ISOLATION, STRUCTURAL ELUCIDATION AND BIOLOGICAL PROPERTIES OF SECONDARY METABOLITES IN SOME PLANTS

THESIS SUBMITTED TO THE UNIVERSITY OF CALICUT
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
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DOCTOR OF PHILOSOPHY IN CHEMISTRY

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CERTIFICATE

This is to certify that this thesis is an authentic record of the research work carried out by **Mrs. Geetha Nambiar M.K.**, in the Department of Chemistry, University of Calicut, under my guidance and 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.

28/11/95

Dr. P. Mohamed Shafi
(Supervising Teacher)

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DECLARATION

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

Geetha
28/1/99

GEETHA NAMBIAR. M.K.

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Geetha Nambiar. M.K.

CONTENTS

PREFACE

Our country has a very rich plant kingdom. Naturally the Indian system of medicine, Ayurveda, makes use of the plant kingdom very effectively and has stood the test of time.

With the modern analytical facilities it is possible to study the plant ingredients in depth. Consequently many active principles in plants have been isolated, identified and put to use in varying fields like medicine and agriculture.

Plant-derived compounds can act as excellent lead to useful and more effective compounds. The structure-activity relationship in pyrethrins have lead to the production of a large number of synthetic pyrethroids with better pesticidal activity and photostability. New informations generated every day in the field of plant chemisry underscores the significance of phytochemical studies.

The work presented in this thesis comprises of the analysis of essential oil from five plants, chemical investigation of leaves of *Sansevieria zeylanica* and antifungal properties of three essential oils.

Chapter I describes the analysis of the essential oil isolated from the dried flowers of *Sphaeranthus indicus,* a medicinal plant. By gas chromatographic analysis (GC) and by the combined gas chromatography-mass spectrometry (GC-MS) analysis 117 compounds of this oil could be identified. The characteristic odour imparted by this oil also was a subject of study. Olfactometric study enabled the identification of the compounds responsible for different odours exhibited by it. An attempt has also been made to correlate the medicinal property of this oil to the known pharmacological properties of its constituent molecules. Based on this work an article entitled, 'Volatile constituents of *Sphaeranthus indicus* L. from South India' has been accepted for publication in the journal *Planta Medica.*

The second chapter deals with the studies on the leaf essential oils of *Vitex negundo* (variety negundo) and *V. negundo* (var. purpurescens). Both these plants are of medicinal importance. By means of GC and GC-MS 89 compounds from the variety negundo and 76 compounds from the variety purpurescens were identified. This investigation illuminated on the similarity of these two plants and also a few dissimilarities in the content of the essential oils. The olfactoric and pharmacological properties also have

been discussed. This work has been accepted for publication in the journal *Acta Pharmaceutica* under the title 'Analysis of the essential oils of the leaves of the medicinal plants *Vitex negundo* var. negundo and *Vitex negundo* var. purpurescens from India.

Curcuma aeruginosa leaf essential oil studies are presented in chapter III. Extensive studies on this plant or related ones are available from our neighbouring countries, while very few are available from India. This essential oil on GC and GC-MS analysis made the identification of fifty three compounds possible. By gas chromatography-sniffing the olfactoric properties also was investigated. Based on this study an article entitled 'Essential oil Analysis of *Curcuma aeruginosa* Roxb. Leaves from South India' has been accepted for publication in *Journal of Essential Oil Research.*

Fourth chapter discusses the study on *Artemisia nilagirica* essential oil. Its analysis by GC and G-MS lead to the identification of fifty nine compounds. By this work it was possible to compare its composition with those reported earlier from other parts of India.

Chapter V is on the phytochemical analysis on the leaves of *Sansevieria zeylanica.* The investigation enabled the identification of eleven

compounds which includ four alkanes, two esters, β-sitosterol and four alcohols. Apart from these two cycloartans were isolated whose exact structure could not be elucidated due to paucity of material. based on this work an article entitled 'Chemical Investigation of *Sansevieria zeylanica* leaves' has been accepted for publication in Asian Journal of Chemistry.

Chapter VI discusses the analysis of leaf essential oils of three plants for their antifungal activity against *Phytophthora capsici,* the foot rot pathogen of black pepper. The leaf oil of *Artemisia nilagirica* was found to be most effective against this plant pathogen. The active ingredients of this oil were also characterised. GC-MS analysis of the oil showed that the major components of this oil were α-thujone and β-thujone. These were also tested for their activity against *phytophthora capsici.* From the results it was proved that the antifungal activity of the leaf essential oil of *A. nilagirica* was due to these components.

CHAPTER I

ANALYSIS OF VOLATILE CONSTITUENTS OF DRIED FLOWERS OF *SPHAERANTHUS INDICUS*

Section 1: Introduction

In our daily practice we see that the fruits, flowers, leaves, stems, barks and roots of nearly all the plants have some pleasant smell. It has been observed that this pleasant smell of the fruits is actually due to the presence of certain steam volatile oils known as essential oils. The essential oils are complex mixtures of hydrocarbons and their oxygenated derivatives. The main constitutents of the essential oils are the terpenoids having carbon atoms up to C_{15} (ie mono and sesquiterpenoids) and their oxygenated derivatives such as alcohols, aldehydes, and ketones. Essential oils, due to their pleasant smelling nature are of commercial importance, particularly in perfumery. Moreover various essential oils show biological activity such as insecticidal, anthelmintic or antiseptic action, so they are also useful in pharmacy.[1]

Isolation

Since essential oils[2] frequently occur as a very small percentage by weight of the original plant material, the processing of large quantities is often required to obtain considerable amount of oil. In general four methods are used for the isolation of essential oils. They are expression, steam distillation, extraction by using volatile solvents and adsorption in purified fats. Steam distillation is the most widely used method for the extraction of essential oils. For that the plant material is macerated and steam distilled when the essential oils go into distillate from which they are extracted by the use of pure organic solvents like diethyl ether.

The function of the essential oil in the plant is not fully understood. They represent the odorous part of the plant material. For flowers the odours are very useful to attract insects involved in pollination and thus may aid in preservation and natural selection. Essential oils are almost always bacteriostats and often bacteriocides. 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. Exudates, which contain essential oils, eg., balsams and resins, act as

protective seals against disease or parasites, prevent loss of sap, and are formed readily when the tree trunks are damaged.

Essential oils contain a large number of volatile components made up of low molecular weight organic molecules of carbon, hydrogen and oxygen and occasionally nitrogen and sulphur. They are mainly terpenoids belonging to mono and sesquiterpenoid groups. Terpenoids are made up of isoprene units joined in a head-to-tail fashion. The monoterpenoids are the simplest among the naturally occurring isoprenoid compounds and form the important constitutents of the essential oils. They are composed of two isoprene units. They may be aliphatic, alicyclic or bi or tricyclic with varying degrees of unsaturation upto three double bonds. Sesquiterpenes contain three isoprene units, diterpenes four, triterpenes six etc. The same species of plant grown in different parts of the world usually contains the same chemical components, but the relative percentages may be different. Climatic and topographical conditions affect plant chemistry and can alter the essential oil content both qualitatively and quantitatively.

Analytical methods

The techniques such as odour or colour comparison was the early methods used for the determination of essential oils. Later analytical

techniques such as specific gravity, refractive index, distillation range, iodine number determination and gas-liquid chromatography etc. are employed for the determination of volatile components of an oil.

The techniques such as capillary gas chromatography [with the more sensitive flame ionisation detector (FID)], gas chromatography-mass spectrometry (GC-MS) and gas chromatography-infra red spectroscopy-mass spectrometry (GC-IR-MS) are the modern analytical methods used for the separation and identification of components of essential oils. Using the above methods, separation of even trace components have become possible. The Fourier-transform GC-IR, high resolution GC-MS, chemical ionisation GC-MS are more powerful and selective characterisation tools for the structure elucidation of components of oils. The combination GC-FTIR-MS is potentially more powerful and it is possible to operate these in series, but chromatographic resolution may deteriorate as the effluent passes along the light pipe in the FTIR instrument enroute to the MS. Therefore the GC effluent is usually split so that about 2 percent goes directly to the more sensitive MS instrument, and the remaining 98% goes to FTIR instrument. Eventhough the informations from IR and MS are complimentary to each

other, their combined ability to identify components in a GC is great indeed.

Gas chromatography[3] 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. It takes advantage of the linear relation between the logarithms of the adjusted retention times of a homologous series (the n-alkanes) and the number of carbon atoms in the molecules. The n-alkanes are used as the reference compounds because of their stability, ready availability, cheapness, 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 the 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

$$I = 100 \times \frac{\log t'_{R(subst)} - \log t'_{R(n\text{-}Cz)}}{\log t'_{R(n\text{-}Cz+1)} - \log t'_{R(n\text{-}Cz)}} + Z$$

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

Sometimes a component with critical odour properties may be present in the oil at ultra trace levels 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.

Gas chromatography-Olfactometry

Aroma chemicals have two sensory odour properties namely intensity and quality.[4] They are usually hydrophobic organic compounds containing a limited number of functional groups. However the presence of a 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 olfactory purity of a

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

Economic Importance

Essential oils have extensive applications in the field of flavour and fragrance industry. Eexamples are mint and cinnamon used in tooth paste, mouth wash or lozenges. Some combinations of essential oils can be found in soaps, detergents, room freshners, paper, printing ink, paint, candles, condiments, floor polishes, etc. Flavour of essential oils are used in baked goods, snack foods, soft drinks, liquors, sauces, gravies, salad dressings and other food products.[5]

Biological Activity of Essential Oils and their Possible Applications

For centuries, essential oils have been known in many different applications.[6] Many of them from ancient times were used as medicaments, disinfectants, insect repellents, fragrances etc. Most commonly they were known for their multiple therapeutic properties. From ancient times to the most modern aromatherapy and pharmacy, essential oils are recognized as medicaments or parapharmaceuticals and are widely applied by most advanced and most traditional doctors. Because of their well-known disinfectant properties they were considered excellent remedies against infections and epidemic diseases. Some times the oils were believed to be a remedy for nearly all health problems. Fragrances in our environments have multiple effects on our life. They are not only for pleasure and seduction, healing and magic products but also for insecticides, agriculture auxliary agents and as mood stimulants. However, little is known about the mechanism of action of essential oils. Their lipoid solubility and therefore their possibility to penetrate into the cells may give rise to influence on the metabolism of the microorganism and thus give an explanation of the effect. It has been observed that the antiseptic activity of many compounds is dependent on their lipoid solubility. The antibacterial

activity of essential oils has been expressed in their phenol-coefficients. It tells us how many times stronger or weaker is the action of the essential oil in question, compared to phenol[6](phenol has the factor one).

Essential oils as Therapeutics

The knowledge of therapeutic properties of essential oils is as old as mankind's use of plants as medicaments. Essential oils are complex mixtures which can act in different complicated ways. From exprience we know that a certain oil was useful in a specific medical case, but a similar illness in another patient cannot be cured with the same result. These effects are different from those achieved with simple chemicals which are much more convenient for evaluation and used in chemotherapy. Therefore individual chemicals isolated from essential oils are more often used than the oils. 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 which in many ways are better than antibiotics, due to their wider spectrum activity. There is also a very interesting phenomenon of synergistic 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 the individual oils. Even more interesting is the synergistic activity of essential oils with antibiotics. It was found that addition of a small quantity of oil to some antibiotic would increase the activity of the antibiotic several times. Bactericide 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. Examples are ointments, syrups, pills for remedies of pains, infections, eczema, bronchitis, skin diseases and many other problems.

Essential oils as Insect repellents and Insecticides

It was known from experience that certain plants growing at proper places can save other plants from dangerous insects. 'Zdrawetz' planted around the rose bushes will prevent lice from attacking the rose buds and also hemps (*Cannabis sativa*) planted around vegetable gardens act as repellents against a variety of worms and insects.

Essential oils also attract insects. They can be used in mixtures with insecticides to increase their effect by gathering the insects to the attractant. In this field of bioactivity of essential oils also synergism can be observed.

For example a mixture of basil and eucalyptus oils will kill 100% of mosquito larvae at a concentration two to six times lower than individual oils. Artemisia oil will kill larvae of anophele at a concentration of 0.016% while the same effect can be obtained with 0.014% DDT. A mixture of peppermint (50%), camphor (25%) and coumarin (25%) is used as a very effective home fumigant. The oil of *chrysantimum balsamita* is a very effective insecticide against lice. Rose oil in 1:1000 dilution will kill earth worms and leeches within 30 to 60 minutes. It is interesting to know that calamus oil will sterilize males of the housefly, so most probably an old custom of decoration of houses in spring with calamus leaves is based on this property of the oil. The few examples given above show the importance of essential oils in the control of insects with safe natural products. careful research and observations of insect behaviour in the presence of essential oils and their constituents can save nature from the use of efficient but dangerous chemicals.

Essential oils in Agriculture

Allelopathy is a phenomena which deals with chemical interactions of plants with each other. It was well-known that some plants grow in perfect harmony with others whereas some others die without any good

reason. There are many reasons for such effects including metabolites exchange in roots, organic, and inorganic products washed from leaves into the soil, interaction 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. There are certain positive interactions and negative interactions. Certain plants produce oils as toxins against their deseases. Tobacco disease *pseudomonas solancearum* will induce synthesis of mixture of sesquiterpenes in tobacco leaves. Another interesting example of allelopathic toxic activity of essential oils of sage and artemisia against grasses. Allelopathy of oil-bearing plants is still an under investigated area with enormous potential. Possible uses of plants or oils as safe natural herbicides, growth boosters, and other agents in agriculture are still open to research scientists.

Section 2: Analysis of Volatile constituents of dried flowers of *Sphaeranthus indicus*

I.1. Introduction

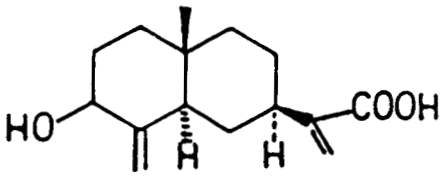
Sphaeranthus indicus belongs to the family Asteraceae, distributed throughout India, Ceylon, Malaysia, China & Africa.[7] *Sphaeranthus indicus* is a much branched herb about 30 cm high, stem and branches cylindric with toothed wings, more or less glandular hairy. The herb has a bitter sharp flavour with a bad taste and various compounds of different parts of this plant are of considerable medicinal importance and used for various treatments.[8] In Indian folk medicine (Ayurvedic system) this plant plays an important role on several deseases like bronchitis, elephantiasis, anaemia, asthma, leucoderma, dysentry, piles, vomitting, urinary discharges etc. It is also used as laxative, emmenagogue and alexipharmic. In Yunani the uses of this plant are as follows. It increases the appetite, enriches the blood, lessens inflammation, cools the brain, and gives lustre to the eye. It is also used as a good remedy for sore eyes, jaundice, scalding of urine, gleet, biliousness, scabies, ringworm of the waist and diseases of the chest. The oil from the root is aphrodisiac, used in prolapsus ani. The root and seeds are considered as anthelmintic. The bark ground and mixed with whey is a valuable remedy for piles. In Java the plant is considered as

a useful diuretic. In Punjab the flowers are highly esteemed as alterative, dipurative, cooling and tonic. Among the Mundas of Chota Nagpur the whole plant bruised is thrown into water to kill fish. It is stuffed into crab's holes to kill them. The plant is pounded with a little water and the juice is expressed and used as a styptic. The juice of the fresh leaves similarly obtained is boiled with a little milk and sugar candy and drunk against cough.[9-12]

I.2. Phytochemical studies so far reported on *Sphaeranthus indicus*

A literature survey on *sphaeranthus indicus* showed the following works reported. Naga Sampagi and co-workers[13] isolated a few 7-hydroxyeudesmanolides and a new sesquiterpene acid, 2-hydroxy costic acid (1) along with a few known compounds, β-eudesmol(2) cryptomeridiol(3), 4-epicryptomeridiol(4) and ilicic acid(5).

Atta-ur-Rahman and co-workers[14] also isolated the same 7-hydroxy frullanolide(6) from this plant which is reported to show a high degree antimicrobial activity. They also isolated[15] an immunostimulating glycoside, sphaeranthanolide(7) and three new eudesmanolides (8), (9) and (10).



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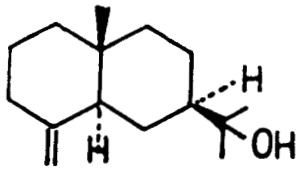

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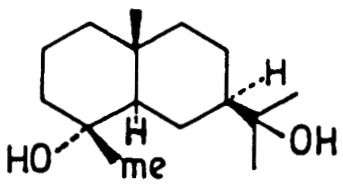

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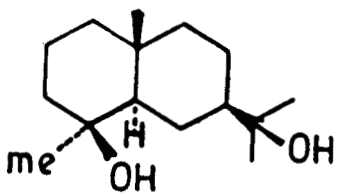

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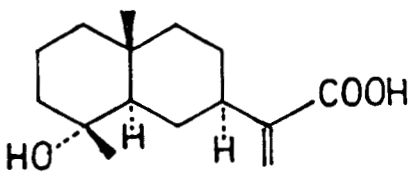

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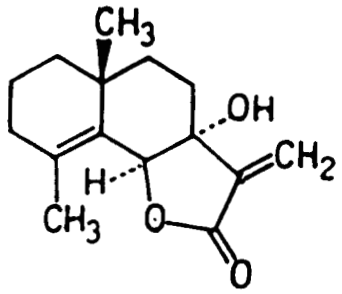

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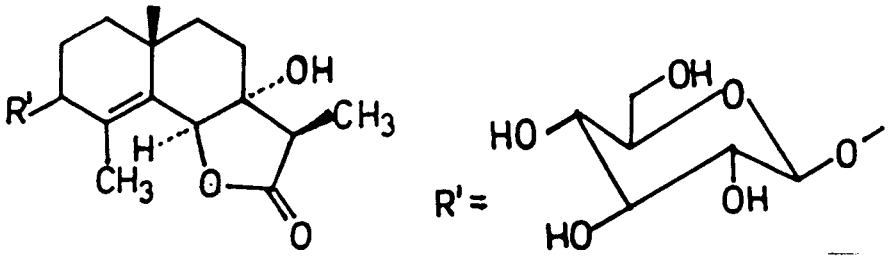
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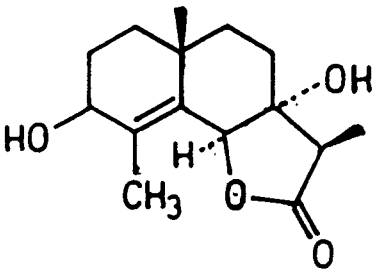
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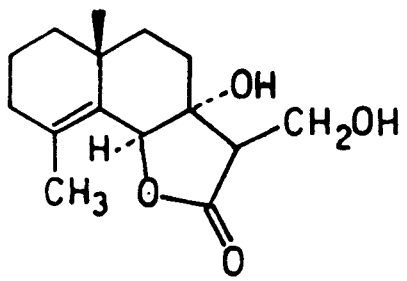
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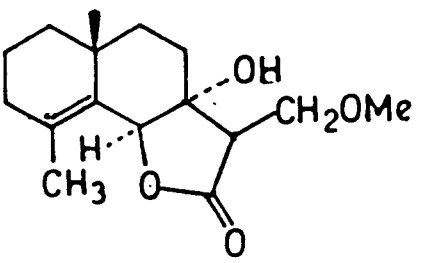
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(10)

The extracts of aerial parts of three different species of sphaeranthus [*S. bullalus, S. kirkii* and *S. suaveolens*] gave eight eudesman-12, 6-β-olides, Seven carvotacetone derivatives, and a thymohydroquinone gluco pyranoside.[16] A few carvotacetone derivatives and myo-inositol esters were also isolated from *S. cyathuloides* and *S. confertifolius*.[17]

So far volatile constituents of three different sphaeranthus species have been reported. The essential oil of *S. cyathuloides*[18] is found to contain trans-dihydrocarvone (67%) and cis-dihydrocarvone (26%) and some small amounts of isodihydrocarveol and neoisodihydrocarveol and limonene. The flower oil contained only small amounts of trans-dihydrocarvone (0.3%) and cis-dihydrocarvone (0.6%).

The essential oil of *S. suaveolens*[19] prepared by hydro-distillation of the dried leaves in which 34 compounds are identified by GC & GC-MS. The oil is marked by a high content of α-pinene (10.6%), isopinocamphone (33.5%) and thymohydroquinone dimethyl ether (16.1%).

The first report on the essential oil constituents of *S. indicus*[20] gave the major constituents as methylchavicol, α-ionone, d-cadinene and p-methoxycinnamaldehyde. Minor constituents are ocimene, α-terpenine,

citral, geraniol, geranyl acetate, β-ionone, sphaerene, indicusene and sphaeranthol. Study of non-saponifiable matter from oil led to the isolation of β-sitosterol, n-triacontanol, phenylurethan, and n-pentacosane. In 1989 Raffaele Tabacchi and Daniel Joulain[21] reported that the oil contains thymoquinol dimethyl ether as major component. Besides it contains several unusual tricyclic sesquiterpenoid hydrocarbons like modephene, silphinene, Isocomene and β-isocomene. The other components are oxygenated sesquiterpenoids, oxides, alcohols, and several 2-methylene butyrolactones and a few aromatic compounds.

Anti microbial activity of *Sphaeranthus indicus*

Several antibacterial principles are isolated from S. indicus by Shaikh and co-workers[22] Garg and co-workers[23] studied the *in vitro* anti bacterial activity of essential oil from S. *indicus* using several bacteria and found that they are generally susceptible to the oil except for *Salmonella paratyphi* and *Shigella sonnei* than the control streptomycin sulphate antibiotic disc.

A sesquiterpene lactone[24] isolated from petroleum ether extract of S. *indicus* exhibit ovicidal and growth disrupting activity against filaria vector. It is also reported that there is a marked effect on the hatching of eggs and

the metamorphosis of larvae of *Culex quinquefasciatus* at a concentration of 50 to 250 ppm.

A bicyclic sesquiterpene lactone is also isolated by Singh and co-workers[25] from petroleum ether extract of the aerial parts of *S. indicus*. The compounds exhibited strong antimicrobial activity against *Staphylococus aurens, S-albus, Escherichia coli, Fusarium* Sp., *Helmintho sporium* Sp. and other micro organisms.

I.3. Present Work

Materials and Methods

Sphaeranthus indicus flowers were collected from the rice fields in Kerala during March-April. The plant was identified by Dr. A.K. Pradeep, Dept. of Botany, Calicut University.

The shade dried flowers (350 g) were powdered in an electric mixer-grinder and subjected to steam distillation for about 5 hrs. The distillate was extracted with diethyl ether (3 x 100mL). The ether portions were pooled together and dried with anhydrous sodium sulphate. Evaporation of the dry ether extract after removal of sodium sulphate, over a water bath yielded 0.5 ml of pale yellow essential oil.

The GC-MS determination was run on a varian 3400 GC fitted with an OPTIC® injector and coupled to a Finnigan ITS40 ion trap mass spectrometer. The column used was an HP ultra 2 (Hewlett packard) (50 m x 0.25 mm; film thickness 0.33 μm). The carrier gas was helium with a flow rate of 1.6 mL/minute and the oven temperature was programmed from 50° to 270°C at 2°C/min.

Quantification and kovats determination were carried out using an HP 5890 GC (Hewlett packard) fitted with an OPTIC® injector and FID detector. The column used was HP-5 (Hewlett packard) (25 m x 0.2 mm, film thickness 0.33 μm). The carrier gas was nitrogen at a flow rate of 0.3 mL/min and the oven temperature was programmed from 30°C to 280°C at 3°/min. All data quoted are % RPA (relative peak area) of all peaks, including unknown.

Identification of the constituents was based on computer matching of the mass spectra against commercial[26] and in-house library spectra and comparison of the retention indices of the constituents with those of authentic reference compounds on the same HP-5 capillary column.

GC-olfactometry was carried out on a specially modified Carlo-Erba GC8000 fitted with an effluent splitter, heated smelling port, and humidified make-up air supply. The column used was an HP-I (25 m x 0.53 mm, film thickness 1.0 μm). The carrier gas was nitrogen at 15 mL/sec and the oven temperature was programmed from 50°C to 280°C at 3°/min.

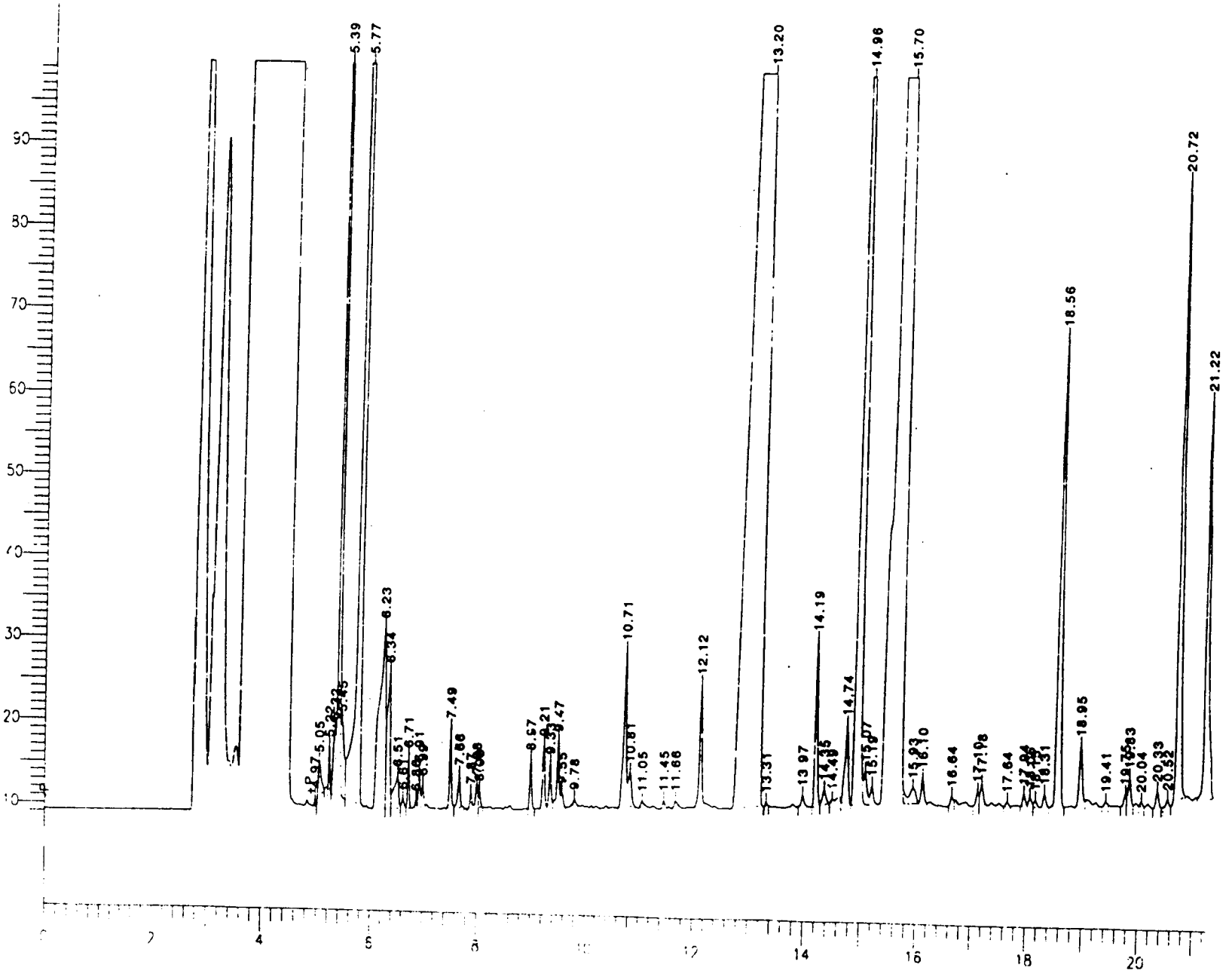
I.4. Results and discussion

a) Identification of Components

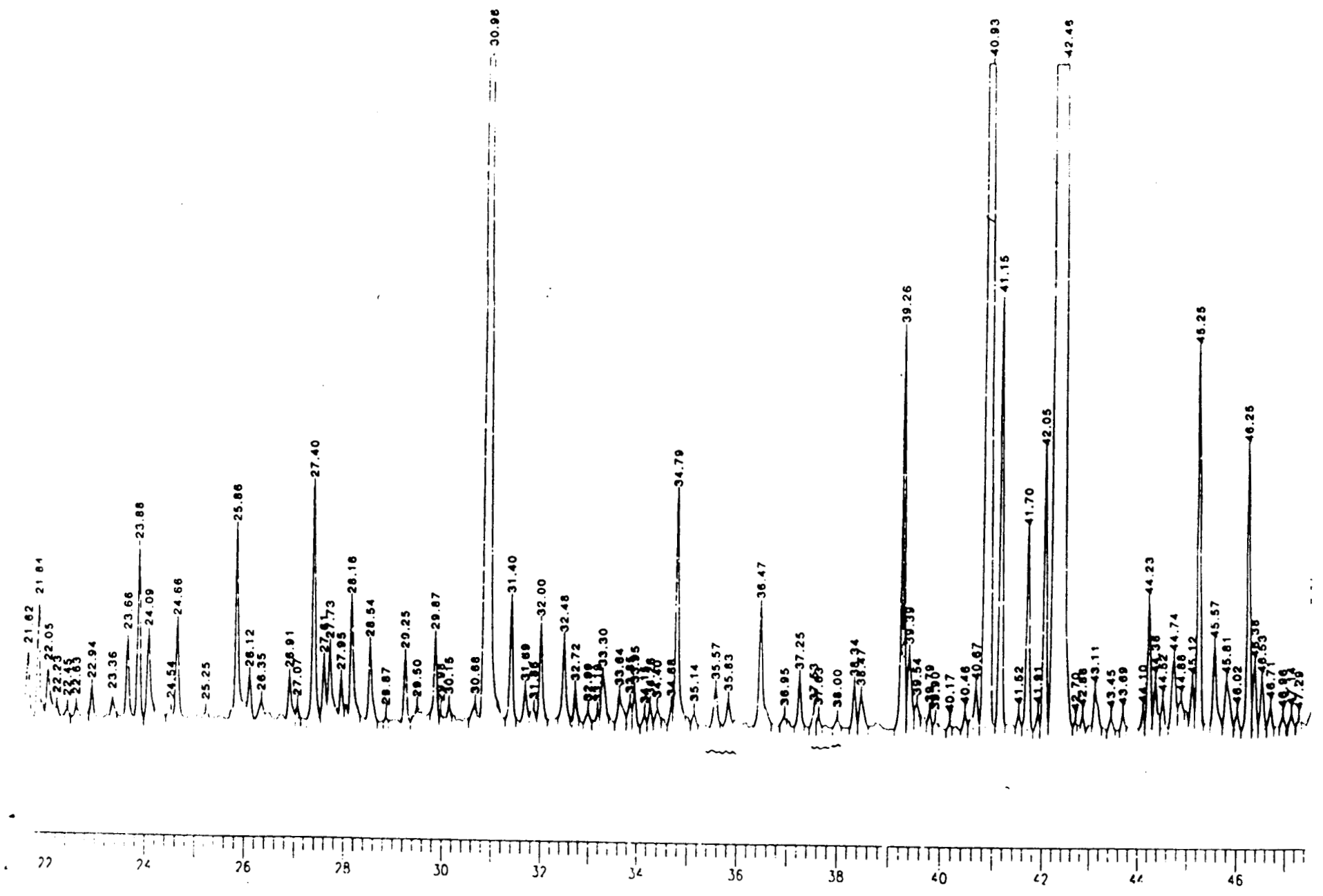
The gas chromatogram of *Sphaeranthus indicus* flower oil showed the presence of at least 258 compounds of which 117 could be reliably identified making up 85% of the total relative peak area (Table I.1). The retention indices of the identified compounds matched very well with the published data. The identified compounds includes 40 monoterpenoids, 22 sesquiterpenoids, 7 aromatic compounds and a few alkane derivatives. The monterpenoid fraction constituted 30.1% RPA and sesquiterpenes that could be identified constitute 31.7%. The remaining 38.2% RPA of the oil comprised of aromatic and alkane derivatives (about 15% RPA) unidentified sesquiterpenes (about 10% RPA) and solvent residues (13% RPA). The main components of the monoterpenoid fraction were 2,5-

dimethoxy-p-cymene (19% RPA), borneol (3.7% RPA) geranyl isovalerate (3.6% RPA) and α-pinene (0.7% RPA). Monoterpenoids detected in trace amounts were trans-pinane, sabinene, α-pinene, γ-terpinene, isoborneol, myrtenol and carvone. The main components of the sesquiterpene group were caryophyllene oxide (17.6%), β-patchoulene (5.4%), β-eudesmol (1.9%), and epi-α-cadinol (1.9%). All the components identified are given in Table I.1. The structures of terpenoids identified are also provided.

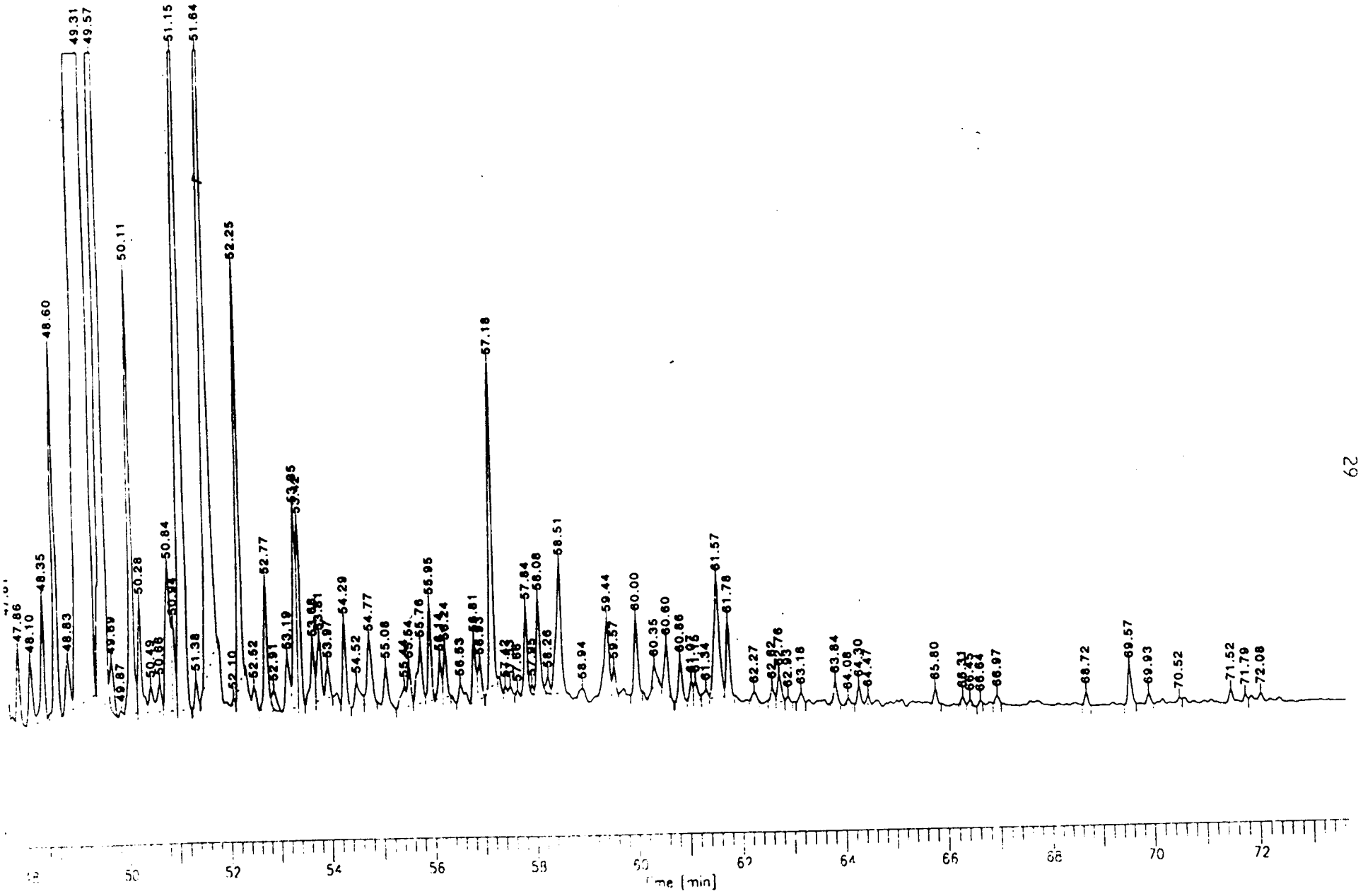
This is the first time that the essential oil from the flowers of *S. indicus* has been analysed by GC-MS. The differences between this analysis and the previous reports can be attributed to the part of the plant extracted, the extraction method and the season and region in which the plant was collected. Several eudesmane derivatives have already been reported in this species to which we can add 11-hydroxy eudesm-4-en-3-one.



Gas Chromatogram of essential oil of dried flowers of *Sphaeranthus indicus*



Gas Chromatogram of essential oil of dried flowers of *Sphaeranthus indicus*



Gas Chromatogram of essential oil of dried flowers of *Sphaeranthus indicus*

Table I.1

Identified Components in the essential oil of *Sphaeranthus Indicus*

	Components	Retention indices	% RPA
1	3-Methyl butanol	734	0.1
2	2-Methyl butanol	737	< 0.1
3	2-Methyl but-3-enol	773	0.1
4	Hexanal	804	0.1
5	Diethylene glycol	825	7.92
6	Iso-valeric acid	841	< 0.1
7	3-Methyl pentanol	848	< 0.1
8	trans-Hex-3-enol	856	0.1
9	trans-Hex-2-enal	856	0.1
10	Cis-Hex-3-enol	861	1.94
11	Trans-Hex-2-enol	876	0.10
12	Hexanol	876	8.4
13	Heptan-2-one	903	< 0.1
14	Heptanal	904	< 0.1
15	Cis-Hex-3-enyl formate	920	< 0.1
16	2,5-Hexanedione	931	0.64
17	α-Pinene	939	0.73
18	Camphene	954	< 0.1
19	Hept-2-enal	957	tr
20	Benzaldehyde	965	< 0.1
21	Trans-Pinane	968	tr
22	Heptanol	973	0.81
23	Hexanoic acid	975	tr
24	Sabinene	977	tr
25	Oct-1-en-3-ol	982	0.57
26	β-Pinene	982	tr
27	6-Methyl hept-5-ene-2-one	990	0.08

28	Octan-2-one	992	tr
29	Myrcene	994	0.2
30	Octan-3-ol	996	0.1
31	Octanal	1002	< 0.1
32	Cis-Hex-3-enyl acetate	1009	< 0.1
33	Hexyl acetate	1012	tr
34	p-Cymene	1030	0.1
35	Limonene	1034	0.22
36	Benzyl alcohol	1038	0.12
37	Ethyl-trans-hex-2-enoate	1047	< 0.1
38	Phenyl acetaldehyde	1049	0.12
39	5-Methyloctan-2-one	1054	tr
40	Oct-2-enal	1061	< 0.1
41	γ-Terpinene	1063	tr
42	Oct-2-en-1-ol	1068	tr
43	Octanol	1073	0.3
44	Cis-Linalool oxide	1078	< 0.1
45	Non-1-en-3-ol	1082	< 0.1
46	Trans-Linalool oxide	1093	0.1
47	P-Isopropenyl toluene	1094	tr
48	3-Nonanol	1096	< 0.1
49	Linalool	1103	0.34
50	Nonanal	1107	0.07
51	Oct-l-en-3-yl acetate	1109	tr
52	Heptyl acetate	1112	tr
53	2-Phenyl ethanol	1119	0.2
54	Cis-2,8-para-Menthadien-1-ol	1127	0.11
55	Menthadienol (isomer)	1141	0.1
56	Pinocarveol	1146	< 0.1
57	Camphor	1154	0.10
58	Nerol oxide	1156	tr

59	β-Pinene oxide	1160	< 0.1
60	Iso-Borneol	1165	tr
61	Borneol	1176	3.68
62	Terpinen-4-ol	1186	0.16
63	p-Cymen-8-ol	1191	< 0.1
64	α-Terpineol	1198	0.14
65	Myrtenol	1203	tr
66	Menthadienol (isomer)	1208	0.10
67	Decanal	1208	< 0.1
68	Octyl acetate	1213	< 0.1
69	Verbenone	1219	< 0.1
70	Carveol	1226	0.07
71	Nerol	1233	0.06
72	Thymyl methyl ether	1238	tr
73	Hexyl-2-methyl butyrate	1240	< 0.1
74	Hexyl-iso-valerate	1244	< 0.1
75	Neral	1247	< 0.1
76	Cuminic aldehyde	1250	< 0.1
77	Benzyl acetone	1250	tr
78	Carvone	1252	tr
79	Carvacryl methyl ether	1252	tr
80	Geraniol	1258	0.34
81	Phenylethyl acetate	1262	tr
82	Geranial	1275	0.06
83	Iso-Bornyl acetate	1295	tr
84	Thymol	1295	0.21
85	Nonyl acetate	1313	0.06
86	Unidentified sesquiterpene	1359	0.60
87	Geranyl acetate	1387	< 0.1
88	β-Patchoulene	1398	5.39
89	Isocomene	1403	0.62

90	Cyperene	1416	0.29
91	E-Caryophyllene	1424	< 0.1
92	β-Isocomene	1425	0.41
93	2,5-Dimethoxy-p-cymene	1435	19.00
94	β-Chamigrene	1459	< 0.1
95	Allo Aromadendrene	1475	< 0.1
96	γ-Decalactone	1478	0.20
97	β-Selinene	1503	0.50
98	α-Selinene	1511	0.13
99	Geranyl isobutyrate	1518	0.10
100	γ-Cadinene	1529	0.43
101	δ-cadinene	1536	0.08
102	Calamenene	1541	< 0.1
103	Elemole	1564	0.16
104	Neryl-2-methyl butyrate	1577	0.12
105	Neryl (iso) valerate	1590	0.56
106	Caryophyllene oxide	1608	17.61
107	Geranyl iso-valerate	1616	3.57
108	Tetra decanal	1619	0.09
109	Caryophyllene oxide (isomer)	1630	0.77
110	Diphenyl amine	1635	0.19
111	Longibornan-9-ol	1641	0.05
112	epi-γ-Cadinol	1658	1.93
113	β-Eudesmol	1672	1.94
114	14-Hydroxy-9-epi (E)-caryo-phyllene	1688	0.61
115	α-Bisabolol	1696	< 0.1
116	Heptadecane	1703	0.23
117	11-Hydroxy-eudesm-4-en-3-one	1915	< 0.1

b) Olfactoric properties

The odour of the oils is described in perfumery terms as woody-herbal. The predominant woody notes are indian woods, cedar wood and patchouli. The herbal notes are eucalyptus and rosemary. Undertones of oakmoss, spice, black pepper, birch tar and cade oil round off the odour.

GC-olfactometry revealed that the characteristic odour would be attributed to the combination of several key components. Borneol, thymol and camphor contributed to the herbal notes, while the predominant woody notes were associated with caryophyllene oxide, β-patchoulene and several minor sesquiterpenes that were not fully characterised. The 2,5-dimethoxy-p-cymene has a dry, woody, and slightly medicinal character.

The floral odour is the combined contribution of mono and sesquiterpenes. Of the monoterpenes, linalool which is present in this essential oil is the most prominent component which imparts floral odour. It is a constituent of rose, jasmine and neroli oil.[27]

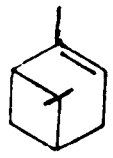
The green note exerted by this oil can be attributed to Hexane derivatives especially to cis-Hex-3-enol.[28] The oil of dried flowers of *Sphaeranthus indicus* contains 1.9% of cis-Hex-3-enol.

c) Pharmacological activity

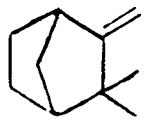
The antimicrobial activity of about one hundred perfume oils on different bacteria and fungi were investigated by J.C. Maruzzella and P.A. Henry.[29] They found that all of them exhibited bactericidal or bacteriostatic and fungicidal or fungistatic activity. As these oils contains volatile components such as terpenoids, the antimicrobial activity of these oils in the above mentioned study confirmed their activity.

It was found that some terpenic compounds such as α-pinene are effective in promoting recovery from fatigue. α-Bisabolol shows antiphlogistic activity. Monoterpenes like camphor and α-terpeneol exert insecticidal activity. The components like terpinen-4-ol, linalool, nerol, geraniol and α-pinene were reported to have antibacterial activity.[30,31]

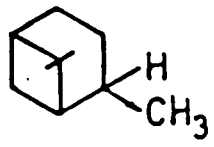
All the compounds mentioned above are constituents of the essential oil of *Sphaeranthus indicus*. So the medicinal property of this plant can be attributed to these compounds especially to 2,5-Dimethoxy-p-cymene (major component). Thus the use of this oil in Indian folk medicine as antibacterial, antifungal, and anthelmintic drug allows the conclusion that not only the less volatile constituents but also the volatiles are responsible for the above mentioned properties.



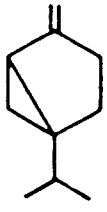
α-pinene



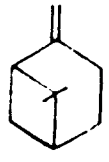
Camphene



Trans-Pinane

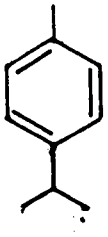


Sabinene

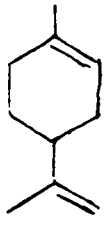


β-pinene

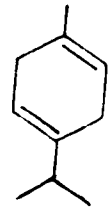
Myrcene



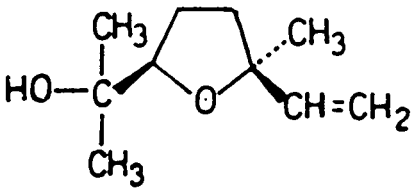
p-cymene



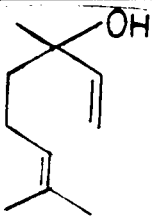
Limonene



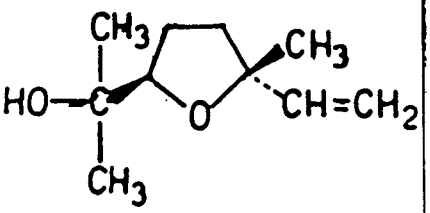
r-Terpinene



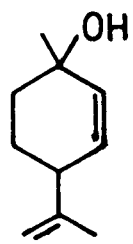
Cis-Linalool oxide



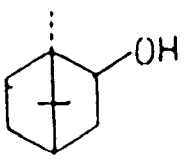
Linalool



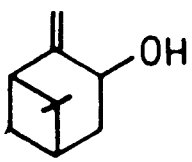
Trans-Linalooloxide



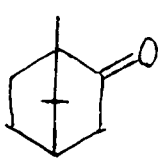
Cis-2,8-para-Menthadien-1-ol



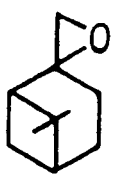
Iso-Borneol



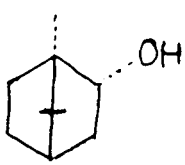
pinocarveol



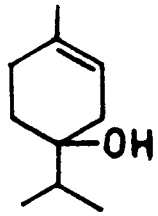
Camphor



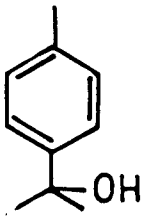
β-pinene oxide



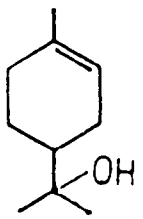
Borneol



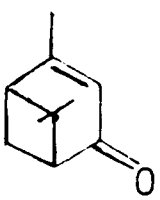
Terpinen-4-ol



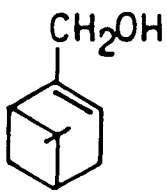
p-cymen-8-ol



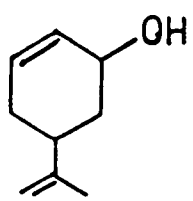
α-Terpineol



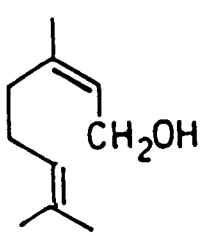
Verbenone



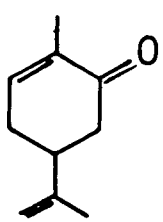
Myrtenol



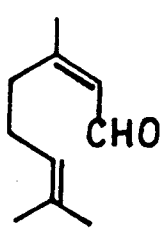
Carveol



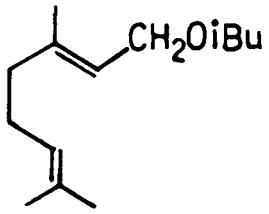
Nerol



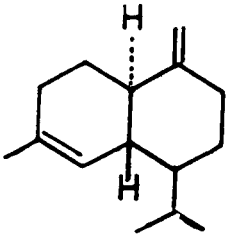
Carvone



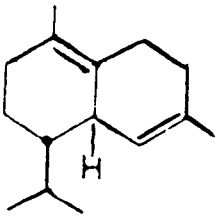
Neral



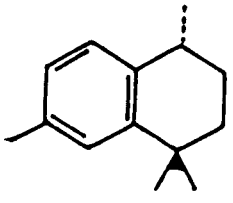
Geranyl isobutyrate



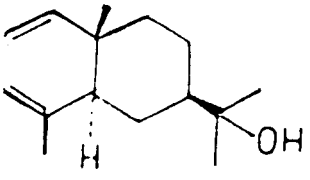
γ-cadinene



δ-cadinene



Calamenene



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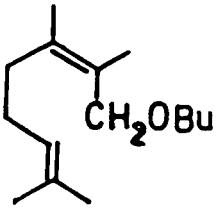
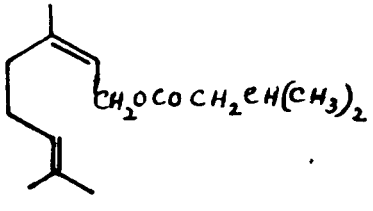
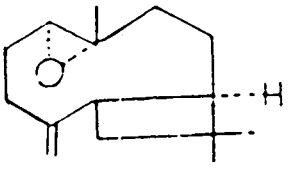


Neryl-2-methyl butyrate



Neryl-iso-valerate



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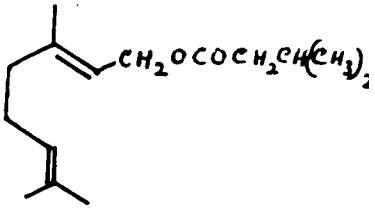

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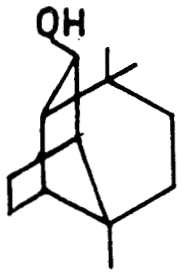
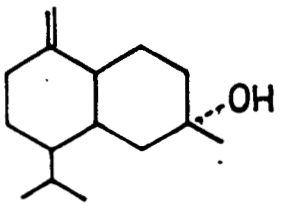
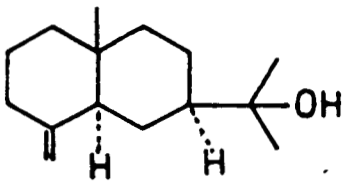


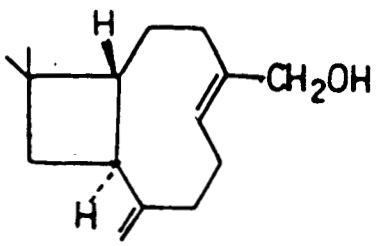
Longibornan-9-ol



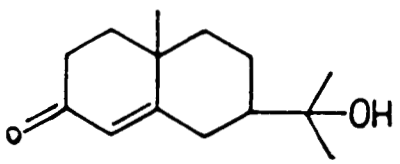
epi-γ-cadinol



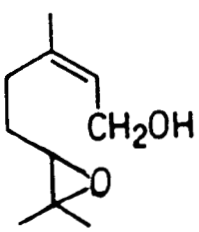
β-Eudesmol



14-Hydroxy-9-epi(E)-caryophyllene



11-Hydroxy-cudesm-4-en-3-one



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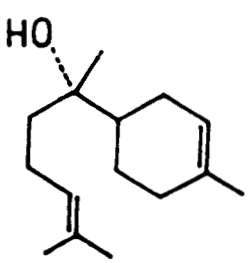
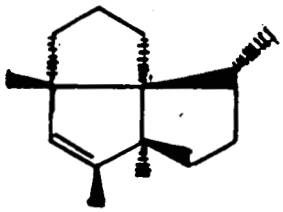
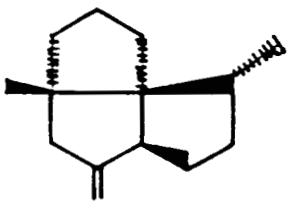


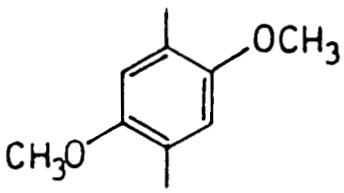
α-Bisabolol



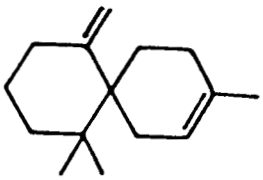
Isocomene



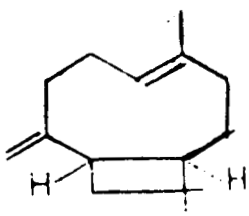
β-Isocomene



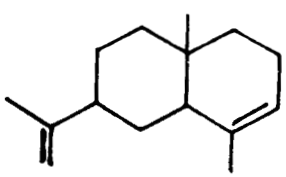
2,5-Dimethoxy-p-cymene



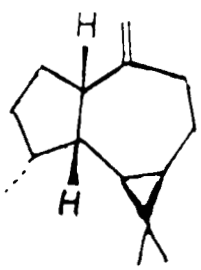
β-chamigrene



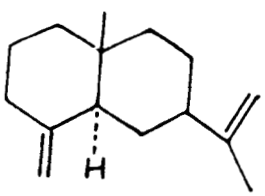
E-caryophyllene



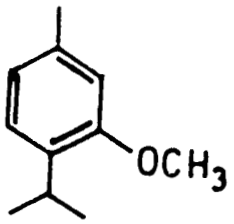
α-Selinine



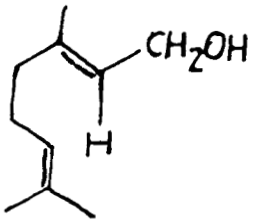
Allo Aromadendrene



β-Selinene



Thymyl methyl ether



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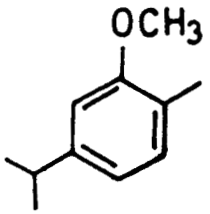

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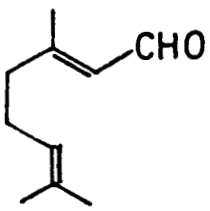
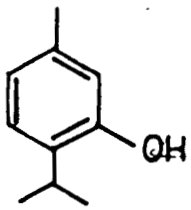


Geranial



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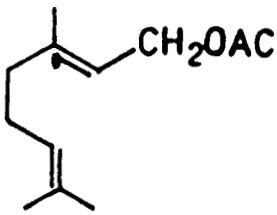
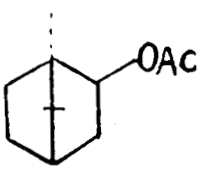
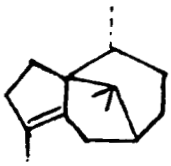


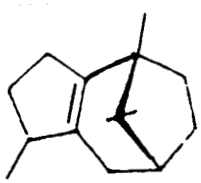
Geranyl acetate



Iso-Bornyl acetate



Cyperene



β-patchoulene

CHAPTER II

ANALYSIS OF ESSENTIAL OILS OF *VITEX NEGUNDO*, VAR. *NEGUNDO* & *VITEX NEGUNDO*, VAR. *PURPURESCENS*

Section I. The Phytochemistry of Vitex Species - A Review

Vitex is one of the genera of the large family verbenaceae[32] which comprises about 100 genera and more than 2600 species. They are found in tropics and subtropics as herbs, shrubs or trees. A few species of this genus are reported to contain essential oil. *Vitex negundo* is found in Pakistan and is also cultivated as an ornamental tree. The two shrubs *V. trifolia* and *V. negundo*,[33] have identical properties, are described by Sanskrit writers under the name '*Nirgundi*'. Some members of this genus are reported to be medicinally important and used for various treatments. Infusion, decoction etc., derived from some of them are used for many therapeutic purposes. Infusion and extract of leaves or bark of *V. peduncularis*[34] are useful in malaria fevers. This drug is nontoxic, not bitter, mildly stimulant and diuretic.

V. agnus-castus[35,36,37] possess anthelmintic properties and its extract stimulated Oogenesis in worker bees. The alcoholic and ethereal extracts of the dried leaves of *V. agnus-castus*[38] inhibited the growth of *Micrococcus*

pyogenes. Infusion of leaves of *V. peduncularis* on administration intramuscularly or orally in rabbits increases the osmotic resistance of the cell and inhibits hemolysis by saponin, cobra venom, bile salts and saline solution. Certain drugs prepared from *V. negundo*[39] exhibited antiarthritic effect in rats. A flavone[40] namely 5,7,3' - trihydroxy-6,8,4' - trimethoxy-flavone isolated from the seeds of *V. negundo* possessed estrogenic properties. The essential oil[41,42] extracted from the plant *V. negundo* was extremely effective in causing knock-down and inactivation of *Sitotroga cerealella* (a pest in stored rice). Combined p-cymene and β-pinene caused the greatest inactivation and mortality of the insects. Some researchers of the University of Philippines[43] developed an analgesic drug from *V. negundo* using the decoction of its leaves. This drug possess curative effect in case of fever, toothache and headache.

The leaves of *V. trifolia* are acrid, pungent, anthelmintic, improve memory and favour the growth of hair. The fruit is emmenagogue, the root is tonic, expectorant and febrifuge. The powdered leaves had been given with success in cases of intermittent fevers and the flowers are prescribed with honey in fevers accompanied with vomitting and severe thirst. The root of *V. negundo* is an antidote to snake venom. The root is also

considered tonic, febrifuge and expectorant. The leaves are aromatic, tonic and vermifuge. A pillow stuffed with the leaves of nirgundi is placed under the head for the relief of headache.[44] Flowers are used in diarrhoea, cholera, diseases of the liver and are also recommended as a cardiac tonic.

In Mysore febrile, catarrhal and rheumatic affections are treated by means of a vapour bath prepared from the leaves of *V. negundo* and also of *V. leucoxylon*.

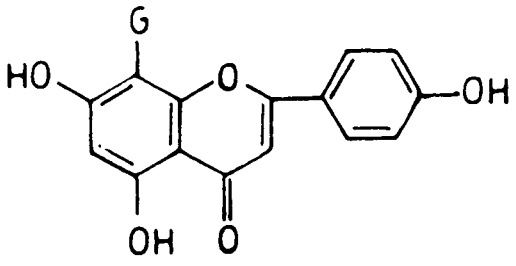
Verbenaceae family as a whole and vitex genus in particular, are rich sources of secondary metabolites of varying categories. They include proanthocyanin, flavones, flavonols, flavonones, and p-coumaric, p-hydroxybenzoic, ellagic and vanillic acids. Some other species contain chalcones, iridoids, and ferulic acid. Some triterpenoids also has been isolated from *V. peduncularis*. The following are the important classes of compounds isolated from various species of vitex.

Flavonoids

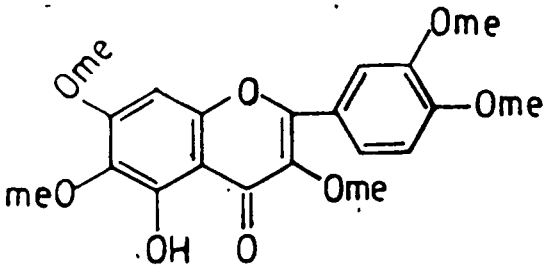
Flavonoids are a group of natural plant pigments. Structurally they are pyrone pigments. Numerous flavonoids have been isolated from various plants belonging to vitex genus.

Table II.1. Flavonoids of vitex species

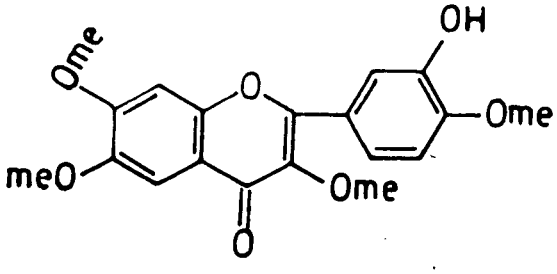
	Name	Structure	Reference	Source
1	5-Hydroxy-3,6,7,3',4' pentamethoxyflavone (Artemetin)	(11)	45 46 47	*V. negundo* *V. rotundifolia* *V. cannabifolia*
2	6-C-Glycosyl-5-O-rhamnopyranosyl trimethoxy wogonin		48	*V. negundo*
3	Acerosin-5-O-glucoside monacetate		48	*V. negundo*
4	5,3'-Dihydroxy-7,8,4' trimethoxy flavanone		49	"
5	5,3'-Dihydroxy-6,7,4' trimethoxy flavanone		49	"
6	3,5-Dihydroxy 3',4',6,7-tetramethoxy flavanol		50	"
7	Vitexin	(12)	51 52	*V. peduncularis* *V. lucens*
8	Casticin	(13)	53 " " 46	*V. agnus-castus* *V. negundo* *V. trifolia* *V. rotundifolia*
9	Luteolin-7-glucoside		54	*V. agnus-castus* *V. negundo* *V. trifolia* *V. lucens*
10	Di-C-glucopyranosyl luteolin		55	*V. lucens*
11	Vitexicaprin (3',5-di hydroxy-3,4'6,7-tetramethoxyflavone)	(14)	56	*V. trifolia*
12	Peduncularisin	(15)	57	*V. peduncularis*


G
HO
O
OH
OH
O

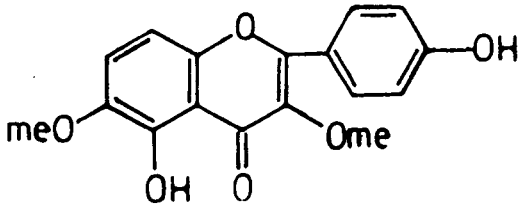
(12)


Ome
Ome
Ome
meO
Ome
O
OH
O

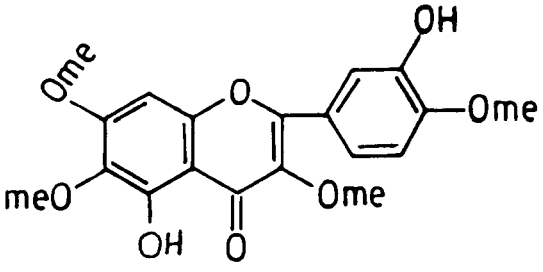
(11)


OH
Ome
Ome
meO
Ome
O

(13)


O
OH
meO
Ome
OH
O

(15)


OH
Ome
O
Ome
meO
Ome
OH
O

(14)

Anthocyanins

Anthocyanins are another group of plant pigments based on benzopyrilium or flavylium chloride structure. These pigments are responsible for the intense colour of flowers, leaves and fruits of various plants. Numerous shades of colours are due to same skeletal structure, but different substitution. A few anthocyanins have been isolated from different vitex species. Subramanian and coworkers[58] isolated two leuco-anthocyanidins from the stem bark of *V. negundo*. They are methyl esters of leucodelphinidin and leucoanthocyanidin-7-0-rhamnoglucoside.

Fatty acids, amino acids, terpenoids and steroids

β-Sitosterol, the commonly occuring plant sterol has been isolated from various members of this genus. Mukherjee and coworkers[59] isolated hentriacontane, β-sitosterol, β-sitosterol acetate and stigmasterol from V. negundo roots. β-Sitosterol also was obtained from the bark of *V. negundo* together with vanillic and p-hydroxybenzoic acid and luteolin.[60] p-Hydroxybenzoic acid also has been isolated from various parts of *V. negundo* and also from the leaves of *V. cannabifolia*.[47] From the leaves of *V. negundo* β-sitosterol was isolated together with 5-hydroxyisophthalic acid

and 3,4-dihydroxybenzoic acid.[61] Gupta and coworkers[62] isolated p-hydroxybenzoic acid from the seeds of *V. negundo.* β-Sitosterol is a common constituent of *V. lucens* and *V. trifolia.* Gupta and coworkers[63] determined the amino acid composition by descending paper chromatography and identified the different amino acids as glycine, alanine, valine, and leucine.

Essential oils

Many members of vitex genus are fragrant due to the presence of essential oils. The constituents of these oils are usually either well known monoterpenes and sesquiterpenes or aromatic compounds. A large number of species are studied for their essential oil components. Jufen and coworkers[64] found that the essential oils from various vitex species grown in china contained α-phellandrene, α-pinene, selinine, 1,8-cineole, β-pinene, β-caryophyllene and caryophyllene oxide. They observed that β-caryophyllene was the main constituent of the essential oil from *V. negundo,* var. cannabifolia, where as α-pinene was the main component in *V-trifolia.*

Higa and coworkers[65] isolated and identified the various terpenoids in the essential oil of the leaves of *V. trifolia* as α-pinene, β-pinene, 1,8-

cineole, and α-terpineol. The first work on the composition of essential oil of *V. negundo* is done by Basu and coworkers.[66] They identified only cineole in the oil. In 1979 Taneja and coworkers[67] investigated the composition of leaf essential oil of *V. negundo* and identified about 20 components in the oil which are α-pinene (0.16%), δ-3-carene(trace: t), limonene (2.84%), camphene (0.86%), β-phellandrene (17%), methylheptenone (3.47%), p-cymene (.76%), linalool (2.27%), camphor (.96%), 1,8-cineole (1.33%), 4-terpineol (5.79%), α-terpineol (t), citral (1.35%), caryophyllene (.33%), geraniol (t), caryophyllene oxide (2.08%), terpenyl acetate (1.59%), geranyl acetate (t), benzaldehyde (0.18%) and cinnamaldehyde (10.95%). In 1982 Manalo and coworkers[68] isolated the following compounds from the essential oil of *V. negundo* growing in Philippines. They are α-pinene, camphene, caryophyllene, citral and two unidentified components. In 1992 G.R. Mallavarapu and coworkers[69] showed difference in the composition of oil of *V. negundo* from earlier reports. They detected the presence of α-guaiene, α-gurjunene, gemacrene-D, elemol, spathulenol, globulol, viridiflorol, bis [1,1-dimethyl] methyl phenol, and abieta-7,13-diene for the first time in *V. negundo* leaf oil. Limonene, 1,8-cineole, citral and cinnamaldehyde which were reported as major constituents of the oil by

earlier investigators could not be detected in this study. β-Caryophyllene was reported as a minor constituent of the oil of Indian origin by Taneja and coworkers where as it was one of the main constituents of the oil in the work of others.

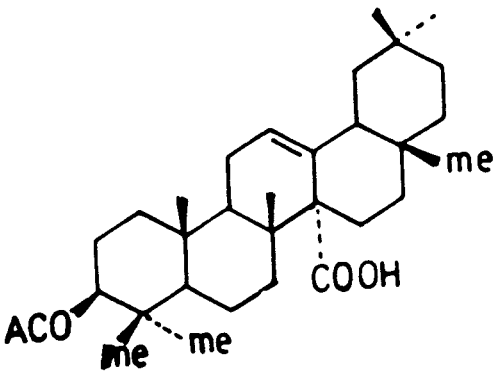
Section 2: Analysis of essential oils of *vitex negundo*, var. *negun do&* *vitex negundo*, var. *purpurescens*

II.1. Introduction

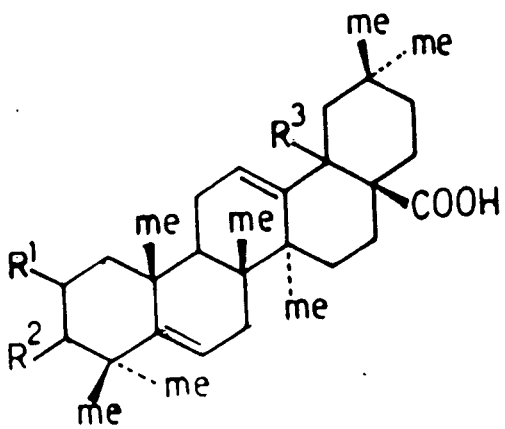
Vitex negundo (Verbenaceae) is a large deciduos shrub or small tree up to 6 m high with whitish-hairy branches and a trunk with a thin gray bark. The leaves are digitate with 3-5 lanceolate long-stalked leaflets, with dense gray-matted hairs beneath, opposite long-stalked. The calyx is bell-shaped, white-wooly, with 5 teeth. The corolla tube is short, hairy, with 5 unequal lobes, stamens, and style exserted.[70] Various compounds of different parts of the plant are of considerable medicinal importance and used for various treatments.[71-76] The leaves are reported to possess insecticidal, antifilarial and fungicidal properties.[77-82] According to literature this plant is used in Indian folk medicine for the treatment of febrile, catarrhal and rheumatic affections.[83] The plant is variously reported to possess insecticidal activity against stored product pests[84,85] mosquito larvae, house flies[86] and tobacco leaf eating larvae[87]. The oily liquid obtained from steam distillation of dry leaves of *V. negundo* showed mosquito repellent activity[88] lasting for a duration of 0.5-1 hr.

The chloroform extract of the defatted seeds of *vitex negundo* exhibited antiinflammatory activity and yielded four triterpenoids[89]. They are 3β-acetoxyolean-12-en-27-oic acid (16), 2α, 3α-dihydroxyoleana-5,12-dien-28-oic acid (17), 2β, 3α-diacetoxyoleana-5,12-dien-28-oic acid (18), and 2α-3β-diacetoxy-18-hydroxyoleana-5,12-dien-28-oic acid (19).

It was also reported that the unsaponifiable matter from the oil of seeds of vitex negundo contains a antiinflammatory diterpene 5β-hydro-8,11,13-abietatrien-6α-ol(20), a triterpene, lanostan-8,25-dien-3β-ol and a flavanoid, artemetin(11).



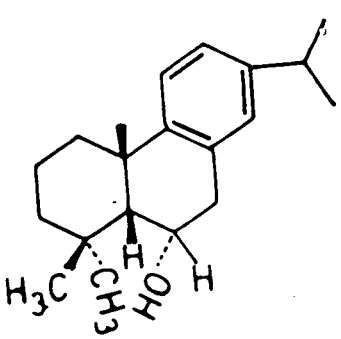
(16)



(17) $R^1=R^2=\alpha$-OH $R^3=H$

(18) $R^1=\beta$-OAC, $R_2=\alpha$-OAC, $R^3=H$

(19) $R^1=\alpha$-OAC, $R^2=\beta$-OAC, $R^3=OH$



(20)

II.2. The aim and scope of present work

Literature survey revealed that no data were available on a comparative investigation on the volatiles of the leaves of *vitex negundo var. negundo* and *V. negundo* var. *purpurescens*. Therefore the aim was to identify the essential oil volatiles of both varieties and to discuss the effects of identified single components on the characteristic odour as well as on the folk medicinal applications.

II.3. Materials and Methods

Fresh leaves were collected from Kerala, South-India. The shade dried leaves (300 g) were powdered in an electric mixer grinder and subjected to steam distillation for 3 hrs. The distillate was extracted with solvent ether (3 x 100 mL). The ether portions thus obtained were pooled together and dried with anhydrous sodium sulphate. On evaporation of the dry ether extract after removal of sodium sulphate, over a water bath yielded [0.32%, (*V. negundo* var. *negundo*) and 0.13% (*V. negundo* var puepurescens] of the essential oils.

Gas chromatographic analysis of the volatiles of the leaf essential oil was carried out using Shimadzu 14A (FID) gas chromatograph with

hydrogen as carrier gas. For GC-MS a Shimadzu 17A GC-QP 5000 instrument with helium as carrier gas was employed. The mass spectra were recorded in the 41-450 amu range (E1 mode, 70 e.v) Hewlett-packard GC-IRD/MSD instrument with IR range of 4000-850 cm^{-1} (MCT detector) was used for the GC-IR-MS analysis, 41-450 amu being the MS range. Columns for all the above analysis were 30 m x 0.32 mm bonded FSOT-RSL-200 fused silica (film thicknes; 0.25 μm, Biorad Co) and 30 m x 0.32 mm bonded stabil wax fused silica (film thickness 0.5 μm, Restek Co).

Temperature programme

The sample was kept at 40°C for 5 min and then heated to 280°C at a rate of 6°C/min.

II.4. Results and Discussion

(a) Identification of components

89 compounds are identified in the gas chromatogram of oil of *Vitex negundo* var. *negundo* and 76 compounds in the variety *purpurescens*. The main constituents of the variety negundo were terpinen-4-ol (34.63%), β-

caryophyllene (10.8%), globulol (9.64%), bis [1,1-dimethyl]methylphenol (4.11%), β-farnesene (3.79%) and sabinene (3.77%).

The main constituents of the variety purpurescens were terpinen-4-ol (25.81%), globulol (16.62%), β-caryophyllene (7.99%), p-cymene (7.06%), α-terpineol (4.84%) and spathulenol (4.81%) . Monoterpenoids constitute about 20% of the total components of the variety negundo and 17% in the variety purpurescens where as sesquiterpenoids constitute 10% in the variety negundo and 13% in the variety parpurescens. All the identified components and their percentages are listed in table II.2 and the structures of terpenoids are also provided.

Table II.2. Essential oil components of the leaves of *Vitex negundo var. negundo* (A) and *Vitex negundo var. purpurescens* (B)

Components	A	B	KI	Identification
Ethyl acetate	0.02	0.01	592	GC, GC-MS
2-Methylfurane	0.01	--	611	GC, GC-MS
2,3-Pentandione	0.02	0.03	678	GC, GC-MS
Butyl acetate	--	0.01	790	GC, GC-MS
(E)-2-Hexenal	0.12	0.09	829	GC, GC-MS
(E)-2-Hexenol	0.18	0.03	852	GC
Hexanol	0.22	0.42	857	GC, GC-MS

2-Acetylfurane	0.04	0.02	888	GC, GC-MS
α-Thujene	0.33	0.51	927	GC, GC-MS
1,-3-Butandiol	0.01	–	933	GC
α-Pinene	0.42	0.83	937	GC, GC-MS
Benzaldehyde	0.21	0.05	943	GC, GC-MS
Camphene	0.17	1.03	950	GC, GC-MS
I-Octen-3-ol	0.42	0.23	964	GC
Sabinene	3.77	1.29	973	GC, GC-MS
β-Pinene	0.82	0.59	981	GC, GC-MS
Myrcene	1.33	0.42	988	GC, GC-MS
α-Phellandrene	0.13	2.73	999	GC, GC-MS
3-Carene	0.09	1.02	1010	GC, GC-MS
α-Terpinene	1.67	0.93	1015	GC, GC-MS
p-Cymene	2.11	7.06	1019	GC, GC-MS
Limonene	1.14	0.78	1025	GC, GC-MS
1,8-Cineole	0.03	143	1029	GC, GC-MS
β-Phellandrene	1.04	0.14	1030	GC
Benzylalcohol	0.52	0.20	1036	GC
(E)-β-Ocimene	0.02	0.22	1039	GC
γ-Terpinene	2.02	0.03	1055	GC, GC-MS
trans-Sabinene hydrate	0.34	–	1060	GC, GC-MS
cis-Linalool oxide	0.23	0.96	1065	GC, GC-MS
trans-Linalool oxide	0.07	0.21	1075	GC, GC-MS
Terpinolene	0.91	0.10	1082	GC, GC-MS

Linalool	1.43	0.66	1090	GC, GC-MS
cis-Sabinene hydrate	0.21	--	1092	GC
Phenylethylalcohol	0.08	0.11	1104	GC, GC-MS
Nonanal	0.55	0.09	1111	GC
cis-p-Menth-2-en-1-ol	0.06	0.18	1113	GC, GC-MS
Camphor	0.42	1.44	1125	GC, GC-MS
Citronellal	0.06	0.42	1143	GC, GC-MS
Borneol	0.03	--	1154	GC, GC-MS
Terpinen-4-ol	34.63	25.81	1175	GC, GC-MS
α-Terpineol	2.22	4.84	1182	GC, GC-MS
Verbenone	0.24	0.04	1186	GC, GC-MS
Citronellol	0.35	0.07	1212	GC, GC-MS
Geraniol	0.58	0.12	1242	GC, GC-MS
Linalyl acetate	0.33	0.05	1249	GC, GC-MS
cis-Sabinyl acetate	0.05	0.21	1262	GC
Bornyl acetate	0.27	0.01	1277	GC, GC-MS
Terpinen-4-yl acetate	0.11	0.08	1281	GC, GC-MS
α-Cubebene	0.04	--	1360	GC
α-Ylangene	0.19	0.02	1369	GC
α-Copaene	0.05	0.74	1378	GC, GC-MS
β-Bourbonene	0.33	--	1386	GC
β-Elemene	0.10	0.22	1399	GC, GC-MS
α-Gurjunene	0.29	0.24	1405	GC, GC-MS
β-Caryophyllene	10.81	7.99	1432	GC, GC-MS

α-Bergamotene	1.09	0.88	1437	GC
β-Farnesene	3.79	0.61	1448	GC, GC-MS
α-Guaiene	0.06	--	1454	GC
α-Humulene	0.67	1.09	1465	GC, GC-MS
Germacrene D	1.22	2.09	1469	GC, GC-MS
β-Selinene	0.10	--	1477	GC
Viridiflorene	0.28	--	1483	GC, GC-MS
γ-Elemene	0.02	0.08	1497	GC
β-Bisabolene	0.14	0.23	1499	GC
γ-Cadinene	0.06	0.88	1506	GC, GC-MS
Elemol	0.77	0.08	1538	GC
(Z)-Nerolidol	0.28	1.42	1543	GC, GC-MS
Spathulenol	1.65	4.81	1562	GC, GC-MS
Globulol	9.64	16.62	1588	GC, GC-MS
Ledol	1.15	2.43	1592	GC, GC-MS
Viridiflorol	1.10	0.49	1596	GC, GC-MS
γ-Eudesmol	0.05	--	1611	GC
T-Cadinol	0.18	--	1624	GC
α-Cadinol	0.14	--	1641	GC, GC-MS
β-Eudesmol	0.07	--	1644	GC
α-Bisabolol	0.04	1.26	1655	GC
β-Bisabolol	0.01	0.26	1667	GC, GC-MS
(Z)-α-Bergamotol	0.06	--	1692	GC, GC-MS

Bis[1,l-dimethyl] methylphenol	4.11	–	1860	GC, GC-MS
Higher hydrocarbons (hexadecane, 9-hexadecene, heptadecane, octadecane, nonadecane, eicosane, α-kaurene and abieta-7, 13-diene (*V. negundo* var. *negundo)*	0.73			
(hexadecane, 9-hexadecene, heptadecane, octadecane, nonadecane, eicosane, α-kaurene, kaurane, and tricosane (*V. negundo* var. *purpurescens)*		1.59		
Fatty acids and their esters myristic acid, palmitic acid and palmitic acid methyl ester (*V. negundo* var. *negundo)*	0.12			
palmitic acid, stearic acid, oleic acid and palmitic acid methyl ester (*V. negundo* var. *purpurescens)*		0.19		

In comparison to data published earlier the essential oils of the leaves of *V. negundo,* var. negundo showed nearly the same qualitative composition (only β-farnesene was not detected earlier) while the concentrations, especially of main compounds, differ significantly. The

concentrations of terpinen-4-ol is 34.63% (in the present case) [13.25%][69], β-caryophyllene 10.81% [13.65%][69], globulol 9.64% [17.27%][69], bis[1,1-dimethyl] methyl phenol 4.11% [7.48%][69] and Sabinene 3.77% [11.19%][69] were observed.

It was found that there is some striking similarities between essential oils of these two plants. Some of the main components which are identical in two varieties are terpinen-4-ol, globulol and β-caryophyllene. But at the same time some differences between the two are also noticed. Some sesquiterpenes like α-cubebene, β-bourbonene, γ-eudesmol, cadinol, and Bis [1,1-dimethyl]methylphenol were found missing in the variety purpurescens. And also the concentrations of p-cymene, α-terpineol, and spathulenol were much higher in the oil of purpurescens variety. Ledol was identified for the first time in an essential oil of *V. negundo* from India.

(b) Olfactoric properties

The odour of the essential oils was described by professional perfumers as follows:- For *vitex negundo* var *negundo*; it was hay-grasslike, herbal, spicy, later sage-clary like.

For *Vitex negundo* var. *purpurescens* it was spicy, weak camphor-like, later sweet woody. In the case of negundo variety the hay-grass like odour impression is attributed to hexane derivatives, herbal-spicy to mono and sesquiterpene hydrocarbons and the sage-clary odour partly to linalool derivatives.

In the case of purpurescens variety a mixture of linalool, pinenes, phellandrenes and γ-terpinene shows a spicy odour impression[90]. The weak camphor note comes from camphene and camphor, and the sweet-woody odour from caryophyllene and further sesquiterpenes.

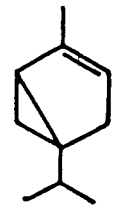
Medicinal Properties

According to folk medicinal system these plants are used in the treatment of febrile, catarrhal and rheumatic affections.[69] Some volatiles of the essential oils of leaves of these vitex species play an important role. Camphene, pinene and terpene derivatives (partly because of their hyperamical potential) are effective in antirheumatic applications while linalool and bisabolol derivatives are well-known antispasmolytics and α-bisabolol shows antiphlogistic activity. The mono and sesquiterpenes having bactericidal properties are linalool, camphor, geraniol and α-

terpineol as well as β-caryophyllene, nerolidol and cadinene derivatives. They also possess fever-depressing and expectorating effects.[5, 6,30,31, 91-93]

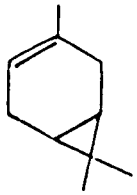
Some identified essential oil volatiles of the leaves of the investigated vitex species may also take part in the insecticidal properties of *Vitex negundo*. The monoterpenes camphor, linalool, α-terpineol and the sesquiterpene β-farnesene respectively have been reported to exert insecticidal and molluscicidal activity.[30,31]

As a result of this comparative investigation of the essential oils of the leaves of *vitex negundo var. negundo* and *vitex negundo* var. *purpurescens* it was found that these oils show different compositions in the quantity (Var. negundo) as well as in quantity and quality (Var. purpurescens). As main compounds of the essential oils the monoterpenes terpinen-4-ol, p-cymene, α-terpineol and sabinene as well as the sesquiterpenes β-caryophyllene, globulol, spathulenol, β-farnesene and bis [1,1-dimethyl] methyl phenol (only in the var. negundo) respectively could be identified in these vitex species. The characteristic odour and the reported folk medicinal use could also be attributed to some volatiles found in the essential oil samples.

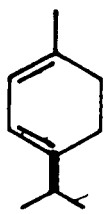


α-Thujene

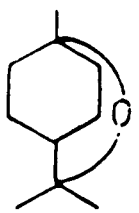
α-Phellandrene



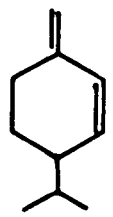
3-Carene



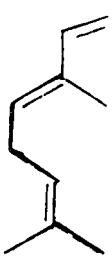
α-Terpinene



1,8-Cineole

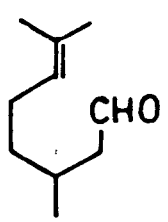


β-Phellandrene

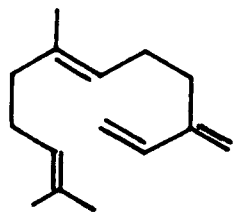


(E)-β-Ocimene

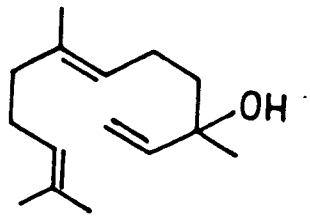
Terpinolene



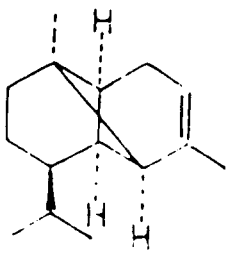
Citronellal



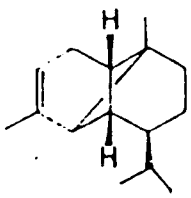
β-Farnesene



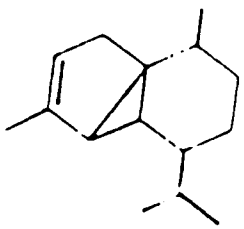
(Z)-Nerolidol



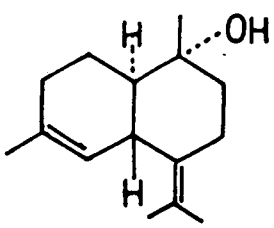
α-Ylangene



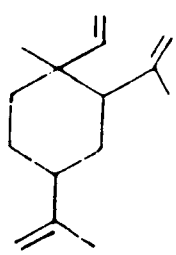
α-Copaene



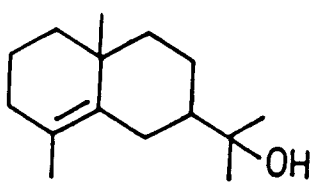
α-Cubebene



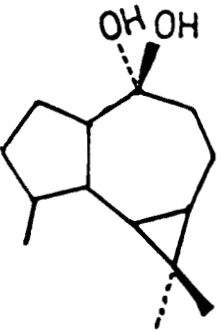
α-Cadinol



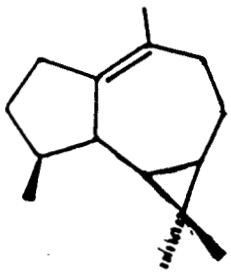
β-Elemene



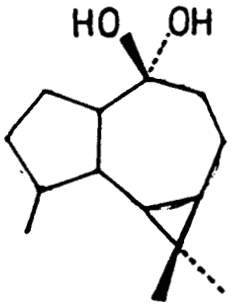
γ-Eudesmole



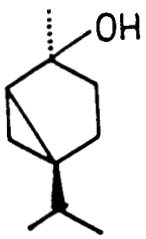
Viridiflorol



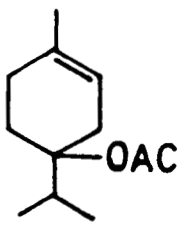
Viridiflorene



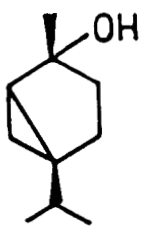
Ledol



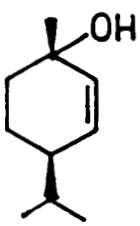
ans.Sabinene hydrate



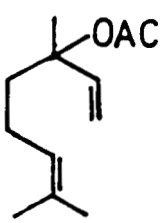
Terpinen-4-yl acetate



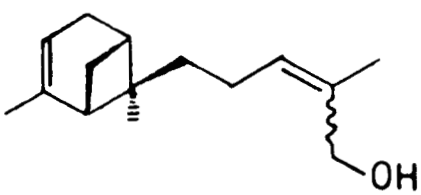
cis-Sabinene hydrate



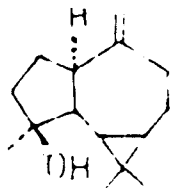
-p-Menth-2-en-1-ol



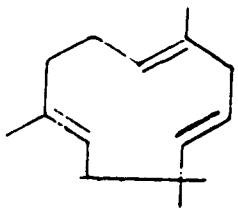
Linalyl acetate



(Z)- α-Bergamotol



Spathulenol



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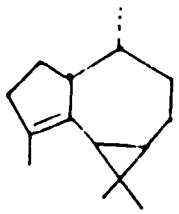

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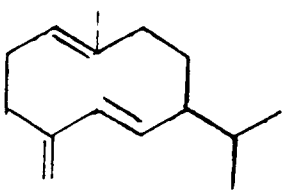

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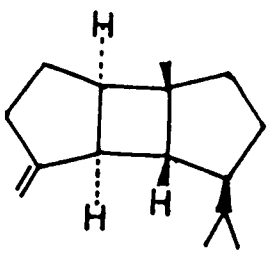
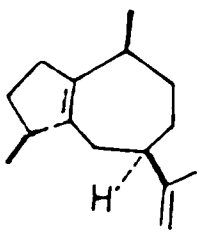
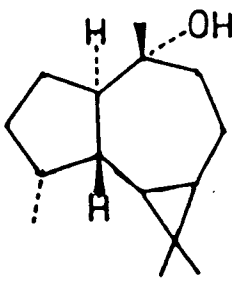


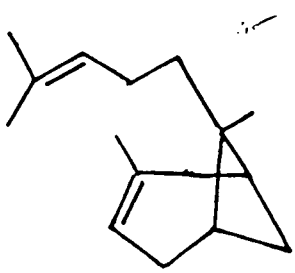
β-Bourbonene



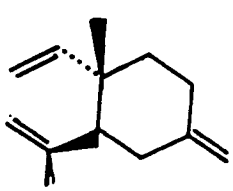
α-Guaiene



Globulol



α-Bergamotene



γ-Elemene

CHAPTER III

ANALYSIS OF *CURCUMA AERUGINOSA* LEAF ESSENTIAL OIL

III. I. Phytochemical studies on *Curcuma* (*Zingiberaceae*) species

A Review

Curcuma is one of the genera of a large family *Zingiberaceae*. They are rhizomatous herbs distributed in India, Japan, Malaysia and Vietnam. About 80 species of this occur in India of which a few are of economic and medicinal importance.[94] Rhizomes of *C. longa, C. zedoaria, C. xanthorhiza* and *C. aromatica* are used as oriental traditional medicines in China, Japan and south eastern Asia.[95]

C. longa syn. *C. domestica* is a perennial herb, 2-3 ft high with a short stem and tufted leaves. The rhizomes are short and thick and constitute the turmeric of commercial importance. Turmeric has been attributed a number of medicinal properties in the traditional system of medicine. It is used both as a colouring material and as a condiment.[94]

Turmeric is an auspicious article in all religious observances in hindu households. It is a normal constituent of condiments, curry powders and

prepared mustards. It is used also for dyeing wool, silk, and unmordanted cotton to which it imparts a yellow shade in an acid bath. It is used as a colouring matter in pharmacy, confectionery and food industry. Turmeric paper is an official reagent in the British pharmacopaeia for testing alkalinity. A diluted tincture of turmeric is suitable for use as a fluorescence indicator even in brown and yellow solutions.

Turmeric[96] is used to some extent as antiseptic cure for poisoning, eliminating body waste products for dyspesia, respiratory disorders and cure for a number of skin diseases including promotion of wound healing.

Curcumin, curcuminoids and essential oils are the major active constituents of turmeric. The main activities have been found to be anti-inflammatory, hepato-protective, antimicrobial woundhealing, anticancer, antitumour and antiviral. A decoction of the rhizome is said to relieve the pain of purulent ophthalmia. Oil of turmeric distilled from the dried rhizomes has feeble antiseptic properties.

It is an antacid and in small doses it act as a carminative, stomachic, appetiser and tonic. In large doses it appears to act as an antispasmodic inhibiting excessive peristaltic movements of the intestines.

Curcumin(diferuloylmethane)[1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] (21) is the major bioactive secondary metabolite found in the rhizomes of turmeric[97] (C. longa).

It is the major yellow pigment and active ingredient of turmeric which has got strong antitumour, anticarcinogenic and antiinflammatory activities.[98]

The essential oil from the rhizomes and leaves of *C. longa* were analysed by Sharma and coworkers.[99] The rhizome oil was found to contain more than 40 constituents, with major components α-turmerone (30-32%), ar-turmerone (17.26%) and β-turmerone (15-18%), where as the leaf oil contained about 60 compounds with major ones being α-phellandrene (18.2%), 1,8 cineole (14.6%) and p-cymene (13.3%).

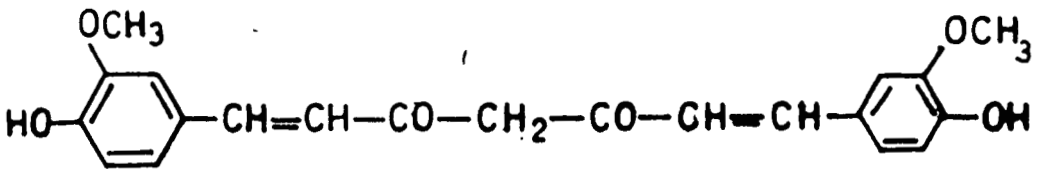
Rhizomes of *C. longa* and *C. aromatica* are well known for their uses. The leaves of both species contain volatile oils. Behura and coworkers[100] analysed the volatile oils of leaves of both species and reported that, the leaves of *C. longa* contains monoterpenes the major constituents being α-phellandrene (38.24%), C_8-aldehyde (20.58%), α-pinene and β-pinene. Where as *C. aromatica* has 1,8 cineole (28.01%), linalool (7.67%), α-pinene

(3.7%) and C_8-aldehyde (2.62%) as major constituents. The rhizomes of *C. aromatica* contain camphene, α-pinene, β-pinene, 1,8-cineole, isofuranogermacrene, borneol, isoborneol, camphor, germacrone and tetramethylpyrazine. α-Curcumene (22) and β-curcumene (23) are also constituents of essential oil of the rhizomes of *C. aromatica*.

Curcuma zedoaria closely resembles *C. longa* in appearence. Its dried rhizome known as *Zedoary* is a crude drug in Japan. A number of novel sesquiterpenoids were isolated from it by Asakawa and coworkers.[101] They include curcumenone (24) (a cyclopropano sesquiterpenoid) curcumanolide A, curcumanolide B, Zedoarol, 13-hydroxygermacrone, and curzeone.

Takeya and co-workers[102] isolated two guaiane sesquiterpene lactones namely zedoalactone A (25) and Zedoalactone B (26) and a guaianolide, zedoarondiol (27) from the dried rhizomes of *C. aeruginosa*.

C. xanthorrhiza Roxb., commonly known as Javanese turmeric in Indonesia[103] has been traditionally used for medicinal purposes and also used as a tonic in Indonesia. The extract of this plant can prevent the liver damage induced by acetaminophen and carbontetrachloride in mice. It has got a broad spectrum hepatoprotective activity.



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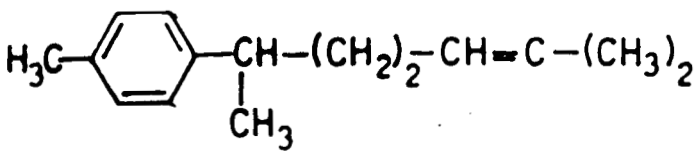

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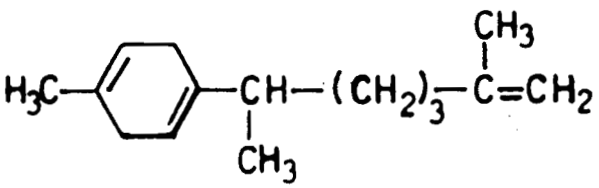

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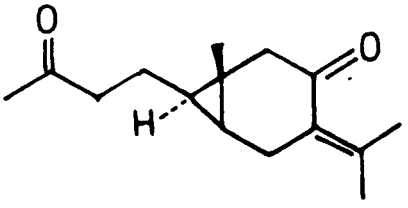

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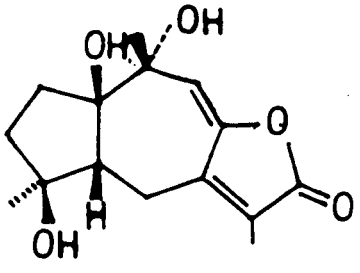

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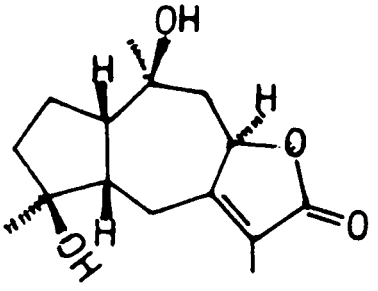

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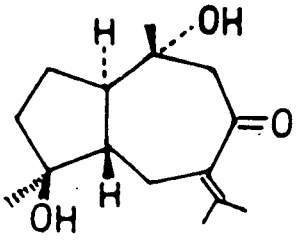


(27)

Moreover several non-phenolic linear 1,7-diarylheptanoids possessing antiinflammatory activity were isolated from *C. xanthorrhiza*.[104]

The same type of diarylheptanoids namely 1,7-diphenyl-5-hydroxy-(1E)-1-heptene, 7-(3,4-dihydroxyphenyl)-5-hydroxy-1-phenyl-(1E)-1-heptene, and 5-hydroxy-7-(4-hydroxyphenyl)-1-phenyl-(1E)-1-heptene were isolated from the ethyl acetate extract of *Curcuma comosa* rhizomes.[105]

Suksamran and coworkers[106] studied the choleretic properties of various extracts of *C. comosa* in rats and results suggest that this plant can be used as a source of choleretic and hypocholesterolemic drugs. A. phloracetophenone glucoside namely 4,6-dihydroxy-2-0-(beta-D-glucopyranosyl) acetophenone which isolated from the ehtyl acetate and n-butanol extracts was found to exhibit choleretic activity in rats. Suksamran and coworkers[107] also studied the hypolipidemic effect of ethyl acetate extract of the rhizome of *C. comosa* and found that it has got a very good hypolipidemic action. They also investigated[108] the uterotrophic activities of various extracts of *C. comosa* in rats and results suggested that the uterotrophic action of *C. comosa* is mediated through weak estrogenic agonistic activity of the plant. Anto and coworkers[109] investigated the antimutagenic and antipromotional activity of three natural curcuminoids

and some synthetic curcuminoids. The natural curcuminoids, curcumin. I. (Diferuloylmethane), curcumin II (feruloyl-p-hydroxycinnamoylmethane) and curcumin III (bis-(p-hydroxycinnamoyl) methane isolated from *Curcuma longa* were found to be potent inhibitors of mutagenesis and crotean oil induced tumour promotion. Among the synthetic curcuminoids, salicyl and anisyl curcuminoids were the most effective inhibitors. Piperonal curcuminoid also exhibited anti-promotional activity.

Two active compounds for programmed cell death (PCD) in human mycloid leukemia cells (HL-60) were isolated from the hexanic extract of the rhizome of C. *longa*.[110] They were identified as ar-turmerone and beta-atlantone.

III.2. The aim and scope of present work

C. aeruginosa Roxb; Zingiberaceae, is a herb native of Burma and also reported from Java and widely cultivated in Malaysia. This plant grows wildly in south India, especially in Kerala and Karnataka. C. *aeruginosa* is also known as C. *caesia*[111] or C. *malabarica*.[112] The leaves were subjected to steam distillation and the oil obtained was analysed by GC and GC-MS. The aim of the work was to identify the compounds present and compare

these with those reported in earlier works. The olfactoric property of this oil also was a subject of study.

III. 3. Materials and Methods

The fresh leaves were collected from the area around the Calicut University Campus. Fresh leaves (950 g) were cut into pieces and made into a paste in an electric mixer grinder. It was subjected to steam distillation for 5 h. The distillate was extracted with diethyl ether (3 x 100 mL). The ether portion after drying with anhydrous sodium sulphate on evaporation gave 0.14% of oil.

The volatiles of the leaf oil of *C. aeruginosa* were analysed by a combination of GC and GC-MS. GC analysis of the oil was carried out on a Shimadzu GC-14A(FID) and on a varian GC-3700 (FID) gas chromatograph using hydrogen as carrier gas. GC-MS analysis was carried out on a Shimadzu GC-17A/QP5000, on a HP-5890GC/HP-5970MSD and on a Finnigan MAT GCQ with helium as carrier gas. The MS range used was 40-450 amu (E1 mode, 70 e.v, and ion-source temperature -200°C).

The columns used for the measurements were 30 m x 0.32 mm bonded unpolar FSOT-RSL-200 (Biorad) and a 30 m x 0.32 mm bonded stabil wax fused silica column (Restek) respectively.

The temperature programme from 40°C for 5 min. to 280°C for 20 min at a rate of 6°C/min. The compound identification was partly possible by coinjection of pure compounds and correlation with published retention time data.

III. 4. Results and Discussion

a) Identification of Components

53 components could be identified in the leaf oil of *C. aeruginosa* by means of GC and GC-MS analysis, by comparing their data with those in the libraries of the respective instruments. Kovats indices of the identified compounds were found to correspond to their reported values.

This is the first report of the analysis of oil from leaves of *C. aeruginosa* from South India. Composition of the volatile oil of leaves of *C. aeruginosa* from Vietnam is already reported[113] and also oil from some of the plant parts, especially rhizomes of various origin (Indonesia, Japan, Malaysia, and Vietnam) were also analysed by various workers.[114] In the present analysis the major components identified were 1,8-cineole (17.7%) curzerenone (10.5%), furanogermenone (7.8%), camphor (7.5%), 3-hexen-1-ol (5.8%), furanodienone (5.1%), curcumenol (4.3%) isocurcumenol (3.7%)

and β-elemene (3.3%). Compared to earlier reports, some striking similarities has been found in the oil especially in the composition of main components (1,8-cineole, camphor and curzerenone). But the other main components (3-hexen-1-ol, β-elemene, furanodienone, isofuranodienone, curcumenol, isocurcumenol and furanogermenone) and some minor constituents differ significantly in composition from earlier reports. All the components identified are given in the table. The structure of terpenoids which are not given earlier are also provided.

Table III.I. **Identified compounds in the steam distilled essential oil of *Curcuma aeruginosa***

	Compound	% RPA
1	(E)-2-Hexenol	0.5
2	(Z)-3-Hexen-1-ol	5.8
3	(Z)-1-Hexen-3-ol	0.2
4	Hexanol	0.3
5	α-Pinene	0.3
6	Camphene	0.5
7	β-Pinene	1.0
8	Myrcene	0.3
9	1,8-Cineole	17.7
10	p-Cymene	0.1
11	cis-β-Ocimene	0.2

12	Limonene	0.2
13	trans-β-Ocimene	0.2
14	γ-Terpinene	0.6
15	cis-Linalool oxide	0.1
16	Terpinolene	0.5
17	Linalool	2.7
18	α-Thujone	1.1
19	Fenchol	0.2
20	trans-Tagetenone	0.1
21	Camphor	7.5
22	trans-Pinocarveol	0.2
23	trans-Verbenol	0.1
24	Isoborneol	0.9
25	Borneol	1.3
26	Terpinen-4-ol	1.0
27	Myrtenal	0.3
28	α-Terpineol	1.4
29	cis-Carveol	0.4
30	Carvone	0.8
31	Pulegone	0.1
32	β-Cubebene	0.3
33	δ-Elemene	0.2
34	β-Elemene	3.3
35	Caryophyllene	2.7
36	trans-β-Farnesene	0.3
37	Allo aromadendrene	0.4

38	α-Selinene	0.8
39	β-Selinene	2.4
40	Curzerenone	10.5
41	Epi-curzerenone	1.0
42	Furanodienone	5.1
43	Isofuranodienone	0.4
44	Germacrone	1.3
45	Curcumenol	4.3
46	Iso curcumenol	3.7
47	Zedoarol	1.2
48	Curdione	1.4
49	Furanogermenone	7.8
50	Curcumanolide A	0.2
51	Curcumanolide B	0.1
52	Curzeone	0.4
53	Phytol	0.9
54	Other compounds	4.6

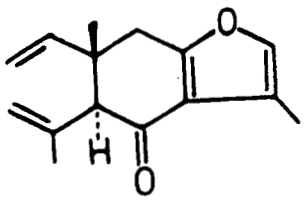
b) Olfactoric properties

The leaf oil was investigated by GC-sniffing technique to get information about the components which were responsible for the characteristic odour impression of the sample. The odour was olfactorically described as fresh-camphoraceous (eucalyptus like), green (fresh leaves),

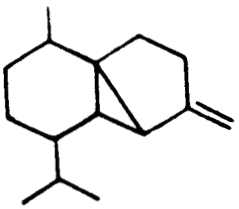
spicy (direction of dill), fine woody and weak aromatic. The correlation substance-peak to odour property showed that monoterpenes like 1,8-cineole was responsible for the dominating fresh-camphoraceous odour notes.

Green note exerted by this oil is attributed to hexane derivatives especially to 1-hexen-3-ol.[28] This oil contains the isomer cis-3-hexen-1-ol (5.8%) which is responsible for the green note exerted by this oil. Green note is an odour which is reminiscent of green foliage such as leaves, stalks and green vegetables and the smell of freshly cut grass. The compound which is released when a lawn is mowed is cis-3-hexen-1-ol.[4] The green odour of the trans-isomer is less pronounced. Generally hexane derivatives impart this odour. The leaf oil of *C. aeruginosa* contains four hexane derivatives including (z)-3-hexen-1-ol (5.8%).

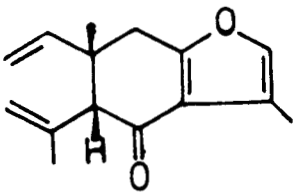
The woody odour describes the odour of not only the essential oils derived from various trees such as cedar wood and sandal wood but also some oils derived from leaves like patchouli and grass like vetiver.[4] In this study pinene derivatives were responsible for the fine woody odour.



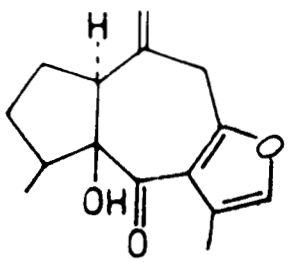
Curzerenone



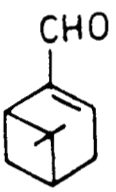
β-Cubebene



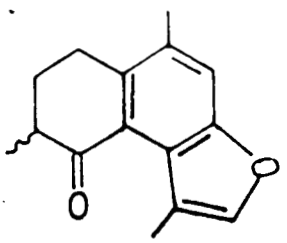
epi-Curzerenone



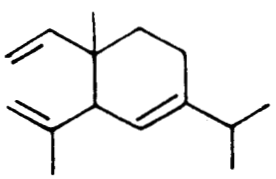
Zedoarol



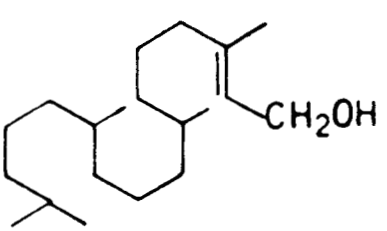
Myrtenal



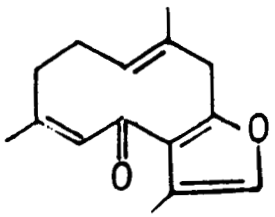
Curzeone



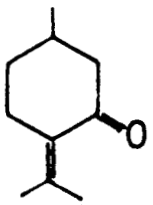
δ-Elemene



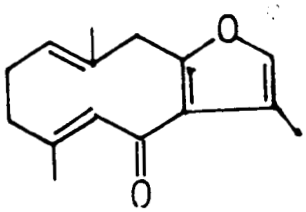
Phytol



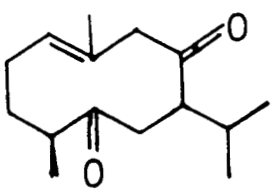
Isofuranodienone



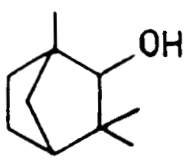
Pulegone



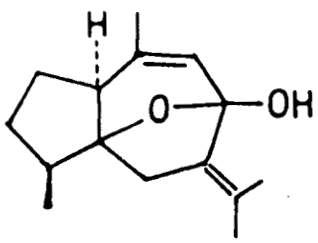
Furanodienone



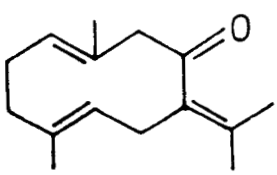
Curdione



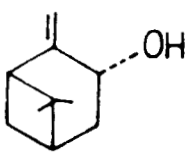
Fenchol



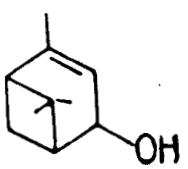
Curcumenol



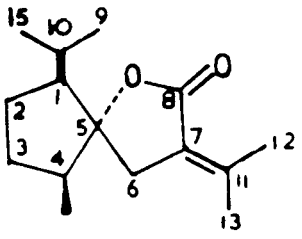
Germacrone



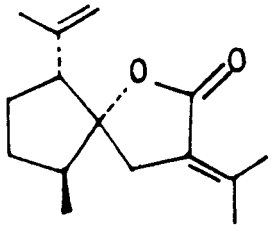
Trans-pinocarveol



Trans-Verbenol



Curcumanolide A



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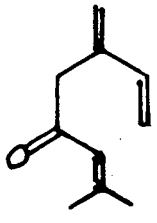
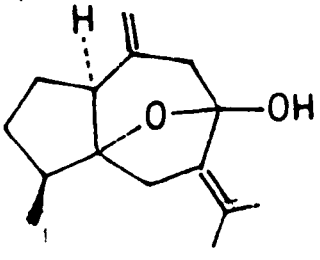
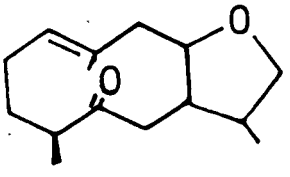


Trans-tagetenone



Isocurcumenol



Furanogermenone

CHAPTER IV

ANALYSIS OF ESSENTIAL OIL OF DRIED LEAVES OF *ARTEMISIA NILAGIRICA*

IV.I. Introduction

The genus *Artemisia* is one of the largest and widely distributed genera of the family *Asteraceae*. They are small herbs and shrubs with more than 300 species found predominantly in the northern temperate regions of the world.[115] Most of its species are aromatic and yield essential oils. During the last decade, the essential oils from leaves and flowers of Artemisia have received much attention because of their significance in perfumery industry and in medicine.

A. nilagirica is a tall aromatic shrubby herb commonly called Indian worm wood found in the hilly regions of India. The herb is considered to be emmenagogue, anthelmintic, and stomachic. Its essential oil is a powerful insecticide and larvicide. This plant exists in the form of several chemotypes. The major components of European chemotypes have been reported to be camphor, 1,8-cineole and linalool.[116] The major compnents of

north Indian chemotypes reported were camphor and 1,8-cineole,[117] where as south Indian chemotype contained high concentration of α-Thujone.[116]

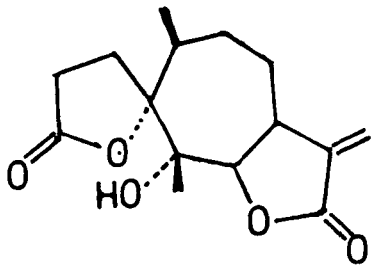
Naqui and coworkers[118] analysed the essential of *Artemisia nilagirica* collected from India and the major components reported are camphor (9.74%), β-eudesmol (7.98%), 1,8-cineole (6.57%), and borneol (5.29%). The volatile constituents of *A. nilagirica* (South Indian chemotype) are known.[119-121] The major components reported are α-thujone (56.3%), β-thujone (7.49%), p-cymene (3.3%), camphor (3%), α-terpineol and β-pinene (2.21% each), geraniol (1.53%), caryophyllene (1.53), γ-cadinene (1.51%), linalool (1.17%), eugenol (1.14%) and trace amounts of limonene, camphene, α-pinene, α-thujene, sabinene, myrcene, borneol, bornyl acetate, terpinolene and aromadendrene.

The essential oil isolated from the leaves of *A. nilagirica* exhibited strong antibacterial activity against several bacteria.[122] Flavanoid type compounds isolated from some species of Artemisia exhibited antitumour activity. For example, artemisetin isolated from *A. absinthium* and *A. Sieversiana,* chrysosplenetin from *A. Sieversiana,* and eriodietyol 7-methyl ester from *A. xanthochora* exhibited marked antitumour activity.[123]

A number of terpenoids which can act as potential pesticides were isolated from several species of Artemisia. Many of these, especially sesquiterpenoid lactones are biologically active as fungicides, herbicides, antimicrobials, insecticides and insect antifeedants. An antimalarial sesquiterpenoid namely *artemisinin*, was isolated from *A. annua*, which is a potent phytotoxin.[124]

Banerji and coworkers[125] isolated capillin [1-phenyl-2,4-hexadiyne-1-ol] from the petrol extract of *A. nilagirica*, which was found to be active against the late 3rd-instar larvae of the mosquito, *culexpipiens quinquefasciatus*.

A large number of phenolics, flavonoids, coumarins, and acetylenes have been isolated from different species of Artemisia, which were found to exhibit antitumour activity against a wide range of tumours.[126] The acetylinic compounds isolated from *A. nilagirica*[127] include, trideca-1,3,5-trien-7,9,11-triyne, tetradeca-4,6-dien-8,10,12-triyne-1-ol, heptadeca-1,7,9-trien-11,13,15-triyne, and tetradeca-6-en-8,10,12-triyne-3-one. Two sesquiterpenoid lactones, namely psilostachyin A(28) and psilostachyin-C(29)also have been isolated[128] from this plant.



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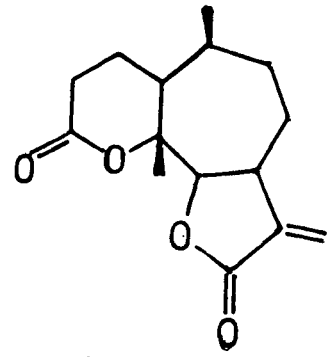

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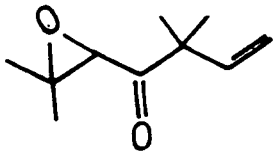
The following are some irregular monoterpenes isolated and identified in the essential oil of *A. nilagirica*.[128] 5,6-Epoxy-3,3,6-trimethyl-1-hepten-4-one(30), 3-methyl-1-(1,1-dimethyl-2-propenyl)-2-butenyl propionate (31), (2E), 2,5-dimethyl-4-vinyl-2,5-hexadienyl propionate(32), (2Z)2,5-dimethyl-4-vinyl-2,5-hexadienylacetate(33), 5-methyl-2-methylidene-3-vinyl-4-hexenylacetate(34), 1,1,4-trimethyl-2-vinyl-3-pentenyl acetate (35), 1,1,4-trimethyl-3-oxo-2-vinyl-4-pentinyl acetate (36) and 3-hydroxy-1,1,4-trimethyl-2-vinyl-4-pentenyl acetate(37).



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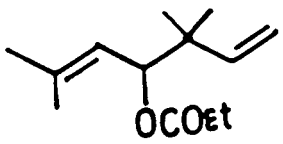

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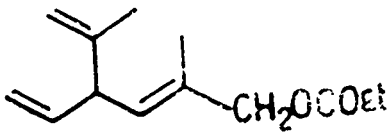

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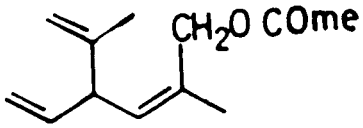

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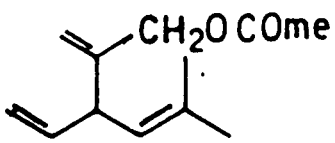

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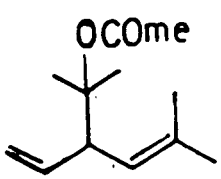

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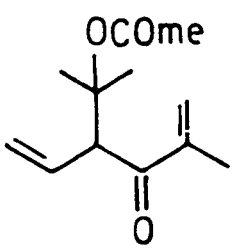

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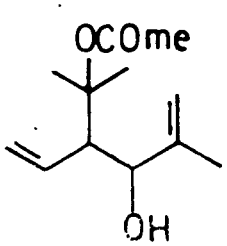

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IV.2. Present work

The essential oil, as explained, of many Artemisia species are known to exhibit biological properties. The aim of this work, therefore was to analyse the essential oil of *A. nilagirica* to identify the compounds present and to relate its antifungal property to the constituent compounds.

The essential oil from the dried leaves of *A. nilagirica* was analysed by GC and GC-MS and 59 constituents were identified.

IV.3. Materials and Methods

The *A. nilagirica* leaves were collected from Kerala, South India and was identified by Dr. A.K. Pradeep, Dept. of Botany, Calicut University.

The leaves were dried in shade and 350g were powdered in an electric mixer grinder and subjected to steam distillation for about 5 h. The distillate was extracted with diethyl ether, (3x100 mL). The ether portions were combined together and dried using anhydrous sodium sulphate. After removing the sodium sulphate, the ether extract was evaporated to get the essential oil (1 mL).

The GC-MS analysis was done on a varian 3400 GC fitted with an OPTIC® injector and attached to a Finnigan ITS40 ion trap mass spectrometer. The column employed for analysis was an HP ultra2 (Hewlett packard) (50 m x 0.25 mm; film thickness 0.33 μm). Helium was used as the carrier gas with a flow rate of 1.6 mL/minute. The temperature programme was from 50°C to 270°C at the rate of 2°C min^{-1}.

Quantification and Kovats determination were carried out using an HP 5890 GC (Hewlett packard) fitted with an OPTIC® injector and flame ionisation detector. A 25 m x 0.2 mm HP-5 column was used for the analysis. Nitrogen was used as the carrier gas at the rate of 0.3 mL/min. The oven temperature was programmed from 30°C to 280°C at the rate of 3°C/min.

The identification of compounds was done on the basis of matching of the mass spectra against commercial and in-house library spectra and comparing their retention indices with those of reference compounds on the same HP-5 capillary column.

IV.4. Results and Discussion

In the gas chromatogram of *A. nilagirica* leaf essential oil, 59 components were identified making a total of 88.89% of the oil (Table IV.1). To some extent this oil showed similarity with the previous analysis.[121-123] But there are marked differences also. In the present analysis the major constituents were found to be α-thujone (41.86%), borneol (10.81%) β-thujone (9.12%) and 1,8-cineole (6.19%). But in previous analysis percentage of α-thujone, the major component was slightly greater being 56.3% and borneol was detected only in trace amounts. At the same time percentage of β-thujone was slightly lower (7.49%). There is some differences in the percentage of β-pinene, p-cymene, linalool, camphor, α-terpineol and eugenol from earlier reports. In the present analysis the percentages of these components are very small, but in the previous analysis percentages are between 1 to 3. The components such as geraniol, γ-cadinene, terpinolene, and aromadendrene reported earlier were not detected in the present analysis.

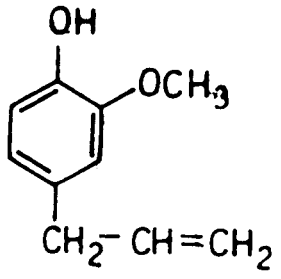
Table IV.1. **Volatile constituents of leaf essential oil of *Artemisia nilagirica***

	Components identified	% RPA	Kovats index
1	3-Methyl butanol	0.07	729
2	2-Methyl butanol	0.02	733
3	Hexanal	0.03	800
4	trams-Hex-2-enal	0.03	851
5	Cis-Hex-3-enol	tr	852
6	Hexanol	tr	865
7	2-Heptanone	tr	891
8	Cis-Hept-4-enal	tr	900
9	Heptanal	tr	901
10	α-Thujene	tr	930
11	α-Pinene	tr	938
12	Camphene	tr	954
13	Sabinene	0.04	977
14	Oct-1-en-3-ol	0.11	978
15	β-Pinene	0.04	982
16	6-Methylhept-5-en-2-one	tr	986
17	Myrcene	0.05	993
18	6-Methylhept-5-en-2-ol	tr	993
19	Dehydro-1,8-cineole	0.05	993
20	Octanal	tr	1003
21	α-Phellandrene	tr	1009
22	α-Terpinene	tr	1021

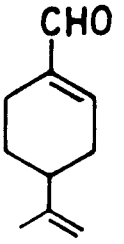
23	p-Cymene	0.13	1029
24	Limonene	tr	1033
25	1,8-Cineole	6.19	1037
26	Phenylacetaldehyde	0.06	1048
27	γ-Terpinene	0.12	1063
28	trans-Sabinene hydrate	0.15	1072
29	Meta-cresol	tr	1075
30	Cis-Linalool oxide	tr	1076
31	trans-Linalool oxide	tr	1092
32	Linalool	0.23	1101
33	Nonanal	tr	1104
34	α-Thujone	41.86	1113
35	Phenylethyl alcohol	tr	1120
36	β-Thujone	9.12	1123
37	Cis-2,8-p-menthadien-1-ol	1.16	1126
38	Pinocarveol	1.46	1148
39	Camphor	0.55	1155
40	Pinocarvone	0.67	1170
41	Borneol	10.81	1176
42	Terpinen-4-ol	3.31	1184
43	Para-cymen-8-ol	0.31	1190
44	α-Terpineol	0.82	1197
45	Myrtenol	0.92	1203
46	Myrtenal	0.33	1206
47	Cuminal	0.21	1249
48	Carvone	0.24	1250

49	Perillaldehyde	0.68	1270
50	iso-Bornyl acetate	0.75	1296
51	Dihydrocuminyl alcohol	0.71	1304
52	Eugenol	0.27	1367
53	Caryophyllene	1.85	1431
54	Humulene	0.29	1472
55	α-Curcumene	0.51	1498
56	Germacrene-D	0.22	1499
57	β-Selinene	0.35	1497
58	Caryphyllene oxide	2.58	1604
59	β-Eudesmol	1.6	1670
	Total	88.89%	

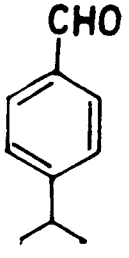
The antifungal property of this oil against *Phytophthora capsici* was evaluated and found to be significant. In order to find out the specific compound responsible for this property, a mixture of the major compounds α-and β-thujones were procured and tested. This experiment proved the fungicidal property of α- and β-thujones, the major components of the essential oil.



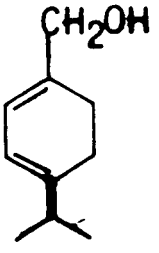
Eugenol



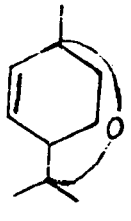
Perillaldehyde



Cuminal



Dihydrocuminyl alcohol



Dehydro 1,8-cineole

CHAPTER V

PHYTOCHEMICAL STUDIES ON *SANSEVIERIA ZEYLANICA* LEAVES

V.1. Introduction

Sansevieria[129] is one of the genera of the family *Liliaceae*. A genus of erect, stiff-leaved perennial herbs with short thick rhizomes; native of tropical Asia & Africa. Some species are important as sources of a useful fibre while many others are ornamentals cultivated for their variegated or mottled decorative leaves. Three species are recorded in India and a few exotics are sometimes grown in gardens as ornamental plants. The species of this genus about 60 in number, have been classified into three groups based on the variations in structure of their leaves, rhizomes, and root system.

The species under group one which are adapted to arid climates and soils have cylindrical leaves and are slow growing and give a low yield of fibre and are thus not suitable for commercial cultivation. Those included in group two show adaptation to a tropical climate of alternating wet and dry seasons, have short tough and coriaceous leaves which yield a higher

percentage of fibre and the plants grow rapidly when planted from rhizomes though not so readily from leaf cuttings. Species included under group three have longer leaves which are narrow or petiolar and rather succulent. They are indigenous to the coastal areas and Islands of the Indian ocean, Africa, Arabia and India and show characteristics of adaptation for a humid climate. They can be propagated easily from leaf cuttings, yield a good return of fibre, and the succulent nature of their leaves makes the extraction of fibre easier and for these reasons they are considered best suited for commercial production.

S. roxburghiana Syn. *S. zeylanica*[130] yield a fibre used for bowstrings, cordage, matting and fine cloth. Rhizomes mucilaginous used in the form of an electuary for cough. Juice of tender shoots is given to children for clearing the phlegm from the throat.

S. trifasciata also yield a fibre used for fishing-lines, nets and bowstrings, but is too coarse for cloth. Juice of leaves is applied to sores. Roots are tonic and stimulant. Pulp remaining after mechanical extraction of fibre from the leaves contain gelling substance. The product finds application as a cosmetic as well as a medicinal base, and is capable of setting meat extracts.[131,132]

V.2. Work so far reported

S. zeylanica is a herb with a creeping root stock found in southern India and ceylon and often cultivated as an ornamental plant. It has leaves 30-60 cm long, deeply channelled above, barred with green and edged with red, inflorescence purple, petals white with purple tips.

This species is very similar to *S. roxburghiana* and has often been confused with it. Both are known by the same vernacular names wherever they occur together. It is a source of fibre of some local importance and like the other species is used for the same purpose in indigenous medicine. It has been used in the past for making some special qualities of paper.

Sansevieria zeylanica has long been prescribed by native physicians in India. According to Dymock, Warden and Hooper[133] it has been used as a remedy for heat of blood, gonorrhoea, heart disease, itch, leprosy, fever, rheumatism, glandular enlargements, and other ailments. The roots of *S. zeylanica*[131] has been used in cough and tuberculosis, and the juice of the tender shoots has been administered to children as an expectorant. The juice of the root and leaves has been used to counteract snake bite.[134]

Dymock and coworkers isolated the crystalline alkaloid "*Sansevierine*" from alkaline ether extract of fresh roots of S. *zeylanica.*

Of the many sansevieria species only two are reported to have been used medicinally. The cells connecting the fibres of sansevieria plants are stated to contains valuable colloidal gelling constituents which are extracted from the residues or pulp remaining after separation of fibers from the leaves. These products are stated to be commercially valuable as cosmetic and medicinal bases.

Fresh leaf juice[135] contains aconitic acid, reducing sugars, inorganic salts and an unstable white amorphous substance. The homogenate of leaves from the yellow-edged variety of sansevieria converts 17-hydroxy-11-deoxy corticosterone to a mixture of 1,4-pregnadiene-17 α, 21-diol- 3,20-dione and pregnane-17α, 21 diol-3,20-dione and testosterone into 1,4-androstadiene-17β-ol-3-one.

Dried rhizomes and roots[136] contain a greenish acid resin, yellow neutral resin, a wax like substance, fructose and the alkaloid sansevierine (.018%).

Davis and Kaffirs[137] used a decoction of the root of *S. thyrsiflora* as a vermifuge and as a remedy for piles. They also found in the rhizome an albumin, globulin and a glycoside identical with glycyrrhizin.

It was reported that tribals of Orissa used *S. zeylanica*[138] as folk remedy for snake bite. Roots are ground with water and given to patient frequently till recovery.

Chemical composition of the leaf fibre[139] is reported as follows:

Moisture 9.2, cellulose-76.1, lignin-11.8, water solubles-2.3 and ash-6%, leaves contain saponin (9.9%).

The pulp and expressed juice of leaves of *S. trifasciata* are applied to ulcers and sores. The leaf is burnt and the fumes inhaled to relieve feverish head ache. Root is credited with tonic and stimulating properties. The plant contains haemolytic sapogenin and organic acids. Onah and Udo[140, 141] studied the antifungal properties and skeletal muscle relaxant properties of aqueous extract of *S. zeylanica.*

V.3. Materials and Methods

Plant Material

The plant material used in this investigation was collected from Calicut University Campus and was authenticated by Dr. A.K. Pradeep, Department of Botany, Calicut University.

Melting point determination

All the melting points of the crystalline isolates were determined using Toshniwal Capillary Melting point apparatus, and are uncorrected.

Infra red absorption spectroscopy (IR)

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

Nuclear Magnetic Resonance Spectroscopy (NMR)

The ^{1}H NMR spectra of the isolates were recorded at 360, MHz in $CDCl_3$ using tetra methyl silane (TMS) as internal standard using Brucker Spectrometers. ^{13}C NMR spectra were recorded at 63 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 (EIMS) were recorded on Finnigan TSQ 70 spectometer. High resolution mass spectra were recorded using Finnigan MAT 8200 under the same condition as above.

Column chromatography (CC)

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

Preparative thinlayer chromatography (pre-TLC)

Preparative thin layer chromatography of the semi purified components were carried out in silica gel-G(Acme) plates prepared using Stahl apparatus (layer thickness 1mm).

Thin Layer Chromatographic Analysis (TLC)

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

Reagents

Liebermann-Burchard Reagent (For triterpenes and Sterols)

The reagent was prepared as follows:

Spray solutions

Acetic anhydride	-	5 mL
Conc- Sulphuric acid	-	5 mL
Ethanol	-	50 mL

Acetic anhydride and 97% sulphuric acid were mixed carefully and with cooling freshly before use and ethanol was added to that mixture with cooling.

V.4. Extraction, fractionation and isolation of compounds from the leaves of *Sanseveria zeylanica*

Dried and finely powdered leaves of *S. zeylanica* (2kg) were extracted repeatedly with petroleum ether (60-80°C, 3 x 6L). The combined extract was then concentrated under reduced pressure to about 500 mL. This extract was then adsorbed on 750 g of silica gel and packed in a column (3 cm x 100 cm; dxl). The column was then eluted with solvents of increasing polarity viz. petroleum ether, benzene, different combinations of benzene-ethyl acetate and ethyl acetate in that order. Several 25mL fractions were collected and each fraction checked by TLC. Identical portions were pooled

together and the solvent removed under reduced pressure. Different compounds isolated from the petroleum ether extract are given in Table V.I.

Table V.I. **Compounds Isolated from petroleum ether extract of *S. zeylanica* leaves**

Fraction	Compounds isolated	Eluent composition	m.p.
1	S_1	Cyclo hexane	58°C
2	S_2	Petroleum ether	66°C
3	S_3	1:1 Petroleum ether-Benzene	292°C
4	S_4	4:1 Benzene-Ethyl acetate	236°C
5	S_5	3:1 Benzene-Ethyl acetate	137°C

The residue after petroleum ether extraction was subjected to extraction with ethyl acetate. The compounds S_1, S_2, S_3, S_4 were also isolated from it together with another compound (S_6) which could not be isolated from petroleum ether extract.

Table V.2. **Compounds isolated from ethyl acetate extract**

Compound	Eluent composition	m.p.
S_6	Benzene	82°C

Isolation of compounds from different chromatographic fraction of petroleum ether extract

Fraction 1, obtained on elution with petroleum ether, yellow in colour, on TLC in petroleum ether gave a single spot at the solvent front, where as TLC in cyclohexane revealed the presence of two compounds.

Column chromatography (3 cm x 100 cm, d x l) over silicagel (60-120 mesh) of this fraction using cyclohexane as eluent gave a white powdery substance. This on repeated recrystallisation from acetone gave 100 mg of a white powdery substance S_1 melting at 58°C. Again an elution with petroleum ether and evporation of petroleum ether eluate gave S_2 as a white crystalline solid (200 mg) melting at 66°C.

Fraction 2, was deep red in colour obtained an elution with a mixture of petroleum ether-benzene (1:1) which on evaporation gave red waxy solid. This was subjected to column chromatography (3 cm x 100 cm, d x l) over silica gel (60-120 mesh) giving white crystalline S_3 (400 mg) which melted at 292°C.

Fraction 3, eluted out with 4:1 benzene-ethyl acetate was green in colour. This on evaporation gave a greenish solid. It was subjected to

column chromatography in a small column over silica gel (60-120 mesh). Benzene was used to develop the column and eluted with benzene and 4:1 benzene-ethyl acetate mixture. The 4:1 benzene-ethyl acetate eluate on evaporation gave a white crystalline substance which on recrystallisation from chloroform yield 100 mg of pure substance S_4, m.p. 236°C.

Fraction 4, deep green in colour on evaporation gave a green mass. It was dissolved in ethyl acetate and adsorbed on silica gel (150 g; 100-200 mesh). After drying it was taken in a chromatographic column and eluted with benzene followed by benzene-ethyl acetate (3:1) mixture. The latter portion on evaporation and recrystallisation from benzene gave shining crystals of S_5 (400 mg, m.p. 137°C).

Fractionation of the Ethyl acetate extract of the leaves of *Sansevieria zeylanica*

Finely powdered leaves of *S. zeylanica,* after extraction with petroleum ether was extracted thrice with Ethyl acetate (3 x 2L). The combined ethyl acetate extract was concentrated under reduced pressure to about 250 mL. It was adsorbed on silica gel (250 g, 100-200 mesh). After drying it was taken in a chromatographic column and eluted with benzene and Benzene-Ethyl acetate mixture. The fraction obtained on elution with

benzene on evaporation gave a white crystalline substance, which on recrystallisation from ethyl acetate yielded 200 mg of pure substance S_6, m.p.82°C.

V.5. RESULTS AND DISCUSSION

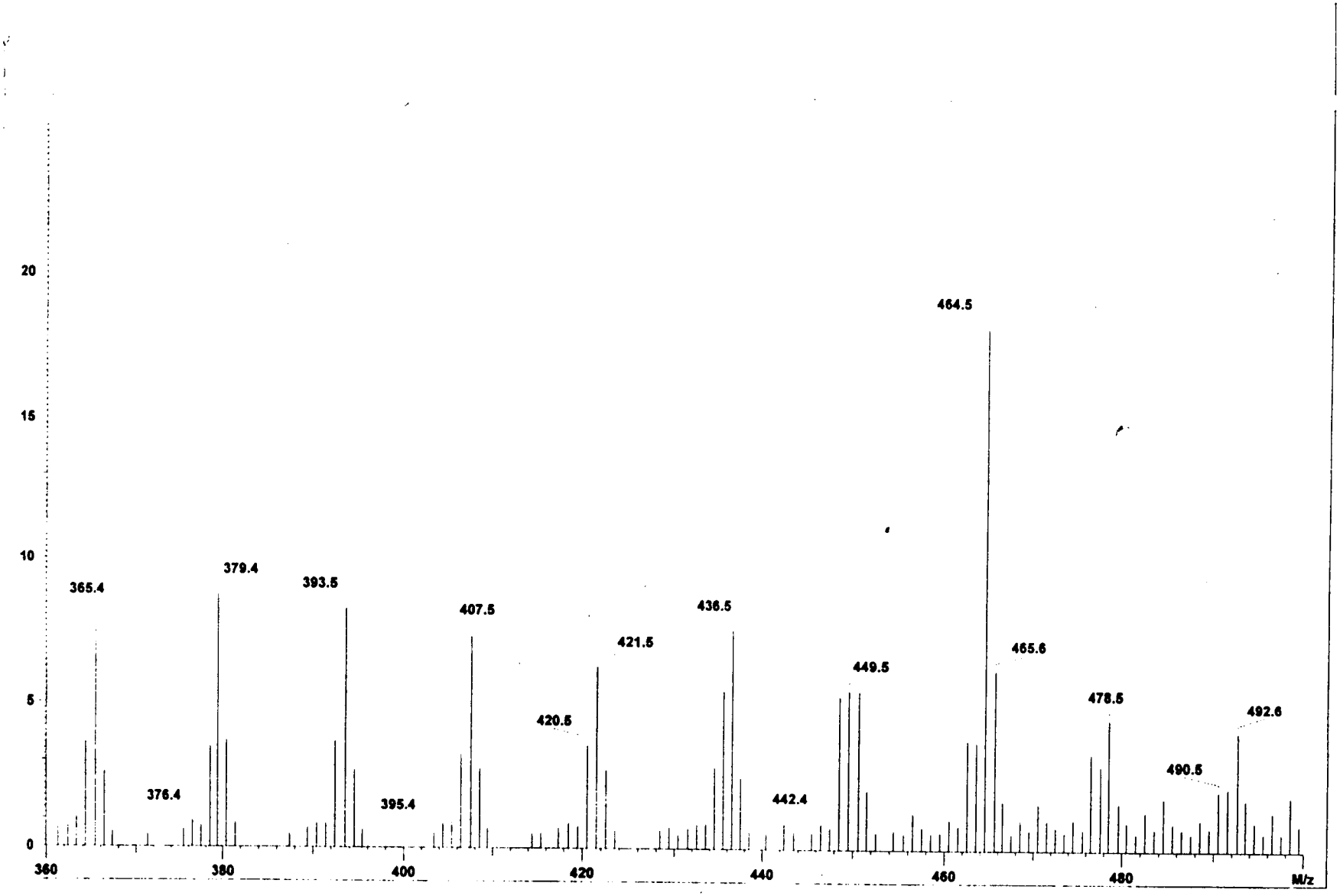
V.5.1. Characterization of S_1

Compounds S_1 and S_2 were obtained together on elution with petroleum ether (60-80°C). They were separated by column chromatography on elution with cyclohexane. Compound S_1 was recrystallised from acetone as a white powdery substance (100 mg) which melted at 58°C.

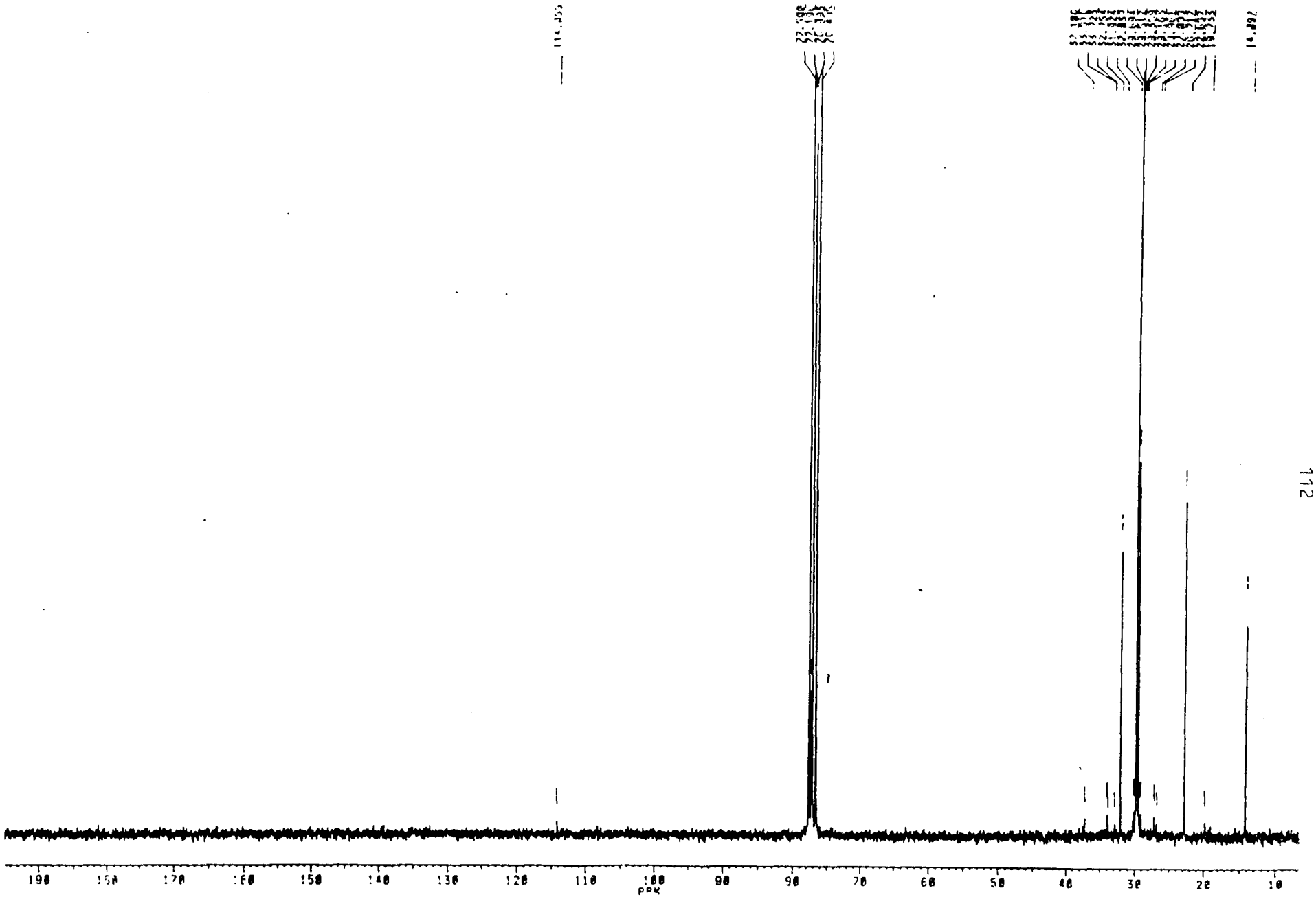
This compound didn't answer Liebermann-Burchard colour reaction, indicating that it was not a triterpenoid or a sterol. It gave a black colour on spraying with H_2SO_4 and strong heating. It moved as a single spot on TLC in petroleum ether and cyclohexane. Absence of decolourisation with dilute $KMnO_4$ solution and bromine water showed its saturated nature. El mass spectrum of this compound showed M^+ at m/z 492.5 and base peak at m/z 57.1. Fragmentation pattern with a regular difference of 14 mass units showed its straight chain saturated hydrocarbon nature. The IR spectrum

gave characteristic absorptions indicating the presence of only C-H and C-C bonds, (2916.7, 2849.2, 1473.8, 1464.1 cm^{-1}). The absorption bands at 728.2 and 719 cm^{-1} were indicative of a long hydrocarbon chain. ^{1}H NMR spectrum (absorption in the region δ 0.9-1.65 and ^{13}C NMR spectrum (absorption at δ14.1, 22.69, 26.7, 27.03 and a number of absorption around 29 and another at 31.93) also supported the straight chain hydrocarbon nature.

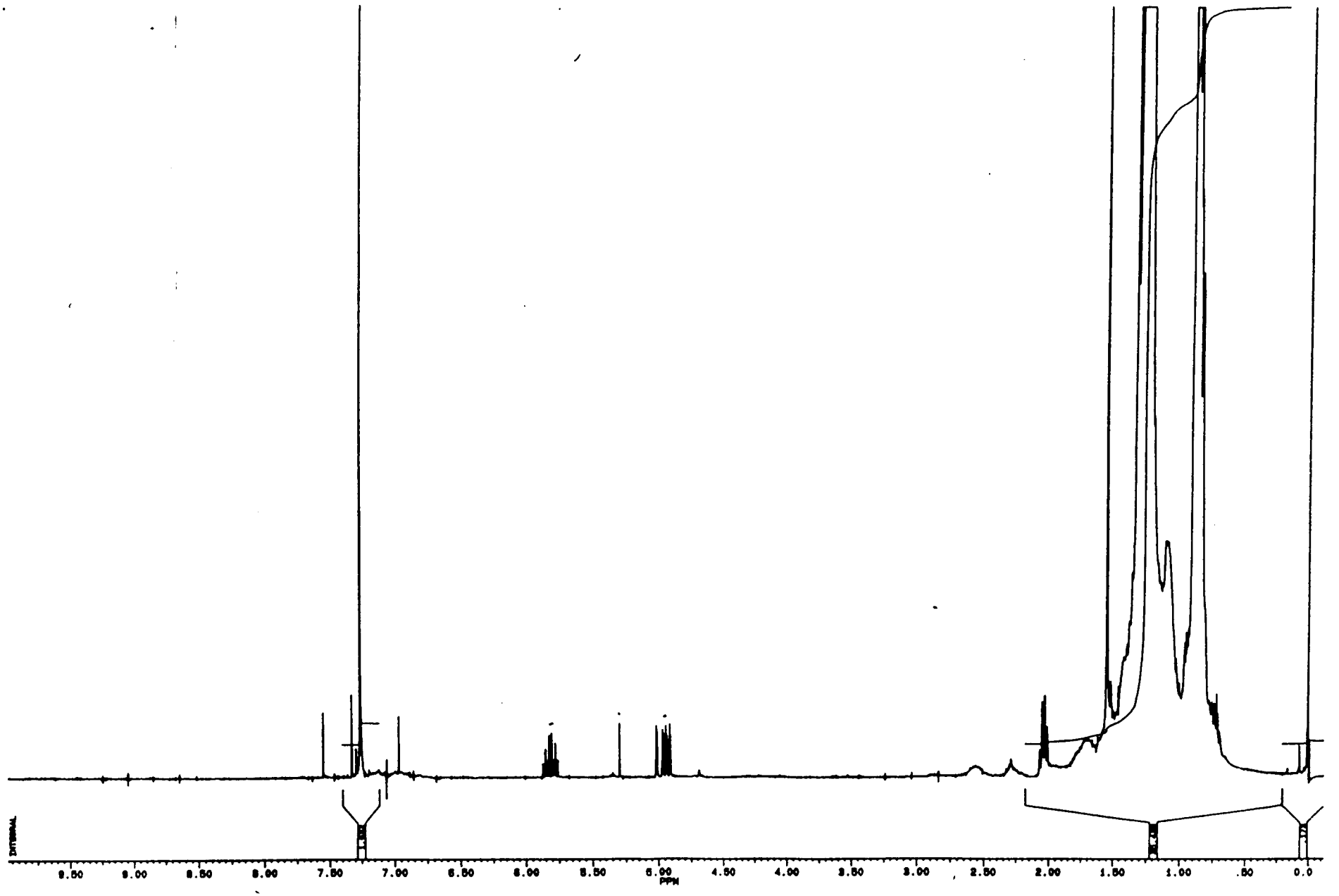
The El mass spectrum of the component had a fragmentation pattern with a regular difference of 14 mass units and the base peak at, m/z 57 and the maximum m/z value at 492.6. The high intensity peaks at m/z 478, 464, 436 did not conform to those of fragment ions, but were indicative of a mixture of compounds, the major compounds being pentatriacontane ($C_{35}H_{72}$ m/z at 492), tetratriacontane ($C_{34}H_{70}$ m/z at 478), tritriacontane ($C_{33}H_{68}$ m/z at 464) and hentriacontane ($C_{31}H_{64}$ - m/z at 436). So the component S_1 was thus identified to be a mixture of hentriacontane, tritriacontane, tetratriacontane and pentatriacontane.



EI - mass Spectrum of S_1 [Mixture of alkanes]



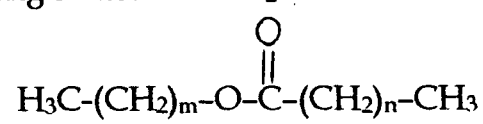
^{13}C NMR Spectrum of S_1 [Mixture of alkanes] [63 MH_2, $CDCl_3$ TMS)



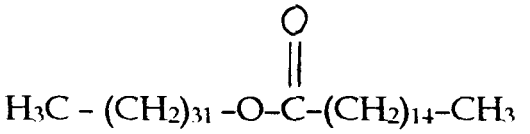
^{1}H-NMR Spectrum of S_1 [Mixture of alkanes] (360 MH_2, $CDCl_3$, TMS)

V.5.2. Characterization of S_2

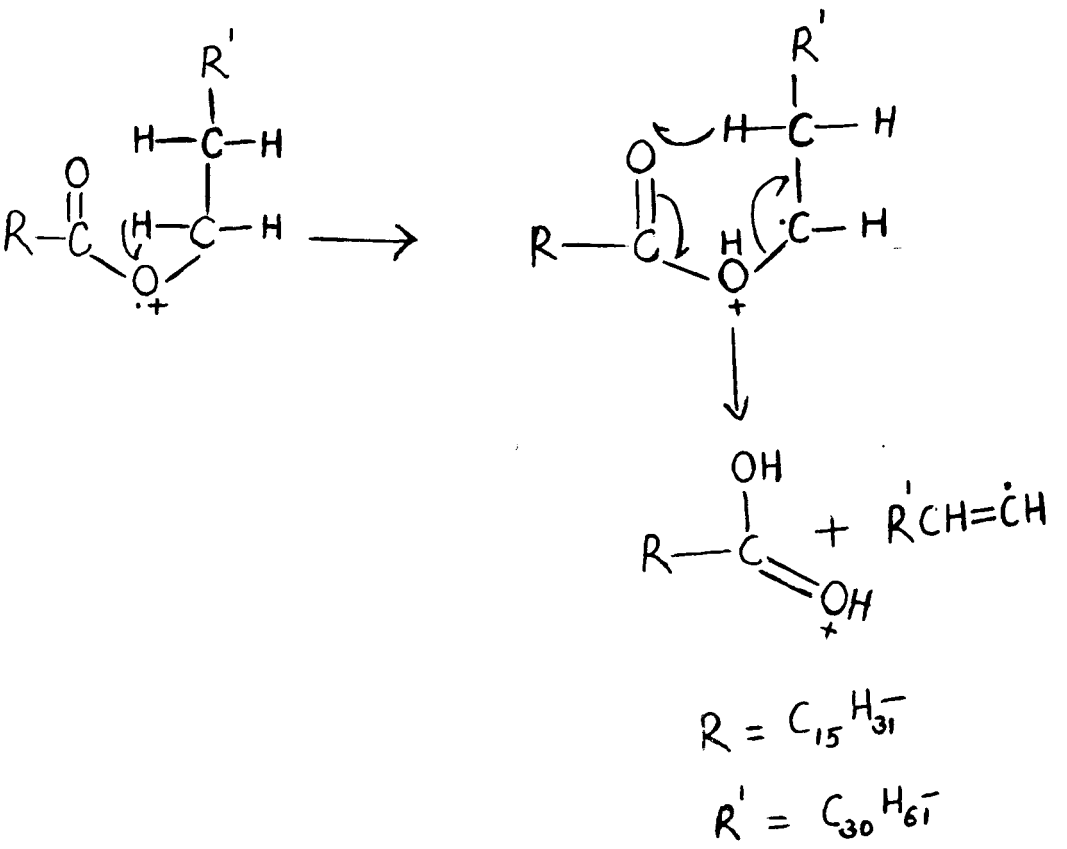
The compound S_2 was isolated from petroleum ether extract and re-crystallised from acetone as a white powdery substance (200 mg). It melted at 66°C. This compound didn't answer Liebermann-Burchard reaction, indicating that it was not a triterpenoid or a sterol. EI mass spectrum of this showed M^+ at m/z 704. Fragmentation pattern with a regular difference of 14 mass units showed its straight chain hydrocarbon nature. The IR spectrum gave characteristic absorption at 1736 cm^{-1} indicating the presence of ester carbonyl group and also other frequencies (734.1 and 719 cm^{-1} of a long chain ester. The ^1H NMR spectrum showed a two proton triplet at δ 4.05 and another two proton triplet at δ 3.65. From the IR, ^1H NMR and mass spectral data it was concluded that S_2 was a mixture of long chain esters with the molecular formula $C_{48}H_{96}O_2$, giving the molecular ion at m/z = 704. The other two prominent peaks at m/z 257 and 285 enabled to propose the following structure for S_2.



Where n is 14 or 16 and 'm' is 29 or 31. When n=14, m=31 the molecular formula was $C_{48}H_{96}O_2$. So the structure was

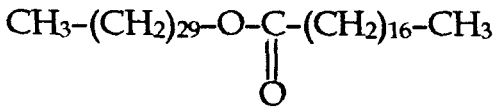


When both the alkyl groups of ester have a γ-hydrogen then a double Mc Lafferty rearrangement or a Double hydrogen transfer (DHT) occurs. The Doble hydrogen transfer (DHT) becomes increasingly common as the alkyl chain of the alcohol moiety lengthens. It can give rise to ions more intense than the Mc Lafferty rearrangement peaks themselves. In the EI mass spectrum of S_2 two intense peaks at m/z 257 and m/z 285 are due to this double hydrogen transfer. This can be illustrated as follows-

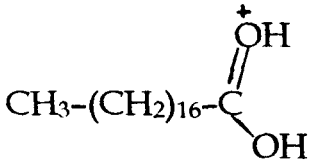


Similarly the peak at m/z 285 could be explained as follows -

when n=16, m =29 the structure would be

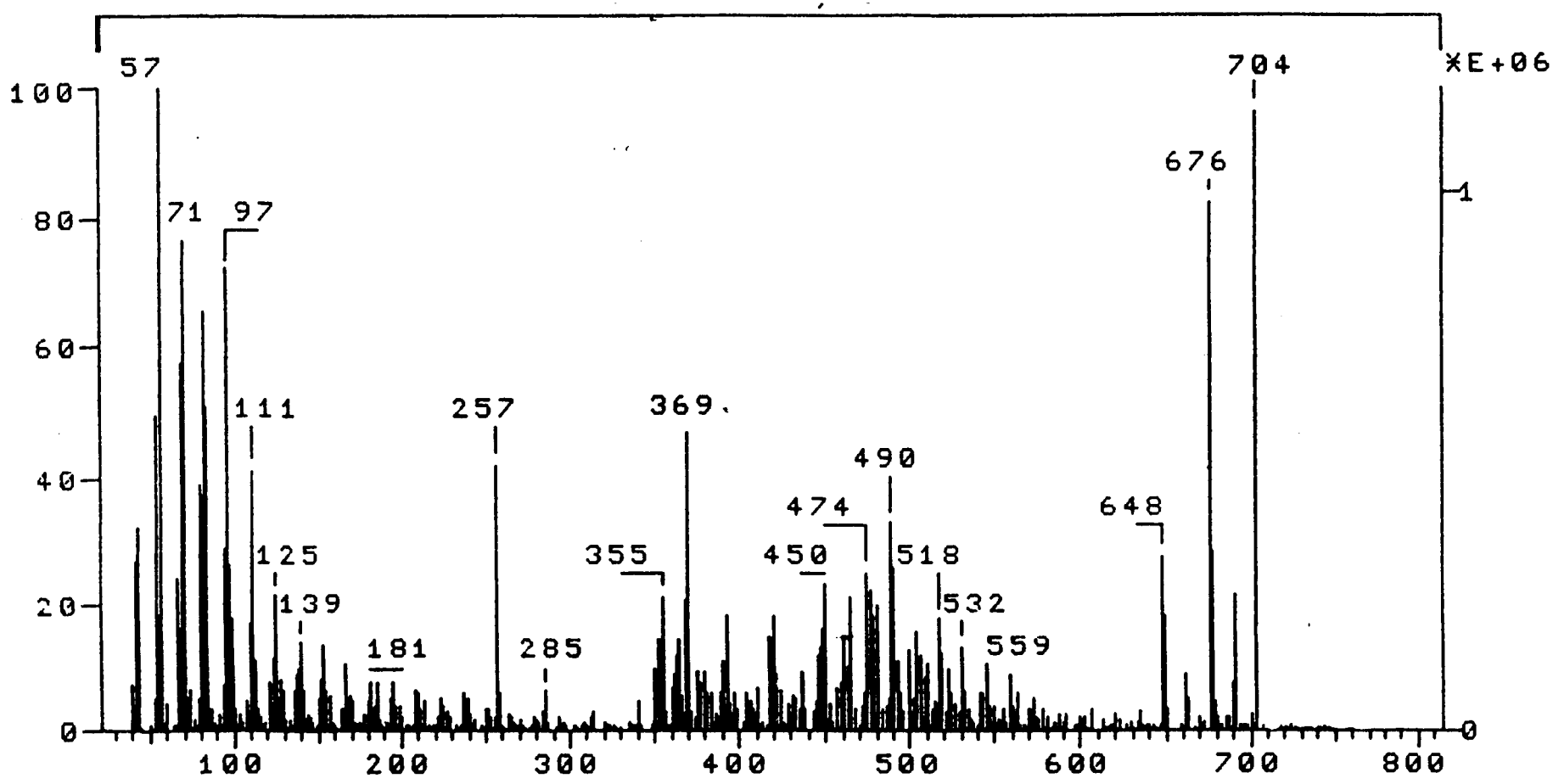


A Double hydrogen transfer would give rise to a molecular ion

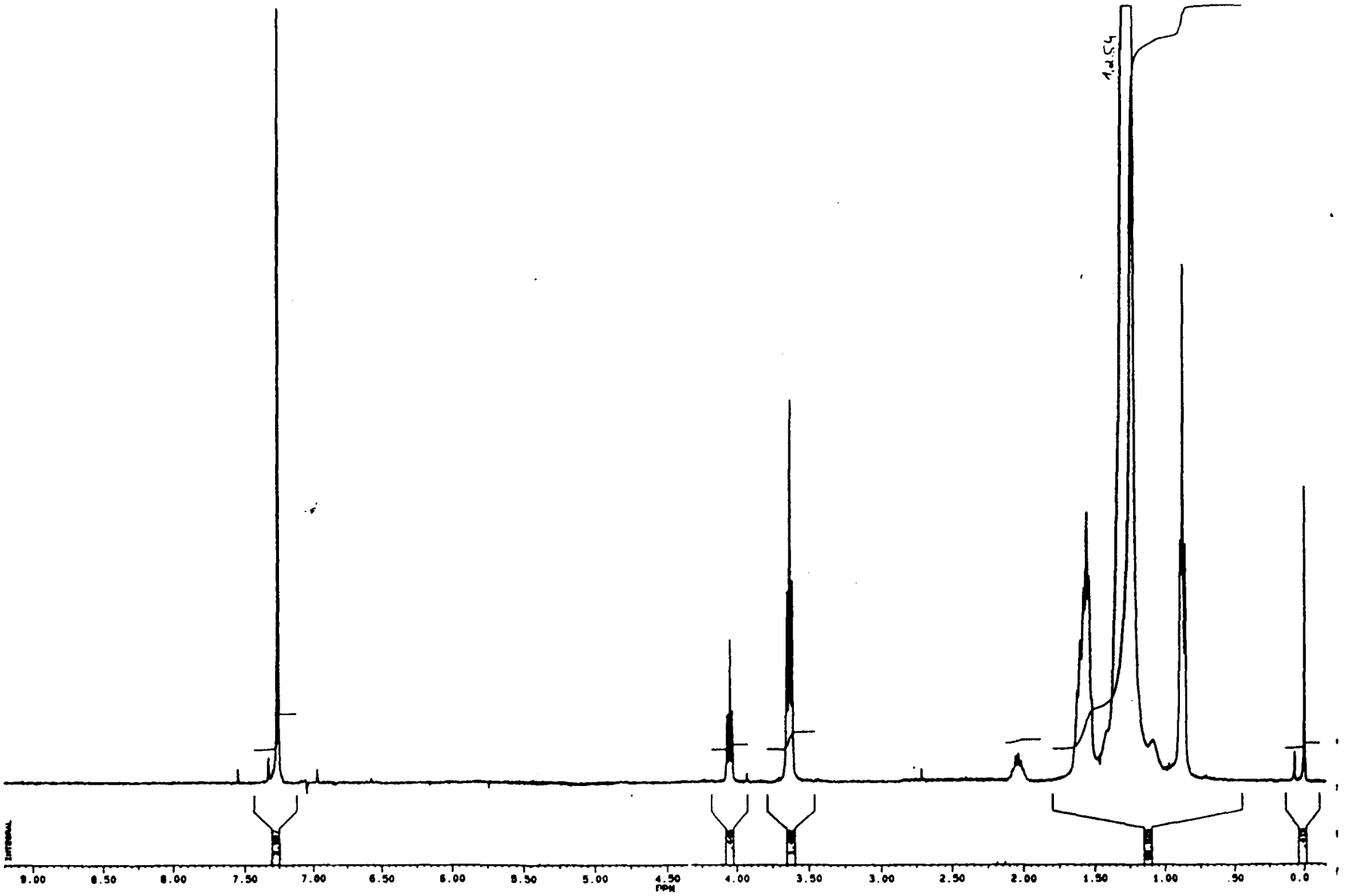
 correspond to m/z 285.

V.5.3. Characterization of S_3 and S_4

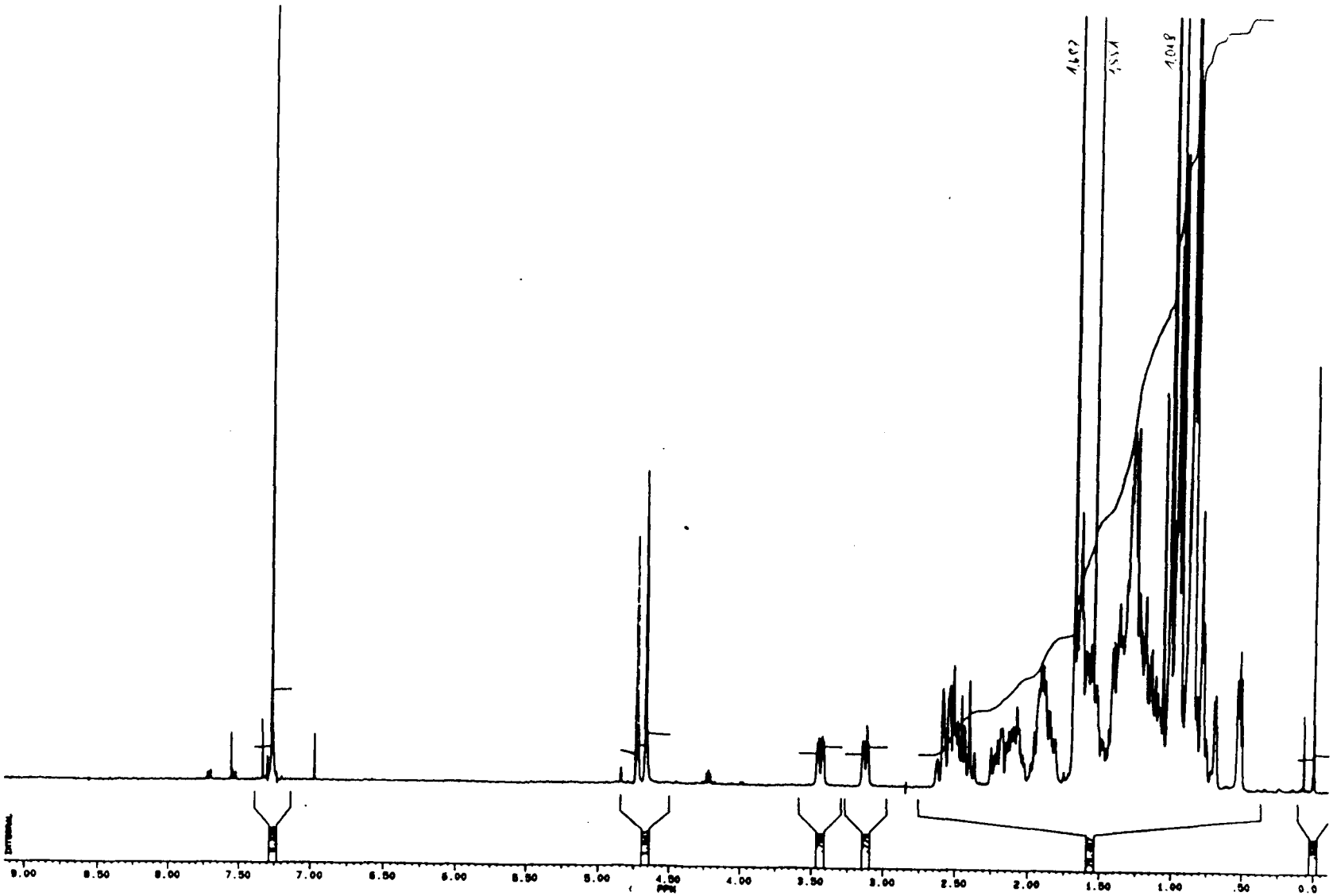
Evaporation of the petroleum ether-benzene (1:1) eluate yielded S_3 (400 mg) melting at 292°C. It gave a positive test with Liebermann-Burchard reagent indicating it to be a triterpenoid. Its ^1H NMR spectrum showed an absorption at low δ value, which is characteristic of cycloartan derivative. The compound S_4, obtained on evaporation of benzene-ethyl acetate (4:1) eluate melted at 236°C. It also gave a positive test with Liebermann-Burchard reagent. The presence of a cyclopropane ring was evident from its ^1H NMR spectrum (absorption at low δ value). Thus the two components S_3 and S_4 were identified to be cycloartans, but the actual structures could not be identified due to paucity of sample.



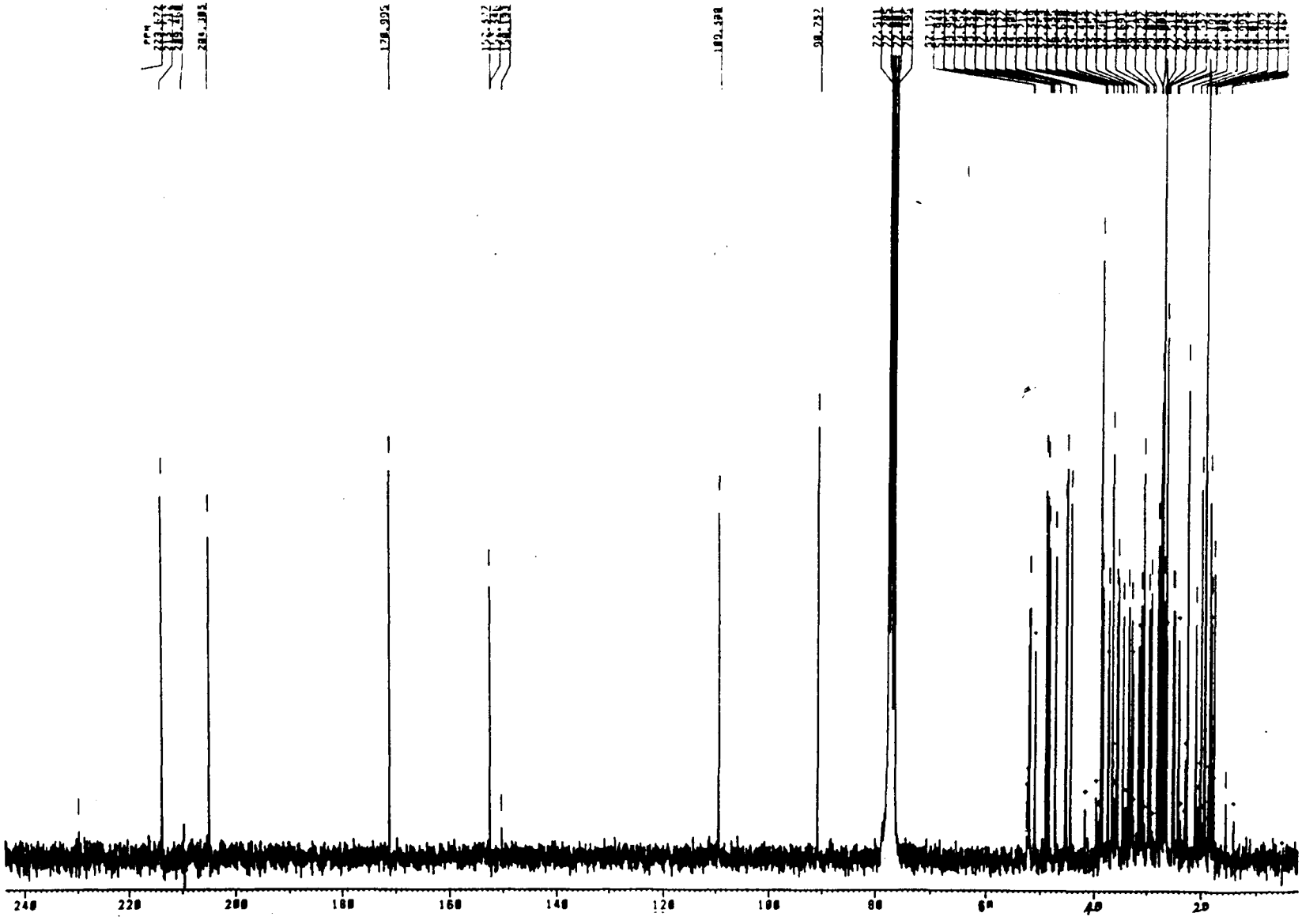
EI-mass Spectrum of S_2 [Mixture of esters]



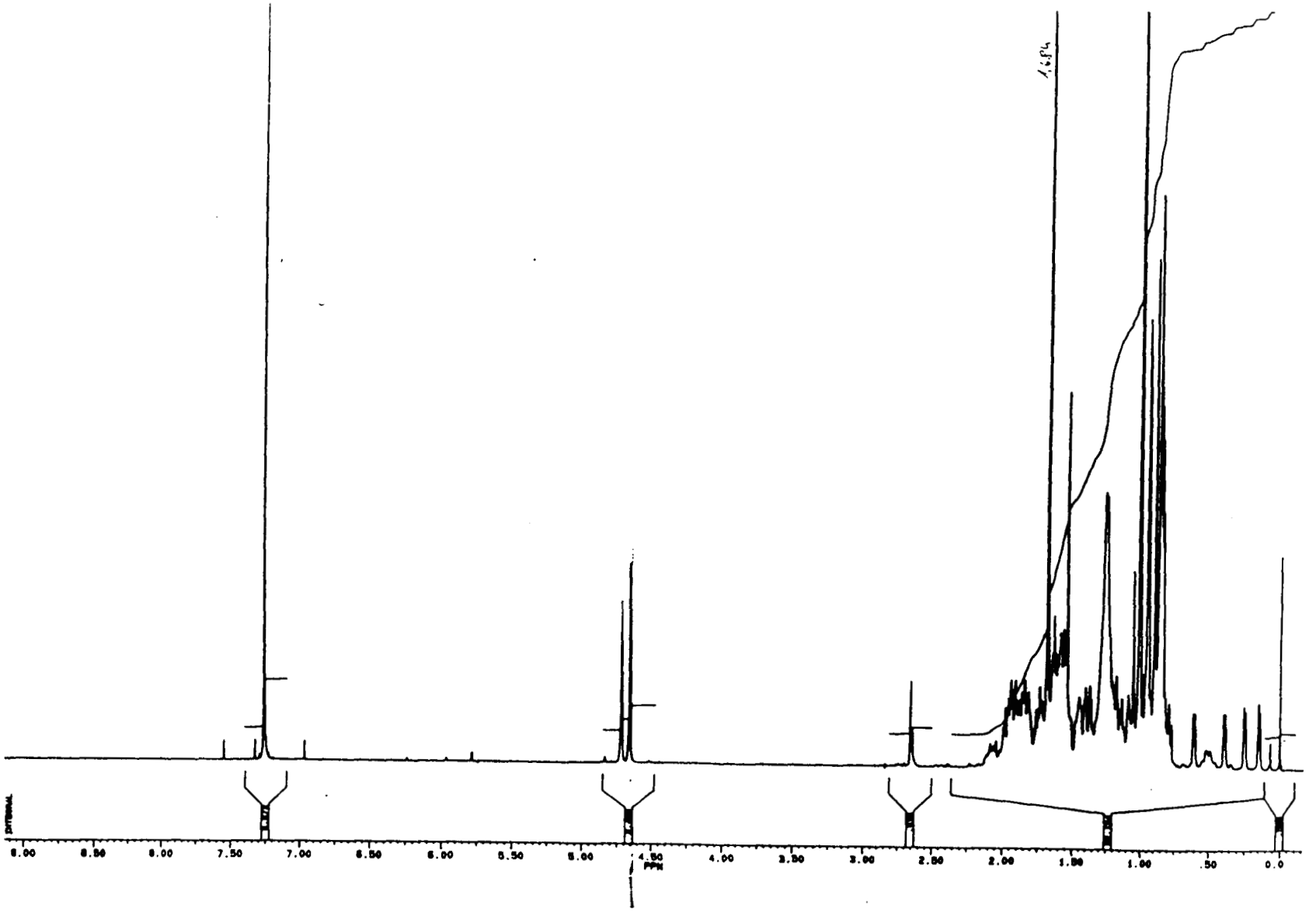
^{1}H-NMR Spectrum of S_2 [Mixture of esters]



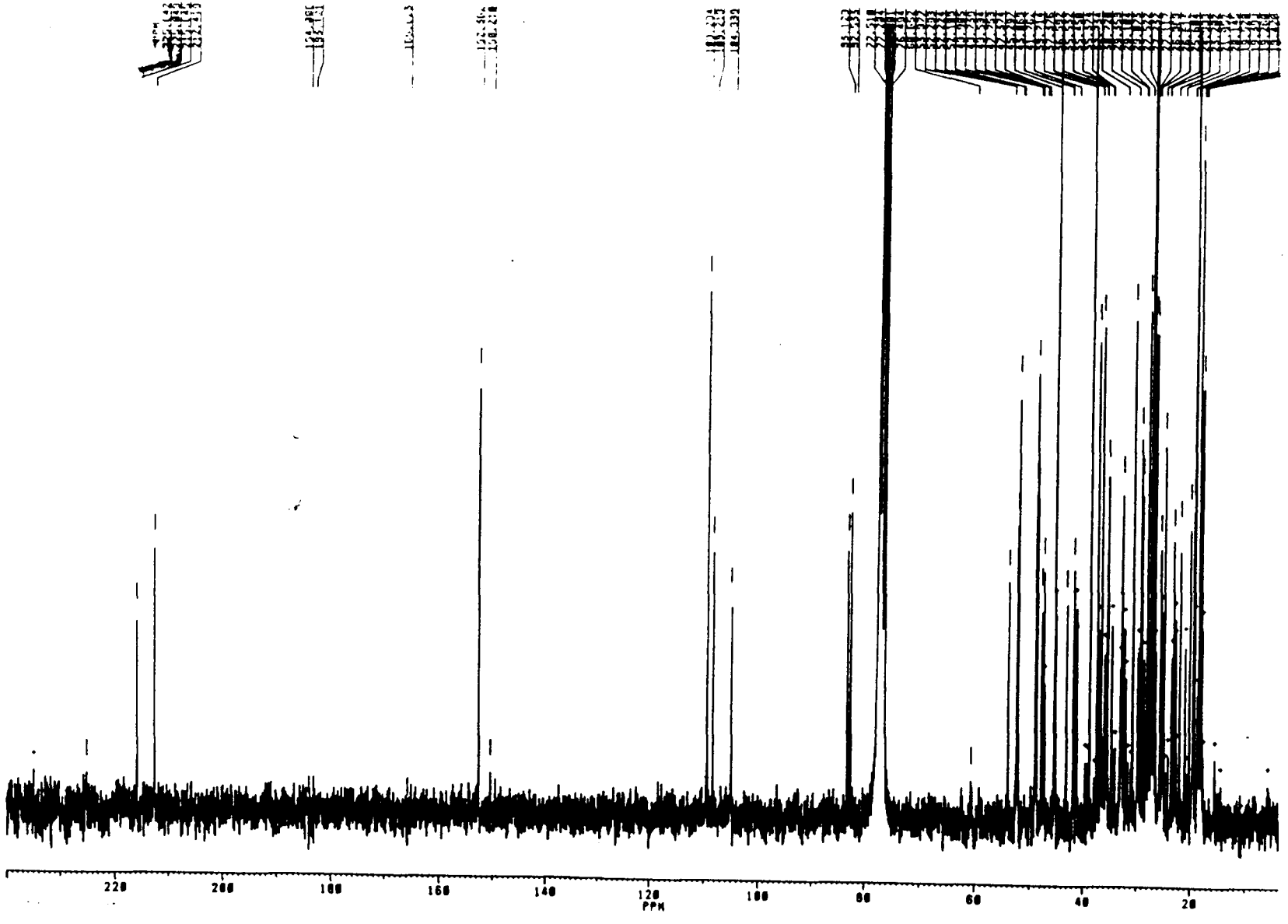
^{1}H NMR spectrum of S_3 (Cycloartan)



^{13}C-NMR Spectrum of S_3 (Cycloartan)



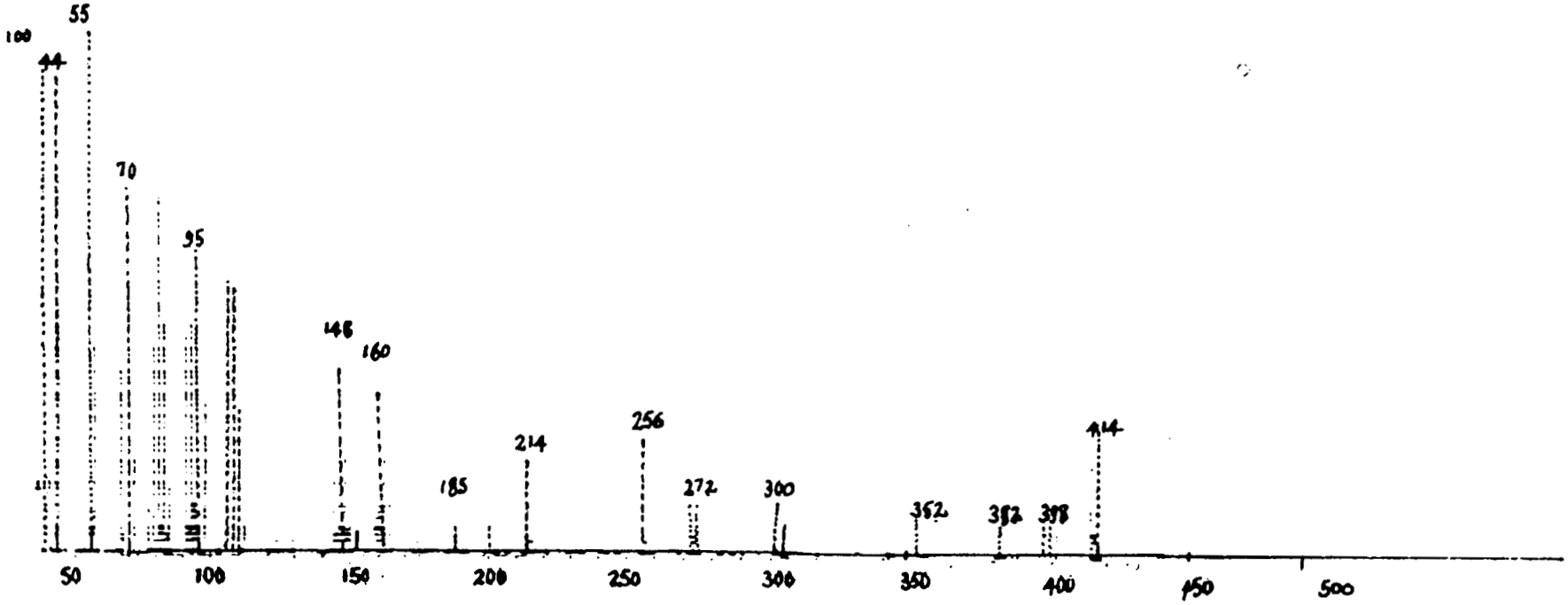
^{1}H NMR spectrum of S_4 (Cycloartan)



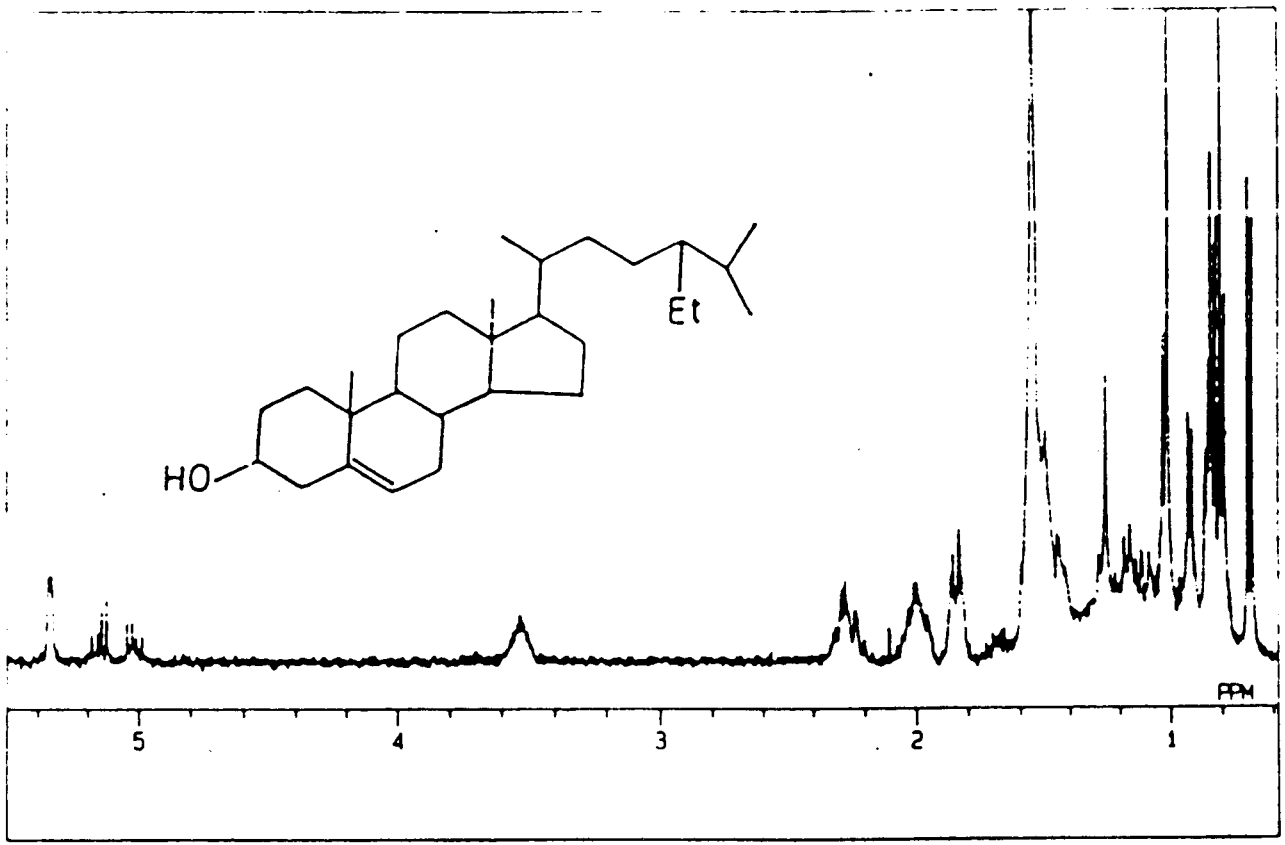
^{13}C NMR spectrum of S_4(cycloartan)

V.5.4. Characterization of S_5 (β-sitosterol)

This compound was isolated from the petroleum ether extract on elution with 3:1. Benzene-ethyl acetate as a white powdery substance. On crystallisation from methanol it yielded colourless needles (600 mg) melting at 137°C. With Liebermann-Burchard reagent it gave a play of colours indicating that it was a sterol. El mass spectrum of this compound showed M^+ at m/z 414 and base peak at m/z 55. IR Absorption spectra showed the presence of a hydroxyl group (broad absorption with maximum at 3441.4cm^{-1}). Bands due to germ dimethyl group [doublet at 1383.1 and 1385.1 cm^{-1}], C–H streching and bending bands [2981, 2816, 1464, 1470 cm^{-1}] were also observed in the spectrum ^{1}HNMR spectrum was quite comparable with that of β-sitosterol. A direct comparison of R_f (0.33 in benzene) with that of an authentic sample established its identity. Prepared the acetate (m.p. 125°C) and benzoate (m.p. 144°C) of this compound and was found to be identical with β-sitosterol acetate (m.p. 127°C) and benzoate (m.p. 145.5°C).[142] Mixed melting point with an authentic sample was undepressed.



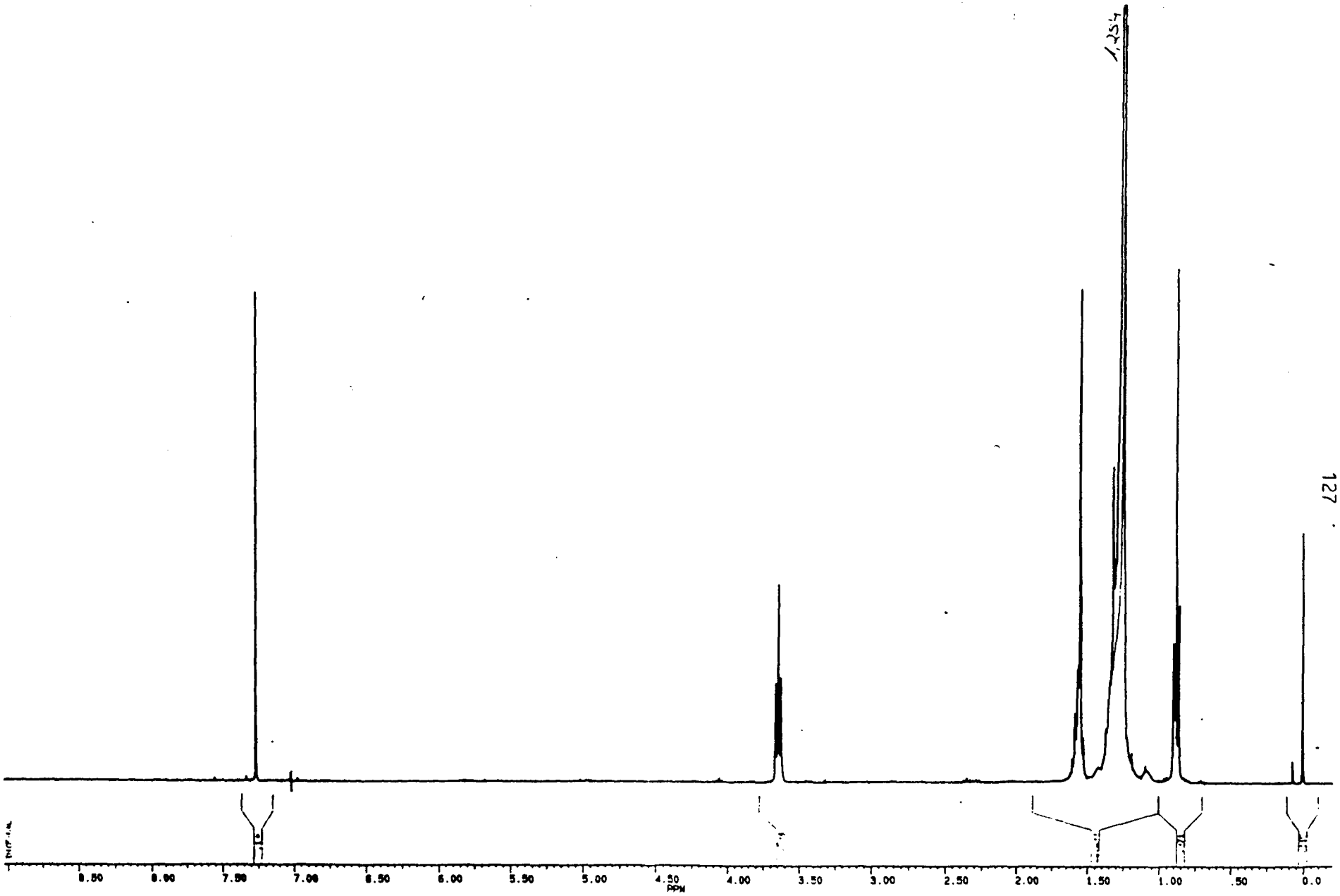
EI-mass Spectrum of S_5 (β-Sitosterol]



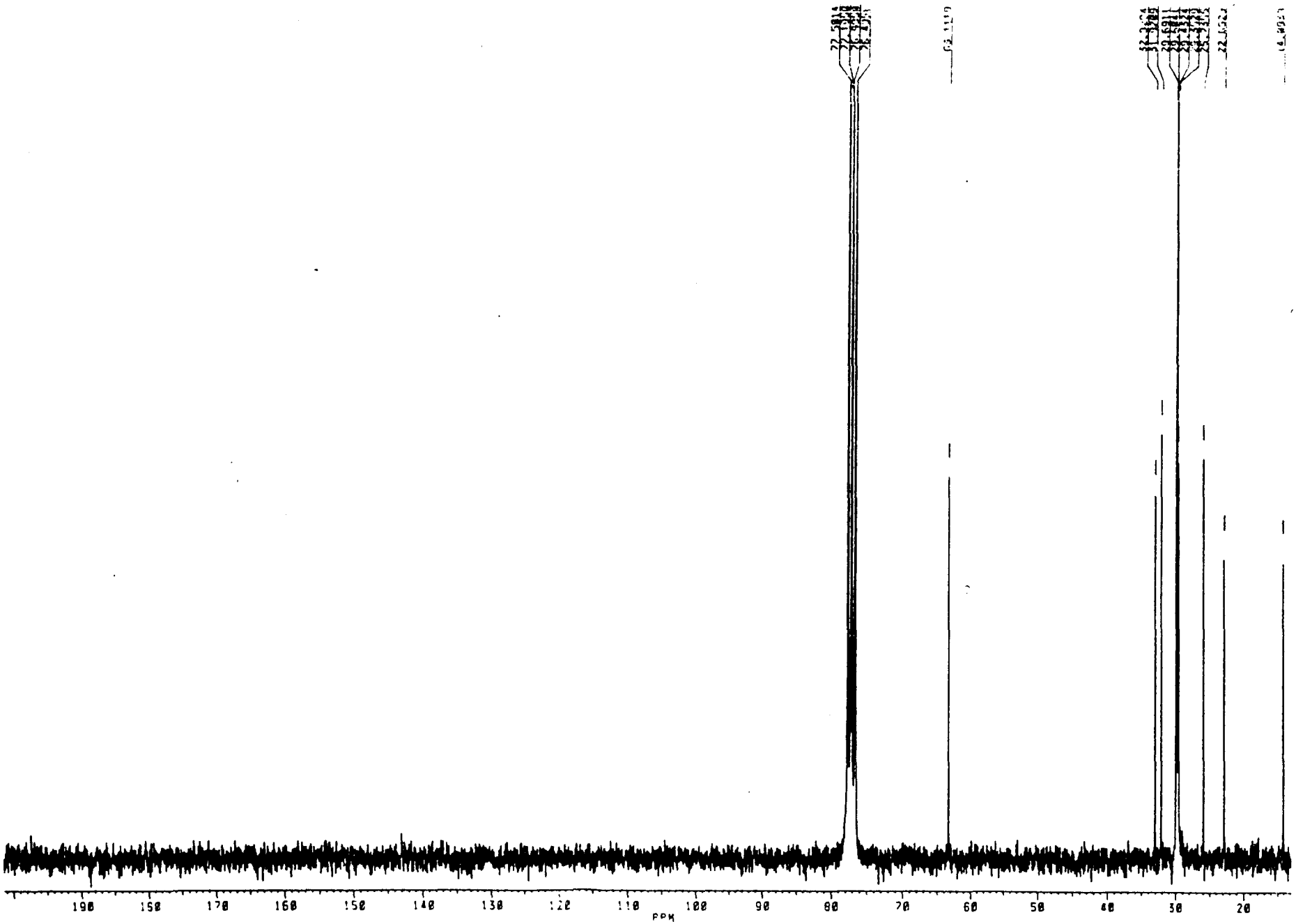
^{1}H-NMR spectrum of S_5 [β-Sitosterol](400MHz, $CDCl_3$, TMS)

V.5.5. Characterization of S_6

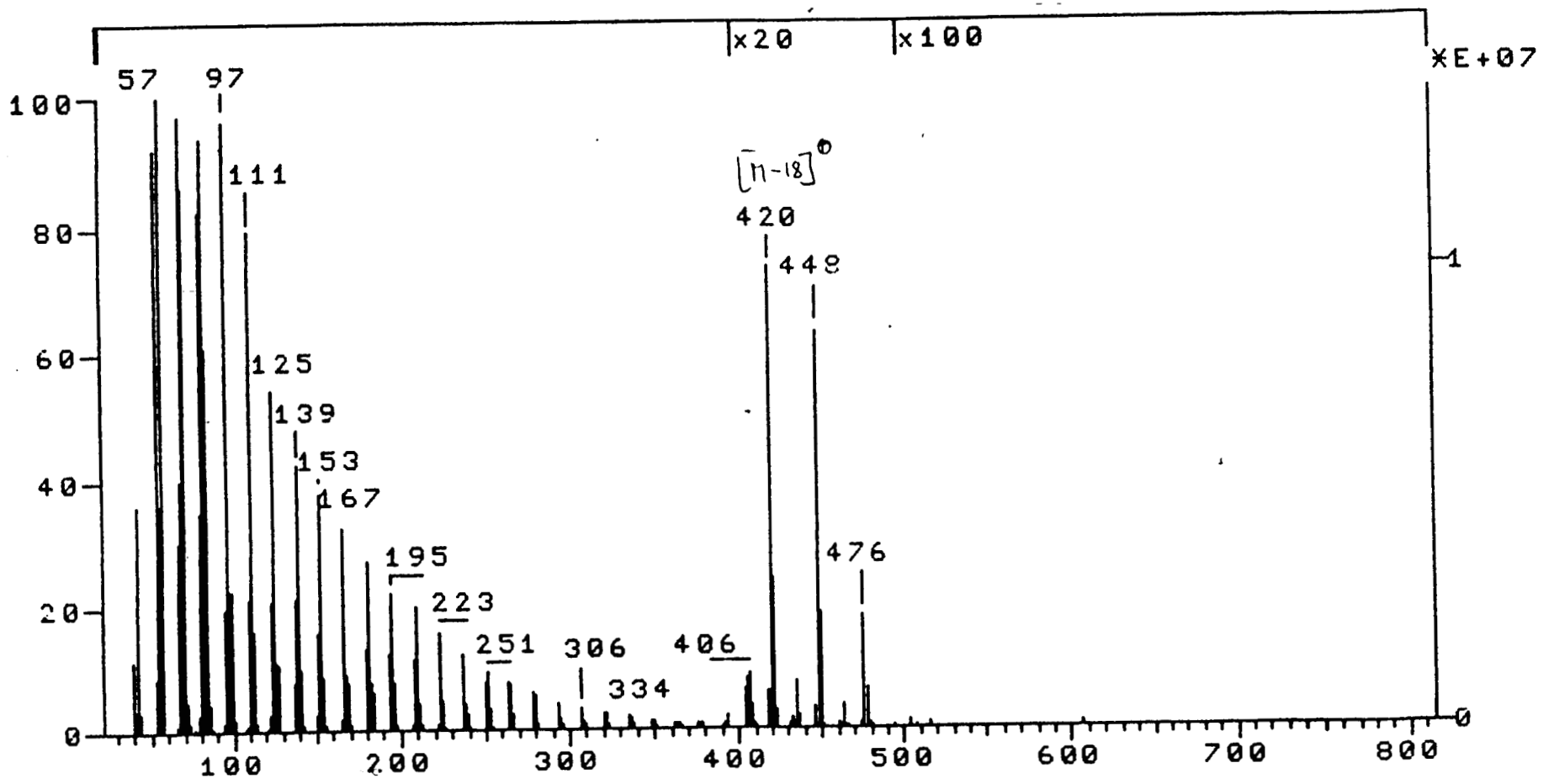
S_6 was isolated from the ethyl acetate extract on elution with benzene as white powdery substance (650 mg), recrystallised from benzene and had a m.p. 82°C. TLC Analysis revealed the polar nature of the component. It didn't answer Liebermann-Burchard test. IR spectrum showed the presence of a hydroxyl group (Broad absorption with maximum at 3450 cm^{-1}). Other prominent absorption bands were at 2918.7, 2849.2 (C-H streching), 1478, 1462.2 (C-H bending) 1061.9 (C-O streching) 734.1 and 719 cm^{-1} (long chain aliphatic compound). ^1H NMR spectrum (absorption in the region δ 0.9 - 1.6) and ^{13}C NMR spectrum (absorption at δ 14.1, 22.65, 25.72, a number of absorptions around 29 and the absorptions at 31.92, 32.31) indicated a long chain hydrocarbon. A triplet at δ3.63 in the ^1H NMR spectrum corresponding to two protons and an absorption at δ63.11 in the ^{13}C NMR indicated the presence of $-CH_2-OH$ group in the compound. The high resolution mass spectrum of S_6 had peaks at m/z 476, 448, 420 and 406. These correspond to $[M-H_2O]^+$ of $C_{34}H_{69}OH$ (tetratriacontanol), $C_{32}H_{65}OH$ (dotriacontanol) $C_{30}H_{61}OH$ (triacontanol) and $C_{29}H_{59}OH$. Thus S_6 was proved to be a mixture of four long chain alcohols.



^{1}H-NMR Spectrum of S_6 [Mixture of alkanols] (360 MH_3, $CDCl_3$ TMS



^{13}C-NMR Spectrum of S_6 [Mixture of alkanols] [63 MH_2, $CDCL_3$, TMS)



EI-mass Spectrum of S_6 [Mixture of alkanols]

V.6. Experimental

1. *Acetylation of S_5 (β-sitosterol)*

100 mg of S_5 was refluxed with 3 mL pyridine and 1 mL acetic anhydride for about 2h. Poured the reaction mixture to cold water filtered, washed with water and them recrystallised from petroleum spirit. The product melted at 133°C.

2. *Benzoylation of S_5 (β-sitosterol)*

100 mg of S_5 was refluxed with 2 mL freshly distilled benzoyl chloride and 5 mL pyridine on a sand bath for 2h. Poured the reaction mixture into cold water filtered, washed with water and recrystallised from benzene. The product of benzoylation of S_5 melted at 144°C.

CHAPTER VI

ANITFUNGAL ACTIVITY OF SOME ESSENTIAL OILS AGAINST *PHYTOPHTHORA CAPSICI*

VI. I. Introduction

The use of synthetic fungitoxic chemicals for controlling plant diseases has made improvement in the field of agriculture. But several of these substances are found to possess side effects such as carcinogenicity, pollutive effects and other residual toxicities. It is encouraging in this context that compounds which are effective as fungitoxicants has been isolated from various plants.[143,144] During the recent years use of plant secondary metabolites for the control of fungi is gaining importance. On account of their potent fungitoxicity, broad fungi toxic spectrum, thermostability, and non phytotoxicity, many of the essential oils can be used as harmless, indigenous and non-pollutive sources of fungicides.[145,146]

The presence of antimicrobial substances in higher plants is well known since ancient times. Egyptians used mixtures of some vegetable oils for the preservation of mummies from protein decomposing bacteria. Greeks and Romans used the juice of green walnut shells against infections

and fungal diseases of skin. Clove oil is used even today to disinfect dental pulp cavities.[147]

A number of plants are known to contain secondary metabolites which are inhibitory to phytopathogenic fungi, bacteria, viruses etc. They include terpenoids [example: iridoids, sesquiterpenoids, saponins], nitrogen or sulphur containing compounds [examples: alkaloids, amines, amides], aliphatics [especially long chain alkanes and fatty acids] , aromatics [phenolics, flavonoids, stilbenes, dibenzyls, xanthones, benzoquinones] essential oils, acetylinic compounds and coumarins.[144,148]

The compounds responsible for antifungal properties can be preformed or can be induced after infection. The preformed ones are called 'constitutive antifungal substances' and that are induced after infection are called induced antifungal constituents or phytoalexins. Constitutive antifungal substances can be either preinfectional or post infectional. Preinfectional plant metabolites are compounds which are present normally in high concentrations and inhibits most fungi. Post-infectional compounds are normally present in plants in an inactive form, but can be converted into active antifungal substance after infection by some chemical reaction such

as enzymic hydrolysis, whereas phytoalexin production may take two or three days after infection.

Some monoterpenoids isolated from essential oils are known to possess antimicrobial activities.[149] For example, thujaplicins from the heartwood of Thuja is an antifungal substance. Several sesquiterpenoids and diterpenoids show antifungal activity, some of these are phytoalexins and some are constitutive antifungal compounds. Aliphatic antifungal compounds include simple alkanones like 2-decanone, long chain acetylinic alcohols and long chain fatty acids especially those having eighteen carbon atoms.

A large proportion of plant substances showing antibacterial and antifungal activities are aromatic in nature. They are mainly alkylated phenols, phenolic acids, coumarins, flavonoids, quinones and xanthones. Benzoic acid[150] isolated from the leaf extract of *Uvaria narum* was reported to be a very good antifungal compound against *Phytophthora capsici,* the common foot rot pathogen of black pepper. The antifungal activity of the essential oil from *Artemisia capillaris* was reported to be due to capillin (1-oxo-1-phenylhexa-2,4-diyne).[151] Coumarins which have a phenyl propanoid nucleus are another group of aromatic substances rich in

antifungal representatives. Many flavonoids, and isoflavonoids has been reported to play a role in plant protection against pathogens, both as preformed antifungal compounds and as phytoalexins. Naturally occuring quinones like juglone (5-hydroxy-1,4-napthaquinone) isolated from the leaves of plants are found to exhibit antifungal activity against fungi.[152]

The essential oils extracted from plants such as *Mentha piperila* L., *Lavandula officinalis* and from roots and flowers of radish are known to exhibit antimicrobial activity.[153] Antimicrobial activity of perfume oils[154], essential oils on wood-destroying fungi and phytopathogenic fungi[155] has been studied in detail by Maruzzella and coworkers. 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.[156,157]

VI.2. Present Work

In the present work leaf essential oils of three plants were analysed for their antifungal activity against *Phytophthora capsici*, the foot rot pathogen of black pepper. The results obtained showed that the leaf oil of *Artemisia nilagirica* was most effective against this plant pathogen. The active ingredient of this oil was also characterised.

Phytophthora capsici

Black pepper (*Piper nigrum*. L.) often referred to as king of spices is an important crop grown exclusively in humid tropics like India, Indonesia, Malaysia, Brazil, Thailand and Srilanka. In India this spice accounts for considerable amount of earnings in foreign exchange. It is commonly cultivated in Kerala, Karnataka, and Tamil Nadu. Besides insect pests and physiological disorders this crop is affected by many fungal, bacterial, viral, mycoplasmal and nematodal diseases. Among the fungal pathogens, *Phytophthora capsici* is the most destructive one. It is responsible for the 'foot rot' disease of pepper known as 'quick wilt' disease, causing severe crop loses in all black pepper producing countries.[158,159] The crop lose due to foot rot in Kerala is estimated to be 10% of the total production.[160]

Phytophthora capsici comes under the class Oomycetes, which contain some most devastating plant pathogens. It 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 spreads 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-September) period in Kerala. This fungus spreads mainly through rain splash and water.[161] 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.[162] This fungus grows luxuriantly at 25-28°C on carrot agar medium[163] and growth is absent at 35°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 systemic fungicides as they are endowed with some unique biochemical and physiological features. In order for a fungicide to be effective against Oomycetes, it should be soluble in water.

For the control of 'foot rot' disease, a number of control measures involving cultural, chemical and biological have been advocated.[164] Foliar spray and swabbing of the collar portions of the vines with Bordeaux mixture is the chemical method for controlling this disease. Drenching of the soil with Bordeaux mixture or copper oxychloride and use of systemic fungicides like metalaxyl, fosetyl-A1 also include the chemical methods. Biocontrol agents like *Gleocladium virens, Trichoderma* spp., *vesicular arbuscular mycorrhiza* (VAM) are also used against this fungus. Nowadays

the effect of botanical pesticides have received enough attention. The root exudates of *Allium* spp. have been reported to be inhibitory to *P. capsici*[165] Soil application of a mixture of garlic and mustard has been reported to be effective against foot rot of black pepper.[166] Water and ethanol extracts of *Piper colubrinum* and *Chromolaena odorata* have been found inhibitory to *P. capsici.*[167] The essential oil from the leaves and leaf extract of *Uvaria narum* inhibit the growth of *P. capsici* at all stages.[168] Benzoic acid and benzyl benzoate were isolated from the leaf extract which are the active ingradients against this fungus.

VI. 3. Materials and Methods

The essential oils used for the experiment were isolated by steam distillation as discussed under the earlier chapters. The essential oil from *Artabotrys odoratissimus* also was extracted similarly.

The *P. capsici* cultures for inoculation were prepared by growing them in carrot agar medium. Discs of 0.5 cm diameter were taken from the growing edges of 48 h old culture plates using sterilized cork borer.

The carrot agar medium was prepared as follows. The carrot was cooked in sterile distilled water untill tender and ground into a paste. Diluted with more distilled water, filtered and added agar into it.

Growth study

In the growth study, different essential oils were incorporated with carrot agar medium in different concentrations. The *P. capsici* was allowed to grow in the above test and control media. The diameter of the colony in both were measured and the percent inhibition to growth was deduced using the following formula.

$$\text{Per cent inhibition} = \frac{a-b}{a} \times 100$$

where 'a' is the radial growth of the colony in the control medium and 'b' the radial growth in the test medium.

VI.4. Results and Discussion

P. 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.

Out of the three essential oils tested, the leaf oil of *Artemisia nilagirica* was found to be highly inhibitory to the fungus. The leaf oil of *Artabotrys* also showed inhibition, but to a lesser extent compared to *Artemisia* oil. All were tested at a concentration of 100 p.p.m of carrot agar medium. Carrot agar with cetone alone served as the control. The results are given in Table VI.I.

Table VI.1. **Effect of essential oils on the growth of *P. capsici***

	Name of plant	Radial growth (m.m)					Inhibition % (a-b) ----- x 100 a				
		1st day	2nd day	4th day	5th day	6th day	1st day	2nd day	4th day	5th day	6th day
1.	Artemisia nilagirica	0	0	0	0	0	100	100	100	100	100
2.	Artabotris odoratissimus	0	0	0	12	31	100	100	100	77	64
3.	Vitex negundo	0	13	26	36	64	100	48	38	33	26
4.	control	15	25	42	54	87					

The above results showed that Artemisia oil was strongly inhibitory to the growth of *P. capsici* at a concentration of 100 p.p.m.

The GC-MS analysis of the Artemisia oil showed that major component present in it is α-thujone (41.86%). In order to find out the activity of α-thujone against this fungus, an essential oil containing α-

thujone (54%), β-thujone (8%), fenchone (14%) and camphor (8%) was tested at different concentrations. (α-Thujone alone is not commercially available. Its separation from the essential oil is also difficult).

A volume of 500 µL of this mixture was added to 100 mL acetone to prepare the stock solution. Different volumes (0.5 mL, 1 mL, 2 mL and 4 mL) were added to carrot agar medium (49.5 mL, 49 mL, 48 mL and 46 mL) to make a total of 50 mL medium of concentrations 50 ppm, 100 ppm, 200 ppm and 400 ppm respectively. Complete inhibition was noticed at a concentration of 100 p.p.m of the medium (Table VI.2).

Table VI.2.

Concentration mL/50 mL	Radial growth in (m.m)			Inhibition % $\frac{(a-b)}{a} \times 100$		
	2nd day	3rd day	4th day	2nd day	3rd day	4th day
0.5 ml	0	15	25	100	62.5	68
1 ml	0	0	0	100	100	100
2 ml	0	0	0	100	100	100
4 ml	0	0	0	100	100	100
Control (acetone)	24	40	81			

Detailed investigation on the effect of leaf oil of *A. nilagirica* on various phases of *P. capsici* showed that it inhibits only mycelial growth.

If the thujones (α-together with β-thujone) are considered to be the only active principles their combined concentration in the oil mixture is only 62%. That is 100 p.p.m of the oil contains only 62 ppm of thujones. By this argument it can be concluded that 100% inhibition of *P. capsici* is possible with 62 p.p.m of thujones. This is really a promising observation.

VI. 5. Experimental

Water used in all the experiments involving fungi was double distilled using a glass apparatus.

Isolation of Essential oils

The isolation procedures of essential oils of *A. nilagirica* and *V. negundo* are provided under chapter IV & II respectively. The shade dried leaves of *Artabotrys odoratissimus* (200 g) were powdered in an electric mixer grinder and subjected to steam distillation for 5 h. The distillate so obtained was extracted with solvent ether (3 x 100 mL). Ether portions were pooled together and dried with anhydrous sodium sulphate. On

evaporation of dry ether extract, after removal of sodium sulphate, yielded the essential oil (0.80 mL).

Preparation of media for antifungal studies

Carrot (200 g) was cooked lightly until tender in 500 mL distilled water. The cooked carrot was ground into a paste using a mixer grinder and added 1000 mL more water. After filtration through a muslin cloth, 15 g of agar (BDH) was added to the carrot preparation and shaken to get a homogeneous mixture.

Antifungal activity of essential oils

Prepared a stock solution of essential oils of 1000 p.p.m by weighing accurately and dissolving it in acetone. In order to get a concentration of 100 p.p.m, 5 ml of this was added to 45 ml of carrot agar medium. Appropriate amount of acetone was added to carrot agar solution for preparing control plates. The Petriplates were autoclaved at 15 psi for 30 minutes and cooled to 40°C. The medium was poured into three Petridishes 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 72h. The radial growth in

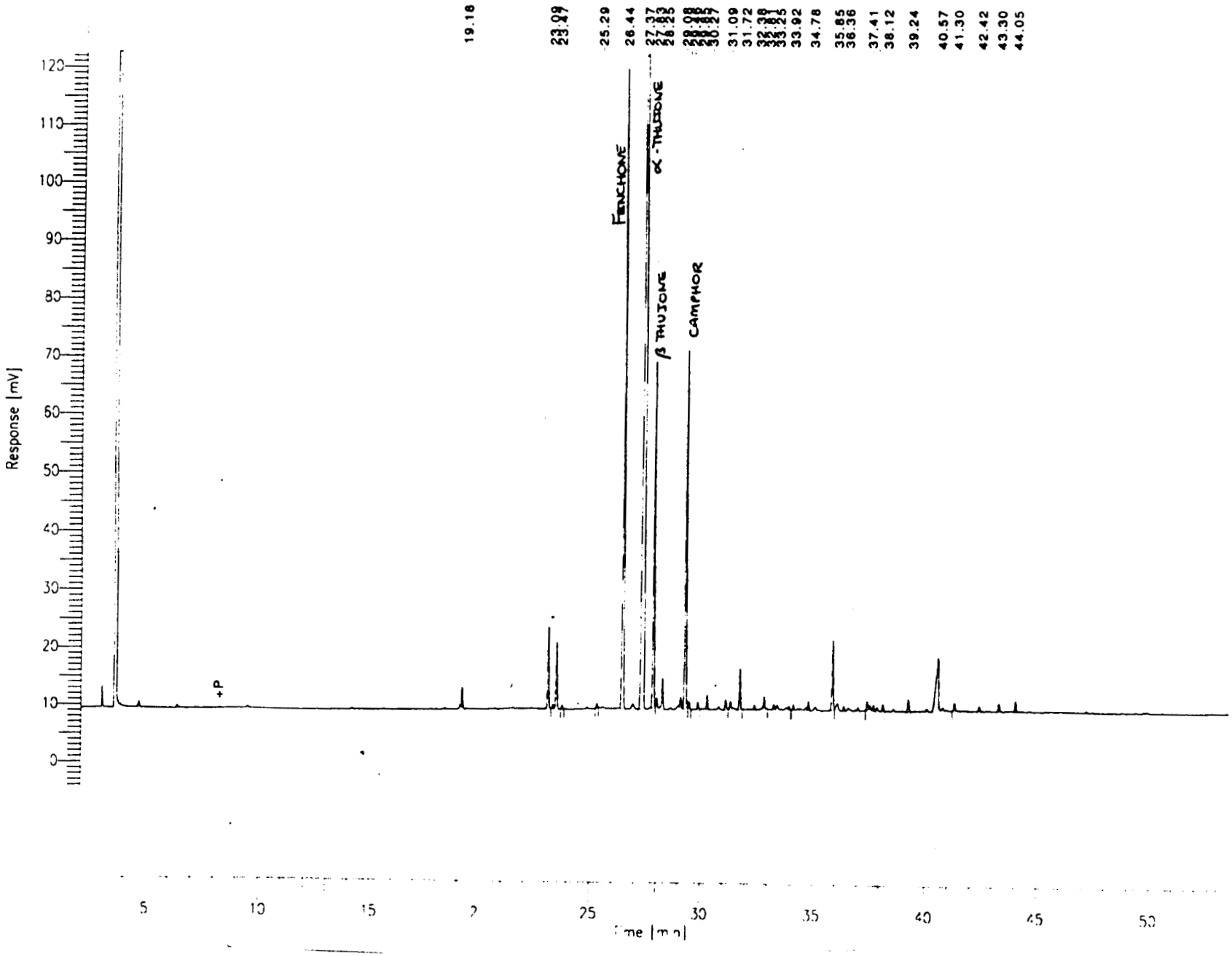
each Petriplate was measured at three points along the diameter of the plates and the mean of these three readings was taken as the radial growth of the colony. The growth of the colony in the control sets were compared with that of various treatments and difference converted into percent inhibition.

Effect of thujones on the growth of *P. capsici*

In order to study the effect of thujone on the growth of *P. capsici*, an essential oil contining 54% α-thujone, 8% β-thujone, 14% Fenchone and 8% camphor was procured from Quest international, England. 0.5 mL of it was dissolved in 100 mL acetone to prepare a stock solution of 5000 p.p.m. From this solution 0.5 mL, 1 mL, 2 mL and 4 mL each were added to 49.5 mL, 49 mL, 48 mL and 46 mL of carrot agar medium to get a final volume of 50 mL medium of concentration 50 p.p.m., 100 p.p.m., 200 p.p.m., and 400 p.p.m. respectively. Control sets were prepared by adding appropriate quantities of acetone. These media were then autoclaved at 15 psi for 30 minutes. After cooling to 40°C, each medium was dispensed in to three Petriplates (90 mm) for replication. Mycelial discs of 0.5 cm diameter taken from the growing edges of 48 h old culture plates of *P. capsici* were placed at the centre of the Petriplates. The plates were incubated in dark at 20-22°C

for 72 h. The radial growth of mycelium in each Petriplate was measured at three points along the diameter of the Petriplates and mean of these three readings gave the radial growth of the colony. Compared the growth of the colony in the control sets with that in various treatments and per cent inhibition was calculated.

From the above experiments it was proved that the antifungal activity of the leaf essential oil of *Artemisia nilagirica* against *P. capsici* was mainly due to the major components α-thujone and β-thujone. The leaf oil of *Artabotris odoratissimus* also showed moderate inhibition, but the constituents responsible for it was not identified.



Gas chromatogram of the commercial essential oil sample used for antifungal studies

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