ISOLATION, STRUCTURE ELUCIDATION, STRUCTURE MODIFICATION AND ANTIMICROBIAL PROPERTIES OF SECONDARY METABOLITES IN PLANTS

Thesis submitted to
University of Calicut in partial fulfillment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY IN CHEMISTRY
under the Faculty of Sciences

By

MOHANAKRISHNAN M.

DEPARTMENT OF CHEMISTRY
UNIVERSITY OF CALICUT
2013

CERTIFICATE

This is to certify that the thesis entitled "**Isolation, Structure Elucidation, Structure Modification and Antimicrobial Properties of Secondary metabolites in plants**" submitted herewith is a *bonafide* record of the research work carried out by **Mohanakrishnan. M** under my supervision and guidance in partial fulfillment of the requirements for the award of Doctor of Philosophy in Chemistry under the Faculty of Sciences, University of Calicut, Kerala. The contents of this thesis have not been submitted to any other Institute or University for the award of any degree or diploma.

University of Calicut,
November, 2013

Dr. P.Mohamed Shafi
(Supervising Teacher)

DECLARATION

It is hereby declared that the thesis entitled **"Isolation, Structure elucidation, Structure modification and Antimicrobial Properties of Secondary metabolites in plants "** submitted herewith is an authentic record of the research work carried out by me under the supervision of **Dr. P. Mohamed Shafi,** Professor, Department of Chemistry, University of Calicut, in partial fulfillment of the requirements for the award of the degree of Doctor of Philosophy in Chemistry under the Faculty of Sciences, University of Calicut, and that this thesis has not been submitted to any other Institute or University for the award of any degree or diploma.

University of Calicut,
November, 2013

Mohanakrishnan. M

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Mohanakrishnan. M

CONTENTS

PREFACE

Chemical constituents present in any plant can be divided into two: namely primary metabolites and secondary metabolites. Primary metabolites are simple molecules or polymers such as carbohydrates, proteins, lipids and nucleic acids . They are essential for the daily survival and structural growth of the parent organism and generally do not possess any therapeutic value.

Secondary metabolites are complex organic molecules biosynthesized from primary metabolites and usually stored in the cell vacuoles. Terpenoids, alkaloids and phenolics are the major types constituting secondary metabolites. These can be further classified into carotenoids, flavanoids, tannins, lignans etc.

In a sense secondary metabolites are not directly involved in the plant life and once considered as the waste products of primary metabolism. Actually they are as important as the primary metabolites for the existence of a plant in an ecosystem with enormous linear and non-linear variables. As part of the biochemical adaptation of the plant they function as anti-herbivore and antifungal defenses , attractants for pollinators and seed dispersers and visible light screens against high energy ultraviolet radiations. Most of them are semiochemicals involved in the plant-plant and plant-insect communications.

From the very ancient time secondary metabolites are an essential part of the human culture. They are used up either in the crude or in the purified form as drugs , flavors, fragrances, stimulants, natural insecticides, herbicides and functional foods. Historical drugs like quinine, morphine, aspirin etc are either natural products or their chemically modified forms. Most of the new age pharmaceuticals and anticancer drugs like taxol owe to phytochemicals as their lead molecules.

Another important category is the plant essential oils which are volatile mixtures mainly of mono and sesqui terpenoids. Essential oils also contain aliphatic and aromatic compounds, certain esters and phenyl propanoids as their constituents. Being used up from the very ancient era of alchemy, the plant volatile oils form the essential basement of multi-dollar flavor and fragrance industry. These oils possess many biocidal potencies and exclusively used in aromatherapy.

Secondary metabolites can be extracted from a plant by mainly two means. In the first method the fresh or dried plant material is extracted by a suitable solvent like alcohol. Concentrated extracts are further subjected to precise methods like chromatography to isolate pure compounds. Essential oils are mainly obtained from the plant parts by techniques like steam distillation, hydro-distillation and cold pressing .

The isolates are characterized by different physical, chemical and spectroscopic methods including NMR and GC-MS (Gas chromatography-Mass spectroscopy) techniques. *In vitro* and *in vivo* bioassays are usually sought for corresponding biological and pharmaceutical studies.

This thesis entitled **"Isolation, Structure elucidation, Structure modification and Antimicrobial Properties of Secondary metabolites in plants"** is divided into 8 chapters. The first chapter is divided into two sections and deals with the analysis of floral volatiles of the timber wood teak (*Tectona grandis* ; Family-*lamiaceae*). In Section I the chemical composition of the volatile oil obtained by the steam distillation of teak flowers is analyzed using GC and GC-MS techniques . Perfumery properties of the volatile components are investigated by GC-olfactory analysis . In section II a preliminary investigation of the chemical ecology of teak flower is attempted against reported pollination syndromes .

Chapter 2 deals with the analysis of essential oil from the areal parts of the aquatic plant *Limnophila repens* (family-*plantaginaceae*) . The oil was obtained by steam distillation and its characterization was done by Gas

chromatography-Mass spectrometry. The medicinal properties are correlated with the major components identified. A preliminary investigation of the olfactory properties of the oil components is also attempted. An article based on this study has been communicated to *Journal of essential oil research.*

Phytochemical investigation of the medicinal plant *Commiphora caudata* (Family- *Burseraceae*) is dealt in Chapter 3. The dried bark of the plant was subjected to solvent extraction and subsequent fractionation using column chromatography. Isolated compounds were characterized by different spectroscopic techniques . Structural modification of the compounds was achieved by simple reactions.

Chapter 4 deals with the volatile oils obtained from the leaves and berries of *Atalantia monophylla*, a *Rutaceae* species of high altitude origin. The oils were obtained from the corresponding parts by steam distillation and characterized by GC-MS analysis. Composition of the leaf oil is compared with those previously reported from the same species, through a chemotaxonomic angle. Medicinal potentials of the key components of berry oil are also discussed.

Analysis of volatile metabolites from the leaves and flowers of an highly threatened species *Cosmostigma racemosum* (Family – *Asclepiadaceae)* is dealt in chapter 5. Properties of the individual constituents are also discussed .

Chapter 6 discusses the analysis of essential oils from the leaves , flowers and rhizomes of an endemic *curcuma* species *C.ecalcerata* (*Zingiberaceae)*. The oils were obtained from the fresh plant parts by steam distillation and characterized by GC-MS analysis . Properties of the major components are also discussed. The chapter also contain an elaborate review on essential oils collected from different plants of the genus. An article based on this work entitled ‘Composition and antifungal activity of the essential oil from *Curcuma ecalcarata* Sivar. & Balach.’ has been published in *Int J Pharm Biomed Sci* 2013, 4(3), 96-99.

Characterization of floral volatiles from two distinct species of the genus *Pogostemon* (*lamiaceae*) is discussed in chapter 7. A preliminary olfactoric profiling of the two oils was also done.

The last chapter deals with the investigation of antimicrobial properties of essential oils against plant pathogenic fungi. Volatile oils collected from five aromatic plants namely *L. repens*, *A. monophyllum*, *C. ecalcerata*, *C.aerugenosa* , and *Costus speciosus* were tested against three soil borne pathogens *Pythium aphanidermatum, Rhizoctonia solani* and *Fusarium oxysporum* . Fungal growth inhibition caused by each oil is discussed both qualitatively and quantitatively in connection with the chemical composition.

CHAPTER 1

PHYTOCHEMICAL INVESTIGATION ON TEAK FLORAL VOLATILES

SECTION I

COMPOSITION AND OLFACTORY PROFILE OF ESSENTIAL OIL FROM TEAK FLOWERS

1.1 Introduction

Teak (*Tectona grandis* Linn.) is one of the most valuable and widely planted tree species in south Asia and other tropical countries. Teak timber is valued for its durability as it is immune to insect and fungus attacks and resistant to wood rot. Being highly adapted to a wide range of climatic and edaphic conditions teak is preferred for large scale plantation programs all over India. Teak is being grown in plantations in around 60 countries in Asia, Africa and Latin America although its natural occurrence is limited to India, Laos, Myanmar and Thailand. Of the estimated global plantations in 2005, about 4% were teak constituting about 75% of the world's high-quality tropical hardwood plantations [1]. It is estimated that approximately 6,000,000 ha have been planted with teak in all over the world [2].

Teak attracted the timber market from its very first formal description by Carl Linnaeus the Younger in his 1782 work 'Supplementum Plantarum' [3]. The major attractions were : easy to grow which needs little attention compared to many agricultural crops, extreme durability and strength , resistance to decay even when unprotected by paints and preservatives, workability and dimensional stability and aesthetic beauty.

It is widely used in making doors, furniture, wooden ceilings , beams and boat decks. The leaves were used in cooking some typical jackfruit items in India, Central Java and Indonesia. In Kerala they were once used as packing materials in local shops. Teak is also considered as a major

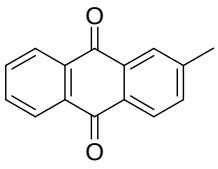
constituent in many folklore medicines. In traditional medicine, a wood powder paste has been used against bilious headache and swellings. They are also used for treating inflammatory swelling [4, 5] .

1.2 Phytochemical studies so far reported on Teak

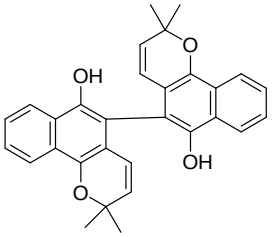
Teak is widely investigated by both industry and academies with agricultural , economical and ecological point of views. A good phytochemical data is also available on this large, deciduous tree. Every part of the plant namely leaves, flowers , fruits, seeds, bark and root was reported by traditional as well as modern studies to possess a wide range of medicinal indications such bronchitis, constipation, anthelmentic, depurative, hyperacidity, dysentery, verminosis, burning sensation, diabetes, leprosy, skin diseases, urinary discharge, diuretic, depurative,anti-inflammatory and burning sensation etc [23].

Teak wood oil :

The preservative nature of teak wood oil which makes the timber termite-proof had been studied recently to identify the anti-rot compounds like tectol[6]. Presence of tectoquinone (b-methyl anthraquinone) in the teak wood oil had been reported back in 1964 [25]. Tectoquinone had been extracted [24] from the saw dust very back in 1987 and was recognized as the compound characteristically responsible for the resistance towards different fungi and termites [22].



Tectoquinone

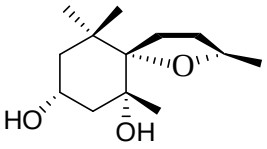


Tectol

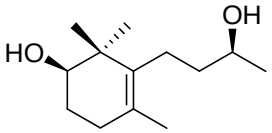
Leaves :

Macías et. al. reported the isolation of one monoterpene, seven apocarotenoids and the dehydrololiolide from the dried leaves. Two of the

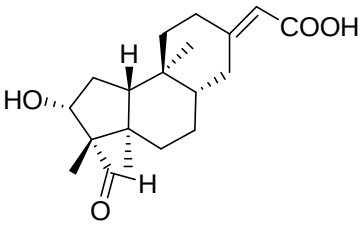
apocarotenoids had been isolated for the first time as natural products – tectoionols A and B [12]. Phytotoxic and allelopathic potencies of these compounds were also examined. Macías et. al. also isolated a new compound, abeograndinoic acid and 21 known terpenoids from the dried leaves [13]. These include the diterpenes 2-oxokovalenic acid and 19-hydroxyferruginol which showed good phytotoxic activity. Two new quinones named naphthotectone and anthratectone were isolated from bioactive leaf extracts by Lacret et.al. The allelopathic activity of the species is attributed to the presence of these quinones [14]. They isolated two new norlignans, tectonoelin A and tectonoelin B, together with ten known compounds from the most bioactive extract [19]correlated with the defense mechanism of the plant.



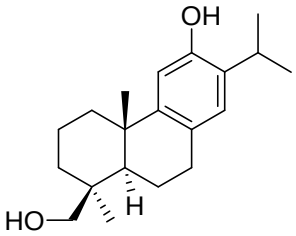
Tectoionol A



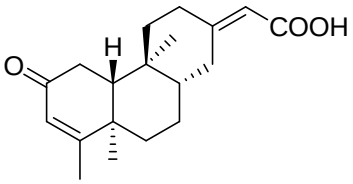
Tectoionol B



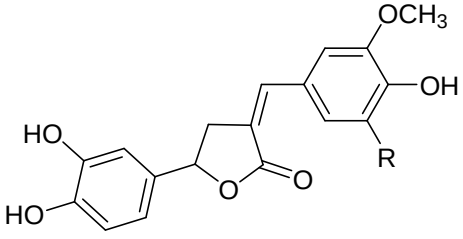
Abeograndinoic Acid



19-Hydroxyferruginol



2-Oxokovalenic Acid



R=H, Tectonoelin A
R=OCH_3, Tectonoelin B

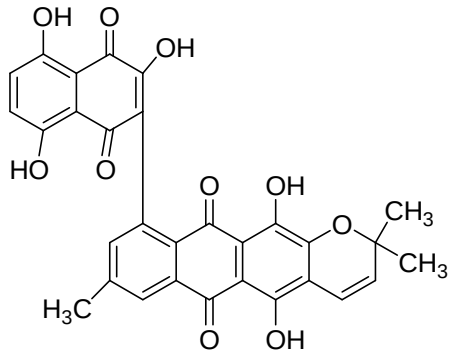
Krishna and coworkers obtained anthraquinones from teak leaves which showed antibacterial , antimycobacterial and antioxidant potencies [10]. Shukla et.al successfully isolated a new anthraquinone (3,8-dihydroxy-2-methylanthraquinone), named tectone along with fourteen known compounds comprised of five terpenoids , four flavonoids , three flavone glycosides and two phenolic glycosides from ethanol extract of the leaves [11]. Tectone was shown to possess good antihyperglycemic activity.

Another new naphthoquinone derivative had been isolated, along with a number of prenylnaphthoquinone congeners, from the heartwood which was characterized as dehydro-α-isodunnione by Gupta et.al. [18 . Derivative of 3,4-dihydroxy benzyl ester, a potent biopesticide with strong antimicrobial activity as well as growth inhibitory activity was isolated from the ethyl acetate fraction of teak leaves by Biswas et. al.[20]. Nayeem and Karvekar reported the isolation of gallic acid, ellagic acid, rutin and quercitin from the methanolic extract of the leaves [21]. Tectograndone, a novel pigment derived from the interaction of two prenylated naphthoquinones was also isolated from teak leaves [17]

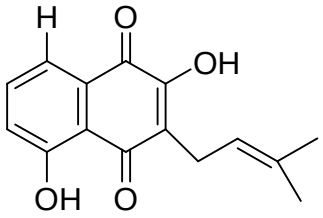
Heart wood :

Khan et. al. isolated a new cytotoxic agent 5-hydroxylapachol along with the known constituents lapachol, dehydro-α-lapachone, methylquinizarin and squalene from the root heart wood [7]. Both 5-hydroxylapachol and lapachol were found to be cytotoxic to *Artemia salina* (brine shrimp).

Khan et.al also isolated a new steroidal glycoside and three new fatty esters along with some known compounds from the stem bark [8]. Singh et.al isolated a new 9,10-dimethoxy-2-methyl anthra-1,4- quinone and several known naptho- and anthra-quinone derivatives from the heartwood [9].



Tectograndone



5-Hydroxylapachol

A phenolic glycoside, verbascoside showing anti-ulcer activity and cytoprotective potential was isolated from the butanol fraction of the plant extract by Singh et. al.[15]. A new naphthoquinone derivative 4′,5′-dihydroxy-epiisocatalponol was isolated from the heartwood of the teak stem by Niamké et. al.[16]. This compound was shown playing a key role in the variability of decay resistance in teak wood. Alsoin '*in-vitro* bioassays the compound showed fungicidial potency against *Trametes versicolor* (white rot).

Flowers

Teak flowers are described as diuretic, depurative and anti-inflammatory in ancient healing methodologies . There is a mention that flower-Oil (no indication of essential oil or volatile oil) was recommended for hair growth and in cases of scabies and eczema [23]. Infusion of flowers were suggested for congestion of liver . A recent study has revealed the medicinal potentials of methanolic extract of teak flowers [26].

1.3 Present Work

Teak belongs to the mint family *lamiacea* (31), which is better known for its aromatic members such as basil, rosemary and lavender all producing economically important essential oils. Even then no work has so far been reported on the steam volatiles of teak, especially from the pleasant smelling

flowers. Here we present the analysis of floral volatiles of teak using GC, GC-MS, and GC-olfactory technique for the first time. A preliminary chemo-ecological analysis is also attempted to address the pollination problems in Teak.

Plant Collection

The teak flowers were collected from Malappuram district, Kerala, India. It was identified by Dr. A.K. Pradeep, Department of Botany, University of Calicut and a specimen voucher is deposited in the specially maintained Herbarium of Chemistry department of University of Calicut.

Extraction

The flowers (500 g) were separated and ground into a paste and subjected to steam distillation for 3 hours. The oil was extracted by diethyl ether from the distillates. The ether extract was dried using anhydrous sodium sulphate and the ether evaporated. The pure oil was stored at 4^{O}C until analyzed.

Gas chromatography/mass spectrometry/olfactometry:

GC/MS/O was carried out using an Agilent 6890 gas chromatograph, fitted with a HP-5 (5 % diphenyl polysiloxane) capillary column (50 m x 0.32 mm x 0.52 µm), with He carrier gas, initial head pressure 15.0 psi (2.0 mL/min) constant flow mode. The column effluent was split between an Agilent 5975N inert MSD spectrometer and an in-house odour detection port *via* a Capillary Flow Technology splitter plate with pressure set to 3.8psi. The injector and odour port transfer line temperatures were held constant at 230°C and 250°C, respectively. Injection of 1µl at 500ng/µl dilution in splitless mode with oven program: 35°C (3min), 15°C/min ramp to 50°C then 5°C/min ramp to 280°C(held 10min). Data was acquired and processed using MSD ChemStation (Rev. D.02.00.275). The odour assessments and description were carried out by experienced perfumers.

Gas chromatography/mass spectrometry:

GC/MS was carried out using the same system but optimised for resolution with a linear temperature ramp of 2°C/min and calibrated for Retention Indices using C7-C28 n-alkanes. The inert MSD was operated with source temp230°C, quad temp 150°C and ionization voltage 70 eV. Target spectra were acquired using the s.tune parameters and compared against in-house and commercial libraries from which identifications were assigned on the basis of both spectral match and retention data [32].

Gas chromatography/flame ionization detection:

GC/FID analysis for quantisation was carried out using an Agilent 6890 gas chromatograph, fitted with an Ultra 2 (5 % diphenylpolysiloxane) capillary column (50 m x 0.2 mm x 0.33 μm), split injection (50:1) with He carrier gas (1.2 mL/min). The oven was programmed from 50–280°C (held for 6 min) at 2°C/min. The injector and detector temperatures were held constant at 230°C and 300°C, respectively. Data was acquired and processed using HP ChemStation software (Rev. A.10.02 [1757]). Quantitative data was obtained from relative peak area (%RPA) without the use of response factors.

1.4 Results and Discussions

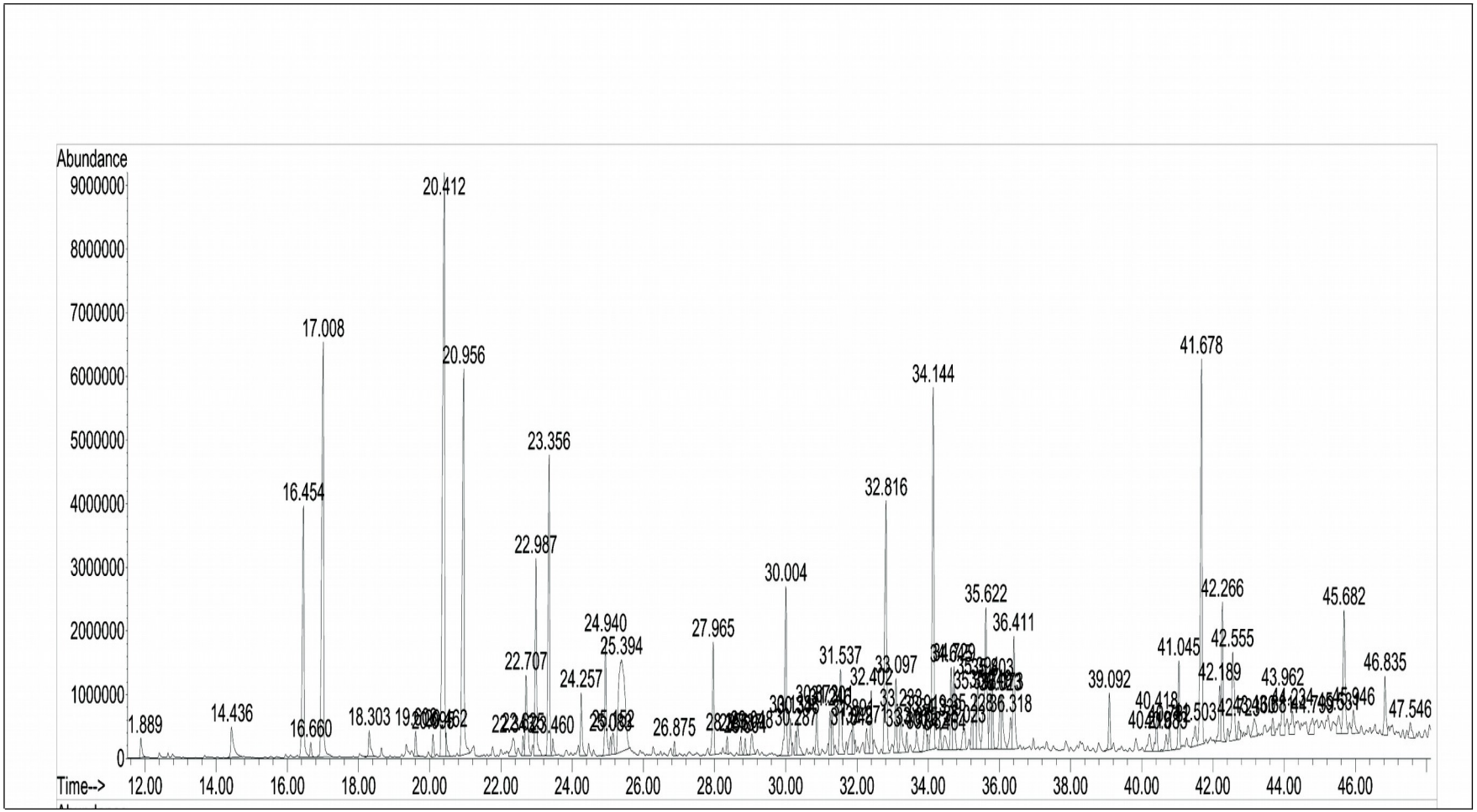
Steam distillation of fresh teak flower afforded an oil with pleasant floral aroma. The product was light yellow in colour and the yield was 0.027% of the fresh weight sample. This is the first authentic report of isolation of essential oil from any part of any of the species T*ectona.*

1.4.1 Chemical composition :

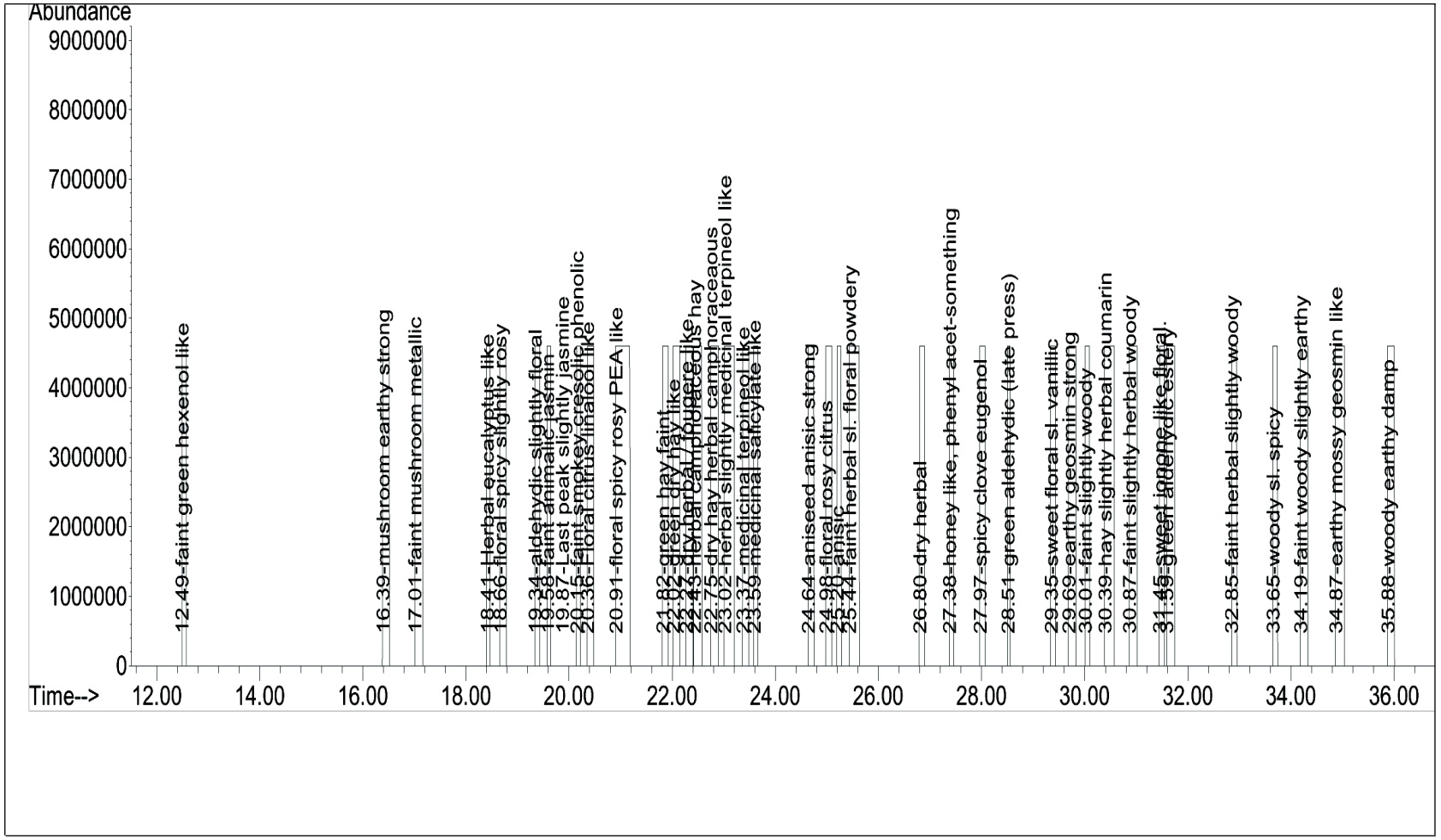
Sixty six components consisting of 84.13% of the oil were identified in the GC-MS analysis. Out of these 52 compounds comprising of 78.8% were oxygenated compounds while only 13 compounds comprising of 20% were various hydrocarbons. Nitrogen containing heterocyclic compound indole was also detected in the oil. Oxygenated compounds mainly consisted of 24

alcohols, 5 aldehydes, 5 ketones, 7 esters and 4 phenyl propanoids. Among the alcohols 17 of them were alcohols of mono-, di- or sesqui- terpenes. The hydrocarbons comprised of 11 sesquiterpenes and 2 diterpenes (Table 1.1).

There were 13 aliphatic compounds among the identified components. C-6 compounds dominated this group with two green leaf volatiles (GLV) Cis-3-hexenol and n-hexanol and four hexenyl esters. Hexenyl ester of benzoic acid was also identified in the oil. Five C-8 aliphatics namely Octan-1-ol, 3-Octanol, Oct-1-en-3-ol, 3-Octanone and 1-Octen-3-one were also identified.



GC Trace of Teak flower essential oil



GC-O Trace of Teak flower essential oil

Table 1.1 Teak Flower Oil – GCMS Analysis with FID quantification.

No	RI(lit)	RI	Name of Compounds	%RPA (FID)	Identification
Hydrocarbons-Sesquiterpens					
1	1374	1383	α –Copaene	0.3	MS, RI(std)
2	1390	1394	7-Epi-Sesquithujene	0.1	MS
3	1417	1429	Caryophyllene	1.9	MS, RI(std)
4	1432	1441	Trans-α-Bergamotene	0.5	MS, RI(lit)
5	1440	1458	Cis-β-Farnesene	0.1	MS, RI(std
6	1452	1463	α-Humulene	0.5	MS, RI(std)
7	1478	1484	γ - Muurolene	0.4	MS, RI(lit)
8	1479	1486	Curcumene	0.2	MS, RI(std)
9	1484	1490	Germacrene D	1.6	MS, RI(std)
10	1522	1530	δ -Cadinene	0.5	MS, RI(std)
11	-	1817	Sesquiterpene hydrocarbon	0.3	MS
Hydrocarbons-Diterpenes					
12	-	1920	Diterpene hydrocarbon	0.6	MS
13	-	1927	Diterpene hydrocarbon	0.7	MS
Alcohols-Aliphatic					
14	850	854	Cis-3-Hexenol	0.5	MS, RI(std)
15	863	866	Hexanol	0.3	MS, RI(std)
16	974	978	Oct-1-en-3-ol	8.0	MS, RI(std)
17	988	995	3-Octanol	12.1	MS, RI(std)
18	1026	1033	Benzyl alcohol	0.4	MS, RI(std)
19	1063	1069	Octan-1-ol	0.4	MS, RI(std)
20	1106	1114	Phenylethyl alcohol	6.3	MS, RI(std)
Alcohols-terpenoids					

No	RI(lit)	RI	Name of Compounds	%RPA (FID)	Identification
21	1095	1100	Linalool	14.6	MS, RI(std)
22	1118	1123	Cis-para-Menth-2-en-1-ol	0.2	MS, RI(std)
23	1136	1141	Trans- para-Menth-2-en-1-ol	0.2	MS, RI(std)
24	1165	1169	Borneol	1.1	MS, RI(std)
25	1174	1180	Terpinen-4-ol	2.3	MS, RI(std)
26	1186	1193	α-Terpineol	3.5	MS, RI(std)
27	1227	1228	Nerol	0.4	MS, RI(std)
28	1249	1254	Geraniol	1.6	MS, RI(std)
29	1561	1566	Trans-Nerolidol	0.5	MS, RI(std)
30	1674	1677	β-Bisabolol	0.7	MS, RI(std)
31	-	1960	Diterpene alcohol	1.2	MS
32	-	1960	Diterpene alcohol	2.0	MS
33	-	1990	Geranyl Linalool isomer	0.5	MS, RI(std)
34	-	1991	Geranyl Linalool isomer	1.1	MS, RI(std)
35	-	2003	Geranyl linalool isomer	0.5	MS
36	-	2006	Geranyl linalool isomer	0.4	MS
37	-	2173	Geranyl Geraniol	0.9	MS, RI(std)
Aldehydes					
38	952	958	Benzaldehyde	0.43	MS, RI(std)
39	1036	1042	Phenylacetaldehyde	0.3	MS, RI(std)
40	1100	1103	Nonanal	0.6	MS, RI(std)
41	1150	1152	2,6-Nonadienal	0.1	MS, RI(std)
42	1247	1254	Anisaldehyde	0.1	MS, RI(std)
Ketones					

No	RI(lit)	RI	Name of Compounds	%RPA (FID)	Identification
43	972	976	1-Octen-3-one	0.1	MS, RI(std)
44	979	985	3-Octanone	1.9	MS, RI(std)
45	1118	1121	Isophorone	0.2	MS, RI(std)
46	1453	1453	Geranyl acetone	0.1	MS, RI(std)
47	1806	1819	Nootkatone	0.3	MS, RI(std)
Esters					
48	1184	1185	Cis-Hex-3-enyl butyrate	0.2	MS, RI(std)
49	1190	1196	Methyl salicylate	0.2	MS, RI(std)
50	1229	1232	Cis-3-Hexenyl 2-methylbutyrate	0.3	MS, RI(std)
51	1254	1256	Phenylethyl acetate	0.3	MS, RI(std)
52	1319	1324	Cis-3-Hexenyl tiglate	0.1	MS, RI(std)
53	1378	1380	Cis-3-Hexenyl hexanoate	0.3	MS, RI(std)
54	1565	1574	Cis-Hex-3-enyl benzoate	0.2	MS, RI(std)
Phenyl Propanoids					
55	1309	1314	4-Vinyl guaiacol	0.1	MS, RI(std)
56	1356	1359	Eugenol	0.9	MS, RI(std)
57	1432	1440	Coumarin	0.6	MS, RI(std)
58	1555	1556	Elemicin	1.2	MS, RI(std)
Other Oxygenated compounds					
59	1067	1073	Cis-Linalool oxide (furanoid)	0.4	MS, RI(std)
60	1084	1089	Trans-Linalool oxide (furanoid)	0.3	MS, RI(std)
61	-	1269	Hydroquinone	1.0	MS, RI(std)
62	1542	1549	Cis-Sesquisabinene hydrate	0.8	MS, RI(std)

No	RI(lit)	RI	Name of Compounds	%RPA (FID)	Identification
63	1577	1585	Trans-Sesquisabinene hydrate	0.2	MS, RI(std)
64	-	1595	β-Caryophyllene epoxide	3.9	MS, RI(std)
65	1608	1620	Humulene epoxide	1.1	MS, RI(std)
Heterocyclic compound					
66	1290	1294	Indole	0.5	MS, RI(std)

RI (std) = by comparison of RI with in-house library of standards
RI (lit) = by comparison of RI with published data
MS = identification by MS match with in-house and commercial libraries (NIST, Adams, Mass finder)

All the hydrocarbons present in the oil were either sesquiterpenes or diterpenes. All the monoterpenes were in the oxidized form; 8 of them were alcohols. It is evident that sesquiterpenes are not readily oxidised as monoterpene hydrocarbons due to their comparatively larger molecular size and lower volatility. Usually the absence of monoterpene hydrocarbons reduces the therapeutic potentials of the oil while the same improves its perfumery value [33].

Among the isolated compounds there were 9 diterpenoids which are the largest and heaviest molecules found in essential oils produced by distillation [33]. These mainly consisted of geranyl geraniol and four isomers of geranyl linalool which can be correlated with the unidentified diterpene hydrocarbons through corresponding biosynthetic pathways [34]. Isomers of linalool oxides (furanoid) were also detected.

The most abundant component of the oil was the monoterpenoid linalool (14.6%) followed by 3-octanol (12.1%) , oct-1-en-3-ol (8%) and 2-phenylethanol (6.3%) . It is common to have linalool as the most abundant component in one of its enatiomeric forms in many flower essential oils (10).

The oil also consisted of nerol, geraniol and terpineol which have the same biosynthetic precursor as linalool. Other compounds of interest were indole (0.5%) and β-caryophyllene epoxide (3.9%) . Indole can be expected as it is a common ingredient of fragrant white flowers such as jasmine and orange blossom [35] while the latter may be one of the resultants of the photo-oxidation of caryophyllene [36]. All these compounds are reported for the first time from this plant to the best of our knowledge.

1.4.2 Olfactoric Properties :

The olfactory evaluation of the oil revealed the presence of a wide spectrum of impressions ranging from spicy-floral to mushroom-metallic and woody-earthy to honey-like. A total of 38 odour impressions were detected and correlated with the corresponding components [Table 1.2]. Twenty five of these impressions corresponded to the compounds detected in GC-FID analysis. Five of the remaining impressions corresponded to compounds which were similar in structure to those of the detected compounds while another five corresponded to undetected compounds. There were three impressions which could not be assigned chemically.

Table 1.2 Teak Flower Oil – Gas-Chromatography-Mass pectrometry -Olfactometry Evaluation

No	Peak Start	Peak End	Comment	RI	Identification
1	12.49	12.57	Faint green hexenol like	855	Cis 3-Hexenol
2	16.39	16.52	Mushroom earthy strong	980	Oct-1-en-3-ol
4	17.01	17.17	Faint mushroom metallic	995	3-Octanol
5	18.41	18.48	Herbal eucalyptus like	1040	1,8-Cineole
6	18.66	18.79	Floral spicy slightly rosy	1048	Phenylacetaldehyde
7	19.34	19.44	Aldehydic slightly floral	1070	Octanol
8	19.58	19.65	Faint animalic jasmine	1075	p-Cresol
9	20.15	20.23	Faint smokey cresolic phenolic	1095	N.I
10	20.36	20.49	Floral citrus linalool like	1102	Linalool
11	20.91	21.19	Floral spicy rosy PEA like	1121	Phenylethyl alcohol
12	21.82	21.92	Green hay faint	1150	Trans p-Menth-2-en-1-ol
13	22.02	22.16	Green dry hay like	1155	Cis-3-Nonen-1-ol
14	22.27	22.41	Dry herbal / fougere like	1162	Non-2-enal
15	22.43	22.59	Herbal camphoraceous hay	1170	Octanoic acid
16	22.75	22.91	Dry hay herbal camphoraceaous	1179	Borneol
17	23.02	23.21	Herbal slightly medicinal terpineol like	1188	4-Terpineol
18	23.37	23.5	Medicinal terpineol like	1201	α- Terpineol
19	23.59	23.67	Medicinal salicylate like	1206	Methyl salicylate
20	24.64	24.76	Aniseed anisic strong	1246	Phenylacetic acid
21	24.98	25.1	Floral rosy citrus	1256	Geraniol

No	Peak Start	Peak End	Comment	RI	Identification
		1			
22	25.2	25.29	Anisic	1263	Anisaldehyde
23	25.44	25.64	Faint herbal sl. Floral powdery	1272	Hydroquinone
24	26.8	26.9	Dry herbal	1320	2,4-Decadienal / Vinyl guaiacol
25	27.38	27.46	Honey like, sweet, Phenylacetaldehyde-like	1348	Heliotropin
26	27.97	28.08	Spicy clove eugenol	1366	Eugenol
27	28.51	28.56	Green aldehydic	1381	Cis-3-Hexenyl hexanoate
28	29.35	29.44	Sweet floral sl. Vanillic	1410	Vanillin
29	29.69	29.84	Earthy geosmin strong	1430	N.P
30	30.01	30.1	Faint slightly woody	1445	Caryophyllene
31	30.39	30.58	Hay slightly herbal coumarin	1458	Coumarin
32	30.87	31.02	Faint slightly herbal woody	1479	Humulene
33	31.45	31.55	Sweet ionone like floral.	1499	β- Ionone
34	31.59	31.75	Green aldehydic estery	1508	Germacrene D + Alkyl thiopene
35	32.85	32.95	Faint herbal slightly woody	1559	Sesquisabinene hydrate / Elemicin
36	33.65	33.74	Woody sl. Spicy	1595	Unidentified Sesquiterpene
37	34.19	34.33	Faint woody slightly earthy	1615	Caryophyllene epoxide
38	34.87	35.05	Earthy mossy geosmin like	1646	N.I
39	35.88	36.01	Woody earthy damp	1688	β - Bisabolol

N.I- not identified. N.P - no peak.

Sesequiterpene hydrocarbons in the oil mainly imparted herbal and woody odour as the base note. Three of the 4 phenylpropanoids stabilize this . Eugenol and an unidentified sesquiterpene hydrocarbon added spicy notes. β-

Bisabolol, cis-sesquisabinene hydrate, β-caryophyllene epoxide, non-2-enal and octanoic acid were the other components enhancing the herbal woody note.

The most abundant component linalool give the fresh floral impression which was enhanced by octan-1-ol, geraniol and phenyl acetaldehyde. The other floral impressions were that of phenylethyl alcohol and sweet floral impression of vanillin and β- ionone (both undetected in GCMS). β -Ionone, the rose ketone having comparatively high relative percentage of odour units is found to be very active even in very low quantities around 0.03 % [37]. The animallic-floral impression observed in the olfactogram corresponded to p-cresol while the similar note expected for indole was absent. All these synergize to give the pleasant floral impression.

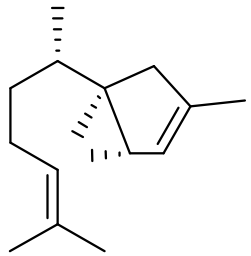
Terpinen-4-ol and α-terpineol imparted a medicinal impression to the oil enhanced by methyl salicylate. Mushroom impressions were added by oct-1-en-3-ol and 3-octanol. A faint 'green-odour' due to hexane derivatives was also observed.

The known grapefruit impression of nootkatone (0.35%) was not observed by the GC-olfactometry analysis. The honey-like, sweet impression of phenylethyl acetate was detected more than once but they corresponded to the retention indices of phenylethyl alcohol and heliotropin. The impression directly from phenylethyl acetate may be masked by other floral notes. There were also unassigned peaks in the olfactogram for faint smokey -cresolic -phenolic and an earthy -mossy-geosmin like impressions. No peak on the MS trace could be observed in the region where the “strong earthy geosmin” impression was smelled, although the measured retention index of 1430 is close to the library value of 1420 for geosmin.

Thus the olfactoric analysis allow the conclusion that the teak flower essential oil can be described as herbal-woody, spicy-floral, medicinal, mushroom-like and earthy geosmin-like . Even though of very low yield, the oil can be suggested for fine perfumery applications where these notes are

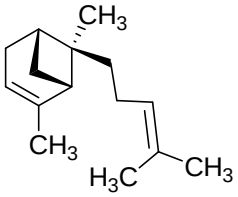
essential.

Structure of compounds from the GC/MS analysis of teak flower essential oil



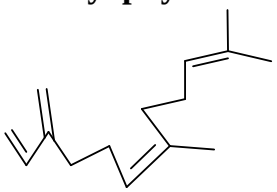
α –Copaene

7-Epi-Sesquithujene



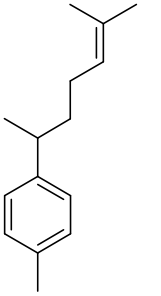
Caryophyllene

Trans-α-Bergamotene



Cis-β-Farnesene

α-Humulene

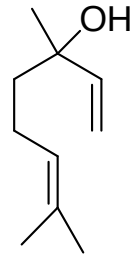


γ - Muurolene

Curcumene

Germacrene D

δ -Cadinene

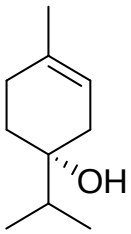


Linalool

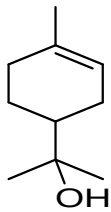
Borneol

Cis-para-Menth-2-en-1-ol

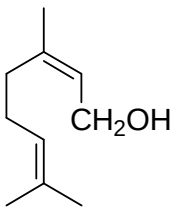
Trans-para-Menth-2-en-1-ol



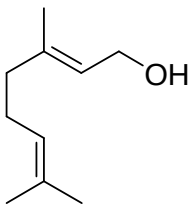
Terpinen-4-ol



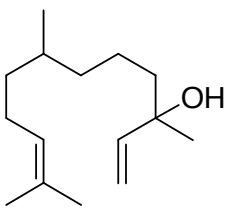
α-Terpineol



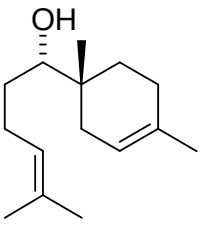
Nerol



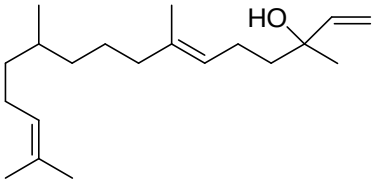
Geraniol



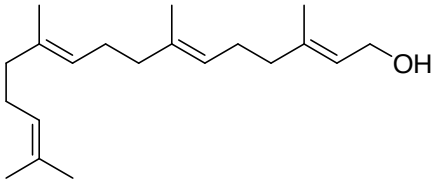
Trans-Nerolidol



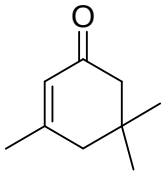
β-Bisabolol



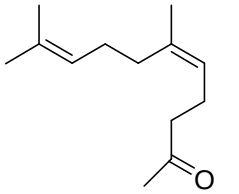
Geranyl Linalool



Geranyl Geraniol



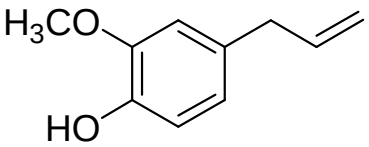
Isophorone



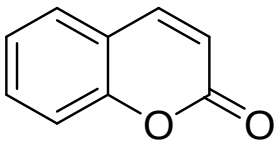
Geranyl acetone

Nootkatone

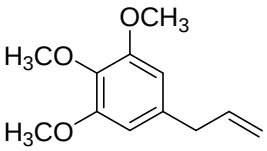
P-vinyl guaiacol



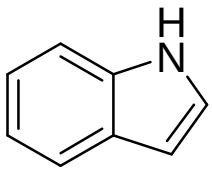
Eugenol



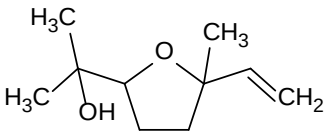
Coumarin



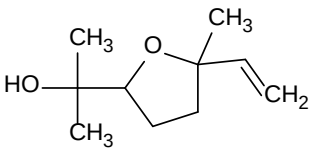
Elemicin



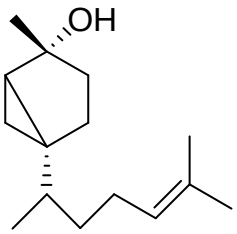
Indole



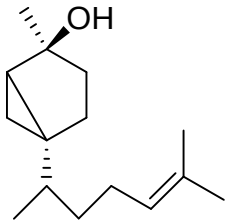
Cis-Linalool oxide (furanoid)



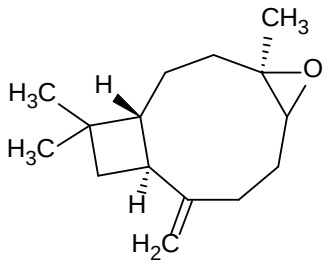
Trans-Linalool oxide (furanoid)



Cis-Sesquisabinene hydrate



Trans-Sesquisabinene hydrate



Caryophyllene epoxide

Humulene epoxide

1.5 References

1. KFRI Documents, Peechi, Kerala.
2. W. Kollert and L. Cherubini, Teak resources and market assessment 2010. FAO Planted Forests and Trees. Working Paper FP/47/E, Rome. 2012.
3. International Organization for Plant Information (IOPI). (http:/ / www. ipni. org/ ipni/ id.
4. P.S Varier, Indian Medicinal Plants: A compendium of 500 species.Vol 5. Orient Longman, Hyderabad, India;. pp. 245-248. 1996
5. C.P Khare. Indian Medicinal Plants: An Illustrated Dictionary. Springer Verlag. Heidelberg;. pp. 649. 2007
6. Sumthong, et.al, Journal of Wood Chemistry and Technology, 28, 247-260 ,2008.
7. Khan et.al. Phytochemistry, 50, 3, 10 ,1999.
8. Khan et.al.,Nat Prod Res.Nat Prod Res. 24,11, 2010.
9. P.Singh, Phytochemistry, 28,4, 1989.
10. M. S. Krishna, J. Nair. International journal of Phytomedicine, 3,1, 2011.
11. Shukla, et. al. Nat Prod Commun. 5,3, 2010.
12. Macías et al. Phytochemistry 69, 2708–2715, 2008.
13. Macías et.al. J Chem Ecol, 36, 396–404,2010.
14. Lacret et.al. J Chem Ecol ,37:1341–1348. 2011.
15. Singh N et. al. Fitoterapia. 81,7, 2010.
16. Niamké et. al. International Biodeterioration & Biodegradation, 74, 2012.
17. Aguinaldo et.al. Phytochemistry, 33, 4, 6 1993.
18. P. K. Gupta and P. Singh, Journal of Asian Natural Products Research , 6, 3, 2004 .
19. Lacret et.al. Phytochemistry Letters, 5, 2, 2012.
20. Biswas et al. Biopestic. Int. 5,1, 24 –34, 2009.
21. N Nayeem and M.D Karvekar, , RJPBCS, 1,2, 2010.
22. Rudman et.al.Nature 181, 1958.
23. Aradhana et. al. Herbal Tech Industry 06, 2010.
24. R. Romanis., j. Chem. Soc. 51,1887.
25. R.M. Beri and M.G. Karnik, Current Science, 1964.
26. Ramachandranet.al., Asian Pac. J. Trop. Med, 4, 8, 2011.
27. K Bryndum and H. T. Silvae, Genetica 18, 33, 1969.
28. Mathew et.al. The Indian Forester, 113,1, 1987.
29. Mohandas et.al. KFRI Research Reprt No 225, ISSN 0970-8103
30. Anmol and Kumar, Ind. For. , 11,8, 1992.
31. International Plant Names Index. International Organization for Plant Information (IOPI). http:/ / www. Ipni.org/ (17 December 2010).
32. R.P. Adams, Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry, 4th ed. Allured Publ. Corp., Carol Stream, IL ,2007.

33. D.Stewart, The Chemistry Of Essential Oils Made Simple. 3rd edn. pp 285, CARE
publications, Marble Hill, MO. 2005.
34. J. Mann, Secondary Metabolism, pp.112. Oxford University Press ,1978.
35. G. Buchbauer, Page no 128, Handbook of Essential Oils: Science, Technology, and Applications., CRC Press. Taylor & Francis Group 2010.
36. Sköld et. al. Food and Chemical Toxicology, 44, 4, 2006.
37. G. Ohloff, Scent and Fragrances, The fascination of odors and their chemical perspectives, Springer-Verlag, Pub., Berlin - Heidelberg, 154-158 , 1994.

SECTION II

CHEMICAL ECOLOGY OF TEAK FLOWERS

1.6 Volatile Metabolites -Introduction and Review

It is estimated that plants may produce over 200, 000 different compounds, the majority of which are classified as secondary metabolites [1].Out of these nearly 2000 volatile compounds have been identified from over 90 families to date [2]. These compounds are released from plant organs above or below the ground sometimes induced by biotic activities . The high vapour pressure and low molecular weight enable the volatile compounds to readily diffuse through the gas phase and within biological systems to serve as signalling molecules (semiochemicals) passing information both within and between organisms. They can function as hormones and as molecular guides in the identification of food, mates, co-specifics, competitors, predators or suitable habitat [3] . Plants produce a huge diversity of different chemicals, which include an array of VOCs (volatile organic compounds) emitted by flowers, foliage, bark, roots and specialised structures [4]. Volatiles produced by plants attract pollinators and seed dispersers. They provide defence against pests and pathogens. Volatiles produced by insects may act as pheromones directing social behaviour or as cues for finding hosts or prey. Chemical ecology or ecological biochemistry is the branch of science investigating the role of these chemical cues in the interaction of organisms with their environment.

The volatile substances involved in the chemical communication of biological systems can be classified into attractants, repellents, kairamones , pheromones and allomones. Attractants are substances that directly drag one species to another while repellents that repel them apart. Pheromones are chemical substances used by animals for communication [6] which is emitted by one individual and received by another individual of the same species to alter its behaviour. These can be further classified as sex pheromones, alarm pheromones and food trail pheromones according to the action. Kairamones

are substances emitted by an organism, which mediates interspecific interactions that benefits an individual of another species which receives it, without benefiting the emitter [7] while allomones are produced and released by an individual of one species that affects the behaviour of a member of another species benefiting exclusively the originator [8]. Production of allomones is a common form of defence, particularly by plant species against insect herbivores. It is to be mentioned that an overlap of more than 80% was found in VOCs produced by plants and insects [9].

Green Leaf Volatiles:

Green leaf volatiles (GLV) are an important class of plant secondary metabolites. They are volatile organic compounds that are released when plants suffer tissue damage or ecological stresses . Some of them act as signalling compounds between either plants of the same or other species, or even with vastly different life forms like insects. Some of them act essentially as plant pheromones [10]. GLVs mainly consist of oxygenated hydrocarbons (especially C-6 alcohols, aldehydes and ketones) produced from the biochemical conversion of linoleic and linolenic acid within plant cells [11].

Green leaf volatiles were identified as important constituents of leaf odours especially the green odour attractive to various phytophagous insects [10] like beetles. As herbivore induced volatiles they are reliable indicators of herbivore presence that other plants can detect these volatiles and modify their defences accordingly . Several volatile metabolites including the green leaf volatiles (E)-2-hexenal, (Z)-3-hexen-1-ol and cis-3-hexenyl acetate, the terpenes myrcene and blended ocimene volatiles and the phytohormones methyl jasmonate, methyl salicylate and ethylene have been reported to function as between and within plant signals [12]. Electrophysiological studies had revealed that the leaf volatiles like C6-alcohols [(Z)-2-hexenol, (E)-2-hexen-ol and (Z)-3-hexenol], aldehyde [(E)-2-hexenal], ester [(Z)-3-hexenyl acetate] and C7-alcohol (1-hepatanol) directly affect the physiology and behaviour of herbivores through its positive (attractive) and negative

(repelling and deterring) responses [13]. In addition to the defensive role, air-borne (Z)-3-hexenol from wounded plants was also proposed to trigger the pre-defence responses in neighbouring un-attacked plants; a phenomenon called priming effect of volatiles [13].

Floral volatiles

Volatile metabolites produced by flowers and inflorescences consist of four major chemical groups namely aromatics, monoterpenes, sesquiterpenes and fatty acid derivatives. They are meant for mainly attracting pollinators of suitable class [14].

In an epic review [15] Jette T. Knudsen presented a list of 1719 chemical compounds identified from headspace samples of floral scent. The list has been compiled from some 270 research papers, including analyses of 991 species of flowering plants and a few gymnosperms. The compounds belonged to seven major categories, of which the aliphatics, the benzenoids and phenylpropanoids, the mono- and sesquiterpenes found to occur in most orders of seed plants. C5-Branched compounds, irregular terpenes, nitrogen compounds, and a group of miscellaneous cyclic compounds were identified in about two-thirds of the orders. Sulfur-containing compounds occurred in one third of the orders, while diterpenes occurred in three orders only. The most common single compounds found in the floral scent were the monoterpenes limonene, (E)-beta-ocimene, myrcene, linalool, alpha-and beta-pinene, and the benzenoids benzaldehyde, methyl salicylate, benzyl alcohol, and 2-phenyl ethanol. These compounds were found to occur occur in 54-71% of the families investigated so far. The sesquiterpene caryophyllene and the irregular terpene 6-methyl-5-hepten-2-one were also common and occurred in more than 50% of the families [15]. However the main classes of floral volatiles are the same as those reported to be released from other parts of the plant. But the overall diversity of vegetative volatiles is less than compared with the floral scent.

1.7 Pollination in Teak

Pollination is the key biological process in higher plant reproduction that involves the transfer of pollen grains (male gametes) to the plant flower carpel (female gamete) [16]. Since self-pollination without the aid of any other organisms is often affected by incompatibility problems, plants have developed cross-pollination strategies. Wind pollination is a major strategy in grasses and sedges; many willows, poplars, oaks, alders, pines and spruces [17]. Other agents involved in carrying out crosspollination are insects, bats, mammals and birds [16].

The major biochemical factors controlling the relationship between pollinators and plants are the scent and colour of the flowers and the nutritional value of the pollen and nectar [18]. It is the floral volatiles playing the chief role in attracting the pollinators from kilometres away [19]. Scent is particularly important in night-blooming species when visual cues become incompetent due to darkness[20]. Different flowers discharge different volatile compounds which are detected by animals which are living in the world of chemical communication [21]. Near the flower it is welcomed by the visual signal (the floral colours) and drawn to the nectar by the visual honey guides on the petal. Finally as pollen is transferred it gets the nutritional reward from the nectar and pollen [21].

Like many other economically important crops teak is mainly an insect pollinated species [22]. Wind pollination is also possible in some cases. Mathew et. al.(1987) studied the insect pollinators of teak in Kerala. They reported 17 species of insect visitors of which 13 were *hymenopterans* and two each of *lepidopterans* and *dipterans* [23]. A publication by L.C Egenti from the Federal Department of Forest Research , Nigeria recorded six insect species belonging to *lepidoptera*, *hymenoptera* and *hemiptera* as the major insect pollinators of Teak. A later investigation by the research group at KFRI records altogether 60 species of insects visiting the teak flower [24]. These belonging to 42 families under 6 orders can be represented as given below:

Lepidoptera (Butterflies, skippers)	-	28 species
Hymenoptera (bees, wasps, ants)	-	15 species
Diptera (True flies, Mosquitos)	-	7 species
Hemiptera (True bugs)	-	4 species
Coleoptera (Beetles)	-	4 species
Thysanoptera (Thunder flies etc)	-	2 species

Even though maximum in numbers *lepidopterans* were not revealed as true pollinators of teak as they were not found carrying pollen grains. Hymenopterans which are considered are as efficient pollinators are second in number. Of these groups the bees were found to be very active but present only in small numbers [23,24].

Flowering season of Teak is from February to August. There will be more than 300 inflorescences on a single teak tree. About 10,000 flowers are produced per inflorescence [23]. Plenty of nectar is available for foraging insects. At this point it is interesting to mention from this report that the visiting frequency of the Indian honey bee, *Apis indica* which is considered as one of the most efficient pollinator was very low in teak flowers. No increase was observed in this value while keeping colonies of Indian bees in the vicinity of trees under investigation. Many local bee-keepers have also reported that honey bees did not approach teak flowers in the corresponding seasons. This observations are to be cross checked with the fact that low fruit production is one of the major problems in teak propagation [25] as only 0.6-1 % of the gigantic teak flowering are being developed into fruits[23,24].

1.8 Present work

In the present work we investigate the pollination syndromes in teak in connection with its floral volatile profile. This chemo-ecological work is the first of this kind carried out in this region especially in the case of an

economically important hardwood timber.

Methodology:

Volatile oil obtained from fresh teak flowers by steam distillation is analysed by GC and GC/MS to identify the individual components (Section I). Each of these components are correlated with the major classes of insect visitors and pollinators of teak with the help of the internet database www.pherobase.com [26] and various literature available. The semiochemical relationships (attractants, kairamones , pheromones and allomones) are tabulated in two tables and the findings are discussed and correlated with the earlier reports from the field.

1.9 Results and Discussions

It is established from the data analysis (Table 1.3, 1.4) that at least 45 out of 66 components in the teak flower oil hold strong semiochemical relationship with one or the other family of insect visitors identified. Of these 38 compounds are in relationship with more than one class. Four of them namely benzaldehyde, linalool, methyl salicylate and caryophyllene have interaction with all the six classes of insects analysed. Thirteen compounds are affiliated with 5 families altogether in one or the other way.

Presence of large number of volatile compounds with diverge semiochemical behaviour may be main reason for the enormous number of insects visiting teak flower which is already rich in nectar.

Of the insect families Hymenopterans have maximum number (36) of affiliated molecules with 20 attractants, 13 pheromones, 1 kairamone and 2 allomones. But the number of *hymenopteran* specie visiting the teak flower was less when compared to *Lepidopterans* with 11 attractants, 12 pheromones and 6 kairamones. This may be due to the presence of following compounds as allomones and alarm pheromones:

i) Benzaldehyde : a defence substance influencing hymenopteran species

like *Leptogenys processionalis* [27]

ii) Benzyl alcohol: a repellent of hymenopterans like *Ceratosolen solmsi marchal* [28]

iii) cis-β-farnesene : cis-β-farnesene which was recognised as a major alarm pheromone of aphids [29] sent out in dangerous situations when having recognized an enemy to warn and save the other members of the species [30].

Coleopterans with 20 attractants, 6 pheromones and 4 kairamone were also found fewer in number. This may be also attributed to the presence of 4 allomones especially benzaldehyde and hydroquinone [31].

Table 1.3

Semio-chemical relationship of Teak floral volatiles and insect visitors [26]

No	Compound	Lepidoptera	Hymenoptera	Hemiptera	Coleoptera	Diptera	Thysano-ptra
1	Cis-3-Hexenol	K	A	A	A	A	-
2	Hexanol	K	P	Al	A	K	-
3	Benzaldehyde	P	Al	P	Al	A	K
4	1-Octen-3-one	-	K	-	K	-	-
5	Oct-1-en-3-ol	-	-	Al	-	-	
6	3-Octanone	-	P	-	A	A	-
7	3-Octanol	-	P	-	-	-	-
8	Benzyl alcohol	A	Al	P	A	A	-
9	Phenylacetaldehyde	A	A	A	K	A	-
10	Octan-1-ol	P	P	P	A	-	-
11	Cis-Linalool oxide	A	A	-	A	-	-
12	t-Linalool oxide	-	-	-	-	-	-

13	Linalool	A	A	P	A	A	K
14	Nonanal	A	P	K	A	A	-
15	Phenylethyl alcohol	P	P	P	P	A	-
16	Isophorone	P	-	-	-	-	-
17	2,6-Nonadienal	P	-	-	Al	-	-
18	Borneol	A	A	-	P	A	-
19	Terpinen-4-ol	K	A	P	P	P	-
20	Z-Hex-3-enyl butyrate	-	A	-	-	A	-
21	α-Terpineol	A	A	P	A	A	-
22	Methyl salicylate	A	A	A	A	A	A
23	Nerol	P	P	P	P	-	K
24	Geraniol	P	P	P	A	-	K
25	Anisaldehyde	-	A	-	A	-	K
26	Phenylethyl acetate	P	A	P	K	-	-
27	Hydroquinone	-	-	-	Al	-	-

28	Indole	P	P	A	A	A	-
29	4-Vinyl guaiacol	-	-	-	K	-	-
30	Eugenol	P	A	-	A	Al	K
31	3-Hexenyl hexanoate	-	A	-	-	-	-
32	α –Copaene	-	A	-	A	A	-
33	7-Epi-Sesquithujene	-	-	-	A	-	-
34	Caryophyllene	P	A	A	A	A	P
35	Coumarin	A	-	-	A	-	-
36	Trans-α-Bergamotene	-	P	-	-	-	-
37	Geranyl acetone	K	P	P	P	A	-
38	Cis-β-Farnesene	-	P	-	-	-	-
39	α-Humulene	K	A	Al	A	P	-
40	γ - Muurolene	A	A	-	-	-	-
41	Curcumene	-	A	P	-	-	-
42	Germacrene D	A	-	-	-	-	-

43	δ -Cadinene	-	P	-	Al	-	-
44	Trans-Nerolidol	K	A	A	P	-	-
45	β-Caryophyllene epoxide	P	A	-	-	-	-
46	β-Bisabolol	-	-	-	A	-	-

A-Attractant Al-Allomone K- Kairamone P- Pheromone

Hemiptera with three allomones and Thysanoptera with fewer semiochemicals (only a total of eight) was also fewer in number. There was a strong back up from six kairamones for Thysanoptera but the presence of B-caryophyllene suspected to be a defense pheromone [32] may have blocked its way.

Diptera with 16 attractants was also less in number clearly due to the presence of eugenol which is a repellant for most of its species [33].

For Lepidopterans there was six kairamones at the same time not any allomone . Presence of phenyl acetaldehyde having a low odour threshold of 4 units[34] and a high potency for attracting of Lepidopterans [35] also favour this class. Thus it became the prevalent group visiting teak flowers.

Table 1.4 Semio-chemicals of Insect visitors of Teak flower

Organism	Attractant	Kairamone	Pheromone	Allomone
Lepidoptera	11	6	12	0
Hymenoptera	20	1	13	2
Hemiptera	6	1	12	3
Coleoptera	20	4	6	4
Diptera	16	1	2	1
Thysanopetra	1	6	1	0

Role of Linalool : Linalool was the most abundant component of the oil. It was also characterised by high volatility and low odour threshold value of 6 units [34]. In the present analysis linalool held positive relationships with all the six insect families. Linalool was recognized as the universal fragrance constituents of white, night-blooming, insect-pollinated flowers worldwide [36]. Familiar examples of such plants are the evening primroses, nocturnal tobaccos, wild gingers ,jasmines and long-spurred orchids. Linalool and its oxides along with the acyclic sesquiterpene nerolidol, heterocyclic compound

indole and certain aromatic esters are detected as important components of the white floral olfactory [36]. All these compounds were identified in teak flower also. Linalool and other mentioned compounds also back up the presence of a large number of insect visitors.

Honey bees and Methyl salicylate: A number of compounds are present in teak flower oil as attractants for honeybees (order hymenoptera) among which phenylacetaldehyde [37] and linalool[38] are most significant .

The phenolic ester methyl salicylate appeared as an attractant for all the six classes under investigation in the tabulation. But when approaching the case of honeybees it was often emerged as a potent repellent [38, 39, 40]. Methyl salicylate in combination with benzyl alcohol also showed repellency towards honeybees [39]. Also in some studies 3-octanone was also recognized as possessing some repellency against honey bees [38]. Thus the presence of methyl salicylate along with octanone and benzyl alcohol is the reason for the low visiting frequency of honey bees in teak flowers.

The high insect population combined with a low visiting frequency of effective pollinators like honey bees result in the destruction of the floral organs, ineffective pollination and low fruit formation in teak. A biomolecular modification of the species aiming higher relative concentration of compounds like linalool and phenyl acetaldehyde and a lower relative concentration of both 3-octanone and methyl salicylate in the floral aroma can be recommended for a more effective pollination.

1.10 References

1. E Pichersky and D. R. Gang. Trends in Plant Science 5, 10, 439-445, 2000.
2. Dudareva et.al. Crit. Rev. Plant Sci. 25, 417-440, 2006.
3. E Pichersky and Gershenzon, J. Curr. Opin. Plant Biol. 5, 237-243.2002.
4. J Kesselmeier and M Staudt M. J Atmos Chem , 33:23-88. 1999.
5. Pichersky et.al., Science, 311, 808-811.2006.
6. T.D Wyatt, Pheromones and Animal Behaviour: Communication by Smell and Taste, pp: 01,Cambridge University Press, 2003.
7. Brown et.al., *BioScience* 20: 21–22. 1970.
8. T.R. Grasswitz, and G.R. Jones "Chemical Ecology". Encyclopedia of Life Sciences. John Wiley & Sons, Ltd. 2002.
9. F. P. Schiestl, Ecology Letters , 13: 643–656, 2010.
10. J. H, Visser and D. A. Ave , Ent. exp. & appl. 24 , 538--549. Ned. EntomoL Ver. Amsterdam Proceedings 4th Insect Host Plant Symposium 1978. www.olfacts.nl
11. Hamilton et.al. Atmos. Chem. Phys. Discuss., 9, 3921–3943, 2009
. www.atmos-chem-phys-discuss.net/9/3921/2009/
12. Bruce et. al. Pesticide Outlook 14: 96–98. , 2003.
13. J. K. Holopainen and J. D. Blande, Chapter 2, Sensing in Nature, edited by Carlos López‐Larrea. Landes, Springer, 2012.
14. H.B Jakobsen and C.E Olsen , Planta 192: 365–371, 1994.
15. Knudsen, et. al. Bot. Rev., 72, 1–120. 2006.
16. Cseke et al.Natural Product Communications ,2 ,12, 2007.
17. A. K. Shukla, M. R. Vijayaraghavan & B Chaudhry , Abiotic pollination. Biology Of Pollen. APH Publishing. pp. 67–69. 1998.
18. J. B. Harborne, Introduction to Ecological Biochemistry, pp-01,Gulf Professional Publishing, 1993 .
19. J. B. Harborne, Nat. Prod. Rep., 18, 361-379, 2001.
20. Jurgens, et.al., Biochem. Syst.Ecol. 30, 383–397. 2002.
21. D. P. Abrol, Pollination Biology: Biodiversity Conservation and Agricultural Production (Google eBook), Springer, 2011 .
22. K Bryndum, and T Hedegart , Silvae Genetica 18, 33, 1969.
23. Mathew et.al. The Indian Forester, 113,1, 1987.
24. Mohandas et.al. KFRI Research Reprt No 225, ISSN 0970-8103
25. Anmol and Kumar, Ind. For. , 118(8) 1992.
26. A.M El-Sayed, The Pherobase: Database of Pheromones and Semiochemicals, 2012. http://www.pherobase.com
27. Fales et.al., J. Chem. Ecol. 18:847-854.1992.
28. Song et.al., J. Chem. Ecol. 27:1929-1942. 2001.

29. Francis et.al., Journal of Applied Entomology, 129, 1, 6–11, 2005.
30. M. Müller and G.Buchbauer, Flavour Fragr. J. , 26, 357–377, 2011.
31. R Classen, and K Dettner, J. Chem. Ecol. 9:201-209.1983.
32. Suzuki et.al., J. Chem. Ecol. 30, 2, 409-423, 2004.
33. Vartak et.al., J. Comm. Dis. 26:156-160.1994.
34. http://www.leffingwell.com/odorthre.
35. S.Maini and G. Burgio, Boll.Ist.Ent. G.Grandi, Univ. Bologna, 45, 157-165, 1990.
36. R. A. Raguso and E. Pichersky, Plant Species Biology , 14, 95-120, 1999.
37. N Theis,. J. Chem. Ecol. 32:917-927. 2006.
38. Henning et.al, Journal of Economic Entomology, 85, 1, 233-239,1992.
39. Sahebzadeh et. al, Journal of Apicultural Research, 48 ,1, 29 – 33, 2009.
40. N. Sahebzadeh, JCPP, 12, 45, 351-363, 2008.

CHAPTER 2

ESSENTIAL OIL FROM *LIMNOPHILA REPENS* BENTH.

2.1 Introduction

Limnophila (meaning pond-loving in Latin) is an important but small genus comprising of 40 species of aquatic or semi-aquatic plants growing in marshes, riversides, forest paths or in similar wet area. They are native to the tropical or subtropical areas of Africa, Asia, Australia and Pacific islands [1]. *Limnophila* plants are widely distributed throughout Indian subcontinent especially in the vast marshy rice fields. The paddy farmers treat them as serious weeds while they are widely used in traditional folk medications [2] . These plants have got mentioned in *Horthus malabaricus* and in ancient Ayurvedic texts.

Limnophila is also commonly known as 'ambulia' or Asian marsh-weed which appears as perennial herbs submersed, emergent and amphibious stem plants. The emerged stems are having leaves longer than the submerged stems and carry single flowers white, pink, purple or blue to lavender. Their fruits are capsules carrying upto 150 seeds, but they reproduce by fragmentation also [14].

L. aromatica , *L. racemosa* , *L. heterophylla* , *L. indica* and *L. repens* are some of the common species of the genus [4].

The plant under investigation *Limnophila repens*. Benth is an aquatic herb distributed mainly in tropical Asia (India, Bangladesh, Indonesia, Malaysia, Philippines, Sri Lanka, Thailand etc) and Australia. In India the species is found in Kerala, Tamil Nadu , Karnataka, Assam, Orissa, West Bengal , Madhya Pradesh ,Maharashtra , and Andhra Pradesh [5]. It is widespread in marshy areas and in paddy fields particularly in low lying areas.

Some years ago the genus has been controversially moved into the family *Plantaginaceae* from *Scrophulariaceae* [6] by certain taxonomists.

2.2 Phytochemistry of *Limnophila*- A Review

The *Limnophila* plants are widely used in traditional and folk medicines from the very ancient time to the present age. A few plants are thoroughly investigated for their chemical composition and pharmaceutical behavior. A good deal of work had already been done on the volatiles from these specie. A number of compounds mainly flavanoids had been isolated from different plants.

Traditional Medicinal Uses : The medicinal properties and uses of *L.aromatica* is described in ancient ayurvedic texts. It is described as sour and slight bitter and reported to be possessing antiseptic, appetizer, digestive, carminative, anthelmintic, anti-inflammatory, diuretic and febrifuge properties. It can be used in vitiated conditions of *pitta*, foul ulcers, galactic impurities, anorexia, dyspepsia, helminthiasis, constipation, inflammations and strangury. The juice from the plant is used as a cooling medicine in fever and pharyngitis and is given to nursing mothers when the milk is sour. Beside this the plant can be used as a spinach, eaten raw or steamed. [7,8]. In Indonesia and Malaysia, the sap of the leaves of *L. aromatica* is used to clean wounds and sores on the legs . A decoction of the leaves is given in fevers but also to promote appetite, and as an expectorant to clear mucus from the bronchial tubes. In Vietnam, the whole plant is used for its diuretic and anti-spasmodic action, against gravel in the kidneys, as a disinfectant for wounds and for haematuria. It is also applied for cough, snakebite and skin diseases. In China, the plant is used for the treatment of intoxication, body pains and for menstrual problems. [9]

L. rugosa (syn. *L. roxburghii*) is widely used in the traditional medicinal systems of India as well as of Philippines. Its juice is administered in diarrhoea, dysentery, and dyspepsia. The juice is also rubbed over the body during pestilent fever and is used as a carminative and a tonic. It is applied on

elephantiasis with coconut oil [10]. . In Indonesia, a decoction of *L.rugosa* is used externally to cure itching eyes, and internally for mild gonorrhoea and impotence [9].

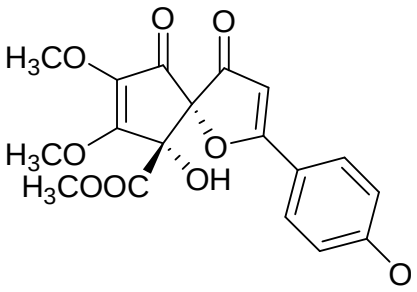
L. indica is a plant with refreshing odour and considered to be carminative and antiseptic. It is applied in elephantiasis as a paste. The plant juice is rubbed over the body in pestilent fever. It is given internally with ginger in dysentery [11]. In Philippines, an infusion of the leaves of *L. indica* is used for dysentery and dyspepsia [9]. *L.aquatica* and *L. racemosa* are also found similar to *L.indica* in medicinal properties.

L. conferta is administered in various types of skin diseases and inflammation in the indigenous system.[12] while *L. gratissima* is described as antiseptic, galactagogue and laxative [13].

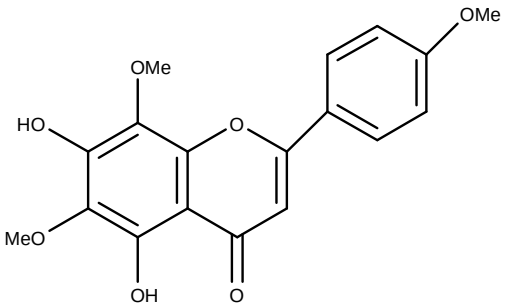
L. repens is described in Ayurvedic texts as having similar medicinal properties as *L. aromatica*. In many instances especially in North Malabar *L. repens* is accepted as the source of required drug in place of *L. aromatica* [12]. Even though identical in the medicinal properties these are considered as two distinct specie of the genus.

Pure compounds: More than 15flavanoids (Table 2.1) and 7 terpenoids (Table 2.2) are reported from the *Limnophila* plants . Some of them are revealed to possess different biological potencies [14]. Isolation of the novel spiro compound Limnophilaspiraketone, a highly oxygenated phenolic derivative from *L.geoffrayi* by Jang et.al [15], may be the latest finding to mention here. Nevadensin (5,7-dihydroxy-6,8,4′-trimethoxyflavone) and isothymusin (6,7-dimethoxy-5,8,4′-trihydroxyflavone), two flavanoids with anti-tuberculosis activity were also isolated from the same plant [16]. Nevadensin was also reported to be showing moderate cytotoxic activity and anti-inflammatory activity [17]. Lacceoroic acid possessing antibacterial activity against gram-negative bacteria was isolated from *L. polystachya* [18].

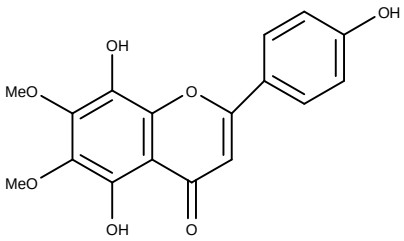
A number of amino acids were isolated from the aerial and submerged leaves of *L. indica* [19].



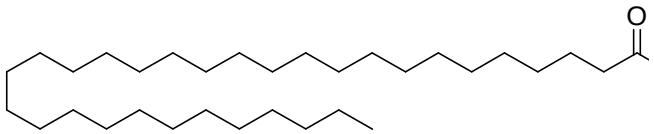
Limnophilaspiraketone



Nevadensin



Isothymusin



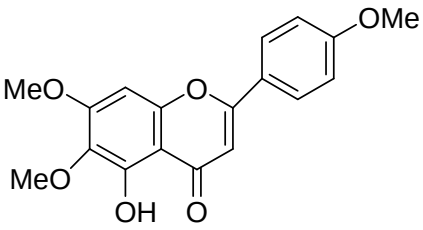
Lacceoroic acid

Crude Extracts : The crude extracts of limnophila plants were investigated for different biological and pharmaceutical activities. Mishra *et al.*[20] studied the antimicrobial activity of the *L. racemosa* and *L. indica* extracts against a number of bacterial species (*Bacillus anthracis* , *Bacillus mycoides*, *Bacillus pumilus* , *Bacillus subtilis* ,*Pseudomonas* sp. , *Salmonella paratyphii* ,. *Staphylococcus albus* , *Xanthomonas campestris and*. *Xanthomonas malvacearum*) and obtained a convincing result to point out that both the extracts of *L. racemosa* and *L. indica* contained certain antimicrobial components. Chloroform extract of the aerial parts of *L. geoffrayi* was found to possess anti-mycobacterial activity and antioxidant activity [16]. The crude extract of *L.conferta* was reported for its anti-inflammatory activity and wound healing activity [17]. Extracts of *L. aromatica* with different solvents also showed considerable antioxidant activity in DPPH scavenging analysis [21].

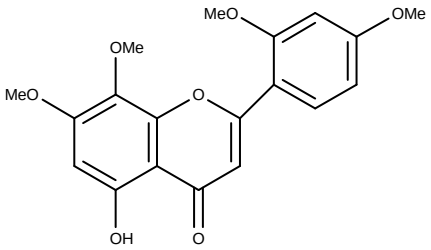
Table 2.1 Flavanoids isolated from Limnophila plants

No	Compound	Plant	Part
1	5-Hydroxy-6,7,4'-trimethoxyflavone (Salvigenin)	*L. rugosa*	aerial parts and roots
2	5-Hydroxy-7,2',4'-trimethoxyflavone	*L. rugosa*	aerial parts and roots
3	5-Hydroxy-7,8,2',4'-tetramethoxyflavone	*L. rugosa* *L. heterophylla*	aerial parts and roots aerial parts and roots
4	5-Hydroxy-6,8-di-methoxy-3',4'-methylene- dioxyflavone	*L. indica*	aerial parts and roots
5	5,7-Dihydroxy-6,8,4'-trimethoxyflavone (Nevadensin)	*L. geoffrayi* *L. heterophylla* *L. rugosa*	aerial parts aerial parts and roots
6	5,8-Dihydroxy-6,7,4'-trimethoxyflavone	*L. indica*	aerial parts and roots
7	5,7-Dihydroxy-8,3',5'-trimethoxyflavone	*L. rugosa*	aerial parts and roots
8	5,2'-Dihydroxy-8,3',4'-trimethoxyflavone	*L. indica*	aerial parts and roots
9	5,2'-Dihydroxy-7,8,4'-trimethoxyflavone	*L. heterophylla*	aerial parts and roots
10	5,7-Dihydroxy-3,6,3',4'-tetramethoxyflavone (7-desmethyl artemetin)	*L. gratissima*	aerial parts and roots
11	5,7-Dihydroxy-6,8,3',4'-tetramethoxyflavone (Hymenoxin)	*L. heterophylla*	-
12	5,8,4'-Trihydroxy-6,7-dimethoxyflavone (Isothymusin)	*L. geoffrayi*	aerial parts
13	5,7,4'-Trihydroxy-6,8-dimethoxyflavone (Demethoxysudachitin)	*L. rugosa*	-
14	5,2',4'-Trihydroxy-7-methoxyflavone (Artocarpetin)	*L. rugosa*	aerial parts and roots
15	3',4'-Ethylenedioxy-5-hydroxy-3-(1-hydroxy-1-methylethyl)-6,7-dimethyl-5'-methoxy-flavone-8-carboxylic acid	*L. indica*	aerial parts and roots
16	5,7,2',5'-Tetramethoxyflavone	*L. indica*	aerial parts and roots

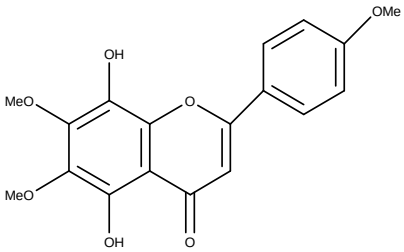
No	Compound	Plant	Part
17	5,7,3',4'-Tetramethoxyflavanone	*L. indica*	aerial parts and roots
18	Nevadensin7-*O*-β-glucopyranoside [32]	*L. aromatica*	-
19	Pilosin [32]	*L. aromatica*	-



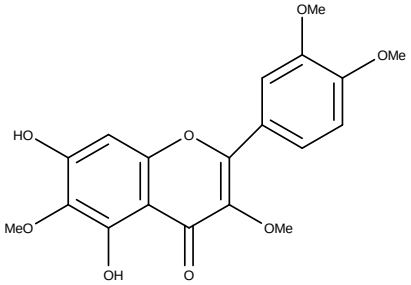
Salvigenin



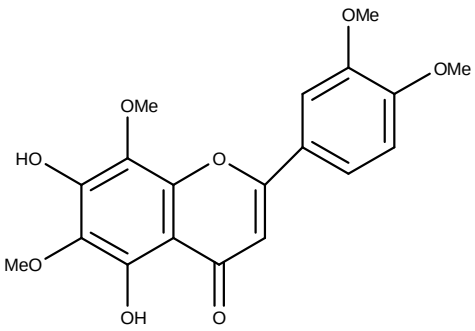
5-Hydroxy-7,8,2',4'-tetramethoxyflavone



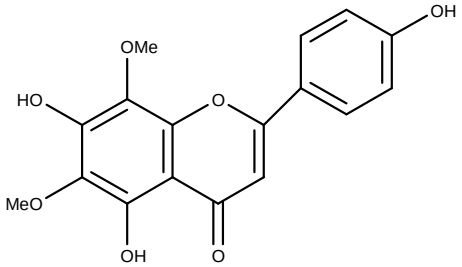
5,8-Dihydroxy-6,7,4'-trimethoxyflavone



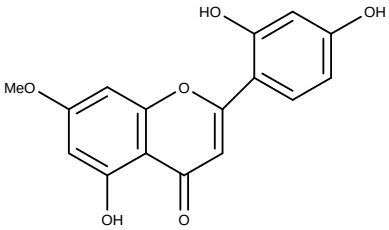
7-desmethyl artemetin



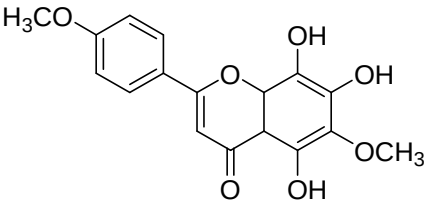
Hymenoxin



Demethoxysudachitin



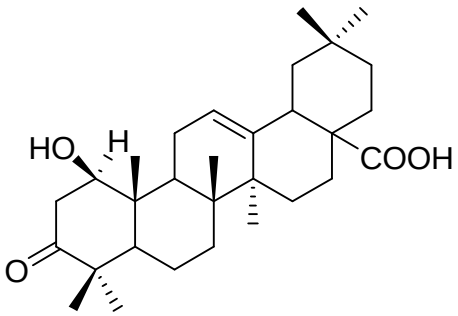
Artocarpetin



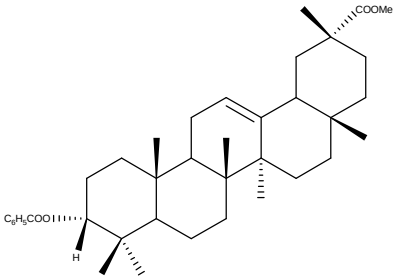
Pilosin

Table 2.2 Terpenoids isolated from Limnophila plants

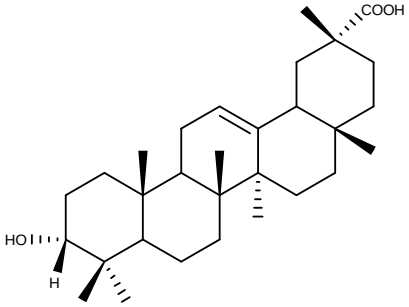
No	Compound	Plant	Part
1	1-β-Hydroxy-3-keto-olean-12-en-28-oic acid	*L. rugosa*	aerial parts and roots
2	Methyl-olean-12-ene-3α-benzoyloxy-29-carboxylate	*L. heterophylla*	aerial parts and roots
3	3α-Hydroxyolean-12-ene-29-oic acid (Katonic acid)	*L. hetero-phylla*	aerial parts and roots
4	Ursolic acid	*L. heterophylla*, *L. rugosa*	aerial parts and roots
5	Betulin	*L. rugosa*	-
6	Betulinic acid	*L. rugosa*	-
7	3β-Hydroxy-lup-20-en-27-oic acid	*L. rugosa*	-



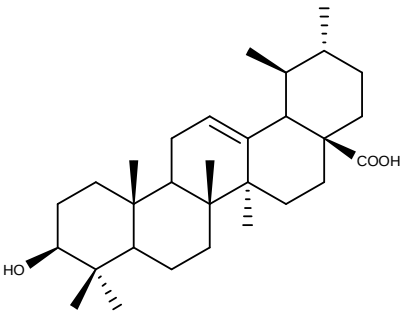
1-β-Hydroxy-3-keto-olean-12-en-28-oic acid



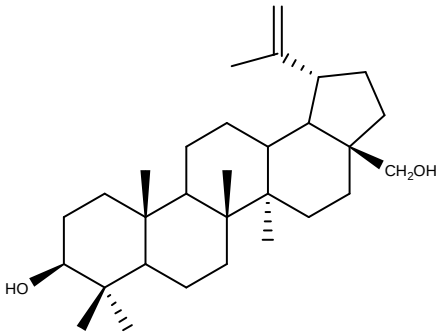
Methyl-olean-12-ene-3α-benzoyloxy-29-carboxylate



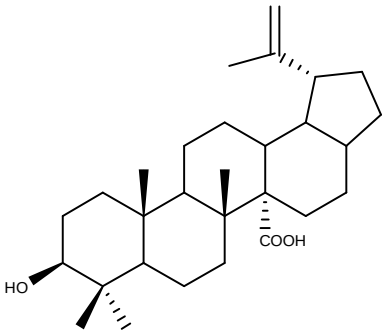
Katonic acid



Ursolic acid



Betulin



3β-Hydroxy-lup-20-en-27-oic acid

Essential Oils : *Limnophila* plants are aromatic herbs with pleasant smelling volatile oils. A good deal of research data is available on the composition and biological properties of *limnophila* essential oils (Table 2.3). Large variations are observed in the composition of these oils for different species ; regional variations are also prominent. Essential oil from *L.aromatica* collected from Bangladesh are rich in z-Ocimene (39%), terpinolene (17%) and camphor (13%) [22]. In another report δ-limonene and δ- perillaldehyde appeared as

the major constituents of the oil which showed bactericidal activity against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* and fungicidal activity against *Aspergillus niger*, *Candida albicans* and *Rhizopus oryzae* [9]. Essential oil from the dried areal parts of *L.geoffrayi* comprising of d-pulgone (27%), perillaldehyde (19%) and limonene (9%) as the major components showed inhibitory activity against microorganisms present in contaminated cosmetic products. [23]. Volatile oils from the leaves and stems of *L. chinensis ssp. aromatica* collected from North American market was dominated by limonene (53%) and cis-4-caranone(12%) [24]. α-Phellandrene (52.2%) and thymol (38.2%) dominated the oil from *L.conferta* which showed considerable anti-inflammatory, antifungal and anthelmintic activity [17]. Oil glands were observed in the anatomy of *L.indica* leaves and the volatile oil was characterized by monoterpenes and long chain fatty acids [25]. Three reports are available on the essential oil of *L.rugosa* . The plant growing in Vietnam yielded an oil possessing antimicrobial activity and containing methylchavicol (71%) and trans-anethole (25%) as the major constituents [26] while those collected from China yielded estragole(17.75%), [1S-alpha.,7.alpha.,8a.beta.)]-1,2,3,5,6,7,8,8a-Octahydro-1,4-dimethyl-7-(1-methylethenyl) azulene (13.24%), caryophyllene (11.29%),Z,Z,Z-1,5,9,9-tetramethy l-1,4,7-cycloundecatriene (10.92%) and eucalyptol(6.78%) as major oil components [27]. The Chinese oil also showed anti-oxidant activity in DPPH scavenging analysis. In another paper Yu Xuejian et. al reported estragole (21.94%) and trans-anethole (76.39%) as the major components in the volatile oil of *L.rugosa* [28]. Essential oil from *L. rugosa* was also reported for its antifungal [12] and antibacterial [29] activities. Volatile oil from *L. gratissima* was reported for its promising antibacterial[17] and antifungal [30] potencies by different authors.

Essential oil from *L. repens* was also got reported more than once. The internet resource PROSEA (Plant Resources of South-East Asia) Foundation, Bogor, Indonesia reported that the essential oil from *L.repens* collected from

south East Asia contained α-phellandrene (52.2%) and thymol (38.2%) as the major components [9]. This report is evidently based on the study by Reddy et.al. [17] on *L.conferta* , which some taxonomists consider as a synonym of *L. repens*. In another study [31] δ-Limonene was reported as the major component which reminds of the essential oil from *L. aromatica* [9].

As these plants are similar in many botanical appearances, occupying the same ecological regions and possessing a similar pungent aroma, minor errors in the plant selection can be suspected. So we decided to re-examine the essential oils from *L. repens* for its chemical composition as well as for antifungal potentials. Olfactory evaluation of the volatile oil was also carried out for the first time for any plant in the genus

Table 2.3 Essential oils from Limnophila plants

Plant	Major components	Biological activity
L.aromatica	1. z-Ocimene , terpinolene, camphor 2. δ-limonene , δ- perillaldehyde	Antibacterial, Antifungal
L.geoffrayi	d-pulgone , perillaldehyde , limonene	Antibacterial
L. chinensis ssp. aromatica	limonene , cis-4-caranone(12%)	-
L.conferta	α-phellandrene , thymol	Anti-Inflammatory, Antifungal, Anthelmintic
L.indica	Monoterpenes, long chain fatty acids	-
L.rugosa	1. chavicol , trans-anethole 2. estragole, Caryophyllene 3. estragole , trans-anethole	Antioxidant, Antibacterial, Antifungal
L. gratissima	-	Antibacterial, Antifungal
L. heterophylla [33]	Limonene, +cadinene, α-pinene	-

2.3 Present Work

Limnophila repens Benth. is called ‘manganaari’ in Malayalam to indicate its smell resembling that of tender mangoes. *Hortus Malabaricus* mentions three different types of *Manganari* namely, *Manganari* (X, t.6), *Cheriya Manganari* (IX, t. 85) and *Valiya manganari* (X. t.40). They are

identified as *L. aromatica* (Lam.) Merr., *L. indica* (Linn.) Druce of Scrophulariaceae and *Wedelia biflora* Linn. of *Asteraceae*, respectively. In ayurvedic texts the former two are described as ‘amragandha’ and ‘anyaamragandha’ again indicating their familiar mango-smell. Amragandha is described as antiseptic, appetizer, digestive, carminative, anthelmintic, anti-inflammatory, diuretic and febrifuge and applicable in vitiated conditions of *pitta*, foul ulcers, galactic impurities, anorexia, dyspepsia, helminthiasis, constipation, inflammations and strangury [7]. Ayurvedic prescriptions are often altered by accepting regional plants of similar properties as the source of drug. *L.repens* is such an effective substitute from Kerala, especially from North Malabar [13]. Sivarajan et.al described this plant as ‘erect or diffuse, fleshy, annual herb; leaves opposite, sessile 2.5 x 1 cm., flowers usually auxiliary, solitary or in racemes, sessile or short [13]. The local medical people report this plant as an efficient anthelmintic drug and an ingredient for anti-inflammatory preparations .

In the present work essential oil from *L.repens* is obtained by hydrodistillation. The oil was subjected to GC and GC/MS for analyzing the chemical composition. Medicinal indications of the plant are examined in co-relation with the constituents. Also an olfactory evaluation of the volatile oil was done carried out for the first time for any plant in the genus. Antimicrobial activity of the oil under *in vitro* conditions was also investigated and is reported in Chapter VIII.

Plant Collection : The fresh plant material was collected from paddy fields of Kannur district, Kerala, India. It was identified by Dr. A.K. Pradeep, Department of Botany, University of Calicut and a specimen voucher is deposited in the specially maintained Herbarium of Chemistry department of University of Calicut.

Extraction of Oil : The aerial parts of the plant (500 g) was washed and ground into a paste and subjected to steam distillation for 3 hours. The oil was extracted by diethyl ether from the distillates. The ether extract was dried

using anhydrous sodium sulphate and the ether evaporated. The pure oil was stored below 4°C until analyzed.

Gas chromatography/mass spectrometry/olfactometry: GC/MS/O was carried out using an Agilent 6890 gas chromatograph, fitted with a HP-5 (5 % diphenyl polysiloxane) capillary column (50 m x 0.32 mm x 0.52 µm), with He carrier gas, initial head pressure 15.0 psi (2.0 mL/min) constant flow mode. The column effluent was split between an Agilent 5975N inert MSD spectrometer and an in-house odour detection port *via* a Capillary Flow Technology splitter plate with pressure set to 3.8psi. The injector and odour port transfer line temperatures were held constant at 230°C and 250°C, respectively. Injection of 1µl at 500ng/µl dilution in splitless mode with oven program: 35°C (3min), 15°C/min ramp to 50°C then 5°C/min ramp to 280°C(held 10min). Data was acquired and processed using MSD ChemStation (Rev. D.02.00.275). The odour assessments and description were carried out by experienced perfumers.

Gas chromatography/mass spectrometry: GC/MS was carried out using the same system but optimised for resolution with a linear temperature ramp of 2°C/min and calibrated for Retention Indices using C7-C28 n-alkanes. The inert MSD was operated with source temp 230°C, quad temp 150°C and ionization voltage 70 eV. Target spectra were acquired using the s.tune parameters and compared against in-house and commercial libraries from which identifications were assigned on the basis of both spectral match and retention data [34].

Gas chromatography/flame ionization detection: GC/FID analysis for quantisation was carried out using an Agilent 6890 gas chromatograph, fitted with an Ultra 2 (5 % diphenylpolysiloxane) capillary column (50 m x 0.2 mm x 0.33 µm), split injection (50:1) with He carrier gas (1.2 mL/min). The oven was programmed from 50–280°C (held for 6 min) at 2°C/min. The injector and detector temperatures were held constant at 230°C and 300°C, respectively. Data was acquired and processed using HP ChemStation

software (Rev. A.10.02 [1757]). Quantitative data was obtained from relative peak area (%RPA) without the use of response factors.

2.4 Results and Discussion

2.4.1 Composition of the essential oil : The hydrodistillation of the plant *L.repens* gave a pale green coloured oil with a yield of about 0.03% of the fresh weight sample. The oil was slightly pungent, but pleasant smelling. From the GC/MS analysis 33 compounds consisting of 91.09% of the oil were identified(Table 2.4). The oil was dominated by 19 monoterpenes followed by 7 aliphatic compounds and 5 sesquiterpenes. Monoterpenes consisted of 12 hydrocarbons and 6 alcohols .

Table 2.4. GC/ MS Analysis with FID quantification of *L.repens* oil

No	RI	RI*	Compound	Percentage
Aliphatic compounds				
1	852.7	852	cis- 3-Hexenol	0.27
2	863.1	863	trans- 2-Hexenol	0.36
3	865.4	865	Hexanol	0.26
4	978.8	978	Oct-1-en-3-ol	7.2
5	985.8	987	Octan-3-one	0.19
6	994.8	995	Octan-3-ol	0.73
7	1840.7	1842	Neophytadiene	0.58
Monoterpenes				
8	927.9	928	α-Thujene	1.09
9	935.4	935	α- Pinene	0.46
10	975.8	975	Sabinene	18.34
11	991.4	993	Myrcene	2.64
12	1006.6	1007	α- Phellandrene	0.38
13	1018.7	1018	α- Terpinene	2.76
14	1026.3	1027	p-Cymene	0.78
15	1030.8	1031	Limonene	1.4
16	1031.6	1032	β- Phellandrene	1
17	1048.7	1048	trans-Ocimene	0.3
18	1061.1	1060	γ- Terpinene	6.39

19	1091.3	1090	Terpinolene	7.29
20	1100.2	1101	Linalool	0.6
21	1124.3	1125	cis-p-Menth-2-en-1-ol	0.82
22	1142.2	1142	trans-p-Menth-2-en-1-ol	0.64
23	1170.4	1172	Borneol	0.57
24	1182.8	1181	Terpinen-4-ol	19.33
25	1194	1194	α-Terpineol	0.9
26	1290.5	1291	Bornyl acetate	0.27
27	1310.3	1308	Ascaridole	0.32
Sesquiterpenes				
28	1430.6	1431	Caryophyllene	4.41
29	1460	1460	trans-β-Farnesene	6.78
30	1464.7	1465	α-Humulene	1.26
31	1503.5	1505	Valencene	1.15
32	1820.4	1822	Nootkatone	1.12
Phenyl propanoid				
33	1361.1	1363	Eugenol	0.5
			Total	91.09

RI- Measured Retention Index **RI*- DB-5 ref.**

It is the presence of ascaridole, the rarely occurring natural organic peroxide and the only natural terpene peroxide to be specially mentioned. It is the first report of this compound from this plant and the whole genus *Limnophila*. Ascaridole , the major component of the Mexican tea plant oil [35] was the first naturally occurring organic peroxide isolated. It imparts the specific flavor to the Chilean tree boldo (*Peumus boldus*) where also it is the major component of the volatile oil [36]. For many years it was a major remedy against intestinal parasites in humans and other household animals [37]. It is interesting to note that the Mexican tea plant itself is traditionally used in tonic drinks and infusions to expel intestinal parasites in folk medicine of North and South Americas, China and Turkey [38]. Biogenesis of ascaridole was proved to be starting from α-terpinene and being catalyzed by a soluble iodide peroxidase [39] in different plants like *Chenopodium ambrosioides.* α-Terpinene was present in our sample while the presence of

peroxidase enzyme is yet to be proved. Even though present in low amount in the essential oil, it will be none other than ascaridole contributing to the traditional anthelmintic property to *L.repens* as in the case of 'worm seed' alias Mexican Tea plant.

The oil consisted of terpinen-4-ol (19.33%) as the major component followed by sabinene(18.34%) , terpinolene(7.29%), oct-1-en-3-ol(7.2%), trans-β-farnesene(6.78%) and γ-terpinene(6.39%). These compounds had been previously reported from *Limnophila* but it is the first time as major components of the essential oil from any plant of the genus. The presence of nootkatone and its precursor valencene, an aroma component of citrus fruits was another peculiarity of this oil. The major components of the earlier reports α- phellandrene, limonene and p-cymene were also present, but in very low concentration. These marked differences make it clear that *L.repens* is a different chemotype of the genus compared to *L. conferta* and *L. aromatica*.

The antimicrobial and medicinal properties of the whole plant and its essential oil can be attributed to the mono and sesquiterpenoids present in the oil to some extent. Oils comprised of α-terpinolene , p-cymene, limonene, linalool, pinenes etc. were shown to be anti-inflammatory in a number of investigations. Oils comprised of α-terpinene, terpinolene, β-caryophyllene, limonene, ocimene etc. showed antioxidant activity in scavenging analysis. Wound healing activities of oils containing these compounds were also tested successfully [40]. Essential oils containing the sesquiterpene caryophyllene and monoterpenes exhibited high antimicrobial activity against a wide range of gram-positive bacteria as well as to some gram-negative bacteria. The antifungal activities of many essential oils especially those containing 4-terpinenol, α-pinene, limonene, sabinene and ascaridole against a large number of pathogens were also investigated successfully [41]. Generally major components are responsible for the biological activity of essential oils, but sometimes the minor components also play major role making the whole

oil more active than the combination of major components in a synergistic effect [42].

2.4.2 Olfactory Evaluation: The odour impressions and odour threshold values of the constituents of the essential oil are given in table 2.5. *Odor threshold value (OTV)* (or *aroma threshold value (ATV)*,) is defined as the most minimal concentration of a substance that can be detected by a human nose. This value varies from substance to substance and expressed as a concentration in water or concentration in air usually in ppb. Generally the odour impressions corresponding to compounds with low OTV dominates in human perception .

Hence, the impressions of α-pinene (pine, turpentine), oct-1-en-3-ol (mushroom), p-cymene (solvent, gasoline), linalool (Fresh, floral) and that of nootkatone (grapefruit) are to be expected as the major notes in the oil. These are expected to be followed by that of limonene (citrus, mint), myrcene (spicy, green mango), eugenol (spicy, clove) and that of trans- ocimene (herbal). Another compound terpinolene is also reported for its typical green mango like aroma [43] ; but the corresponding note is not expected to be perceived due to its high OTV . However the familiarities with tender mangoes in the region of plant collection made the recognition of the specific mango-note first and above all other impressions to name the plant as 'manganaari' (mango-smelling). The flavor of mango ginger (*Curcuma amada* Roxb.) another herb with mango-like odour was attributed in some extent to *cis*-ocimene [44] which is also present in this oil enhancing the specific impressions.

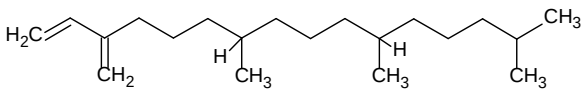
Table 2. 5 Odour impressions and O.T Values of E.oil components of *L.repens*

No	Compound	Odour Impression	OTV in water (ppb) [45]
1	cis- 3-Hexenol	Faint green	70
2	trans- 2-	Green, leafy	400

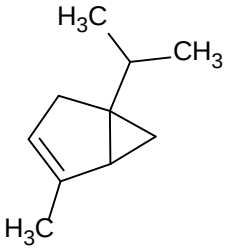
No	Compound	Odour Impression	OTV in water (ppb) [45]
	Hexenol		
3	Hexanol	resinous, green	2500
4	α-Thujene	wood, green, herbal	-
5	α- Pinene	pine, turpentine	6
6	Sabinene	pepper, turpentine, wood	37
7	Oct-1-en-3-ol	Weak, mushroom	1.2
8	Octan-3-one	Herbal, resinous	28
9	Myrcene	balsamic, spicy, green mango	13-15
10	Octan-3-ol	faint mushroom metallic	110-130
11	α- Phellandrene	turpentine, mint, spice	200
12	α- Terpinene	lemon	260
13	p-Cymene	solvent, gasoline, citrus	6.2
14	Limonene	citrus, mint	10
15	β- Phellandrene	mint, terpentine	200
16	trans-Ocimene	sweet, herb	34
17	γ- Terpinene	gasoline, turpentine	260
18	Terpinolene	Floral, sweet, slightly green mango, sour	200
19	Linalool	Fresh, floral	6
20	cis-p-Menth-2-en-1-ol	Herbal	N.A
21	trans-p-Menth-2-en-1-ol	green hay faint	N.A
22	Borneol	Earthy , Camphorous	140
23	Terpinen-4-ol	Medicinal, turpentine, nutmeg	1200
24	α-Terpineol	oil, anise, mint	330
25	Bornyl acetate	Woody, camphoreous, spicy	75
26	Ascaridole	pungent	NA
27	Eugenol	Spicy, clove	6-30
28	Caryophyllene	Woody, spicy	64
29	trans-β-Farnesene	Woody, sweet, citrus	NA
30	α-Humulene	woody	NA
31	Valencene	Green, oily	NA
32	Nootkatone	Citrus, grapefruit - woody	0.8-1

No	Compound	Odour Impression	OTV in water (ppb) [45]
33	Neophytadiene	Not specific	NA

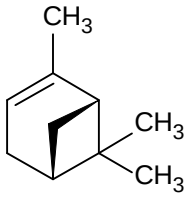
Structure of compounds identified in the GC/MS analysis of essential oil from *L.repens*



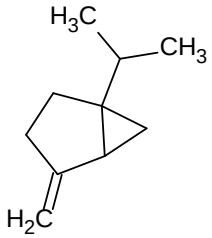
Neophytadiene



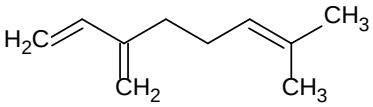
α-Thujene



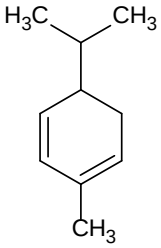
α- Pinene



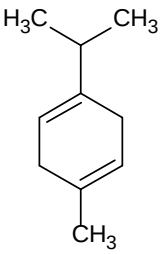
Sabinene



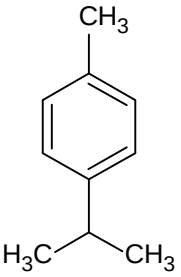
Myrcene



α- Phellandrene



α- Terpinene



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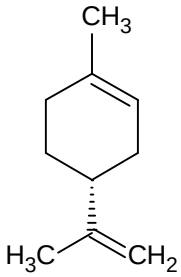
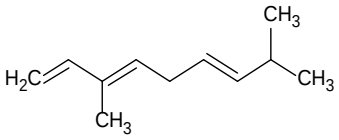
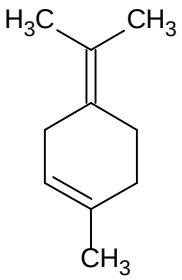


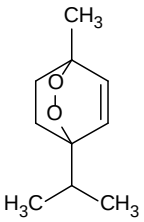
Limonene



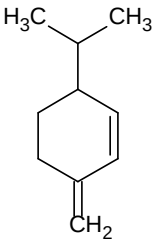
trans-Ocimene



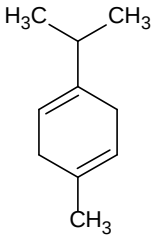
Terpinolene



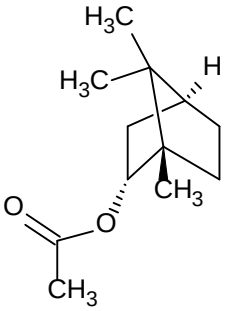
Ascaridole



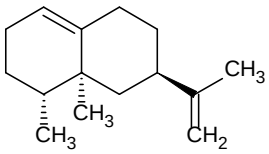
β- Phellandrene



γ- Terpinene



Bornyl acetate



Valencene

2.5 References

1. D. Philcox, A Taxonomic Revision of the Genus Limnophila R.Br. (Scrophulariaceae). Kew Bulletin 24, 1, pp.101-170, 1970.
2. L.M.Perry, Medicinal plants of East and Southeast Asia. Attributed properties and uses. MIT Press, Cambridge, Massachusetts, United States & London, UK. 1980.
3. CDK Cook, Water Plants of the World: A Manual for the Identification of the Genera of Freshwater Macrophytes, pp: 524, Springer, 1974.
4. L.W.S Shi,. Flora of China, , 18, 26-28, 1998.
5. www. indiabiodiversity.org/species/227593
6. D Estes and R. L. Small, Systematic Botany, 33, 1, 176–182, 2008.
7. P. K. Warrier, V. P. K. Nambiar and C. Ramankutty, Indian Medicinal Plants: A Compendium of 500 Species, Volume 3, Orient Blackswan, 1995 .
8. S. P. Ambasta,. The Useful Plants of India, (Editor-in –chief), Publications & Information Directorate, CSIR: New Delhi-110012,1986.
9. G.H. Schmelzer, Limnophila R.Br.[Internet] Record from Proseabase. Van Valkenburg, J.L.C.H. and Bunyapraphatsara, N. (Editors). PROSEA (Plant Resources of South-East Asia) Foundation, Bogor, Indonesia (2001). http://www.proseanet.org
10. R.N Chopra, S.L Nayar and I.C Chopra. Glossary of Indian Medicinal Plants; CSIR: New Delhi, 1956.
11. N.P Aashan, Ayurveda Prakashika,; Vol. 3, 1950.
12. The Wealth of India (Raw Materials); CSIR: New Delhi, 1962.
13. V.V. Sivarajan and I Balachandran, Ancient Science Of Life, 5, 4 , 1986.
14. G. Brahmachari, The Open Natural Products Journal, 1, 34-43 , 2008.
15. Jang et.al., J. Nat. Prod. 68, 7., 2005
16. Suksamrarn et.al., Archives of Pharmacal Research, 26, 10, 2003.
17. Reddy et.al., Pharmaceutical Biology, 29, 2,1991.
18. Kalimuthu et.al., Asian Journal Of Chemistry, 23, 2 2011.
19. A Ghani. Medicinal plants of Bangladesh with chemical constitutions and uses. Asiatic Society of Bangladesh, 2003.
20. Mishra et.al., Bull. Bot. Soc. Univ.Saugar, 27,1980.
21. Sribusarakum et.al. Thai Journal of Phytopharmacy 11, 2, 2004.
22. Bhuiyan et.al., Bangladesh J. Pharmacol., 5, 2010.

23. Thongdon and Inprakhon, World Journal of Microbiology & Biotechnology,25,8, 2009.

24. Tucker et.al., Journal of essential oil research, 14, 3 , 2002.

25. Sandhya et.al., Journal of Pharmacy Research; 4 , 11, 2011.

26. N.T Linh and L.N Thach, Journal of Essential Oil Bearing Plants, 14 ,3, 2011.

27. Xiao-Dong,et.al., Journal of Quanzhou Normal University , 02, 21-27, 2011.

28. Y.X.C Biqiang, Acta Botanica Yunnanica, 01, 1986.
29. Kapil et.al., Bull. Med. Ethnobot. Res.,4,1983.
30. Rao et.al., Fitoterapia, 60, 1989.

31. T Rudrappa, Glimpses in Plant Research, Vol. 13, Indigenous medicinal Plants including microbes And Fungi, 2nd Edition Edited by Purshotam Kaushik, 2009.

32. Buia et.al., Biochemical Systematics and Ecology, 32, 10, 2004.
33. R.P Rastogi and B.N Mehrotra,. Eds.; Compendium of Indian Medicinal Plants; CDRI and NISCOM: New Delhi, Vol. 4,1998.
34. R.P. Adams, Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry, 4th ed. Allured Publ. Corp., Carol Stream, IL, 2007.
35. E. K. Nelson , A Chemical Investigation Of The Oil Of Chenopodium. J. Am. Chem. Soc., 33 ,8, 1911 .
36. Verdeguer , et.al. Essential Oils from Chile Molecules, 16,1,403-411, 2011.
37. W. H Brown, C. S Foote, B. L Iverson and E. V Anslyn,. Organic Chemistry. Cengage Learning. pp: 967, 2009.
38. Chenopodium ambrosioides. Medicinal Plants for Livestock. Ithaca, NY, USA: Cornell University, Department of Animal Science.2008. www.ansci.cornell.edu.
39. M.A. Johnson and R. Croteau. Archives of Biochemistry and Biophysics, 235, 1, 1984.
40. G. Buchbauer. In Handbook of Essential oils. Science, Technology and Applications, CRC Press, Taylor and Francis: London, 235–280, 2010.
41. G Lang and G Buchbauer, Flavour Fragr. J. 27, 2012,
42. R.G. Berger (Ed.), Flavours and Fragrances Chemistry, Bioprocessing and Sustainability. Springer-Verlag, pp.88-91, 2007.
43. Boonbumrung et.al. Food Sci. Technol. Res., 7, 3, 2001.
44. S.A Gholap and C Bandyopadhyaya . J. Agric. Food Chem. 32 ,57−59, 1984
45. Odor Detection Thresholds - Leffingwell & Associates. www. leffingwell.com

CHAPTER 3

PHYTOCHEMICAL INVESTIGATION OF *COMMIPHORA CAUDATA* (WIGHT & ARN.) ENGL.

The genus *Commiphora* belongs to *Burseraceae* with more than 150 plant species. It is distributed in the tropical and subtropical regions, especially in northeastern Africa, southern Arabia and India[1] and characterized as small trees or shrubs with spinescent branches, pale-gray bark and reddish-brown resinous exudates. Over 200 *Commiphora* species were identified around Read Sea in East Africa, 20 species in Madagascar 6 in India [2] and recently 10 new species in Somalia [3].The important species of the genus are given below :

C .myrrha (Nees) Engl.	*C. abyssinica (Berg)*
C. madagascarensis Jacq.)	*C. africana (A. Rich.)*
C. Guidottii	*C. mukul (Hook ex Stocks) Engl.*
C. wightii (Arnott.) Bhanol.)	*C. opobalsamum*
C. erythraea (Ehrenb.) Engl.	*C. aprevali (Baill) Guill*
C. Boiviniana Engl.	*C. merkeri Engl.*
C. Pervilleana Engl.	*C. gardoensis*
C. Stellatopubescens	*C. chiovendana*
C. murraywatsonii	*C.caudata (Wight & Arn.) Engl.*

Of these *C. Myrrha* and *C. Mukul* may be the most investigated specie while *C. Caudata* will be one of the least studied.

3.1 Phytochemistry of the genus *Commiphora*-A Review

The resinous exudates from the bark *Commiphora* plants are

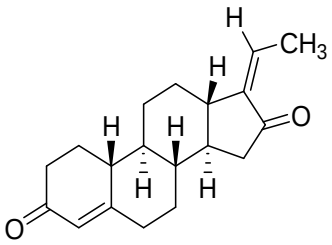
important indigenous medicines. They have a long medicinal application in Ayurvedic other traditional medical systems for arthritis, hyper-lipidemia, pain, wounds, fractures and blood stagnation. Phytochemical investigation of these plants has resulted in the identification of more than 300 secondary metabolites. The isolated metabolites and crude extracts have exhibited a wide range of pharmacological effects both *in vitro* and *in vivo*. These include anti-proliferative, antioxidant, anti-inflammatory and antimicrobial activities [4].

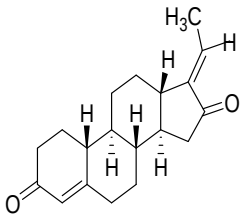
Myrrh : Myrrh is the aromatic resin of a number of small, thorny tree species of the genus *Commiphora*. It is an *oleoresin* commonly harvested from the species *Commiphora myrrha*, and also from *Commiphora molmol*. The Bible records myrrh showing up three times in the life and death of Jesus Christ. Myrrh consists of a water-soluble gum, alcohol-soluble resins and a volatile oil. The gum contains polysaccharides and proteins and the volatile oil is composed of steroids, sterols and terpenes. Myrrh's characteristic odour is due to the presence of furanosesquiterpenes [5]. The constituents of essential oil in myrrh and the gum were analyzed by GC-MS. The main constituents of myrrh was furanoeudesma-1,3-diene out of 15 compounds and that of gum out of 33 compounds was trans-β-ocimene[6] . A number of compounds present in myrrh had already been isolated and identified for their potentials.

The use of myrrh medicinally was recorded in China in A.D. 600 and still it is used in Chinese medicine to treat wounds, relieve painful swelling, and to treat menstrual pain due to blood stagnation. It has long been used in the Ayurvedic system of medicine also. The gum myrrh has been used as incense for many centuries. In more recent times, the gum has found medical usage as an antiseptic, the tincture being applied to inflammatory and ulcerated conditions of the throat and mouth. [5].

Guggul : The gum-resin of *C. mukul* and *C. wightii* is called guggul. Guggul is produced by drying the milky-white sap of the tree for one year. It was

found that guggul resin is a complex mixture of various classes of chemical compounds, such as lignans, lipids, diterpenoids and steroids [5] . Guggulsterons (steroids) and guggulsterols are two important classes of compounds isolated from this gum resin [7, 8]. The Atharva Veda, one of the four well-known holy scriptures of the Hindus, is the earliest reference to the therapeutic properties of guggul. The actions, uses, and indications as well as the varieties of guggul have been described in the Ayurvedic treatises, Charaka (1000 B.C.), Sushruta Samhita (600 B.C.), and Vagbhata (7^{th} century A.D.) Traditionally it has been indicated for healing bone fracture to inflammation, arthritis, cardiovascular conditions, obesity, and lipid disorders. Several external and internal uses for guggul have been described in folklore and ethnomedicine . Ayurvedic physicians extensively used guggul for treating arthritis and related conditions for centuries. Lipid-lowering property (hypolipidemic activity) of guggul had been investigated by several researhers [9, 10] and from 1988 onwards gugulipid was available as a hypolipidemic agent on the Indian market. Modern therapeutic uses of guggul include nervous diseases, skin diseases, hemiplegia, leprosy, marasmus, muscle spasms, neuralgia, ophthalmia, pyelitis, pyorrhea, spongy gums, ulcerative pharyngitis, scrofula,, ischaemia, hypertension, hemorrhoids, and urinary tract disorders [23].





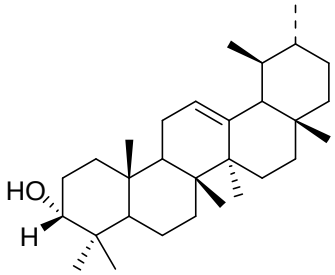
Z-Guggulsterone **E-Guggulsterone**

Terpenoids : From the volatile oils obtained from the plants ,monoterpenoids including α-pinene, camphene, β-pinene, myrcene and limonene have been detected[4]. Composition of volatile oil from different *Commiphora* species varies largely where sesquiterpenoids with

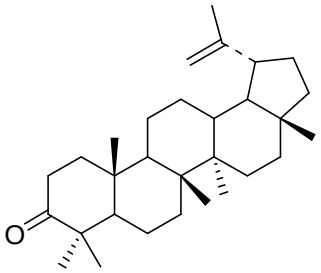
low degree of oxidation are the major constituents. β-Elemene, α-copaene, α-humulene, β-selinene and germacrene B are the widely distributed sesquiterpenoids in these oils . The structures of sesquiterpenoids from the genus are mainly classified into germacrane, eudesmane, guaiane, cadinane, elemane, bisabolane and oplopane groups. The presence of furanosesquiterpenoids mainly furanogermacrane, furanoeudesmanes, furanoguaiane, furanocadinane and furanoelemane is an important characteristic of this genus [4]. Several sesquiterpene lactones including germacranolide , eudesmanolides , elemanolide , guaianolide , and cadinanolide had been isolated from the resin of *C. opobalsamum* and *C. Myrrha* [11, 12, 13]. Diterpenes like camphorene, cembrane diterpenoids, verticillane diterpenoids, pimarane diterpenoids and abietane diterpenoids were also reported from *C. Mukul* and *C. Myrrha* [14, 15].

Commiphora plants are rich in dammarane triterpenoids are. More than twenty-one dammarane triterpenoids had been isolated from the resins of *C. Kua* [16,17], *C. dalzieli* [18], *C. Confuse* [16, 19] and *C. Myrrha* [12]. Ten cycloartane triterpenoids with novel substitution at C-2 position have been isolated from *C. opobalsamum* and *C. myrrha* recently [20, 4]. Polypodane triterpenoids were also isolated but only from the species of *C. Mukul* [4].

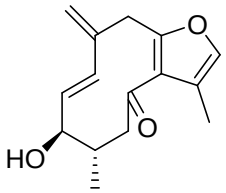
Steroids : A number therapeutically important sterols and steroids were isolated solely from *C. Mukul* [4]. Sukhdev et al had isolated Z-guggulsterone, E-guggulusterone, β-hydroxyprogesterone and three new sterols viz. guggulsterols I, II & III [7]. Two more new sterols guggulsterol-IV and guggulsterol- V had also been isolated from the same plant [8, 21] . The crude gum guggul was found to contain 2% guggulsterones while the ethyl acetate extract contains 4% to 4.5% guggulsterones. The neutral subfraction contains 4.2% to 4.7% guggulsterone while its acetone subfraction contains 35% to 40% from which the E- and Z- guggulsterones were derived [22].



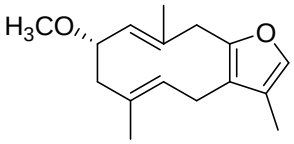
3-epi-α-amirin



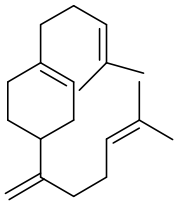
lupeone



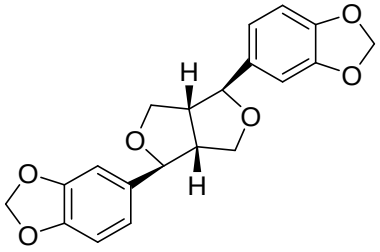
1,10(15)-furanodien-6-one



2-methoxyfuranodiene



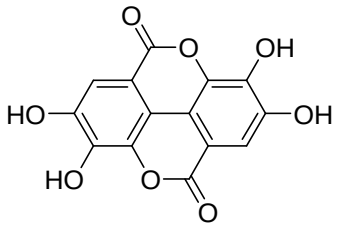
Camphorene

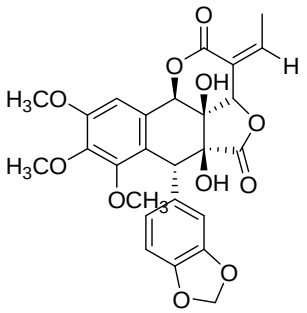


(+)-sesamin

Many other secondary metabolites such as flavonoids, flavanol glycosides, lignans and long chain aliphatic derivatives were also isolated from the genus. Flavanoids are not found in the resinous exudates but in stem , bark and flowers [24].

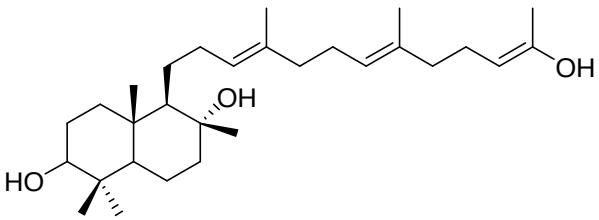
Carbohydrates had been reported from the gum in the form of polysaccharide [25]. 1,2,3,4-tetrahydroxy long chain aliphatic derivatives occur in the gum in the form of ferrulic acid ester or glycoside [4, 20].





Ellagic acid (flavanoid) **Erlangerin A (lignan)**

Pharamacological activites : Studies carried out on crude extracts and pure metabolites provided supporting evidences in favour of the traditional uses, revealing this genus to be a valuable source for medicinally important molecules [4]. Triterpenoids and diterpenoids are mainly responsible for anti-inflammatory property, sesquiterpenoids for antimicrobial, smooth muscle-relaxing and analgesic effects, lignans for the cytotoxic and toxicity, and steroids for antiproliferative, anti-inflammatory, hypolipidemic and antidiabetic activities. Guggulsterones exhibited potent inhibitory effect on tumor cells in *invitro* and *in vivo*, as well as anti-inflammatory property in *in vivo*. Myrrhanol A a polypodane triterpenoid from *C.mukul* [4], exhibited more potent anti-inflammatory effect than hydrocortisone in adjuvant-induced air pouch granuloma model. All finding support the traditional medicinal uses of the plants of the genus.



Myrrhanol A

A clinical study indicated that the resin extract of *C. mukul* had significant improvements of osteoarthritis [26]. The volatile oil of *C. molmol*

was shown to inhibit the production of IL-1b-stimulated IL-6 and IL-8 in human gingival fibroblasts cells [27].

Antimicrobial potential of the leaf and stem extracts of ten *Commiphora* plants against four bacteria and two yeasts had been examined by Paraskeva et al. [28] while inhibitory effects of *C. swynnertonii* had been investigated by Bakari et al [29].

The essential oil of *C. myrrha* exhibited potent singlet oxygen quenching activity. Three furanosesquiterpenoids from *C. myrrha* showed DPPH radical scavenging activity [30]. The stem extract of *C. schimperi*, *C. neglecta*, *C. tenuipetiolata*, *C. Edulis* and *C. berryi*a also exhibited antioxidant effect in the DPPH assay [30, 31].

The resin of *C. mukul* and the active guggulsterone were inhibitors of low density lipoprotein(LDL) oxidation, and beneficial in the treatment of atherogenesis [32]. Guggulsterones were identified as the most potent hypolipidemic substances in the *C. mukul* resin [33]. Ethanol resin extract of *C. mukul* exihibited antihyperglycemic activity [34] which can be utilized in treatment of diabetes. The aqueous extract of *C. molmol* resin provided dose-dependent anti-ulcer and gastric mucosa protective effects in rats. The resin extracts of *C. opobalsamum* and *C. berryi* also displayed similar protective effects in different mice ulcer models[4].

***C. Caudata* :** As mentioned earlier only a few works had so far been reported from *C. Caudata*. Traditionally it is used for anti-arthritic preparations. Different extracts of this plant were found to be possessing interesting bioactivities. Ethanolic extracts of the leaves showed comparable free radical scavenging activity in *in-vitro* assays [35]. Alcoholic extracts of the leaves also showed potent anti-inflammatory and analgesic activity justifying its uses in traditional medicine [36]. The ethyl acetate and methanol extracts of *C. caudata* showed moderate antimicrobial activity with an MIC of more than 2.0mg mL^{-1} against *P. Aeruginosa* [37].

Antiulcer activity of bark extract and gum exudates of *C. caudata* on aspirin induced ulcer in rats were successfully studied [38]. Both ethyl acetate extract and methanolic extract of *C.caudata* (200 mg/kg of body weight) exhibited significant anti-inflammatory activities in rats [39]. Analgesic and anti-inflammatory activity of the alcoholic (70%) and aqueous extracts of leaves were also evaluated with rodents [40]. Ethyl acetate and methanolic extracts of the species (200 mg/kg of body weight) exhibited diuretic activity (increase in urine volume and increase in Na+ excretion) in rats [41]. Anti-hyperlimidic activity of the ethanolic extracts were investigated in rats and obtained encouraging results [42].

The ethanolic extract of the bark was evaluated for its cytoprotective activity against ethanol-induced gastric lesions in rats and obtained beneficiary results [43]. The methanolic extracts of dried bark also showed moderate cytotoxic activity against a human mammary carcinoma cell line (MCF-7), with values IC_{50} of 82.6 and 88.4μgmL^{-1}, respectively [37].

3.2 Present work

The present work consists of the phytochemical investigation of the dried bark of *C.caudata*. This include the extraction of the plant material using different solvent systems, the fractionation and isolation of the chemical constituents , their purification and characterization by various physical, chemical and spectral techniques.

Plant material

The bark of *C.caudata* were collected from Malappuram district, Kerala. It was identified by Dr. A.K Pradeep, Department of Botany, University of Calicut. A voucher specimen was deposited in the Herbarium, Department of Botany, University of Calicut.

3.3 Materials and Methods

All **melting points** were determined on Toshniwal melting point apparatus and are uncorrected. **UV spectra** were obtained in a JASCO UV spectrometer. **IR spectra** (KBr) were taken on a JASCO FT-IR spectrometer. The **^{1}H-NMR spectra** of the isolates were recorded under room temperature on Bruker ARX 500 instrument at 500MHz in DMSO-*d6*. TMS was used as the internal standard. The **^{13}C-NMR** was obtained at 125 MHz. **Column chromatography** were carried out using Silica gel (100-200, mesh size, MERCK) . **GC-MS** analysis was done by Varian 4000 GC-MS. The columns were prepared as slurry with suitable solvents and a gradient elution was carried out by mixing of solvents with different polarity. **Thin layer chromatographic plates** were prepared using TLC grade silica gel (Merck).

Spray tests for TLC

20% aqueous sulphuric acid (20% H_2SO_4) : The reagent is sprayed on the plate and heated to 110^{0}C until spots are visible. Compounds (tri-terpenes and sterols) develop brown, pink, purple or yellow colour.

Liebermann-Burchard test: It is prepared by carefully mixing 5ml acetic anhydride with 5ml conc. sulphuric acid under cooling. The mixture is then added cautiously to 50ml absolute ethanol. The reagent is sprayed on the plate and heated to 100^{0}C for 10 minutes and observed in UV light.

2,4-Dinitrophenylhydrazine reagent : Suspended 2,4-Dinitrophenyl-hydrazine (2g) in methanol (100 ml) and then conc. Sulphuric acid (4 ml) added slowly and filtered. The reagent is used for detecting ketones and aldehydes .

3.4 Isolation of compounds from the Petroleum ether extract of *C.caudata*

Dried and coarsely powdered bark of *C.caudata* (4kg) was extracted with petroleum ether (bp ; 60-80^{o}C, 3x 7L). The combined extract was concentrated under reduced pressure to about 500 ml of dark coloured

solution. This solution was heated , absorbed on silica gel (200 g, 60-120 mesh) and dried to free flowing. It was loaded in a chromatographic column (3cm x 60 cm ; d x l) and eluted with solvents of increasing polarity viz . P. ether (1L), 10:1 P. ether-ethyl acetate (500 ml), 8:1 P. ether-E. acetate (500 ml), 6:1 P. ether-E. acetate (500 ml), 1:1 P. ether-E. acetate (500 ml), E. acetate (500 ml) and methanol (500 ml). Several 50 ml fractions were collected and each fraction was checked by TLC. Fractions were pooled together according to their homogeneity judged from TLC analysis. Following fractions were found distinctive and analysed by various techniques for further characterization;

Fraction	Eluent composition	Characteristics	Further analysis
I	10:1 P. ether-E. acetate	Yellow solid +solution	UV spectroscopy
II	8:1 P. ether-E. acetate	Yellow oily, semisolid	Chemical methods, GC-MS
III (CC1)	7:1 P. ether-E. acetate	White solid	Chemical methods, IR, ^{1}HNMR, C^{13}NMR
IV (CC2)	5:1 P. ether-E. acetate	White solid	Chemical methods, IR, ^{1}HNMR, C^{13}NMR

3.4.1 Analysis of fraction I

Yellow solid obtained in fraction I is filtered and washed with petroleum ether. UV spectrum with petroleum ether as the solvent gave two characteristic peaks at 472 nm and 446 nm with corresponding absorbance at 0.0473 and 0.0778 respectively. Presence of α-carotene in the fraction was identified by a close comparison with the reported data [44] .

3.4.2 Analysis of fraction II – Mixture of fatty acids and lipids

The fractions on elution with petroleum ether and ethyl acetate in the ratio 8:1 on concentration yielded a yellow syrupy semi-solid (about 4g). It showed large number of spots on TLC examination. The sample responded

positively to saponification test giving a clear solution. It was then subjected to the following analyses adopting known methodologies with proper modification [45].

a) Determination of saponification value

Saponification value or saponification number represents the number of milligrams of potassium hydroxide required to saponify 1g of fat or oil under the conditions specified. It is a measure of the average molecular weight (or chain length) of all the fatty acids present.

About 1 gm of the oil was weighed into an RB flask and added 25 ml alcoholic potassium hydroxide solution. The flask was connected with a reflux condenser and heated on a water bath for 1 hour, boiled gently and steadily until the sample was completely saponified, as indicated by absence of any matter and appearance of clear solution. After the flask and condenser have cooled, inside of the condenser was washed down with about 10 ml of hot ethyl alcohol. About 1 ml of phenolphthalein indicator solution was added, and titrated with 0.5 N standard hydrochloric acid. A blank analysis was conducted at the same time.

Saponification value = 40 x (B-S) x N/W

Where B = volume of standard HCl solution required for the blank, S = volume of standard HCl solution required for the sample, N = normality of hydrochloric acid solution and W = weight in g of the sample

b) Determination of acid value

Acid value is the mass of potassium hydroxide (KOH) in milligrams that is required to neutralize one gram of fat or oil. It is a measure of the amount of carboxylic acid groups present in the mixture.

About 1 g of the oil was weighed accurately in a 250 ml conical flask. 50 ml of freshly neutralized hot ethyl alcohol and about 1 ml of 1% phenolphthalein indicator was added. Boiled the mixture for about 2 minutes and titrated while hot with about 0.1 N standard aqueous potassium hydroxide solutions, with vigorous shaking.

Acid value = t x N x 56.1/W

Where t = titer value , N = normality of KOH solution, W= weight in g of the sample.

c) Estimation of iodine value

Iodine value is the number of grams of iodine consumed by 100 g of fat or oil. It is measure of the degree of unsaturation in fatty acids.

About 0.4 g of the oil was weighed accurately in a clean stoppered bottle to which 10 ml of pure carbon tetrachloride was added. 25 ml of 0.1N iodine monochloride solution was added, shaken well and allowed to stand for 1 hour in dark. Added about 10 ml of 15% potassium iodide solution, shaken well and added 10ml freshly boiled and cold water, washing down any free iodine on the stopper. Liberated iodine was titrated against 0.1 N sodium thiosulphate solution with continuous shaking. When the colour of the solution turned to straw yellow 1 ml of starch solution was added as indicator and continued the titration until the blue colour completely disappeared. A blank was also carried out.

Iodine value = (B-S) x 0.0127 x 1000/W

Where, B = volume of thiosulphate solution required for the blank

S = volume of thiosulphate solution required for the sample.

N = normality of sodium thiosulphate solution and W = weight in g of the sample

d) Ester value

Ester value is the number of mg of potassium hydroxide required to saponify the esters in 1.0 g of the fat or oil. It can be calculated as the difference between saponofication value and acid value.

Results

Saponification Value	79.05
Acid Value	15.05
Iodine Value	213.90
Ester value	64.00

The high value of acid number indicates that the oil is not edible .The higher iodine value also suggest that the oil contains fatty acid esters with high degree of unsaturation. The lower saponification value indicates that the oil contain long chain fatty acids.

e) Preparation fatty acid methyl ester (FAME)-transmethylation

About 2 g of oily fraction was saponified with 25 ml 10 % alcoholic KOH solution. Potassium salt of fatty acids (soap) solution was cooled and transferred into a separating funnel, added 25 ml water followed by the addition of 10 ml Petroleum ether. Inserting the stopper it was shaken vigorously for 1 minute and allowed to settle until both layers were clear. The lower layer containing the soap solution was transferred to another separating funnel and repeated the ether extraction 6 times using 10 ml P. ether for each extraction. All the extracts were collected in a separating funnel, washed the combined extract 3 times with 25 ml portions of aqueous alcohol (10 % ethanol) solution , shaken vigorously and drawn off the alcohol-water layer after each washing. It was diluted with 25 ml water and neutralized with conc. HCl. The precipitated mixture was extracted with P. Ether (40-60^{o}C), and the solvent evaporated to yield about 1 g of lipids.

The mixture (about 1 g) was esterified using a mixture of methanol, toluene and sulfuric acid in the ratio 88:10;02. The solution was heated for about 1 hr at 80^{o} C and was extracted with P. ether (40-60, 2x20 ml) . It was dried over anhydrous sodium sulphate. The organic solvent was removed under reduced pressure to yield about 0.5 g fatty acid methyl ester (FAME) along with other lipids.

GC-MS analysis of the FAME- lipid mixture is carried out by using a Varian 4000 instrument equipped with a flame ionization detector. A VF5 silica column (30m x 0.25 µm x 0.25mm) was utilized. Helium was used as the carrier gas at a flow-rate of 0.8 ml/min and the temperature is programmed as 40^{o}C -280^{o}C. Individual components were identified both by the library search and by comparison with reported data [46]

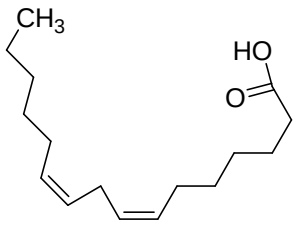
Table 3.1 GC/MS analysis of methyl ester of fatty oil

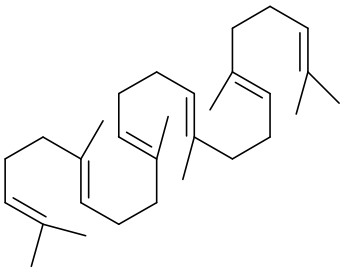
No	**RT**	**Compound**	**Amount %**
1	7.54	Methyl undecaonate	0.1
2	14.36	cis-7-Tetradecenoic acid methyl ester	1.76
3	14.60	Methyl Myristoleate	0.26
4	17.38	Iso-Pentadecanoic acid methyl ester (Iso-methyl palmitate)	1.38
5	19.49	7(Z),10(Z)-Hexadecadienoic acid Methyl Ester	18.82
6	19.68	Iso-Hexadecanoic acid methyl ester	0.81
7	21.27	9,12,15 -hexadecatrienoic acid methyl ester	8.33
8	23.79	cis-13-Octadecenoic acid methyl ester	0.1
9	24.57	cis-9,12-Octadecadienoic acid methyl ester (Methyl Linoleate)	0.11
10	25.91	cis-9,12,15-Octadecatrienoic acid methyl ester (Methyl Linolenate)	3.17
11	27.00	Pentacosane	3.97
12	27.73	Methyl Arachidate	4.20
13	28.51	Tetracosanal	9.80
14	29.45	Heptacosane	3.3
15	29.83	Iso-Methyl Heneicosanoate	3.72
16	30.31	Squalene	14.42
Total			74.25

Out of 16 components identified in the lipid mixture 12 were methyl esters of various fatty acids. Majority of them (total 7) corresponded to acids with high degree of unsaturation confirming the higher iodine value. Methyl

Ester of 7(Z),10(Z)-hexadecadienoic acid was the most abundant component (18.82%) followed by squalene (14.42%), tetracosanal (9.8%) and 9,12,15 - hexadecatrienoic acid methyl ester (8.33%). cis-7-Tetradecenoic acid methyl ester , methyl myristoleate, cis-13-octadecenoic acid methyl ester , methyl linoleate and methyl linolenate corresponded other unsaturated acids.

7(Z),10(Z)-Hexadecadienoic acid is a conjugated dienoic fatty acid metabolite of conjugated linoleic acid, which in turn possessing potential beneficial effects on atherosclerosis, carcinogenesis or obesity in human [47]. Another acid present in the sample cis-9,12,15-Octadecatrienoic acid was earlier reported for its antiinflammatory, hypocholesterolemic, cancer preventive, hepatoprotective and antiarthritic potentials [48]. The second abundant component squalene is a novel triterpenoid possessing various therapeutic potentials [49]. Squalene was well recognized for its promising cancer preventive and cholesterol lowering properties. It was also identified as an efficient sink for the elimination of highly lipophilic xenobiotics from the body. Presence of these novel lipids establishes the medicinal potential of *C.caudata.*





7(Z),10(Z)-Hexadecadienoic acid **Squalene**

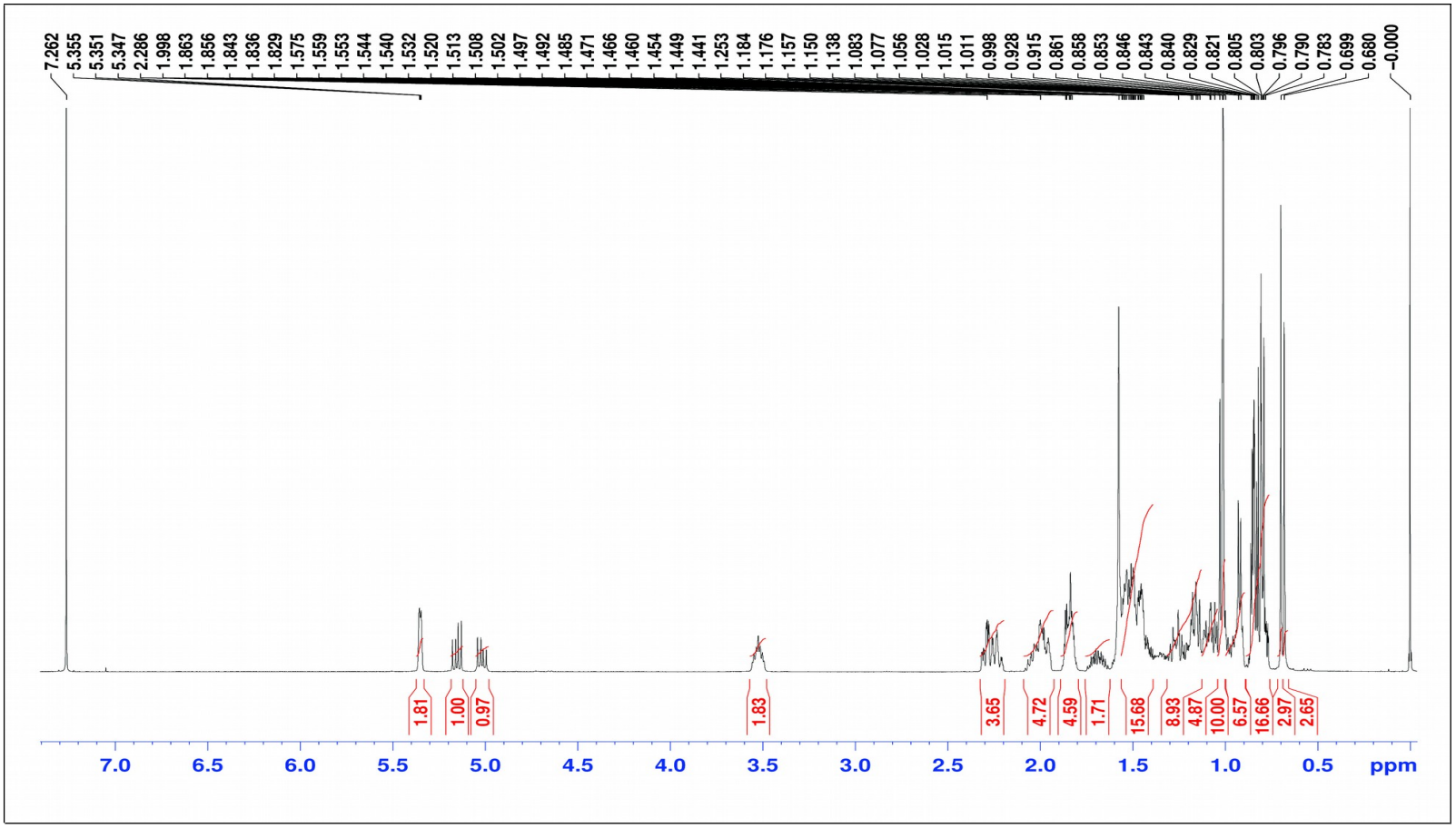
3.4.3 Analysis of CC1

Fraction obtained on elution of the extract with 7:1 P. ether-E. acetate gave a white solid on evaporation. It was recrystallized from ethanol and its melting point was obtained as 123^{o}C. TLC showed a pink colour typical of

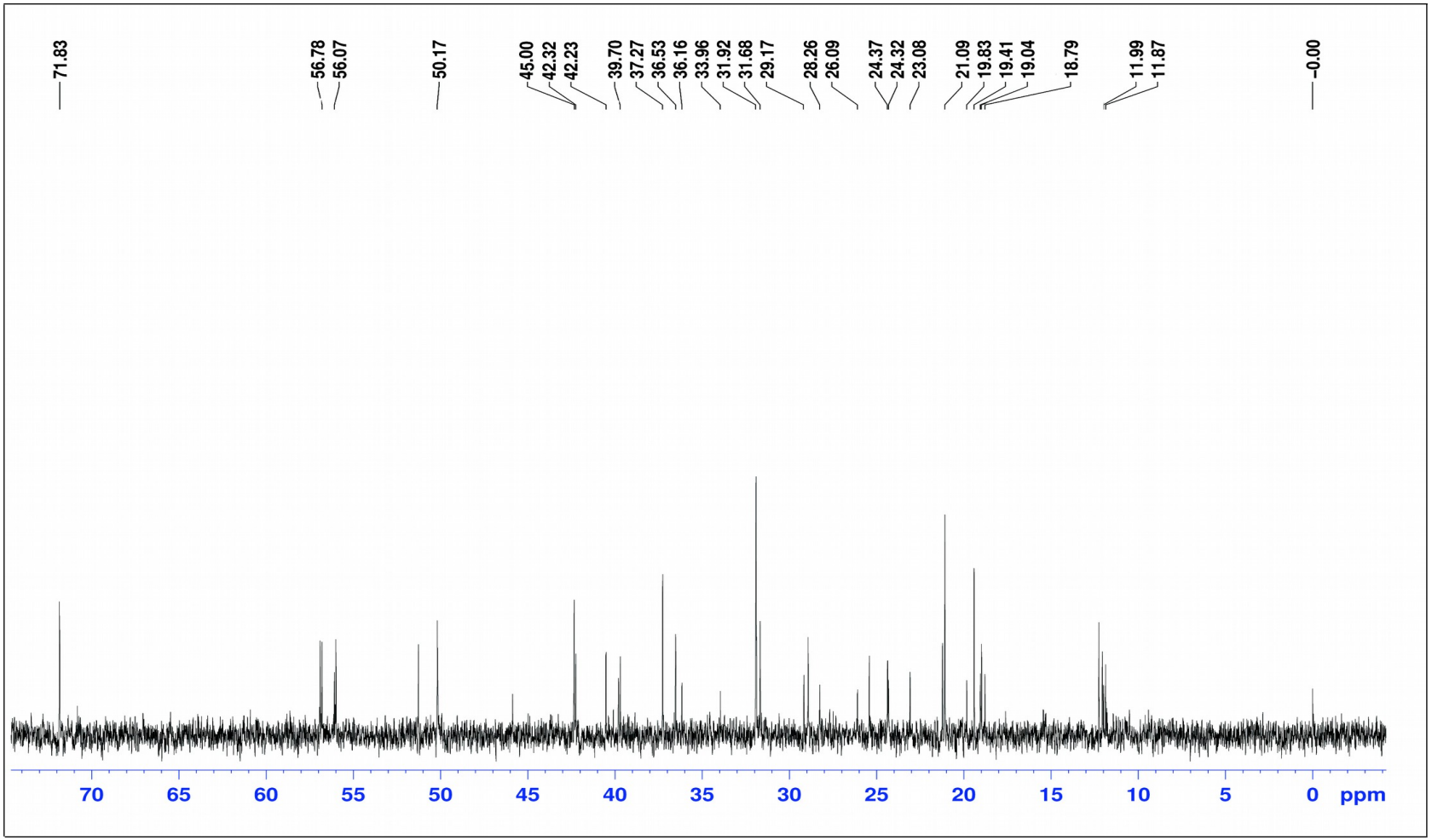
triterpenes on spraying 10% H_2SO_4 and heating to 100^0C. Liebermann-Burchard reagent gave a bluish green colour indicating it was a sterol. Bayers reagent was decolourised indicating unsaturation. Mass spectrum of the compound showed m+ at m/z= 414. IR absorption spectra showed the bands due to hydroxyl group (3441 cm^{-1}, broad), C=C group (1610 cm^{-1}) and gem dimethyl groups (1390, 1380 and 1308 cm^{-1}) . In TLC experiment Rf of the compound was comparable with that of an authentic sample of β-sitosterol.

A comparison with the reported data [50] showed that ^{1}H NMR spectrum of the compound was identical to that of lawsaritol, an isomer of β-sitosterol. A doublet obtained at δ 5.35 corresponding to hydrogen at C-4, a multiplet at δ 3.52 corresponding to the axial hydrogen at C-3 interacting with C-2 axial, C-2 equatorial and C-4 protons were the characteristic peaks. The downfield shift of the C-3 proton at 3.52 in comparison to the C-3 proton of β-sitosterol(δ 3.25), and the appearance of the olefinic proton at 5.35 as a doublet suggested a double bond between C-4 and C-5 in ring A. Other peaks in the spectrum were also found identical to those previously reported .

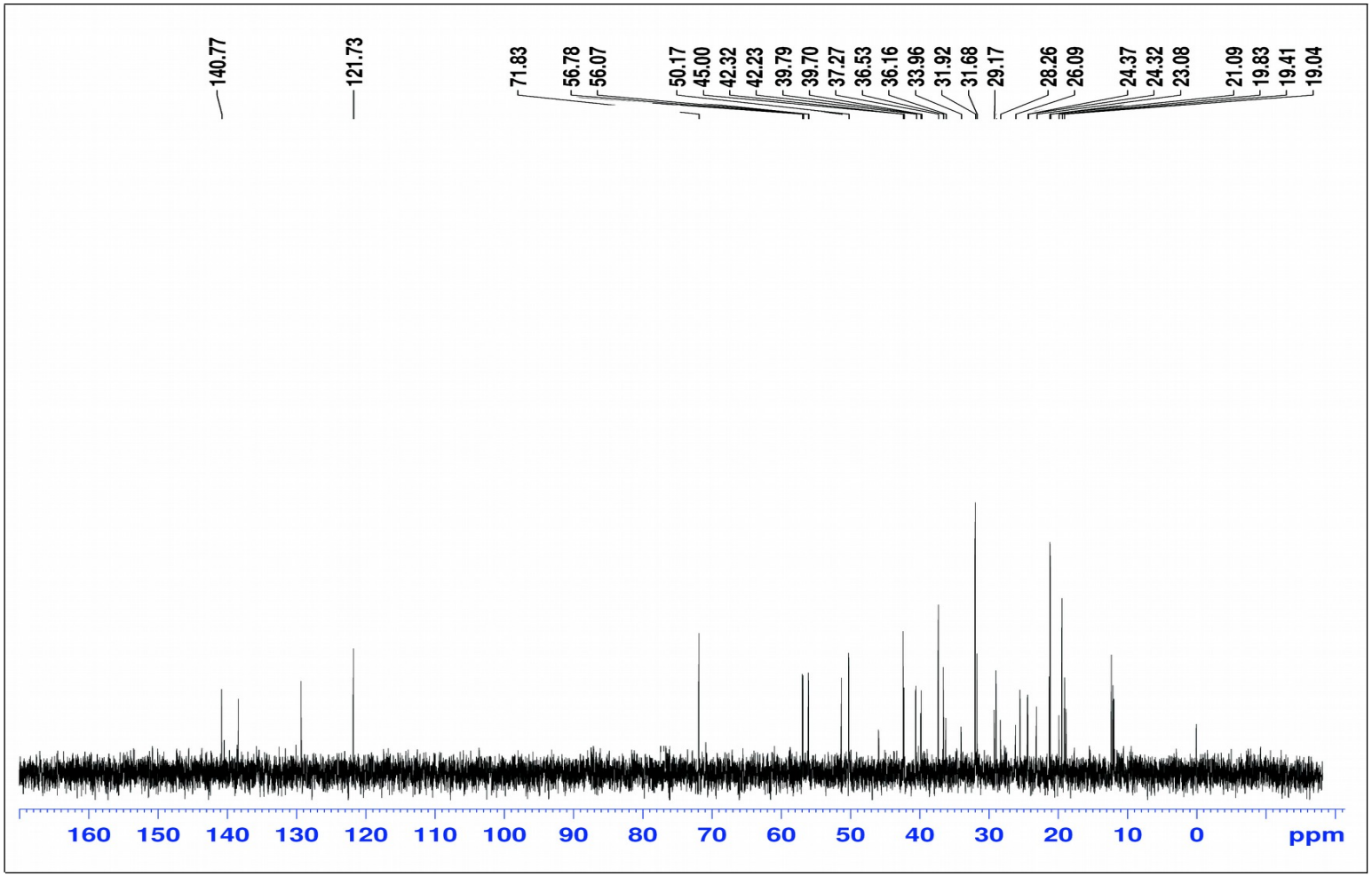
In the ^{13}C NMR spectrum signals at δ 140.77, 121.73 and 71.83 corresponded to the unsaturated carbons C-5, C4 and C-3 respectively. Other signals were also found matching with those previously reported for lawsaritol [51] (table 3.2). Due to the presence of slight impunity more signals were present in the NMR Spectra.



^{1}H NMR spectrum of lawsaritol



^{13}C NMR spectrum of lawsaritol



^{13}C NMR spectrum of lawsaritol

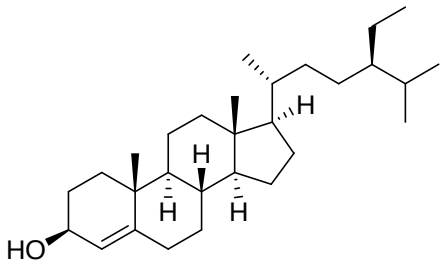
Acetylation of lawsaritol : 50mg of the compound was refluxed with 1 ml acetic anhydride in pyridine for 2 hours. The reaction mixture is poured into cold water , filtered, washed with water and recrystallised from petroleum ether. Melting point of the product was 117^{o}C which had been earlier reported for monoacetyl derivative of lawsaritol [50].

Thus the compound CC1 was identified as lawsaritol. This compound had been earlier isolated from the plants like *Lawsonia inermis* [50] and this is the first report of this sterol from *C.caudata.*

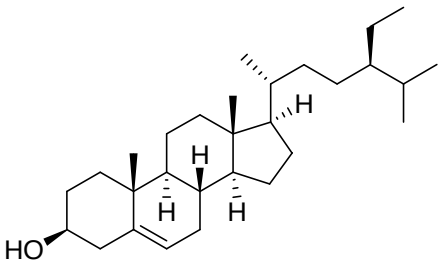
Table 3.2 ^{13}C NMR signals of lawsaritol

Carbon atom No	**^{13}C NMR shift of the compound**	**^{13}C NMR shift From literature**
1	37.27	37.2
2	31.68	31.6
3	71.83	71.8
4	121.73	121.7
5	140.77	140.7
6	24.32	24.3
7	33.96	33.9
8	31.92	31.9
9	50.17	50.1
10	36.53	36.5
11	21.09	21.0
12	42.32	42.4
13	42.23	42.3
14	56.78	56.7
15	24.37	24.3
16	28.26	28.1
17	56.07	56.1
18	11.99	11.9
19	19.83	19.8
20	36.16	36.1
21	18.79	18.7
22	39.70	39.3
23	26.09	26.1
24	45.00	45.8
25	29.17	29.2
26	19.41	19.3
27	19.04	19.0
28	23.08	23.1

Carbon atom No	^{13}C NMR shift of the compound	^{13}C NMR shift From literature
29	11.87	11.8



lawsaritol



β-sitosterol

3.4.4 Analysis of CC2

Fraction obtained on elution of the extract with 5:1 P. ether-E. acetate gave a white solid on evaporation. It was recrystallized from 1:1 petroleum ether-ethyl acetate mixture and its melting point was obtained as 242^{o}C. On TLC the compound gave a red-violet spot on heating to 100^{o}C with 20% H_2SO_4 and a pink colour with Liebermann-Burchard reagent, both typical of triterpenes. Bayers reagent was not decolourised indicating the saturated nature of the compound.

This compound gave an orange colour with 2,4-ninitrophenylhydrazine reagent indicating the presence of carbonyl group. Presence of carbonyl group was also confirmed by the IR spectrum (strong absorption at 1711.6 cm^{-1}). Mass spectrum of the compound showed m+ at m/z= 426. ^{13}C NMR spectrum indicated the presence of 30 carbon atoms in the molecule. From these the molecular formula of the compound was deduced as $C_{30}H_{50}O$. The formula suggested 6 equivalents of hydrogen deficiency which could be attributed to the carbonyl oxygen and a pentacyclic ring. All these results were similar to those obtained for the triterpene friedelin isolated from *Cissus glauca* [52] earlier in our lab. A

direct comparison of Rf with an authentic sample confirmed the identity of the compound.

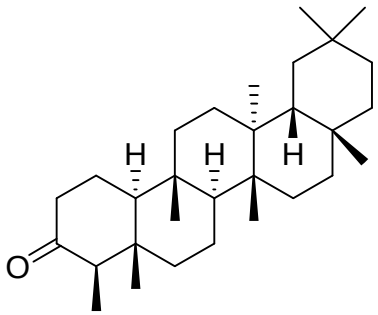
The ^{1}H NMR spectrum showed seven tertiary methyl groups at δ 0.725, 0.885, 0.954, 1.008, 1.050, 1.215 and 1.255. Secondary methyl group appeared as a doublet at δ 1.001. In the ^{13}C NMR spectrum the most down-shielded signal corresponded to the carbonyl carbon at δ 213.23. Other signals were also well corresponded to those in the reported data [53] (table 3.3). Due to slight impurity more signals were present in the NMR spectra.

Table 3.3 ^{13}C NMR signals of friedelin

Carbon atom No	**^{13}C NMR shift of the compound**	**^{13}C NMR shift From literature**
1	22.29	22.3
2	41.54	41.5
3	213.23	213.2
4	58.24	58.2
5	42.16	42.1
6	41.30	41.3
7	18.25	18.2
8	53.11	53.1
9	37.45	37.4
10	59.49	59.4
11	35.63	35.6
12	30.52	30.5
13	39.71	39.7
14	38.31	38.3
15	32.43	32.4
16	36.02	36.0
17	30.01	30.0
18	42.80	42.8
19	35.35	35.3
20	28.18	28.1
21	32.78	32.7

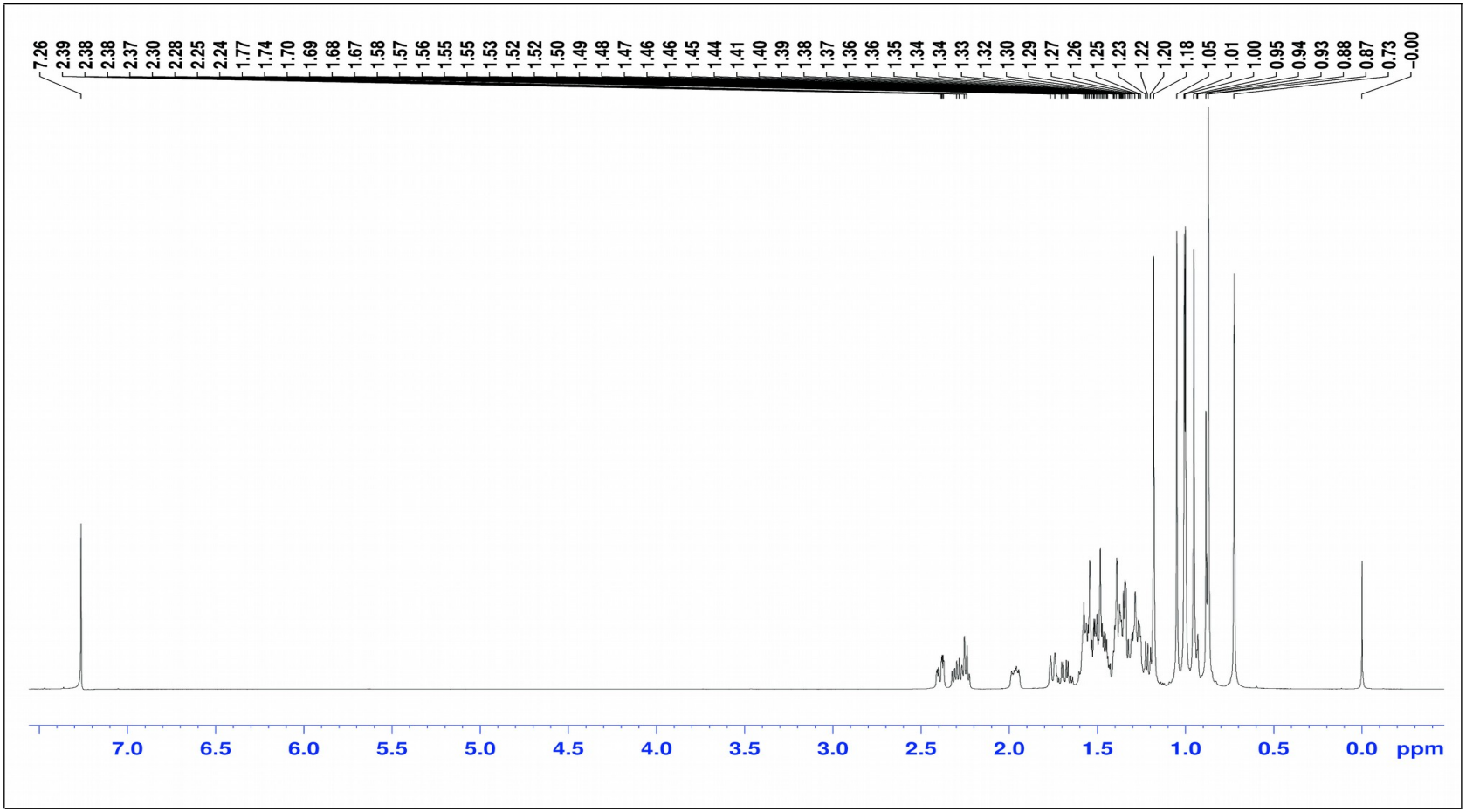
22	39.26	39.2
23	6.83	6.8
24	14.67	14.6
25	17.95	17.9
26	20.27	20.2
27	18.67	18.6
28	32.10	32.1
29	31.79	31.8
30	35.03	35.0

Thus the compound CC2 was identified as the pentacyclic triterpenoid friedelin.

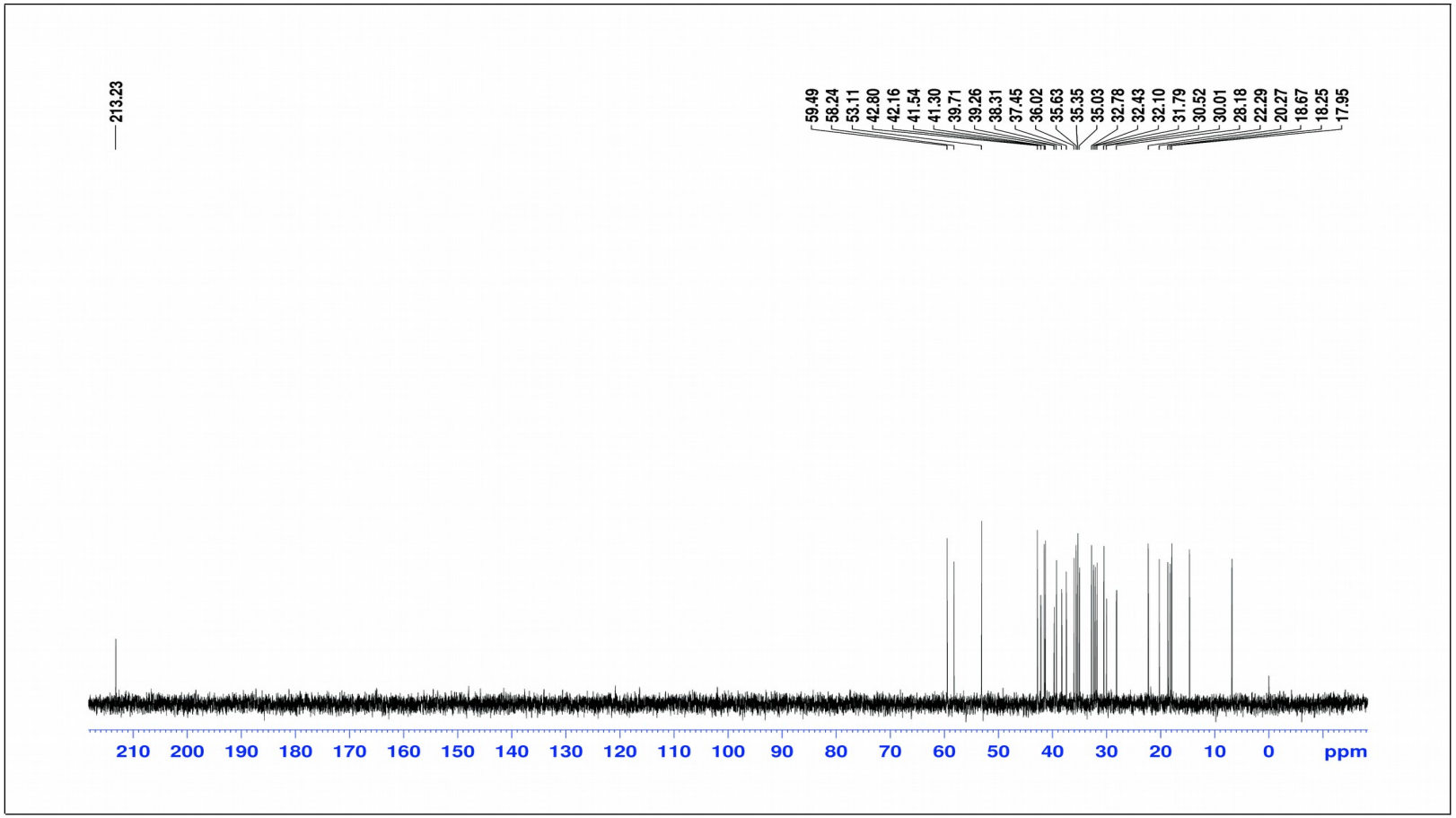


friedelin

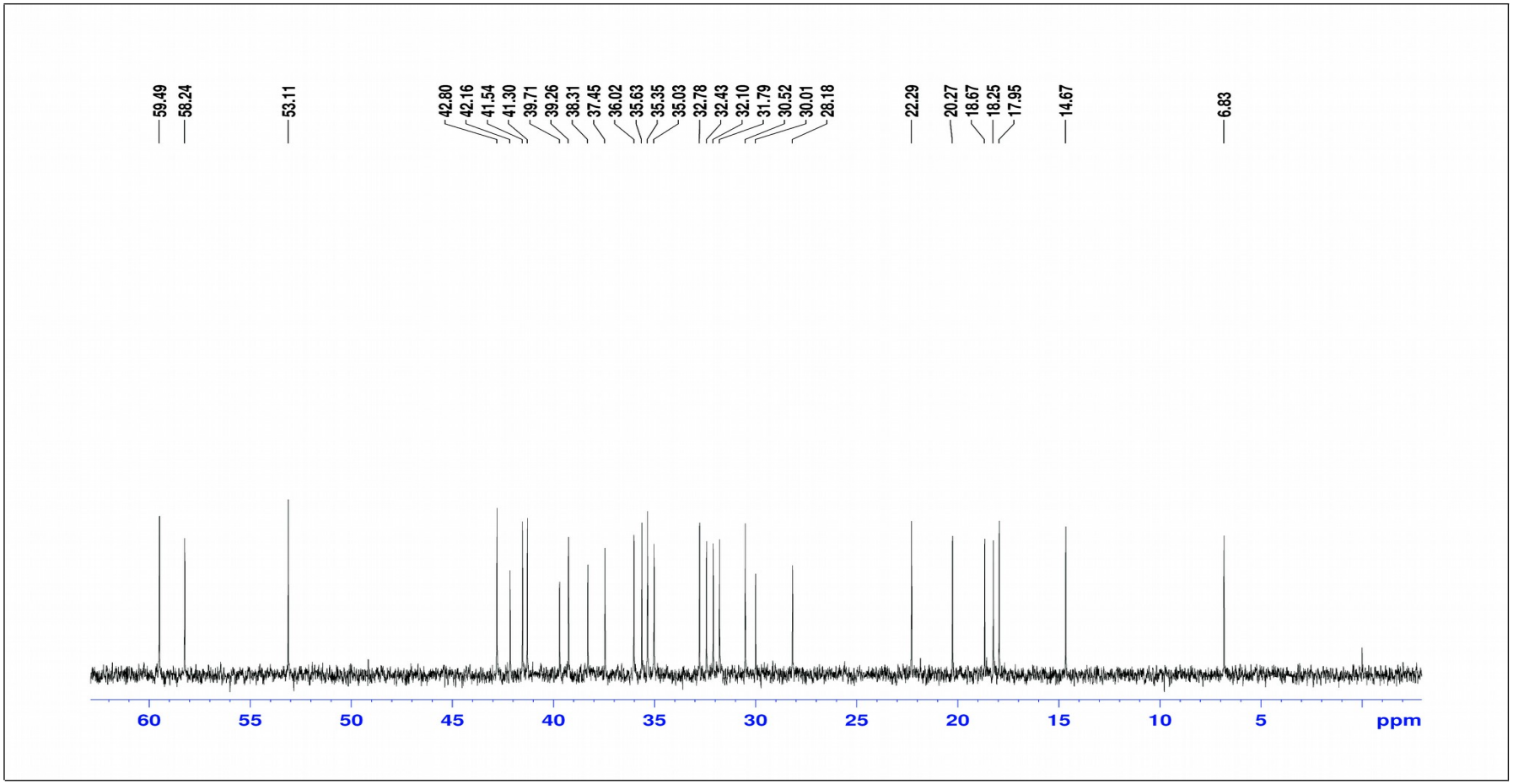
The same compound had been earlier isolated from *C. opobalsamum*, another species belonging to the same genus [54], but for the first time from *C.caudata.*



^{1}H NMR spectrum of friedelin



^{13}C NMR spectrum of friedelin



^{13}C NMR spectrum of friedelin

3.5 References

1. J.H Langenheim, Plant Resins: Chemistry, Evolution, Ecology and Ethnobotany. Timber Press, Portland, Cambridge. 2003.
2. R Pernet, Phytochimie des Burseracees. Lloydia 35, 280– 287, 1972.
3. M Thulin , Nord J Bot 20, 395–411, 2000.
4. Shen et al. Journal of Ethnopharmacology 142 , 319–330, 2012.
5. Hanuš et. al., Biomed. Papers 149, 3–28 ,2000.
6. Jingai and Shangwei , Zhongguo Zhongyao Zazhi 21, 235–237, 1996.
7. Patil et.al., Tetrahedron 28, 2341–2352, 1972.
8. Purushothaman and Chandrasekharan , Indian J Chem, Sect B 14, 802–804, 1976.
9. Satyavati, PhD thesis, Banaras Hindu University (BHU). 1966.
10. Kuppurajan et.al., Journal of the Association of Physicians of India 26, 367–373, 1978.
11. Shen et.al., Helvetica Chimica Acta, 91, 881–887, 2008.
12. Shen et. al. Helvetica Chimica Acta 92, 645–652, 2009.
13. Xu et.al. Planta Medica 77, 2023–2028, 2011.
14. A Bhati, Journal of the Indian Chemical Society 27, 436–440, 1950.
15. Su et. al. Planta Medica 75, 351–355, 2009.
16. Dekebo et.al., Bulletin of the Chemical Society of Ethiopia 16, 81–86, 2002.
17. Manguro et.al., Chemical and Pharmaceutical Bulletin 51, 479–482, 2003.
18. Waterman and Ampofo, Phytochemistry 24. 2925–292, 1985.
19. Manguro et.al., Chemical and Pharmaceutical Bulletin 51, 483–486, 2003.
20. Shen et.al. Phytochemistry 68, 1331–1337, 2007.
21. Raldugin et.al., Khim. Prir. Soedin, 1, 108 -109, 1976.
22. Mesrob et.al., J. Chromatogr. B 720(1-2), 189-196, 1998.
23. AyuHerbal.com. Undated. Herb of the week: Guggul :Available from: Commiphora mukul. www.ayuherbal.com.
24. H.K. Kakrani, Fitoterapia 52: 221-223. 1981.
25. Hough et.al., Journal of the Chemical Society, 796–800, 1952.
26. Singh et.al., Alternative Therapies in Health and Medicine 9, 74–79, 2003.
27. Tipton et.al., Toxicology in Vitro 17, 301–310, 2003.
28. Paraskeva et.al., Journal of Ethnopharmacology 119, 673–679, 2008.
29. Bakari et.al., Research Journal of Biological Sciences, 6, 175–179, 2011.
30. Fraternale et.al.,Fitoterapia 82, 654–661, 2011.
31. Kumari et.al, Natural Product Research 25, 1454–1462, 2011.
32. Wang et.al., Atherosclerosis 172, 239–246, 2004.
33. K.G Ramawat and J. M Merillon, Bioactive Molecules and Medicinal Plants.

pp.101. Springer, Heidelberg, Berlin, 2008.

34. Bellamkondaet.al, Pathophysiology 18, 255–261, 2011.

35. Deepa et al. African Journal of Biotechnology 8 ,8, 1630-1636, 2009.
36. Annu et. al. Indian Journal of Natural Products and Resources1, 44-48, 2010.
37. Kumari et. al. Natural Product Research: Formerly Natural Product Letters, 25,15, 2011.
38. Nanthakumar et.al., Pharmacognosy Research, 1:375-80, 2009.
39. Sivakumar et. al. Asian Journal of Chemistry 21 , 5 , 4130-4132, 2009.
40. Mohan et. al. Pharmacology online, 2, 991-998. 2009.
41. Sivakumar et.al. Int. J. Chem. Sci.: 6,1, 447-450,2008.
42. Kodali and Seru . IJBPR.; 4, 4, 250-255, 2013.
43. Chandrasekar et.al., Journal of Pharmacy Research 2009 Vol. 2 No. 4 pp. 701-703 .
44. J.B Harborne, Phytochemical methods –A Guide to modern techniques of plant analysis, 3rd Edn. pp.145, Chapman and Hall, UK, 1998.
45. M. Arif, PhD Thesis, Univesity of Calicut, 2007.
46. B.L Applegate , Short description of FAME Analysis, University of Alaska, Anchorage , 2007.
47. Müller et.al., Biochim Biophys Acta. 15,145-51. 2005.
48. Mohan et.al., International Journal of Applied Biology and Pharmaceutical Technology ,3, 4, 112, 2012.
49. Kelly GS., Altern Med Rev. 4, 29-36. 1999.
50. Gupta et.al., Phytochemistry, 31, 2558-2560, 1992.
51. M. Ali, Techniques in Terpenoid Identification, pp. 490, Birla Publications, India, 2002.
52. G.T. Abraham, PhD Thesis, University of Calicut, 2008.
53. S.B. Mahato and A.P Kundu, Phytochemistry, 37, 6, 1537, 1994.
54. Abbas et. al.,Natural Product Research, 21:5, 383 – 391, 2007.

CHAPTER 4

ESSENTIAL OILS FROM THE LEAVES AND BERRIES OF *A. MONOPHYLLA* DC. GROWING IN WESTERN GHATS

Rutaceae, commonly known as the citrus family is a family of flowering plants placed in the order *Sapindales* [1]. Species belonging to the family range from herbs to shrubs and small trees. There are approximatively 160 genera and 1600 species in the family [2]. The family is of high economic importance in warm temperate and sub-tropical regions for its numerous edible fruits like the orange, lemon, calamansi, lime, kumquat, mandarin and grapefruit. Non-citrus fruits such as White sapote (*Casimiroa edulis*), Orangeberry (*Glycosmis pentaphylla*), Clymenia (*Clymenia polyandra*) and Limeberry (*Triphasia trifolia*) are also to be specially mentioned. *Ruta, Zanthoxylum* and *Casimiroa* species are having medicinal potencies. Several plants lke *Boronia megastigma* are used by the fragrance industry.

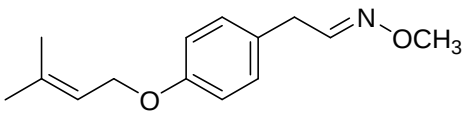
Atalantia is a small genus of 65 species of flowering plants in the family . *A. racemosa, A. ceylanica, A.wightii* and *A. monophylla* are the important members of the genus [3].

4.1 Phytochemical studies so far reported from *Atalantia*

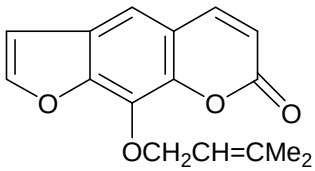
The several closely related species of the genus *Atalantia* are found in India and other south east Asian countries. Morphology of the genus resemble citrus in general charecteristics which are small to medium trees, spinous, bearing white fragrant flowers and small globose fruits similar to diminutive greenish-yellow limes or oranges [18]. In South India, the genus is represented by three species namely *A.monophylla* (Roxb.) DC., *A.racemosa* Wight. and *A. wightii* Tanaka. Both the former two are widely distributed in south India, while *A.wightii* is endemic to Shola forests of Western Ghats

[19]. Atalantia-plants had been subjected to hectic phytochemical investigations from different angles a few of which are reviewed here .

A.ceylanica : The limonoid glucoside isocycloatalantin 17-β-d-glucopyranoside and two aglycones cycloatalantin and dehydrocycloatalantin were isolated from *A.ceylanica* seeds [4]. The glucoside was a 6β-hydroxy-7-keto analogue of cycloepiatalantin, and the aglycones, were the 7-epimer and the 7-ketone, respectively, of the latter. Ceylantin (7,8-dimethoxy-5,6-pyranocoumarin) and xanthotoxin had also been isolated from the heartwood of *A. ceylanica* [5]. Two new oximes, ataloxime A and B, and the known furanocoumarins bergapten, xanthotoxin, heraclenin, oxypeucedanin and imperatorin were isolated from lipophilic seed extract of *A. ceylanica* [6]. The oximes displayed contact toxicity against freshly hatched larvae of the pest insect *Spodoptera littoralis.* The isolation of two bi-acridone alkaloids, atalanine and ataline had also been reported from *A.ceylanica* [7].

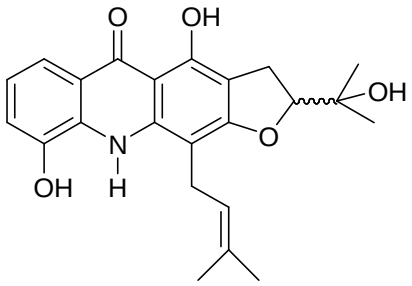


Atalaoxime B



Imperatorin

A.buxifolia : Two new acridone alkaloids, 3-methoxy-1,4,5-trihydroxy-10-methylacridone and 2,3-dimethoxy-1,4,5-trihydroxy-10-methylacridone were isolated from the ethanol extract of the branches of *A.buxifolia* [8]. These compounds exhibited significant antibacterial activity against *Staphylococcus aureus* . Ethanol extract from the aerial parts of *A. buxifolia* had led to the identification of a new acridone alkaloid buxifoliadine, along with known compounds citrusinine – I , *N*-methylatalaphylline , Severinolid and cycloseverinolide [9].



Buxifoliadine

A. racemosa: From the aerial parts of the plant *A. racemosa* two new pyranoflavones, namely atalantoflavone [8,8-dimethyl-5-hydroxy-2-(4′-hydroxyphenyl)-4H,8H-benzo-(1,2-*b*: 3, 4-*b*′) dipyran-4-one] and racemoflavone [8,8-dimethyl-5-hydroxy-2-(4′-hydroxy-3′-methoxyphenyl)-4H,8H-benzo (1,2-*b*: 3,4-*b*′) dipyran-4-one] had been isolated [10]. Seven biogenetically related coumarin derivatives namely, xanthyletin, luvangetin, recemosin, xanthotoxin, umbelliferone, rutarin, rutaretin and a triterpene, friedelin had also been isolated along with this [10].

A.wightii: Two flavones racemoflavone and atalantoflavone and four acridones atalaphylline 3, 5-hydroxynoracronycin , citrusinine-I, and citrusinine-II had been isolated and identified from the leaves of *A.wightii* along with the triterpene epi-friedelinol [11].

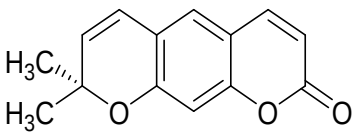
A. retusa: The dichloromethane extract of the air-dried leaves of *A. retusa* (Philippine endemic shrub with high antinociceptive and anti-inflammatory activities) had afforded a new triterpene, retusenol along with friedelin , dischidiol , 5,7-dimethoxy-8-(3-methyl-2-oxybutyl)coumarin , humulene and β -caryophyllene [12] .

A. monophylla : Various parts of *A. monophylla* have been extensively used in folk medicine in different conditions such as the treatment of rheumatoid pain and glandular swelling [20]. The root is believed to be antispasmodic [21] and a decoction of the leaves is often applied for itching and other skin complaints [22]. Besides its medicinal use *A. monophylla*

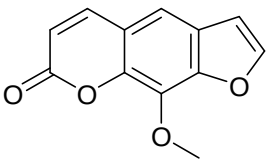
leaves are employed as flavoring agent, berries are used for making pickles, and wood as timber. It is also useful as a rootstock for breeding new cultivars of Citrus Linn. [24].

Hexane, chloroform and ethyl acetate extracts of the plant showed antifeedant, larvicidal and pupicidal activities against *Helicoverpa armigera* [23] while the chloroform extract of leaves had shown antiviral activity [29]. The methanolic extract of the leaves had been found to possess various activities, such as larvicidal, pupicidal, and insect growth regulation properties against the three mosquito species *Culex quinquefasciatus*, *Anopheles stephensi*, and *Aedes aegypti* in the laboratory [26]. Leaf extracts also exhibited ovicidal activity against *Spodoptera litura* [27] Methanolic extract of the bark showed good *in-vitro* antioxidant activity of scavenging DPPH radical, nitric oxide radical and hydrogen peroxide radical [28].

Phytochemical investigation of the roots and stems of *A. monophylla* had led to the isolation and identification of sixteen known compounds including nine acridone alkaloids six coumarins and a limonoid [13]. From the aerial parts of the plant the coumarin, racemosin had been earlier isolated along with known compounds xanthyletin, xanthotoxin and friedelin [25].



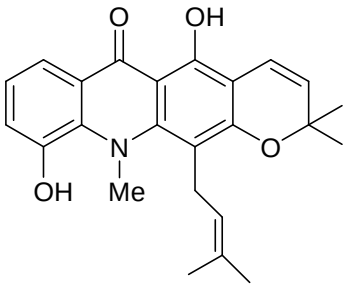
Xanthyletin



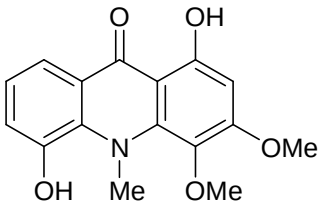
Xanthotoxin

Acridone alkaloids, cycloatalaphylline-A , N-methylcycloatalaphylline-A and N-methylbuxifoliadine-E were isolated from the root of *A. monophylla* along with eight known acridone alkaloids namely buxifoliadine-A , buxifoliadine-E , N-methylatalaphylline , atalaphylline , citrusinine-I ,N-methylataphyllinine , yukocitrine and junosine and two known coumarins: auraptene and 7-O-geranylscopoletin . Their structures were elucidated on the basis of spectroscopic analyses. Some of these acridone alkaloids

exihibited appreciable anti-allergic activity in RBL-2H3 cells model with IC(50) values of 40.1, 6.1 and 18.7 microM, respectively [14].



N-Methylcycloatalaphylline



Citrusinine-I

A new alkaloid N-methylbicycloatalaphylline [15] and a new acridone alkaloid atalaphylline 3,5-dimethyl ether [16] had also been isolated from the same plant. Another new alkaloid 5-hydroxydictamnine, a furoquinoline type, along with β-sitosterol had been isolated from the heartwood of the plant [17].

Novel bioactive compounds 'pyropheophorbide A' and 'pyropheophorbide A methyl ester' were isolated from the leaves of *A.monophyllum*. The former compound was established as the possible antiviral active principle against herpes simplex virus type 2 (HSV-2) [23].

Isolation of compounds like carpachromene and 5-hydroxyarborinine [49], atalaphylline and N- methylatalaphylline from the root bark [50], copane, trans-bergamotene, bisabolene [51] etc also from the root bark [51] and severine palmitate from the fruits [52] are also to be mentioned.

Essential oil studies: Plants of the genus A*talantia* yield volatile oil reminiscent of the pleasant citrus aroma. The essential oil obtained from the branches and leaves of *A. roxburghiana* had been analyzed by GC and GC/MS [30]. The main constituents of the oil were monoterpene hydrocarbons namely γ-terpinene (38.3%), p-cymene (15.7-%), β-pinene (5.2%) and α-pinene (4.7%).

Essential oil from the leaves of *A. racemosa* was dominated by sesquiterpenes where the major components identified were T-cadinol (11.08%), caryophyllene oxide (9.78%), β- caryophyllene(9.20%), spathulenol (7.21%), β-phellandrene (5.67%) and decanal (4.01%) [19].

β-caryophyllene (16.37%), D-limnonene (12.15%), decanal (10.49%), β-myrcene (7.67%), tetradecanal (6.99%), caryophylleneoxide (6.29%) and hexadecylene oxide (5.87%) were the major compounds identified in the essential oil obtained from the leaves of *A. wighlii* [19].

The major components reported in the leaf essential oil of *A. citroides* were Caryophyllene(49.7%) terpenyl acetate (7.7%) and terpineol (16.6%) while that of *A.missionis* were tridecanol(38%) and hexadecnoic acid (16%) [47]

Earlier reports are available on the essential oil obtained from *A.monophylla* also. Nayak and Guha were the first to study the volatile oil (say AMO 1) from the leaves back in 1951. They had obtained sabinene (38%), linalool(14%), linayl acetate (17%) as the major components along with some higher terpene esters of azulene group [31]

In 1988 Prasad and Rajendra obtained essential oil (AMO 2) from the leaves by hydrodistillation which was further analyzed to identify almost the same components with comparable quantification ; sabinene (41.05 %), linalool (17.34%) and linalyl acetate (14.63 %) as the major components. d-Limonene (8.02), citral (1.34), chamazulene (14.42) and guaiol (4.22) were also identified in the oil [32] which exihibited antimicrobial activities against both gram-pos. and gram-neg. organisms and against some pathogenic fungi.

In 1992 Sharma et.al obtained an oil (AMO 3) with a lemon-like odor and a floral note from the leaves. They identified methylisoeugenol (32.38%) and sabinene (28.51%) as the major components [33]. Oil was found possessing fungicidal property.

In 2002 Manimaran et. al identified methyl eugenol (36.46%), elemicin (24.89%), sabinene (24.61%) and ethyl safranate (2.36%) as the principal constituents of the volatile oil (AMO 4) obtained from the fresh leaves [34] of the plant growing in Narrtha hills, Pudukkottai, Tamilnadu.

And rather recently Das and Swamy have reported the identification of α-asarone or cis-isoelemicin (28.82%), sabinene (13.19%), eugenol methyl ether (12.71%), 1,2-Dimethoxy-4-(2-methoxyethenyl)benzene (11.63%) and β-Pinene (5.3%) as the dominating components of the leaf oil (AMO 5) . They had collected the leaves from hills of Madurai district, Tamil Nadu [19].

The variations in the results indicates the possibility of more than one chemotype existing within the species. So we decided to revisit the leaf essential oil of *A.monophylla* by collecting the plant from another region, the Nelliampathy forests in Western Ghats.

Also we are examining the chemical composition of volatile oil from the berries which is for the first time from any plant of the whole genus .

4.2 Present work

*Atalantia monophylla (*L.*)* Corr. Serr. locally known as 'kaattunaranga' (Wild lime) is a shrub or small flowering tree distributed in the Indo-Srilanka-Malaysian region [35]. In India the species is mainly distributed in hill slopes and high altitude semi -ever green forests of Kerala, Tamil Nadu, Karnataka Andhra Pradesh and Maharashtra [36]. In Kerala it is found along the Western Ghats in Idukki, Wayanad and Palakkad districts [35] .

The species is described as small glabrous armed tree- spines to 2 cm long- leaf apices acute-notched-flowers in axillary racemes, fragrant, cream-fruits-yellowish green[35,37]. Flowering and fruiting period of the plant was recorded as December-March [35].

Plant Collection

Fresh leaves and berries of *A. monophylla* were collected from the Nelliampathy forest region of Western ghats. It was identified by Dr. A.K. Pradeep, Department of Botany, University of Calicut and specimen vouchers were deposited in the specially maintained Herbarium of Chemistry department of University of Calicut.

Extraction of Oils

The leaves and berries were cleaned with water and ground into paste. They were separately subjected to steam distillation for 3 hours. The oils were extracted by diethyl ether from the distillates. The ether extract was dried using anhydrous sodium sulphate and the ether evaporated. The pure oils were stored below 4^{o}C until analyzed.

Analysis of the oils :

GC-FID analysis was carried out using a Perkin Elmer Clarus 500 GC equipped with a 30 m × 0.32 mm Elite-5MS capillary column (0.32 μm film thickness). 1 μl of each sample was diluted with 300 μl of Et_2O and injected (0.5 μl) in the "split" mode (1:30) with a column temperature programme of 40°C for 5 min, then increased to 280°C at 4°C/min and finally held at this last temperature for 10 min. Injector and detector were set at 250 and 300°C, respectively, and the carrier gas was He with a head pressure of 12.0 psi.

GC/MS analyses were carried out using a Perkin Elmer Clarus 500 GC equipped with a Clarus 500 mass spectrometer using the same capillary column and chromatographic conditions as for the GC/FID analysis. Mass spectra were acquired over 40-500 amu range at 1 scan/sec with ionizing electron energy 70 eV, ion source 200°C. The transfer line was set at 300°C, while the carrier gas was He at 1.0 ml/min.

Identification and quantification of the oil components :

The identification of essential oil components was performed by means of their retention indices (AI), by a peak matching library search (8) and by comparison with authentic reference compounds as well as with published mass spectra (39, 40). Retention indices (AI) were calculated using a n-alkane series (C_6-C_{35}) under the same GC conditions as for the samples. The relative amount (%) of individual components of the oil is expressed as percent peak area relative to total peak area from the GC/FID analyses of the whole extracts.

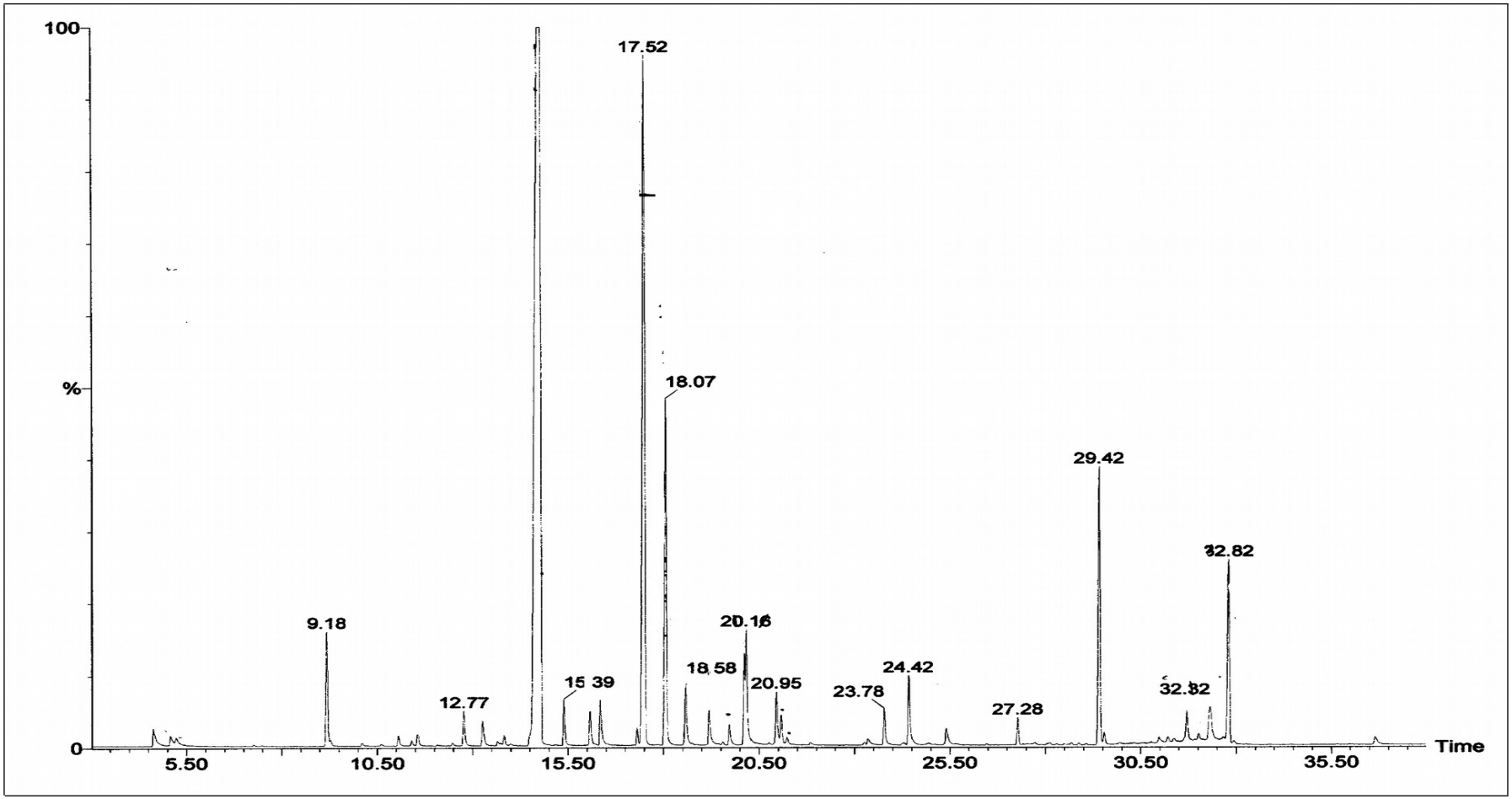
4.3 Results and Discussion

4.3.1 Leaf oil : The steam distillation of the leaves of the plant *A.monophyllum* gave a colourless oil with a yield of about 0.06 % of the fresh weight sample. The oil possessed a fresh pleasant odour with a slight citrus note.

GC/MS analysis lead to the isolation and identification of 33 components consisting of 98.63% of the oil . The oil was over dominated by 20 monoterpenes, especially oxygenated monoterpenes. Contribution of oxygenated monoterpenes was 82.44 % towards the overall composition of the oil while that of non-oxygenated was only 3.29%. Sesquiterpenes (6 Nos) and aliphatic compounds (7 Nos) were also low in number and amount ; 10.12 and 2.78 percentage respectively.

Major components : Monoterpene alcohol linalool (51.65%) was identified as the most abundant component followed by terpinen-4-ol (15.91 %), α-terpineol(6.58%), elemol(4.82%) and α-bisabolol (3.33%). The oil also contained small amounts of sabinene and linalyl acetate (2 and 1.08% respectively) which had been earlier reported as the major components in AMO1 and AMO2 [31,32].

Linalool is responsible for the sweet, pleasant fragrance of the oil which is esteemed by the flavor and fragrance industry [41] . As one of the universal constituent of the night blooming flowers it attracts a number of pollinators and insects including bees to the host plant[42]. The high linalool content in the leaf essential oil indicates a huge number of insect visitors either as pollinators or forager on this species.



GC Trace of essential oil from the leaves of *A.monophyllum*

Table 4.1. GC/ MS Analysis with FID quantification of essential oil from the leaves of *A.monophyllum*

#	Compound	AI[a]	AI[b]	%
Aliphatic compounds				
1	cis-3-Hexanol	850	847	0.45
2	trans-2-Hexanol	854	861	0.09
3	Hexanol	867	865	0.09
4	6-Methyl octanol	-	1144	0.75
5	Nona-2-en-1-ol	-	1170	0.25
6	Decanol	1266	1272	0.89
7	Dodecanal	-	1417	0.26
Monoterpenes				
8	Sabinene	969	966	2.00
9	β-Myrcene	987	987	0.07
10	α-Terpinene	1014	1010	0.18
11	p-Cymene	1020	1019	0.08
12	β-Phellandrene	1025	1023	0.24
13	γ-Terpinene	1054	1053	0.57
14	cis-Sabinene hydrate	1065	1065	0.45
15	Terpinolene	1086	1079	0.15
16	Linalool	1095	1098	51.65
17	trans-Sabinene hydrate	1098	1102	tr
18	cis-p-Menth-2-en-1-ol	1121	1119	0.69
19	trans-p-Menth-2-en-1-ol	1136	1137	0.56
20	Terpinen-4-ol	1174	1175	15.91
21	α-Terpineol	1186	1190	6.58
22	Oxygenated Monoterpene	-	1204	1.01
23	Nerol	1227	1221	0.62
24	Linalyl acetate	1254	1248	1.08
25	Geraniol	1249	1249	2.16
26	Neryl acetate	1359	1356	0.59
27	Geranyl acetate	1379	1376	1.14
Sesquiterpenes				
28	γ-Muurolene	-	1477	0.45
29	Elemol	1548	1548	4.82
30	γ-Eudesmol	1630	1629	0.46
31	epi-α-Cadinol	1638	1640	0.11
32	β-Eudesmol	1649	1651	0.95
33	α-Bisabolol	-	1668	3.33
	total			98.63

[a]Arithmetic indices (AI) on Elite-5MS capillary column; [b]Arithmetic indices (AI) from Adams 2007, Identification of essential oil components by gas chromatography/mass spectrometry, 4th Ed, A[ll]ured Publishing Corporation Carol Stream, IL, USA.

Of the many biological activities of linalool reported the possible synergistic effect in phytocannabinoid-terpenoid interactions is very

significant in the treatments of pain, inflammation, depression, anxiety, addiction etc[43]. Cannabinoids are chemical compounds that activate the receptors on cells to repress neurotransmitter release in the brain. [44]. It was also reported that intra plantar injection of linalool reduced paclitaxel (a chemotherapy agent) induced pain in mice [45]. Inhalation of linalool was found inhibiting stress-induced effects on the profiles of blood cells and gene expression in rats [46]. So the linalool rich essential oil from *A.monophyllum* leaves growing in Nelliampathy forests can be recommended for aromatherapy and combinational herbal therapy to reduce the side effects and psychological problems associated with chemotherapy.

Comparison of compositions : Beside the terpenes and aliphatic compounds there was not any other type of compound including aromatics or phenyl propanoids detected in this oil. This can be taken as a uniqueness of this oil as there were either or both phenyl propanoids or aromatic compounds in the oils previously extracted from this species. Phenyl propanoids namely methylisoeugenol, eugenol and elemicin, isoelemicine and methyl eugenol were the major components in the oils AMO3, AMO4 and AMO5 respectively. Aromatic compounds like chamazulene (azulene derivatives) were an integral part of the oils AMO1 and AMO2. But the presence of linalool (the chief component) and even though in low amounts that of sabinene and linalyl acetate point towards a correspondence with AMO1 and AMO2.

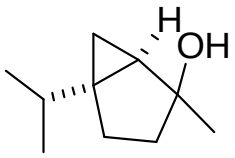
Sabinene was present in almost all the previously studied oils and also in the new sample which is a common phenomena with the plants belonging to various *Rutaceae* genus like *citrus, murraya and zanthoxylum* (53, 54,55).

Table 4.2 Comparison of the compositions of leaf essential oils from *A.monophyllum*

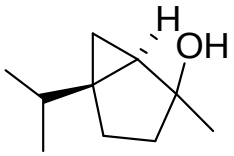
Sample	Major compound categories	Significant compounds	Common characteristic compounds	Region
AMO1	Monoterpenes, Ar.compounds	Sabinene, Linalool, Linalyl Acetate	Azulene derivative	-
AMO2	Monoterpenes, Ar. compounds	Sabinene, linalool Linalyl acetate	Azulene derivative	-
AMO3	Monoterpenes, Phenyl propanoids	Methylisoeugenol Sabinene	Eugenol derivative	-
AMO4	Monoterpenes, Phenyl propanoids	Methyl Eugenol , Elemicin ,sabinene	Eugenol derivative	Narrtha hills, Tamilnadu.
AMO5	Monoterpenes, Phenyl propanoids	Cis-Isoelemicin Sabinene , Methyl Eugenol	Eugenol derivative	Hills of Madurai Tamil Nadu
NEW SAMPLE	Monoterpenes	Linalool, terpinen-4-ol, α-terpineol	Linalool	Nelliampathy , Western ghats

The difference in chemical constitution of essential oils are frequently attributed to the environmental variations and genetic factors, possibility of chemotypes and the nutritional condition of the plants [56]. Here it is clear from the comparison (Table 4.3) that there exists two chemotypes in *A.monophyllum* . One of them gives leaf essential oils containing sabinene, linalool , linayl acetate and azulene derivatives as the major and common components-ie AMO1 and AMO2. It can be taken as an azulene chemotype. The other group proposes phenyl propanoids as the major components and eugenol or its derivatives as the common component- ie. AMO3, AMO4 & AMO5. It can be established as a eugenol chemotype. To ascertain whether the species taken from Nelliampathy forests a third chemotype or a distinct variety of the first category some more investigations are essential.

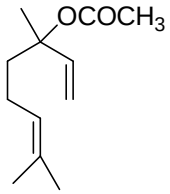
Structure of compounds identified in the leaf essential oil of A. monophylla



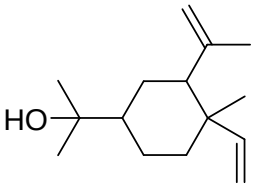
Cis-sabinene hydrate



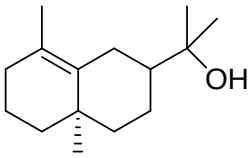
trans-sabinene hydrate



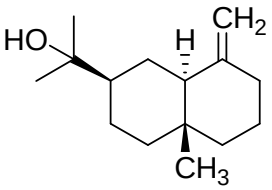
linalyl acetate



Elemol



γ-eudesmol



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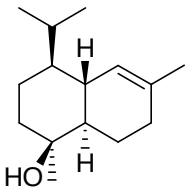


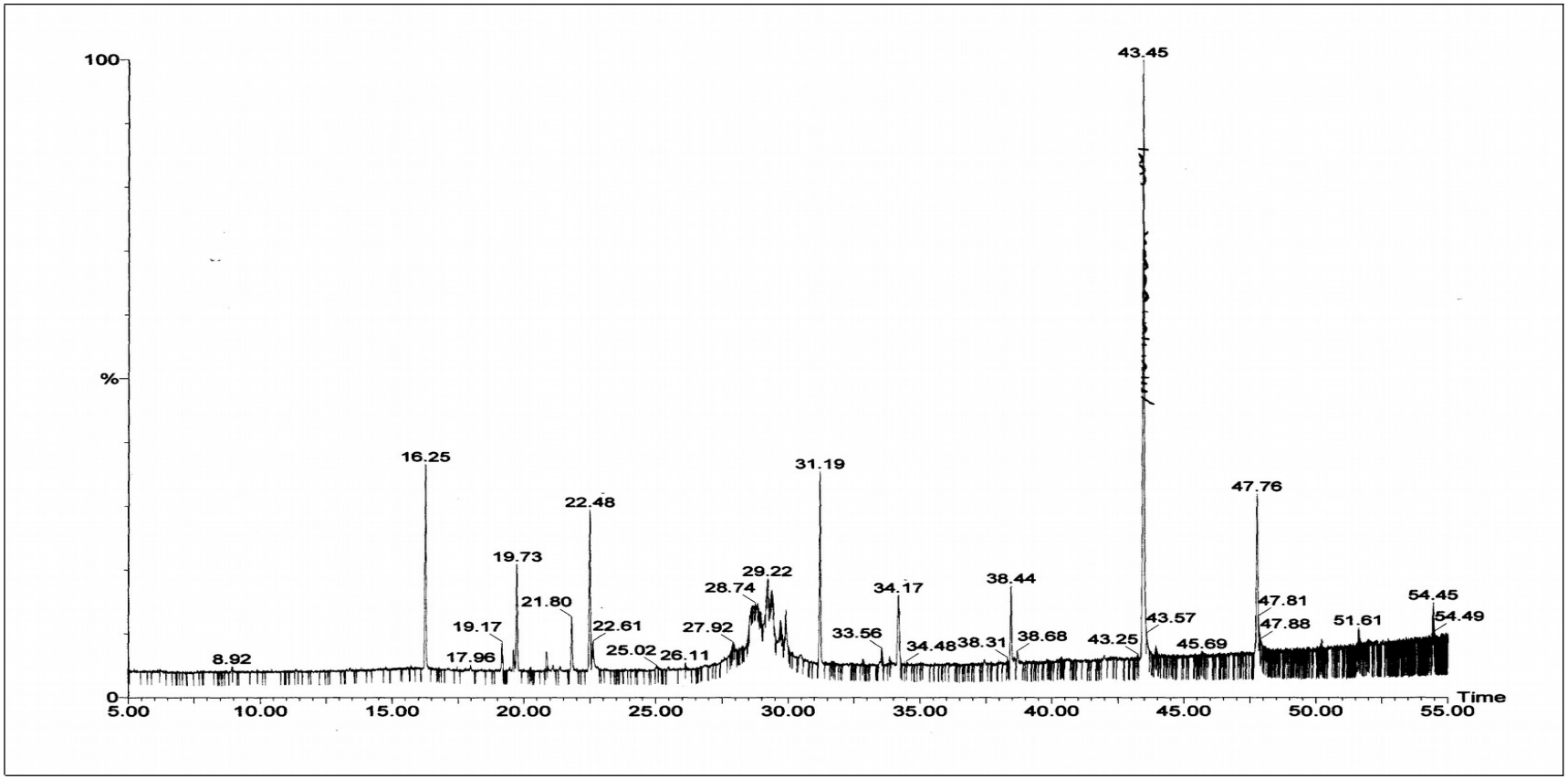
epi-α-cadinol

4.3.2 Berry oil

The steam distillation of the berries gave a pleasant smelling colourless oil with a yield of about 0.1 % of the fresh weight sample. Nine components comprising 98.37 % of the berry oil were isolated by GC/MS analysis out of which all except one were identified .

Table 4.5 GC/MS with FID quantification of essential oil from the berries of *A.monophylla*

	Compound	AI	AI*	%
1	Linaool	1098	1095	10.00
2	α-terpineol	1191	1186	5.24
3	Geraniol	1249	1249	3.50
4	Hydroquinone	1286	-	8.69
5	α-Cadinene	1542	1538	10.34
6	α-Cadinol	1647	1651	5.67
7	Unidentified	1805	-	5.80
8	Methoxsalen	2009	-	36.17
9	Methoxy methoxsalen or Pimpinellin	2201	-	12.96
	Total			98.37



GC Trace of essential oil from the berries of *A.monophylla*

The oil consisted of three monoterpene alcohols linalool, geraniol and α-terpineol, two sesquiterpenes α-cadinene and α-cadinol and hydroquinone. But the major components of the oil were two furanocoumarins namely methoxsalen (36.17 %) and methoxy methoxsalen or pimpinellin(12.96%). Those monoterpenes were present in the leaf oil also; where the amount of linalool was much higher while that of geraniol and terpineol was comparabale. The sesqiterpenes present in the berry oil are also comparable with those in the leaf oil. The results are tabulated in table 4.5.

Furanocoumarins : Furanocoumarins are a typical category of secondary metabolites whose structure consists of a furan ring fused to coumarin [57]. They are classified mainly into : 1) linear furanocoumarins - psolaren, xanthotoxin, bergapten and isopimpinellin and 2) angular furanocoumarins - angelicin, sphondin, and pimpinellin. Plants belonging to *rutaceae* are a major source of linear furanocoumarins or psoralens along with *apiaceae* ,*moraceae* , *compositae*, *solanceae*, and *leguminose* [58]. The angular furanocoumarins are less widely distributed and confined to *apiaceae* and *leguminosae* [59,60].

Linear furanocoumarins can act as photosensitizers towards UV light causing skin problems[78]. But this property is made use in the phototherapy of skin ailments like psoriasis.

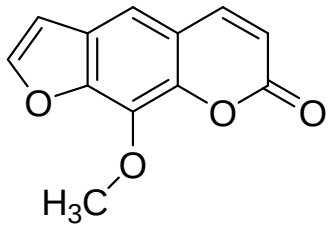
A number of furanocoumarins had been previously isolated from the genus *atalantia* . These are presented with their reported biological properties in table 4.4 .

Table 4.4 Some Important Furanocoumarins isolated from the genus Atalantia

Compound	Species [47]	Biological / Medicinal potency
Angelicin	*A. monophylla*	Antiviral activity [61], Inducing apoptosis [62]
Bergapten (5-methoxypsoralen)	*A. ceylanica*	Anti tumor activity against melanoma cells [63] Effective in PUVA therapy for psoriasis [64]
Heraclenin	*A. ceylanica*	Nematicidal activities [65] Inducing apoptosis in leukemia cells [66]
Imperatorin	*A. ceylanica* *A.missionis*	Inducing apoptosis in leukemia cells [66] Inhibition of nicotine [67]
Isopimpinellin	*A.missionis*	Insecticidal [68], Antimicrobial [69]
Marmesin	*A. monophylla*	Hepatoprotective potential [70]
Oxypeucedanin	*A. ceylanica*	Antiproliferative with UV on melanoma Cells [71]
Psoralen	*A. monophylla*	Historical photoactive agent in UV based therapy of psoriasis [72]
Xanthyletin	*A. monophylla* *A.missionis* *A. racemosa*	Antiinflammatory [73], Anticancer activity [74]
Xanthotoxin (8-methoxypsoralen)	*A. ceylanica* *A.missionis* *A. racemosa*	Photoactive agent in UV based therapy of psoriasis [75] and vitiligo [76] Photochemotherapy [77]

Methoxsalen: It is for the first time methoxsalen (xanthotoxin, 8-methoxypsoralen) was identified from *A.monophylla*. It had been earlier isolated from *A. ceylanica , A.missionis* and *A. racemosa* [47]. The parent

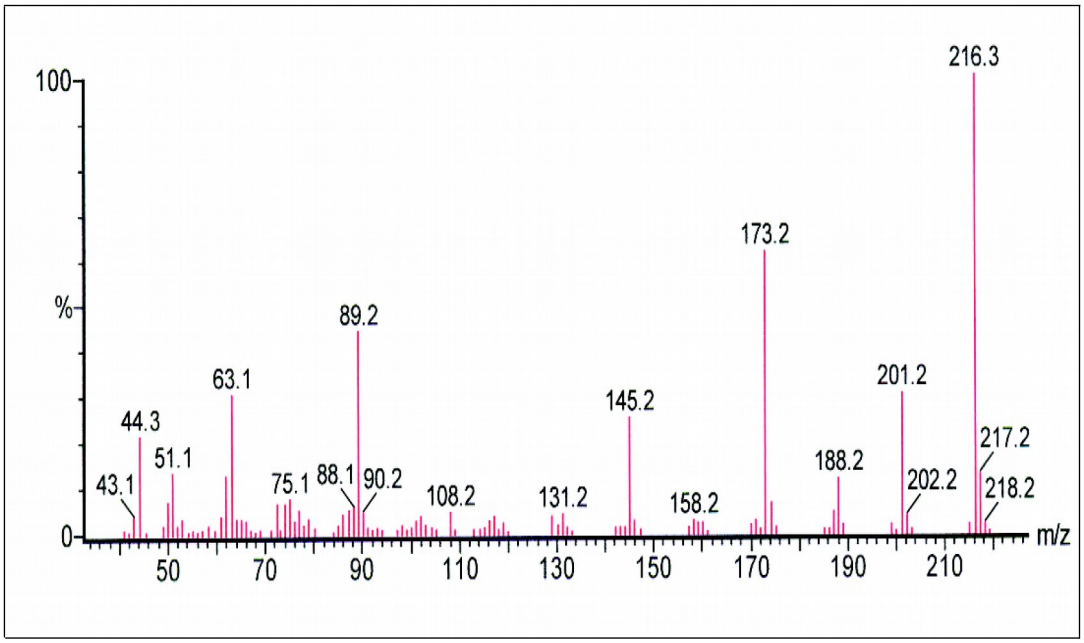
compounds psoralen and angelicin (isopsoralen) had been previously isolated from *A.monophylla* itself [47]. The compound was totally absent in the leaf oil.



Methoxsalen / xanthotoxin / 8-methoxypsoralen

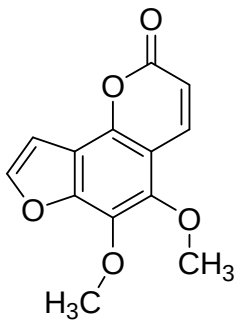
As a linear furanocoumarin methoxsalen sensitizes long-wave UV which may lead sunburn or serious blistering[78] . This property of methoxsalen is channelized in UV assisted therapy for psoriasis. It is used as a successful photoactive agent in UV based therapy of moderate to severe forms of psoriasis [74] . It is being used for skin repigmentation in patients with vitiligo [75] and in the photochemotherapy of the malignancy mycosis fungoides[76]. In combination with ultraviolet light the compound is also employed for its antineoplastic effects and for treating certain skin disorders, including alopecia, cutaneos T-cell lymphoma, excema and lichen planus [78].

Methoxsalen had also been identified as an effective and selective inhibitor of human hepatic cytochrome P-450 (CYP)2A6 [79] which is involved in nicotine metabolism and corresponding mutagenic activation of pro-mutagens and pro-carcinogens[80]. Further studies had revealed that the compound completely inhibited nicotine metabolism in mice[81] and lead to a reduced smoking tendency by its oral administration in human patients [82]. The compound was also recognized as a strong chemopreventive against lung tumorigenesis [80], lung adenoma and adenocarcinoma development [83].

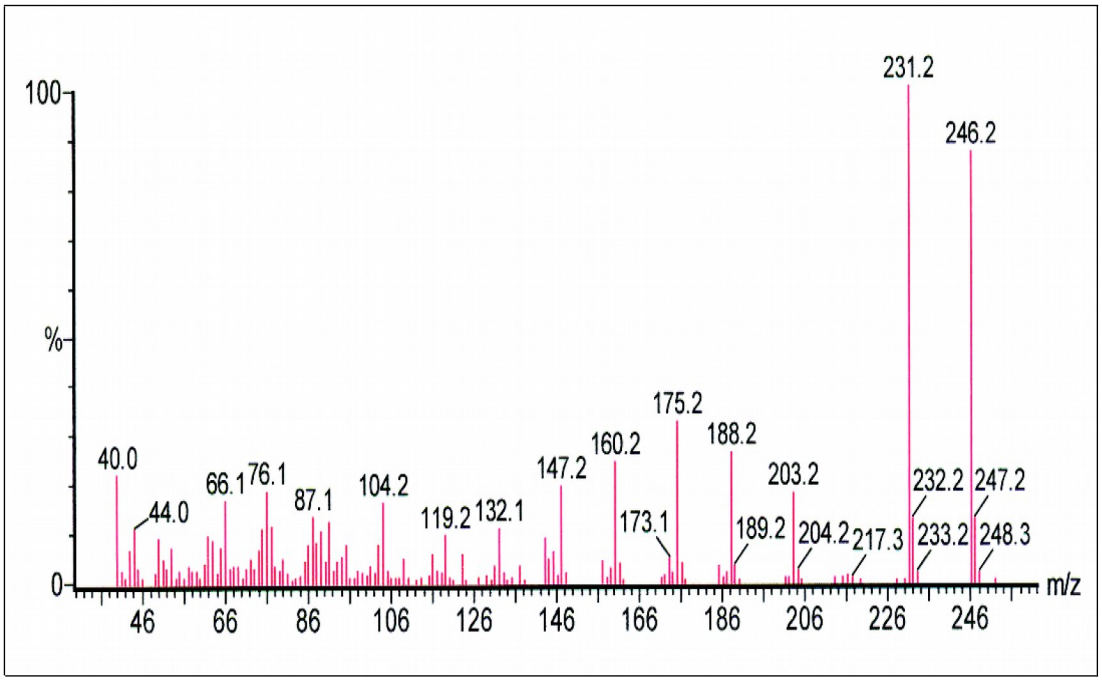


Mass spectrum of Methoxsalen

Methoxy methoxsalen or Pimpinellin : Pimpinellin belongs to the group of angular furanocoumarins which are not so widely distributed [78]. The compound was found to be effective against the fourth instar larvae of *Aedes aegypti*. L.[84] but only moderately phototoxic [85]. It was for the first time this compound being identified from any plant of the whole genus *Atalantia*.



Pimpinellin



Mass spectrum of Pimpinellin

Hydroquinone : The berry oil also contained considerable amount (8.69%) amount of hydroquinone. Of the many of its potencies the inhibition of melamine synthesis [86] is significant in this context. It is a common ingredient in the skin lightners [87] which had been proved to be effective in the treatment for post-inflammatory hyperpigmentation [88] and for hypermelanosis [89].

Hyperpigmntation had been identified as one of the severe side effects of prolonged Psoralen Photochemotherapy or PUVA [90]. At the same time UV induced pigmentations in guinea-pig skin were found to be interrupted by the treatment with hydroquinone [91].

An appropriate blend of melanin stimulators like methoxsalen and melanine inhibitors like hydroquinone will be an improved therapeutic agent with balanced side effects .

Volatile oil from the berries of *A.monophylla* containing the above mentioned compounds can be recommended as an excellent mediator for a more harmonious photo-chemotherapy. It can also be taken as a new natural source for the novel compounds methoxsalen and pimpenellin.

4.4 References

1. http:/ / www. plantsystematics. org/ taxpage/ 0/ family/ Rutaceae. html
2. RUTACEAE (http:/ / www. botanical-dermatology-database. info/ BotDermFolder/ RUTA. html) in BoDD – Botanical Dermatology Database
3. http://www.theplantlist.org/browse/A/Rutaceae/Atalantia/
4. Bennett et. al., Phytochemistry , 36, 1, 1994.
5. Ahmad et.al. Phytochemistry, 23, 9, 21 1984.
6. Bacher et.al. Phytochemistry 50,6, 1999.
7. Fraser and Lewis J. Chem. Soc., Chem. Commun., 615-616, 1973.
8. Yang et.al. Journal of Asian Natural Products Research , 15,8, 2013.
9. Shan et.al. Natural Product Research: Formerly Natural Product Letters , Published online: 31 May 2013.
10. Banerji et.al. , Phytochemistry, 27, 11, 1988.
11. S Kumar, K. Raj and P.Khare, Indian Journal of Chemistry B, 48, 2009.
12. Ragasa et.al., Z. Naturforsch. 67b, 426 – 432, 2012.
13. Sripisut et.al., Planta Med 79 - PN11, 2013.
14. Chukaew et.al., Phytochemistry. 69(14):2616-20, 2008.
15. D. Basu and S. C. Basa , J. Org. Chem.,37 ,19, 1972.
16. G. H. Kulkarni and B.K. Sabata, Phytochemistry,20,4, 1981.
17. Kumar et.al., Natural Product Research, 24,16,2010.
18. Ranade et.al., Physiol.Mol. Biol. Plants, 15, 211-223 ,2009.
19. A. K. Das and P.S. Swamy, Journal of Environmental Biology, 34, 569-571, 2013.
20. Sankaranarayanan et.al., J. Med. Plants Res., 4, 1089-1101, 2010.
21. K.R Kirtikar and B.D. Basu, Indian Medicinal Plants. Bishen Singh Mahendra Pal Singh Publication, Dehradun, pp. 1655-1656, 1999.
22. H Panda, Handbook on Medicinal Herbs with Uses. Asia Pacific Business Press, Inc., Delhi. pp. 166-167 , 2004.
23. Baskar et.al.,Chemosphere, 75, 355-359 ,2009.
24. D.N Guha Bakshi, P. Sensarma and D.C. Pal: A lexicon of medicinal plants in India. Vol. 1, Naya Prokosh Publications, Culcutta, pp.212 ,1999.
25. Joshi et.al., Proc.Indian Acad.Sci., 87A(Chemical sciences-3) No 6, 173-179, 2000.
26. N Sivagnaname and M Kalyanasundaram, Mem Inst Oswaldo Cruz, Rio de Janeiro, 99, 1, 115-118, 2004.
27. Baskar et.al., Asian Pac J Trop Biomed. 2,12, 987-91. 2012.
28. Kumar. et al.,International Journal of Biological & Pharmaceutical Research. 3, 1,122-125. 2012.
29. Chansakaow et.al., Chem Pharm Bull (Tokyo). 44, 7, 1415-7, 1996.
30. Minh et.al., Journal of Essential Oil Research ,22, 1, 2010.
31. Nayak and Guha, J.Indian Chem Soc, 23, 713-16, 1951.
32. Prasad and Rajendra, Parfuemerie und Kosmetik, 69, 7, 418-19, 1988.
33. Sharma et.al., Parfuemerie und Kosmetik, 73, 5, 336-8, 340, 1992.

34. Manimaran et.al., Indian Perfumer, 46, 4, 341-342, 2002.
35. N Sasidharan, http://indiabiodiversity.org/species/show/31059
36. G.Renu, S.J Thilakar, and D. Narasimhan, http://indiabiodiversity.org/species/
37. T. Pullaiah, Biodiversity in India , Vol 4, pp-144, , Daya Books, 2006.
38. NIST/EPA/NIH Mass Spectral Database, Version 2.1 Perkin-Elmer Instrument LLC, Copyright © 2000.
39. D. Joulain and W.A. Konig, The atlas of spectral data of sesquiterpene hydrocarbons. E.B. Verlag, Hamburg , 1998.
40. R.P. Adams, Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry, 4th ed. Allured Publ. Corp., Carol Stream, IL , 2007.
41. G Ohloff Scent and fragrances. The fascination of odors andtheir chemical perspectives. (Translation by W. Pickenhagen & B. M. Lawrence) Springer-Verlag, Berlin, 1994.
42. R. A. Raguso and E. Pichersky, Plant Species Biology , 14, 95-120, 1999.
43. E. B Russo, British Journal of Pharmacology ,163, 1344–1364, 2011.
44. P Pacher, S Batkai, and G Kunos Pharmacol Rev.58 , 3, 389–462. 2006.
45. S Katsuyama , Biomed Res. 33, 3, 175-81. 2012
46. J. A Nakamura . Agric. Food Chem., 57 (12), pp 5480–5485, 2009.
47. Pavithra, et.al., Journal of Medicinal and Aromatic Plant Sciences, 31, 63-72, 2009
48. Badam et.al., Indian J.Med.Res. 87, 379-83, 1988.
49. Saraswathy et.al., Fitoterapia 69, 463-464, 1998
50. Govindachari et.al, Tetrahedron, 26,2905-2010, 1970.
51. Shringarpure and sabata, Indian J Chem 13, 24-28, 1975.
52. Dreyer et.al, Tetrahedron, 36, 827-829, 1980.
53. Ruberto, et.al. J. Agric. Food Chem., 45, 3206-3210 ,1997.
54. P.M. Shafi., A.saidutty and R.A. Clery, J.Essent Oil Res.12 , 179, 2000.
55. Mallavarapu, et.al., Journal of Essential Oil Research , 12, 6, 766-768, 2000.
56. M Ozcan,. and J.C. Chalchat. Czech J.Food Sci., 20, 223-228 ,2002.
57. IUPAC, Compendium of Chemical Terminology, 2nd ed. (the "Gold Book") (1997). Online corrected version: 2006.
58. Murray et. al, The Natural Coumarins, Wiley & Sons, UK, 1982
59. Berenbaum et.al., J Chem Ecol. 17, 207-215. 1991.
60. Bourgaud et.al., Psoralea species: in vitro culture and production of furanocoumarins and other secondary metabolites. Y.P.S. Bajaj (Ed.), Medicinal and aromatic plants, vol. VIII, Springer Verlag, Berlin, 388-411. 1995.
61. Cho et. al., Antiviral Res. 25;100(1):75-83. 2013.
62. Rahman et.al. Mol Cell Biochem. 369(1-2):95-104. 2012 .
63. Sumiyoshi et.al., J .Nat Med. 7. [Epub ahead of print] 2013.

64. Calzavara-Pinton et.al., .J Am Acad Dermatol. ,36 (6 Pt 1):945-9. 1997.
65. Wang et.al., Nat Prod Res. 20;22(8):666-71. 2008.
66. Appendino et.al., J Nat Prod. 67(4):532-6. 2004
67. Budzynska et.al. Physiol Behav. 30;122C:46-55. [Epub ahead of print] 2013.
68. Miyazawa et. al. J Agric Food Chem. 14;52(14):4401-5. 2004
69. Ngwendson et.al., Pharmazie. 58(8):587-9. 2003.
70. Jain et. al, J Pharm Pharmacol. 64(6):888-96. 2012.
71. Kimura et al., Photochem Photobiol. 89(5):1216-25. 2013 .
72. Almutawa et.al., Am J Clin Dermatol. 14(2):87-109. 2013.
73. Chan et.al., Chem Pharm Bull (Tokyo). 58(1):61-5. 2010.
74. Gunatilaka et.al., J Nat Prod. 57(4):518-20. 1994.
75. Bond et.al., Am J Hosp Pharm. 38(7):990-5. 1981.
76. J. A. Kenney Jr., Arch Dermatol. 103(5):475-480, 1971.
77. Barbara and Gilchrest, Et.al., Cancer, 38, 2, 683–689, 1976.
78. K Szewczyk and A Bogucka - Kocka In Phytochemicals - A Global Perspective of Their Role in Nutrition and Health, Dr Venketeshwer Rao (Ed.), InTech, 2012. Available from: http://www. intechopen.com/ books/
79. Kharasch et.al., Drug Metab Dispos. 28(1):28-33. 2000.
80. Takeuchi et.al., Cancer Res. 15;63(22):7581-3. 2003.
81. Damaj et.al., J Pharmacol Exp Ther. 320(1):250-7, 2007.
82. Sellers et.al., Nicotine Tob Res. 5(6):891-9. 2003.
83. Takeuchi et.al., Mol Med Rep. 2(4):585-8. 2009.
84. de Oliveira et. al., Pharmaceutical Biology, 43, 1 , 53-57, 2005.
85. Kavli et.al., Contact Dermatitis. 9(4):257-62. 1983.
86. Siddique et.al., Journal of Cosmetics, Dermatological Sciences and Applications, 2, 224-228 , 2012.
87. AP DeCaprio . Crit Rev Toxicol. 29(3):283-330. 1999
88. Chandra et.al., Acta Derm Venereol. 92(3):232-5. 2012
89. Fitzpatrick et.al., Arch Dermatol., 93(5):589-600. 1966.
90. K Wolff Journal of Dermatology, 122: 117–125, 1990.
91. Imokawa et.al., Arch Dermatol Res. 278(5):352-62. 1986.

CHAPTER 5

VOLATILE METABOLITES OF *COSMOSTIGMA RACEMOSUM (*ROXB.*)* WIGHT (GREEN MILKWEED CREEPER)

5.1 Introduction

The milkweeds or *Asclepias* L. is a genus of herbaceous perennial, dicotyledonous plants that contains about 150 known species. Once belonged to the family *Asclepiadaceae*, is now classified as the subfamily *Asclepiadoideae* of the huge family *Apocynaceae*[1] . The genus name, was taken in honor of the Greek God Asclepius (God of medicine) because of the many folk-medicinal uses for the milkweed plants.

Milkweed is named for its milky juice comprised of a latex containing alkaloids and several other complex compounds including cardenolides. Milkweeds are ecologically significant being an important nectar source for bees and other insects, and a larval food source for monarch butterflies and a variety of other herbivorous insects including beetles, moths, and true bugs. At the same time milkweeds use three primary defenses to limit damage caused by herbivore: hairs on the leaves, latex fluids and cardenolide toxins [2]. Also milkweeds are found beneficial to nearby plants by repelling some pests like wireworms and by using chemical defense against many predators [3]. Many milkweeds also contain cardiac glycoside poisons to inhibit animal cells from maintaining a proper K^+, Ca^+ concentration gradient. Many tribals of South America and Africa use arrows poisoned with these glycosides in hunting. Some of the milkweeds may cause death when animals consume the plant more than 10% of their body weight [3].

5.2 Phytochemistry of Milkweeds- A Review

More than 800 plant names were recorded under the genus *Asclepias* [4] from which a few important species are reviewed below for their phyto-chemical characteristics.

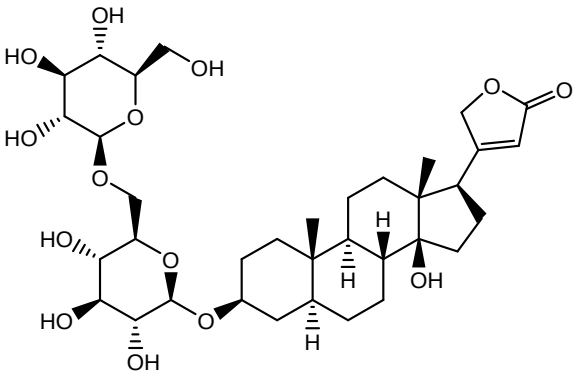
Asclepias plants and their latex have been extensively used as folk medicines especially by the American Indian tribes [5]. *Asclepias* latex, the protoplasmic content derived from the laticiferous cell which characterizes this genus, was proved to be containing novel substances including cardiac glycosides, proteolytic enzymes , the toxic compounds like asclepione and other substances poisonous to livestock [6]. Different plants of the genus were reported to be relevant even in the treatment of various forms of cancer [7].

'Somalatha' (*Starcostemma acidum*) the legendary plant belonging to this family whose essence was believed to be the most favourite herb of the gods in the vedic period was considered as a wonder herb alleviating all the three doshas according to Indian medicine [8].

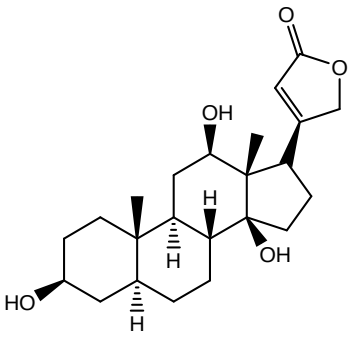
One of the important species *A. procera* known as 'giant milkweed' had been extensively used to cure several ailments like leprosy, ulcers, bronchial asthma, skin infections, piles and disorders of the spleen, liver and abdomen in the native systems of India, Sudan, Unan, and Arab [9]. A multitude of biological potencies like spasmogenic, antidysentric, antisyphilitic, antirheumatic,antifungal, mullusccide, diaphoretic had been ascribed to different parts of this useful plant [10]. Another species the heart-leaf milkweed (*A. cordifolia*) had been used as a contraceptive and snakebite remedy [11]. Different tribes of north America have been using *A. cryptoceras* as a herbal remedy for headache , sores and for ringworm while *A.verticillata* for treating snakebite and throat problems and to increase breast milk in nursing mothers [12].

Extracts from different parts of common milkweed have been used in folk medicine as emetics, anti-asthmatics expectorants and antimicrobial agent [13]. Tinctures of the plant are used in homeopathy [14]. Every part of the plant was revealed to be containing different cardinolides like syrioside,

syriobiocide, uzarin, desglucouzarin, uzarigenin, xysmalogenin and syriogenin [15]. A number of phenolic acids like gallic, vanilic, p-coumaric and p-hydroxybenzoic acid were identified in the leaves and flowers [13]. Resins, waxes, terpenes, hydrocarbons and different enzymes were identified as the main components of the latex [16].



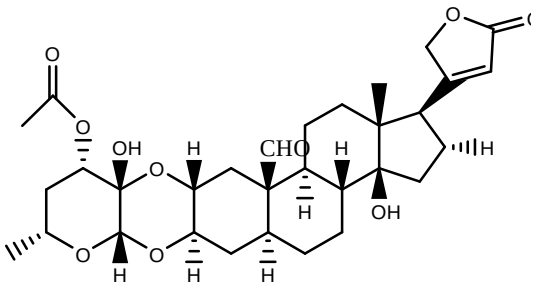
Uzarin



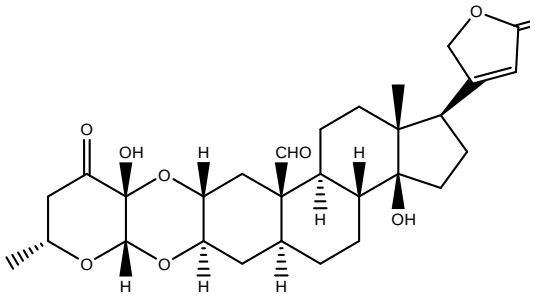
Syriogenin

Structure of two typical cardinolides isolated from milkweeds

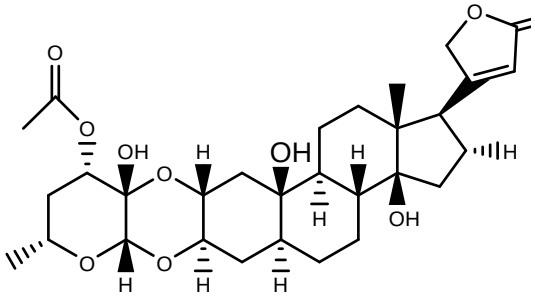
A large number of glycosides with cardenolide , pregnane, oxypregnane, acylated, lignan moieties had been isolated from different plants of the genus like *A. curassavica* [17,18, 19, 24] , *A. speciosa* [20], *A. incarnata* L.[21,22], *A. tuberose* [23], *A. eriocarpa* and *A. Labriformis* [25], *A. asperula* [26], *A. amplexicaulis* [27] and of *A. glaucescens* [28]. Cytotoxic activities of these compounds had also been well established.



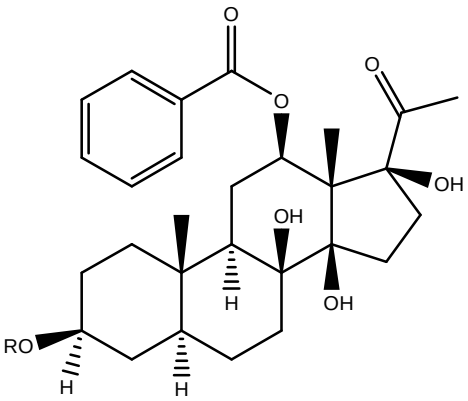
Asclepin



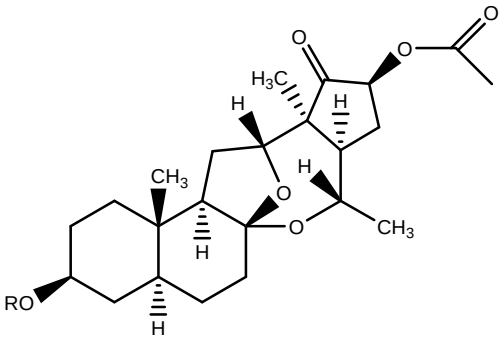
Uscharidin



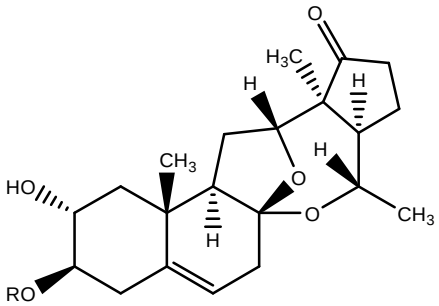
19-nor-10b - hydroxyasclepin.



Curassavioside A
(aglycon)



Tuberoside A3
(aglycon)



Tuberoside B8
(aglycon)

Structure of some glycosides isolated from milkweeds

The species under investigation *Asclepias racemosum* or *Cosmostigma racemosum* is also an important herb used in Indian traditional medicine. The leaves of the plants are used to cure ulcerous sores. Root bark is given internally as an efficient cholagogue in dyspepsia due to torpidity of the liver [29]. A preliminary investigation of the roots had revealed the presence of some crystalline fatty acid, a glucosidal acid resin, a sugar similar to dextrin and compound suspected to be an alkaloid [29]. But no such investigation had ever reported on the aerial parts.

The chemical investigation of the secondary metabolites of leaves and flowers of *Cosmostigma racemosum* , the green milkweed creeper is significant in these perspectives.

5.3 Present work

Cosmostigma racemosum belonging to an unpopular sub-genus of *Asclepiadoideae* is a climbing herb endemic to Western ghats [30]. It is said to be distributed in the south Indian states of Kerala, Karnataka and Tamilnadu and also in southern Maharashtra. In Malayalam it is called ‘Vattuvalli’.

The species is described as : climbing herb, stem glabrous, Leaves 6-10 x 3-5 cm, Racemos to 2.5 cm long. Flowers 8 mm across, many together, slender, pubescent; corolla greenish-yellow with brown dots, outer corona lobes orbicular, emarginated, membranous; inner corona similar to outer [31]. The general habitat of the plant was recognized as moist deciduous forests, scrub jungles and sacred groves [31] . The flowering and fruiting season is April to June [31]. The leaves and flowers generate a gentle aroma which can be compared with that of curry leaves.

Six species had so far been approved under the genus *cosmostigma* namely *C. acuminatum* Wight , *C. cordatum* (Poir.) M.R.Almeida, *C. hainanense* Tsiang , *C. philippinense* Schltr. , *C. racemosum* Wight and *C. racemosum* var. *glandulosum* Costantin [4]. But not much phytochemical work has so far been reported on any of these specie to the best of our knowl-

edge. Here we present the chemical examination of secondary metabolites from the leaves and flowers of *C. racemosum for the first time* by GC and GC/MS analysis. Biological properties and ecological implications of major and important compounds are also discussed along with this.

Plant Collection : The fresh plant material was collected from Calicut University campus, Kerala, India. It was identified by Dr. A.K. Pradeep, Department of Botany, University of Calicut and a specimen voucher is deposited in the specially maintained Herbarium of Chemistry department of University of Calicut.

Extraction of Volatile oil:

The leaves and flowers of the plant were separated and washed with clean water. Each of them (250 g) were ground into a paste and subjected to steam distillation for 3 hours. The volatile oils were extracted by diethyl ether from the distillates. The ether extract was dried using anhydrous sodium sulphate and the ether evaporated. The pure volatile oils were stored below 4^{o}C until analyzed.

Analysis of the Volatile oil:

GC-MS analysis of the samples were carried out on a S*himadzu* GC-17A with QP 5050 and the data system Compaq-proLinea (softwatre), a hewlett-Packard GC-HP 5890 with HP-5970 MSD ans PC-Pentium (Bohm Co; Chemstation –Software) and a FinniganMAT GCQ with data system Gateway-2000-PS75 (Siemens Co. , GCQ software).An apolar 30 m OV-5 type column (0.32 i.d and 0.25 µm film thickness) with helium as carrier gas were used. Injector temperature : 250^{0}C ; interface heating : 300^{0}C ; ion source heating : 200^{o}C, EI –mode; scan range : 1-450 amu. For compound identification Wiley, NBS and NIST library spectra (online) were used, as well as reference MS data[32-34].

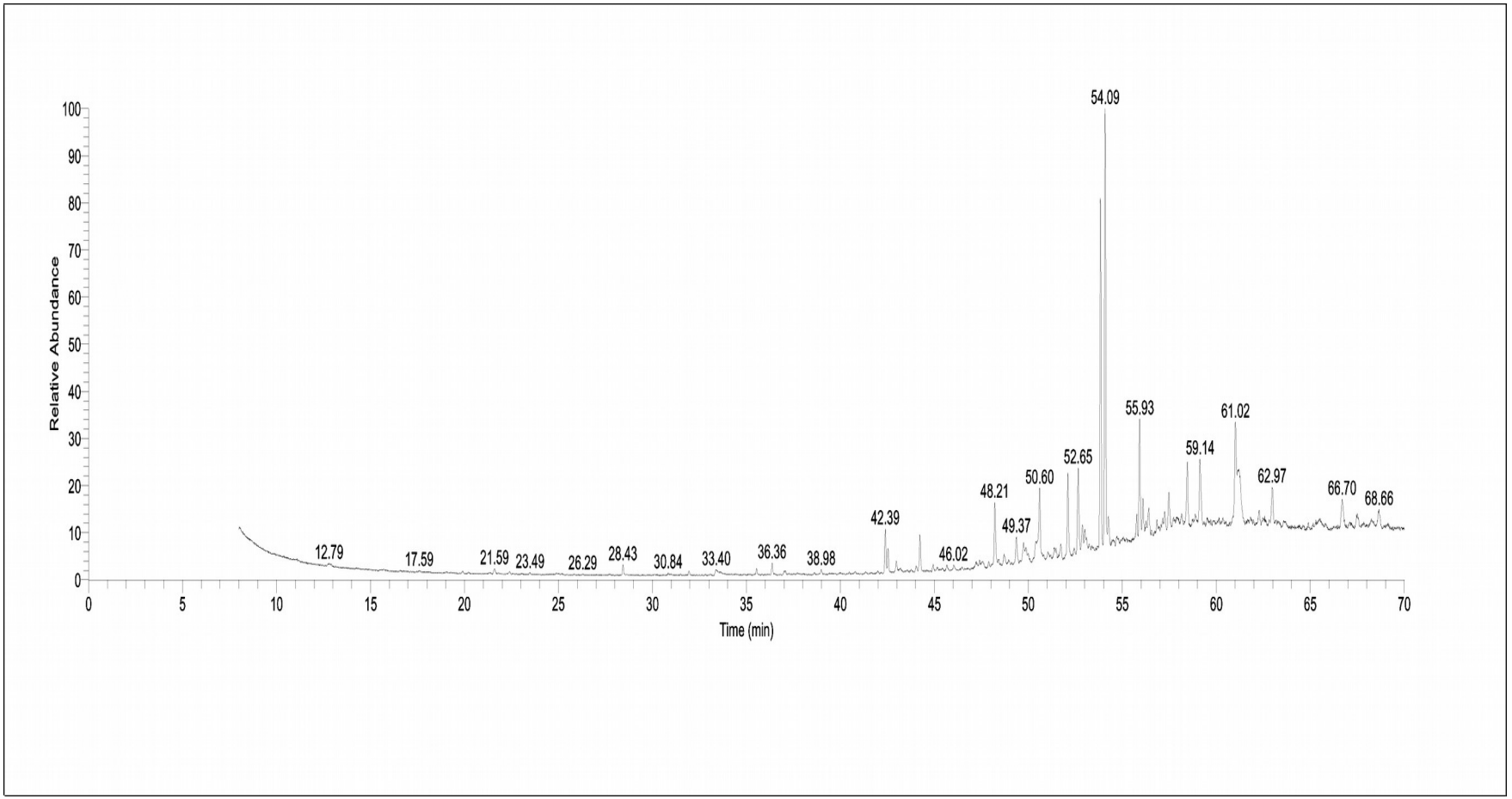
GC-FID analysis was carried out using a Shimadzu GC-14A with FID

and the integrator CRA-Chromatopac and a Varian GC-3700 (F'ID) and the integrator C-R1B-Chromatopac. The same column used for GC-FID. Carrier gas: hydrogen; Injector temperature : 250^{o}C and detector temperature 320^{o}C ; temperature program : 40^{0}C/5min to 280^{o}C/ 5 min with a heating rate of 6^{o}C/ min. Quantifications were made by relative % peak-area calculations.

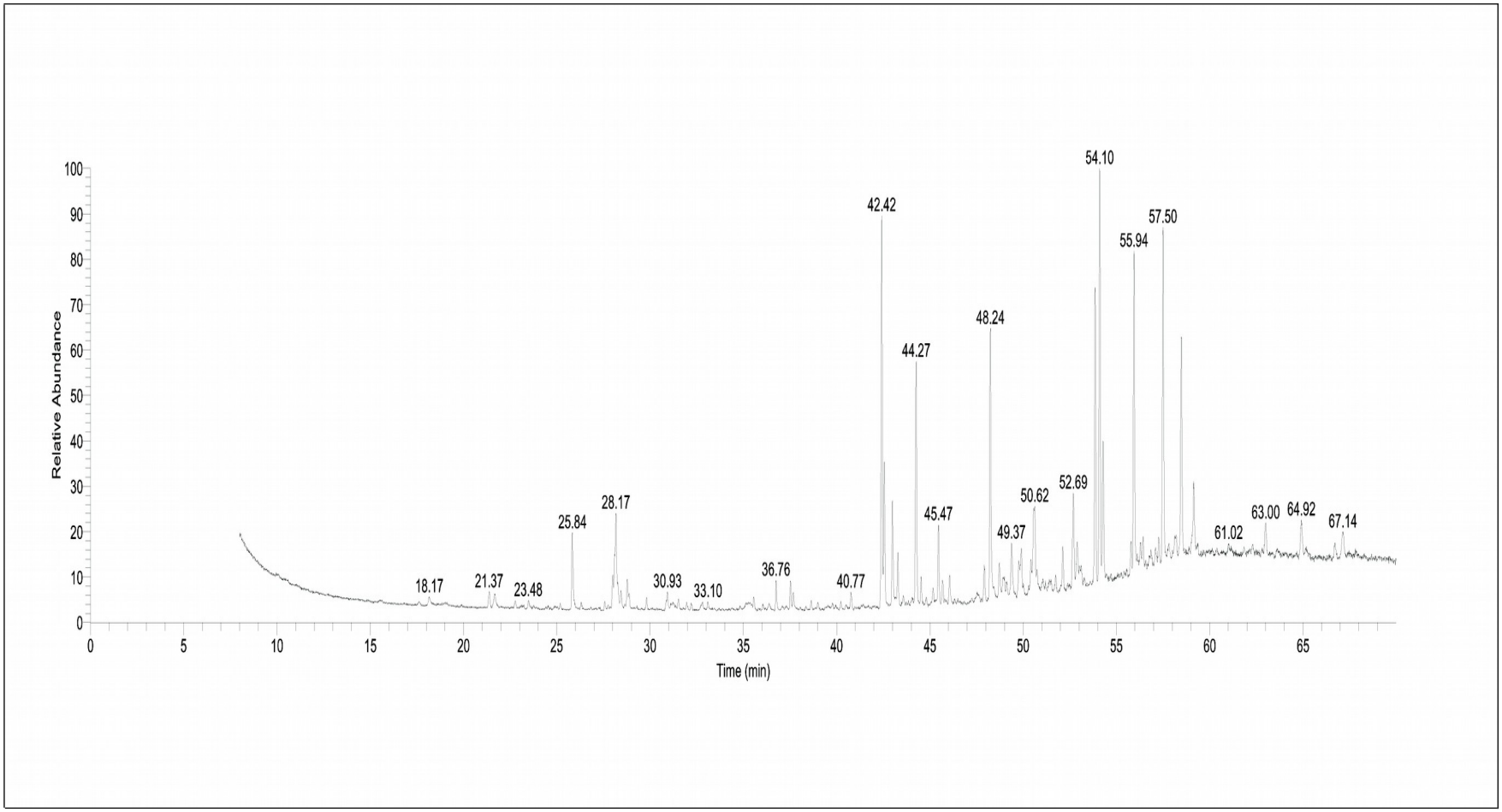
5.4 Results and Discussion

Steam distillation of the leaves and flowers afforded colourless oils, each with a yield of about 0.001% of the fresh weight sample. The GC and GCMS analysis of the two volatile oils revealed the identity of 48 compounds in total. Thirty four compounds comprising 80.72 % were identified in the leaf oil while 35 compounds comprising 74.76 % were identified in the flower oil. Out of these 21 compounds were present as common constituents. Both the oils were dominated by sesquiterpenes of various categories. Results are tabulated in Table 5.1.

Isomer of davana ether (18.27 %) was identified as the most abundant component in the leaf oil followed by bicyclohumulenone (14.86%), crispatanolide (4.94%) maaliol (7.98%), eudesm-11-en-4-α-ol (3.86%), methyl palmitate (3.79%), isolongifolol (3.69 %) and caryophylla-3(15),7(14)-dien-6-ol (3.00 %) ; all being sesquiterpenoids. Longifolol (2.85 %) and the diterpene, torulosol (2.66 %) were the other significant compounds in the leaf oil.



GC Trace of volatile oil from leaves of *C. racemosum*



GC Trace of volatile oil from flowers of *C. racemosum*

In the flower oil also davana ether isomer (8.27%) was identified as the most abundant component. It was followed by 7-hydroxyeudesm-4-en-6-one(7.97%), crispatanolide (6.48%), bicyclohumulenone (6.14%), longifolol (6.1%) , italicene ether (5.2%), eudesm-11-en-4-α-ol (3.57 %) , caryophylla-3(15),7(14)-dien-6-ol (3.52 %) and nootkatol (3.07%). Polygodial (2.94 %) , cadalene (2.48%) and aristolone (2.24%) were the other significant compounds present in the flower oil.

Davana ether had been earlier established as the principal component of the aromatic plant *Artemisia pallens* [35] belonging to *Asteraceae* . It is the first report of this compound from any species other than *A. pallens* as a most abundant component.

Bicyclohumulenone is well known as one of the principal chemical constituents of bryophytes (non-vascular plants) like *Plagiochila sciophila* [36]. Bicyclohumulenone possesses strong mossy note and is applicable in the manufacturing of compounding perfume [37]. It is also the first time this rare compound got reported from a vascular plant.

Table 5.1 GC/ MS Analysis with FID quantification of volatile oils from leaves and flowers of *C. racemosum*

No	RT	Compound	Leaves	Flower
1	17.59	E-2-Heptenal	0.1	-
2	18.17	Hexanoic acid	-	0.13
3	21.37	2E-Nonen-1-al	-	0.42
4	21.59	Limonene	0.33	-
5	23.48	Acetophenone	-	0.25
6	25.84	Phenyl ethyl alcohol	-	1.90
7	28.17	2E-Nonenol	-	1.94
8	28.43	Benzaldehyde	0.1	-
9	30.84	Myrtenal	0.1	-
10	30.93	2Z-Nonenol	-	0.27
11	33.2	Trans-p-Menthan-2-one	-	0.11
12	33.4	Hydroquinone	1.05	-
13	36.36	Unidentified	0.5	-
14	36.76	4E-Decenal	-	0.56
15	38.98	Eudesma-1,4(15),11-triene	0.1	-
16	40.77	γ-Terpineol	-	0.33
17	42.40	7-Hydroxyeudesm-4-en-6-one	1.75	7.97
18	42.56	Nootkatol	0.95	3.07
19	42.97	Aristolone	0.51	2.24
20	43.28	Damascone	-	1.13
21	44.27	Italicene ether	1.57	5.20
22	45.47	Myliol	-	1.56
23	46.02	10-Hydroxy-4-oplopanone	0.11	-
24	48.22	Longifolol	2.85	6.1
25	48.43	Longicamphenilol	0.1	0.19
26	49.37	Guaia-6,10(14)-diene-4-β-ol	1.22	1.29
27	49.90	T-Muurolol	0.56	0.83
28	50.41	Alpha-cadinol	0.75	0.7
29	50.62	Eudesm-11-en-4-α-ol	3.86	3.57
30	52.11	Isolongifolol	3.69	0.97

No	RT	Compound	Leaves	Flower
31	52.65	Maaliol	4.29	-
32	52.69	Cadalene	-	2.48
33	52.89	Elemenone	0.45	1.07
34	53.85	Bicyclohumulenone	14.86	6.14
35	54.1	Davana ether Isomer	18.27	8.27
36	54.25	Polygodial	0.98	2.94
37	55.78	3-Acetoxyamorpha-4,7(11)-dien-8-one	0.70	0.59
38	55.94	crispatanolide	4.94	6.48
39	56.09	Eudesma-4(15),7(11),9-trien-12-olide	1.34	-
40	56.43	Albicanol	0.50	0.57
41	58.47	Caryophylla-3(15),7(14)-dien-6-ol	3.00	3.52
42	59.14	Methyl palmitate	3.79	1.09
43	62.15	Heptacosane	1.91	-
44	62.98	1-Heptatriacotanol	1.5	0.2
45	64.92	Isopropyl hexadecanoate	-	0.46
46	66.7	Torulosol	2.66	-
47	67.14	Docosanal	-	0.22
48	68.66	2-α-acetoxy-Amorpha-4,7-dien-8-one	1.33	-
		Total	80.72	74.76

Another peculiar compound present in both the oils is italicene ether. This compound was detected as an important aroma component of the essential oils from the curry plant (*Helichrysum italicum*) [42]. Presence of italicene ether may be the basis of curry like odour of *C.racemosum*.

The tricyclic sesquiterpenol longifolol is significant as a lead structure for the design of inhibitors of the human UDP-glucuronosyltransferase (UGT) 2B7 [38]. A closely related compound longicamphenilol was also identified in both the oils. Longicamphenilol was recently reported as one of the autoxidation products of longifolene which is stored as a precursor of plant self defensive compounds [39].

Beside these both the oils contained various compounds especially sesquiterpenes and diterpenes in trace amounts (Table 5.2). These sesquiterpenes mainly belonged to the categories eudesmane, brasilane, amorphane, drimane, maaliane and guaine .

Diterpenes are the largest and heaviest molecules found in volatile oils produced by distillation [40]. In our samples most of the diterpenes were of the labdane type which is well known for their antibacterial, antifungal, antiprotozoal, and anti-inflammatory activities [41]. Many labdanes were found to be exhibiting significant cytotoxic and cytostatic effects against leukemic cell lines of human origin and interferring with the biochemical pathways of apoptosis and the cell cycle phases [43]. Larixol , 3-alpha-hydroxy-Manool, 7-alpha-hydroxy –Manool, Coronarin E, Copalol , Torulosol , Methyl daniellate and Neo-Abietol were the labdanes mainly present in these oils.

All the above mentioned compounds are obtained for the first time from *C.racemosum* and even from any species of the genus. These results suggest that the leaves and flowers of *C.racemosum* are rich in various important metabolites possessing significant biological and medicinal potencies.

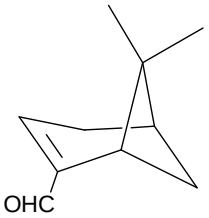
According to the IUCN red list corresponding to many parts of the world[44] *C.racemosum* is one of the highly threatened species . The present work, identifying the potent phytoconstituents of the plant, underlines the need for taking serious measures to protect its existence at any cost.

Table 5.2 Compounds detected in trace amounts

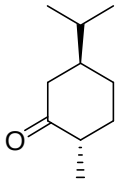
Sesquiterpenoids	Diterpenes	Miscellanous
Fokienol Laciniata furanone G and H	E-15,16-Bisnorlabda-8(17),12-dien-14-al Labda-8(17),14-dien-6,13-diol	p-vinyl-Guaiacol Z-Isoeugenol Methyl linoleate

Artedouglasia oxide A	Sclareol	Salicylaldehyde
2,7,10-Bisabolatrien-1-ol-4-one	Cembrenol	Methyl anthranilate
5-neo-Cedranol	Larixol	Theaspirane (Isomer)
Brasila-1(6),5(10)-diene	3-alpha-hydroxy-Manool	Salvial-4(14)-en-1-one
Eudesma-1,4(15),11-triene	7-alpha-hydroxy –Manool	
Germacrene B	Coronarin E	
E,E-Germacradiene-11-ol	Cembrene	
g-Gurjunene	10-a-Hydroxy-12-prenylguai-11-ene	
Isoafricanol	Copalol	
5-Guaiene-11-ol	3-alpha-acetoxy- Manool	
14-oxy-alpha-Muurolene	Z-Biformene	
Guaia-6,10(14)-diene-4-b-ol	Methyl labdanolate	
Germacra-4,5,10-trien-1-a-ol	Methyl daniellate	
4-epi-Marsupellol	Neo-Abietol	
5-Hydroxymarsupellyl acetate		
Plagiochilline T		
5-Hydroxymarsupellol		
g-Bicyclofarnesal		

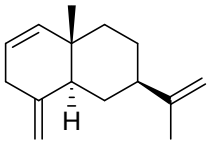
Structures of compounds identified in the volatile oils from C.racemosa



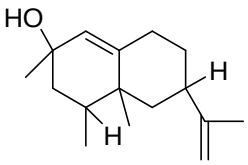
Myrtenal



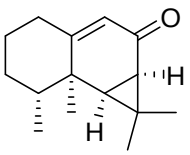
Trans-p-Menthan-2-one



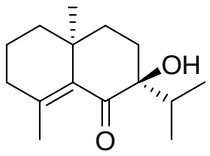
Eudesma-1,4(15),11-triene



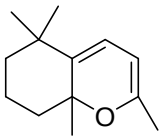
Nootkatol



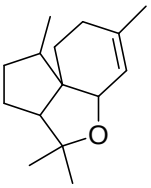
Aristolone



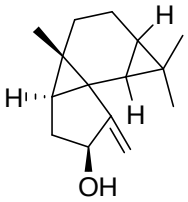
7-Hydroxyeudesm-4-en-6-one



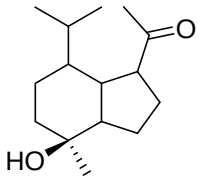
Cyclo-β-ionone



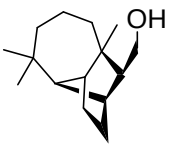
Italicene ether



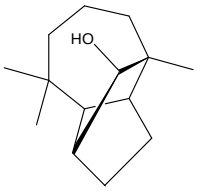
Myliol



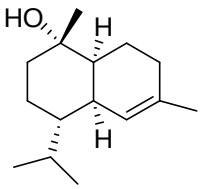
10-Hydroxy-4-oplopanone



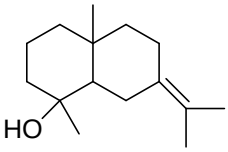
Longifolol



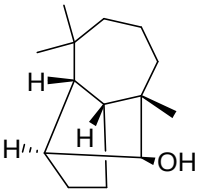
Longicamphenilol



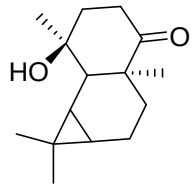
T-Muurolol



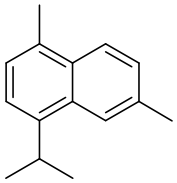
Eudesm-11-en-4-α-ol



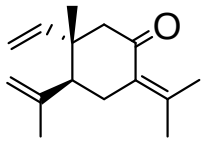
Isolongifolol



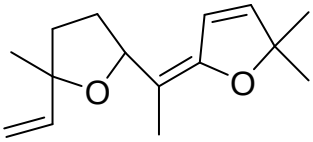
Maaliol



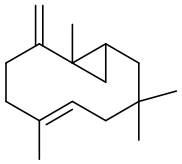
Cadalene



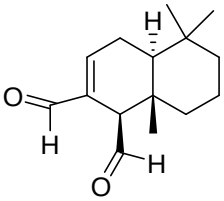
Elemenone



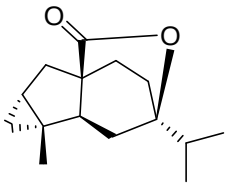
Davana ether



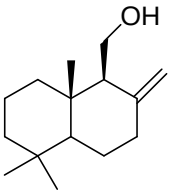
Bicyclohumulenone



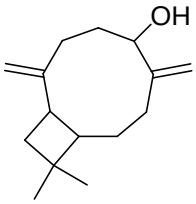
Polygodial



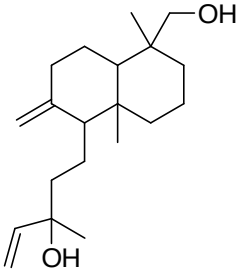
crispatanolide



Albicanol



Caryophylla-3(15),7(14)-dien-6-ol



Torulosol

5.5 References

1. ME Endress and PV Bruyns , *The Botanical Review* 66 ,1, 1–56, 2000.
2. S.B Malcolm,. *Chemoecology* 5, 6, 101-117, 1995.
3. M. Séquin, Chapter 5, The Chemistry of Plants: Perfumes, Pigments, and Poisons, Royal Society of Chemistry, 2012 .
4. http://www.theplantlist.org.
5. E.D Keoke and K.M Porterfield. Encyclopedia of American Indian Contributions to the World:15,000 Years of Inventions and Innovations, pp: 175, Infobase Publishing , 2009.
6. Mahadevan et al. Int J Pharm Pharm Sci, 3, 4, 70-72, 2011.
7. J.L Hartwell. Plants used against cancer. A survey. Lloydia, 30: 379-436, 1967.
8. SKR Rao, Encyclopaedia of Indian Medicine: Materia medica - herbal drugs, Popular Prakashan, 1985.
9. Ahmed et.al.,. Pharmacog. Mag.,1 ,2, 48-52, 2005.
10. Ahmad et.al. Journal of Medicinal Plants Research ,5,19, 4879-4887, 2011.
11. K Wiese. *Sierra Nevada Wildflowers*. Globe Pequot. p. 20. 2000.
12. http://plants.usda.gov/
13. M Sikorska and I Matlawska, Acta Polonia Pharmaceutica-Drug research, 57,4, 321-324, 2000.
14. A Hoppe , Drgenkunde. 1, Walter Gruyter, Berlin. Newyork , 1975.
15. Brown,. et.al., 334th communication. HCA, 62: 412–441. 1979.
16. K.R Lynn, Phytochemistry, 28, 1345, 1989.
17. T Warashina and T Noro., Chem. Pharm. Bull. (Tokyo). 56,3, 315-22,2008 .
18. Li et. al., Bioorg Med Chem Lett. 19,7, 1956-9,2009.
19. T Warashina et.al, Chem Pharm Bull (Tokyo). ,56,8, 1159-63. 2008.
20. V. Emon and J. N. Seiber, Economic Botany, 39,1,47-55, 1985 .
21. T Warashina and T Noro. Chem Pharm Bull (Tokyo).48(4):516-24. 2000.
22. T.Warashina and T. Noro, Phytochemistry , 53, 4, 485-498, 2000.
23. T Warashina and T Noro ., Chem Pharm Bull (Tokyo). ,58(2):172-9, 2010.
24. Li et. al. Steroids. 73(6):594-600, 2008.
25. Seiber et.al., Phytochemistry, 17, 5, 967–970, 1978.
26. Martin et.al., Phytochemistry, 30,12, 3935–3939, 1991.
27. Ahsan et.al., Experientia 29,7, 788-789, 1973.
28. Fonseca et.al., *J. Nat. Prod.*, *54* ,3, pp 860–862, 1991.
29. K. M. Nadkarni, Indian materia medica. Volume 2, pp-385, Popular Prakashan, 1994
30. Flora of Tamil Nadu, VOL. II, 1987.
31. N Sasidharan, http://indiabiodiversity.org/species/show/229341
32. N. W. Davies, J. Chromatogr., *503*, 1-24 ,1990.
33. W. Jennings and T. Shibamoto, *Qualitative Analysis of Flavor and Fragrance volatiles by Glass Capillary* Gas *Chromatography.*

Academic Press, New York , 1980.
34. F. W. McLafferty and D. B. Staufen, *The Wiley NBS Registry of Mass Spectral Data*, John Wiley, New York, 1989.
35. R.N. Kulkarni , pp : 104, Artemisia edited by Colin W. Wright, CRC Press, 2004.
36. Y. Asakawa, L.Agnieszka and F.Nagashima, Chemical Constituents of Bryophytes: Bio- and Chemical Diversity, Biological Activity, and Chemosystematics(Google eBook), Springer, 2012.
37. Patent no:4720354, Asakawa, Yoshinori ,Aroma composition,January, 1988, freepatentsonline.com.
38. Bichlmaier e t.al., ChemMedChem. 2,6, 881-9.2007.
39. Ashitani et.al., ,Proceedings of the 26th Annual Meeting, International society of chemical ecology, 2010.
40. D.Stewart, The Chemistry Of Essential Oils Made Simple. 3rd edn. pp 285, CARE publications, Marble Hill, MO. 2005.
41. Studies in Natural Product Chemistry : Bioactive Natural Products, Part F, Atta- Ur- Rahman (Editor), Elsevier, 2003.
42. Tucker et.al., J. Essent. Oil, Res,, 9,583-565 , 1997.
43. Costas Demetzos and Konstantinos S. Dimas, Studies in Natural Products Chemistry, 25, F, 235-292, 2001.
44. IUCN Sri Lanka and the Ministry of Environment and Natural Resources (2007), The 2007 Red List of Threatened Fauna and Flora of Sri Lanka, Colombo, Sri Lanka.xiii+148pp.

CHAPTER 6

ESSENTIAL OILS FROM A NEW ENDEMIC CURCUMA SPECIES

6.1 Introduction

Zingiberaceae or the ginger family constitutes a vital group of rhizomatous medicinal and aromatic plants [1] characterized by the presence of volatile oils and oleoresins of export value. Their volatile oils are widely used as ingredients in perfumery, flavour, fragrance and pharmaceutical products. The family has great ethno-botanical importance as described in many indigenous medical systems. Many members of *Zingiberaceae* are used in Ayurvedic, Unani, and Homoeopathic systems of medicine. The ancient Indian books on medicines describe the wonderful curative properties of members of *Zingiberaceae* especially *Zingiber* and *Curcuma* due to their chemical principles [2]. The medicinal and aromatic qualilties of Indian *Zingiberaceae* members are well described in Materia Indica also. Recently some members are successfully employed in aromatherapy, a branch of herbal medicine, which exploits the therapeutic properties of herbs and herbal oils to cure many ailments.

Curcuma is an important genus of the family having originated in the Indo-Malayan region[3] and distributed as wild and cultivated forms mainly in the tropical and sub tropical Asia, Africa and Australia from sea level to altitude as high as 2000 m in the Western Ghats and Himalayas. The name is derived from Arabic word *kurkum* meaning "turmeric". As many as 133 types of curcuma has been identified worldwide out of which 92 are accepted as separate specie. About 20 species are reported from South West India, of which 12 are endemic [1] .

6.2 Review on curcuma Essential oils

The species curcuma has being studied for decades for their chemical and biological properties. The foremost isolation of a pure compound which is none other than curcumin was back in 1815 whose structure could only be identified in 1910 [4]. Isolation of new compounds with excellent medicinal properties is still going on with these plants. Isolation of two guaiane derivatives from the rhizomes of *C.aeruginosa* [5], diaryl derivatives from the root tuber of *C.longa* [6], novel unsymmetrical sesquiterpene-dimers (Parviflorenes) with cytotoxic activities from *C. parviflora* [7], three new bisabolocurcumin ethers from the rhizomes of C. *longa* [8], four new sesquiterpenes from *C. wenyujin* with inhibitory effects on nitric-oxide production [9], anti-inflammatory sesquiterpenes from *C. zedoaria* [10] , anti-babesial compounds from *C. xanthorrhiza*[11] and labdane diterpenes from *C. comosa* with fetal hemoglobin induction potency [12] are a few recent developments to be mentioned.

Most of the curcuma plants carry volatile oils with specific aroma. Oils are being isolated from rhizome, leaves and flowers and widely used in perfumery, flavouring and aromatherapy. Chemical composition of these oils were found to be comparable among identical specie but at the same time regional variations are unpredictable within same species . Many of the oils were proved to be possessing bactericidal, fungicidal, herbicidal and antioxidant properties. These are summed up in table 6.1.

Table 6. 1 Essential oils from different curcuma species

No	Sample Description	Oil yield %	Composition (Major components & Percentage)
I. *C. aerugenosa*			
1	India,Rhizome*[13]*	0.94	ar-tunnerone (37. 85), anthorrhizole (8.68) curzerenone (6.58), camphor (6.14)
2	Thailand,Rhizome*[14]*	-	curzerenone (41.63), 1,8-cineol (9.64) α-pinene (7.71)
3	Malaysia,Rhizome*[15]*	0.6	curzerenone (24.6), 1,8-cineole (11) camphor (10.6), zedoarol (6.3) isocurcumenol(5.8), curcumenol(5.6) furanogermenone (5.5)
4	Malaysia,Rhizome*[16]*	0.8	1,8-Cineole (25.2) , curzerenone (30.4) camphor (6.8)
5	Indonesia,Rhizome*[17]*	-	curcumanolides A,B (11.4), curcumenol (9.9) dehydrocurdione (9.4), isocurcumenol (8.5) β-eudesmol (6.5), curdione (3.6) curcumenone (1.9)
6	India,Leaves*[18]*	0.14	1,8-cineole (17.7), curzerenone (10.5) furanogermenone (7.8), camphor(7.5)
II. ***C. angustifolia***			
7	*Central India* , Rhizome*[19]*	0.8	xanthorrhizol (12.7), methyl eugenol (10.5), palmitic acid (5.2), camphor (4.2
8	*South India*,Rhizome*[20]*	0.014	germacrone (12.8), camphor (12.3) isoborneol (8.7) , curdione (8.4), 1,8-cineole (4.8)
III. ***C. amada***			
9	India,Rhizome [13]	1.53	ocimene (41.26) , linalool (3.73) terpineol (3.35), citronellal (3.02) curzerenone(2.84), camphor

			(1.81)
10	India,Rhizome*[20]*	0.01	ar-curcumene (28.1), b-curcumene (11.2) camphor (11.2), curzerenone (7.1) 1,8-cineole (6.0)
11	N. India,Leaves*[21]*	0.15	Camphor (17.90), epi-curzerenone (10.76) curzerenone (9.53), isoborneol (7.30) curzerene (3.95)
12	N.India,Rhizome*[21]*	0.50	myrcene (88.84) , β-pinene (3.74) (E)-β-ocimene (2.61)
13	Eastern India , Fresh rhizome*[22]*	1.3	(Z)-β-farnesene (21.9), guaia-6,9-diene (19.8) α-longipinene (14.8), α-guaiene (14.5)
IV. ***C. aromatica***			
14	India,Rhizome*[13]*	1.93	camphor (18.33), 1,8cineole (9.93), bomeol (4.44), curzerene (5.32), ar-turmerone (3.92), a-pinene (3.77), ar-curcumene (2.55), zingiberene (1.89),
15	Northeast India, Leaves*[23]*	0.4	camphor (28.5), ar-turmerone (13.2) curzerenone (6.2), 1,8-cineole (6.0) α-turmerone (2.5)
16	Northeast India , Rhizome*[23]*	1.7	camphor (32.3) , curzerenone (1 l.0), α-turmerone (6.7) , ar-turmerone (6.3) 1,8-cineole (5.5).
17	Eastern India , Leaves*[24]*	-	p-Cymene (25.2), 1,8-cineole (24.0) 2-oxabicyclo (3.2.1) octane 1.4-dimethyl-8-methylene (8.1), p-cymen-8-ol (4.6)
18	Indonesia ,Rhizome*[18]*	-	xanthorrhizol (25.7), β-curcumene (25.5) *ar*-curcumene (18.6)
19	Thailand ,Rhizome*[14]*	-	camphor (26.94), ar-curcumene (23.18) xanthorrhizol (18.70)

20	Japan , Dried rhizome*[30]*	7.11	curcumol (35.77), 1,8-cineole (12.22) ar-Turmerone (6.98)
V. ***C. caesia***			
21	India ,Rhizome*[13]*	1.41	1.8-cineole (25.03), curzerenone (22.83) camphor (9.6) , ocimene (1.95)
22	North India , Rhizome*[25]*	1.5	camphor (28.3) , *ar*-turmerone (12.3) (*Z*)-β -ocimene (8.2), *ar*-curcumene (6.8) 1,8-cineole (5.3), β -elemene (4.8) borneol (4.4), γ-curcumene (2.82)
23	Eastern India , Leaves*[26]*	0.8	1,8-cineole (27), camphor (16.8) borneol (8.7), α-terpineol (5.2), β-pinene (6.3)
VI. ***C. decipiens***			
24	India ,Rhizome *[13]*	1.2	eugenol(38.15), l,8- cineole (26.37) camphor (2.44), a-pinene (l .35)
VII. ***C. domestica***			
25	Malaysia ,Rhizome *[16]*	5.1	α-tumerone (45.3), linalool (14.9) β-tumerone (13.5), 1,8-cineol (1.9)
26	Indonesia ,Rhizome *[17]*	-	*ar*-turmerone (24.7), turmerone (29.5) turmerol (20.0), α-atlantone (2.4)
VIII ***C. Haritha***			
27	South India , Rhizome*[27]*	0.30	Camphor (36.0), 1,8-cineole (13.9) Isoborneol (10.6), camphene (5.7) curdione (6.9), linalool (4.7) furanogermenone (3.3), germacrone (2.8)
28	South India ,Leaves*[28]*	0.14	Curdione (18.3), 1,8-cineole (11.8) camphor (11.8 , furanogermenone (8.6) furanodiene (8.9)
IX. ***C. harmandii***			
29	Vietnam,	-	curdione (36.8, 25.3)

	leaf and stem*[29]*		1,8-cineole (13.5 , 21.8 Germacrone (11.5, 15.5)
30	Vietnam, Small and large Rhizomes*[29]*	-	β-pinene (22.6, l.2), 1,8-cineole (12.5, 4.5) germacrone (9.0, 20.5), β-elemene (11.3, 6.5) isocurcumenol (3.7, 13.4)
31	Vietnam, Roots*[29]*	-	germacrone (24.4), isocurcumenol (12.9) curcumenol (10.8)
32	Vietnam, Flower*[29]*	-	Curdione (27) Curcumol (7.2) Linalool (5.8)
X. ***C. heyneana***			
33	Malaysia , Rhizome*[31]*	-	curcumanolide (19.6), dehydrocurdione (17.2) isocurcumenol (16.5), curcumenol (13.7) curcumenone (6.4), germacrone (5.0)
34	Indonesia , Rhizome*[17]*	-	1,8-cineole (14.2), curcumanolides A,B (13.1) dehydrocurdione (10.2), isocurcumenol (7.4) β-eudesmol (4.7), curcumenone (2.3)
XI. ***C. inodora***			
35	Malaysia, Rhizomes and leaves*[32]*	0.23, 0.14	curzerenone (20.8, 16.9), germacrone (11.1, 7.5) curdione (7.5, -), 1,8-cineole (5.3, 5.3)
XII. ***C. kwangsiensis***			
36	China*[33]*	trans-ethyl-p- methoxycinnamate -	(55.29), 3,4-dimethoxycinnamic acid(8.57) anethole (6.29), ethyl cinnamate(5.23)
XIII. ***C. leucorhiza***			
37	North east India , Rhizome and Leaves[34]	0.53 , 0.50	germacrone (9.6, 19.7), curdione (19.1, 19.5) camphor (7.2, 8.1), 1,8-cineole (4.0, 7.4) curzerene (3.0,5.7), linalool (5.2, 5.4) neo-curdione (2.8, 4.6), isobor-neol (2.0, 3.8)
XIV. ***C. longa***			

38	India ,Rhizome*[13]*	3.6	ar-turmerone (25.44) ,p-tunnerone (l4.64) sabinene (4.60), 1,8-cineole (4.25) zingiberene (3.93), ar-curcumene (2.59)
39	North India , Rhizome*[35]*	0.8	α-turmerone (44.1) , β-turmerone (18.5) ar-turmerone (5.4)
40	North India , Leaves*[35]*	0.65	α-phellandrene (53.4), terpinolene (11.5) 1,8-cineole (10.5)
41	Butan , Rhizome*[36]*	2 - 5.5	α-turmerone (30-32) , ar-turmerone *(17-26)* β-turmerone*(15-18).*
42	Butan, Leaves *[36]*	0.37 - *0.42*	α-phellandrene (18.2), 1,8-cineole *(14.6)* p-cymene (13.3)
43	Reunion Island , Rhizome*[37]*	1.1	α-turmerone (21.4), zingiberene (1 1.8) terpinolene (15.8), β-sesquiphellandrene (8.8) ar-turmerone (7.7) , β-turmerone (7.1)
44	Reunion Island , Leaves*[37]*	0.5	Terpinolene (77), 1,8-cineole (4.6) α-terpinene (3.7)
45	Reunion Island , Flower*[37]*	0.15	Terpinolene (67.4), 1,8-cineole (4.6) α-terpinene (4.4), α-phellandrene (3.6)
46	North India , Leaves*[38]*	-	Terpinolene(71.2), 1,8-cineole (6.2) p-cymen-9-ol (4.2)
47	North east India , Leaves[26]	1.41	myrcene (48.8), terpinolene (10.1) 1,8-cineole (6.4), p-cymene (4.3)
48	Japan, Dried rhizome*[30]*	9.85	ar-turmerone (49), humulene oxide (17) , β-selinene (10.18)
49	Nigeria ,Rhizome*[39]*	1.24	isabolene (13.9), trans-ocimene (9.8) myrcene (7.6), 1,8-cineole (6.9) thujene (6.7) , thymol (6.4)

XV. ***C. malabarica***			
50	Rhizome*[13]*	0.86	1.8-cineole (30.27), camphor (17.86) ar-turmerone (1 1.27) , b-pinene (3.14) limonene (l .44)
XVI. ***C. mangga***			
51	Malaysia , Rhizome*[40]*	0.15	myrcene *(78.6)* , (E)-β-ocimene (5.1) β-pinene *(3.7)*, α-pinene (2.9)
52	Malaysia , Rhizome*[41]*	-	myrcene (46.5) , β-pinene (14.6)
XVII. ***C. neilgherrensis***			
53	India*[42]*	-	ar-turmerone, β-cymene, curlone
XVIII. ***C. ochrorhiza***			
54	Malaysia , Rhizome*[43]*	0.43	furanogermenone (53.14), germacrone (9.62) β-elemene (8.84), camphor (6.31)
XIX. ***C.oligantha***			
55	India , Rhizome*[44]*	3.2	Cinnamyl cinnamate (48.9), n-hexenal(14) n-octadecane(10.1)
XX. ***C. petiolata***			
56	Rhizome*[45]*	0.13	2-methyl-5-pentanol, 1H-pyrrol-1-amine, 2-(4-methoxyphenyl)-n,n,5-trimethyl, curcumol
XXI. ***C. pierreana***			
57	Vietnam, Flower*[46]*	0.08	isoborneol *(27.3)*, camphor *(24.1)* isobornyl acetate *(7.3)*, *C*amphene *(6.7)* *α*-pinene (5.1)
58	Vietnam, Rhizome and stem*[47]*	-	isoborneol (22.9, 12.4) isobornyl acetate (18.8, 14.4) β-caryophyllene (0, 10.4),(Z)-β-farnesene (0,10.8).
59	Vietnam ,Leaves*[47]*	-	camphor (13.0), isoborneol (12.8)
XXII. ***C. pseudomontana***			
60	*[42]*	-	ar-turmerone, β-cymene, curlone
XXIII. ***C. purpurascens***			

61	Indonesia , Rhizome[48]	4.90	ar-tumerone (31.60)
XXIV. ***C.raktacanta***			
62	India , Rhizome[13]	1.36	camphor (17.98), 1.8-cineole (l3.64), zingiberene (4.24), curzerenone (7.93)
XXV. ***C. sichuanensis***			
63	China ,Rhizome*[30]*	3.21	ar-turmerone (43.52), β-selinene (13.36) δ-cadinene (13.22)
XXVI. ***C. trichosantha***			
64	Vietnam ,Rhizome[49]	-	curdione (47.4), curcumol (7.0) germacrone (6.1)
XXVII. ***C.wenyujin***			
65	China ,Rhizome*[50]*	2.11	1,8-cineole (15.26), camphor (10.12) germacrone (6.86), β-elemene (6.33) curzerene (6.70), β-elemenone (5.23) curzerenone (4.52)
66	China ,Rhizome*[51]*	-	germacrone (9.07), curcumenol (8.53) isocurcumenol (7.48), *ar*-zingiberone(5.06), curzerenone (4.98)
XVIII. ***C. xanthorrhiza***			
67	Malaysia , Rhizome*[16]*	6.7	xanthorrhizol(44.5), zingiberene (10.2) a-curcumene (7.6) , germacrone (5.2**)**
68	Indonesia , Rhizome*[17]*	-	*ar*-curcumene (41.4), xanthorrhizol (21.5)
69	Thailand , Rhizome*[14]*	-	1,8 -cineol (37.58), curzerenone (13.70)
XXIX. ***C. zedoaria***			
70	India ,Rhizome[13]	4.76	Curcumene (41.21), xanthorrhizol(12.60) curzerenone (5.79), camphor (5.06) curzerene (4.68) , zingiberene(3.71)
71	India ,Leaves[52]	0.2	selina-4(15),7(11)-dien-8-one (9.4) dehydrocurdione (9), α-Terpinyl acetate(8.4) isoborneol (7)
72	North east India ,	0.36	Curzerenone (22.3), 1,8-cineole

	Rhizome[53]		(15.9) germacrone (9.0), Camphor (7.8)
73	Thaiwan , Rhizome[54]	-	Epicurzerenone(24.1), Curzerene(10.4)

6.3 Present Work

Curcuma ecalcarata Sivar. & Balach. is endemic to Kerala and distributed widely in the Western Ghats and midlands of Kerala [55]. It grows as undergrowth in forests, margins of forests at low and high altitudes, in coconut groves and various plantations in plains. Its flowering season is August to October. It is easy to get them interpreted as *C.aerugenosa* growing alongside with analogous leaves, but can be distinguished by the small conical rhizomes which are yellow inside and also by the long inflorescence. *C.ecalcarata* is regarded as con-specific to *C.aurantiaca* Zijp[56].

Rameshkumar and George reported the isolation of compounds like β-sitosterol, curcumin and 5,7-dihydroxy flavanone from the species in a preliminary phytochemical investigation [57]. They also analyzed the essential oils from the plant which is taken for comparison in a later session .

In the present work essential oils are collected from fresh rhizomes, flowers, and fresh leaves of *C.ecalcarata* by steam distillation. The oils are subjected to GC and GC/MS for analyzing the chemical composition. The characteristics of the major compounds are briefly outlined . Antimicrobial activity of the rhizome oil under *in vitro* conditions was also investigated and is reported in Chapter VIII.

Plant Collection : The fresh plant was collected from a rubber plantation in north Kerala. It was identified by Dr. A.K. Pradeep, Department of Botany, University of Calicut and a specimen voucher was deposited in the specially maintained Herbarium of Chemistry department of University of Calicut.

Extraction of Oil : The rhizome, leaves and flower of the plant were separated and washed with clean water. Each of them were ground into a paste and subjected to steam distillation for 3 hours. The oils were extracted by diethyl ether from the distillates. The ether extract was dried using anhydrous sodium sulphate and the ether evaporated. The pure oils were stored below 4^{o}C until analyzed.

Analysis of the oils : GC-FID analysis was carried out using a Perkin Elmer Clarus 500 GC equipped with a 30 m × 0.32 mm Elite-5MS capillary column (0.32 μm film thickness). 1 μl of each sample was diluted with 300 μl of Et_2O and injected (0.5 fKl) in the "split" mode (1:30) with a column temperature programme of 40°C for 5 min, then increased to 280°C at 4°C/min and finally held at this last temperature for 10 min. Injector and detector were set at 250 and 300°C, respectively, and the carrier gas was He with a head pressure of 12.0 psi.

GC/MS Analyses were carried out using a Perkin Elmer Clarus 500 GC equipped with a Clarus 500 mass spectrometer using the same capillary column and chromatographic conditions as for the GC/FID analysis. Mass spectra were acquired over 40-500 amu range at 1 scan/sec with ionizing electron energy 70 eV, ion source 200°C. The transfer line was set at 300°C, while the carrier gas was He at 1.0 ml/min.

Identification and quantification of the oil components : The identification of essential oil components was performed by means of their retention indices (AI), by a peak matching library search [58] and by comparison with authentic reference compounds as well as with published mass spectra [59, 60]. Retention indices (AI) were calculated using a n-alkane series (C_6-C_{35}) under the same GC conditions as for the samples. The relative amount (%) of individual components of the oil is expressed as percent peak area relative to total peak area from the GC/FID analyses of the whole extracts.

6.4 Results and Discussion

The GC and GC-MS analysis of the three oils revealed the identity of 33 compounds in total. Thirty compounds were identified in the rhizome oil while 32 compounds were identified both in flower and leaf oils. Twenty nine compounds were common in all the three oils. In the earlier investigation [57] only 22 compounds were identified from the rhizome oil while 26 and 29 compounds from leaves and flower oils respectively. All the three oils were rich in monoterpenoids and cyclic compounds. The amount of aliphatic as well as aromatic (phenolic) compounds were very low. The results are tabulated in Table 6.3 and 6.3.

Rhizome Oil : Essential oil obtained from fresh rhizomes was characterized by high monoterpene content (almost 92%). Including these monoterpenoids the oil contained 94% of cyclic compounds. It contained only trace amount of aliphatic and aromatic compounds. A total of 30 compounds consisting of 98.84 % were characterized in the rhizome oil against 22 compounds in the earlier study. Piperitenone (46.11 %) was identified as the most abundant component followed by 1,8-cineole (28.24 %) and terpinolene (11.61%). Piperitenone was identified in the earlier study (about 65 %) whose presence is once again confirmed in our work. The variation in amount may be due to seasonal variations. But the other two abundant components previously reported were camphor (5.66 %) and d-nerolidol (4.86 %) which were detected in our sample also but with 1.09 and 1.64 % only. Coahuilensol methyl ether and the aliphatic aldehydes tetradecanal and pentadecanal were not detected in the rhizome oil but were present in leaf and flower oils . *α*-Phellandrene was present only in the rhizome oil.

Flower oil : Flower oil was also rich in monoterpenoids (81%) and cyclic compounds (more than 80%). Quantity of sesquiterpenoids and aliphatics were high when compared with the other two oils . Thirty two compounds consisting of 97.21 % were identified against the previously reported 29 components. Piperitenone (47.42 %) was the most abundant component followed by 1,8-cineole (27.42 %) and *trans*-β-farnesene (8.18 %) . It is

comparable with the previous report in which piperitenone (65.91 %), 1,8-cineole (7.83 %) and d- nerolidol (6.34 %) were the major constituents.

Leaf oil : Leaf oil was identical in composition with flower oil. Both contained the same compounds but with a slightly different percentage. Thirty two compounds consisting of 97.71 % were identified against the previously reported 26 components. Once again Piperitenone (47.28 %) was the most abundant component followed by 1,8-cineole (34.75 %) and *trans-b*-farnesene (4.63 %). It can be compared with the previous report in which the major components were piperitenone (61.00 %), 1, 8- cineole (21.16 %), and d- nerolidol (4.86 %) .

Table 6.2

GC/ MS Analysis with FID quantification of Essential oils from C. ecalcerata

No	AI tab	AI	compound	Leaves	Flowers	Rhizome
Acyclic Compounds						
1	850	855	*cis*-3-Hexenol	0.74	3.24	tr
2	988	990	β-Myrcene	0.04	0.12	0.20
3	1095	1101	Linalool	1.17	1.50	0.16
4	1454	1452	*trans*-β-Farnesene	4.63	8.18	2.80
5	1561	1560	*trans*-Nerolidol	2.33	2.98	1.64
6	-	1613	Tetradecanal	0.11	tr	-
7	-	1714	Pentadecanal	0.76	0.16	-
Cyclic Compounds-Monoterpenoids						
8	932	929	α-Pinene	0.07	0.12	0.26
9	946	944	Camphene	0.04	0.06	0.41
10	974	972	β-Pinene	0.21	0.27	0.15
11	1002	1002	α-Phellandrene	-	-	0.15
12	1008	1004	δ-3-Carene	0.04	0.07	0.32
13	1014	1014	α-Terpinene	0.02	0.01	0.65
14	1020	1023	p-Cymene	0.11	0.15	0.27
15	1026	1030	1,8-Cineole	34.75	27.42	28.24

No	AI tab	AI	compound	Leaves	Flowers	Rhizome
16	1054	1056	α-Terpinene	tr	0.06	0.20
17	1086	1083	Terpinolene	0.27	0.32	11.61
18	1141	1142	Camphor	0.41	0.69	1.09
19	1155	1159	Isoborneol	0.07	0.14	0.14
20	1165	1168	Borneol	0.49	0.42	0.35
21	1174	1177	Terpinen-4-Ol	0.55	0.49	0.28
22	1186	1193	α-Terpineol	1.86	1.61	1.16
23	1219	1219	Coahuilensol methyl ether	0.12	0.08	-
24	1239	1243	Carvone	0.18	0.17	0.05
25	1340	1342	Piperitenone	47.28	47.42	46.11
26	1359	1359	Piperitenone oxide	0.12	0.07	0.03
Cyclic compounds-Sesquiterpenoids						
27	1417	1412	β-Caryophyllene	0.47	0.53	2.07
28	1452	1448	α-Humulene	0.03	0.04	0.12
29	1511	1513	δ-Amorphene	0.03	tr	0.19
30	1582	1574	Caryophyllene oxide	0.26	0.35	0.17
31	1605	1596	Curzerenone	0.26	0.06	tr
Cyclic compounds-Phenolics						
32	1289	1288	Thymol	0.10	0.26	tr
33	1309	1307	p-Vinyl guaiacol	0.19	0.22	0.02
			Total	97.71	97.21	98.84

Table 6.3 Overall composition Essential oils from *C. ecalcerata*

Class of compounds	% in Leaves	% in Flowers	% in Rhizome
Aliphatics	1.61	3.4	Tr
Monoterpenes	87.8	81.19	91.83
Sesquiterpenes	8.01	12.04	6.99
Aromatics	0.29	0.48	0.02
Acyclic compounds	9.78	16.18	4.8

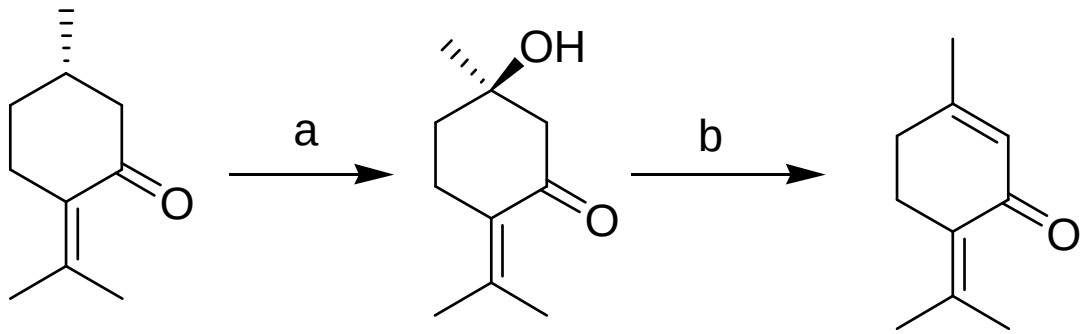
Cyclic compounds	87.93	81.03	94.04

Piperitenone : All the three oils contained about 50% of piperitenone which suggests that the plant can be used as a source for this compound. This compound previously reported for its antimicrobial activities [68] can also be used as starting material for different 3-oxygenated p-menthanes which have various medicinal potentials. Piperitenone also called p-Mentha-1,4(8)-dien-3-one was occasionally detected as one of the major constituent of essential oils from many *Mentha* species [62]. *C. ecalcerata* is the only curcuma plant containing this compound as the major component. So this species can be treated as piperitenone chemotype. Also it is a new chemotype in the genus.

Piperitenone had been revealed as one of the major metabolite of R(+)-pulegone which is present in many mentha essential oils. It was suggested that the latter is subjected to stereoselective hydroxylation at C-5 position to form hydroxypulegone, which upon dehydration , yields piperitenone (Scheme I) [63] . A biosynthetic route for pulegone from linalool was also suggested in which piperitenone appeared as an intermediate [64]. A bio-transformation of pulegone into piperitenone by the plant pathogen *Botrytis allii* was also reported [65] . It was observed in one of the *Mentha* species that the young leaves are characterized by piperitenone while the older ones by both pulegone and piperitenone [66].

Pulegone was also reported in the essential oils from many *curcuma* species including *C. aerugenosa* [18] but not in the species under investigation. More detailed study can reveal the origins of these closely related compounds.

Scheme I



Pulgeone **Hydroxy Pulgeone** **Piperitenone**

a) Stereoselective hydroxylation b) Dehydration

1,8-cineole : Eucalyptol or 1,8-cineole was detected in every curcuma essential oil. Many times it appeared as one of the most abundant components [13-54]. All the three oils in our study contained about 30 % of 1,8-cineole ; leaf oil consisting the maximum of 34.75%. The compound characterized by fresh camphor-like smell and a spicy cooling taste is extensively used in flavorings, fragrances, and cosmetics including baked goods, confectionery, meat products, cigarettes and beverages[67]. It is a common ingredient in many brands of mouthwash and cough suppressant and can be used as an insecticide and insect repellent [69]. It was found to reduce inflammation and pain [70] and to control airway mucus hypersecretion and asthma [71] and effective in treatment for nonpurulent rhinosinusitis [72]. 1,8-cineole was also found to kill leukaemia cells of two cultured human leukemia cell lines[73]. The high cineole content confirms the medicinal potentials of these oils.

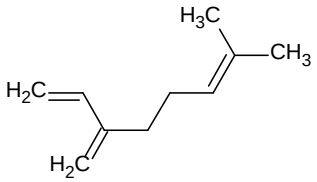
Terpinolene : Terpinolene was present in all the three oils but only the rhizome oil contained a good amount (more than 10%). The increased amount of the compound in the rhizome may be intended to inhibit the pathogens in the rhizosphere as it was reported to be sensitive for fungi like *Phytophthora cinnamomi* [74].

trans-β-Farnesene and trans-Nerolidol : These compounds play significant roles in the interaction of the plant with herbivores. Farnesene isomers are

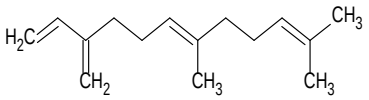
insect semiochemicals ; they act as alarm pheromones in termites . It is also released by aphids as an alarm pheromone upon death to warn away other aphids. Several plants, including potato species, have been shown to synthesize this pheromone as a natural insect repellent [75, 76]. Nerolidol is produced as an antifeeding sesquiterpene alcohol for folliators like gypsy moth larvae [77] and in response to species like spidermite in many leaves. It has been believed as the precursor for the biosynthesis of DMNT ((3*E*)-4,8-dimethyl-1,3,7-nonatriene, 4,8-dimethyl-1,3(*E*),7-nonatriene) which has a key role in attracting natural enemies to herbivore injured plants. [78]. A large number of herbivores might be getting attracted to the leaves and the large colorful inflorescence of *C. ecalcereata*. The production of these compounds are evidently aimed for controlling these group from attacking the plant. Concentration of both these compounds were found maximum in the flowers (and leaves next) which is in accordance with the above facts.

The higher content of monoterpenoids assures the therapeutic potential of these oils. These oils can be readily recommended for aromatherapy as they also contained compounds having pleasant floral impressions like linalool and nerolidol along with those having herbal notes like camphor. The rhizome oil with maximum monoterpenoid content will be the best choice.

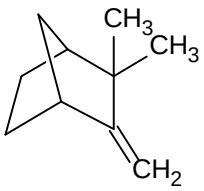
Structure of compounds identified in the essential oils from C.ecalcerata



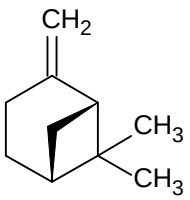
β-myrcene



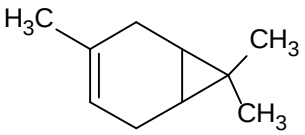
***trans*-β-farnesene**



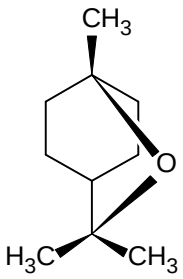
camphene



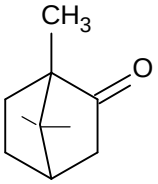
β-pinene



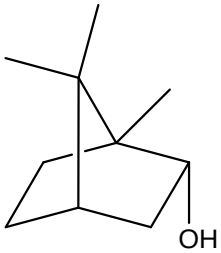
δ-3-carene



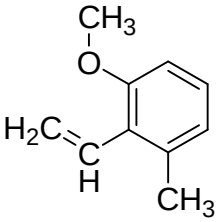
1,8-cineole



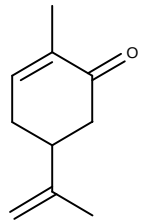
camphor



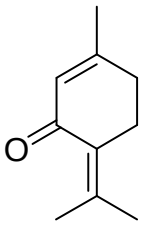
Isoborneol



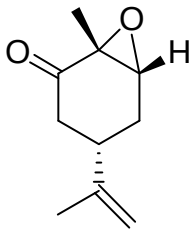
coahuilensol methyl ether



carvone

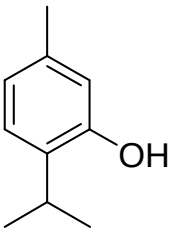


piperitenone



piperitenone oxide

δ-amorphene



thymol

6.5 References

1. M Sabu, Zingiberaceae and Costaceae of South India, Indian Association for Angiosperm Taxonomy: Calicut, India, 2006.
2. K. R Kirtikar and B. D Basu, Indian Medicinal Plants. Vol. IV, Lalit Mohan Basu, Leader Road, Allahabad ,1933.
3. J.W Purseglove, Tropical crops Monocotyledons. Longman, London. 1968.
4. Milobedzka et.al., Structure. Chem Ber , , 43, 2163. 1910.
5. Takano et.al. Phytochemistry, 40, 4, 1197 1200, 1995.
6. Wang et al. Biochemical Systematics and Ecology, 36, 5-6, 2008.
7. Toume et al. Tetrahedron , 60 ,2004.
8. Xiao et. al. Helvetica Chimica Acta , 95 ,2012.
9. Loua et. al. Chemistry & Biodiversity – Vol. 7 , 2010.
10. Makabe et.al., Natural Product Research: Formerly Natural Product Letters, 20:7,2006.
11. Matsuura , et.al. Natural Product Research: Formerly Natural Product Letters, 21:4, 2007.
12. Chokchaisiri et.al. J. Nat. Prod., 73, 4, 2010.
13. Major Essential oil Constituents in Zingiberaceae, Digital Archieves, Mahathma Gandhi University, Kottayam, Kerala.
14. Jarikasem et.al., ISHS Acta Horticulturae 675: III WOCMAP Congress on Medicinal and Aromatic Plants - Volume 1: Bioprospecting and Ethnopharmacology.
15. Sirat et.al. , Journal of Essential Oil Research, 10:4, 1998.
16. Ibrahim et.al., Journal of Essential Oil Research, 11:6, 1999.
17. J. H. Zwaving and R. Bos,. Flavour and Fragrance Journal, 7 1, 19–22, 1992.
18. Jirovetz et.al., Journal of Essential Oil Research,12, 47-49, 2000.
19. Srivastava et.al., Flavour Fragr. J. 21, 2006.
20. Srivastava et.al., Journal of Essential Oil Research, 13,1, 2001.
21. Padalia et.al., Journal of Essential Oil Research, 25,1, 17-22, 2013.
22. Mustafa et.al., Journal of Essential Oil Research, 17,5, 490-491, 2005.
23. Bordoloi et.al., Journal of Essential Oil Research,11,5, 537-540, 1999.
24. Singh et.al., Journal of Essential Oil Bearing Plants, 7,3,258-263, 2004.
25. A.K Pandey and A. R Chowdhury, Flavour Fragr. J. 18, 463–465, 2003.
26. S. Behura and V. K Srivastava 16:2, 109-110, 2004.
27. Raj et. al. Flavour Fragr. J. 23, 348–352, 2008.
28. Raj et.al., Journal of Essential Oil Bearing Plants, 14 ,2, 185 – 191, 2011 .
29. Dũng et.al., Journal of Essential Oil Research, 9, 6, 677-681, 1997.
30. Tsai et al. Asian Journal of Arts and Sciences, 2, 1, 57-66, 2011.
31. M.S Hasnah and M.L Lay Malaysian Journal of Science , 28 , 3, 323-328, 2009.
32. Malek et.al., Journal of Essential Oil Research, 18,3, 281-283, 2006.

33. Xue-mei, et.al., Chinese Journal of Experimental Traditional Medical Formula, 19, 2011.
34. Devi et.al., Journal of Essential Oil Research, 24,6, 533-538, 2012.
35. Raina et.al., Journal of Essential Oil Research, 17, 5, 556-559, 2005.
36. Sharma et.al., Journal of Essential Oil Research, 9, 5, 589-592, 1997.
37. Chane-Ming et.al., Journal of Essential Oil Research, 14,4, 249-251, 2002.
38. C Pande and C. S Chanotiya, Journal of Essential Oil Research, 18, 2, 166-167, 2006.
39. L.A. Usman,. World Journal of Chemistry 4 ,2, 178-181, 2009.
40. Wong et.al., Journal of Essential Oil Research, 11, 3, 349-351, 1999.
41. Wahab et.al., Chem Biodivers. 8, 11, 2005-14, 2011.
42. M Maridass, International Journal of Essential Oil Therapeutics 3 , 2/3 , 109-114, 2009.
43. Hasnah et.al., Journal of Essential Oil Research, 9, 3, 351, 1997.
44. Ahamad et. al. IJRAP, 3,5, 2012.
45. Thakam et al., Advanced Materials Research, 506, 393, 2012.
46. Dũng et.al., Journal of Essential Oil Research, 10:5, 527-528, 1998.
47. Dũng et. al. Journal of Essential Oil Research , 7, 3, 1995.
48. Lestari et al. , (eds); Bogor, Biopharmaca Research Center, Bogor Agricultural University, 2004; pp : 298-313. Proceedings, International Symposium on Biomedicines, Bogor, 2003.
49. P.T Ky, Journal of Essential Oil Research , 6, 2, 1994.
50. Liu et. al. .Molecules, 17, 2012.
51. Cao et.al., Analytica Chimica Acta, 561, 1–2, 88–95, 2006.
52. Garg et.al., Journal of Essential Oil Research, 17, 1, 29-31, 2005.
53. Purkayastha et.al., Journal of Essential Oil Research, 18, 2, 2006.
54. Mau et. al. Food Chemistry, 82, 4, 2003 .
55. Sivarajan and Indu, Notes. Roy. Bot. Gard. Edinburgh 41: 321, 1983.
56. Leong-Škorničková et. al. Taxon 59 ,1, 2010.
57. http://210.212.24.72/~kscsteuser/digital-library/digital/KSC/ksc19/02-Chemical%20Sciences/02-General/02-06%20(K.B.Rameshkumar).pdf.
58. NIST/EPA/NIH Mass Spectral Database, Version 2.1 Perkin-Elmer Instrument LLC, Copyright © , 2000.
59. D. Joulain and W.A. Konig, The atlas of spectral data of sesquiterpene hydrocarbons. E.B. Verlag, Hamburg , 1998.
60. R.P. Adams, Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry, 4th ed. Allured Publ. Corp., Carol Stream, IL , 2007.
61. K. V. Peter (Editor), Handbook of herbs and spices , 3 , 503. CRC Press, England, 2006.
62. B.M Lawrence, Mint: The Genus Mentha, CRC Press, 2010.
63. K. M Madyastha, and P.C Raj, Xenobiotica, 23 ,5, 1993.
64. M.J Murray and D.E. Lincoln, Genetics. 65, 3, 457–471. 1970.
65. L. N Mander and H Liu(Editors), Comprehensive Natural Products II: Chemistry and Biology, Elsevier Science & Technology Books, 2010 .

66. R.J Singh, Genetic Resources, Chromosome Engineering, and Crop Improvement: Medicinal Plants, Volume 6 pp: 709, CRC Press, 2012.
D. J Boland, J.J Brophy, and A.P.N House, Eucalyptus Leaf Oils: Use, Chemistry, Distillation and Marketing. Melbourne: Inkata Press. 1991.
67. Romaganoli, . et.al. Protoplasama, 225, 1-2, 2005.
68. Klocke, et.al., Journal of Chemical Ecology 13, 12, 1987.
69. F. A Santos, V. S Rao, Phytotherapy Research, 14, 4, 240–244, 2000.
70. Juergens et.al., Respiratory Medicine, 97 , 3, 250–256, 2003.
71. Kehrl et.al., The Laryngoscope114, 4, 738–742, 2004.
72. Moteki et.al., Oncology Reports 9,4, 757–760, 2002.
Y.R. Dommergues, Interactions Between Non-Pathogenic Soil Microorganisms And Plants, **Elsevier , 1978.**
75. Šobotník, et. al. Journal of Chemical Ecology 34, 4, 2008.
76. R. W Gibson and J. A Pickett, Nature , 302 , 5909, 1983.
77. R. W. Doskotch, Journal of Chemical Ecology ,6,4, 1980.
78. R.T Cardé and J. G. Millar, Advances in Insect Chemical Ecology, Cambridge University Press, 2004

CHAPTER 7

ANALYSIS OF ESSENTIAL OILS FROM TWO *POGOSTEMON* SPECIES

The *Pogostemon Desfontaines* is an important genus of the tribe *Pogostemoneae*, subfamily *Lamioideae* of the family *Lamiaceae* [1] . It is globally represented by 96 species [2] distributed mainly in tropical and subtropical Asia with another five species endemic to Africa [3]. India has the highest number of *Pogostemon* species in the world,with 56 taxa (53 species and 3 varieties). Of which 19 species and 3 varieties are endemic.

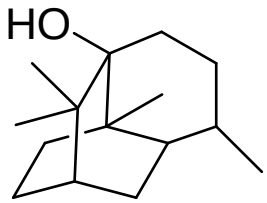
A number of pogostemons like *P. nilagiricus, P. mollis, P. Vestitus, P. petiolaris, P. rotundatus* and *P. paludosus* are distributed in western ghats , its vallyes and nearby plains. New speciee like *Pogostemon rajendranii* [4] and *Pogostemon raghavendranii* [5]have been discovered from this region recently. Most of them are aromatic and widely used for extracting essential oils.

7.1 Phytochemistry of *Pogostemon*- A Review

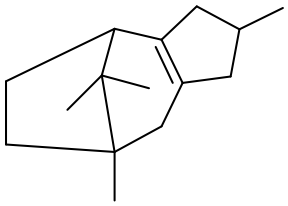
Essential oils extracted from leaves and flowers of different speciee of the genus *Pogostemon* were analysed for their chemical composition, olfactoric properties and antimicrobial properties by different researchers. The major findings are discussed below.

Essential oil from patchouli (*P.cablin*) is the most studied essential oil from any plant of this genus. Patchouli oil is used widely in modern perfumery and scented industrial products such as paper towels, laundry detergents, and air fresheners. It was suggested as a all-purpose insect repellent [6]. In all the investigations patchoulol or patchouli alcohol was obtained as the most abundant component present in the oil [7,8, 9]. Patchoulol is the sesquiterpene alcohol whose (-)optical isomer is mainly responsible for the typical patchouli scent. Isomers of patchoulene, α-

bulnesene , α-guaiene and caryophyllene were also present as minor components. Patchouli oil exihibited antimicrobial potencies[10] against many organisms in invitro and in vivo conditions. The oil was proved to be insecticidal and repellent against urban ant species [8] and repellent and pupicidal against human vector mosquitoes [11]. Fractionation of the essential oil from *P. cablin* guided by inhibitory action against PAF-induced platelet aggregation led to the isolation of the alpha-bulnesene [12] which was one of the major constituents.



Pachoulol



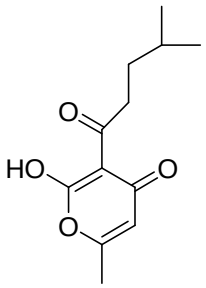
Pachoulene

Chemical constituents of involatile moiety of *P.cablin* were isolated and purified by repeated column chromatography [13]. Nine compounds have been isolated and identified as epifriedelinol , 5-hydroxymethol-2-furfural , succinic acid , beta-sitosterol , daucosterol , crenatoside , 3'''-O-methylcrenatoside , isocrenatoside , and apigenin-7-O-beta-D-(6''-p-coumaryl)-glucoside .

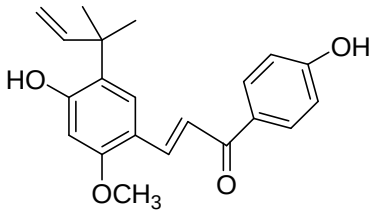
Several known and new compounds were isolated from *P. Cablin* previously. Eight flavonoids were isolated from the alcoholic extract of the whole plant [14]. Their structures were elucidated as 5- hydroxy 7, 3′, 4′ trimethoxyfla vanone , 5- hydroxy 7, 4′ dimethoxyflavanone , 3, 5- dihydroxy -7, 4′- dimethoxyflavone, 5- hydroxy- 3, 7, 4′ -trimethoxyflavone, 5 -hydroxy- 3, 7, 3′, 4′- tetramethoxyflavone , 5, 4′- dihydroxy- 3, 7, 3′- trimethoxyflavone , 5, 4′ dihydroxy-7- methoxyflavone and 3, 5, 7, 3′, 4′- pentahydroxyflavone . These compounds exihibited antifungal activity in *invitro* studies.

Four new patchoulol derivatives, 8*α*,9*α*-dihydroxypatchoulol , 3*α*,8*α*-dihydroxypatchoulol , 6*α*-hydroxypatchoulol , and 2*β*,12-dihydroxypatchoulol

, were isolated from the aerial part of *P. cablin* , together with nine known compounds [15]. The metabolite pogostone, serving as the effective component of the antimicrobial activity was isolated from the plant along with other flavanoids [16]. Licochalcone A, ombuin , and 5,7-dihydroxy-3′,4′-dimethoxyflavanone were isolated from the aerial parts of *P. cablin* by cytotoxicity-guided fractionation [17]. Licochalcone A showed cytotoxicity and PI-PLCγ1 inhibition activity in *in vitro* studies.



Pogostone



Licochalcone A

The volatile oil of the leaves of *P. heyneanus* Benth. was analyzed by GC and GC-MS [18]. The major components of the oil were acetophenone (51.0%), patchouli alcohol (14.0%), β-pinene (5.3%)and (E)-nerolidol (5.4%).

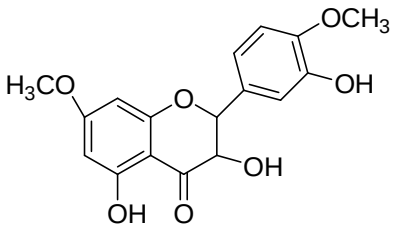
The essential oil of *P. travancoricus* Bedd. var. travancoricus collected from the Agasthyamalai region (Tamil Nadu, India) was analyzed by GC and GC-MS [19]. Bicyclogermacrene (16.0%), cis-calamenene (6.3%), germacrene B (11.1%), spathulenol (7.6%), viridiflorol (5.8%), alpha-cadinol (4.2%) and pogostone (9.9%) were the major components of the oil . Calamenene, cadina-1,4-diene and seychellene were identified as the major components in the essential oil from *P. Travancoricus* collected from western ghats [19].

Essential oils from the leaf and inflorescences of *P.benghalensis* (Burm.f.) Kuntze were investigated by gas chromatography–mass spectrometry [20]. The inflorescences oil are rich in trans-caryophyllene (8.52%), germacrene B (4.50%), δ-cadinene (4.37%) and β -ocimene (4.30%)

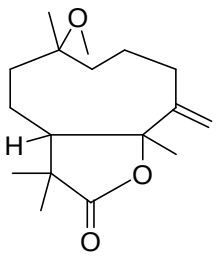
, while leaf oil is rich in cadinene isomer (2.615%), elemol (1.458%), α-bulnesene (2.184%) and χ-elemene (2.118%) .

The essential oil from the leaves of *P. paniculatus* (Willd.) Benth. Collected from Karnataka was characterized by nineteen compounds constituting 85.36% of the total oil [21] . Patchouli alcohol (30.65%), α-guaiene (10.67%), β-guaiene (9.09%), caryophyllene (8.64%) and eicosene (5.27%) were identified as the major constituents . The flowers of the same plant collected from Kerala yielded an essential oil [22] rich in cis-b-farnesene (45.3%), farnesene epoxide (41.7%) and germacrene D (4.4%).

A new sesquiterpene stemonolone (5α-hydroxy-10β-selina-1,4(15),7-trien-6-one) was isolated from *P. plectranthoides*(Desf) and characterised by spectroscopic and *X*-ray crystallographic evidence [23]. Epoxyparvinolide which belongs to a new class of sesquiterpenoid lactones, the secocaryophyllanolides, was isolated from *P. parviflorus* along with known compounds [24]. Epoxyparvinolide was characterized as 4,5-epoxy-9,10-secocaryophyllen-9β,10-olide.



Ombuin



Epoxyparvinolide

No phytochemical studies have been reported so far on the species *P. pubescens* Benth. *and P. quadrifolius (Benth.)* F.Muell. In this chapter the essential oils from the flowers of these two plants are investigated by GC and GC/MS for their chemical composition.

7.2 Present Work

Pogostemon quadrifolius (Benth.) is an aromatic herb native to India,

Bangladesh and Myanmar [25]. It was reported from different locations of Meghalaya, Assam, and Orissa in the north. In Kerala it is disributed in Kasaragode, Thiruvananthapuram, Kollam, Malappuram and Kozhikkode districts. It is a common herb found in the rocky regions, especially laterite hillocks of moderate altitude and moderately hot weather. It is called 'Naithumba' in the native language. The plant is described by as large erect herb, 1-2 m high; stem angled and ridged; whole plant densely grey-pubescent. Leaves usually 4 in a whorl, densely gray-pubescent on both sides. Spikes terminal, 10-15 cm long. Flowers clustered at axils of bracts. [26]. Both the leaves and flowers of the species carry a distinct aroma which is moderately pungent.

Pogostemon pubescens Benth is an aromatic shrub native to India and Srilanka [27]. It is mainly distributed in the evergreen forests and shola forests of Western Ghats along Kerala, Thamilnadu and karnataka. In Kerala the species is disributed in the high altitude and low temperature regions of Idukki, Kollam, Palakkad, Kannur, Thiruvananthapuram and Wayanad districts. Its flowering and fruiting occurs during November to December. The species is described as follows: Shrubs. Leaves to 8 x 4 cm, ovate, apex acute, base cuneate, doubly crenate membranous, sparsely hirsute; petiole to 3.5 cm. Panicles axillary and terminal; tooth 1 mm, pubescent; corolla tube 3 mm, filaments 6 mm, bearded [26]. The leaves and flowers of *P. Pubescens* bear a pleasant floral odour.

Plant Collection :

The flowers of *P.quadrifolius* were freshly collected from the laterite hillock near Calicut Universty campus while the flowers of *P. Pubescens* was collected from the Nelliampathy forest region of Western ghats. Both were identified by Dr. A.K. Pradeep, Department of Botany, University of Calicut and specimen vouchers were deposited in the specially maintained Herbarium of Chemistry department of University of Calicut.

Extraction of Oil :

Flowers of both the plants were separately ground into paste and subjected to steam distillation for 3 hours. The oils were extracted by diethyl ether from the distillates. The ether extract was dried using anhydrous sodium sulphate and the ether evaporated. The pure oils were stored below 4°C until analyzed.

Analysis of the oils :

GC-FID analysis was carried out using a Perkin Elmer Clarus 500 GC equipped with a 30 m × 0.32 mm Elite-5MS capillary column (0.32 μm film thickness). 1 μl of each sample was diluted with 300 μl of Et_2O and injected (0.5 μl) in the "split" mode (1:30) with a column temperature programme of 40°C for 5 min, then increased to 280°C at 4°C/min and finally held at this last temperature for 10 min. Injector and detector were set at 250 and 300°C, respectively, and the carrier gas was He with a head pressure of 12.0 psi.

GC/MS analyses were carried out using a Perkin Elmer Clarus 500 GC equipped with a Clarus 500 mass spectrometer using the same capillary column and chromatographic conditions as for the GC/FID analysis. Mass spectra were acquired over 40-500 amu range at 1 scan/sec with ionizing electron energy 70 eV, ion source 200°C. The transfer line was set at 300°C, while the carrier gas was He at 1.0 ml/min.

Identification and quantification of the oil components :

The identification of essential oil components was performed by means of their retention indices (AI), by a peak matching library search (28) and by comparison with authentic reference compounds as well as with published mass spectra (29, 30). Retention indices (AI) were calculated using a n-alkane series (C_6-C_{35}) under the same GC conditions as for the samples. The relative amount (%) of individual components of the oil is expressed as percent peak area relative to total peak area from the GC/FID analyses of the whole extracts.

7.3 Results and Discussion

7.3.1 Chemical composition of the oils : The steam distillation of the plant *P. Pubescens* gave a colourless oil with a yield of about 0.03% of the fresh weight sample. The oil was pleasant smelling. From the GC/MS analysis 37 components consisting of 90.25 % of the oil were isolated from which 24 compounds were identified. Coumarin(43.67 %) was identified as the major component followed by eugenol (7.47%) and linalool (4.38%). Except sabinene all the monoterpenoids present were oxygenated. Only two sesquiterpenoids namely (E,E)-α-Farnesene and its derivative hexahydrofarnesyl acetone were identified in the oil. Twelve aliphatic compounds including 4 long chain hydrocarbons were also present in this oil.

The steam distillation of the plant *P. quadrifolia* also gave a colourless oil with a yield of about 0.035% of the fresh weight sample. The oil was slightly pungent. From the GC/MS analysis 17 compounds (97.37%) were isolated from which 15 compounds consisting of 94.72% of the oil were identified. α-Muurolol (34.02%) and caryophyllene oxide (26.92 %)were detected as the major components. Borneol was the only monoterpenoid identified while 14 sesquiterpenoids were identified . β--Caryophyllene (4.77 %) and its derivatives Caryopylla-(12),8(13)-dien-5-β-ol (5.9%), Caryopylla-(12),8(13)-dien-5-α-ol(2.6%), 14-Hydroxy-9-epi-(E)-caryophyllene (4.42%), α-Caryophyllene (α-humulene) with humulene epoxide dominated the sesquiterpene part. α-Cadinol which is the stereoisomer of muurolol was also detected upto 1.8%. Aliphatic and aromatic compounds were not detected in this oil.

Both caryophyllene and farnesene had been previously reported from the genus . But coumarin and muurolol are getting reported for the first time from any plant of the genus *pogostemon* as the major components to the best of our knowledge.

7.3.2 Olfactory analysis :

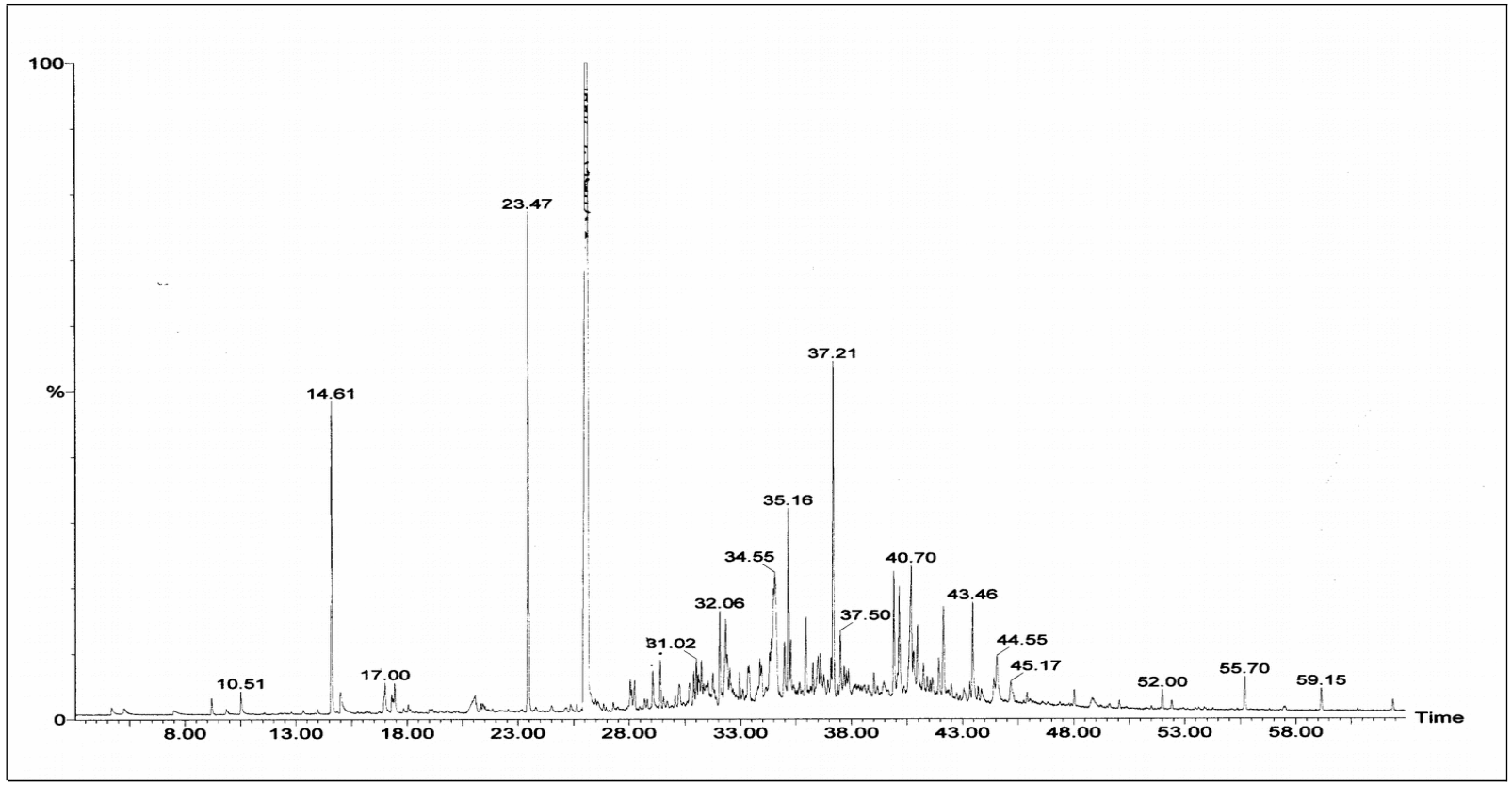
The two oils exhibit completely different odour profiles according to a preliminary olfactroic analysis [31] . The oil from *P. Pubescens* was characterized by floral impressions due to linalool, phenyl ethanol, lavandulol, neryl acetate, geranyl acetate,(E,E)-α-farnesene and hexahydrofarnesyl acetone. Of these neryl acetate and geranyl acetate add a rosy note also while lavandulol , α-farnesene and hexahydrofarnesyl acetone add a herbal note. The major components coumarin and eugenol imparts sweet and spicy notes respectively. The medicinal note of Terpinen-4-ol was also considerable. These are in support to the pleasant odour of *P. Pubescens* flowers and corresponding essential oil . The oil can be suggested for fine perfumery applications where sweet spicy floral notes are essential. Abundance of monoterpenes improves the therapeutic potential [32] of the oil that it can also be recommended for aromatherapy.

The oil from *P.quadrifolia* was characterized by spicy notes from β-caryophyllene (woody spicy), caryophyllene oxide , humulene epoxide and 1,10-di-epi-cubenol (all herbal spicy). Woody impressions were improved by α-humulene ,γ-cadinene and α-cadinol (herbal woody). Earthy mossy impressions were imparted by isomers of caryopylla-(12),8(13)-dien-5-ol. These support the spicy pungent nature of *P.quadrifolia* flowers and its essential oil. The abundance of sesquiterpenes enhances the perfumery property [32] of the oil that it can be used in fine perfumery applications where herbal woody spicy earthy notes are essential.

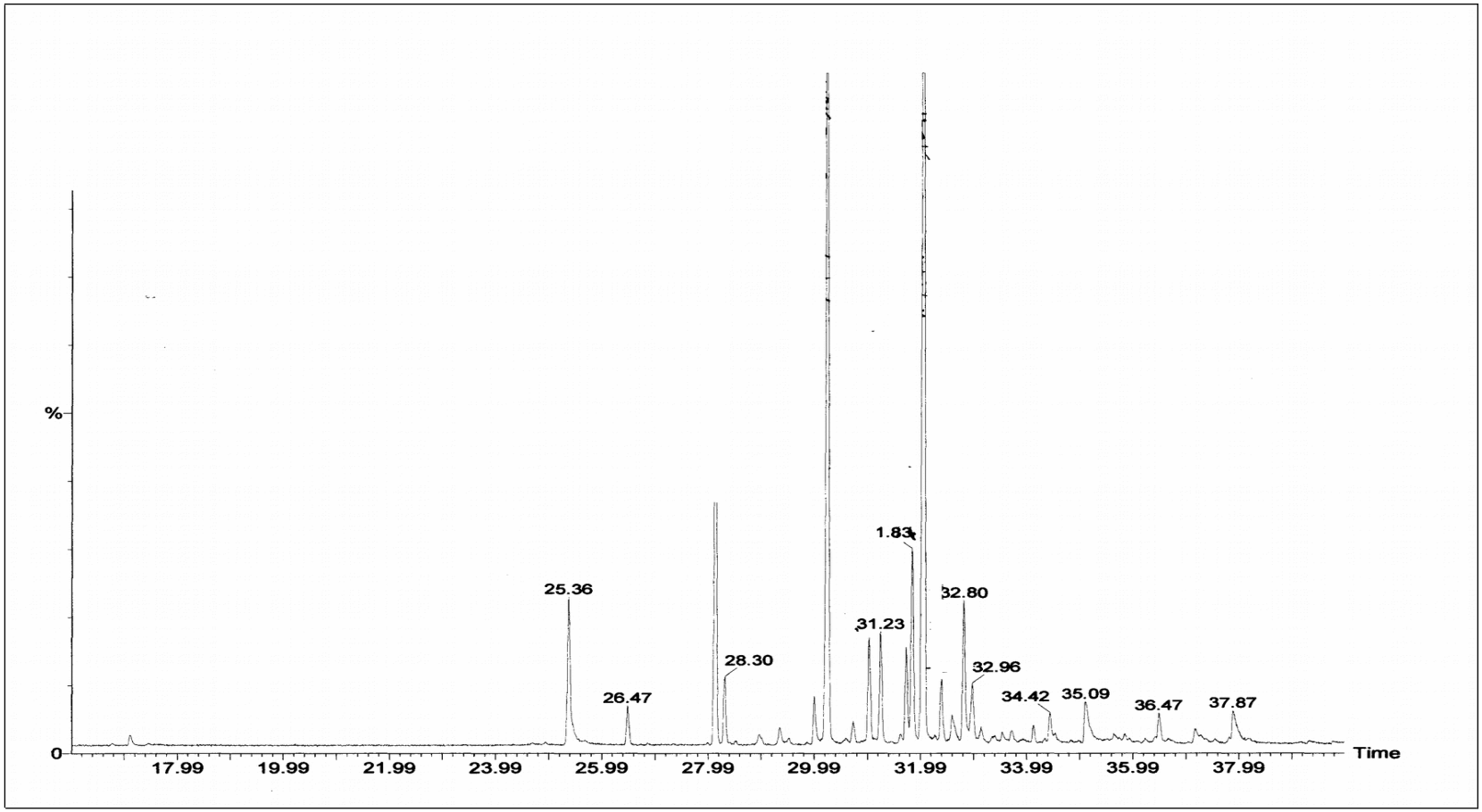
Comparison :

A close comparison revealed that the two oils differed both qualitatively and quantitatively. Not a single compound was detected as a common component either major or minor. The essential oil from *P. Pubescens* was pleasant smelling and characterised by the abundance of aliphatic compounds and monoterpene alcohols. The oil from *P.quadrifolia* was slightly pungent characterised by oxygenated sesquiterpenes. Phenyl propanoids were the major compounds in the former oil while they were

absent in the latter. Farnesene, which is an alarm pheromone for aphids and termites[33] was present in the former oil which was also absent in the latter. Olfactroic profiles of the two oils are also considerably different. These differences can be attributed to the variations in the climate, altitude and soil composition associated with the two specie. Ecological factors like insect population in the habitat may also have its role in the production of specific volatile metabolites.



GC Trace of essential oil of *P. Pubensens*



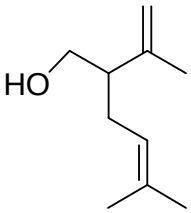
GC Trace of essential oil of *P.quadrifolia*

Table 7.1 GC/ MS Analysis with FID quantification of essential oil components of P. Pubensens and P.quadrifolia

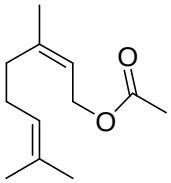
No	AI[b]	AI[c]	Compound	*P. pubensens*	*P.quadrifolia*
Aliphatic compounds					
1	847	850	cis- 3-Hexenol	tr	-
2	865	863	Hexanol	tr	-
3	982	-	Oct-1-en-3-ol	tr	-
4	997	-	Octan-3-ol	0.3	-
5	1108	1106	Phenyl ethanol	0.82	-
6	1170	-	Nona-2-en-1-ol	0.4	-
7	1275	-	Nona-2-enoic acid	0.35	-
8	1965	-	Palmitic acid	2.03	-
9	2300	2300	n-Tricosane	0.24	-
10	2499	2500	n-Pentacosane	0.29	-
11	2698	2700	n-Heptacosane	0.54	-
12	2899	2900	n-Nonacosane	0.34	-
Aromatic compound					
13	927	-	p-Benzoquinone	0.15	-
Monoterpenoids					
14	966	969	Sabinene	0.25	-
15	1098	1095	Linalool	4.38	-
16	1162	1165	Lavandulol	0.19	-
17	1165	1164	Borneol	-	0.28
18	1173	1174	Terpinen-4-ol	0.47	-
19	1189	1186	α-Terpineol	0.17	-
20	1357	1359	Neryl acetate	0.14	-
21	1377	1379	Geranyl acetate	tr	-
Sesquiterpenoids					
22	1417	1415	β-Caryophyllene	-	4.77
23	1452	1441	α-Humulene	-	1.16
24	1498	1505	(E,E)-α-Farnesene	0.29	-
25	1513	1510	γ-Cadinene	-	1.94

26	-	1568	Sesquiterpene $C_{15}H_{22}O$ MW = 218	-	1.36
27	1582	1577	Caryophyllene oxide	-	26.92
28	1609	1604	Humulene epoxide II	-	3.14
29	1618	1611	1,10-di-epi-Cubenol	-	3.18
30	1639	1629	Caryopylla-(12),8(13)-dien-5-α-ol	-	2.6
31	1639	1633	Caryopylla-(12),8(13)-dien-5-β-ol	-	5.9
32	1644	1641	α-Muurolol	-	34.02
33	1652	1653	α-Cadinol	-	1.8
34	-	1660	Sesquiterpene $C_{15}H_{22}O$ MW = 218	-	1.15
35	1668	1668	14-Hydroxy-9-epi-(E)-caryophyllene	-	4.42
36	-	1674	Sesquiterpene $C_{15}H_{22}O$ MW = 218	-	2.08
37	1836	-	Hexahydrofarnesyl acetone	1.09	-
Phenylpropanoids					
38	1347	1366	Eugenol	7.47	-
39	1431	1432	Coumarin	43.67	-
40			Isolated but unidentified	26.67	2.65
Total				90.25	97.37

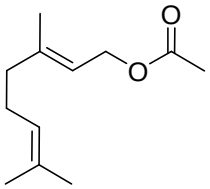
Structure of compounds identified in the essential oils from P. pubensens & P.quadrifolia



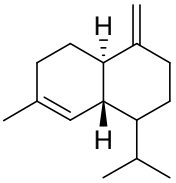
Lavandulol



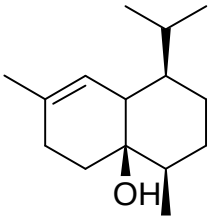
Neryl acetate



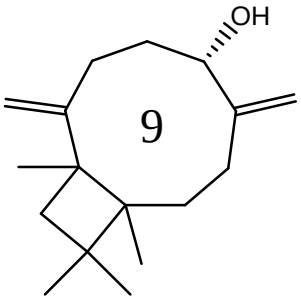
Geranyl acetate



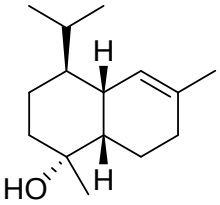
γ-cadinene



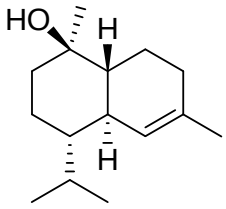
1,10-di-epi-Cubenol



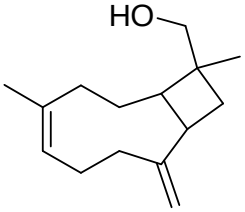
Caryopylla-(12),8(13)-dien-5-ol



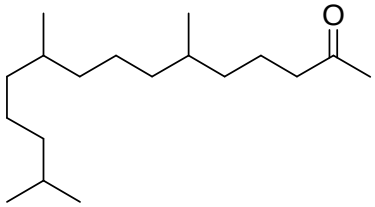
α-muurolol



α-cadinol



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



Hexahydrofarnesyl acetone

7.4 References

1. Harley. et. al. Labiatae. In : Kubitzki, K. & Kadereit, J. W. (eds.) The families and genera of vascular plants 7. Springer, Berlin & Heidelberg, pp. 167–275.2004.
2. http://www.theplantlist.org
3. G.R. Bhatti and M. Ingrouille. Systematics of Pogostemon (Labiatae). Bull. Nat. Hist. Mus.London (Bot.). 27,2, 77 – 147. 1997.
4. Ramamoorthy et al Asian J. Plant Sci. Res., 2 ,4, 515-517. 2012.
5. R. Murugan and C. Livingstone, Rheedea, 20 ,1, 21-24, 2010.
6. Trongtokit et.al. Phytotherapy Research 19 ,4, 303–309.2005.
7. Hu et al. Journal of Pharmaceutical and Biomedical Analysis 42 , 200–206, 2006.
8. Albuquerque,et. al. Acta Trop. 127,3, 181-6. 2013.
9. Sundaresan et al. Journal of Essential Oil Research Vol. 21, 2009.
10. Hammer et. al. Journal of Applied Microbiology, 86, 985–990,1999.
11. Gokulakrishnan et al. Asian Pac J Trop Dis; 3,1, 26-31, 2013.
12. Ying-Chieh Tsai et. al. Fitoterapia , 78,1,7-11, 2007.
13. Huang L et. al. Zhongguo Zhong Yao Za Zhi. 34,4, 410-3, 2009.
14. Guang wen et.al. Chinese Traditional and Herbal Drugs, 32,10. 2001.
15. Zhou et. al. Helvetica Chimica Acta, 94,2, 2011.
16. Li et.al. J. Liq Chromatogr. Relat. Technol. January 1; 34,15, 2011.
17. Park et. al. Planta Med., 64,5, 1998.
18. Murugan et. al. Nat Prod Commun. 5,12, 1961-4. 2010.
19. R Murugan, and GR Mallavarapu.Nat Prod Commun. 7,1,87-8, 2012.
20. Bhuiyan et. al. International Research Journal of Plant Science, 2,9, 2011.
21. Manoj et.al., Nat Prod Res. 26, 22, 2012.
22. M , Arif., PhD Thesis, University of Calicut, 2007.
23. Anil. et. al. J. Chem. Soc., Perkin Trans. 1, 937 – 939. 1984.
24. Nanda et.al. , Phytochemistry, 24 ,11, 1985.
25. IUCN Red List of Threatened Species. www.iucnredlist.org
26. http://indiabiodiversity.org/species/show/251235
27. Flora of Tamil Nadu, VOL. II, 1987.
28. NIST/EPA/NIH Mass Spectral Database, Version 2.1 Perkin-Elmer Instrument LLC, Copyright © 2000.
29. D. Joulain and W.A. Konig, The atlas of spectral data of sesquiterpene hydrocarbons. E.B. Verlag, Hamburg ,1998.
30. R.P. Adams, Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry, 4th ed. Allured Publ. Corp., Carol Stream, IL , 2007.
31. http://www.flavornet.org
32. D. Stewart, The Chemistry Of Essential Oils Made Simple. 3rd edn. pp 285, CARE publications, Marble Hill, MO. 2005.
33. Šobotník, et. al. Journal of Chemical Ecology 34 (4), 2008.

CHAPTER 8

ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS AGAINST PLANT PATHOGENIC FUNGI

8.1 Introduction

In India about 73% of the population is engaged in agriculture contributing about 40% of nation's income. The climate, soil, topography and vegetation within the country varies far and wide where the farming mainly depends on the two monsoons (the south - west and north – east) . As a necessary and sufficient condition to be the 'Indian' the plant diseases also exhibit aggressive diversity [1]. Plant diseases, other enemies of plants and methods of their control had been recorded even in the ancient Indian books like *Rigveda and Atharva Veda* (1500-500 BC) [2]. 'Vriksha Ayurveda' written by Surapal is in 11th century considered as the first Indian book systematically dealing with plant diseases [3].

Every economic crop cultivated in India is affected by at least one pathogen and even by a large number of pathogens. Of the 30,000 plant infections reported from different parts of the world more than 5000 are found in India. Of these 5000 more than 1000 are directly affecting the economic crops .This include the devastating diseases like blast and bacterial blight of rice, bunchy top of banana, tristeza of citrus, ring rot of potato and many other seed borne diseases [4]. The resultant losses are beyond imagination and of manifold for a developing nation like ours.

It was estimated that of the above mentioned plant diseases about 800 are of fungal origin [5]. Of these majority of them are air-borne specie belonging to *Cercospora, Alternaria, Oidium, Septoria* etc. These mainly infect stem and leaves and are comparatively easy to control. The other group, the seed-borne pathogens are really dreadful affecting most of the ornamental plants and economic corps like sugarcane. Many times the chemical and

physical treatments given to the seed destroy the beneficial organisms associated with the plant [5]. It can be found that the soil-borne pathogens attacking through roots are the most complicated to combat and control. Scientists were always found baffled along with the farmers in front of these organisms.

Fusarium, Rhizoctania, Cephalosporium, Pythium, Fomes, Ganoderma and *Ceratocystis* are the major genera whose some species causing serious root diseases. These also persist in the soil as saprophytes [6] along with many micro-organisms beneficial to the host plant. Any attempt to destroy these pathogens often upsets the microflora of the soil also. Another misery is that many of the disease like rusts of wheat, blast of rice, leaf fall of citrus and early blight of potato found to occur year after year causing continuous damage to the agriculture economy.

Important fungal diseases occurring in various types of crops cultivated in India with their characteristics , estimated economical impacts and control measures are presented in table 8.1. It is estimated that plant diseases alone cause monetary losses of worth Rs. 1560/- crores annually [7].

Crop protection by chemical methods

Methods used to control plant diseases varies according to the host plant, the kind of pathogen, the extent of their interaction and other environmental factors [9]. These methods can be generalized into three namely cultural methods, methods using host plant resistance and chemical methods [10]. Cultural control method considered as the foundation of crop protection includes host eradication, crop rotation, sanitation, irrigation, tillage and improving growth condition [9].Host plant resistance is defined as the collectable, heritable characteristics by which a plant species diminish the possibility of exploitation of that plant as a host by a pest or pathogen [11]. Use of resistant cultivars is one of the simplest and most convenient method of crop protection.

Table 8.1 Important Fungal diseases of crops cultivated in India [7]

Crops & Disease	Pathogen	Infected parts	Significance
Rice, Blast of rice	*Pyricularia oryzae*	leaf collar, culm, culm nodes, and panicle neck ,	In India, more than 266,000 tons of rice were lost [8]
Wheat, Leaf rust	*Puccinia recondita*	leaves and stalks	Million tons of wheat is damaged due to rusts only
Sugar cane, Red rot	*Colletotrichum falcatum*.	Leaves, whole cane	Epidemic fashion in India
Citrus, Gum disease	*Phytophthora citrophthora*.	Trunks, branches, leaves, fruits	Prevalent in Gujarat, Maharashtra, Andhra Pradesh, Mysore
Mango, , Anthracnose	*Colletotrzchum gleosporzdes*.	Leaves, twigs, fruits	Common in the states of Punjab and Uttar Pradesh
Banana , Panama	*Fusarium oxysporum*.	Leaves	Economic loss
Ginger, Soft rot	*Pythium aphanidermatum.*	Leaves , rhizomes	Prevalent in south India
Turmeric Rot	*Pythium graminicolum*.	Leaves ,roots rhizomes	SouthIndia
Cotton wilt	*Fusarium oxysponum*	Seedlings	
Coffee Rust	*Hemilia vastatrix.*	Leaves	Loss
Tea Blister	*Exopasidium vexans*.	Leaves, stems	Darjeeling and Assam

The control of crop diseases by chemical methods has a long history of more than a century [12]. It extends from the first fungicide sulphur [13] used against powdery mildew in grapes to the latest arrival named ‘Emerald’ (Boscalid, an Anilide) labeled for turf grass diseases like dollar spot and bentgrass dead spot [14]. Chemical agents especially fungicides belonging to

the categories of Benzimidazoles , Cadmium Compounds , Carbamic Acid Derivates , Copper Compounds , Halogenated Benzenes, Organomercury Compounds and Phthalimides have become an integral part of the crop production in many parts of the world resulting in increased yield and income [12]. But later numerous environmental and ecological problems aroused by the over usage of fungicides [15]. For example Methyl bromide, the effective soil fumigant for control of nematodes, fungi, insects and weeds in more than 100 crops worldwide had been later recognized as Class 1 stratospheric ozone depletory [16].

Many synthetic fungicides were found to generate side effects in non-target organisms especially in soil affecting cell membrane components, protein synthesis, signal transduction, respiration, cell mitosis, and nucleic acid synthesis [17]. They are also found affecting earthworms which are the most important members of the soil biota [18] . Fungicide residues have been found on food for human consumption, mostly from postharvest treatments [19] . Regular use of fungicides was realized as a serious menace to the environment, particularly when the residues persist in the soil or migrate to enter waterways to affect both terrestrial and aquatic ecosystems. For instance, long term use of copper-based fungicides result in the accumulation of copper in the soil which would adversely influence soil organisms and long-term fertility of the soil [20] of some of the commonly used fungicides. A large number of fungicides had been reported for their adverse effects on domestic animals, wildlife, birds, honey bees, fish and aquatic invertebrates which is a serious issue as there is no specific treatment for fungicides poisoning in domesticated animals and humans [21]. Above all the development or evolution of resistance in target organisms against fungicides pose a severe problem in agriculture , ecology and in all related fields [22]. All the above discussed issues speed up the research for developing new antifungal agents which are cheap, devoid of side effects to non targets and above all eco-friendly and green. Besides providing flavor and fragrance plant

essential oils readily offer themselves as one of the best alternative in this regard [23].

8.2 Review on antifungal potencies of essential oils

Several *in vitro* and *in vivo* studies have been published confirming the effect of essential oil and their major compounds on plant and human pathogenic fungi. Shukla *et al*, have reviewed the antifungal studies on essential oils from 1959 to 2010, especially in the management of post harvest fungal pests [44]. Nuzhat Tabassum and Vidyasagar have reviewed the antifungal investigations on essential oil in accordance with the plant families correlating with major constituents [24]. Some of the important findings are extracted and presented here.

The antifungal activity of essential oil of flowerheads of garland chrysanthemum *Chrysanthemum coronarium* L. was found active both in contact and headspace in vitro assays and produced hyphal growth inhibition of 12 agricultural pathogens [25]. The main compounds in the oil were camphor, α- and β-pinene and lyratyl acetate. The essential oil of *Tagetes patula* L. exerted good antifungal activity against two phytopathogenic fungi, *Botrytis cinerea* and *Penicillium digitatum*, providing total growth inhibition [26].

The essential oil from the epicarp of *Citrus sinensis* (L.) Osbeck exhibited absolute fungitoxicity against ten post-harvest pathogens [27]. The antifungal activities of essential oils from *Citrus limon* (L.)Burm.f., *C. paradise* Macfad , *C. sinesis* were reported against five phytopathogenic fungi [28]. Bergamot oil (*Citrus hystrix* DC.) showed high activity against seven species of economically important rice pathogenic fungi *Alternaria brassicicola, Aspergillus flavus, Bipolaris oryzae, Fusarium moniliforme, F. proliferatum, Pyricularia arisea* and *Rhizoctonia solani* [29].

Essential oils obtained from the well known members of lamiaceae like *Ocimum basilicum, Thymus daenensis,* sage (*Salvia officinalis* L.), lavender

(*Lavendula angustifolia* Mill.), *Origanum vulgare* L., *Rosmarinus officinalis* L. showed considerable fungicidal activities against many human and plant pathogens including food poisoning fungi [24].

The antifungal activity of the essential oils from several aromatic species from the Lauraceae family, *Aniba rosaedora* Ducke, *Laurus nobilis* L., *Sassafras albidum* (Nutt.) Nees and *Cinnamomum zeylanicum* Blume. were investigated [30]. The oils were rich in Linalool , 1, 8-cineole , safrole and trans-cinnamaldehyde and found active against seventeen micromycetes including food spoilage fungi, plant and animal pathogens. Oil obtained from *Cinnamomum osmophloeum* Kaneh. had significant antifungal activity against wood decay fungi[31]

Volatile oil from the leaves of *Calocedrus formosana* Florin. (Cupressaceae), whose timber is recognized for its natural resistance to decay displayed activity against four fungi *namely, Lenzites betulina*, *Pycnoporus coccineus, Trametes versicolor* and *Laetiporus sulphurous*. α-cadinol and murolol were identified as the active components of the oil. [32].

Oil of fennel *(Feoniculum vulgare* Mill, family-apiaceae) showed higher inhibition against *Alternaria alternate, Fusarium oxysporum* and *Aspergillus flavus*. The major component trans-anethole was identified as the active principle [33].

The antifungal activities of essential oils from *Cymbopogon citrates (Graminaceae)* was found inhibiting five phytopathogenic fungi [28]. *In vitro* antagonistic activity of volatile oil from tea tree(*Melaleuca alternifolia*-Family Theaceae) against seven species of economically important rice pathogenic fungi was also reported *[29]*.

Several reports have been made on the fungicidal potential of essential oil from neem (*Azadirachta indica* (L.)Adelb. Family-Meliaceae) which was found maximum against *Alternaria alternata, Aspergillus niger* and *Fusarium oxysporum* [34, 35].

Oil from from clove(*Syzygium aromaticum* , family -Myrtaceae*)* when mixed with that of cinnamon at appropriate ratios was reported as a potent inhibitor of the postharvest decaying fungi of grapes like, *Aspergillus niger* and *Rhizopus stolonifer* [36]. The active principles of clove was idenfied as Eugenol and carvacrol [37].

Essential oil from *Piper nigrum* L. (Piperaceae) was also reported as potent inhibitor of *Fusarium graminearum* commonly found on cereal grains, most commonly on wheat and barley [38].

Oils from caraway, clove, fennel and thyme had been found exhibiting growth inhibition potencies against six plant pathogenic fungi including *R.solani* and *F. oxysporum* in in vitro studies. Among the individual constituents of the oils monoterpenes like thymol and carvacol showed highest activity [39]. Essential oil from the aerial parts of *Artemisia sieberi* Bess with alpha-thujone, camphor, verbenol and p-mentha-1,5-dien-8-ol as the major components was found to be highly effective against the soil born pathogen *R. solani* [40].

Another report is that the essential oils from three medicinal plants *Zataria multiflora*, *Thymusvulgaris* and *Thymus kotschyanus* were found inhibiting the mycelial growth of four pathogenic fungi namely *Pythium aphanidermatum, Rhizoctonia solani* (AG4), *Fusarium graminearum* and *Sclerotinia sclerotiorum* [41]. Mustard essential oil was reported as a green substituent for methyl bromide by possessing the capacity to control some the of the soil born pathogens in the nursery independent of the soil texture [42].

Cosic et.al. tested the in vitro antifungal efficiency of Eleven essential oils (clove, rosemary, cinnamon leaf, sage, scots pine, neroli, peppermint,aniseed, caraway, lavender and common thyme) on twelve plant pathogenic fungi *(Fusarium graminearum, F. verticillioides,F. subglutinans, F. oxysporum, F. avenaceum, Diaporthe helianthi, Diaporthe phaseolorum var. caulivora, Phomopsis longicolla, P. viticola, Helminthosporium sativum,*

Colletotrichum coccodes and *Thanatephorus cucumeris).* They found that all oils except scots pine and neroli was active against some or all tested fungi while common thyme, cinnamon leaf, clove and aniseed oils were highly active [43].

In total approximately 30% of essential oils examined are inhibitory to bacteria, and more than 60% of have been shown to be inhibitory to fungi [44]. More possibilities are still open for further investigations with newly reported endemic plants and chemotypes.

8.3 Present work

Antifungal potencies of five essential oils against three plant pathogens in '*in vitro'* conditions are investigated in the present work. These essential oils are collected from the aromatic plants *Limnophila repens*, *Atalantia monophyllum*, *Curcuma ecalcerata*, *Curcuma aerugenosa* , and *Costus speciosus* **.** Pathogens selected for this study are *Pythium aphanidermatum, Rhizoctonia solani* and *Fusarium oxysporum* which cause serious diseases to various plants. A brief description of these soil fungi is given in the table 8.2[45,46]

Experimental

Plants under investigation were collected from different parts of Kerala, south India. Essential oils from each of them are extracted by hydrodistillation . The composition (identified by GC and GC/MS) of the oils from *L. repens*, *A. monophyllum* and *C.ecalcerata* have been discussed in chapter 2, 4 and 6 respectively while that of oils from *C.aerugenosa* , and *Costus speciosus* gets referred to a previous works in our research lab[47, 48].

Analysis of Antifungal Activity by *in vitro* bioassay

A definite amount of each oil was accurately weighed out in to a clean standard flask and made up to 100 ml using diethyl ether. Various concentrations of test solutions were prepared in PDA (Potato Dextrose Agar)

medium to obtain concentrations such as 5.0ml/100ml, 10.0ml/100ml, 15.0ml/100ml and 20ml/100ml of the ether solution before autoclaving the medium. It was also tested that, whether the activities were lost during autoclaving, and it was confirmed that, the oil is thermo-stable. In addition, two controls were (control with respective concentrations of diethyl ether as control and without any test solution or diethyl ether as absolute control) also maintained. For each treatment, three replications were maintained.

Table 8.2 Description of three Soil borne pathogens under investigation

Pythium aphanidermatum [45]	
Host range	Annuals and bedding plants
Existence	wet soils, Warm temperatures
Plant part infected	Leaves, rhizomes
Diseases	Damping off, root and stem rots, and blights of grasses and fruit
Affected crops	Economic crops - beets, peppers, cucurbits, cotton & turf grasses
Importance	Affects crops produced even in greenhouses and soilless culture
Rhizoctonia solani [46]	
Host range	herbaceous plants
Existence	warm wet weather
Plant part infected	Parts below ground such as the seeds, hypocotyls, and roots.
Diseases	Damping off of the seed, collar rot, root rot
Affected crops	Turfgrass, potatoes ,cucumber, sugar beet, rice and many cerals
Importance	Major yield losses of 25%-100%, increased soil tare
Fusarium oxysporum [45]	
Host range	Broad- animals, arthropods , humans as well as plants
Existence	Dormant for 30 years before resuming virulence
Plant part infected	Root, seed
Diseases	Fusarium wilt (plant death without any outward sign of infection)
Affected crops	Banana, ginger, cotton, sweet potato
Importance	Affect every crop without discrimination, Tough to eradicate

The cultures of three fungi, for the evaluation studies were obtained from Culture Collection Centre, PG Department of Botany, The Zamorin's Guruvayoorappan College, Calicut. The cultures were maintained in its pure form in PDA slants and being maintained in BOD. The pure cultures were sub-cultured in PDA plates and fresh cultures (48 hours old) used for the evaluation studies. Culture discs of 0.5cm collected from actively growing margins were used for inoculation. The inoculated plates were incubated at room temperature for 72 hours. The radial growth of fungus in each plate was measured and recorded. Percentage of inhibition and half maximal inhibitory concentration (IC_{50}) (concentration which inhibits growth of 50% of the pathogens) corresponding to each pathogen-essential oil set is calculated. The data were analyzed statistically (MSTATC) and results discussed.

8.4 Results and Discussion

Each oil analyzed was found inhibiting one or the other organism to some extent. Activity of individual oils can be correlated with their chemical composition . The results are tabulated in tables: 8.3-8.14. Activity of each oil is discussed separately below.

Activity of *L.Repens* oil :

The oil showed good growth inhibitory effect on *P. aphanidermatum* . It was less active on *F.oxysporum* and even less on *R.solani*. At the lowest concentration of 05 ml/100ml the microbial growth was inhibited up to 40% in the case of *Pythium*. About 70% inhibition was shown at a concentration of 20ml/100ml . IC50 value of LR oil for *Pythium* was 111.08 mg/100ml while that for *Fusarium* was 566.04 mg/100ml. Increase in concentration had no considerable role in the activity. A critical difference (C.D) of 1.353 was obtained from the statistical analysis (DMRT) indicating that the inhibition is significant for at least this much difference between two consecutive values.

Table 8.3 Effect of *L.repens oil* on the pathogens *in vitro* – (Radial growth in mm)

Concentrations	*Pythium*			*Rhizoctonia*			*Fusarium*		
	24 hrs	**48 hrs**	**72 hrs**	**24 hrs**	**48 hrs**	**72 hrs**	**24 hrs**	**48 hrs**	**72 hrs**
05.0ml/ 100ml	06.167	15.83	21.83	11.17	38.50	45.00	05.17	09.33	13.33
10.0ml/100ml	06.00	15.67	25.50	16.50	45.00	45.00	04.00	10.83	17.67
15.0ml/100ml	05.33	08.00	14.83	07.33	26.83	45.00	01.50	07.00	12.83
20ml/100ml	00.66	07.83	12.33	10.67	33.83	45.00	01.00	06.50	11.83
Control	11.17	28.00	40.00	26.33	45.00	45.00	03.67	10.00	15.10
Absolute control	07.00	20.67	28.17	22.67	45.00	45.00	02.00	08.17	13.67
CD at 5% = 1.353									

Fungicidal activity of LR oil can be attributed to the monoterpenes terpinen-4-ol, sabinene, terpinolene and γ-terpinene which are its major components. Terpinen-4-ol had been several times reported for its similar biological properties [49, 50]. Antifungal activity terpinolene [51] and γ-terpinene [52] had also been reported.

It is to be mentioned that essential oil from *L.repens* collected from south east Asia had been earlier reported to be sensitive against *Aspergillus niger*, *Candida albicans* and *Rhizopus nigricans* [56].

Table 8.4 Percentage Inhibition at various concentrations by Essential oil of L.repens.

On *Pythium*			
Concentration	**After 24 hrs**	**After 48hrs**	**After 72 hrs**
05.0ml/ 100ml	44.79	43.46	45.43
10.0ml/100ml	46.28	44.04	36.25
15.0ml/100ml	52.28	71.43	62.93
20ml/100ml	94.09	72.04	69.18
On *Rhizoctania*			
Concentration	**After 24 hrs**	**After 48hrs**	**After 72 hrs**
05.0ml/ 100ml	57.58	14.44	0.00
10.0ml/100ml	37.33	0.00	0.00
15.0ml/100ml	72.16	40.38	0.00
20ml/100ml	59.48	24.82	0.00
On *Fusarium*			
Concentration	**After 24 hrs**	**After 48hrs**	**After 72 hrs**
05.0ml/ 100ml	0	6.70	11.72
10.0ml/100ml	0	0	0
15.0ml/100ml	59.13	30.00	15.03
20ml/100ml	72.75	35.00	21.66

Activity of *A.monophylla* oil :

Essential oil from the leaves of *A.monophylla* also showed good inhibition on *P. aphanidermatum* but a very low activity on *F.oxysporum* and *R.solani*. A critical difference of 1.496 obtained from statistical analysis indicated that the inhibition is significant for at least this much difference between two consecutive values. IC50 value of AM oil for *Pythium* was 329.04 mg/100 ml which was higher than that in the case of LR oil. This oil

was rich in compounds previously reported for its fungicidial potencies linalool [53] and terpinen-4-ol.

Table 8.5 Effect of *A.monophyllum* oil on the pathogens *in vitro* – (Radial growth in mm)

Concentrations	*Pythium*			*Rhizoctonia*			*Fusarium*		
	24 hrs	**48 hrs**	**72 hrs**	**24 hrs**	**48 hrs**	**72 hrs**	**24 hrs**	**48 hrs**	**72 hrs**
05.0ml/ 100ml	08.33	17.50	30.17	15.83	45.00	45.00	04.00	08.33	12.67
10.0ml/100ml	14.33	25.00	40.67	19.33	45.00	45.00	02.00	14.50	15.83
15.0ml/100ml	13.47	25.17	41.00	17.17	45.00	45.00	02.88	14.67	18.67
20ml/100ml	06.33	17.17	26.17	14.00	45.00	45.00	02.00	09.83	14.17
Control	11.17	28.00	40.00	26.33	45.00	45.00	03.66	10.00	15.10
Absolute control	07.00	20.33	28.17	22.67	45.00	45.00	02.00	08.33	13.76
CD at 5% = 1.496									

Both LR oil and AM oil had monoterpene alcohols as their major components . Formation of hydrogen bonds between the hydroxyl groups of the components and the active sites of enzymatic systems related with the synthesis of microbial cells had been recognized as one of the major methodology of fungitoxic activity of essential oil [54]. This mechanism may be working well on *P. aphanidermatum* but not on *F.oxysporum* and *R.solani*. The difference in activity may be due to the higher percentage of monoterpene hydrocarbons in LR oil (42.83%) compared to that AM oil (3.29 %) . The basis of fungitoxicity of monoterpene hydrocarbons was explained by their ability to diffuse into pathogens to damage the cell membranes and membrane constituents [55]. A previous report of *F.oxysporum* being inhibited by the ethanol extract of leaves of *A.monophylla* [57] is also to be mentioned here.

Table 8.6 Percentage Inhibition by Essential oil of *A.monophylla.*

On *Pythium*			
Concentration	**After 24 hrs**	**After 48hrs**	**After 72 hrs**
05.0ml/ 100ml	25.43	37.50	24.58
10.0ml/100ml	0	10.71	0
15.0ml/100ml	0	10.11	0
20ml/100ml	43.33	38.68	34.58
On *Rhizoctania*			
Concentration	**After 24 hrs**	**After 48hrs**	**After 72 hrs**
05.0ml/ 100ml	39.88	0.00	0.00
10.0ml/100ml	26.59	0.00	0.00
15.0ml/100ml	34.79	0.00	0.00
20ml/100ml	46.83	0.00	0.00
On *Fusarium*			
Concentration	**After 24 hrs**	**After 48hrs**	**After 72 hrs**
05.0ml/ 100ml	0	16.70	16.09
10.0ml/100ml	45.36	0	0
15.0ml/100ml	21.31	0	0
20ml/100ml	45.36	1.70	6.16

Activity of *C.ecalcerata* oil :

Essential oil from the leaves of *C.ecalcerata* showed the highest activity on all the three organisms tested. A critical difference of 0.801 was obtained from statistical analysis indicated that the inhibition is significant for at least this much difference between two consecutive values. IC50 values obtained for *P. aphanidermatum* , *F.oxysporum* and *R.solani* were very low ; 4.39 mg, 1.28mg and 2.4 ml per 100 ml respectively indicating a higher potency . Both *Rhizoctania* and *Fusarium* were inhibited very effectively . Also the oil showed a very high sensitivity towards *Rhizoctania*.

Table 8.7 Effect of *C. ecalcarata* essential oil of on the pathogens (Radial growth in mm)

Concentrations	*Pythium*			*Rhizoctonia*			*Fusarium*		
	24 hrs	**48 hrs**	**72 hrs**	**24 hrs**	**48 hrs**	**72 hrs**	**24 hrs**	**48 hrs**	**72 hrs**
05.0ml/ 100ml	10.00	10.00	40.83	00.00	00.00	10.50	4.66	05.50	10.33
10.0ml/100ml	17.83	20.67	38.00	01.00	01.66	08.16	05.33	05.66	10.5
15.0ml/100ml	14.50	17.83	21.17	01.00	00.83	08.33	05.33	05.33	07.33
20ml/100ml	17.67	21.50	21.00	01.00	01.00	05.83	03.16	04.16	07.50
Control	22.00	24.00	45.00	08.00	10.17	20.50	07.66	10.83	24.50
Absolute control	23.17	30.67	45.00	08.00	10.00	21.17	07.83	11.67	26.67
CD at 5% =0.801									

Table 8.8 Percentage Inhibition by Essential oil of *Cecalcerata.*

On *Pythium*			
Concentration	**After 24 hrs**	**After 48hrs**	**After 72 hrs**
05.0ml/ 100ml	54.55	58.33	9.27
10.0ml/100ml	18.95	13.88	15.56
15.0ml/100ml	34.09	25.71	52.96
20ml/100ml	19.68	10.42	53.33
On *Rhizoctania*			
Concentration	**After 24 hrs**	**After 48hrs**	**After 72 hrs**
05.0ml/ 100ml	100.00	100.00	48.78
10.0ml/100ml	87.50	83.68	60.20
15.0ml/100ml	87.50	91.84	59.37
20ml/100ml	87.50	90.17	71.56
On *Fusarium*			
Concentration	**After 24 hrs**	**After 48hrs**	**After 72 hrs**
05.0ml/ 100ml	39.16	49.22	57.84
10.0ml/100ml	30.42	47.74	57.14
15.0ml/100ml	30.42	50.78	70.08
20ml/100ml	58.75	61.59	69.39

The higher fungitoxicity of CE oil can be accredited to the most abundant components and 1,8-cineole and piperitenone. These oxygenated monoterpenes had been earlier reported for their antifungal properties as part of many essential oils [24, 62]. Potency of piperitenone against phytopathogenic fungi had been earlier reported [58]. The corresponding oxide which occur together in most of the piperitenone rich oils was also found fungitoxic [59]. 1,8-cineole was found to be highly capable of eliminating the fungal pathogens by altering the structure and moisture of mucous membranes of fungal cells and interrupting the respiratory processes [60]. Various monoterpnes with antimicrobial potencies like camphor [61] also seem to be contributing to the total efficiency.

It was for the first time the antimicrobial efficiency of *C.ecalcerata* against any pathogen, that also with very promising results being reported.

Activity of *C. aerugenosa* oil :

Essential oil from the leaves of *C.aeruginosa* also inhibited all the three fungi tested to an noticeable extent. Compared to the other two the inhibition was much higher on *Rhizoctania* , comparable with the activity shown by CE oil . IC50 value obtained for *Rhizoctania* to be exact 2.09 mg/100ml was also close with that obtained with CE oil. IC50 values obtained for *Pythium* (72.32) and *Fusarium* (213.83) were too higher to compare with those obtained with CE oil.

Table 8.9 Effect of of *C. aerugenosa* essential oil on the pathogens– (Radial growth in mm)

Concentrations	*Pythium*			*Rhizoctonia*			*Fusarium*		
	24 hrs	**48 hrs**	**72 hrs**	**24 hrs**	**48 hrs**	**72 hrs**	**24 hrs**	**48 hrs**	**72 hrs**
05.0ml/ 100ml	12.67	23.67	39.67	04.37	04.66	07.66	04.66	05.83	17.00
10.0ml/100ml	09.83	19.50	33.67	04.50	04.16	07.66	05.00	06.00	14.00
15.0ml/100ml	09.66	20.00	35.17	03.33	04.16	07.33	04.83	05.66	20.33
20ml/100ml	10.33	16.83	27.50	02.50	03.16	06.83	04.83	05.83	17.83
Control	22.00	24.50	45.00	08.83	10.17	20.33	07.63	10.67	26.50
Absolute control	23.67	30.67	45.00	08.83	10.17	21.17	07.83	11.83	24.50
CD at 5% =1.094									

Major compounds like 1,8-cineole and camphor may be primarily contributing to activity of this oil. Comparison with other oils show that the low monoterpene content may be one reason for its lower activity against *Pythium* and *Fusarium*. 1,8-Cineole and camphor are the main common components of CE and CA oil. An earlier report [63] presented that essential oils with these two compounds as the major components showed higher activity against *R.solani* while lower acivity against *F.oxysporum*. So it can be finalized that the compound effect of 1,8-cineole and camphor is the basis of higher activity of both CE and CE oils on *Rhizoctania* .

It can also derived that it is none other than piperitenone making *C.ecalcerata* oil exclusively active on *f. oxysporum* among all the oils tested.

Table 8.10 Percentage Inhibition by Essential oil of *C.aeruginosa*

On *Pythium*			
Concentration	**After 24 hrs**	**After 48hrs**	**After 72 hrs**
05.0ml/ 100ml	42.41	3.39	11.84
10.0ml/100ml	55.32	20.41	25.18
15.0ml/100ml	56.09	18.37	21.84
20ml/100ml	53.05	31.31	38.89
On *Rhizoctania*			
Concentration	**After 24 hrs**	**After 48hrs**	**After 72 hrs**
05.0ml/ 100ml	50.51	54.18	62.32
10.0ml/100ml	49.04	59.10	62.32
15.0ml/100ml	62.29	59.10	63.94
20ml/100ml	71.69	68.93	66.40
On *Fusarium*			
Concentration	**After 24 hrs**	**After 48hrs**	**After 72 hrs**
05.0ml/ 100ml	38.93	45.36	35.85
10.0ml/100ml	34.47	43.77	47.17
15.0ml/100ml	36.70	46.95	23.28
20ml/100ml	36.70	45.36	32.72

Activity of *Costus speciosus* oil :

Essential oil from the rhizomes of *Costus speciosus* showed higher inhibition of all the three pathogens. The inhibition was maximum for *Pythium*. . A critical difference of 0.983 was obtained from statistical analysis indicated that the inhibition is significant for at least this much difference between two consecutive values. The IC50 values obtained for *P. aphanidermatum* , *F.oxysporum* and *R.solani* were respectively 97.06, 113.31 and 50.89 mg per 100 ml which were a bit high in comparison.

Also this oil showed a very high sensitivity towards *Pythium* and *Rhizoctania.*

Table 8.11 Effect of of *C.speciosus* essential oil on the pathogens (Radial growth in mm)

Concentrations	***Pythium***			***Rhizoctonia***			***Fusarium***		
	24 hrs	**48 hrs**	**72 hrs**	**24 hrs**	**48 hrs**	**72 hrs**	**24 hrs**	**48 hrs**	**72 hrs**
05.0ml/ 100ml	11.00	11.17	15.00	01.66	01.66	17.50	04.16	04.83	10.50
10.0ml/100ml	07.83	08.00	10.83	00.00	00.00	04.33	02.33	04.00	07.33
15.0ml/100ml	13.00	25.17	42.33	03.16	04.16	11.33	02.66	03.16	17.33
20ml/100ml	00.00	00.00	03.00	00.00	00.00	04.50	01.33	03.00	05.00
Control	22.00	24.00	45.00	08.83	10.00	20.33	07.66	10.67	24.00
Absolute control	23.67	30.67	45.00	08.83	10.17	21.17	07.83	11.83	26.67
CD at 5%=0.983									

Table 8.12 Percentage Inhibition by Essential oil of *Costus speciosus.*

On *Pythium*			
Concentration	**After 24 hrs**	**After 48hrs**	**After 72 hrs**
05.0ml/ 100ml	50.00	53.46	66.67
10.0ml/100ml	64.41	66.67	75.93
15.0ml/100ml	40.91	0	0
20ml/100ml	100.00	100.00	93.33
On *Rhizoctania*			
Concentration	**After 24 hrs**	**After 48hrs**	**After 72 hrs**
05.0ml/ 100ml	81.20	83.40	13.92
10.0ml/100ml	100.00	100.00	78.70
15.0ml/100ml	64.21	58.40	44.27
20ml/100ml	100.00	100.00	77.87
On *Fusarium*			
Concentration	**After 24 hrs**	**After 48hrs**	**After 72 hrs**
05.0ml/ 100ml	45.69	54.73	56.25
10.0ml/100ml	69.58	62.51	69.46
15.0ml/100ml	65.27	70.38	27.79
20ml/100ml	82.64	71.88	79.17

The major component of this oil zerumbone had earlier been identified as a strong fungitoxic agent against *R.solani* [64]. Essential oils containing the other two components humulene [65] and camphene [66] were also reported to be active against many pathogens. The high amount of zerumbone make the oil highly active despites its low monoterpene content.

Major components are usually found to be responsible for the antimicrobial activities of essential oils. But sometimes the minor and trace components may also play significant role in synergistic and antagonistic interactions to influence the total activity of the oil [67].

Table 8.13 IC50 Values (mg/ 100 ml) of essential oils for the three pathogens

Pathogens	***Oils***				
	LR	**AM**	**CE**	**CA**	**CS**
Pythium	111.08	329.04	4.39	72.32	97.06
Rhizoctonia	-	-	1.28	2.09	113.31
Fusarium	566.04	-	2.4	213.83	50.89

LR-*L. repens*, AM-*A.monophyllum*, CE-*C. ecalcerata*, CA-*C.aerugenosa* ,
CS- *C.speciosus*

Table 8.14 Overall composition and Inhibitory effect of essential oils

Oil	MT HC %	MT O %	ST %	Major components with percentage	Inhibition		
					P	R	F
LR	42.83	23.45	14.72	Terpinen-4-ol (19.33) Sabinene (18.34) Terpinolene (7.29) γ-Terpinene (6.39)	++	-	+
AM	3.29	82.44	10.12	Linalool-51.65 Terpinen-4-ol-15.91 α-Terpineol-6.58	+	-	-
CE	0.8	87.0	8.01	Piperitenone (47.28) 1,8-Cineole (34.75)	+++	+++	+++
CA	4	35.9	47.8	l,8-Cineole (17.7) Curzerenone **(**10.5**)** Camphor (7.5)	+	+++	++
CX	7.74	2.22	83.91	Zerumbone (55.11) Humulene (20.55) Camphene (4.96)	+++	+++	+++

LR-*L. repens*, AM-*A.monophyllum*, CE-*C. ecalcerata*, CA-*C.aerugenosa* , CS- *C.speciosus*
P- *Pythium aphanidermatum*, R-*Rhizoctonia solani* , F-*Fusarium oxysporum*
MTHC- Monoterpene hydrocarbon, MTO- Oxygenated monoterpenes, ST- Sesquiterpenes
+ : Inhibition, ++ : High Inhibition, +++ : Very high Inhibition, - : No effect

8.5 Conclusions

1. All the essential oils were found inhibiting the growth of one or the other pathogen tested.

2. *P. aphanidermatum* was inhibited by all the essential oils tested.

3. Essential oil from *A.monophylla* showed the least potential by showing activity only against *P. aphanidermatum* .

4. Oil from *L.repens* was moderately active on all the pathogens.

5. Essential oils from *C. ecalcerata*, *C. aerugenosa* , and *Costus speciosus* inhibited all the three pathogens significantly.

6. Most significant inhibition of *R.solani* was shown by *C. ecalcerata* and *C. aerugenosa* while that of *F.oxysporum* was by *C. ecalcerata*

7. Oil from *Costus speciosus* showed maximum percentage of inhibition throughout the experiments but offered a bit higher IC50 values.

8. Essential oil from *C. ecalcerata* exhibited the highest potency with the lowest IC50 values for all the pathogens.

9. The inhibition of organisms belonging to *Pythium* and *Fusarium* may be mainly due to monoterpens which are acting either through the cell membranes or by forming hydrogen bonds with enzymes.

10. Special compounds like piperitenone, 1,8-cineole or zerumbone is essential for the inhibition of *Rhizoctania*.

Making use of these results new antifungal agents with an increased efficiency and reduced side effects can be formulated against different plant pathogenic fungi. Compounds like piperitenone, 1,8-cineole and zerumbone can be taken as the lead molecules for further *in vitro* and *in vivo* studies.

8.6 References

1. Raychaudhuri et.al., Ann.Rev.Phytopath., 10, 21-36, 1972.
2. D.V. Singh , PLANT PATHOLOGY, Introductory Plant Pathology ,New Delhi ,2007, http://nsdl.niscair.res.in/
3. A.V.S.S. Sambamurty, A Textbook of Plant Pathology, pp : 01, I. K. International Pvt Ltd, 2006.
4. G. Rangaswami, and A. Mahadevan, Diseases Of Crop Plants In India, 4th edn, 1-8, PHI Learning Pvt. Ltd,1998.
5. G. Rangaswami, and A. Mahadevan, Diseases Of Crop Plants In India, 4th edn, 482, PHI Learning Pvt. Ltd,1998.
6. Naik, Manjunath and G.S.Devika Rani, Advances in Soil Borne Plant Diseases, pp : 55, New India Publishing, 2008.
7. K T. Chandy, Booklet No. 342, Plant Disease: Control: PDCS – 4, http://www.inseda.org
8. http://www.knowledgebank.irri.org
9. Dale Walters, Disease Control in Crops: Biological and Environmentally-Friendly Approaches, pp – 07, John Wiley & Sons, 2009.
10. Dale Walters, Disease Control in Crops: Biological and Environmentally-Friendly Approaches, pp – 03, John Wiley & Sons, 2009.
11. D. Dent, Integrated Pest Management, pp-56, Springer, 1995.
12. U. Gisi, Ilan Chet and Maria Lodovica Gullino, Recent Developments in Management of Plant Diseases, (Google eBook), Springer, 2009.
13. http://www.melpat.com.au/documents/ TechNote_SulphurAsAFungicide_001.pdf
14. http://buckeyeturf.osu.edu/
15. Hung-Chang Huang and Min-Tze Wu, Plant Pathology Bulletin 18: 1-12, 2009.
16. World Meteorological Organization (WMO). Scientific assessment of ozone depletion: 2002 Global Ozone Research Monitoring Project Report #47,Geneva., Switzerland, 498 pp. 2003
17. Chao Yang et. al., ISRN Ecology, 2011 http://dx. doi.org/10.5402/ 2011/ 130289
18. Clive A. Edwards and Patrick J. Bohlen, Reviews of Environmental Contamination and Toxicology, 125, , pp 23-99,1992.
19. G.T Brooks, & T.R Roberts, Pesticide Chemistry and Bioscience. Published by the Royal Society of Chemistry. 1999.
20. Wightwick et.al., Environmental Risks of Fungicides Used in Horticultural Production Systems, Fungicides, Odile Carisse (Ed.), http://www.intechopen.com/books/fungicides/environmental-risks-of-fungicides-used-in-horticulturalproduction-systems 2010.
21. Hasan. and Oruc Fungicides and Their Effects on Animals, Fungicides, Odile Carisse (Ed.), 2010. http://www.intechopen.com/books/ fungicides/fungicides-and-theireffects-on-animals

22. Keith Brent in Fungicide Resistance in Crop Protection: Risk and Management edited by Tarlochan S. Thind pp 1-13, CABI, 2012 .
23. K.A. hammer and C.F. Carson In Chapter 11, Lipids and Essential Oils as Antimicrobial Agents edited by Halldor Thorma John Wiley & Sons, 2010.
24. Vidyasagar et al. Int J Pharm Pharm Sci, Vol 5, Suppl 2, 19-28, 2013.
25. Alvarez -Castellanos et.al. J. Phytochemistry. 57(1):99-102.2001.
26. Romagnoli et.al., Protoplasma. 225(1-2):57-65. 2005.
27. N Sharma and A Tripathi World J Micro Biotech. 22: 587-593. 2006.
28. OL Sun et.al., Plant Path J.; 23(2): 97-102. 2007.
29. Pitipong et.al.,Asian J Food Ag-Ind.; Special Issue, S374-S380. 2009.
30. Simic et.al., . Phytother Res. 18(9):713-7. 2004.
31. Wang et.al., Biores Tech. 96(7):813-8. 2005.
32. Cavaleiro et.al., J Appl Microbiol. 100:1333-1338. 2006.
33. Shabnam et.al.,. African J Biotech.;11(94):16065-16070. 2012.
34. SAR Kazmi et.al.,Pak J Bot.; 27(1): 217-220. 1995.
35. Locke JE. Fungi. In: The Neem Tree, source of Unique National Products for Integrated pest Management, Medicine, Industry and Other proposes. (Ed.): H. Schmutterer. V.C.H, Weinheim, Germany.; 118-125. 1995.
36. U Sukatta et.al.,Kasetsart J (Nat. Sci).; 42: 169–174. 2008.
37. H. Martini, et.al., Italian J Food Sci.; 1:63-7. 1996.
38. .G Singh et.al., J Sci Food Agric. 2004a; 84: 1878-1884.
39. S.R. El-Zemity and S.M. Ahmed, J.Pest cont& Enviorn. Sci. 13,1,61-72, 2005.
40. M. Farzaneh, et.al., Commun. Agric. Appl. Biol. Sci. **2006**, 71, 1327–1333.
41. M. Amini et.al., Trakia Journal Of Sciences, Vol. 10, No 1, 2012
42. O.D. Dingra et.al., Fitopatol. bras. 29(6), 2004.
43. J. Cosic et.al., POL. J O PRIVREDA 16: (2) 25-28, 2010.
44. Shukla et al, Current Discovery 1(1) page 33-45, 2012.
45. Charles R. Lane, Paul Beales, Kelvin Hughes, Fungal Plant Pathogens, CABI, 2012. Google books.
46. B. Sneh, Rhizoctonia Species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control, Springer, 1996 . Google books.
47. Mity Thampi, PhD Thesis, University of Calicut, 2012.
48. Jirovetz et.al., Journal of Essential Oil Research, 12:1, 47-49, 2000.
49. Terzi et.al., Lett Appl Microbiol. 44(6):613-8. 2007.
50. Ramage et.al., Front Microbiol. ; 3: 220. 2012.
51. Faruq etal., Z Naturforsch C. Sep-Oct;57(9-10):863-6. 2002.
52. Vale-Silva et.al. J Med Microbiol. ;61(2) 252-60. 2012.
53. Duman et.al., Nat Prod Commun. 5(6):969-74. 2010.
54. Daferera et.al., J. Agric. Food Chem., 48: 2576-2581, 2000.
55. Sikkema, J., J.A. de Bont and B. Poolman, Microbiol. Mol. Biol. Rev., 59: 201-222,1995.
56. G.H. Schmelzer, Limnophila R.Br.[Internet] Record from Proseabase.

Van Valkenburg, J.L.C.H. and Bunyapraphatsara, N. (Editors).PROSEA (Plant Resources of South-East Asia) Foundation, Bogor, Indonesia (2001). http://www.proseanet.org.
57. P.S.Pavithra,N. Sreevidya and Rama.S. Varma, Journal of Medicinal and Aromatic Plant Sciences, 31, 63-72, 2009.
58. C Romagnoli et.al., Protoplasma. 225(1-2):57-65. 2005.
59. B. Marinkovié etal, Bocconea 16(2) – 2003.
60. V.T Andriole.. J Antimicrobial Chemo. 44:151-162. 1994.
61. V. Moleyar and P. Narasimham, Food Microbiology, ,3,331-336. 1986.
62. Gudrun Lang and Gerhard Buchbauer, Flavour Fragr. J., 27, 13–39, 2012.
63. Pitarokili et.al., J Agric Food Chem. 21;51(11):3294-301. 2003.
64. N. Kishore and R. S. Dwivedi, Mycopathologia, 120,3, 155-159, 1992.
65. Edziri et.al., Nat Prod Res. 24(9):789-96. 2010.
66. Magwaet.al., J Ethnopharmacol. 3;103(1):85-9. 2006.
67. Ismail et.al., Science International, 1: 148-154, 2013.