavonoids. New York: Springer-Verlag, 1970, p.271

42 Markham, K.R., Techniques of Flavonoid Identification. London: Academic Press, 1982, p.75.

43 Dey, P.M., Harborne, J.B., Methods in Plant Biochemistry Vol. I. New York: Academic Press, 1989, p.314.

44 Liping, S., Shangzhen, Z., Jinxin, W., Xuwei, S., *Indian Journal of Chemistry* **41B(6)**, 2002, 1319.

45 Harborne, J.B., Mabry, T.J., Helga, M., The Flavonoids. London: Chapman and Hall, 1975, p.63.

46 Mabry, T.J., Markham, K.R., Thomas, M.B., The Systematic Identification of Flavonoids. New York: Springer-Verlag, 1970, p.261.

47 Markham, K.R., Techniques of Flavonoid Identification. London: Academic Press, 1982, p.76.

48 Harborne, J.B., Mabry, T.J., Helga, M., The Flavonoids. London: Chapman and Hall, 1975, p.68

49 Mabry, T.J., Markham, K.R., Thomas, M.B., The Systematic Identification of Flavonoids. New York: Springer-Verlag, 1970, p.267

50 Dey, P.M., Harborne, J.B., Methods in Plant Biochemistry Vol.I. NewYork: Academic Press, 1989, p.316.

51 Markham, K.R., Techniques of Flavonoid Identification. London: Academic Press, 1982, p.78.

52 Clark-Lewis, J.W., Jackman, L.M., Spotswood, T.M., *Australian J. Chem.* 17, 1964, 632.

53 Markham, K.R., Techniques of Flavonoid Identification. London: Academic Press, 1982, p.83.

54 Mabry, T.J., Markham, K.R., Thomas, M.B., The Systematic Identification of Flavonoids. New York: Springer-Verlag, 1970, p.269.

55 Dey, P.M., Harborne, J.B., Methods in Plant Biochemistry. New York: Academic Press, 1989, p.320.

56 Mabry, T.J., Markham, K.R., Thomas, M.B., The Systematic Identification of Flavonoids. New York: Springer-Verlag, 1970, p.253

57 Harborne, J.B., Mabry. T.J., Helga, M., The Flavonoids. London: Chapman and Hall, 1975, p.62.

58 Dey, P.M., Harborne, J.B., Methods in Plant Biochemistry Vol.I. New York: Academic Press, 1989, p.318.

59 Dey, P.M., Harborne, J.B., Methods in Plant Biochemistry Vol.I. New York: Academic Press, 1989, p.319.

60 Dey, P.M., Harborne, J.B., Methods in Plant Biochemistry Vol.I. New York: Academic Press, 1989, p.317.

61 Dey, P.M., Harborne, J.B., Methods in Plant Biochemistry Vol.I. New York: Academic Press, 1989, p.224.

62 Markham, K.R., Techniques of Flavonoid Identification. London: Academic Press, 1982, p.80.

63 Markham, K.R., Techniques of Flavonoid Identification. London: Academic Press, 1982, p.84.

64 Haq, A., Malik, A., Khan, S. B., *Polish J. Chem.* **78**, 2004,1851.

ZANTHOXYLUM RHETSA

CHAPTER II

SECTION I : INFLUENCE OF THE EXTRACTION METHODS ON THE ESSENTIAL OIL COMPOSITION OF *ZANTHOXYLUM RHETSA* SEEDS

II.1 General Introduction

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. They are so named because of their odour or essence. The natural essential oils and their aroma are perhaps the most remarkable products of plant metabolism. Younger plants produce more oils than older ones, but old plants are richer in more resinous and darker oil because of continuing evaporation of lighter fractions of the oil. The oils are stored as micro droplets in the glands of plants. After diffusing through the walls of the glands, the droplets spread over the surface of the plant before evaporating and filling the air with perfume.The most odoriferous plants are found in the tropics, where solar energy is the greatest. Climatic and topographical conditions affect plant chemistry and alter the essential oil content qualitatively and quantitatively. India is considered to be the ancient home of perfumes and aromatic plants, because it is blessed with a wide variety of soil and climatic conditions that support the enormous plant wealth.

Essential oils are complex mixtures of hydrocarbons and their oxygenated derivatives. The main constituents of essential oils are the terpenoids having carbon atoms up to C_{15} (i.e., mono and sesquiterpenes) and their oxygenated derivatives such as alcohols, aldehydes, ketones etc. Essential oils due to their pleasant smell are commercially important especially in perfumery. Moreover, various essential oils show biological activity such as insecticidal, anthelmintic or antiseptic action, so they are also useful in pharmacy.[1]

II.2 Extraction of Essential Oils

Essential oils frequently occur as a very small percentage by weight of the original plant material, so the processing of large quantities is often required to obtain considerable amount of oil.[2] In general, four methods are used for the isolation of essential oils. They are steam distillation, expression (pressing), solvent extraction and enfleurage (extraction by using fat). Of these steam distillation is the most commonly used method. At first the plant material is crushed or ground to reduce the particle size and to rupture some of the cell walls of oil bearing glands. The crushed or ground mass is then steam distilled when the essential oils go into distillate from which they are extracted using pure organic solvents like diethyl ether. Steam distillation may change or modify the components of essential oils. Liquid carbon dioxide extraction[3] retains the true nature characteristic of essential oils and extracts all the desirable components, without drastic treatments, because of its strong solvating power and low viscosity.

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

II.3 Analytical Techniques

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

Two types of gas chromatography are encountered Gas-Solid Chromatography (GSC) and Gas-Liquid Chromatography (GLC). Gas-Liquid Chromatography is based upon the partition of the analyte between a gaseous mobile phase and a liquid phase immobilized on the surface of an inert solid. GLC finds widespread use in all fields of science, where its name is usually shortened as Gas Chromatography (GC). Gas-Solid Chromatography is based upon a solid stationary phase in which retention of analytes is the consequence of physical adsorption.[4]

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

1. Squalene (Non polar)
2. Silicone oils (poly dimethyl siloxane, poly (phenyl methyl) dimethyl siloxane) (Intermediate polarity).
3. Polyethylene glycols or PEG 400 (Highly polar).
4. Polyethylene glycol succinate PEG-S (Highly polar).

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

Relative Retention

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

The adjusted retention time

$$t_R' = t_R - t_M$$

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

$$t'_R\,(s) = t_R(s) - t_M.$$

The relative retention

$$r\,(a,s) = \frac{t_R'(a)}{t_R'(s)} = \frac{t_R(a) - t_M}{t_R(s) - t_M}.$$

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

Retention Indices

Gas chromatography is essentially an analytical technique commonly used for qualitative analysis by comparing the retention data of the analyte with those of the compound which it is thought to be. Simple retention times are not very reproducible and it is better to use relative retentions or retention indices. The most, useful system of retention indices is the one due to Kovats.[5] It takes advantage of the linear relation between the logarithms of the adjusted retention times of a homologous series (n-alkanes) and the number of carbon atoms in the molecule. The n-alkanes are used as the reference compounds because of their stability, ready availability, low cost and wide range of boiling points. The retention of any analyte is compared with the two n-alkanes which elute nearest to it. The adjusted retention time of the analyte is measured at the same time as those of n-alkanes which elute in front and behind it (containing 'z' and 'z+1' carbon atoms respectively) and the retention index of the analyte, I is then defined by

$$I = 100\left[\frac{\log t'_R\,(\mathrm{subst}) - \log t'_R\,(\mathrm{n}-\mathrm{C}_z)}{\log t'_R\,(\mathrm{n}-\mathrm{C}_{z+1}) - \log t'_R\,(\mathrm{n}-\mathrm{C}_z)} + Z\right]$$

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

Columns

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

Detectors:

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

1. Thermal Conductivity Detector (TCD)

In TCD, a heated filament is placed in the emerging gas stream. The amount of heat lost from the filament by conduction to the detector walls depends on the thermal conductivity of the gas phase.

2. Flame Ionisation Detector (FID)

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

3. Mass Spectrometer

Gas chromatography is often coupled with the selective techniques of spectroscopy, thus giving so-called hyphenated methods that provide the chemist with powerful and pragmatic tools for identifying the components of complex mixtures. Gas chromatography-Mass spectrometry (GC-MS) and Gas Chromatography– Infrared Spectroscopy-Mass Spectrometry (GC-IR-MS) are the modern analytical methods used for the separation and identification of components of essential oils. When we use GC-MS, the mass spectrometer is a universal detector for gas chromatographs since any compound that can pass through a gas chromatograph is converted into ions in the mass spectrometer. At the same time the highly specific nature of a mass spectrum makes the mass spectrometer a very specific gas chromatographic detector. Gas chromatography is an ideal separator whereas mass spectrometry is excellent for identification.

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

II.4 Gas Chromatography-Olfactometry

Aroma chemicals have two sensory odour properties namely intensity and quality.[6] They are usually hydrophobic organic compounds containing a limited number of functional groups. However the presence of functional group is not a pre-requisite for odour. The two odour properties are very difficult to measure objectively with physical instruments. Olfactometers simply generate and deliver an odorous air sample of known concentration to a human subject for assessment. The technique gas chromatography-olfactometry, more commonly referred as GC sniffing is a more useful method to check the olfactoric purity of a sample. Provided that the GC conditions adequately separate the components of a mixture, each component can be smelt at the exit of the port of a GC column in olfactorily pure state. Repeated analysis of the same sample at successively high dilutions is a methodology commonly used to identify the components that contributed towards the overall odour of the sample. An advantage of the method is its ability to analyse minute quantities of a sample and to assess pure components. Due to the absence of a universal odour perception, sometimes the odour description from two laboratories may be slightly different.

Gel Permeation Chromatography[7] (GPC) is a useful supplementary method for the separation, identification and carbon skeleton determination of mono and sesquiterpenes of essential oils having same molecular formula but different carbon skeletons on the basis of their molecular size.

II.5 Economic Importance

Essential oils have extensive applications in the field of flavour and fragrance industry. Examples are mint and cinnamon used in toothpaste, mouthwash or lozenges. Some combination of essential oils can be found in soaps, detergents, room freshners, papers, printing ink, paint, candles, condiments, floor polishes etc. Flavour essential oils are used in baked foods, snacks, soft drinks, liquors, sauces, gravies, salad dressings and other food products.[8]

II.6 Biological Activity of Essential Oils and Their Applications:

Since time immemorial, essential oils have been known in many different applications.[9] Many of them from ancient times were used as medicaments, disinfectants, insect repellents, fragrances etc. Because of their well-known disinfectant properties they were considered excellent remedies against infections and epidemic diseases. Sometimes the oils were believed to be remedy for nearly all health problems.

Even though many microbiological studies have been performed on essential oils, only little is known about the mechanism of action of essential oils. Their lipoid solubility and their possibility to penetrate into the cells may influence the metabolism of the microorganisms and thus give an explanation of the effect.[10] It has been observed that the antiseptic activity of many compounds is based on their lipoid solubility. The antibacterial activity of essential oils has been expressed in terms of phenol-coefficient. It tells us how many times stronger or weaker is the action of the essential oil in question, compared to phenol[9] (phenol has the factor one).

II.7 Therapeutic Applications of Essential Oils

The knowledge of therapeutic properties of essential oils is as old as mankind's use of plants as medicaments. Essential oils, being complex mixtures can act in different complicated ways. Therapeutic property of each essential oil is specific. Therefore individual chemicals isolated from essential oils are more often used than the oils themselves for the treatment. There are however more and more research works on the therapeutic properties of essential oils treated as standard medicaments and subjected to full pharmacological evaluation. The most thoroughly examined are antimicrobial properties of essential oils that in many ways are better than antibiotics, due to their wider spectrum of activity. There is also very interesting phenomenon of synergic activity of two or more essential oils against bacteria in which the addition of one oil to another will increase the original bactericidal activity of individual oils. Even more interesting is the synergic activity of essential oils with antibiotics. It was found that the addition of a small quantity of oil to some antibiotic would increase the activity of the antibiotic several times.[11] Bactericidal activity of essential oils can also be applied in food preservation.

Apart from the above few examples of uses of essential oils, there are so many preparations using essential oils as their constituents. "Analgetica" the pain killers contain cinnomon, clove, eucalyptus, jasmine, mint, sage and pepper oils. Cold remedies "Antigrippe" include basil, cinnamon, pine, rosemary and thyme.

II.8 Psychological Effect of Essential Oils

Volatile components of essential oils can affect human body and mind positively and creatively. They are very powerful to create good feeling and also to calm and stimulate mind. Essential oils can be used for the treatment of mental and physical disorders. The accepted application of essential oils in aromatherapy are sniffing, inhalation, oral use and skin penetration.

In Europe, hop pillows were used in folk medicine as sedatives. Head-space analysis of dried hops showed the presence of dimethyl vinyl carbinol that is proved to have a distinct sedative-hypnotic effect. Rose oil is found to stimulate the central nervous system of psychotic patients. The oil increases the ability of concentration of healthy subjects, accelerates the working rate and improves the capacity to do work.

II.9 Essential Oils and Insects

It was known from experience that certain plants growing at proper places could save other plants from dangerous insects. Hemps (*Cannabis sativa*) were planted around vegetable gardens as repellents against a variety of worms and insects. Calamus oil can sterilise males of houseflies, so most probably an old custom of decorating houses in spring with calamous leaves is based on this property of the oil. Camphor, citronella, eucalyptus, clove and cinnamon oils show insecticidal property. Oils of laurel, thyme and coriander attract insects. Rose oil in 1:1000 dilutions will kill earthworms and leeches.

II.10 Essential Oils in Agriculture

Allelopathy deals with the chemical interaction of plants with each other. Some plants grow in perfect harmony with others, but some others perish without any apparent reason. There are many reasons for such effects including metabolites exchange in roots, organic and inorganic products washed away from leaves into the soil, interactions between plants and soil microflora and the presence of volatile plant products in the air and soil. Essential oils can dissolve in leaves of the plants and migrate into their roots. Certain plants produce oils as toxins against their diseases. Tobacco disease *Pseudomonas solancearum* will induce the synthesis of mixture of sesquiterpenes in tobacco leaves. Allelopathy of oil-bearing plants is still an under-investigated area with enormous potential.

In short essential oils have an important role in fields like medicine, agriculture, perfumery, flavour and food industry.

CHAPTER II

SECTION 2: COMPARISON OF ESSENTIAL OIL COMPOSITION OF *ZANTHOXYLUM RHETSA* SEEDS ON STEAM DISTILLATION FROM AQUEOUS, ALKALINE AND ACID MEDIA

II.11 Introduction

Zanthoxylum is a large genus of over 200 species of aromatic plants. Most of the plants of this genus are dioecious, pantropical in distribution and bear sharp thorns on either the stem or the foliage. *Zanthoxylum* spp. are used in the Americas, India, China, Japan and Africa for medicinal and culinary purposes. About 13 species are recorded from India.[12]

Zanthoxylum rhetsa D.C. syn. *Zanthoxylum budrunga* Wall and *Z. limonella* (Dennst.) Alston is a lofty, deciduous tree, up to 35 m. tall, commonly found in the evergreen monsoon forests of the foothills of Assam and Meghalaya, Eastern ghats especially Rumpa[*] and Western ghats of peninsular India.[13-15] In Kerala it is seen everywhere. This tree belongs to the family Rutaceae. Its stems and branches are covered with conical spines or prickles of 2 to 3 cm length. It is called 'Ashwagra' in Sanskrit, 'Munnina' in Kannada, 'Badrang' in Hindi, 'Triphal' (Trifla) in Marathi, 'Iratchai' in Tamil and 'Mullilam' in Malayalam.[16] In Malabar, people call it as 'Kumitty', 'Kuili' and 'Karimurikku'.

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[*] The specific name rhetsa[5] is given by the hillmen of Rumpa, which is probably the locality. Rhetsa means " committee" that under the shade of this tree hillmen assembled for panchayat.

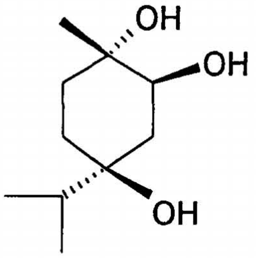
The tree is essentially of spice value. The bark is bitter and aromatic. It has the taste of the rind of orange and is pickled. The fruits are digestive and appetizing and the tender leaves are cooked and eaten in Assam. In western and southern India, they are used as a condiment in both sweet and savoury preparations, especially in the preparation of fish curries. The pericarp of the unripe fruits is pleasantly aromatic and tastes like the rind of a fresh orange, the seeds are pungent in taste, more or less like black pepper.[14,17] The dried fruits are used as an ingredient in the preparation of spice blends like 'garam masala' powder.[17] The fruits yield an essential oil called Mullilam oil, which is obtained by steam distillation of the dried ripe fruits. The oil has pleasant odour resembling that of sweet orange and tangerine.[13,17] The oil is used as an antiseptic and disinfectant. It produces some degree of irritation when given intradermally or instilled in the eye in a concentration of 5%. The fruits are prescribed in atrabiliary dyspepsia. They are also used in asthma and bronchitis, heart troubles, toothache, and rheumatism.[18-19] The essential oil, obtained from the fruits, the main component of which is (-) sabinene (30-90%) is used in the treatment of cholera in the indigenous system of medicine.[19] Its effectiveness in inflammatory dermatosis on topical application has been chemically proved.[20] Local anaesthetic activity has also been proved (maximum potency in 2% concentration). The drug possesses a wide margin of safety[21] (LD_{50} 1000 mg/kg).

II.12 Previous studies on the essential oil of *Zanthoxylum rhetsa*

There are a number of scientific publications on this plant describing the medicinal and essential oil aspects. The constituents of the oil have been the subjects of investigation by many workers. Earlier independent works on the chemical composition of the essential oil by various groups[22-25] of scientists led by Rao, Naves and Mathur, revealed the presence of alpha and beta-pinenes, (-) sabinene, alpha-terpinene, beta-phellandrene, (+) – terpinen-4-ol, mullilam diol, 1,4-cineole, cuminic aldehyde, octanal, decanal, (-) cryptone and phloroacetophenone dimethyl ether. Although l-sabinene (30-90%) is the chief or one of the chief constituents of all the oil samples distilled from the fruits, wide variation is observed in other constituents. Some oils contain phloroacetophenone dimethylether, suggesting that the fruits at some stage of ripening contain this compound. Joy and co-workers[26] detected 32 components, from the laboratory sample and 31 components from commercial sample. Sabinene constitutes the major portion of the oil (approx. 50%), terpinen-4-ol (14.1%), alpha-pinene (10.0%) and beta-pinene (7.5%) are the other major components.

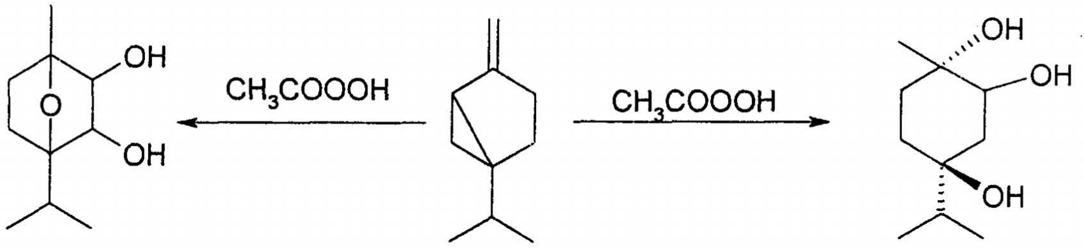
Ruangrungsi[27] and co-workers found that, the fruit of *Z. budrunga* on maceration in ethanol, followed by extraction with $CHCl_3$ yielded xanthoxylin, a new constituent in this genus, and the two alkaloids arborine and dictamnine.

A new monoterpene triol occurring in the volatile oil from *Z. budrunga* fruits was isolated by Thappa and co-workers[28] and its structure determined as (1s, 2s, 4s)-trihydroxy-p-menthane.



(1s, 2s, 4s)-trihydroxy-p-menthane

Paknikar and Kamat[29] have revised the structure of so-called 'mullilam diol', a crystalline compound obtained from *Zanthoxylum rhetsa* fruit oil, which was formerly formulated as p-menthane-2, 3-dihydroxy-1, 4-oxide (I) and assigned the new structure, (±)-p-menthane-1α, 2β, 4β-triol (II). Mullilam diol has been isolated from the fruit oil along with the major constituent sabinene by Mathur and co-workers.[24] It is found to be identical with the compound prepared by Henderson and Robertson[30] by the action of peracetic acid on sabinene. Hikino[31] re-investigated the reaction and conclusively proved the structure (II); instead of structure (I) for the Henderson and Robertson compound. This is further verified by the work of Halsall and co-workers.[32] Paknikar and Kamat[29] isolated the crystalline compound (m.p. 171°C) from mullilam oil and established its identity with an authentic sample of mullilam diol isolated by Mathur.[25] They chemically and spectroscopically verified that the structure of mullilam diol is (II), with molecular formula, $C_{10}H_{20}O_3$ and not (I) having molecular formula $C_{10}H_{18}O_3$. They also suggested to replace the name 'mullilam diol' by the name of structure (II) from literature to avoid confusion.



I Sabinene II

Shankaracharya and co-workers[33] analysed the dried fruits of five commercial samples for their physical and chemical composition. The colour of the fruit varies from dull brown to dark brown, essential oil content obtained by clevenger method, varies from 1.14% to 4.05% while that obtained by steam distillation method varies from 1.0% to 3.0%. The seeds are found to contain a small quantity of essential oil (10.35%) and the carpels are found rich in essential oils. As the seeds are poor in essential oil and rich in crude fibre (ca. 50%) they may be separated from their husks and discarded if the bulk is not required for the preparation of final product like spice powders or essential oil.

Chatterjee and co-workers[35] isolated quinazolone alkaloids rhetine, chelerythrine, rhetsine, evodiamine and rhetsinine (hydroxy evodiamine) from trunk bark of *Z. rhetsa.* The bark is aromatic and contains an essential oil, resin, and alkaloids. Pepuline hydrochloride isolated from the stem bark had a stimulating action on the mammalian heart and a spasmolytic effect on smooth muscles. The bark also contains lupeol (0.43-0.7%) and xanthosterol.

Nazrul Islam and co-workers[36] isolated 3,5-dimethoxy-4-geranyl oxycinnamyl alcohol, 8-methoxy-N-methyl findersine, xanthyletin and sesamin from the petroleum ether extract of the stem-bark of *Z. rhetsa.* The petroleum ether extract and 8-methoxy-N-methyl findersine showed cytotoxicity on brine shrimp nauplii.

Kalyani and co-workers[37] studied the *in vitro* anthelmintic activity of the fruit oil against tapeworms (*Taenia solium*), round worms (*Ascaridia galli*) and earthworms (*Pheritima postuma*) using piperazine phosphate as reference substance. [38] The anthelmintic activity was assessed following the method of Gaind and Budhiraj.[39] They found that the oil is more active on earthworms than on both types of intestinal worms. For tapeworms and round worms, the correlation between the death rate and concentration is exponential up to a concentration of 4% and linear beyond this concentration. Further, the activity of essential oil on all types of worms is higher than that of reference substance, piperazine phosphate.

The essential oil is found to be leavorotatory and poor in aldehyde content. The oil has high specific gravity and high evaporation residue indicating that the oil does not evaporate easily when exposed to air. Also the oil does not dissolve easily in 90% alcohol indicating that the oil is mainly composed of terpenoid hydrocarbons, sabinene being the major component. Its composition varies from 35.73% to 67.73%. The other major compounds are β-pinene, myrcene, γ-terpineol, α-terpinene and terpinen-4-ol. The analyses show that there are differences in the composition of clevenger (hydro) and steam distilled oils. The steam distilled oils have more amount of sabinene and alpha-pinene compared to clevenger distilled oils.[33]

II.13 Present work

Earlier works on *Zanthoxylum rhetsa* seed essential oil showed that it is rich in monoterpenes, especially sabinene.[22-26,29,33,37,40] Essential oil composition varies depending upon the method of extraction.[41-45] Dependence of the sabinene content in essential oils on the pH of the extraction medium is well known.[46,47] The present work was aimed to examine the essential oil composition of *Z. rhetsa* seeds, which contain considerable amount of sabinene, extracted from aqueous, alkaline and acidic media. Also olfactoric properties of the seed oil obtained under these three different conditions were studied and the odour-compound relations were correlated.

II.14 MATERIALS AND METHODS

The fresh greenish black seeds of *Z. rhetsa,* used for three different extractions, were collected in the same week (November 2001) from the same tree, from Malappuram district of Kerala. These seeds (300 g each) were steam distilled from water (500 ml), alkaline solution (500 ml of 10% Na_2CO_3), and acid solution (500 ml of 4% H_2SO_4) for 3h., without powdering or maceration. The distillate was collected in each case, extracted with diethyl ether (2x100 ml) and dried over anhydrous sodium sulphate. The solvent on evaporation yielded 5.5g (1.83%), 4.5g (1.5%) and 3.26g (1.08%) of oil, based on fresh weight of the seed.

Gas Chromatography (GC)

Quantification by GC-FID was carried out using HP 5890 GC (Hewlett Packard) fitted with an OPTIC® injector and FID detector. A HP5 (Hewlett Packard) capillary column (25m x 0.2 mm; film thickness, 0.33 µm) was used with nitrogen as carrier gas, at a flow rate of 3 ml/min. The column was temperature programmed from 30°C to 280°C at a rate of 3°C/min. The injector and detector temperatures were programmed at 270°C and 280°C respectively. The quantitative data quoted are percentage relative peak areas (RPA).

Gas Chromatography-Mass Spectrometry (GC-MS)

The GC-MS analyses were carried out at Quest International using Varian 3400 GC fitted with an OPTIC® injector and coupled with a Finnigan ITS 40 ion trap mass spectrometer. The column used was a HP ultra 2 (Hewlett Packard, 50 m x 0.2 mm, film thickness, 0.33 µm). Injector temperature 270°C; carrier gas: helium at 1.6 ml/min; temperature programme from 50-270°C at 2°C/min; mass spectra: EI mode 70 eV; scan range: 35-450 amu. Component identification was made by comparison with in-house references libraries, commercial libraries and known retention indices.

II.15 RESULTS AND DISSCUSSION

a. Identification of Components

The GC-MS analysis of the *Z. rhetsa* seed essential oil, obtained under three different conditions contained practically, the same components in different proportions. A total of 43 compounds were identified, of which monoterpenes and sesquiterpenes were the major classes of compounds. The compounds were identified by comparison of mass spectral data with those in the computer database of the instrument. These compounds and their percentages are listed in Table II.1.

Table II.1: Comparison of essential oil composition of *Z. rhetsa* seeds on steam distillation from aqueous, alkaline and acid media

Components	*RI	% RPA		
		Aqueous	Alkali	Acid
α-Thujene	930	0.47	0.39	0.34
α-Pinene	938	6.08	6.08	2.52
α-Fenchene	951	-	-	0.03
Camphene	954	0.09	0.09	0.12
Sabinene	977	66.74	72.7	3.31
β-Pinene	982	6.47	6.59	1.37
Myrcene	992	1.48	1.6	0.96
Octanal	1003	0.99	0.84	0.04
α-Phellandrene	1009	0.07	0.06	2.14
α-Terpinene	1021	0.74	0.04	23.68
p-Cymene	1029	0.03	t	1.13
Limonene	1033	1.95	2.07	4.67
1,8-Cineole	1037	t	t	2.05
trans-Ocimene	1049	0.08	0.09	0.17
γ-Terpinene	1063	1.36	0.1	23.06
p-2,4(8)-Menthadiene	1089	0.35	0.06	3.38
Terpinolene	1091	--	--	5.66

Linalool	1101	0.2	0.2	0.01
Nonanal	1104	t	t	0.02
cis-p-Menth-2-en-1-ol	1126	0.26	0.01	0.02
Terpinen-1-ol	1140	--	--	3.01
Terpinen-4-ol	1184	4.29	0.25	1.38
α-Terpineol	1197	0.9	0.77	1.99
Decanal	1206	2.04	1.81	0.72
Octyl acetate	1211	0.06	0.06	--
Piperitone	1264	t	t	0.03
Dec-2-enal	1265	0.09	0.03	--
Decanol	1272	0.05	0.04	0.05
Isobornyl acetate	1296	0.03	0.03	-
Undecanal	1308	t	t	t
α-Cubebene	1359	0.06	0.08	0.16
α-Copaene	1385	0.31	0.43	0.65
β-Copaene	1400	0.3	0.4	t
γ-Elemene	1403	0.65	0.65	0.42
Dodecanal	1410	0.23	0.21	0.34
Caryophyllene	1431	0.2	0.26	0.12
Humulene	1471	0.04	0.04	0.04
Alloaromadendrene	1472	0.03	0.04	0.06
Germacrene D	1499	0.52	0.68	0.19
β-Selinene	1502	--	--	0.73
α-Selinene	1505	0.41	0.48	1.34
γ-Cadinene	1528	0.06	0.13	--
δ-Cadinene	1536	0.16	0.14	1.61

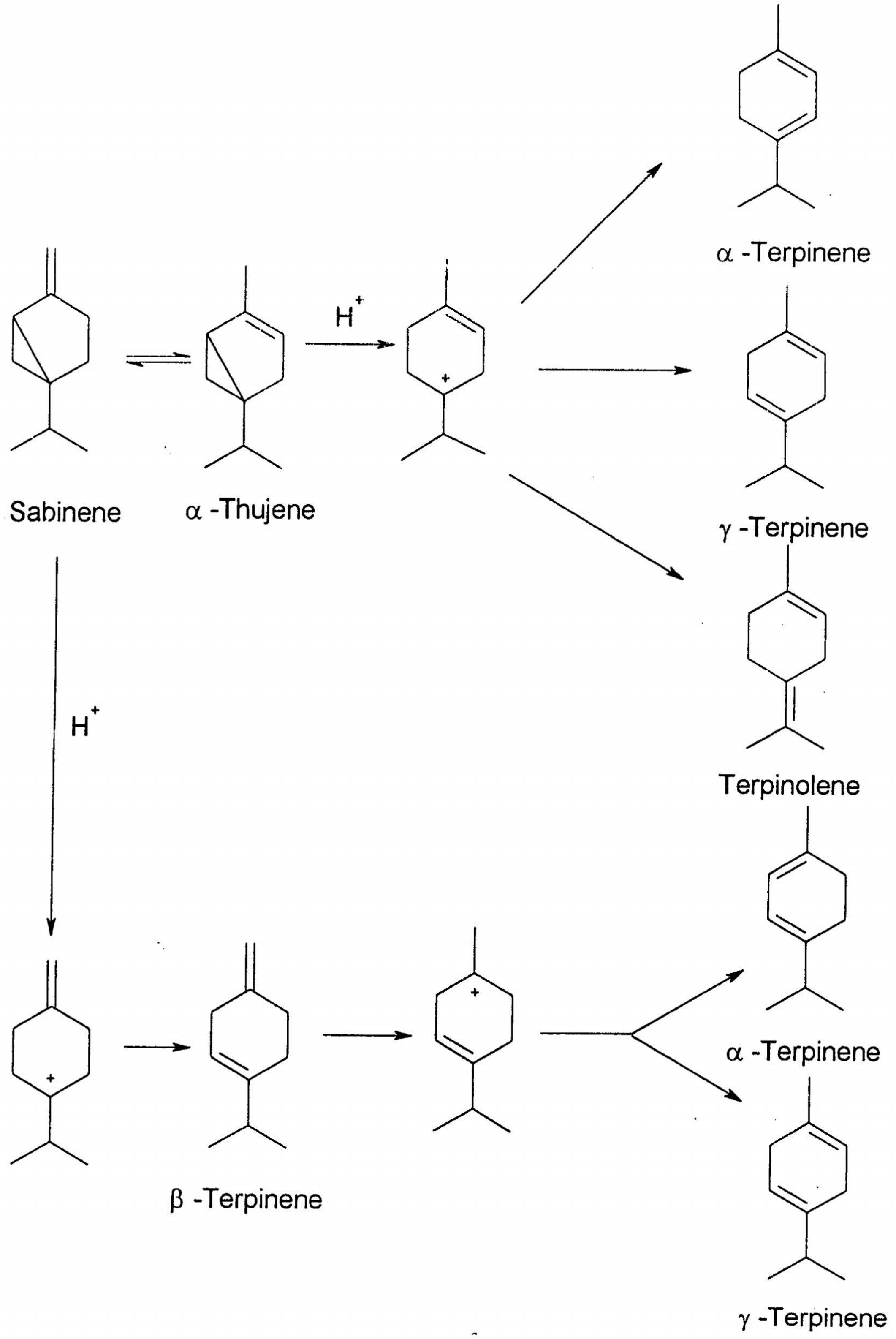
*RI = Retention index, carried out on HP-5 capillary column, t = trace (<0.01% RPA).

The concentration of monoterpenes in the seed oil, obtained by steam distillation from aqueous and alkaline media, [48] was as high as 91.59% and 91.13% respectively. This is because they contained sabinene (66.74% and 72.7%), β-pinene (6.47% and 6.59%), α-pinene (6.08% and 6.08%) as the major constituents. However, the percentage of monoterpenes in the acid distilled sample, was comparatively low as 81.39%, due to decrease in sabinene content. The percentage of sesquiterpenes in aqueous, alkali and acid distilled samples were 2.68%, 3.33% and 5.66% respectively.

Sabinene (66.74% and 72.7%), β-pinene (6.47% and 6.59%), α-pinene (6.08% and 6.08%), myrcene (1.48% and 1.6%), limonene (1.95% and 2.07%) and decanal (2.04% and 1.81%) were the major components obtained from aqueous and alkaline media respectively, while, α-terpinene (23.68%), γ-terpinene (23.06%), terpinolene (5.66%), limonene (4.67%), p-2,4(8)-menthadiene (3.83%), terpinen-1-ol (3.01%), α-phellandrene (2.14%), 1,8-cineole (2.05%) and α-terpineol (1.99%) were the major constituents from acid medium. Here, sabinene content was distinctly low (3.31%), and α-pinene (2.52%), β-pinene (1.37%) also showed remarkable reduction in percentage composition. α-Fenchene (0.03%), terpinolene (5.66%), terpinen-1-ol (3.01%) and β-selinene (0.73%) were present in addition to the other components, whereas, octyl acetate, dec-2-enal, isobornyl acetate and γ-cadinene were found to be absent in the acid extract. The refractive indices of the seed essential oils obtained from aqueous and alkaline media were the same, 1.464 and that of the oil obtained from acidic medium was 1.530.

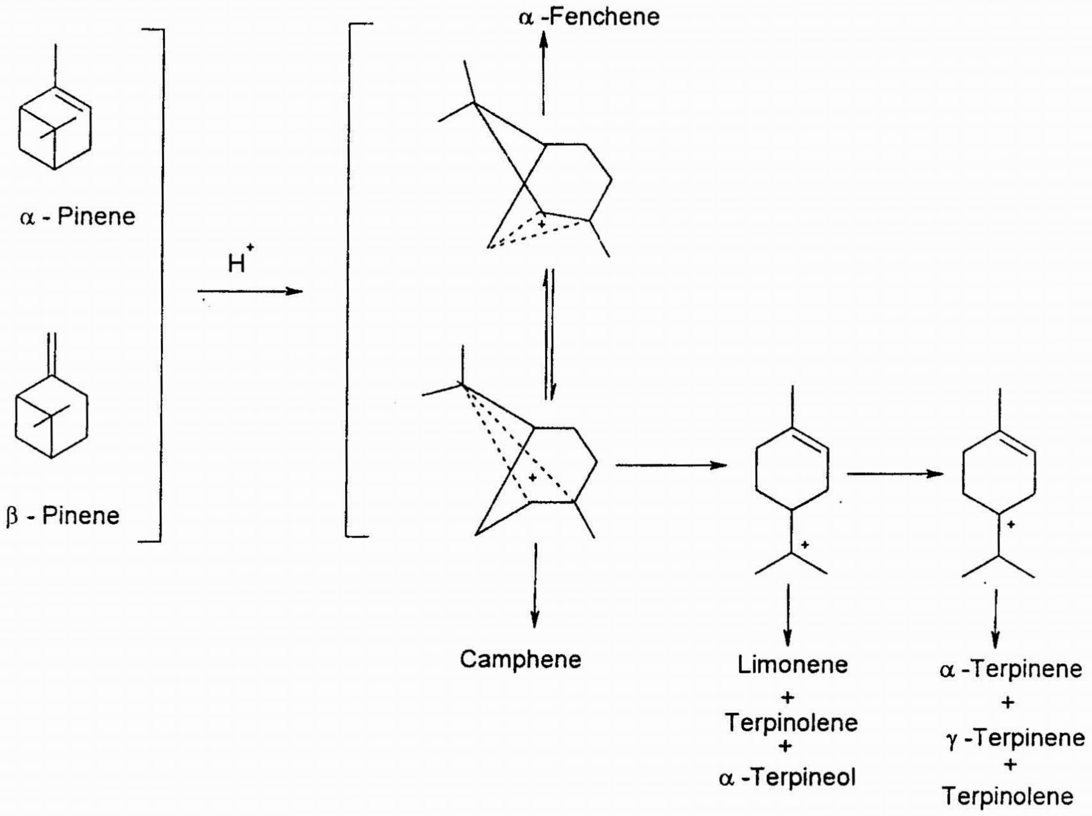
Sabinene is known to undergo acid catalysed transformation to α-terpinene, γ-terpinene, terpinolene and terpinen-4-ol.[46] Accordingly, sabinene contents were 72.7%, 66.7% and 3.3% in the oils obtained from alkaline, aqueous and acidic media respectively. The percentage of α-terpinene, γ-terpinene, terpinolene and limonene were considerably high in the oil distilled from acid medium. The α- and β-pinene content (6.08% and 6.47%) was reduced in the acid extract and the limonene content increased. Transformation of pinenes to limonene follows known pathways.[49]

The acid catalysed transformation of sabinene is as follows.



The rearrangements of both α-thujene and sabinene are catalysed by acids.[50] From the above transformation we can very well account for the increase in the concentration of α-terpinene (23.68%), γ-terpinene (23.06%) and terpinolene (5.66%) in acidic conditions.

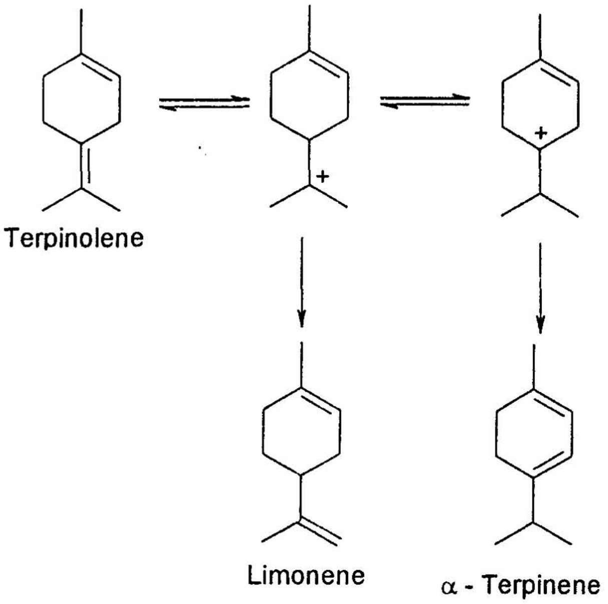
Both α-pinene and β-pinene undergo rearrangements in acidic medium. Acid catalysed reactions of α-pinene and β-pinene proceed as shown below.

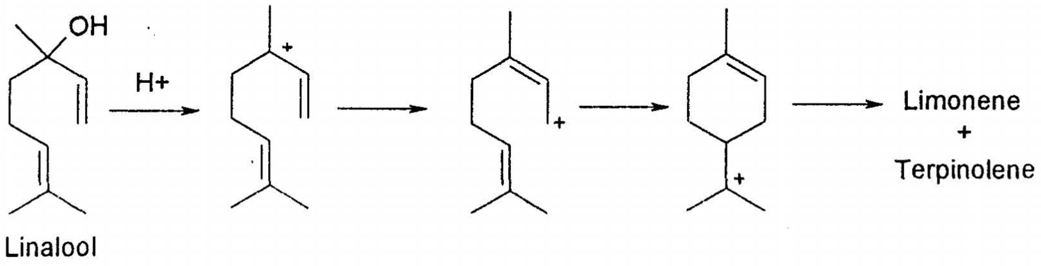


α-Pinene and β-pinene, give identical product mixtures in dilute acid, β-isomer reacting more rapidly.[49] The above mechanism clearly explains the decrease in the percentage of α-pinene (2.52%) and β-pinene (1.37%) and also the increase in limonene content (4.67%), in presence of acid.

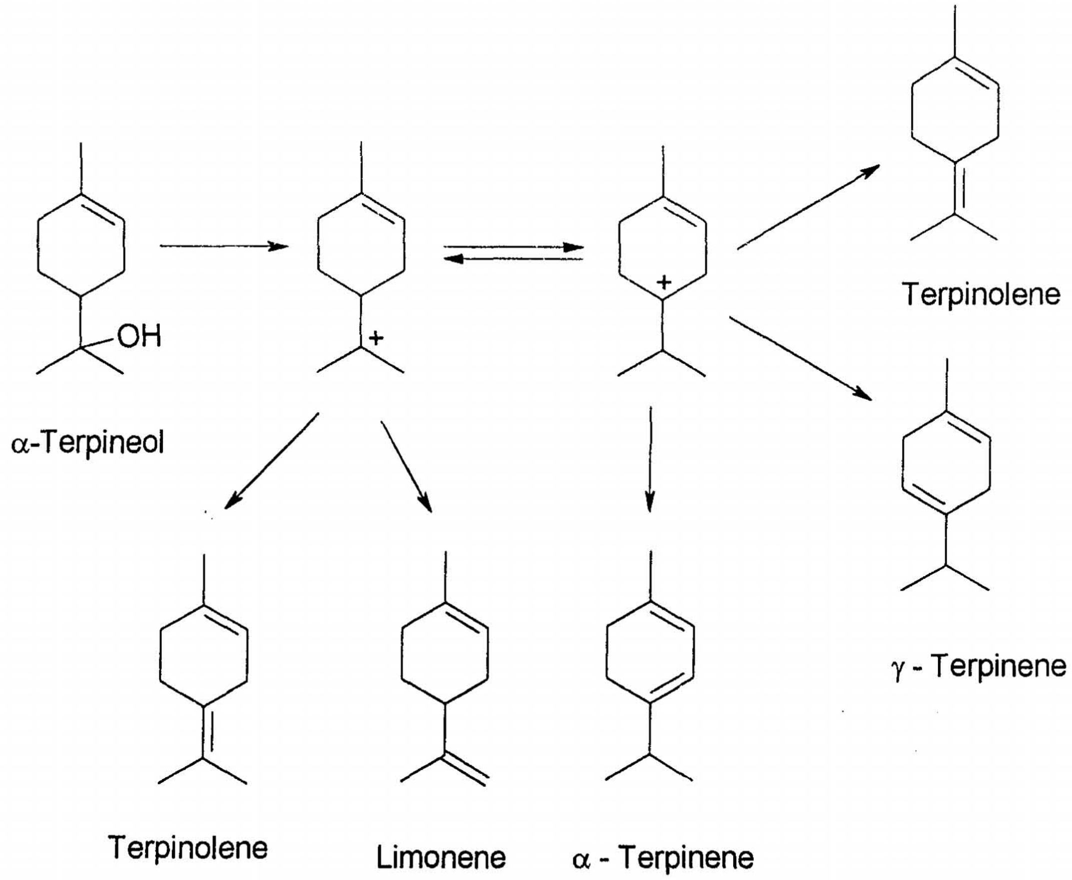
The increase in the limonene content in the acid distilled sample, can also be understood from the following:

Acid catalysed rearrangement of terpinolene and linalool, resulted in the formation of limonene and α-terpinene.





α-Terpineol, on dehydration with acids, forms a mixture of 1,8-cineole, plus dienes consistent with the formation of the ions, [51]



In this connection it is also of interest to note the Briggs and Sutherland's[52] findings that sabinene is found in all the oils in which terpinen-4-ol occurs. However, Koedam and co-workers[47] are of the view that it is probably more realistic to formulate the reverse. Conversion of sabinene to alpha and gamma terpinenes, terpinolene and terpinen-4-ol were brought about by the acidity of water rather than by high temperature conditions, as the experiments were performed in aqueous solutions. The rearrangements of the above type will be catalysed by the organic acids present in the plant material. The observed decrease in sabinene content in the seed essential oil obtained by steam distillation from acidic medium is due to the presence of mineral acid, 4% H_2SO_4, which catalyses the ring opening of sabinene. The increase in sabinene content in the oil obtained from alkaline medium is due to the neutralization of the plant acid by Na_2CO_3 solution, so that acid catalysed ring opening of sabinene is forbidden. The fruits contain plant acids

(100 g of fruits were heated under reflux in 100 ml of distilled water for 0.5 h . Its pH was then found to be 5). Therefore, it may be suggested that, if a commercial source of sabinene is required, the *Z. rhetsa* seeds may be steam distilled from alkaline medium.

b. Olfactoric studies

The seed essential oils of *Z. rhetsa* obtained under three different conditions were olfactorically evaluated. The odour of the essential oils of seeds of *Z. rhetsa* were described by professional perfumers as spicy in the direction of pepper, herbal-earthy, weak fruity like lemon and sweet orange, weak floral, pinene and terpinene like with woody-smokey notes in the back ground. The observed odour characteristics are correlated with identified chemical compounds as follows.

The seed oil obtained by steam distillation from alkaline and aqueous solution exhibit strong spicy odour, similar to that of pepper. This was caused by the presence of hydrocarbons like sabinene and pinenes as well as by monoterpene alcohols, terpinen-4-ol, α-terpineol and linalool. These compounds dominate in the essential oil of *Piper nigrum*.[53]

The ester functionality is not necessarily a criterion for fruity odour. Out of 85 fruity odourants studied by Mc Gugan[54] 41% are esters, 24% ketones, 9% aldehydes, 7% lactones and the remaining miscellaneous. The intense fruity lemon-like, sweet orange-like odour of *Z. rhetsa* seed oil obtained under acidic condition compared to the other two cases, was due to the high percentage content of limonene, α-terpinene, linalool and γ-terpinene which are among the constituents of lime oil, [55,56] orange juice and orange peel oil.[57]

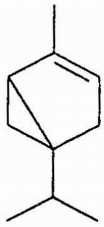
The faint floral odour of the seed oils obtained under three different conditions were contributed by linalool, α-terpineol, nonanal, octanal etc.

The floral odour is the combined contribution of monoterpenes and sesquiterpenes. Of the monoterpenes linalool, which is a constituent of rose, jasmine and neroli oils [58,59] imparts a floral odour.

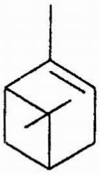
Sabinene, terpinen-4-ol, selinenes, δ-cadinene were responsible for the herbal-earthy and woody-smokey notes of the oil obtained from aqueous and alkaline media. δ-cadinene adds the smokey tarry tonality to the odour bouquet.[60]

In conclusion, it is possible to report that the essential oil of seeds of mullilam obtained by steam distillation from aqueous and alkaline media may be an interesting alternative to traditional pepper oil, for flavouring food as the oils of *Z. rhetsa* and *P. nigrum* seeds have a similar, but not identical, chemical composition. In the acid distilled oil, the sabinene, α-pinene, β-pinene content is distinctly lower. However, due to the formation of artefacts by exposure to acid, the content of limonene, α-terpinene, γ-terpinene, p-cymene, 1,8-cineole and α-terpineol are higher. Probably these components, enriched in the distillation process, contribute to the "typical" lime aroma.[61]

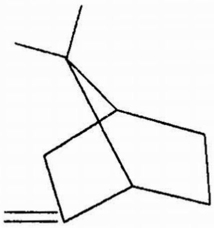
Structures of the compounds identified in the essential oil of *Zanthoxylum rhetsa*



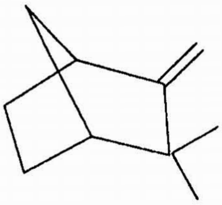
α-Thujene



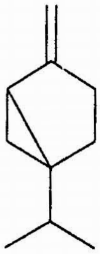
α-Pinene



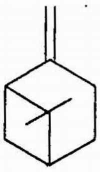
α-Fenchene



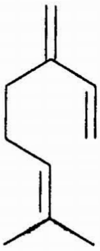
Camphene



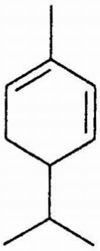
Sabinene



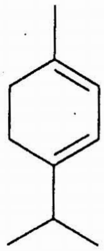
β-Pinene



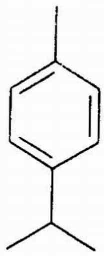
β-Myrcene



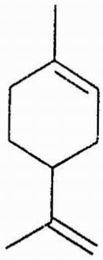
α-Phellandrene



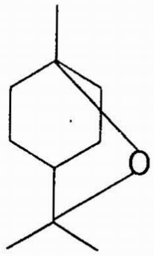
α-Terpinene



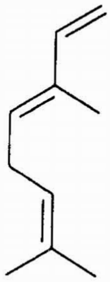
p-Cymene



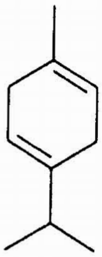
Limonene



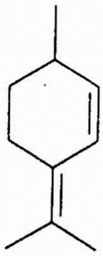
1,8-Cineole



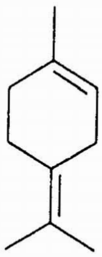
trans-β-Ocimene



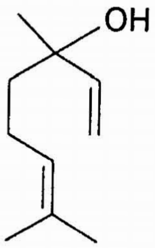
γ-Terpinene



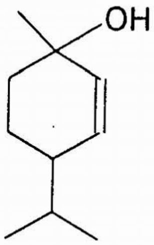
p-2,4(8)-Menthadiene



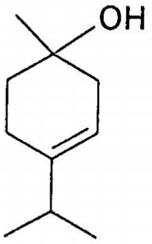
Terpinolene



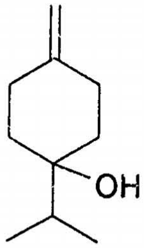
Linalool



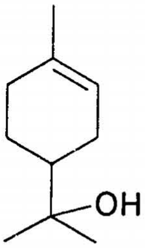
cis-p-Menth-2-en-1-ol



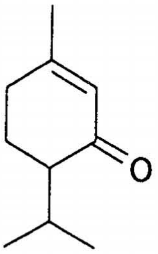
Terpinen-1-ol



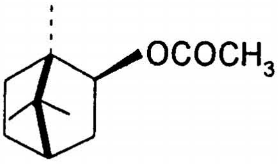
Terpinen-4-ol



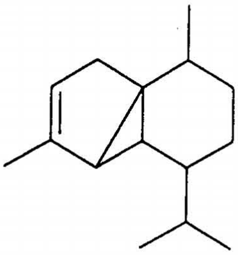
α-Terpineol



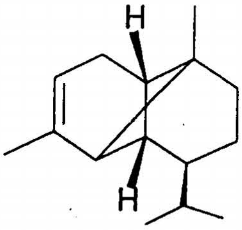
Piperitone



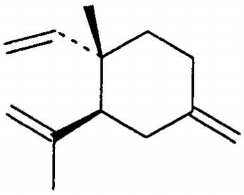
Isobornyl acetate



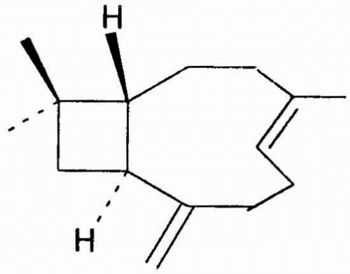
α-Cubebene



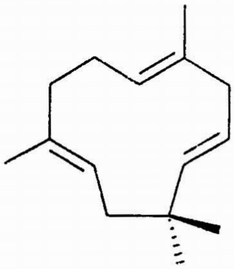
α-Copaene



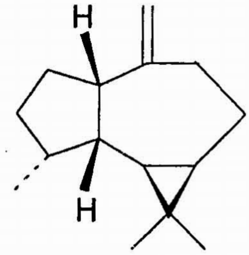
γ-Elemene



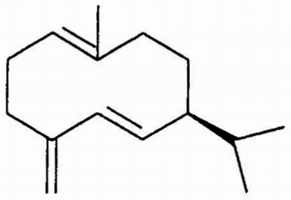
β-Caryophyllene



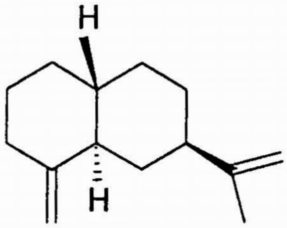
α-Humulene



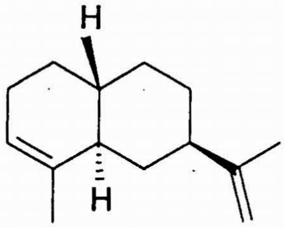
Alloaromadendrene



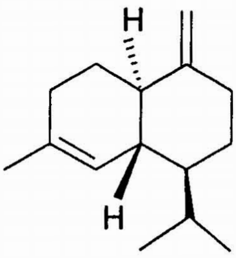
Germacrene D



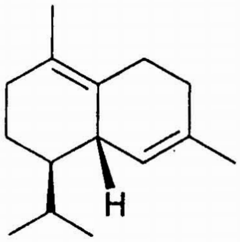
β-Selinene



α- Selinene



γ-Cadinene



δ-Cadinene

SECTION 3: CHEMICAL TRANSFORMATION OF *ZANTHOXYLUM RHETSA* DC. SEED ESSENTIAL OIL

II.16. Introduction

Zanthoxylum rhetsa DC. is a small or moderate sized tree with pale corky bark, covered with conical prickles on stems and branches. Seeds are globose, bluish black, smooth, shining, tasting of black pepper.[62]

Paknikar and Kamat[29] isolated a crystalline compound from *Z. rhetsa* fruit oil and assigned its structure as 1S, 2S, 4S-trihydoxy-p-menthane. It was found to be identical with the compound prepared by Hikino[31] by the action of peracetic acid on sabinene. In Hikino's work sabinene was stirred at 55°C with acetic acid and 30% H_2O_2 for 100 h. It was then neutralized with Na_2CO_3 and extracted with ethyl acetate. The residue obtained after the evaporation of the solvent, was refluxed with 5% ethanolic KOH for 1 h. On extraction with ethyl acetate it gave p-menthane-1, 2, 4-triol.

In the present work, *Z. rhetsa* seed essential oil obtained by steam distillation from alkaline medium, with 72.7% of sabinene content, on treatment with peracetic acid became highly viscous. It was then subjected to GC-MS analysis. Comparison of the mass spectral data with those available in the in-house library revealed the presence of p-menthane-1, 2, 3-triol as the major component. This is in contrast with Hikino's observation. The present work was therefore envisaged to investigate further on this.

II.17. EXPERIMENTAL

Extraction of essential oil from the seeds of *Z. rhetsa* and its analysis by GC and GC-MS are described in section 2.

Preparation of peracetic acid [63]

A well-stirred mixture consisting of 69 ml acetic anhydride (97.6%) and 50 ml H_2O_2 (30%, W/V) was maintained at 40°C with external cooling. After the mixture reacted for about four hours at 40°C and stood overnight at room temperature (30°C), the concentration of peracetic acid reached a maximum (about IM, 60-80% yield). This solution is quite stable and may be stored for several weeks at 5°C without appreciable reduction in the peracetic acid content.

Action of peracetic acid on *Z. rhetsa* seed essential oil

It has been observed that if the temperature is maintained between 20° and 25°C, the consumption of peracetic acid by olefins with non-terminal, isolated double bonds will complete in 2-4 h. whereas terminal unsaturated compounds require about 24 h.[64]

Five ml (4.37g) of *Z. rhetsa* seed essential oil, obtained by steam distillation from alkaline medium, was stirred with 36 ml of peracetic acid, at room temperature (30°C) for 24 h. The solution was poured into cold water, which turned milky. This was extracted with diethyl ether (2 x 100 ml) and neutralized with 100 ml 10% Na_2CO_3. The ether layer was washed with water and dried using anhydrous sodium sulphate. The solvent on evaporation yielded a highly viscous, pale yellow oil (3.32g, 76%). It was then column chromatographed (3cm x 100cm; d x l) on silica gel (60-120 mesh), using petroleum ether, 10:1 mixture of petroleum ether-acetone and acetone as eluents. 50 ml fractions were collected and homogeneity of each fraction was checked by tlc assay. Identical fractions were pooled together and concentrated by evaporation. The highly viscous yellow oil obtained by elution with acetone was used for further study.

Reaction with 50% orthophosphoric acid

Three ml (2.924g) of viscous yellow oil obtained by elution with acetone was refluxed with 10 ml 50% H_3PO_4 for 3h. It was cooled, extracted with diethyl ether, washed the etherial layer with water, dried and removed the solvent by evaporation. The orange red oil 1.5g (51.3%) thus obtained, was chromatographed (3cm x 100cm; d x l) over silica gel (60-120 mesh) and eluted with petroleum ether followed by a mixture of petroleum ether-ethyl acetate. Fractions 1 to 10 collected using 8:1 petroleum ether-ethyl acetate mixture were concentrated by evaporation. The deep orange yellow coloured oil thus obtained gave positive test with Borche's reagent, suggested the presence of aldehydes/ketones. The IR spectrum of the compound gave absorption at 1670.6cm^{-1}, indicating the presence of conjugated carbonyl group. Further purification of this compound was carried out by treatment with saturated sodium sulphite solution and shaking well for half an hour. The solid bisulphate product was washed with ether and then decomposed by dil. HCl. It was again extracted with diethyl ether and washed till acid free, dried and concentrated by evaporation of the solvent. The yellow oil 0.5g (17%) thus obtained, having pleasant odour, comprises mainly of aldehydes/ketones were analysed by GC-MS.

II. 18. RESULTS AND DISCUSSION

The GC and GC-MS analysis of the *Z. rhetsa* seed essential oil, obtained by steam distillation from alkaline medium, on reaction with peracetic acid, showed the presence of 23 compounds, of which p-menthane-1, 2, 3-triol was the major component (36.65%). Other predominant compounds are 6-undecanone (10.07%), sabina ketone (6.26%) and decanal (5.18%) (Table II.2).

Table II.2

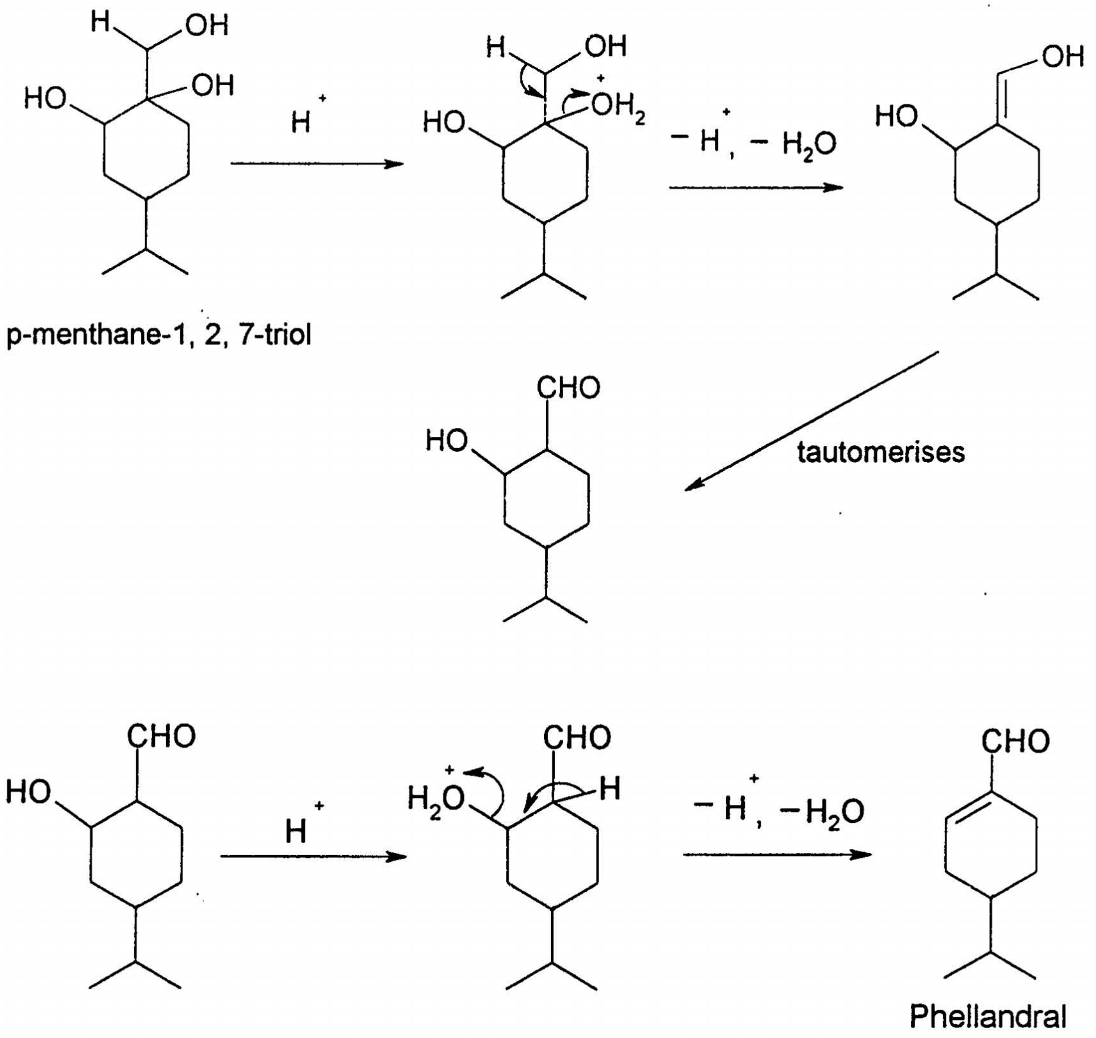
Composition of *Z. rhetsa* seed essential oil on reaction with peracetic acid

Components	% Composition
1, 4-Cineole	0.26
1, 8-Cineole	3.10
p-Cymene	1.87
Octanal	1.18
trans-Linalool oxide (furanoid)	2.28
Octyl acetate	0.59
cis-Linalool oxide (furanoid)	1.95
Fenchyl acetate	0.58
Decanal	5.18
Octanol	2.89
Fenchol	1.20
2, 5-Octane dione	2.32
Sabina ketone	6.26
2, 9-Decane dione	0.80
Borneol	0.77
Epoxy linalool	0.49
Decanol	0.39
Hydroxy-cineole	0.44
P-Menthane-1, 2, 3-triol	36.65
6-Undecanone	10.07
Undecanol	0.43
Cuminol	2.02
P-Menthane-1, 2, 4-triol	0.62

Sabinene, the main component present in the *Z. rhetsa* seed essential oil, on reaction with peracetic acid has got converted into a triol. It may be assumed that, peracetic acid functions as an oxidizing agent in exactly the same way as perbenzoic acid to yield epoxy compounds, but that in acetic acid solution the epoxy ring is opened and mixtures of hydroxy-acetoxy compounds are obtained.[63] The product after peracetic acid treatment containing the triol was made to react with 50% H_3PO_4 as explained in the experimental section. The product obtained, on GC-MS analysis contained phellandral (p-menthane-1-en-7-al) as major component. The formation of phellandral in this reaction is possible only if p-menthane-1, 2, 7-triol is present in the starting material, while p-menthane-1, 2, 3-triol cannot yield this product. The compounds identified and their percentage compositions are given below.

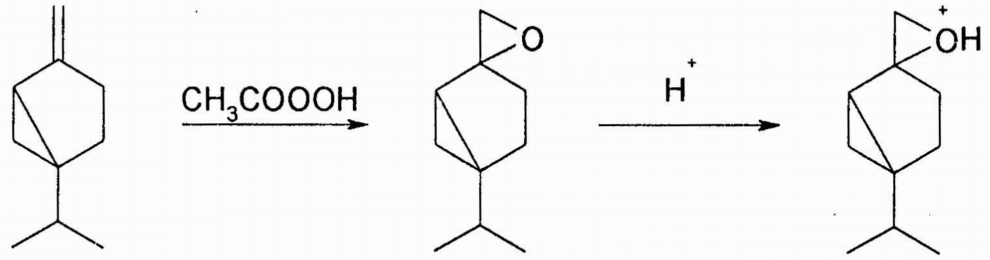
Components	**% Composition**
Phellandral (p-Menth-1-en-7-al)	82.13
Cuminic aldehyde	12.52
2-Caren-10-al	2.01
2, 6-di (t-Bu)-4-Methyl phenol	1.79
Cuminyl acetate	1.56

The conversion of p-menthane-1, 2, 7-triol to phellandral can be accounted as follows.



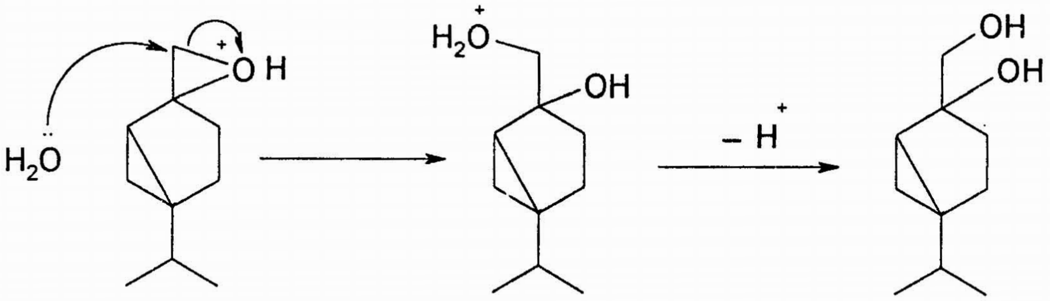
The probable mechanism of formation of p-menthane-1, 2, 7-triol from sabinene is as follows.

i) Epoxidation of sabinene followed by protonation.



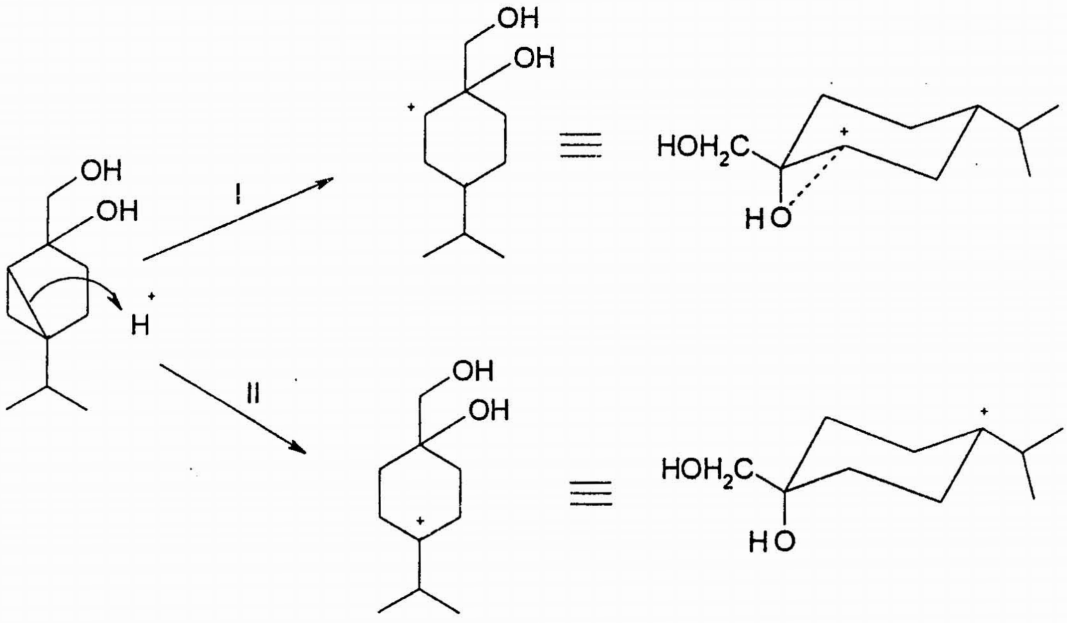
Sabinene

ii) Attack by water molecule and removal of H$^+$.



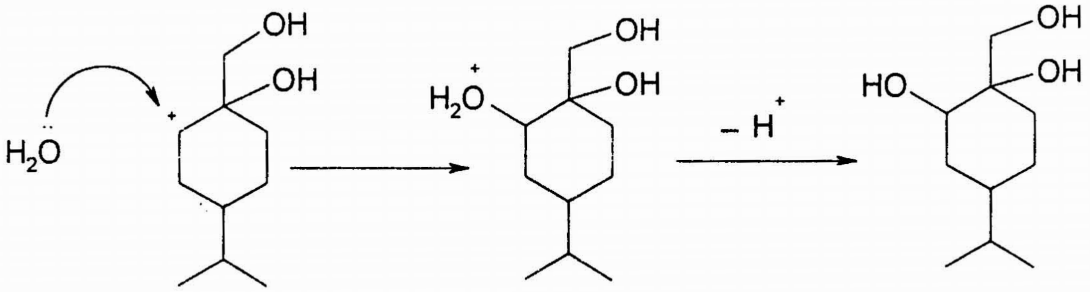
Diol

iii) Protonation of the cyclopropane ring in the diol.



Though a 3^0carbocation is more stable than a 2^0carbocation, pathway (I) leading to a 2^0carbocation is stabilized by the presence of adjacent — OH group as shown, while pathway (II) cannot be stabilized by this way.

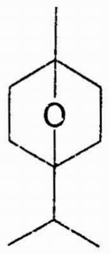
iv) Carbocation formed in pathway (I), on reaction with water leading to the formation of p-menthane-1, 2, 7-triol.



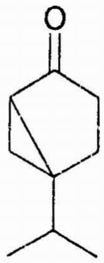
p-Menthane-1, 2, 7-triol

This work has generated enough proof that peracetic acid treatment of sabinene gives p-menthane-1, 2, 7-triol that is in contradiction to earlier findings. Cuminic aldehyde was also one of the compounds found in the final product that possesses a pleasant odour and therefore it can be used in perfumery.

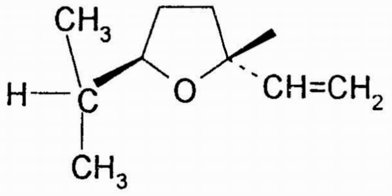
The structures of the compounds identified are given below:



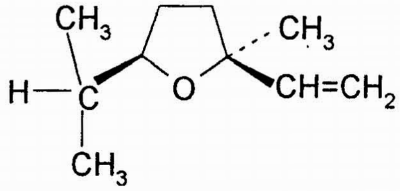
1, 4-Cineole



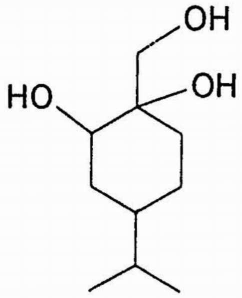
Sabina ketone



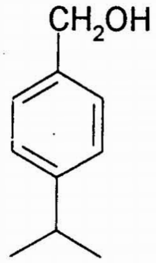
(E)-Linalool oxide



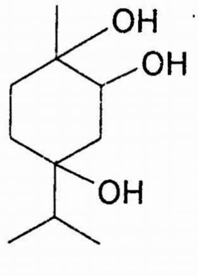
(Z)-Linalool oxide



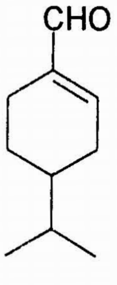
p-Menthane-1, 2, 7-triol



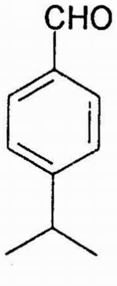
Cuminol



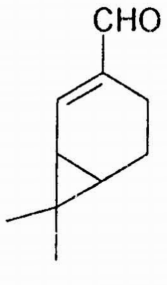
p-Menthane-1, 2, 4-triol



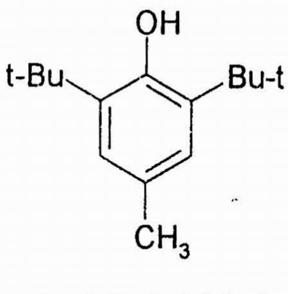
p-Menth-1-en-7-al



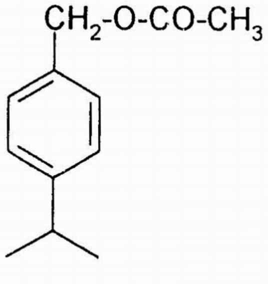
Cuminic aldehyde



2-Caren-10-al



2,6-di- (t-Bu)-4-Methyl phenol



Cuminyl acetate

REFERENCES

1. Agarwal, O.P., *Chemistry of Natural Products* **1**, 1994, 405.
2. *Kirk Othmer Ency. of Chem. Tech,* 4th edition **17**, 1996, 605.
3. Moyler, D.A., in *Proce. II^th^ Int. Congress of essential oils, Fragrances and Flavours* **1,** 1989, 63.
4. Willard, H.H., Merritt, L.L., Dean, J.A., Settle, F.A. (eds). Instrumental Methods of Analysis. New Delhi: CBS, 1986, p.540.
5. Willett, J.E., *Gas Chromatography* 1991, 163.
6. Rossiter, K.J., *Chem. Rev.* **96,** 1996, 3201.
7. Hisashi, K., Nanao, H., in *Proce. 11th Int. Congress of essential oils; Fragrances and Flavours* **5**, 1989, 57.
8. Brud, W.S., Gora, J., in *Proce. Int. Congress of essential oils, Fragrances and Flavours* **2**, 1989, 13.
9. Schilcher, H., *Deutsche Apotheker Zeitung* **124**, 1984, 1433.
10. Svendsen, A.B., Scheffer, J.J.C. (eds). Essential Oils and Aromatic Plants. Drodrecht: Martinus Nijhoff, 1985, p.220.
11. Brud, W.S., Gora, J., in *Proce. 11th Int.Congress of essential oils, Fragrances and Flavours* **2**,1989, 18.
12. Ramidi, R., Ali, M., Velasco-Negueruela, A., Perez-Alonso, M, J., *J. Essent. Oil. Res.* **10**, 1998, 127.
13. The Wealth of India, Raw Materials Vol. XI. New Delhi: CSIR, 1976, p.21.

14. Krishna, S., Badhwar, R.L., *J. Sci. Industr. Res.* **7(6),** Supplement|: 1948, p. 113.

15. Gamble, J.S., A manual of Indian Timbers. Dehradun: Bischensingh Mahendrapalsingh, 1972, p. 123.

16. Brandis, D. in *Indian Trees* ed. by Bischensingh Mahendrapalsingh Dehradun, 1971, p. 118.

17. Rao, R.M., Bhave, Y.M., *Indian Perfumer* **3**, 1959, 99.

18. Ambasta, S. P. (ed). The useful plants of India, New Delhi: CSIR, 1986, p. 698.

19. Joshi, C. G., Magar, N. G., *Indian J. Pharm.* **15**, 1953, 312.

20. The Wealth of India, Raw Materials Vol. XI. New Delhi: CSIR, 1972, p.22.

21. Agshikar, N.V., Abraham, G.J.S., *Ind. J. Med. Res.* **60**, 1972, 22.

22. Rao, B.S., Sudborough, J.J., Watson, H.E., *J. Indian Inst. Sci.* **8A**, 1925, 174.

23. Naves, Y.R., Azdizio, P., *Bull. Soc. Chim. Fr.* **41**, 1950, 673.

24. Naves, Y.R., *Perfume. Essent. Oil Res.* **41**, 1950, 441.

25. Mathur, R.K., Ramaswamy, S.K., Rao, A.S., Bhattacharya S.C., *Tetrahedron* **23**, 1967, 2495.

26. Joy, M.T., Verghese, J., Retamar, J.A., Talenti, E.C.J., Catalan, C.A.N., Gros, E.G., *Flav. Frag. J.* **1**, 1986, 165.

27. Ruangrungsi, N., Tantivatana, P., *J. Sci. Soc. Thailand* **7(3)**, 1981, 123.

28. Thappa, R.K., Dhar, K.L., Atal, C.K., *Phytochemistry* **15**, 1976, 1568.

29. Paknikar, K., Kamat, V.P., *J. Essent. Oil Res.* **5**, 1993, 659.

30. Henderson, G.G., Robertson, A., *J. Chem. Soc.* 1923, 1849.

31. Hikino, Y., *J. Pharm. Soc. Japan* **85**, 1965, 477.

32. Garside, P., Halsal, T.G., Hornby, G.M., *J. Chem. Soc.* C, 1969, 716.

33. Sankaracharya, N.B., Puranaik, J., Nagalakshmi, S., Rao, L.J., *Pafai Journal* **16(1)**, 1994, 15.

34. Saidutty, A. Ph.D. Thesis, Calicut University, 1999, p. 46.

35. Chatterjee, A., Bose, S., Ghosh, C., *Tetrahedron* 7, 1959, 257.

36. Nazrul Islam, S.K., Asham, M., Zaman, T.A., Hasan, C.M., Ito, C., *Fitoterapia* **71(6)**, 2000, 697.

37. Kalyani, G.A., Aithal, K.S., Srinivasan, K.K., *Fitoterapia* LX, No.2, 1989, 160.

38. Garg, L.C., Atal, C.K., *Indian J. Pharm.* **29**, 1967, 127.

39. Gaind, K.N., Budhiraj, R.D., *Indian, J. Pharm.* **29**, 1967, 127.

40. Jirovetz, L., Buchbauer, G., Shafi, M. P., Saidutty, A., Z. *Lebensm. Unters Forsch A* **206**, 1998, 228.

41. Dahoun, A., Derriche, R., Belabbes, R., *Riv. Ital. Eppos* **4(10)**, 1993, 29.

42. Amaia, A., Ines, A., Gustavo, R., Juan, D.B., *Z. Lebensm. Unters Forsch A* **209(2)**, 1999, 126.

43. Robert, G., Mina, G., Jamal, B., *Biruniya* **4(2)**, 1988, 117.

44. Sorensen, J. M., Katsiotis, S.T., *Planta Medica* **66(3)**, 2000, 245.

45. Maria, T., Bela, S., Vendel, I., Sandor, P., Daood, H., Klara, S., *Olaj- Szappan. Kozmetika* **45**, 1996, 75.

46. Koedam, A., Looman, A., *Planta Medica* Supplement: 1980, 22.

47. Koedam, A., Scheffer, J.C.J., Svendsen, A.B., *Perfumer & Flavorist* **5**, 1980, 56.

48. Radhamani, K.T., M.Phil. Thesis, Calicut University, 2002, p.22.

49. Whittaker, D., In *Chemistry of terpenes and terpenoids. The monoterpenes*. Newman, A.A. (ed). New York: Academic Press, 1972, p. 60.

50. Whittaker, D., In *Chemistry of terpenes and terpenoids. The: monoterpenes*. Newman, A.A. (ed). New York: Academic Press, 1972, p.51.

51. Whittaker, D., In *Chemistry of terpenes and terpenoids. The: monoterpenes*. Newman, A.A. (ed). New York: Academic Press, 1972, p.28

52. Briggs, L.H., Sutherland, M.D., *J. Org. Chem.* **7**, 1942, 397.

53. Karl, C. in *Die naturlichen und Kustlichen Armen.* Ziegler, E. (ed). (Huthig, Heidelberg), 1982, p. 85.

54. Mc Gugan, W.A., *Food Res. Rep.* **4(1)**, 1980, 13.

55. Inoma, S., Miyagi, Y., Akieda, T., *Kanzei Chuo Bunsekishoho* **29**, 1989, 87.

56. Fleisher, A., Biza, G., Secord, N., Dono, J., *Perfumer and Flavorist* **12(2)**, 1987, 57.

57. Hunter, G.L.K., Brodgen, Jr., W.B., *J. Food. Sci.* **30**, 1965, 1.

58. Mukherjee, B.D., Trenkle, R.W., Technical data, *International Flavors & Fragrances Inc.* Union Beach N.J., 1967.

59. Boelens, M.N., Oporto, A., *Perfumer Flavor.* **16(6)**, 1984, 1.

60. Arctander, S., in *Perfume and Flavour Chemicals.* New York: Arctander Publications, 1969.

61. Kurt, B., Garbe, D., Common Fragrance and Flavor Materials. Weinheim: VCH Publishers, 1985, p.118.

62. Kirtikar, K.R., Basu, B.D. Indian Medicinal Plants. Vol. I. Bischensingh Mahendrapalsingh. Dehra Dun, 1984, p. 464.

63 Findley, T.W., Swern, D., Scanlan, J.T., *J. American Chemical Soc.* **67**, 1945, 412.

64 Swern, D., *Chem. Rev.* **45(1)**, 1949,16.

BIOPHYTUM SENSITIVUM

CHAPTER III

SECTION 1: ANALYSIS OF THE ESSENTIAL OIL OF AIR-DRIED *BIOPHYTUM SENSITIVUM* (L.) DC.

III.1 Introduction

Biophytum sensitivum (L.) DC. Syn.*Oxalis sensitivum* Linn. belongs to the family Oxalidaceae.[1] The genus *Biophytum* is known to contain about 60 species of herbs growing throughout the tropical regions of south Asia, Africa and Medagascar. About 8 species are seen in India.[2,3] *Biophytum sensitivum* (L.) DC is a variable annual herb, 2.5-25 cm in height, found as a weed in open habitats during the rainy season throughout the hotter parts of India, ascending to 1800m in the Himalayas.[4] The plant is commonly known as 'Mukkutti' in Malayalam, 'Jalapushpam' in Sanskrit and 'Lajalu' in Hindi. Leaves are of 3.8-12.7 cm long, and sensitive to touch, flowers are yellow, seeds minute with obliquely transverse tubercled ridges.[1] There is some information about the chemical composition of this plant, but studies of the volatiles from *Biophytum sensitivum* could not be found yet.

III.2 Medicinal Properties and Uses

The plant is reported to possess tonic and stimulant properties and is used in chest complaints, insomnia, convulsions, cramps and inflammatory tumours, and its ash in stomach ache.[5,6] The whole plant is dried, powdered and given internally to cattle, to stop excess salivation. A decoction of the roots is reported to be given in fevers, gonorrhoea and lithiasis. In Philipines, the powdered seeds are used as a vulnerary; along with butter they are applied to abscesses to promote suppuration.[7] The juice of the plant is reportedly taken as a remedy for malarial fever among the Mahalia in the Chotanagpur region of Bihar. The whole plant is eaten as an aphrodisiac among the tribal inhabitants of northeastern Madhya Pradesh.[8]

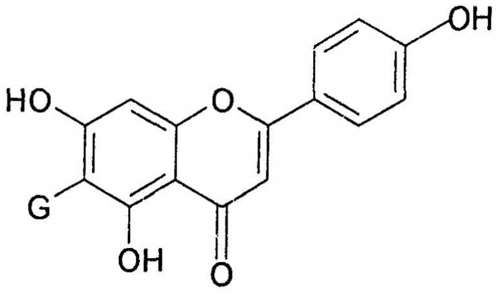
The leaves are diuretic and possess astringent and antiseptic properties; the pounded or bruised leaves or their juice is used in dressing burns and contusions. A paste of the leaves is applied to wounds and cuts; it is also a tonic. A decoction of the leaves is given in asthma and phthisis. An aqueous extract of the fresh leaves, partially inhibited the growth of *Mycobacterium tuberculosis* 607.[1] The mature leaves contain an insulin-like principle and are recommended in diabetes. A saline extract of the leaves showed hypoglycaemic activity in rabbits.[9] The boiled leaves are used by the Bhils and Dhankas of Gujarat to relieve rheumatic pain. The leaf juice is applied for temporary relief of scorpion stings by the Gonds of northern Andhra Pradesh.[8]

Literature survey revealed that no study has so far been carried out on the essential oil of *Biophytum sensitivum*. More over, the plant is well-known for its medicinal properties. The objective of the present investigation is therefore to analyse the essential oil of the air-dried *Biophytum sensitivum* by gas chromatographic-spectroscopic (GC-FID and GC-MS) and olfactoric methods and to identify those components responsible for the characteristic aroma impressions, their possible biological effects as well as applications in food-flavoring, perfumery and cosmetics.

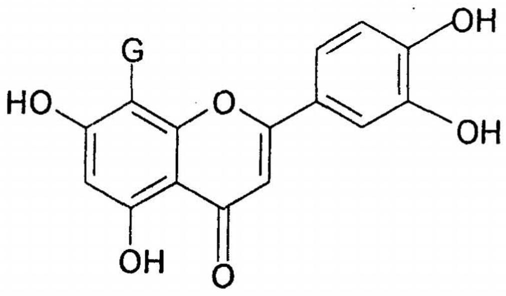
III.3 Previous Work

Seal and co-workers[10] reported that *Biophytum sensitivum* is known to accumulate oxalic acid and fix $14CO_2$ into oxalic acid photosynthetically with one minute of exposure to gas.

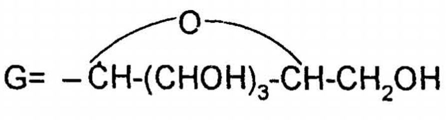
Bucar and co-workers[11,12] isolated C-glycosyl flavones and proanthocyanidins from *Biophytum sensitivum*. Orientin, isoorientin, isovitexin, isoorientin 7-O-glucoside, isoorientin 2"-O-rhamnoside and isovitexin 2"-O-rhamnoside were isolated from the methanol extract of the leaves. Only flavones but no flavonols could be identified in *Biophytum sensitivum.* For isoorientin and orientin, radical scavenging activity and a moderate inhibition of xanthine oxidase is reported.[13,14] From the acetone-water percolate of the roots, (-)-epicatechin and proanthocyanidin B2 were isolated. Proanthocyanidins have shown to exhibit anti-inflammatory activity *in vivo*[15] and to inhibit COX-I and COX-2 catalysed prostaglandin biosynthesis when tested *in vitro*·[16,17] The highest amounts of C-glycosyl flavones were found in leaves. The 2"-O-rhamnosides were present in higher amounts than the corresponding C-glycosides. The highest total content of proanthocyanidins was found in roots, followed by stems and leaves.[18]



Isovitexin

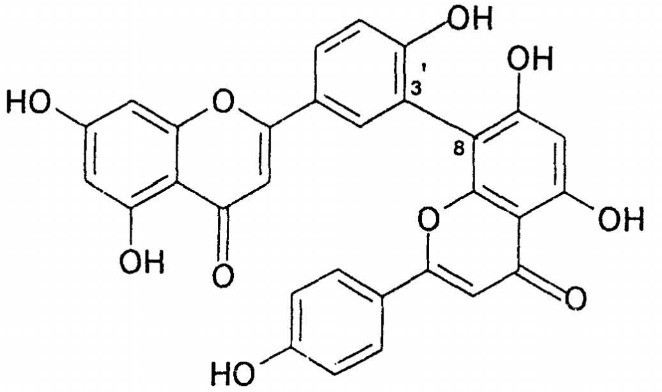


Orientin



$$\mathrm{G= -\overset{}{C}H\text{-}(CHOH)_3\text{-}\overset{}{C}H\text{-}CH_2OH}$$ (with $\mathrm{-O-}$ bridging the two CH)

Amentoflavone, a biflavonoid was isolated from the roots of *Biophytum sensitivum* by Bucar and co-workers.[19] Its isolation represents the first finding of a biflavonoid in the family Oxalidaceae. Amentoflavone has been reported to possess anti-inflammatory effects *in vivo,* [20,21] in addition *in vitro* studies have revealed activity of amentoflavone in processes with relevance to inflammation.[14,22-24] In order to evaluate a further possible mechanism of action for the anti-inflammatory activity of amentoflavone it was tested for inhibition in cyclooxygenase COX-I and cyclooxygenase COX-2 catalysed prostagladin biosynthesis by *in vitro* assays. It was shown to be a selective inhibitor of COX-I catalysed prostaglandin biosynthesis with an IC 50 value 12.4 μM (Std: indomethacin, IC 50=1.1 μM). Doses of up to 37μM showed only a slight inhibition in the corresponding COX-2 assay. Quantification of amentoflavone was carried out by reversed phase HPLC in methanolic and aqueous extracts of the roots, stems and leaves. Highest amounts of amentoflavone were detected in methanolic extracts of roots and stems (0.26-0.35%) while considerably lower amounts were detected in the corresponding water extracts.[19,25]



Amentoflavone

Jachak and co-workers[26] evaluated the anti-inflammatory activities of aqueous and methanol extracts of aerial parts, an aqueous extract of roots as well as ultra filtration fractions of a methanol extract of roots of *Biophytum sensitivum*, in the carrageenin induced rat paw oedema model. All the extracts except the methanol extract of aerial parts exhibited anti-inflammatory activity, but inhibition of oedema was found to be maximum with aqueous extracts.

Paul and co-workers[27] put forward a novel report on non-legume nodule system in *Biophytum sensitivum.* The bacteria in the nodules of *Biophytum sensitivum* belong to the genus *Rhizobium* and utilize fructose, arabinose, mannitol, xylose, glucose, sucrose, lactose, galactose, maltose and sodium citrate. They produce exopolysaccharides and was also found to be acid producers and fast growers.

Puri[28] studied the insulinotropic activity of the leaf extract of *Biophytum sensitivum.* An acute effect of the extract on fasting plasma glucose (fpg) levels and serum insulin response was examined in non-diabetic and alloxan diabetic rabbits. The results prove that the plant material has significant hypoglycaemic effect, which is possibly due to pancreatic beta cell stimulating action.

III.4 MATERIALS AND METHODS

Plant Material

The fresh plants of *Biophytum sensitivum* were collected from the Calicut University Campus in August 2002 and the plant material was identified by Dr. A.K. Pradeep, Department of Botany, Calicut University. A voucher specimen has been deposited in the specially maintained Herbarium of the Department of Chemistry at Calicut University (voucher No.41). The plants were air-dried for one month at room temperature (30°C) and powdered using an electric grinder.

Essential oil Extractions

The air-dried and powdered *B. sensitivum* (250 g) were steam distilled for 2 h. The essential oil was extracted with diethyl ether (2 x 100 ml) and dried over anhydrous sodium sulphate. After removal of the sodium sulphate and evaporation of the ether, a yield of 0.01 g (0.004% of the fresh weight of sample) pale yellow-green essential oil was obtained.

Olfactoric Evaluations

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

Gas Chromatography

GC analysis was 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, one 30 m x 0.32 mm bonded non polar FSOT-RSL-200 fused silica (film thickness 0.25 μm, Biorad Co.) and another 30 m x 0.32 mm bonded polar stabil wax (film thickness 0.50 μm; Restek Co.) were used. Carrier gas used was hydrogen. Injector temperature: 250°C, detector temperature: 320°C. Temperature programme was 40°C/ 5 min to 280°C/ 5 min with a heating rate of 6°C/ min. Quantifications were done by Percent Peak Area calculations (non polar column). Some single components could be identified by co-injection of pure compounds and correlation of their retention times (using Kovats indices) with published data.[29-32]

Gas Chromatography – Mass Spectrometry

The sample was analysed by GC-MS system also. Shimadzu GC-17A with QP 5000 and the data system Compaq-ProLinea (Class 5k-software) Shimadzu GC-17A with QP 5050 and data system Pentium-II (Böhm Co., Class 5k-software) Hewlett-Packard GC-HP 5890 with HP 5970 MSD and PC-Pentium (Böhm Co., Chem station-software and Finnigan MAP GCQ with data system Gateway-2000-PS75 (Siemens Co., GCQ-software) were used for analysis.

Carrier gas used: helium, injection temperature: 250°C, interface-heating: 300°C, ion-source heating: 200°C, EI mode, scan range: 41-450 amu. For compound identifications Wiley, NBS and NIST library spectra (on-line) as well as reference MS spectral data were used.[31-41]

III.5 RESULTS AND DISCUSSION

The essential oil of air-dried *Biophytum sensitivum* plant from southern India was olfactorically evaluated as spicy, earthy-woody (direction of mushrooms and cedar wood), tobacco and leather-like, floral (direction of bergamote and lavender), fatty-fruity and sweet-creamy (vanilla-note).

Using gas chromatographic spectroscopic systems 68 components could be identified in this sample with benzene derivatives, such as 1,4-dimethoxybenzene (24.9%), 1, 2-dimethoxybenzene (10.6%) and 2-methoxy-4-methylphenol (3.5%), the monoterpenes (Z)-linalool oxide (8.1%), (E)-linalool oxide (5.2%) and linalyl acetate (3.4%) as well as 1-octen-3-ol (9.5%) and isophorone (3.1%) as main compounds (concentrations higher than 3% calculated as % peak area using GC-FID with a non polar column). The compounds identified and the corresponding concentrations (%) are given in the following Table III.1.

Table III.1: Composition of the essential oil (eo) of air-dried plants of *Biophytum sensitivum* from South India.

Compounds[1]	eo[2]	KI[3]
3-Methyl butanol	tr[4]	733
2-Methyl butanol	tr	737
Hexanal	0.1	778
(E)-2-Hexenal	tr	831
(Z)-3-Hexenol	0.1	845
(E)-2-Hexenol	tr	854
Anisole	0.5	917
α-Thujene	tr	929
α-Pinene	0.1	951
Camphene	0.1	953
β-Pinene	0.1	957
1-Octen-3-ol	9.5	964
Sabinene	tr	975
6-Methyl-5-hepten-2-ol	2.5	992
Octanal	tr	1001
p-Cymene	0.1	1012
Limonene	0.1	1023
β-Phellandrene	tr	1028
1,8-Cineole	0.1	1030
(E)-Linalool oxide (furanoid)	5.2	1061
(Z)-Linalool oxide (furanoid)	8.1	1064
1-Nonen-3-ol	tr	1068
Linalool	1.9	1085

Nonanal	tr	1087
β-Phenylethyl alcohol	tr	1107
Isophorone	3.1	1121
4-Ketoisophorone	1.6	1143
Camphor	0.6	1146
(Z)-3-Nonen-1-ol	1.7	1156
1,2-Dimethoxybenzene	10.6	1168
(Z)-Linalool oxide (pyranoid)	2.3	1171
Terpinen-4-ol	1.6	1174
Menthol	0.1	1176
1,3-Dimethoxybenzene	0.2	1178
α-Terpineol	1.3	1185
2-Methoxy-4-methylphenol	3.5	1187
1,4-Dimethoxybenzene	24.9	1191
Safranal	0.4	1197
Decanal	0.2	1206
Geraniol	tr	1253
Linalyl acetate	3.4	1258
Decanol	0.7	1269
para-Menth-1-en-7-al	1.2	1276
Safrole	0.6	1285
Thymol	1.3	1289
Carvacrol	0.3	1298
Eugenol	2.1	1358
Dihydro eugenol	0.3	1369
1,1-Dimethoxy decane	2.3	1379
α-Copaene	tr	1399

Methyl eugenol	0.4	1407
Carvone hydrate	2.4	1422
β-Caryophyllene	tr	1424
α-Ionone	0.2	1429
(E)-β-Farnesene	tr	1448
Isoeugenol	0.4	1452
Decanol	0.7	1468
β-Ionone	1.0	1486
δ-Cadinene	tr	1505
6-Methyl-α-ionone	0.2	1522
Eugenyl acetate	0.3	1524
(Z)-Nerolidol	0.3	1536
(E)-Nerolidol	0.2	1551
Dimethyl ionone	0.1	1566
Caryophyllene oxide	tr	1574
τ-Cadinol	tr	1649
(E, E)-Farnesol	tr	1733
Phytol	0.8	2080

[1] in order of their elution

[2] concentrations as % peak area calculated by GC-FID analysis

[3] Kovats indices from apolar FSOT-RSL column

[4] not detected

[5] trace compound (less than 0.1%)

The gas chromatographic-spectroscopic data was correlated with olfactometric data and it was found that eugenol derivatives and 1,4-dimethoxybenzene were responsible for the spicy aroma impressions (direction of clove and fennel), while earthy-woody odour notes from 1-octen-3-ol, isophorone and partly from linalool oxide. Isophorone and 2-methoxy-4-methylphenol imparted tobacco and leather-like odour to the oil. The floral aroma (direction of bergamote and lavender) can be attributed to linalool and its derivatives, such as (Z)- and (E)- linalool oxide as well as linalyl acetate. 1,4-Dimethoxybenzene, 1,2-dimethoxybenzene, 2-methoxy-4-methylphenol and isophorone possess fatty-fruity and sweet-creamy odour notes and were therefore responsible for these aroma impressions of the *Biophytum sensitivum* sample. 1,4-Dimethoxybenzene had a warm-herbaceous and sweet, medicinal odour and the medicinal note of the oil can be attributed to the presence of this compound.[42-46]

1,4-Dimethoxybenzene is found to be the major component present in the volatiles of lotus flower. The content of this compound increases day by day during anthesis and contribute to the pollination of the lotus flower as insect attractants. It is found to be one of the significant components responsible for the scent of the flower[47]. Hence, it can be suggested that the pollination of *B. sensitivum* also is aided by the presence of 1,4-dimethoxybenzene, as it acts as an insect attractant .

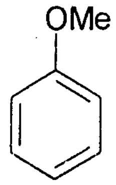
Referring to the biological activities of the identified constituents of the essential oil of air-dried *Biophytum sensitivum*, phenolic compounds as well as linalool and its derivatives are known for their antimicrobial effects. In addition, linalool and its derivatives should be mentioned for their mild sedative and spasmolytic activity.[48-52] Therefore, the essential oil of air-dried *Biophytum sensitivum* may be used as natural drug against convulsions, inflammatory tumours, burns, fevers and insomnia.

1,2-Dimethoxybenzene, isophorone and 2-methoxy-4-methylphenol are some of the main compounds present in *B. sensitivum* essential oil, with sweet-creamy-fatty notes (vanilla-coconut-and hazelnut-notes) and it can be possibly used in food flavouring (candies, non-alcoholic beverages and bakeries[45]) industry, but needs testing for non toxicity.

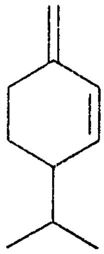
In relation to perfumery it can be stated that the essential oil of air-dried *Biophytum sensitivum* could be useful in perfume industry when earthy-woody and sweet-creamy fatty odour notes are required (Those odour notes are recommended in deodorants, soaps, perfumes and lotions of any type).

In summary, the essential oil from air-dried *Biophytum sensitivum* possesses a characteristic pleasant odour, with benzene derivatives (1,4-dimethoxybenzene, 1,2-dimethoxybenzene, 2-methoxy-4-methylphenol), monoterpenes (linalyl acetate, (Z)-and (E)- linalool oxide), 1-octen-3-ol and isophorone as main compounds. These constituents are especially responsible for the complex odour impression, a possible use as natural drug against different diseases, for the flavoring of various foods as well as aroma-intense agents in some perfumery and cosmetic products.

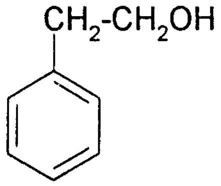
The structures of the identified compounds of *Biophytum sensitivum* (structures given in earlier chapters are omitted) are given below.



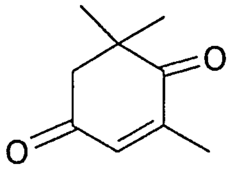
Anisole



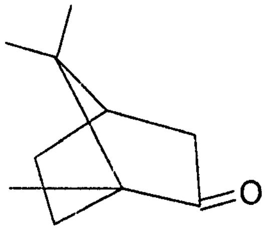
β-Phellandrene



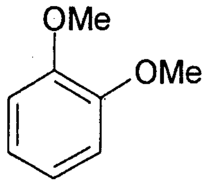
β-Phenylethyl alcohol



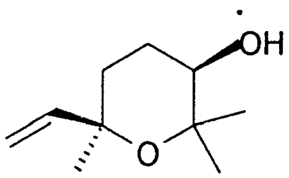
4-Ketoisophorone



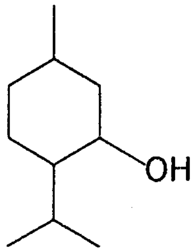
Camphor



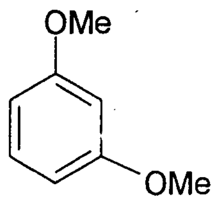
1,2-Dimethoxybenzene



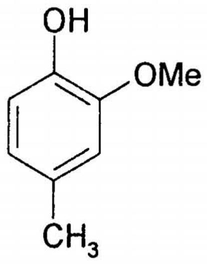
cis-Linalool oxide



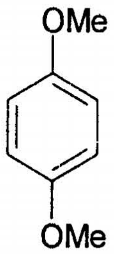
Menthol



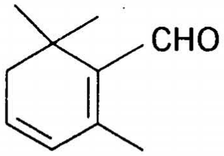
1,3- Dimethoxybenzene



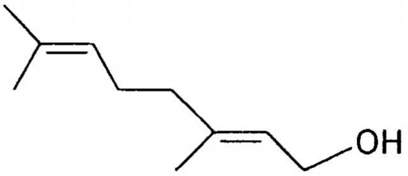
2-Methoxy-4-methyl phenol



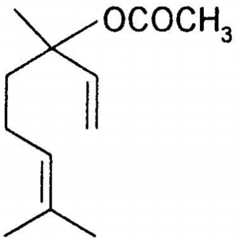
1,4- Dimethoxybenzene



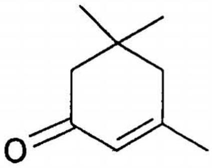
Safranal



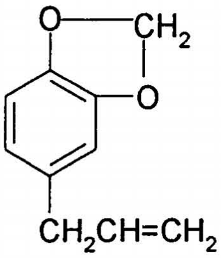
Geraniol



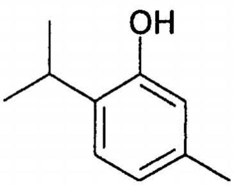
Linalyl acetate



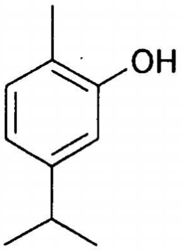
Isophorone



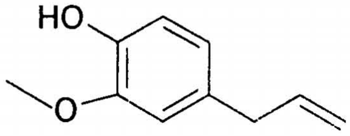
Safrole



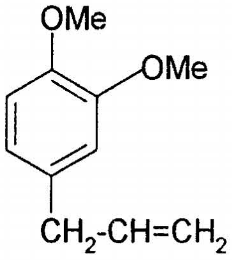
Thymol



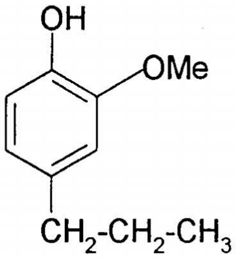
Carvacrol



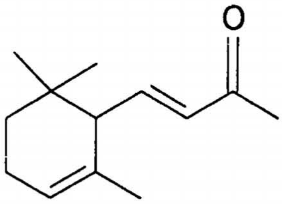
Eugenol



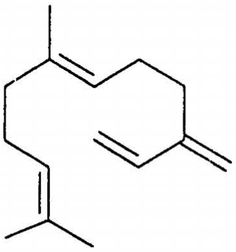
Methyl eugenol



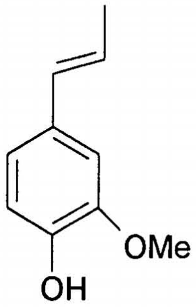
Dihydroeugenol



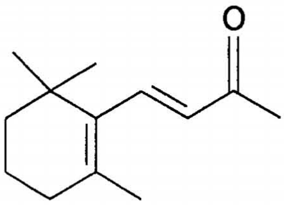
α-Ionone



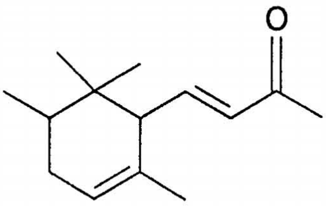
(E)-β-Farnesene



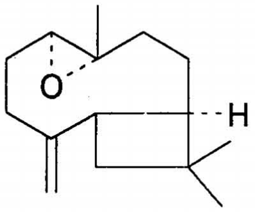
Isoeugenol



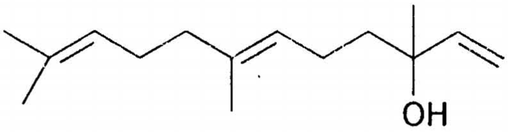
β-Ionone



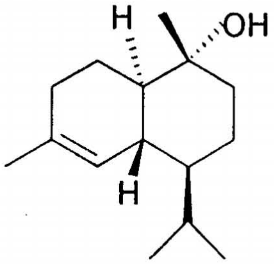
6-Methyl-α-ionone



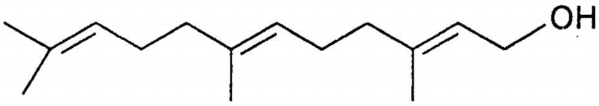
Caryophyllene oxide



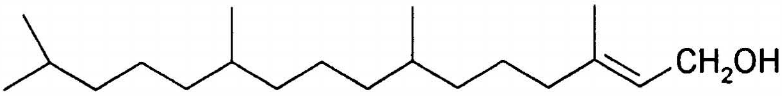
Nerolidol



T-Cadinol



(E, E)-Farnesol



Phytol

SECTION 2: PHYTOCHEMICAL STUDIES ON *BIOPHYTUM SENSITIVUM* (L.) DC.

III.6 Introduction

Biophytum sensitivum (L.) DC, native to the tropical areas of south Asia and Africa, is used in traditional medicine, for various purposes as seen in section I. The aim and scope of the present work is to investigate further on this and isolate the compounds responsible for it. Only two compounds could be successfully isolated from this.

III.7 MATERIALS AND METHODS

The plant material used in this investigation was collected during August 2002, from the Calicut University Campus and was identified by Dr. A.K. Pradeep, Dept. of Botany, Calicut University.

Experimental set up for tlc, column chromatography, IR spectra and mass spectra were the same as that described in chapter I. ^{1}H NMR spectra were recorded using Bruker spectrometer at 400 MHz in $CDCl_3$ using TMS as internal standard. The melting points of all the crystalline isolates were determined using Toshniwal Capillary Melting Point Apparatus.

III.8 Extraction, fractionation and isolation of compounds from *Biophytum sensitivum*

III.8.1. Fractionation of the petroleum ether extract of *Biophytum sensitivum*

Dried and finely powdered plant tissue (2 kg of whole plant) was extracted thrice with petroleum ether (3 x 2.5L; 60-80°C). The combined extract was then concentrated under reduced pressure to get 8 g of viscous dark material, which was then subjected to column chromatography (3 cm x 100cm; d x l) over silica gel (60-120 mesh). The column was developed using petroleum ether and eluted with solvents of increasing polarity (petroleum ether, petroleum ether – ethyl acetate). Several 100 ml portions were collected and each fraction was checked by tlc. Identical portions were pooled together, and the solvent removed under reduced pressure. The compounds isolated from petroleum ether extract are given in Table III. 2.

Table III.2. Compounds isolated from petroleum ether extract of *Biophytum sensitivum*

Fractions	Eluent composition	Compounds
1	Petroleum ether	S_1
2	7:1 Petroleum ether- ethyl acetate	S_2

III.8.2. Isolation of compounds from different chromatographic fractions of petroleum ether extract

Fraction 1, obtained on elution with petroleum ether was colourless, on tlc analysis in petroleum ether and cyclohexane revealed the presence of two compounds. It was then filtered, washed with cyclohexane and recrystallised from ethyl acetate gave 100 mg of compound S_1, m.p. 77°C.

Fraction 2, obtained on elution with 7:1 mixture of petroleum ether-ethyl acetate, gave pale green solid. It was washed with cyclohexane and the solid formed was found to be the same as S_1.

III.8.3. Fractionation of the Ethyl acetate extract of *Biophytum sensitivum*

The residue after petroleum ether extraction was subjected to extraction with cold ethyl acetate twice. The ethyl acetate extract, on evaporation, yielded dark green coloured solid, insoluble in petroleum ether. It was washed repeatedly with petroleum ether and then boiled with it and filtered. The solid residue, after 2 days, gave dark glittering flakes. These brown flakes were found to be insoluble in cold ethyl acetate, but soluble in hot ethyl acetate. So it was heated with ethyl acetate and allowed to cool. Brown solid formed was filtered off and the yellow filtrate, on tlc analysis showed the presence of two compounds. It was adsorbed on silica gel (150 g, 60-120 mesh) and taken in a chromatographic column and eluted with 8:1 mixture of petroleum ether-ethyl acetate. Several 100 ml fractions were collected and each fraction was checked by tlc. Identical fractions were collected together and solvent removed under reduced pressure. Compounds isolated from it are given in Table III.3.

Table III.3. Compounds isolated from ethyl acetate extract of *Biophytum sensitivum*

Fractions	Eluent composition	Compounds
1	8:1 Petroleum ether- ethyl acetate	S_3
2	"	S_4

III.8.4. Isolation of compounds from different chromatographic fractions of ethyl acetate extract

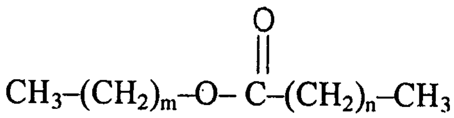
Fraction 1, obtained on elution with 8:1 mixture of petroleum ether-ethyl acetate, on evaporation, gave white powdery solid S_3, with m.p. 85°C.

Fraction 2, obtained on elution with 8:1 mixture of petroleum ether – ethyl acetate, on evaporation yielded white crystalline solid S_4 with m.p. 75°C.

III.9 RESULTS AND DISCUSSION

III.9.1. Characterization of S_1

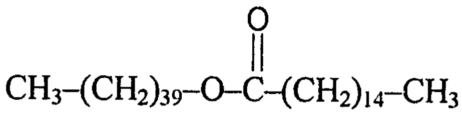
The compound S_1 isolated as described in section III.8.2 was a white crystalline solid having m.p. 77°C. This compound didn't answer Liebermann – Burchard colour reaction, indicating that it was not a triterpenoid or a sterol. It gave a black colour on spraying with H_2SO_4 and strong heating. It moved as a single spot on tlc in petroleum ether and cyclohexane. Absence of decolourisation with dilute $KMnO_4$ solution and bromine water showed its saturated nature. On spraying with Borche's reagent, the compound gave an orange red colour, indicating the presence of $>C{=}O$ group. IR spectrum showed absorption due to –C–H bending (2918.7, 2849.2 cm^{-1} and 1472.8, 1464.1 cm^{-1} respectively). A strong absorption at 1738.1cm^{-1} suggested the presence of ester group. A long chain hydrocarbon nature was indicated by the absorptions at 731.1 and 719.5 cm^{-1}. The absorption at 1172.9cm^{-1} can be attributed to C–O stretching. Mass spectrum of this compound showed M^+ at m/z 816 and base peak at m/z 57. Fragmentation pattern with a regular difference of 14 mass units showed its straight chain saturated hydrocarbon nature. The molecular ion peak at m/z 816 suggested the molecular formula of the compound as $C_{56}H_{112}O_2$, and can be represented as follows.



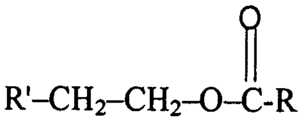
$$CH_3-(CH_2)_m-O-\overset{\overset{O}{\|}}{C}-(CH_2)_n-CH_3$$

The prominent peaks at m/z 257, 285, 313 can be accounted as follows.

When m=39, n=14

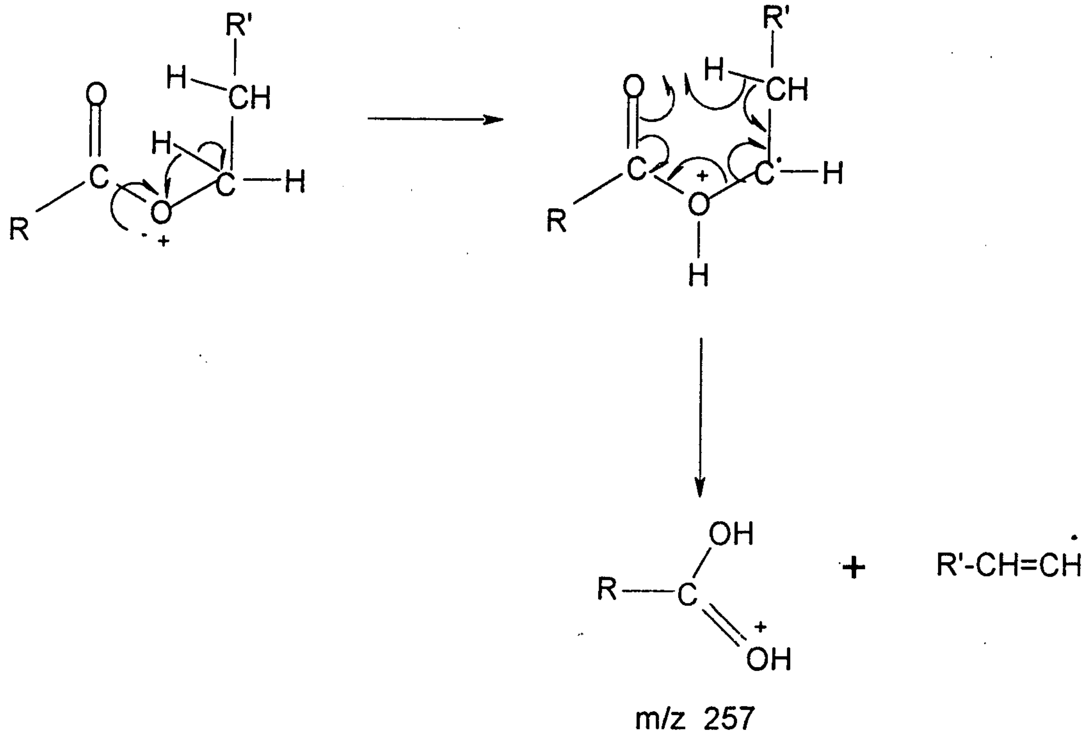


$$CH_3-(CH_2)_{39}-O-\overset{\overset{O}{\|}}{C}-(CH_2)_{14}-CH_3$$



$$R'-CH_2-CH_2-O-\overset{\overset{O}{\|}}{C}-R$$

Where $R = C_{15}H_{31}$ and $R' = C_{36}H_{73}$



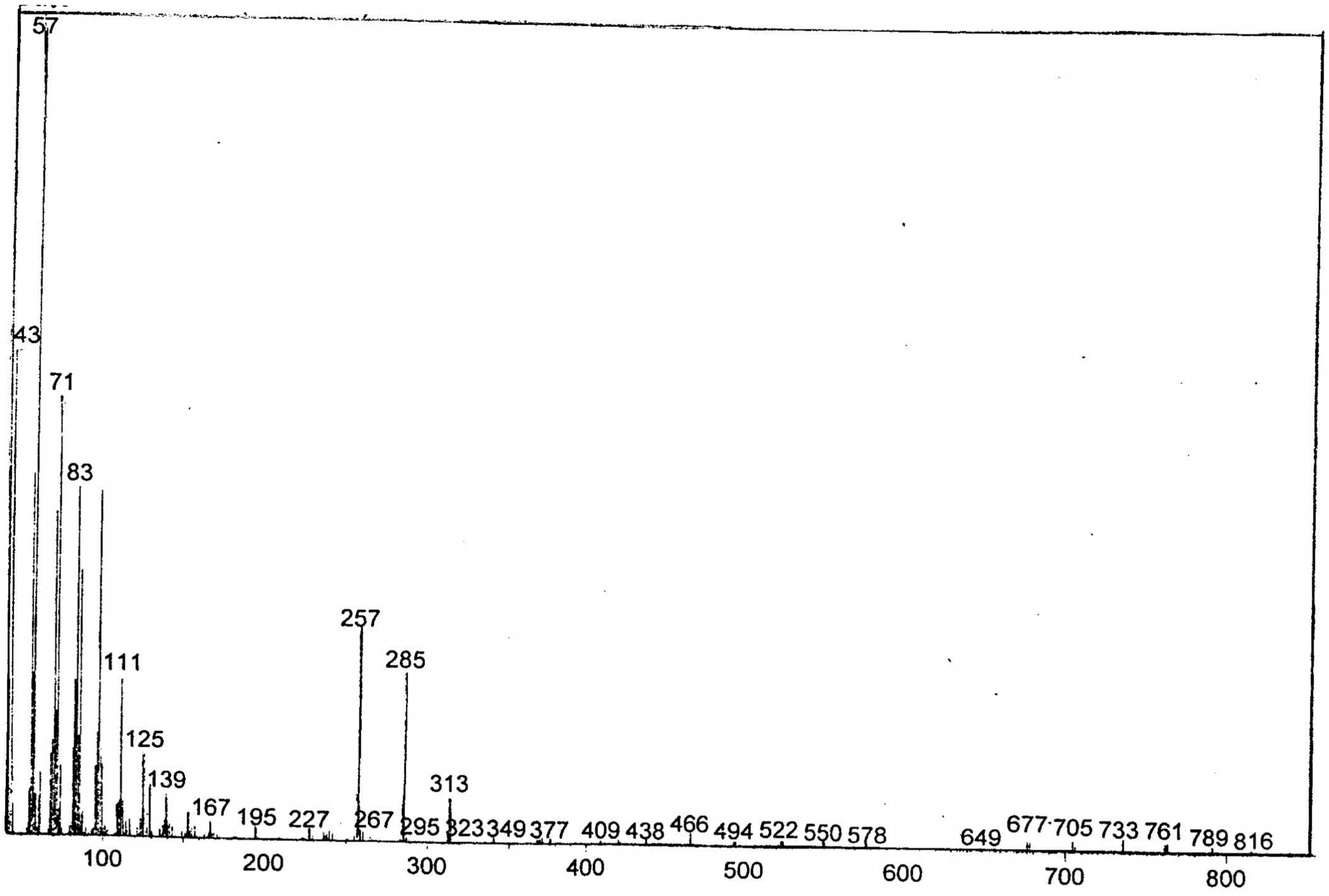
similarly, when

m = 37 n = 16 m/z becomes 285

m = 35 n = 18 m/z becomes 313

m = 33 n = 20 m/z becomes 341

Fragmentation pattern showed difference of 14 mass units up to m/z 649, but peaks at 578, 377 did not conform to those of fragment ions but are indicative of a mixture of compounds. Due to the paucity of the sample further analysis could not be done.



Mass spectrum of S_1 [Mixture of esters]

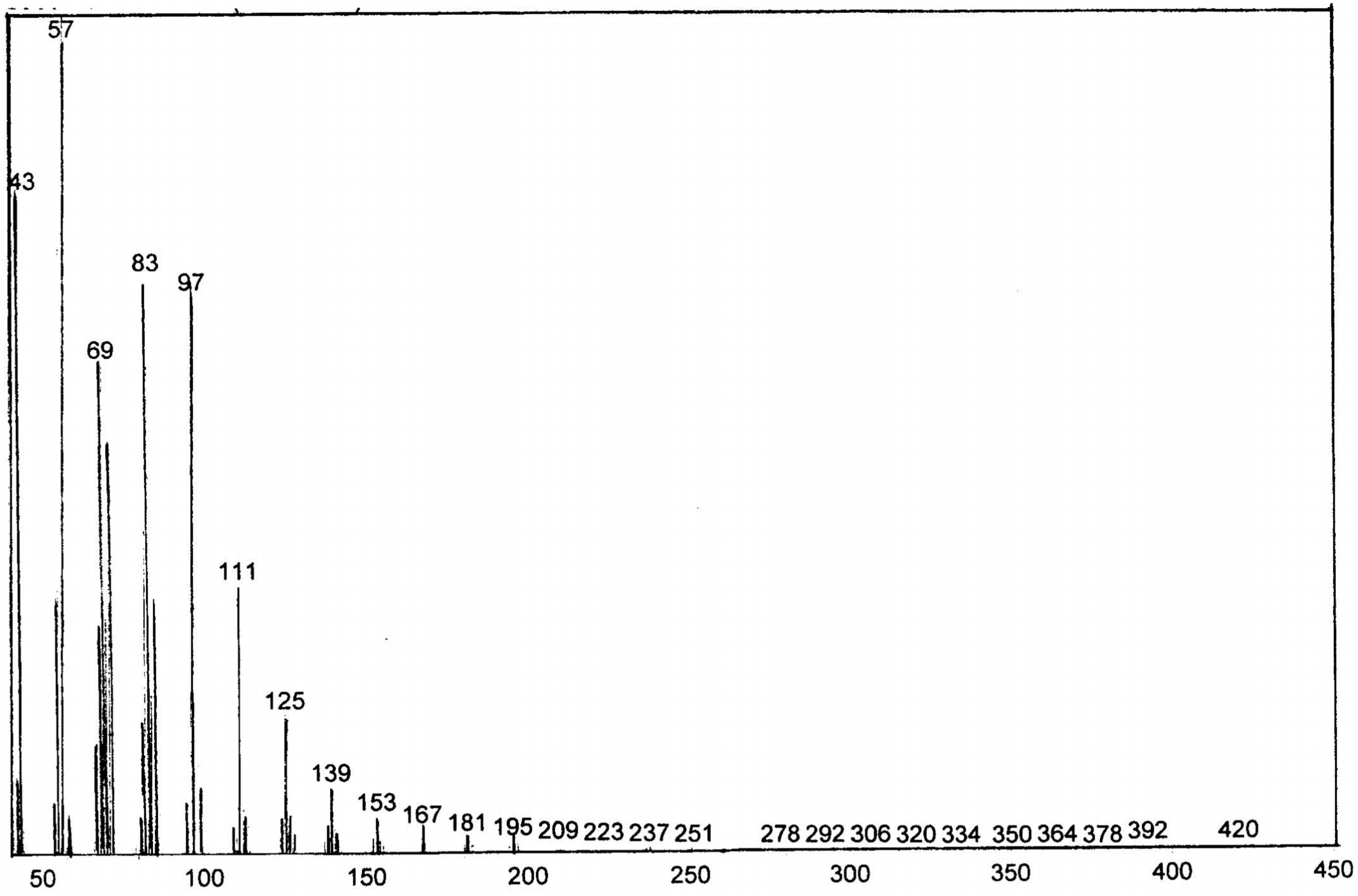
III.9.2. Characterization of S_3

The compound S_3 isolated as described in section II.2.4, was white powdery solid with m.p. 85°C. Mass spectrum showed molecular ion peak at m/z 475. Infrared spectrum showed absorption due to C–H stretching and –C–H bending (2918.7, 2849.2 cm^{-1} and 1472.8, 1464.1 cm^{-1} respectively). Absorptions at 1736.1 cm^{-1} and 1718.8 cm^{-1} suggested the presence of ester group and keto group. Due to the insolubility of the sample in almost all solvents, its NMR spectrum could not be taken, and the structural elucidation was not possible.

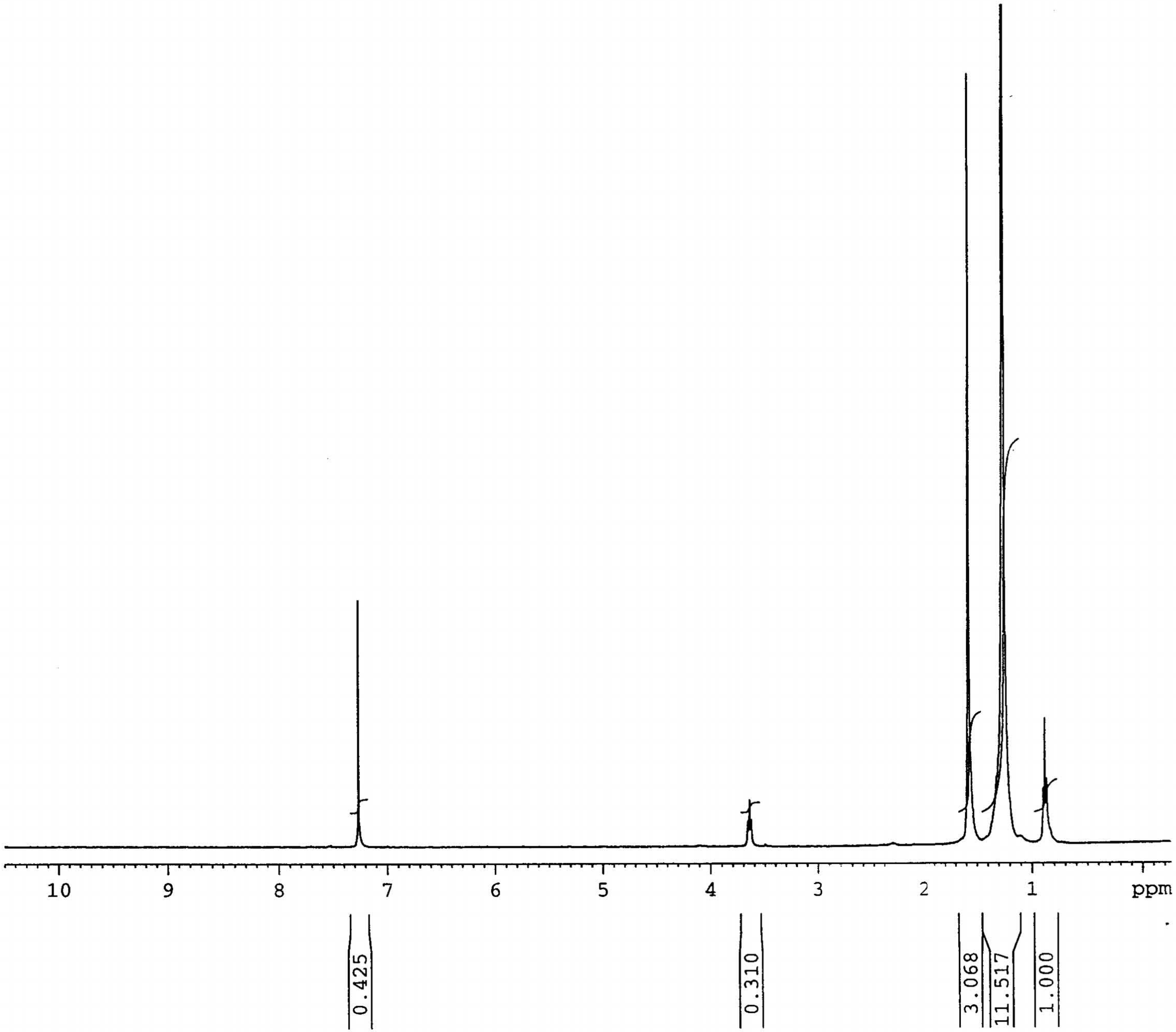
III.9.3. Characterization of S_4

The compound S_4 isolated from the ethyl acetate extract, on elution with 8:1 mixture of petroleum ether-ethyl acetate, as white crystalline solid (100 mg) melted at 75°C. It answered negatively towards Liebermann – Burchard reagent for triterpenoids and sterols. The IR spectrum indicated the presence of a hydroxyl group (Broad absorption with maximum at 3450 cm^{-1}, –O–H stretching) and 1062.9cm^{-1} (C–O stretching). Absorptions at 732cm^{-1} and 719.5 cm^{-1} showed its long chain hydrocarbon nature. The presence of a primary hydroxyl group was also evident from the triplet at δ 3.64 in the ^1H NMR spectrum, and the ^1H NMR absorption in the range δ 0.86 – 1.32 confirmed the straight chain nature. The mass spectrum of S_4 had molecular ion peak at m/z 420. This corresponds to $(M-18)^+$ peak of triacontanol ($C_{30}H_{61}OH$). Fragmentation pattern with regular difference of 14 mass units suggested its saturated straight chain nature.

But the peaks at m/z 334 and 251 did not follow the regular fragmentation pattern and is indicative of a mixture of compounds. The reported melting point of triacontanol was 88 ^0C [53] and the melting point of S_4 was found to be 75^0C, showing the presence of a mixture of alkanols, of which triacontanol is one of the components.



Mass spectrum of S_4 [Mixture of alkanols]



^{1}**H-NMR spectrum of S_4 [Mixture of alkanols] (400 MHz, $CDCl_3$, TMS)**

REFERENCES

1. The Wealth of India, Raw Materials, Vol. II-B. New Delhi: CSIR, 1988, p.151.

2. The Wealth of India, Raw Materials Vol.I. New Delhi: CSIR, 1948, p.187.

3. Marles, R.J., Farnsworth, N.R., *Phytomedicine* **2**, 1995, 137.

4. Parrotta, J.A., Healing Plants of Peninsular India. New York: CABI Publishing, 2001, p.557.

5. Heywood, V.H., In: Blütenpflanzen der Welt. Basel: Birkhäuser–Verlag, 1982, 209.

6. Neuwinger, H.D., African Traditional Medicine. Stuttgart: Medpharm Scientific Publishers, 2000, p.69.

7. Kirtikar, K.R., Basu, B.D., Indian Medicinal Plants. Vol.I. Dehra Dun: Bischensingh, Mahendrapalsingh, India, 1984, p.441.

8. Parrotta, J.A., Healing Plants of Peninsular India. New York: CABI Publishing, 2001, p.558.

9. Puri, D., Baral, N., *Indian J. Physiol. Pharmacol.* **42**, 1998, 401.

10. Seal, S.N., Sen, S.P., *Plant and Cell Physiology* **11(1)**, 1970, 119.

11. Bucar, F., Jachak, S.M., Kartnig, T., Schubert – Zsilavecz, M., *Pharmazie* **53(9)**, 1998, 651.

12. Lin, Y.L., Wang, W.Y., *Chinese Pharm. J.* **55**, 2003, 71.

13. Hayashi, T., Sawa, K., Kawasaki, M., Arisawa, M., Shimizu, M., Morita, N., *J. Nat. Prod.* **51**, 1988, 345.

14. Huguet, A.I., Manez, S., Alcaraz, M.J., Z. *Naturforsch* **45C**, 1990. 19.

15. Blazso, G., Gabor, M., Rohdewald, P., *Pharmazie* **52**, 1997, 380.

16. Bucar, F., Jachak, S.M., Kartnig, T., Noreen, Y., Bohlin, L., Schubert-Zsilavecz, M., In: International Symposium on Bioassay Methods in Natural Products Research and Drug Development, Abstracts volume, Uppsala, 1997, p.49.

17. Noreen, Y., Separation and Bioassay in Identification of Cyclooxygenase Inhibitors from Plants, with Emphasis on Flavonoids, Thesis, Acta Universitatis, Upsaliensis, Uppsala, 1997.

18. Bucar, F., Jachak, S.M., Kartnig, T., Schubert-Zsilavecz, M., *Pharmazie* **53(9)**, 1998, 652.

19. Bucar, F., Jachak, S.M., Noreen, Y., Kartnig, T., Perera, P., Bohlin, L., Schubert-Zsilavecz, M., *Planta Med.* **64(4)**, 1998, 373.

20. Gambhir, S.S., Goel, R.K., Gupta, D.G., *Indian J. Med. Res.* **85**, 1987, 689.

21. Della, L.R., Sosa, S., Tubaro, A., Morazzoni, P., Bombardelli, E., Griffini, A., *Fitoterapia* **67**, 1996, 257.

22. Tordera, M., Ferrandiz, M.L., Alcaraz, M.J., *Z. Naturforsch* **49C**, 1994, 235.

23. Alcaraz, M.J., Mas, J.A., Ubeda, A., Manez, S., *Planta Med.* **57**, Supplement: 2A, 1991, p.110.

24. Amellal, M., Bronner, C., Briancon, F., Haag, M., Anton, R., Landry, Y., *Planta Med.* **51**, 1985, 16.

25. Ravishankara, M.N., Pillai, A.D., Padh, H., Rajani, M., *J. Planar Chromatogr.* **16**, 2003, 201.

26. Jachak, S.M., Bucar, F., Kartnig, T., *Phytotherapy Research* **13(1)**, 1999, 73.

27. Paul, S., Bhowmik, G., Baruah, P., Roy, M.K., *Journal of the Assam Science Society* **37(3)**, 1995, 185.

28. Puri, D., *Journal of Ethnopharmacology* **78(1)**, 2001, 89.

29. Davies, N.W. J., *Chromatogr.* **503**. 1990, 1.

30. Kondjoyan, N., Berdaque, J.L., Compilation of Relative Retention indices for the Analysis of Aromatic compounds. Edition du Laboratorie Flaveur, Saint Genes Champanelle, 1996.

31. Jennings, W., Shabamoto, T., Qualitative Analysis of Flavour and Fragrance Volatiles by Glass Capillary Gas Chromatography. New York: Academic Press, 1980.

32. Jirovetz. L., Private retention time library.

33. Joulian, D., Konig, W.A., The Atlas of spectral Data of sesquiterpene Hydrocarbons. Verlag Humburg 1998.

34. Mc Lafferty, F.W., Staufer, D.B., The Wiley NBS Registry of Mass spectral Data. New York: John Wiley, 1989.

35. Schmaus, G., Untersuchungen Uber die Zusammenset Zung der atherischen Wurzelole Verschiedener mittelund Westeuro Paischer Peudecanum Palustre (L) Moench and Peucedanum lancifolium Lange. Ph.D. Thesis, University of Wuerzburg, 1998.

36. Cornu, A., Massot, R., Compilation of Mass Spectral Data Vol.I and II. London: Heyden and Sons, 1975.

37. Eight Peak Index of Mass Spectra, Vol.I and II. Mass Spectrometry Data Centre (distribution) Reading, 1970.

38. Stenhagen, E., Abrahamson, S., Mc Lafferty, F.W., Registry of Mass Spectral Data, Vol.I-IV. New York: John Wiley, 1974.

39. Private Mass Spectra Libraries.

40. Adams, R.P., Identification of essential oil components by gas chromatography / quadrupole mass spectroscopy. Carol Stream: Allured Publishing Corp., 2001.

41. Pfleger, K., Maurer, H.H., Weber, A., Mass Spectral and GC Data of Drugs, Poisons, Pesticides, Pollutants and Their Metabolites. Weinheim: Wiley-VCH, 2000.

42. Arctander, S., Perfume and Flavor Chemicals. New York: Arctander Publications, 1969.

43. Bauer, K., Garbe, D., Surburg, H., Common Fragrance and Flavour Material. Weinheim: VCH-Verlagsges. m.b.H., 1997.

44. Fazzalari, F.A., Compilation of Odor and Taste Threshold Values Data. Philadelphia: American Society for Testing and Materials, Data series 48d, 1978.

45. Furia, T.E., Bellanca, N., Fenaroli's Hand book of Flavour Ingradients. Cleveland: CRC Press, 1975.

46. Sigma-Aldrich., Flavours and Fragrances, The Essence of Excellence. Milwaukee: Sigma – Aldrich Co., 2001.

47. Omata, A., Yomogida, K., Nakamura, S., Ohta, T., Izawa,Y., In: *Proce. 11th Int. Congress of essential oils, Fragrances and Flavours*, Vol. IV, New Delhi: Oxford & IBH, 1989, p.43.

48. Buchbauer, G., Jager, W., Jirovetz., L., Ilmberger, J., Dietrich, H., Therapeutic properties of Essential Oils and Fragrances. In: Bioactive Volatile Compounds from Plants, ACS-Symposium-Series 525. Washington: American Chemical Society, 1993, 159.

49. Buchbauer, G., Jirovetz, L., Jager, W., Plank, C. Dietrich, H., Fragrance Compounds and Essential Oils with Sedative Effects upon Inhalation. *J. Pharm. Sci.* **82**, 1993, 660.

50. Buchbauer, G., Jirovetz, L., Aromatherapy – Use of Fragrances and Essential Oils as Medicaments. *Flavour Frag. J.* 9, 1994, 217.

51. Jirovetz, L., Buchbauer, G., Fleischhacker, W., Ngassoum, M.B., Yonkeu, S., Analysis of the Essential Oil of Stems and Leaves of *Diplolophium africanum.* Turcz. from Cameroon. *Acta Pharm.* Turcica, **40**, 1998, 185.

52. Jirovetz, L., Buchbauer, G., Fleischhacker, W., Analysis of the Essential Oil of the Leaves of the Medicinal Plant *Chenopodium ambrosioides var. anthelminticum* (L.) A. Gray from India. *Sci. Pharm.* **68**, 2000, 123.

53. John, A.M., Alcohols-Their Chemistry, Properties and Manufacture. New York: Reinhold Book Corporation, 1968, p.192.

ANISOCHILUS CARNOSUS

CHAPTER IV

SECTION 1: ANALYSIS OF THE ESSENTIAL OIL VOLATILES OF *ANISOCHILUS CARNOSUS* WALL. SPIKES, USING GC-FID, GC-MS AND OLFACTOMETRY

IV.1 Introduction

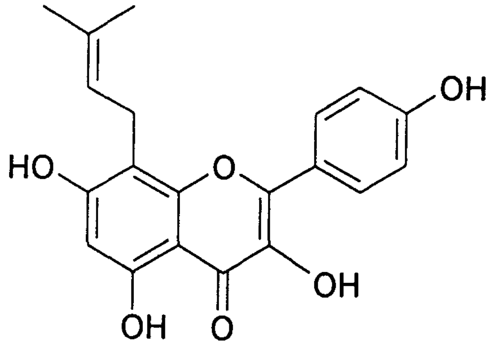
Anisochilus carnosus (L.f) Wall. ex Benth., Syn. *Lavandula carnosa* (L.f)., belongs to Lamiaceae (Labiatae) family which consists of about 200 genera and 3200 species. Lamiaceae family is also known as aromatic or mint family. Economically this family is important as a source of volatile aromatic essential oils and garden ornamentals. Some of them important for essential oils are, salvia, lavender, rosemary and mint. In addition to many of the above, others serve as important culinary herbs valued for the flavour or aroma imparted to food.[1] *Anisochilus carnosus* is an erect, annual herb up to 60cm tall, with stout, quadrangular stems, often tinged with a red colour. It is found almost throughout India, in dry, rocky habitats, ascending to an altitude of 2400m in the western Himalayas. Leaves are 2.5-6.3cm long, and fruits are smooth and compressed. *A. carnosus* is known on the peninsular India under various common names, such as thick-leaved 'lavender' (English), 'Padukurkka' (Malayalam), 'Karpuravalli' (Tamil), 'Sitaki' (Hindi)[2]. This plant is used in Indian folk medicine especially for its stimulant, expectorant and diaphoretic effects.[3-8]

The juice of the fresh leaves of the plant, is credited with cooling properties and is used to relieve urticaria associated with liver disorders and other allergic manifestations. With sugar-candy, it is used as a reputed domestic remedy for coughs and cold. Mixed with sugar and gingelly oil, it is extensively used as a cooling liniment for the head. In Siddha, the leaf is used for treating cough, digestive disorders and dropsy. The leaves can be eaten as vegetable. In Bihar, the herb is used to dress wounds. The reddish brown oil, obtained by steam distillation of the leaves of *A. carnosus*, is found to have muscle relaxant action and also antibacterial and fungicidal properties. The plant is used as a cure for cold and fever.[9-13]

IV.2 Previous work

Subramanian and co-workers[12] reported the isolation of luteolin and apigenin from the leaves of *Anisochilus carnosus.*

Ramani and co-workers[14] isolated 3, 5, 7, 4'- tetrahydroxy-8-isoprenyl flavonoid from the ethyl acetate extract of the aerial parts of the plant.



3, 5, 7, 4'- tetrahydroxy-8-isoprenyl flavonoid

Senatore and co-workers[15] studied the composition and antibacterial activity of the essential oil of the aerial parts of *A. carnosus* obtained by hydrodistillation. They identified 28 compounds and found that oil contained carvacrol (27.9%), camphor (14.1%) and α-cis-bergamotene (10.2%) as the most abundant components. The oil showed activity against gram positive and gram negative bacteria.

The anti-anaphylactic effect of the essential oil of *A. carnosus* was tested by Sirsi and co-workers[10]. The results showed that *A. carnosus* might be useful in intestinal manifestations of allergic and anaphylactic disorders.

IV.3 Present work

The objective of the present work is to identify the volatiles of the essential oil of *Anisochilus carnosus* spikes and to discuss the constituents responsible for the characteristic aroma impressions of this oil, their biological effects, as well as applications in perfumery and cosmetics.

IV.4 EXPERIMENTAL

The spikes of *A. carnosus* were collected from the neighbouring village of Calicut University Campus in December 2002. The plant material was identified by Dr. A.K. Pradeep, Department of Botany, Calicut University, Kerala. A voucher specimen (No.40) has been deposited in the specially maintained herbarium of the Department of Chemistry at Calicut University.

Essential oil Extraction

The fresh spikes (1.8 kg) of *Anisochilus carnosus* were cut into pieces and ground by means of an electric grinder, into a paste, which was steam distilled for 2 h. The distillate was extracted with diethyl ether (2x100 ml) and dried over anhydrous sodium sulphate. After evaporation of the solvent, 0.36g (0.02% of the fresh weight) of an orange-red essential oil was obtained.

Olfactoric Evaluation

The essential oil of *Anisochilus carnosus* spikes was olfactorically evaluated by professional perfumers.

Gas Chromatography

GC analysis was 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, one 30m x 0.32mm bonded non polar FSOT-RSL-200 fused silica (film thickness 0.25µm, Biorad Co., Germany) and another 30m x 0.32mm bonded polar Stabilwax (film thickness, 0.50 µm, Restek Co., USA) were used. Carrier gas used was hydrogen. Injector temperature 250°C, detector temperature 320 °C. Temperature programme was 40°C/5min. to 280°C/5 min. with a heating rate of 6°C/min. Quantifications were done by Percent Peak Area Calculations (non polar column). Some single components could be identified by co-injection of pure compounds and correlation of their retention times (using Kovats indices) with published data.[16-22]

Gas Chromatography-Mass Spectrometry

The sample was analysed by GC-MS system also. Shimadzu GC-17A with QP 5000 and the data system Compaq-Pro Linea (Class 5k- software), Hewlett-Packard GC-HP 5890 with HP 5970 MSD and PC-Pentium (Bohm Co., ChemStation-Software) and Finnigan MAT GCQ with data system Gateway 2000-PS 75 (Siemens Co., GCQ-Software) were used for analysis.

Carrier gas used: helium, injection temperature: 250°C, interface-heating: 300°C, ion-source-heating: 200°C, EI mode: 70eV, scan range: 41-450 amu. The columns were 30m x 0.32mm bonded FSOT-RSL-200 fused silica, with a film thickness of 0.25µm (Biorad Co., Germany) and 30m x 0.32mm bonded polar Stabilwax with a film thickness of 0.50µm (Restek Co., USA). For compound identifications Wiley, NBS and NIST library spectra as well as reference MS spectra data were used.[18, 19, 21-29]

IV.5 RESULTS AND DISSCUSSION

The essential oil was olfactorically analysed as follows. The essential oil was diluted with dichloromethane (1:10) and 10µl of the sample was placed on a commercial odour strip (Dragoco Co., Germany) and was evaluated by professional perfumers as herbal, spicy, warm-woody and weak fatty.

Using gas chromatographic-spectroscopic systems, hundred and eleven components could be identified in the spike essential oil of *Anisochilus carnosus*. The main compounds (concentrations higher than 2% calculated as percent peak area of GC-FID analyses) were the sesquiterpenes such as caryophyllene oxide (18.7%), β-caryophyllene (4.8%), (Z)-nerolidol (4.5%), β-eudesmol (4.4%), caryophyllene alcohol (2.5%), γ-eudesmol (2.3%) and (E, E)-farnesol (2.3%) and some monoterpenes such as eugenol (2.8%), linalool (2.5%), geraniol (2.4%), and geranyl acetone (2.1%) as well as nonanol (2.7%), (Z)-Jasmone (2.2%) and benzyl alcohol (2.1%). The compounds identified, their concentration (%), and the odour elicited by them are given in the Table IV.1

Table IV.1

Composition of the essential oil of *Anisochilus carnosus* spikes from southern India

Compounds[1]	Con.[2]	KI[3]	Aroma impression[4]
3-Methyl butanol	tr[5]	733	fusel-like
Hexanal	0.3	778	green-fatty
(E)-2-Hexenal	0.1	831	green-vegetable
(Z)-3-Hexenol	0.1	845	fresh-green
(E)-2-Hexenol	tr	854	green-leafy
Hexanol	0.2	859	alcoholic-herbal
Heptanal	tr	883	fatty-nutty
α-Thujene	0.1	944	warm-herbal
α-Pinene	0.2	951	piney
β-Pinene	0.1	957	piney-woody
Heptanol	1.4	960	herbal
1-Octen-3-ol	0.1	964	mushroom-like
6-Methyl-5-hepten-2-one	0.3	968	herbal green
(E)-3-Hexenyl acetate	tr	982	green
3-Octanone	1.1	984	herbal
Myrcene	tr	987	sweet-balsamic
2-Octanone	0.6	991	herbal
(E)-2-Hexenyl acetate	tr	995	green
Hexanoic acid	0.3	999	cheesy-fatty
Octanal	tr	1001	fatty, citrus-note
p- Cymene	0.1	1012	weak citrus-note
Limonene	0.1	1023	citrus-like, herbal
(Z)-β-Ocimene	tr	1027	warm-herbal

Benzyl alcohol	2.1	1030	faint aromatic
(E)-β-Ocimene	tr	1033	herbal, spicy
(E)-Linalool oxide (furanoid)	1.1	1055	floral
Octanol	0.8	1062	fatty, citrus-note
(Z)-Linalool oxide (furanoid)	0.7	1064	floral
(Z)-2-Nonen-1-ol	0.1	1068	waxy, melon-note
Terpinolene	0.1	1080	plastic-note
Linalool	2.5	1085	floral
Nonanal	0.2	1087	fatty, citrus-note
2-Nonanone	0.5	1090	fruity, floral, herbal
Phenylethyl alcohol	1.1	1107	floral, rose-like
Citronellal	0.7	1135	fresh, lemon-like
Nonanol	2.7	1158	fatty, fruity
Borneol	tr	1169	herbal-woody
Terpinen-4-ol	0.8	1174	peppery, woody
Verbenone	tr	1177	spicy, minty
α-Terpineol	0.1	1185	floral, liliac-like
Dihydrocarveol	0.6	1189	minty
Dodecane	0.2	1200	weak fatty
Linalyl formate	1.2	1214	green, herbal
Citronellol	1.5	1225	rose-like
Nerol	tr	1229	floral
Neral	tr	1235	floral
Geraniol	2.4	1253	floral, rose-like
Decanol	1.3	1258	floral, fruity, waxy
(E)-2-Decenol	0.4	1262	orange-like, fatty
Geranial	tr	1265	floral
6-Undecanol	0.4	1281	lemon-note, sweet

Nonanoic acid	0.9	1288	fatty, cheesy
Eugenol	2.8	1358	spicy, clove-like
α-Cubebene	tr	1362	weak woody
Undecanol	0.3	1364	citrus-like, sweet
Geranyl acetate	tr	1379	floral, fruity
4,6-Dimethyl dodecane	0.2	1384	weak waxy
(E)-Jasmone	0.3	1390	floral, jasmine-like
(Z)-Jasmone	2.2	1393	floral, jasmine-like
α-Copaene	tr	1399	woody
Tetradecane	0.7	1400	weak fatty
β-Cubebene	tr	1402	woody, herbal
β-Elemene	0.2	1410	sweet-woody
β-Bourbonene	tr	1414	woody, spicy
β- Caryophyllene	4.8	1424	green-woody spicy
Aromadendrene	0.7	1427	woody
α- Ionone	0.8	1430	floral, violet-like
(E)-α-Bergamotene	tr	1435	warm woody
(E)-β-Farnesene	tr	1448	sweet-warm-mild
Geranyl acetone	2.1	1451	rose-like, green
α-Guaiene	0.1	1455	sweet, woody
α-Humulene	0.3	1462	woody
(E)-2-Dodecenal	0.6	1466	herbal, floral
Germacrene D	tr	1468	dry woody, spicy
Alloaromadendrene	tr	1474	weak woody
β-Ionone	0.2	1487	balsamic-floral
(E, E)-α-Farnesene	tr	1490	mild-sweet-warm
2-Tridecanone	1.9	1496	warm-herbal-spicy

Pentadecane	0.7	1500	weak fatty
δ-Cadinene	0.3	1515	dry woody, spicy
γ-Cadinene	0.3	1522	warm-woody
(Z)-Nerolidol	4.5	1536	green-fruity-waxy
(E)-Nerolidol	1.2	1551	apple-like, green
Spathulenol	0.8	1553	weak woody
Caryophyllene alcohol	2.5	1557	spicy
Longifolene	0.1	1566	woody
Tridecanol	1.5	1571	sweet-fruity
Caryophyllene oxide	18.7	1574	spicy-woody note
Guaiol	1.2	1584	balsamic-woody
Hexadecane	1.4	1600	weak fatty
Humulene oxide	0.9	1603	weak woody
Cedrol	0.2	1609	balsamic-woody
γ-Eudesmol	2.3	1631	sweet-woody
β-Eudesmol	4.4	1647	sweet-woody
τ-Cadinol	1.2	1649	woody
α-Cadinol	0.5	1652	spicy
α-Eudesmol	0.3	1654	sweet-woody
β-Bisabolol	0.1	1663	warm-woody
Tetradecanol	1.4	1672	weak fruity, fatty
2-Pentadecanone	1.3	1680	herbal-spicy
Heptadecane	0.9	1700	weak fatty
α-Guayl acetate	tr	1727	balsamic-woody
(E, E)-Farnesol	2.3	1733	sweet-green-floral
Lanceol	1.0	1761	mild-woody
γ-Eudesmyl acetate	0.3	1784	woody
β-Eudesmyl acetate	1.2	1792	woody

α-Eudesmyl acetate	0.2	1795	woody
Octadecane	0.3	1800	weak fatty
Haxadecanol	0.9	1873	floral-waxy
Abietatriene	0.3	2057	weak woody
Phytol	0.6	2080	floral-balsamic

[1] in order of their elution

[2] concentrations as %-peak-area calculated by GC-FID analysis

[3] Kovats indices from unpolar FSOT-RSL column

[4] aroma impressions from published data elsewhere [30-35]

[5] trace compound (less than 0.1%)

The correlation of the olfactoric data [30-35] with gas chromatographic – spectroscopic data revealed that the herbal scent of the essential oil of *A. carnosus* spikes were due to 2-tridecanone (1.9%), 2-pentadecanone (1.3%), 3-octanone (1.1%), heptanol (1.4%), minor esters, mono and sesquiterpenes. The spicy notes observed of this oil can be attributed to caryophyllene oxide (18.7%), β-caryophyllene (4.8%), caryophyllene alcohol (2.5%) and eugenol (2.8%).

The woody aroma-notes of the oil is the combined contribution of caryophyllene oxide (18.7%), β-caryophyllene (4.8%), β-eudesmol (4.4%) and γ-eudesmol (2.3%).

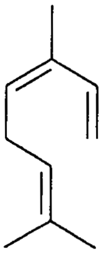
(Z)-nerolidol (4.5%), nonanol (2.7%), decanol (1.3%), hexadecane (1.4%), tetradecanol (1.4%) are responsible for the characteristic fatty-waxy notes of the essential oil.

Some major and minor sesquiterpene components of *Anisochilus carnosus* spike essential oil, such as caryophyllene, nerolidol, eudesmane, farnesene and cadinene derivatives as well as monoterpenes eugenol, linalool, geraniol, pinene and terpinene derivatives are known for their antimicrobial effects.[36-47] Moreover, linalool and geraniol in medium concentration (2.5% and 2.4% respectively) showed mild sedative and spasmolytic activities.[36-38] Earlier works reported the antibacterial activity of *A. carnosus* essential oil.[15] Caryophyllene oxide is well-known for its antifungal activity.[48] Since the *A. carnosus* spike essential oil contained a high proportion of this compound, it may be used as an antifungal agent to protect plants against fungal diseases.

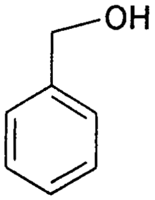
The predominant constituents of the oil, especially sesquiterpenes such as caryophyllene oxide, β-caryophyllene, caryophyllene alcohol and nerolidols, find wide use in food flavouring (candies, non-alcoholic beverages and bakeries[33]) industry. The herbal-spicy top-notes of the essential oil of *A. carnosus* spikes may be useful in the flavouring of ready-to-cook food products (e.g.soups and sauces). The woody-herbal spicy aroma-notes of the oil, seemed to be of interesting application in perfumery and cosmetics as odour systems in soaps, shower products, lotions and skin-care-products. Oil of *Anisochilus carnosus* is used as an ingredient in anti-dandruff hair cleanser, which contains blend of several oils and herbs with powerful therapeutic properties. It stimulates the hair bulbs to enhance hair growth.

It can be concluded that mono and sesquiterpenes, as well as nonanol, (Z)-Jasmone, benzyl alcohol are responsible for the characteristic odour impressions and cited biological activities of the essential oil of fresh spikes of *Anisochilus carnosus*. In relation to perfumery, it can be stated that the spike essential oil of *Anisochilus carnosus* could be useful in the perfume industry when herbal-spicy-woody odour notes are required (These odour notes are recommended in after-shaves, toilet-water etc.)

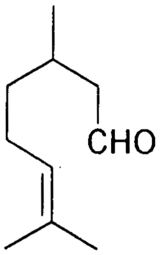
Structures of the identified compounds and not included in earlier chapters are as follows:



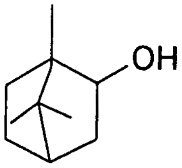
(Z)-β-Ocimene



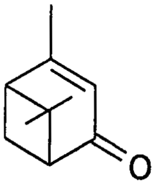
Benzyl alcohol



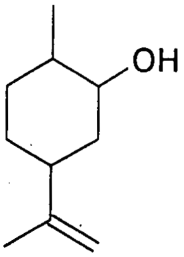
Citronellal



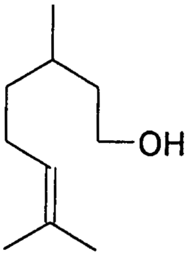
Borneol



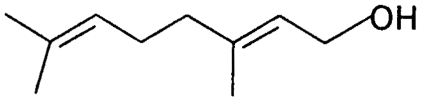
Verbenone



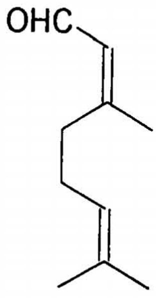
Dihydrocarveol



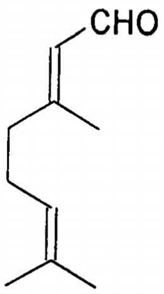
Citronellol



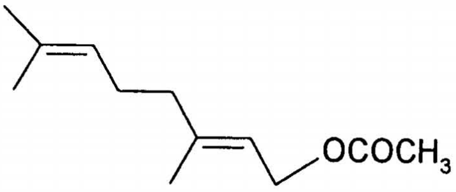
Nerol



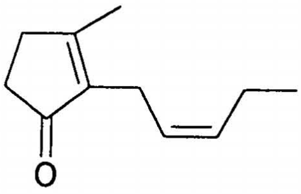
Neral



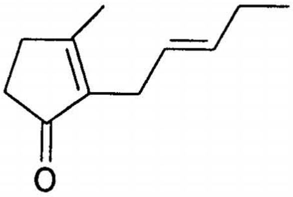
Geranial



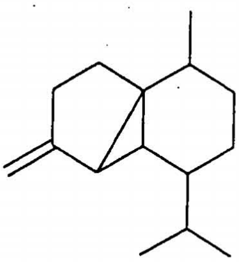
Geranyl acetate



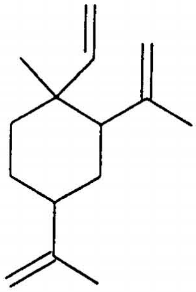
cis-Jasmone



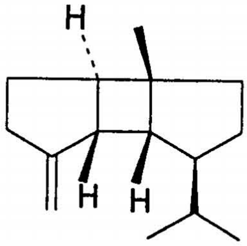
trans-Jasmone



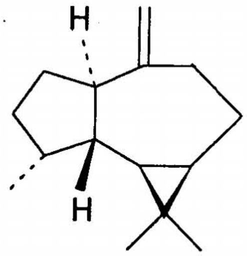
β-Cubebene



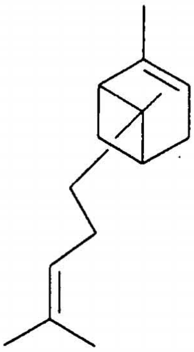
β-Elemene



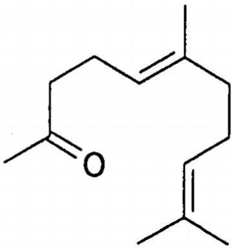
β-Bourbonene



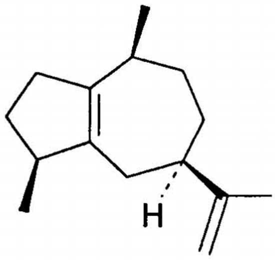
Aromadendrene



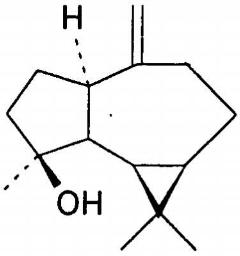
(E)-α-Bergamotene



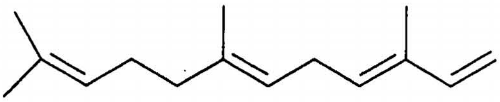
Geranyl acetone



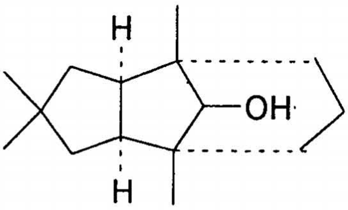
α-Guaiene



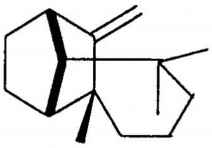
Spathulenol



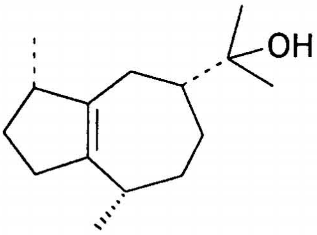
(E, E)- α-Farnesene



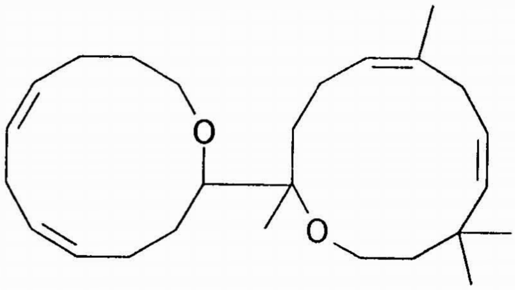
α-Caryophyllene alcohol



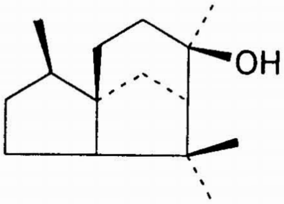
Longifolene



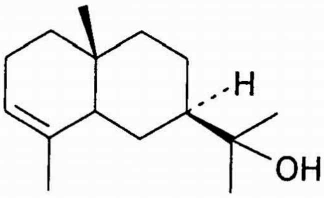
Guaiol



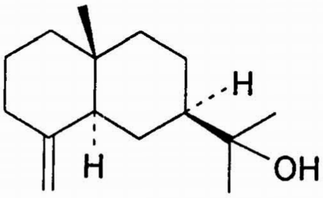
Humulene oxide



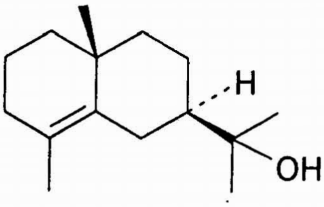
Cedrol



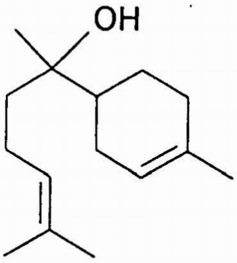
α -Eudesmol



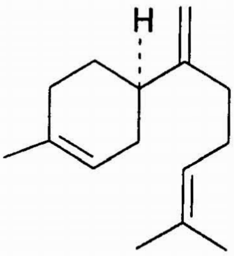
β-Eudesmol



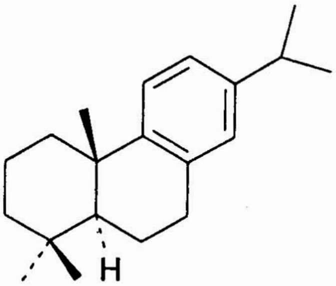
γ- Eudesmol



β-Bisabolol



Lanceol



Abietatriene

SECTION 2: SOLID PHASE MICRO EXTRACTION (SPME) OF *ANISOCHILUS CARNOSUS* WALL. ROOT ESSENTIAL OIL

IV.6 Introduction

Anisochilus carnosus Wall. native to southern Asia from Pakistan to Sri.Lanka and Myanmar (Burma) is used in Indian folk medicine, for various purposes as explained in section 1. Although *Anisochilus carnosus* is known for folk medicinal use since many years, very few works has been so far reported on the chemical composition of this plant and no data about volatiles from *Anisochilus carnosus* roots has been published yet.

In the present study, the aroma compounds of the essential oil of fresh roots of *A. carnosus* as well as four corresponding SPME- samples of the oil (two SPME-headspace samples and the other two SPME-samples obtained by introducing SPME-needle directly into the root essential oil) were analysed by GC-FID, GC-MS and olfactoric methods.

IV.7 EXPERIMENTAL

Plant Material

Fresh roots of *A. carnosus* were collected from Calicut University Campus in December 2003 and the plant material was identified by Dr. A.K. Pradeep, Department of Botany, Calicut University.

Essential Oil Extraction

The fresh roots (300g) of *A. carnosus* were cut into small pieces and ground to a paste using an electric grinder. It was then subjected to steam distillation for 3 h. About 1L of the distillate was collected and extracted with diethyl ether as solvent (2 x 100 ml). The ether portions were pooled together and dried over anhydrous sodium sulphate. Evaporation of the dry ether extract after the removal of sodium sulphate, over a water bath, yielded 0.15g (0.05% of fresh weight of the sample) of a viscous, orange-red essential oil.

In the solid phase micro extraction of the essential oil of *A. carnosus* roots, the oil was deposited in a dark brown 5ml flask, and the aroma compounds extracted with a 2cm-50/30μm Divinyl benzene / Carboxen / polydimethylsiloxane- Stable flex coated glass fiber (Supelco, USA Cat-No.5-7348) for 30 and 60 minutes (SPME-1 and SPME-2) respectively. SPME-samples 3 and 4 were obtained by introduction of the SPME-needle directly into the essential oil for 1 second (SPME-3) and 10 seconds (SPME-4). Immediately after these extraction procedures, the trapped volatiles on the fiber were directly analysed by GC-FID and GC-MS.

IV.8 Solid Phase Micro Extraction (SPME)

Headspace sampling

Beyond the conventional gas chromatographic analysis of gases and low viscosity liquids, some situations are more effectively handled by head-space sampling. This is true only when the vapour above the sample is of interest as with perfumes or food products. In order to obtain the concentration of the volatile components solid phase micro extraction (SPME) is employed. More volatile components can be analysed by this method.

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

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

Olfactoric Evaluations

The *Anisochilus carnosus* root essential oil was olfactorically evaluated by professional perfumers.

GC-FID and GC-MS

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

IV.9 RESULTS AND DISSCUSSION

Anisochilus carnosus root essential oil was olfactorically evaluated as herbal-spicy, sweet-balsamic, warm-woody, aromatic-medicinal-smokey odour with weak green-fresh top-notes as well as weak floral-fruity background notes.

Using GC-FID and GC-MS (both with two columns of different polarity) sixty-nine volatiles could be identified in this sample with 13-C hydrocarbons megastigma-4, 6(Z), 8(E)-triene (26.9%), megastigma-4, 6 (E), 8(E)-triene (7.7%), megastigma-4, 6(E), 8(Z)-triene (6.9%) and megastigma-4, 6(Z), 8(Z)-triene (4.9%), the sesquiterpene hydrocarbons β-gurjunene (10.5%) and α-cadinene (4.2%) as well as the phenolic compound p-vinyl guaiacol (5.2%) as main compounds (concentrations higher than 4%, calculated as % peak area using GC-FID with a non polar column).

One interesting feature of the chemical composition of the *A. carnosus* root essential oil is the presence of all four isomers (E, E; E, Z; Z, E; Z, Z) of the megastigma-4, 6, 8-trienes, with isomers megastigma-4, 6(Z), 8(E)-triene and megastigma-4, 6(E), 8(E)-triene predominating. The megastigma-4, 6, 8-triene nucleus is 1-(but–2-enylidene)-2, 6, 6-trimethylcyclohex-2-ene, and the semitrivial nomenclature is used here to facilitate stereochemical descriptors. All four compounds have been reported previously as important trace aroma components of purple passion-fruit[49] and star fruit[22]. Megastigmatrienes are thought to be derived in nature from β-ionone via the readily dehydrated β-ionol.[49,50] β-Ionone has a well-known floral odour and is derived from β-carotene degradation.[51, 52] It appeared therefore that the carotenoids of *Anisochilus carnosus* roots are the precursors of megastigma-4, 6, 8-trienes.

To get more information on the aroma compounds responsible for the characteristic odour of the essential oil, the volatiles in the headspace of

A. carnosus root essential oil was trapped using SPME-needle and analysed by means of GC-FID and GC-MS. This is logical as any odour is elicited when the concerned compound reaches the olfactory buds in the nose. This is possible only when the compound is volatile. The composition of the two SPME-headspace samples (SPME-1 and SPME-2) is significantly different from that of the essential oil quantitatively, but not qualitatively. The sesquiterpenes β-gurjunene (45.3% and 46.1%) and β-caryophyllene (12.6% and 12.9%) were identified as main compounds of these headspace samples, with megastigma-4, 6(Z), 8(E)-triene (5.3% and 5.4%), geranyl acetate (2.2% and 3.8%), (E)-α-bergamotene (2.3% and 2.9%) and β-elemene (2.2% and 2.3%) in medium amounts.

The SPME-samples of *A. carnosus* with the introduction of the needle directly into the root essential oil (SPME-3 and SPME-4) were found to be rich in megastigma-4, 6(Z), 8(E)-triene (19.9% and 19.2%), β-gurjunene (15.9% and 15.3%), megastigma-4, 6(E), 8(E)-triene (7.0% and 6.3%), megastigma-4, 6(E), 8(Z)-triene (6.2% and 5.5%), α-cadinene (6.1% and 5.2%) and β-caryophyllene (4.9% and 5.2%) and β-elemene (3.6% and 4.0%).

It has been found that 13-C hydrocarbon megastigma-4, 6(Z), 8(E)-triene, is the chief component of *A. carnosus* root essential oil as well as SPME-3 and SPME-4 samples, whereas SPME-headspace samples are found to be rich in sesquiterpene β-gurjunene and the total percentage of sesquiterpene hydrocarbons seemed to increase in the headspace samples of the oil. Compounds of the essential oil of the fresh roots of *A. carnosus* and the corresponding solid phase micro extraction (SPME) samples of the essential oil in the order of their Kovats indices (KI) using non polar column is given in the following Table IV. 2.

Table IV.2

Composition of the essential oil of *Anisochilus carnosus* roots and corresponding SPME- samples from southern India (EO essential root oil, SI-S4 SPME samples)

Compounds[1]	EO[2]	S1	S2	S3	S4	Kl[3]
3-Methyl butanol	tr[5]	nd[6]	nd	nd	tr	733
Hexanal	0.1	0.2	0.3	0.1	0.1	778
(E)-2-Hexenal	tr	0.1	0.2	0.1	0.1	831
(Z)-3-Hexenol	0.4	0.5	0.6	0.3	0.3	845
(E)-2-Hexenol	tr	tr	tr	tr	tr	854
Hexanol	0.2	0.2	0.3	0.1	0.1	859
Heptanal	tr	tr	tr	tr	tr	883
α-Pinene	0.2	0.2	0.3	0.2	0.2	951
β-Pinene	0.1	0.7	0.8	0.2	0.2	957
Heptanol	tr	tr	tr	tr	tr	960
Benzaldehyde	0.1	1.3	0.4	0.2	0.2	962
1-Octen-3-ol	0.3	0.4	0.5	0.3	0.4	964
(E)-3-Hexenyl acetate	tr	tr	tr	tr	tr	982
Myrcene	0.2	0.3	0.4	0.3	0.4	985
(E)-2-Hexenyl acetate	tr	tr	tr	tr	tr	995
Octanal	tr	tr	tr	tr	tr	1001
p-Cymene	0.1	1.5	0.6	0.2	0.3	1012
Limonene	0.3	1.7	0.9	0.4	0.5	1023
(Z)-β-Ocimene	tr	0.4	0.7	0.2	0.5	1027
(E)-β-Ocimene	tr	0.1	0.1	0.1	0.1	1033
1-Nonen-3-ol	0.1	0.2	0.2	0.1	0.1	1068
Terpinolene	nd	tr	tr	tr	tr	1080
Linalool	0.2	0.4	0.5	0.3	0.4	1085
Nonanal	0.1	0.1	0.1	0.1	0.2	1087
Guaiacol	2.3	0.6	0.6	2.6	1.7	1090
Fenchol	0.3	0.4	0.6	0.3	0.4	1117
1,3-Dimethoxybenzene	0.8	2.3	1.5	1.9	1.0	1165

Borneol	tr	tr	tr	tr	tr	1169
Terpinen-4-ol	nd	0.1	0.2	tr	tr	1174
α-Terpineol	0.1	0.2	0.2	0.1	0.1	1185
Fenchyl acetate	0.1	0.3	0.4	0.2	0.3	1220
p-Cumenol	0.2	0.9	1.3	0.6	0.7	1227
Cumin aldehyde	1.3	2.5	1.7	1.4	1.5	1242
Geraniol	0.4	0.7	0.9	0.5	0.6	1253
Anisaldehyde	0.7	2.2	1.4	0.9	1.1	1262
Geranial	tr	tr	tr	tr	tr	1265
p-vinyl Guaiacol	5.2	1.6	1.7	2.5	2.6	1291
Megastigma-4, 6(Z), 8(Z)-triene	4.9	0.2	0.7	1.9	1.4	1318[4]
Megastigma-4, 6(E), 8(E)-triene	7.7	0.9	1.1	7.0	6.3	1328[4]
Megastigma-4, 6(E), 8(Z)-triene	6.9	0.7	1.0	6.2	5.5	1344[4]
Megastigma-4, 6(Z), 8(E)-triene	26.9	5.3	5.4	19.9	19.2	1349[4]
Eugenol	0.8	0.2	0.3	1.5	1.4	1358
α-Cubebene	tr	tr	tr	tr	tr	1362
Geranyl acetate	0.7	2.2	3.8	0.9	1.3	1379
α-Copaene	tr	tr	tr	tr	tr	1399
α-Gurjunene	0.1	0.3	0.7	0.4	0.5	1407
β-Elemene	3.2	2.2	2.3	3.6	4.0	1410
β-Caryophyllene	3.2	12.6	12.9	4.9	5.2	1424
β-Gurjunene	10.5	45.3	46.1	15.9	15.3	1431
(E)-α-Bergamotene	1.9	2.3	2.9	1.7	1.8	1435
(E)-β-Farnesene	tr	tr	tr	tr	tr	1448
α-Humulene	0.3	0.5	0.5	0.3	0.4	1462
Germacrene-D	tr	0.2	0.2	0.1	0.1	1468
γ-Gurjunene	tr	tr	tr	tr	tr	1477
ar-Curcumene	tr	0.2	0.2	0.1	0.1	1481
(E, E)-α-Farnesene	tr	tr	tr	tr	tr	1490
δ-Cadinene	nd	tr	tr	tr	tr	1505

γ-Cadinene	0.8	0.3	0.3	2.9	2.2	1510
(Z)-Nerolidol	2.5	0.9	0.8	2.6	2.6	1536
α-Cadinene	4.2	1.0	1.1	6.1	5.2	1539
(E)-Nerolidol	1.2	0.1	0.2	1.3	1.3	1551
Spathulenol	0.8	0.3	0.4	2.4	1.7	1553
Caryophyllene alcohol	1.5	0.2	0.2	1.7	1.8	1557
Caryophyllene oxide	1.7	0.9	0.9	1.8	1.9	1574
τ-Cadinol	0.2	tr	tr	0.2	0.2	1649
α-Cadinol	0.5	0.1	0.1	0.7	0.9	1652
β-Bisabolol	0.1	tr	tr	tr	tr	1663
(E, E)-Farnesol	2.3	0.2	0.2	0.9	1.2	1733
Phytol	2.6	nd	nd	0.2	0.2	2080

EO essential root oil.

S1 SPME- headspace 30 min.

S2 SPME-headspace 60 min.

S3 SPME essential oil 1 sec.

S4 SPME essential oil 10 sec.

[1] in order of their elution.

[2] concentrations as %-peak-area calculated by GC-FID analysis.

[3] Kovats indices from unpolar FSOT-RSL column (~DB5)

[4] aroma impressions from published data elsewhere.[22, 23]

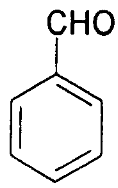
[5] trace compound (less than 0.1%).

[6] not detected.

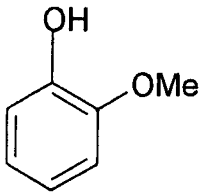
The correlation of the olfactoric data with gas chromatographic-spectroscopic data showed that the olfactoric description of the *A. carnosus* root essential oil was qualitatively and quantitatively more attached to the composition of the SPME-headspace samples and lesser to that one of the SPME-samples with introduction of the needle directly into the oil. The sesquiterpenes β-gurjunene, β-caryophyllene, (E)-α-bergamotene, β-elemene and cadinenes, the aromatic compounds benzaldehyde, 1,3-dimethoxybenzene, guaiacol, p-vinyl guaiacol, p-cumenol, cumin aldehyde and anisaldehyde as well as the monoterpenes β-pinene, myrcene, (Z)-β-ocimene and eugenol are responsible for the complex base-aroma (herbal-spicy, sweet-balsamic, warm-woody and aromatic-medicinal-smokey) of the essential oil. The green-fresh odour of the oil can be attributed to hexane derivatives, limonene, fenchol and fenchyl acetate, whereas the floral-fruity notes are due to megastigma-4, 6, 8-trienes, geraniol and geranyl acetate.[30-33,35]

It can be stated that solid phase micro extraction is an excellent method to trap aroma-active compounds of the essential oil before identification using GC and GC-MS. The SPME-headspace technique is more effective to analyse the aroma-compounds from an essential oil than introducing the needle directly into the oil.

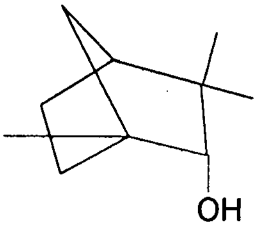
The structures of the identified compounds of *A. carnosus* root essential oil are given below:



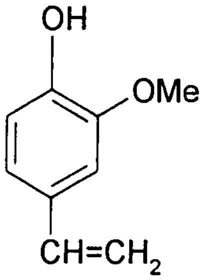
Benzaldehyde



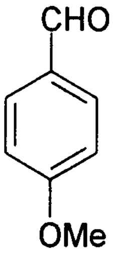
Guaiacol



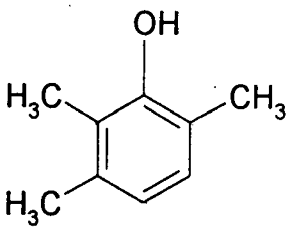
α-Fenchol



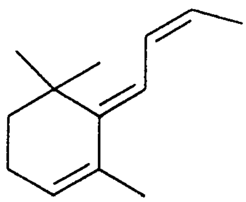
p-vinyl Guaiacol



Anisaldehyde

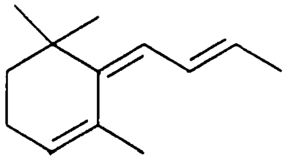


Cumenol



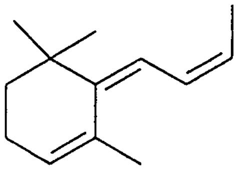
Megastigma

-4, 6(Z), 8(Z)-triene

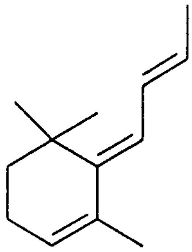


Megastigma

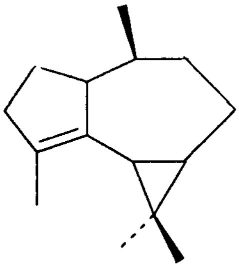
-4, 6(E), 8(E)-triene



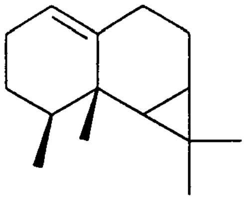
Megastigma-
4, 6(E), 8(Z)-triene



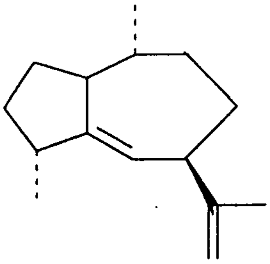
Megastigma-
4, 6(Z), 8(E)-triene



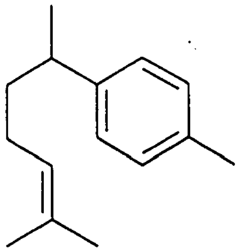
α-Gurjunene



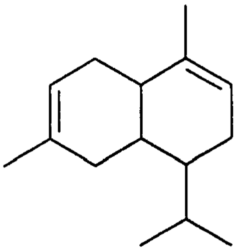
β-Gurjunene



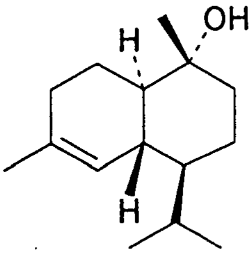
γ-Gurjunene



ar-Curcumene



α-Cadinene



α-Cadinol

10. Sirsi, M., Rao, *Indian J. Med. Res.* **44**, 1956, 283.

11. Sirsi, M., Narayanamurthy, N.L., Shah, C.N., *J. Indian Inst. Sci.* **37A**, 1955, 98.

12. Subramanian, S.S., Nair, A.G.R., *Phytochemistry* **11(1)**, 1972, 452.

13. Jirovetz, L., Buchbauer, G., Shahabi, M., Shafi, P.M., Jose, B., *J. Essent. Oil. Bearing Plants* **6**, 2003, 78.

14. Ramani, V.A., Arunachalam, T., Antony, T.V., Amaladasan, M., *Asian Journal of Chemistry* **14(1)**, 2002, 247.

15. Senatore, F., Lentini, F., Venza, F., Bruno, M., Napolitano, F., *Flavour and Fragrance Journal* **18(3)**, 2003, 202.

16. Davies, N.W., *Chromatogr.* **503**, 1990,1.

17. Kondjoyan, N., Berdaque, J.L., A compilation of Relative Retention Indices for the Analysis of Aromatic Compounds. Edition du Laboratoire Flaveur, Saint Genes Champanelle, 1996.

18. Jennings, W., Shibamoto, T., Qualitative Analysis of Flavor and Fragrance Volatiles by Glass Capillary Gas Chromatography. New York: Academic Press, 1980.

19. Adams, K.P., Identification of essential oil components by gas chromatography/quadrupole mass spectroscopy. USA: Allured Publishing Corp. Carol Stream, 2001.

20. Forsblom, I., Wahlberg, I., Nordfors, K., Eklund, A.M., *Flavour Science and Technology*, Proc.III. Weurman Flavour Research Symposium. Bessiere, Y., Thomas, A.F. (eds). John Wiley & Sons Ltd., Chichester, 1990, 61.

21. MacLeod, G., Ames, J.M., *Flavour Science and Technology*, Proc. III. Weurman Flavour Research Symposium. Bessiere, Y., Thomas, A.F. (eds). John Wiley & Sons Ltd., Chichester, 1990, 81.

22. MacLeod, G., Ames, J.M., *Phytochemistry* **29**, 1990,165.

23. Cornu, A., Massot, R., Compilation of Mass Spectral Data, Vol. I-II, London: Heyden & Sons, 1975.

24. Eight Peak Index of Mass Spectra, Vol. I-II, Mass Spectrometry Data Centre (distribution), Reading, 1970.

25. Mc Lafferty, F.W., Staufer, D.B., The Wiley NBS Registry of Mass Spectral Data, Vol.II. New York: John Wiley, 1989.

26. Schmaus, G., Ph.D.Thesis, University of Wurzburg, Germany, 1988.

27. Stenhagen, E., Abrahamsson, S., Mc Lafferty, F.W., Registry of Mass Spectral Data, Vol.I-IV. New York: John Wiley, 1974.

28. Private Mass Spectra Libraries.

29. Woerdenbag, H.J., Bos, R., Salomons, M.C., Hendriks, H., Pras, N., Malingre, T.M., *Flavour and Fragrance Journal* **8**, 1993, 131.

30. Arctander, S., *Perfume and Flavor Chemicals*. New York: Arctander Publications, 1969.

31. Bauer, K., Garbe, D., Surburg, H., *Common Fragrance and Flavor Materials*. Weinheim: VCH-Verlagsges, m.b.H, 1997.

32. Falazzari, F.A., *Compilation of Odor and Taste Threshold Values Data,* American Society for Testing and Materials. USA: Philadelphia, 1978.

33. Furia, T.E., Bellanca, N., Fenaroli's Handbook of Flavour Ingredients. Cleveland: CRC Press, 1975.

34. Ohloff, G., *Perfumer & Flavourist* 3(2), 1978, 11.

35. Sigma-Aldrich., *Flavors & Fragrances. The Essence of Excellence*, Sigma-Aldrich Co., Milwaukee, 2001.

36. Brud, W.S., Gora, J., *Proc. 11th Int. Congress Essent. Oils, Fragrances and Flavours*, Mohan Primlani, New Delhi: Oxford & IBH Publishing, 1989, p.13.

37. Buchbaur, G., Book of Abstracts, 33rd International Symposium on Essential Oils, Centro de Biotechnologia Vegetal-Fundacao da Faculdade de Ciencias de Lisboa, Lisboa, 2002, p.23.

38. Hansel, R., Atherische Ole-Anspruch und Wirklichkeit, Wiss. Verlagsges, m.b.H, Stuttgart, 1993.

39. Harborne, J.B., Baxter, H., Moss, G.P., Phytochemical Dictionary-A Handbook of Bioactive Compounds from Plants 2nd ed. Taylor and Francis, London, 1999.

40. Kaufmann, P.B., Cseke, L.J., Warber, S., Duke, J.A., Brielmann, H.L., Natural Products from Plants. Boca Raton: CRC Press, 1999.

41. Kubo, I., Muroi, H., Himejima, M., *J. Agric, Food Chem.* **40**, 1992, 245.

42. Lis-Balchin, M., Deans, S.G., Eaglesham, E., *Flavour and Fragrance Journal* **13**, 1998, 98.

43. Nigg, H.N., Seigler, D., Phytochemical Resources for Medicine and Agriculture. New York: Plenum Press, 1992.

44. Rucker, G., *Dtsche. Apoth. Ztg* **113**, 1973, 1291.

45. Schilcher, H., *Dtsche. Apoth. Ztg* **124**, 1984, 1433.

46. Teuscher, E., Biogene Arzneimittel, Wiss. Verlagsges. m.b.H, Stuttgart, 1997.

47. Tringali, C., Bioactive compounds from Natural Sources, Taylor & Francis, London, 2001.

48. Cakir, A., Kordali, S., Zengin, H., Izumi, S., Hirata, T., *Flavour Frag. J.* **19**, 2004, 62.

49. Whitfield, F.B., Sugowdz, G., *Aust. J. Chem.* **32**, 1979, 891.

50. Tressl, R., Engel, K.H., in *Instrumental Analysis of Foods: Recent Progress.* Charalambous, G., Inglett, G. (eds). New York: Academic Press, 1983, p.153.

51. Enzell, C.R., in *Flavour 81.*, Schreier, P.(ed). Berlin: Walter de Gruyter, 1981, p.449.

52. Enzell, C.R., in *Flavour 81.*, Schreier, P.(ed). Berlin: Walter de Gruyter, 1981, p.649.

CHAPTER V

SECTION 1: EVALUATION OF ANTIMICROBIAL PROPERTIES OF *ZANTHOXYLUM RHETSA* SEED ESSENTIAL OIL

V.1 Introduction to Antimicrobial Studies

Microorganisms are universally associated with the lives of humans, other animals and plants. Some of them are beneficial and others are detrimental. Microorganisms play an important role in the food and pharmaceutical industry. They are involved in the making of yogurt, cheese, wine, butter milk and in the production of antibiotics. Baked goods are made using yeast. Sauerkraut, pickles and some sausages also owe their existence to microbial activity. Microorganisms are essential for the digestive process in ruminant animals such as cattle and sheep. Legumes, which live in close association with special bacteria that form nodules on their roots. In these root nodules, atmospheric nitrogen is converted to fixed nitrogen compounds that the plants can use for growth. Besides their role as a beneficiary, microoganisms can cause disease, spoil food and deteriorate materials like iron pipes, glass lenses and wood pilings. There is no field of human endeavour, whether it be in industry or agriculture or in the preparation of food and the combating of disease, where the microbe does not play an important and often dominant role.

Each kind of microorganisms has specific growth requirements. Many of them can be grown in the laboratory culture medium containing necessary nutrients for their growth and multiplication. Some of them require a supply of inorganic salts, particularly the anions, phosphate and sulphate, and the cations sodium, potassium, iron, etc. whereas others can grow in a medium containing organic compounds (amino acids, vitamins or coenzymes) in minute quantities. Some others require complex natural substances (peptone, blood, serum, etc.) and microorganisms like rickettsias cannot be grown in an artificial laboratory medium. On solid culture media, microbial cells can grow and form visible masses called colonies.

BACTERIA

The bacterium is a single-celled organism, that does not have intracellular membrane-bound organelles such as a nucleus, golgi bodies, endoplasmic reticulum or mitochondria. Therefore, the bacterium's essential metabolic and biosynthetic activities must be carried out within the cytoplasm and the cell envelope. Bacteria lack a true nucleus and are classified as prokaryotes. One of the most important cytological features of bacteria is their reaction to a simple staining procedure called the Gram-stain. The procedure involves staining the cells with the dye crystal violet, and a mordant known as Lugol's solution (3% I_2/KI) is added to set the stain. The bacteria are next decolourised with alcohol. Finally, the bacteria are counterstained with safrain. Gram-positive bacteria retain the crystal violet, whereas Gram-negative bacteria, which lose the crystal violet, on counterstaining by safranin appear red in colour.[1] The most plausible explanation for this difference in behaviour lies in the relative differences between the cell walls of the above two types of bacteria.

Bacterial cell wall is made up of peptidoglycan, an insoluble, porous, heteropolymer of alternating N-acetylglucosamine and N-acetyl muramic acid units. The cell wall in Gram-positive bacteria has a relatively thick layer of peptidoglycan 20 to 80 nm across. The peptidoglycan layer is closely attached to outer surface of the cell membrane. Chemical analysis shows that 60 to 90 percent of the cell wall of a Gram-positive bacterium is peptidoglycan. The thick cell walls of Gram-positive bacteria retain such stains as the crystal violet-iodine dye in the cytoplasm.

The cell wall of a Gram-negative bacterium is thinner but more complex than that of a Gram-positive bacterium. Only 10 to 20 percent of the cell wall is peptidoglycan; the remainder consists of various polysaccharides, proteins and lipids. Gram-negative bacteria fail to retain the crystal violet-iodine dye during the decolourising procedure partly because of their thin cell walls and partly because of the relatively large quantities of lipoproteins and lipopolysaccharides in the wall.[2]

V.2 Modes of Action of Antimicrobial Agents

Microorganisms can be inhibited or killed by various physical and chemical agents. The agents that kill or destroy the organisms are referred as 'cidal' whereas the one that merely halts the growth of the microorganism is called ‘static’. If a static agent is removed from a culture, the organism will resume growth, but the effects of cidal agents are irreversible. The manner in which antimicrobial agents inhibit or kill can be attributed to the following kinds of actions.[3]

Several types of chemical agents damage the cell wall by blocking its synthesis. Some of them will disrupt the cell membrane, so that the cell loses its selective permeability and can neither prevent the loss of vital molecules nor bar the entry of damaging chemicals. Some others will inhibit the enzyme action and will damage the microbial life. Chemicals such as strong solvents (alcohols, acid and phenolics) coagulate bacterial proteins; some agents disrupt or denature proteins. Such losses in normal protein function can arrest bacterial metabolism, thereby inhibit the growth or kill them.

Antibiotics

They are chemical substances produced by certain microorganisms that inhibit or kill other microorganisms. An antibiotic that acts on both Gram-positive and Gram-negative bacteria is called a broad-spectrum antibiotic, whereas narrow-spectrum antibiotics, act on only a specific group of organisms. The widespread use of antibiotics has increased the number of pathogenic microorganisms that display antibiotic resistance. Drug resistance, may be due to the pre-existing factor in the microorganism or it may be due to some acquired factors. For example, penicillin resistance may result from the production of penicillinase by resistant organisms, which convert penicillin to inactive penicilloic acid.

FUNGI

Unlike bacteria, which are prokaryotes, fungi are eukaryotes. Each fungus has a golgi apparatus, mitochondria, nucleus, ribosomes, endoplasmic reticulum and a cell membrane, making it difficult to develop antibiotics that are selectively toxic for fungi.

The habitates of fungi are quite diverse. Some are aquatic, living primarily in fresh water, and a few marine fungi are also known. Most fungi however, have terrestrial habitats, in soil or dead plant matter. A large number of fungi are parasites of terrestrial plants. Indeed, fungi cause the majority of economically significant diseases of crop plants.

Fungal cell walls resemble plant cell walls architecturally, but not chemically. Although, cellulose is present in the walls of certain fungi, many fungi have non cellulosic walls. Chitin, a polymer of the glucose derivative, N-acetyl glucosamine, is a common constituent of fungal cell walls. The cell wall is composed of cross-linked polysaccharides, proteins and glycoproteins and it provides the fungus with osmotic stability and rigidity. The polysaccharides, which make up about 80% of the cell wall are polymers of simple sugars and include, chitin, glucan, mannan, etc. Cellulose and chitosan, two other polysaccharides, make the fungal wall resistant to the degradative effects of acids and alkalies. The fungal cell membrane contains ergosterol and zymosterol.

Filamentous fungi are called moulds. Each filament grows mainly at the tip, by extension of the terminal cell. A single filament is called a hypha. Hyphae usually grow together across a surface and form compact tufts; collectively called mycelium. The mycelium arises because the individual hyphae form branches as they grow, and these branches intertwine, resulting in a compact mat.

Fungi can reproduce asexually or sexually. During reproduction, all fungi generate specialized reproductive structures called spores or conidia.

In fungi, the asexual spores arise from a portion of the hyphae are referred to as blastic conidia, e.g., sporangiospores. Sporangiospores form within sac like structures known as sporangia. Sexual spores of fungi are usually resistant to drying, heating, freezing and some chemical agents. Either an asexual or a sexual spore of a fungus can germinate and develop into a new hypha and mycelium.

A major ecological activity of many fungi, is the decomposition of wood, paper, cloth and other products derived from natural sources.

V.3 ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS

Essential oil mainly comprises of mono and sesquiterpenes. Many of the monoterpenes and their derivatives are important agents of insect toxicity. In corn, cotton and other species, certain monoterpenes and sesquiterpenes are produced and emitted only after insect feeding has already begun. These substances attract natural enemies including predatory and parasitic insects that kill plant feeding insects and so help to minimize further damage. Thus volatile terpenes are not only defenses in their own right but provide a way for plants to enlist defensive help from other organisms.

Recently carvone, a monoterpene isolated from the essential oil of *Carum carvi* has shown its ability to inhibit the sprouting of potatoes during storage as well as fungicidal activity in protecting tubers from rooting without exhibiting mammalian toxicity.[4]

Essential oil of *Salvia officinalis* has also shown practical potency in enhancing the storage life of some vegetables by protecting them from fungal rootings.[5] There are several reports on the antifungal activity of essential oils. Essential oils from different plant species are known to exhibit various kinds of biological activities including antifungal, antimicrobial, cytostatic, insecticidal, allelopathic, antioxidant and bioregulatory actions.[6]

The volatile oils of black pepper and clove were assessed for antibacterial activity against twenty-five different genera of bacteria. These included animal and plant pathogens and spoilage bacteria.[7] Essential oils extracted from plants such as mentha, *Lavandula officinalis* and from roots and flowers of radish are known to exhibit antimicrobial activity.[8] The oils from onion and garlic exhibit high activity. Pine oil is known to inhibit various species of fungi and has been used in fungicidal preparations.[9,10]

SECTION 2: ANTIBACTERIAL ASSAY OF *ZANTHOXYLUM RHETSA* SEED ESSENTIAL OIL

V.4 Introduction

Microbiological assays are used for the quantitative determination of antibiotics and inhibitory chemical agents and also the determination of the sensitivity of microorganisms to these agents. The development of bacterial resistance to presently available antibiotics has necessitated the search for new antibacterial agents.[11] The Gram-positive and Gram-negative bacteria can be inhibited by antibiotics, either by blocking the protein synthesis or peptidoglycan synthesis in bacterial cell wall.

The antimicrobial property exhibited by an essential oil is the combined effect of its constituents. The activity of individual components will invariably be different from one another, however, the isolation of each and every component from an essential oil, is practically impossible. In this work, the antibacterial property of the *Zanthoxylum rhetsa* seed essential oil was checked against four bacteria namely *Staphylococcus aureus, Escherichia coli, Proteus vulgaris* and *Klebsiella pneumoniae*. The Gram-positive bacteria, *Staphylococcus aureus* are mainly responsible for post-operative wound infection, toxic shock syndrome and food poisoning. The Gram-negative bacterium *E. coli* is present in human intestine and causes lower urinary tract infection, coleocystis or septicemia. Gram-negative bacteria such as *Klebsiella pneumoniae* cause pneumonia and urinary tract infections. *Proteus vulgaris*, the Gram-negative bacteria, cause wound infections and pneumonia. The *Zanthoxylum rhetsa* seed oil showed significant activity against these organisms probably due to the synergic effect of the components present in the oil.

V.5 WORK REPORTED

The dried, ripe seeds of *Zanthoxylum rhetsa*, on steam distillation yielded an essential oil. The oil is used as an antiseptic and disinfectant. The seeds are also used for the treatment of asthma, bronchitis, tooth ache and rheumatism.[12-14] In the indigenous system of medicine the seed essential oil is used in the treatment of cholera.[14] It has been reported that [15] ethyl acetate and ethyl acetate-methanol (9:1) fraction of *Z. limonella* stem bark extract exhibited significant antimicrobial activity against all tested strains of bacteria, fungus and yeast.

V.6 MATERIALS AND METHODS

Zanthoxylum rhetsa seeds were collected from the Calicut University Campus, and the seed essential oil was extracted by steam distillation from aqueous, alkaline and acidic media, as explained in chapter II. These oil samples were examined for their antibacterial activity against Gram-positive bacterium, *Staphylococcus aureus* and Gram-negative bacteria such as *Escherichia coli, Proteus vulgaris* and *Klebsiella pneumoniae*, cultured at Life Sciences Department, Calicut University. Gentamicin, inhibiting bacterial cell wall biosynthesis, was used as standard for comparison.

MEDIA USED AND THEIR COMPOSITION

1. Nutrient agar

Nutrient agar medium was used for maintaining pure bacterial culture and also to make lawn cultures of bacteria for the study of antibacterial activity.

Peptone : 3 g
Sodium Chloride : 1.5 g
Beef extract : 0.9 g
Agar : 6 g
Distilled water : 300 ml
pH : 7.2 - 7.5

2. Nutrient broth

This was used for making suspension of test bacteria.

Peptone : 0.5 g
Sodium Chloride : 0.25 g
Beef extract : 0.15 g
Distilled water : 50 ml
pH : 7.2 - 7.5

Antibacterial Activity Assay

The antibacterial activity was assayed by 'cut well method'. The essential oils used for screening were dissolved in DMSO, which did not influence the growth of bacteria. Gentamicin was used as a positive control.

The nutrient agar medium was prepared and sterilised by autoclaving at 15 lbs pressure (121°C for 20 min). The medium was poured into sterile petridishes and allowed to solidify. Inoculum was prepared by suspending a loop full of bacterial cultures into about 10 ml of nutrient broth and was incubated at 37° C for 12-14 h. About 0.1 ml of this bacterial suspension was taken and poured into petriplates containing 20 ml nutrient agar medium. Using an L-shaped sterile glass spreader bacterial suspensions were spreaded to get a uniform lawn culture.

The wells of diameter 9 mm (0.9 cm) were dug on the inoculated nutrient agar medium with sterile cork borer and 50 μl of the seed essential oils (at various concentrations) were added in each well. The essential oils of required concentrations 10%, 5% and 1% were prepared by dissolving the oils into appropriate quantities of DMSO, which was used as a negative control. The plates were then incubated at 37°C overnight and examined for zone of inhibition. The diameter of the inhibition zone was measured in mm. The standard antibiotic drug gentamicin was also screened under similar conditions for comparison.

The antibacterial spectra of the *Z. rhetsa* seed essential oil obtained by steam distillation from aqueous, alkaline and acid media, showing zone of inhibition in millimetres, for Gram-positive and Gram-negative bacteria are shown in Table V.1, V .2 and V.3 respectively.

TABLE V.1: Antibacterial activity of *Z. rhetsa* seed essential oil steam distilled from acid medium

Concentration of the oil	Zone of inhibition in mm			
	Gram-positive		Gram-negative	
	Staphylococcus aureus	*Escherichia coli*	*Proteus vulgaris*	*Klebsiella pneumoniae*
100%	47	29	42	37
10%	39	22	26	20
5%	23	19	20	19
1%	19	17	11	15
DMSO (negative control)	Nil	Nil	Nil	Nil
Gentamicin 200 μg (positive control)	25	23	34	23

TABLE V.2: Antibacterial activity of *Z. rhetsa* seed essential oil steam distilled from alkaline medium

Concentration of the oil	Zone of inhibition in mm			
	Gram-positive		Gram-negative	
	Staphylococcus aureus	*Escherichia coli*	*Proteus vulgaris*	*Klebsiella pneumoniae*
100%	33	26	20	27
10%	23	15	15	13
5%	15	12	13	11
1%	13	9	11	9
DMSO (negative control)	Nil	Nil	Nil	Nil
Gentamicin 200 μg (positive control)	25	23	34	23

TABLE V.3 : Antibacterial activity of *Z. rhetsa* seed essential oil steam distilled from aqueous medium

Concentration of the oil	Zone of inhibition in mm			
	Gram-positive		Gram-negative	
	Staphylococcus aureus	*Escherichia coli*	*Proteus vulgaris*	*Klebsiella pneumoniae*
100%	31	27	23	30
10%	23	19	17	16
5%	13	15	13	13
1%	12	13	11	11
DMSO (negative control)	Nil	Nil	Nil	Nil
Gentamicin 200 μg (positive control)	25	23	34	23

V.7 RESULTS AND DISCUSSION

Evaluation of antibacterial activity of the *Z. rhetsa* seed essential oil steam distilled from aqueous, alkaline and acidic media revealed that, the acid medium extract exhibited moderate activity towards the tested microorganisms. The activity of the *Z. rhetsa* seed essential oils differ considerably with the microorganisms used. All the oil samples showed only moderate activity towards the Gram-negative bacteria *Escherichia coli*. Both alkaline and aqueous medium extract showed moderate activity against all the tested microorganisms, at all concentrations and their results were quite comparable. The *Z. rhetsa* seed essential oil obtained by steam distillation from acid medium, was found to be highly active against all the tested bacteria especially with *Staphylococcus aureus, Proteus vulgaris* and *Klebsiella pneumoniae* at a concentration of 100%. Significant activity was also shown by the acid medium extract at a concentration of 10%, 5% and 1%.

The GC-MS analysis of the *Z. rhetsa* seed essential oil obtained by steam distillation from aqueous, alkaline and acid media showed that α-terpinene (23.6%), γ-terpinene (23.06%) and terpinolene (5.66%) are the main constituents present in the acid medium extract, while sabinene (66.74% and 72.7%), β-pinene (6.47% and 6.59%), α-pinene (6.08% and 6.08%) and myrcene (1.48 and 1.6%) were the major components obtained from aqueous and alkaline media respectively. Terpinenes and terpinene derivatives are reported to have antibacterial activities.[16-18] α- and γ-terpinenes and terpinolene are the chief constituents of acid extract and the antibacterial activity of the oil can be attributed to the presence of these components. The combined concentration of terpinenes (α-and γ-terpinenes) and terpinolene in the oil is only 52.4%. For the antibacterial assay, 100%, 10%, 5% and 1%

solutions of oil were taken. As 1 ml of oil (taken as 100%), corresponds to 0.8740 gm, 50 μl 100% oil contains 43.7 mg oil. Similarly, 50 μl each of 10%, 5% and 1% solution contains 4.37, 2.185 and 0.437 mg of oil respectively. It is clear from the GC analysis that, 100 mg oil contains 52.4 mg of terpinenes and terpinolene. Accordingly, 100%, 10%, 5% and 1% of the oil contain 22.89 mg, 2.28 mg, 1.14 mg and 0.228 mg of terpinenes and terpinolene respectively.

Among the three samples of essential oils screened for antibacterial properties, the acid medium extract showed higher order of activity against the microorganisms. Aqueous and alkaline medium extract also displayed moderate activity and the activity of all the three extracts were tested at various concentrations. The lowest concentration of each extract dilution series that prevents bacterial growth is considered to be the MIC (minimum inhibitory concentration) of the extract. As the acid extract showed superior activity, its activity at the MIC (i.e., 1% of oil that contains 228 μg of terpinenes and terpinolene) was studied and it was found to be comparable with that of the standard, gentamicin, at a concentration of 200 μg.

The zone of inhibition shown by the *Z. rhetsa* seed essential oil at different concentrations proved that the activity is concentration dependent. The poor antibacterial activity of alkali and aqueous extract revealed that, sabinene, the chief constituent of the extracts, was a poor antibacterial agent.

Because of the notable antimicrobial activity coupled with pleasant odour, the essential oil extracted from *Z. rhetsa* seeds, by steam distillation from acid medium, can be used as a potential antiseptic in prevention and treatment of antibacterial infections such as skin diseases, at a concentration of 1%.

SECTION 3 : EVALUATION OF ANTIFUNGAL ACTIVITY OF *ZANTHOXYLUM RETSA* SEED ESSENTIAL OIL AGAINST *PHYTOPHTHORA CAPSICI*

V.8 Introduction

Black pepper (*Piper nigrum* L.) that belongs to the family Piperaceae, christined as the 'king of spices' and 'black gold' is the most important and widely used spice in the world, occupying a position that is supreme and unique. It is an important crop grown exclusively in humid tropics like India, Indonesia, Malaysia, Brazil, Thailand and Sri Lanka. In the past, black pepper ranked with gold and was used as barter money. It was the lure of Indian pepper that brought the western world to the Indian sub-continent.

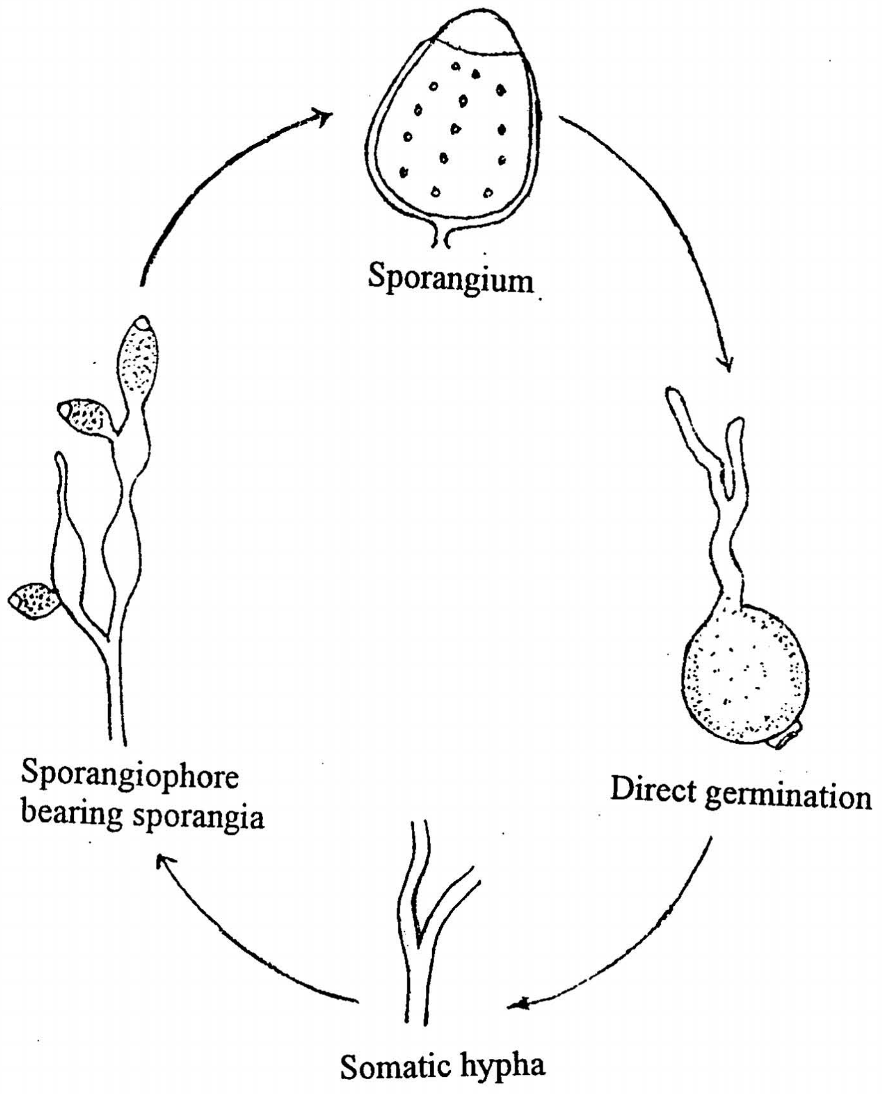
The spice with its characteristic pungency and flavour, is an ingredient in many food preparations. For the civilized western people, it is a spice, an essential additive to their food; for the ancient Egyptians it was an ingredient in the embalming mixture, for ancient Aryans, it was a valuable drug, and now for the common Indians pepper is a spice as well as a medicine, a sure cure for cold and fever and a component of many traditional Ayurvedic drugs.

Black pepper is affected by many fungal, bacterial and viral, mycoplasmal and nematodal diseases. Among the fungal pathogens, *Phytophthora capsici*, is the most destructive one. It is responsible for the devastating disease 'foot rot' also known as 'quick wilt' of pepper causing severe crop loses in all black pepper producing countries.[19-20] The crop loss due to foot rot in Kerala is estimated to be 10% of the total production.[21]

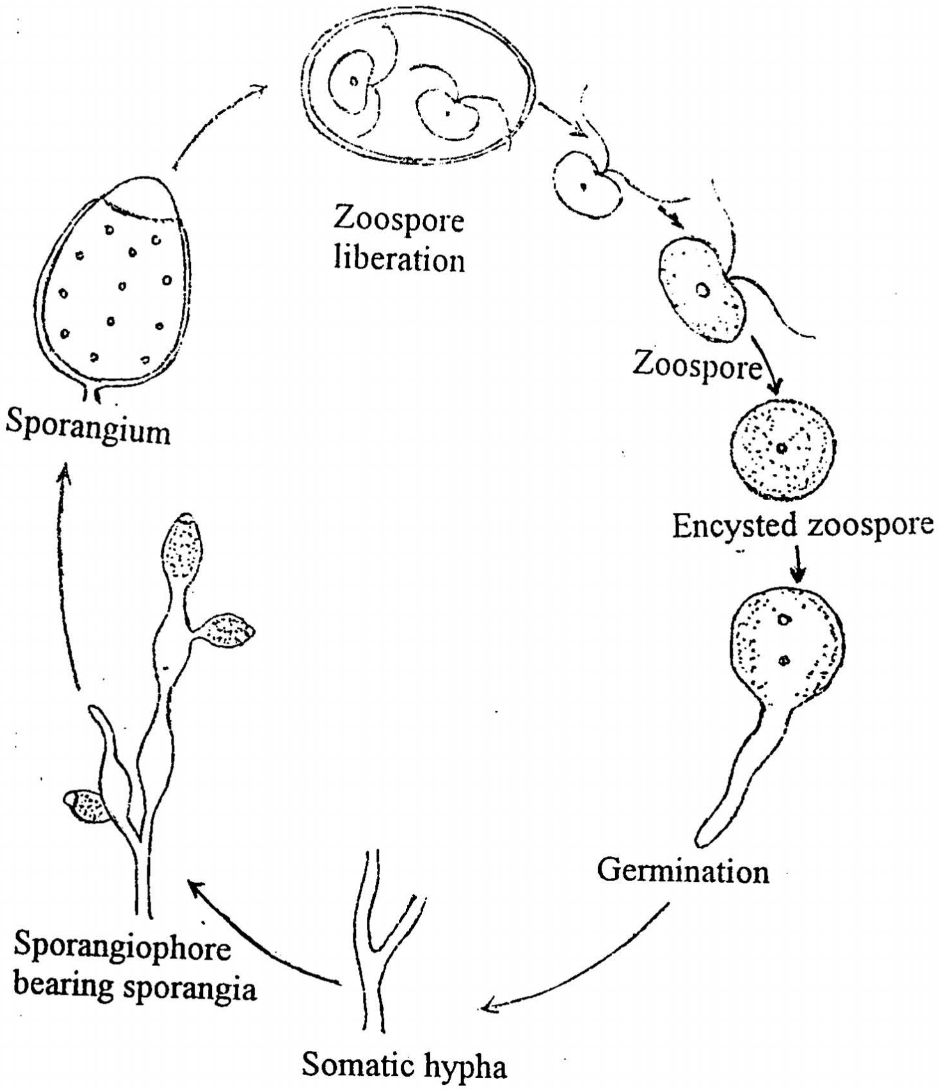
Phytophthora capsici, comes under the class Oomycetes, which contain some most devastating plant pathogens. Classic examples of the diseases they cause include late blight of potato and downey mildews of different crops. Among Oomycetes, the *Phytophthora* spp. include plant pathogens causing an array of diseases ranging from foliar and fruit diseases to stem and root diseases.

Phytophthora capsici affects all parts of the pepper vine. Its infection on underground parts namely roots and collar (foot) results in root rot and foot rot. Infection on the aerial parts like leaves, spikes and stems occur and spread rapidly under favourable conditions causing defoliation and complete destruction of vines in severe cases. *P. capsici* is a wet weather pathogen and infection occurs mainly during south west monsoon (June-Sept.) period in Kerala. This fungus spreads mainly through rain splash and water.[22] High rainfall and microclimatic conditions like high relative humidity, low temperature and shorter duration of sunshine prevailing during this period are known to favour the increase in disease.[23] This fungus grows luxuriantly at 25-28°C on carrot agar medium[24] and growth is absent at 35°C. Sporangial formation is found to be maximum at pH 6 and absent at pH 3.

In *P. capsici*, spores are contained within the sporangium. Germination of this sporangium is governed by temperature and moisture. At low temperature (below 15°C), and wet conditions, indirect germination occurs. In this case, cleavage of cytoplasmic contents results in the formation of uninucleate zoospores. Liberated zoospores swim in water film on the host, come to rest by withdrawing their flagella, get encysted and each produces a germ tube. The germ tube penetrates to the host. The sudden disastrous effects of the *Phytophthora* infection may be due to this indirect germination under favourable conditions.



Direct germination of *P. capsici*



Indirect germination of *P. capsici*

High temperature and dry condition favour direct germination of sporangia. In this case sporangium behaves as a single spore and germinates directly by forming a multinucleate germ tube and zoospores are not formed. Optimum temperature for direct germination is 24°C.

For a long time, the control of Oomycetes fungi remained a tough challenge to the plant pathologists. They were not amenable for the control with many earlier systematic fungicides as they are endowed with some unique biochemical and physiological features.

Water solubility is considered to be the most important attribute of fungicides in order to be effective against Oomycetes that thrive in aqueous environments. For the control of 'foot rot' disease, a number of control measures involving cultural, chemical and biological have been advocated.[25] Chemical control measures include foliar spray and swabbing of the collar portions of the vines with Bordeaux mixture besides drenching of the soil with Bordeaux mixture or copper oxychloride and use of systematic fungicides like metalaxyl, fosetyl–Al. Biocontrol agents like *Gleocladium virens*, *Trichoderma* spp, Vesicular arbuscular mycorrhiza (VAM) are also used against this fungus. However, the effect of botanical pesticides hasn't received enough attention. During recent years, use of plant secondary metabolites for the control of fungi is gaining importance.[26,27] The root exudates of *Allium* spp. have been reported to be inhibitory to *P. capsici.*[28] Inhibitory effect of ajoene, a component of garlic on *P. drechsleri* has also been reported [29] . Soil application of a mixture of garlic and mustard has been reported to be effective against foot rot of black pepper.[30] Water and ethanol extracts of *Piper colubrinum* and *Chromolaena odorata* have been found inhibitory to *P. capsici.*[31] The root bark oil of *Uvaria narum* is reported to possess antimicrobial activity against many fungi and bacteria.[32,33] The inhibitory effect of the aqueous extract of *Uvaria narum* leaves, on the growth of *P. capsici* has been reported recently.[34] The essential oil from the leaves of *Artemisia nilagirica* (Clarke) Pamp has been found to be fungicidal against *P. capsici.* Thujones, the well-known antifungal agent, is the chief component present in the oil.[35]

V.9 PRESENT WORK

The present work is aimed at the study of antifungal activity of *Zanthoxylum rhetsa* seed essential oil steam distilled from aqueous, alkaline and acid media, against *Phytophthora capsici*, the foot rot pathogen of black pepper. The active ingredients of these oils were also characterized.

V.10 EXPERIMENTAL

Water used in all the experiments involving fungi was double distilled using a glass apparatus. Isolation of *Z. rhetsa* seed essential oil, used for the antifungal assay, by steam distillation from aqueous, alkaline and acidic media, was described earlier.

Preparation of *P. capsici* cultures

The pure culture of *P. capsici* was placed at the centre of petriplates containing carrot agar medium and incubated in dark at 20-22°C. Culture discs for experiment were cut out from the growing edge after 48h.

Preparation of media for antifungal studies

Carrot (200g) was cooked lightly until tender in 500 ml distilled water. The cooked carrot was ground into a paste using a mixer grinder and diluted with more water. After filtration through muslin cloth, 17g of agar (BDH) was added to the carrot preparation and diluted to 1L with sterile double distilled water. It was then shaken well to get a homogeneous mixture.

Activity of *Z. rhetsa* seed essential oil on the growth of *P. capsici*

In the growth study, 0.5 g of each of the steam distilled essential oil from the seeds of *Z. rhetsa*, obtained under three different conditions were dissolved in acetone and made up to 100 ml in a standard flask. After autoclaving the carrot agar medium at 15 psi for 30 minutes and cooled to 40°C, different volumes (0.25, 0.50, 1.0, 2.5 and 5 ml) of the above solutions were added to 60 ml of the medium to obtain desired concentrations (0.02, 0.04, 0.08, 0.20 and 0.38 mg) of the test compound per ml of the carrot agar medium. For control sets appropriate quantities of acetone were added to the medium in place of test solutions. The medium was then dispensed into three petriplates for replication. Mycelial discs of 0.5 cm taken from the growing edges of 48 h old culture plate of *P. capsici* were placed at the centre of these petriplates. Incubated in the dark at 20-22°C for 72 h. The radial growth in each plate was measured at three points along the diameter of the plates and the mean of these three readings was taken as the radial growth of the colony. The radial growth of the colony after 48h. and 72h. was measured. Compared to the growth of the colony in the control sets, percent inhibition was calculated using the formula[36]

$$\text{Percent inhibition} = \frac{a-b}{a} \times 100, \text{ where}$$

a = radial growth of the colony in the control medium

b = radial growth of the colony in the test medium

Effect of *Z. rhetsa* seed essential oil on the sporulation of *P. capsici*

In the sporulation study, 0.5 of each of the *Z. rhetsa* seed essential oil, steam distilled under three different conditions were dissolved in acetone and made up to 100 ml in a standard flask. 2.5 ml and 5 ml of these oil samples were then added to 60 ml of sterile double distilled water to obtain the concentration of 0.2 and 0.38 mg of the test compound per ml of the medium. Mycelial discs of 0.5 cm taken from the growing edges of 48h old culture plate of *P. capsici* were placed at the centre of petriplates, keeping five discs per plate. The above solutions (15 ml) were placed on these discs. In control, the discs were covered with sterile double distilled water containing appropriate quantities of acetone. All the petriplates were then incubated under luminescent light for sporulation at 20-22°C for 48 h. Number of sporangia formed per microscopic field were counted (magnification 15/14 x 4, Nikon). The average of three microscopic fields per discs for five replications were counted and made comparison with that of control, the percent inhibition was calculated using the formula

$$\text{Percent inhibition} = \frac{a-b}{a} \times 100, \text{ where}$$

a = number of sporangia in the control medium

b = number of sporangia in the test medium

Effect of *Z. rhetsa* seed essential oil on zoospore liberation of *P. capsici*

To study the effect of *Z. rhetsa* seed essential oil on the zoospore release (indirect germination of sporangia) of *P. capsici*, mycelial discs of 0.5 cm diameter were cut out from the growing edges of 48h. old culture plates and placed at the centre of the petriplates. These discs were then allowed to sporulate by incubating under luminescent light for 48h. The *Z. rhetsa* seed essential oil of various concentrations were prepared as explained under sporulation of *P. capsici*. To the sporulating discs in the petriplate, *Z. rhetsa* seed essential oil of different concentrations (0.2 and

0.38 mg per ml of the medium) were added. These treated discs were then given a cold shock at 10°C to facilitate indirect germination and later incubated at room temperature at 28°C for 30 minutes. Control sets were prepared by mixing appropriate quantities of acetone with sterile double distilled water. Due to cold shock, zoospores were released from sporangia. The number of empty sporangia was counted from three microscopic fields per discs, five discs per plate and for five replications. The average was taken and compared with control, the percent inhibition was calculated using the formula

$$\text{Percent inhibition} = \frac{a-b}{a} \times 100, \text{ where}$$

a = number of empty sporangia in the control medium

b = number of empty sporangia in the test medium

Effect of *Z. rhetsa* seed essential oil on the zoospore germination of *P. capsici*

In order to evaluate the effect of *Z. rhetsa* seed essential oil on zoospore germination, sporulating discs of *P. capsici* were subjected to cold shock at 10°C for 10 minutes, as described under zoospore liberation and thus prepared the zoospore suspension. *Z. rhetsa* seed essential oil of concentrations 0.2 and 0.38 mg per ml of the medium were prepared as described under sporulation of *P. capsici*. Total number of zoospores in 200 µl of zoospore suspension was counted, and solutions of *Z. rhetsa* seed essential oil of different concentrations added to it. After incubating for one day at room temperature, zoospore germination per microscopic field was recorded. Zoospore suspension in sterile double distilled water containing appropriate quantities of acetone served as control. The percentage of zoospore germination and inhibition of zoospore germination etc. were calculated by comparing with the control using the formula

$$\text{Percent inhibition} = \frac{a-b}{a} \times 100, \text{ where}$$

a = number of zoospore germinated in the control medium
b = number of zoospore germinated in the test medium

V.11 RESULTS AND DISCUSSION

Phytophthora capsici has four different phases of growth namely vegetative growth, sporangial formation, zoospore liberation and zoospore germination. If any compound inhibits any one of these four phases, it could be useful as an antifungal agent against this fungus.

Effect of *Z. rhetsa* seed essential oil on the growth of *P. capcisi*

The *Z. rhetsa* seed essential oil obtained by steam distillation from acidic, alkaline and aqueous media were examined for antifungal activity. Out of the three essential oils tested, the oil extracted from acidic medium was found to be highly inhibitory to the growth of the fungus. Acid extract showed 100% inhibition to the growth of *P. capsici*, at a concentration of 0.38 mg/ml of the medium. Seed oil obtained by steam distillation from aqueous medium, also showed inhibition, but to a lesser extend compared to acid extract at the same concentrations. At a concentration of 0.38 mg/ml of the medium, the inhibition percent (after 48 h.) of aqueous extract on the fungus was 46.75. *Z. rhetsa* seed oil steam distilled from alkaline media, found to facilitate the growth of the fungus at low concentrations, but at a concentration of 0.38 mg/ml of the medium, it showed an inhibition of 49.36%. The radial growth of the fungus measured after 48 h is given in the TableV.4

Inhibitory effect of *Z. rhetsa* seed essential oil on the growth of *P. capsici* was also measured after 72 h. The results are given in the TableV.5.

Table V.4

Effect of *Z. rhetsa* seed essential oil on the growth of *P. capsici* (48 h.)

Essential oil dose (mg/ml)	Control		Essential oil of *Z. rhetsa* seeds					
			Acid extract		Alkali extract		Aqueous extract	
	(ml/60 ml)	Radial growth (mm) (a)	Radial growth (mm) (b)	% inhibition $\left[\frac{a-b}{a}\right] \times 100$	Radial growth (mm) (b)	% inhibition $\left[\frac{a-b}{a}\right] \times 100$	Radial growth (mm) (b)	% inhibition $\left[\frac{a-b}{a}\right] \times 100$
0.02	0.25	28.33	25.67	9.38	31.33	Promotes growth	27	4.69
0.04	0.5	27.67	24.67	10.84	29.33	"	26.67	3.61
0.08	1.0	27.0	19.33	28.40	27	0	25.67	4.92
0.20	2.5	26.0	18.33	29.50	26	0	23.33	10.26
0.38	5	25.67	0	100	13	49.36	13.67	46.75

Table V.5

Effect of Z. rhetsa seed essential oil on the growth of *P. capsici* (72h.)

Essential oil dose (mg/ml)	Control		Essential oil of Z. *rhetsa* seeds					
			Acid extract		Alkali extract		Aqueous extract	
	(ml/60 ml)	Radial growth (mm) (a)	Radial growth (mm) (b)	% inhibition $\left(\frac{a-b}{a}\right)$ x 100	Radial growth (mm) (b)	% inhibition $\left(\frac{a-b}{a}\right)$ x 100	Radial growth (mm) (b)	% inhibition $\left(\frac{a-b}{a}\right)$ x 100
0.02	0.25	42	38.67	7.93	43.67	Promotes growth	41.67	0.79
0.04	0.50	40.33	35.67	11.55	43.33	"	39.67	1.64
0.08	1.0	40	27	32.50	41.67	"	35.0	12.5
0.20	2.5	38.33	22.33	41.74	39.33	"	30.33	20.87
0.38	5.0	32	7.33	77.09	17.3	45.94	24.6	23.13

At a concentration of 0.38 mg/ml of the medium, the percent inhibition (after 72 h.) of acid and alkali extract on the growth of *P. capsici* were 77.09 and 45.94 respectively. However, at low concentrations, alkali extract promotes the growth of the fungus. Aqueous extract showed poor inhibition at all concentrations. It has been found that percent inhibition measured after 72 h was less than that recorded at 48 h. Hence, all further studies were conducted after 48 h.

Effect of *Z. rhetsa* seed essential oil on sporulation of *P. capsici*

Acid extract inhibited the sporulation of *P. capsici*, but to a lesser extent. The inhibition percent was 39.44 at 0.2 mg/ml and 62.69 at 0.38 mg/ml of the medium. Alkali extract supports sporulation of the fungus at all tested concentrations. Aqueous extract, at a concentration of 0.38 mg/ml showed 66.16% inhibition whereas at low concentration it promotes sporulation. The results are given in Table V.6.

Effect of *Z. rhetsa* seed essential oil on zoospore liberation of *P. capcisi*

Zoospore liberation of *P. capsici* was supported by acid extract. At low concentration, alkali extract promotes spore liberation, but at a concentration of 0.38 mg/ml of the medium, it inhibits spore liberation to an extent of 36.86%. Aqueous extract showed poor inhibition at all concentrations. The results are shown in TableV. 7.

Table V. 6

Effect of *Z. rhetsa* seed essential oil on sporulation of *P. capsici*

Essential oil dose (mg/ml)	Control		Essential oil of *Z. rhetsa* seeds					
			Acid extract		Alkali extract		Aqueous extract	
	(ml/60 ml)	No. of sporangia/ microscopic field (a)	No. of sporangia/ microscopic field (b)	% inhibition $\left(\frac{a-b}{a}\right) \times 100$	No. of sporangia/ microscopic field (b)	% inhibition $\left(\frac{a-b}{a}\right) \times 100$	No. of sporangia/ microscopic field (b)	% inhibition $\left(\frac{a-b}{a}\right) \times 100$
0.2	2.5	48.66	29.47	39.44	58.40	Promotes sporulation	52.70	Promotes sporulation
0.38	5	45.30	16.90	62.69	55.53	"	15.33	66.16

Table V.7

Effect of *Z. rhetsa* seed essential oil on zoospore liberation of *P. capsici*

Essential oil dose (mg/ml)	Control		Essential oil of *Z. rhetsa* seeds					
			Acid extract		Alkali extract		Aqueous extract	
	(ml/60 ml)	No. of sporangia liberated (a)	No. of sporangia liberated (b)	% inhibition $\left(\frac{a-b}{a}\right)$ x 100	No. of sporangia liberated (b)	% inhibition $\left(\frac{a-b}{a}\right)$ x 100	No. of sporangia liberated (b)	% inhibition $\left(\frac{a-b}{a}\right)$ x 100
0.2	2.5	98.1	188.33	Promotes zoospore liberation	127.7	Promotes zoospore liberation	88.8	9.48
0.38	5	90.6	91.5	"	57.2	36.86	63.1	30.35

Effect of *Z. rhetsa* seed essential oil on zoospore germination of *P. capsici*

Alkali extract was found to be highly effective against *P. capsici*, at this stage. At 0.2 mg/ml, the inhibition percent was 75.64. At 0.38 mg/ml, the inhibition percent increased to 100. Acid extract also inhibited the zoospore germination of *P. capsici*. Inhibition percent was found to be 33.85 and 98.27 at 0.2 mg/ml and 0.38 mg/ml respectively. Aqueous extract showed poor inhibition against zoospore germination of the fungus.

Percentage of zoospore germination calculated from 200 µl zoospore suspension and the effect of *Z. rhetsa* seed essential oil on zoospore germination of *P. capsici* are given in Tables V. 8 and V. 9 respectively.

Table V. 8

Percentage of zoospore germination

Essential oil dose (mg/ml)	Essential oil of *Z. rhetsa* seeds								
	Acid extract			Alkali extract			Aqueous extract		
	Total no. of spores in 200 µl	Zoospore germinated (number)	% of germination	Total no. of spores in 200 µl	Zoospore germinated (number)	% of germination	Total no. of spores in 200 µl	Zoospore germinated (number)	% of germination
0.2	135	89.3	66.15	78	19	24.36	67	59.7	89.10
0.38	77	1.33	1.73	87.33	0	0	59.7	52.7	88.27

Table V. 9

Effect of *Z. rhetsa* seed essential oil on zoospore germination of *P. capsici*

Essential oil dose (mg/ml)	% of germination (control)	*Z. rhetsa* seed essential oil					
		Acid extract		Alkali extract		Aqueous extract	
		% of germination	% of inhibition	% of germination	% of inhibition	% of germination	% of inhibition
0.2	100	66.5	33.85	24.36	75.64	89.10	10.90
0.38	100	1.73	98.27	0	100	88.27	11.73

The results showed that *Z. rhetsa* seed essential oil obtained by steam distillation from acid medium was strongly inhibitory to the growth of *P. capsici*, at a concentration of 0.38 mg/ml of the medium.

The GC-MS analysis of the oil showed that α-terpinene (23.68%), γ-terpinene (23.06%) and terpinolene (5.66%) are the major components present in it. In order to find out the activity of these components against *P. capsici*, the oil was tested at different concentrations. (The separation of these components from the essential oil is difficult, and is not commercially available also). The combined concentration of terpinenes (α- and γ-terpinenes) and terpinolene in the oil is only 52.4%. That is 100 mg oil contains only 52.4 mg of above compounds. i.e. 0.38 mg of oil contains 0.199 mg of terpinenes and terpinolene. By this argument, it can be concluded that 100% inhibition on the growth of *P. capsici* is possible with 0.199 mg/ml of terpinenes and terpinolene.

The poor antifungal activity of alkali and aqueous extract against *P. capsici* revealed that, sabinene, the major component of these extracts, was a poor antifungal agent against *P. capsici*. Yet, alkali extract showed 100% inhibition of zoospore germination of *P. capsici*, but the constituent responsible for it was uncertain.

REFERENCES

1. Sistorm, W.R., Microbial Life. New Delhi: Amerind Publishing, 1970, p.15.

2. Black, J.G., Microbiology, Principles and Explorations, 5th edn. New York: John Wiley & Sons, Inc. 2002, p.80.

3. Talaro, K., Talaro, A. (eds). Foundations in Microbiology, 2nd ed., London: Wm.C. Brown Publishers, 1996, p.503.

4. Hartmans, K.J., Diepenhorst, P., *Industrial Crops and Products* **4**, 1995, 3.

5. Bang, U., in Proceedings of the 25th International Symposium on Essential Oils. Turkey: Ekisehir, 1997.

6. Khanuja, S.P.S., *Journal of Medicinal and Aromatic Plant Sciences* **22**, 2000, 336.

7. Hubert, S.H., Richter, T.E., Axtell, J.D., Bennetzen, J.L., *Proc. Natl. Acad. Sci.* USA, **87**, 1990, 4251.

8. Mohmood, Z., Ahmad, S., Mohammad, F., *Indian Perfum.* **43**, 1999, 164.

9. Tokin, B.P., *Rev. Appl. Mycol.* **41**, 1962, 364.

10. Raychaudhury, S.P., *Bull. natn. Inst. Sci.* India, **24**, 1962, 143.

11. Davis, J., *Science* **264**, 1994, 375.

12. The Wealth of India, Raw Materials Vol. XI. New Delhi: CSIR, 1976, p. 22.

13. Ambasta, S.P.(ed). The Useful Plants of India. New Delhi: CSIR, 1986, 698.

14. Joshi, C.G., Magar, N.G., *Indian J. Pharm.* **15**, 1953, 312.

15. Mallikarjuna, P., Uma Maheswara Rao, V., Satyanarayana, T., *Indian Drugs* **36(7)**, 1999, 476.

16. Buchbauer, G., Jirovetz, L., *Flav. Frag. J.* **9**, 1994, 217.

17. Recio, M.C., Rios, J.L., Villar, A., *Phytotherapy Research* **3**, 1989, 117.

18. Kubo, I., Muroi, H., Himejima, M., *J. Agric. Food Chem.* **40**, 1992, 245.

19. Anandaraj, M., Ramachandran, N., Sarma, Y.R. 'Black Pepper diseases,' Sarma, Y.R., Premkumar, T. (eds). National Research Centre for spices, Calicut, 1991, p.114.

20. Holliday, P , Mowat, W.P., 'Phytopathological paper no.5', Common wealth Mycol. Institute, Kew Surrey, 1965, p.62.

21. Prabhakaran, P.V., *J. of Spices and Aromatic Crops* **6(1)**, 1997, 31.

22. Sarma, Y.R., Nambiar, K.K.N., 'Proc. of Workshop on *Phytophthora* Diseases of Tropical Cultivated Plants,' Nambiar, K.K.N. (ed). Central Plantation Crops Research Institute, Kasargod, Kerala, India, 1982, p.204.

23. Ramachandran, N., Sarma, Y.R., Anandaraj, M., Jose, A., *J. Plant. Crops* **16**, 1988, 110.

24. Santhakumari, P., Ph.D. Thesis, University of Agricultural Sciences, Dharward, 1987, p.139.

25. Sarma, Y.R., Anandaraj, M., Venugopal, M.N. 'Advances in Horticulture,' Chadha, K.L., Rethinam, P. (eds). New Delhi: Malhotra Publishing House, **10**, 1994, p.1015.

26. Dixit, S.N., Dubey, N.K., Tripathi, M.N. Recent Advances in Plant Pathology', Hussain, A., Singh, B.P., Agnihotri, V.P. (eds). New Delhi: (CBS), 1983, p.248.

27. Singh, J., Tripathi, N.N. *J. Ind. Bot. Soc.* **73**, 1993, 1.

28. Monohara, D., Kasim, R., Sitepu, D. 'Proceedings of the International Workshop on Black pepper Diseases,' Research Institute for Spices and Medicinal Plants, Bogor, Indonesia, 1992, p.144.

29. Singh, U.P., Chauhan, V.B., Wagner, K.G., Kumar, A., *Mycologia* **84**, 1992, 105.

30. Pailoor, S., 'Spice India', 991, p.24.

31. Dennis, L., Webster, J., *Trans. Br. Mycol. Soc.* **57**, 1971, 25.

32. Nanda, A., Narayana, C.S., Kulkarni, D.R., Iyengar, M.A., *Indian Drugs* **23**, 1986, 647.

33. Padmaja, V., Thankamany, V., Hisham, A.K., *Journal of Ethnopharmacology* **40**, 1993,181.

34. Bindu, T. K., Shafi, M., Rajan, P.P., Sarma, Y.R., *Allelopathy Journal* **5(1)**, 1998, 67.

35. Mohamed Shafi, P., Geetha Nambiar, M. K., Robin, A. Clery., Sarma, Y. R., Veena, S.S., *J. Essent. Oil Res.* 16, 2004, 377.

36. 'Preparative Organic Chemistry', Hilgetag, G., Martini, A. (eds). New York : John Wiley and Sons, 1972, p.332, 334.