ISOLATION, STRUCTURE ELUCIDATION AND PROPERTIES OF SECONDARY METABOLITES IN PLANTS

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By

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Forwarded

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CERTIFICATE

This is to certify that the thesis entitled "*Isolation, Structure elucidation and properties of secondary metabolites in plants*" is an authentic record of the research work carried out by **Muhammed Arif M.**, in the Department of Chemistry, under my supervision in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry, under the Faculty of Science of the University of Calicut and that no part thereof has been presented earlier for any other degree.

05/10/07

C.U Campus,

Dr. P. Mohamed Shafi
(Supervising Teacher)

DECLARATION

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

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Muhammed Arif M.

CONTENTS

PREFACE

During the last few decades natural products research has advanced tremendously through the field of chemistry, food science and materials sciences. Comparison of natural products from microorganisms, lower eukaryotes, animals, higher plants and marine organisms are now well documented.

Natural products are ubiquitous in our everyday life. Some are active constituents of many medicines, vitamins, food additives flavor and fragrance, agrochemical and pesticides. Indeed natural products are essential components of our life itself, which include carbohydrates, nucleic acids, lipids, vitamins, hormones, steroids, prostaglandins etc.

With the advent of improved chromatographic separation techniques, the separation of various natural products including positional and stereo isomers is achieved routinely. Newer spectroscopic techniques such as two-dimensional high resolution Nuclear Magnetic Resonance Spectroscopy, Infrared and Raman Spectroscopy, X-ray crystallography, high-resolution electron microscopy and mass spectrometry have simplified the structural elucidation of new natural products.

The secondary metabolites function as medicines, fungicides, perfumes and flavoring agents. Many compounds from plants and their derivatives were incorporated into modern medicine. The healing action of traditional medicine

such as Ayurveda can be scientifically explained on the basis of phytochemical studies.

The work presented in this thesis deals with chemical investigation of the barks and seed oils of *Macaranga peltata,* analysis of essential oil from leaves of three varieties of *Piper betle,* analysis of essential oil of flowers of *Hyptis capitata* and *Pogostemon Paniculatus* , analysis of essential oil and evaluations antimicrobial properties of *Litsea lavigata* fruit essential oil and its column chromatographic fractions.

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

The first chapter is divided into two sections. Section 1 deals with the extraction of 3-acetylaleuritolic acid from both male and female plants of *Macaranga peltata.* The acid catalysed lactonisation of 3-acetyaleuritolic acid is also investigated. In section 2, single crystal X-ay analysis of 3-acetylaleuritolic acid is presented.

The second chapter deals with the analysis of fatty oils present in the seeds of *Macaranga peltata.* Important chemical parameters such as acid value, unsaponifiable matter, iodine value, saponification value, acetyl value, Reichert-Meissl value, Polenske value and peroxide value and physical parameters such as specific gravity, refractive index, melting point, titer and viscosity were determined. The composition of the fatty oil was determined by the GC-MS analysis of the fatty acid methyl ester prepared from the oil.

The third chapter consists of analysis of essential oils from leaves of three different cultivars of *Piper betle* namely, nadan, selan and kuzhikkodi by GC and GC-MS. Forty, thirty eight and forty three compounds respectively could be identified in them. Safrole was the major component in nadan and kuzhikkodi while in selan it was eugenol. This work has been published in papers entitled "Analysis of Essential oil of *Piper betle L.* Leaves from South India Using GC/FID, GC/MS and Olfactometry" Scientia Pharmazeutia, Austria. **67**, 305-312 (1999) and "piper betle –Composition of leaf oils in three varieties from Kerala" Indian perfumer **45** (3) 255-257

The fourth chapter consists of the essential oil composition of flowers of *Pogostemon paniculatus* and *Hyptis capitata* were analysed by GC-MS and presented in chapter four. Twenty compounds representing 94.6% of essential oil of flowers of Pogostemon paniculatus were characterized; *cis*-β-farnacene and farnacene epoxide were found to be the major components. In the essential oil of *Hyptis capitata* 46 compounds representing 66.0% of the oil were identified and the major compounds were oct-1-en-3-ol and linalol. The essential oil of *H.capitata* also noted by the presence of hydroquinone

The fifth chapter consists of two sections. In the first section the essential oil analysis of *Litsea laevigata* (LL) and its fractions are included. Twenty six compounds representing 99.2% of the oil were identified in the essential oil sample LL of which monoterpenes were the major class of compounds (59.3%). The major compounds were α-pinene, β-pinene, α-terpineol, fenchol, limonene and 1,8-cineole. The percentage of sesquitertpene was about 37.4 % were as nonterpenoid compounds constitute

only 2.5%. The important sesquiterpenes are *trans*-α-bergamotene, α-copaene and β-santalene.

The second section consists of the anti-microbial study of the *Litsea laevigata* essential oil and its fractions. The essential oil exhibited concentration dependent activity. The oil is very active against gram-positive bacteria such as *Streptococcus albus* and fungi such as *Aspergillus niger*. The essential oil fractions LLP and LLD were less active against all micro organisms when compared with the original essential oil. This shows a synergic action of molecules in anti-microbial activity. The polar fraction which contains more oxygenated compounds showed slightly higher anti-microbial activity than the nonpolar fraction. This observation is well known. The minimum inhibitory concentration is low for gram positive bacteria such as *Staphylococcus albus* and gram negative bacteria such as *Escherichia coli.* The two fungi C*andida albicans* and *Aspergillus niger* also showed lower MIC. The antimicrobial activity exhibited by the oil is fairly good even though it does not contain phenolics. The attractive odour of this essential oil along with its promising anti-microbial property makes it a valuable material for a possible therapeutic use.

CHAPTER - I

SECTION 1:

PHYTOCHEMICAL STUDIES ON *MACARANGA PELTATA* Muell.

1.1.1 Introduction

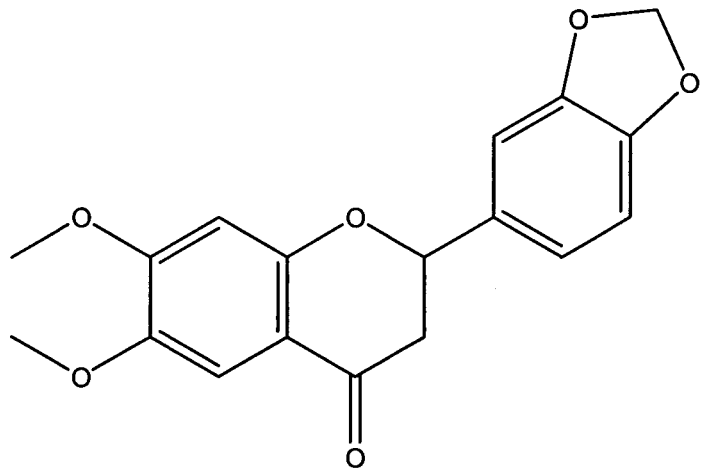
Macaranga peltata Muell. is a small tree commonly found in Indian forests.[1] It is found in Bengal, Bihar, Orissa and the Deccan Peninsula, mostly in the hills.[2] It is found throughout Kerala.[4] These are small dioecious trees; h-12 m, d-30 cm. Bark 10-15 mm thick, surface pale, grayish brown, mottled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

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

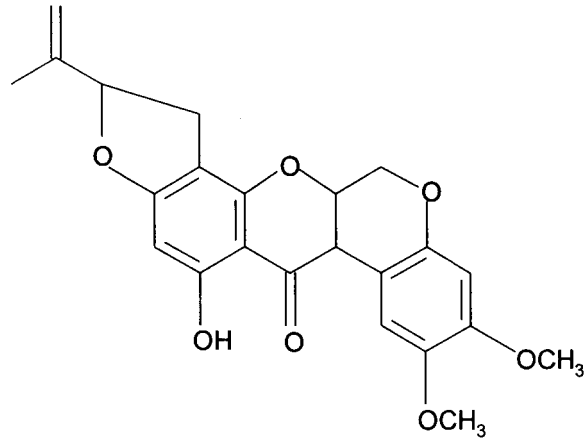
A reddish brown gum (kino) exudes from the cut branches, bases of petioles, young shoots and fruits. It is partly soluble in water and is available in the form of hard tears or agglutinated masses with a shiny luster and little or no taste. It is used for sizing paper and for taking impressions of leaves, coins, medallions etc; it is used also as a substitute for gum arabic.[5] The gum powder from *Macaranga peltata* has been used in Indian medicine for the treatment of venereal diseases.[6] A decoction of leaves and bark is used as a wash for ulcers. The fruit is eaten during the periods of scarcity. The wood of *M. peltata* is pale brown with a mottled appearance and is reported to be suitable for making pencil, matches and paper pulp.[7]

1.1.2 Work so far reported on *Macaranga* species

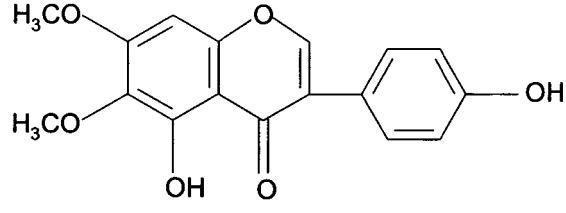
Only three species, apart from *M. peltata* of *Macaranga* genus have been chemically examined so far.[8-10] *Macaranga tenarus* has been previously examined and the isolation of a new diterpene, macaranganol and some other terpenoids were reported.[11] A new flavanone, 6,7-dimethoxy-3',4'-methylenedioxyflavanone,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



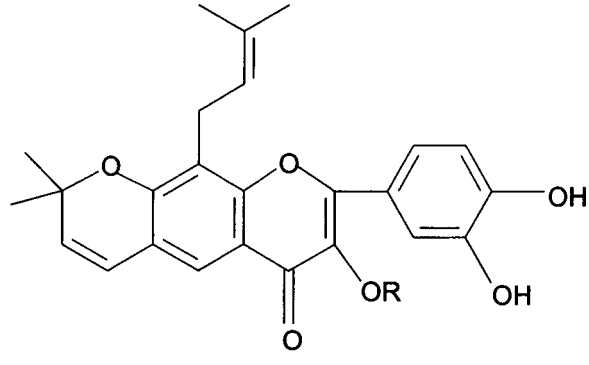
6,7-dimethoxy-3',4'-methylenedioxyflavanone



Sumatrol



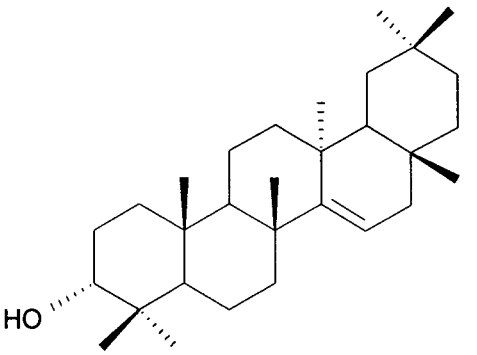
7-methyltectorigenin



R=H Macaflavone I

R = Me Macaflavone II

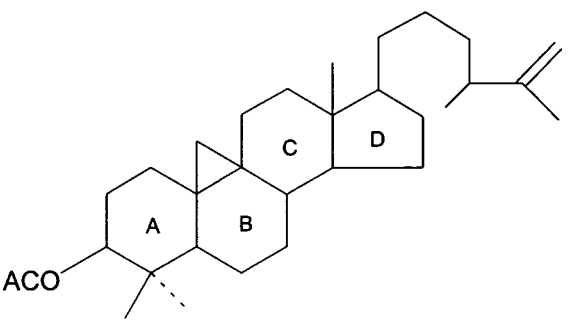
A triterpene 3-epitaraxerol was isolated from *M. denticulata* along with taraxerone and β-sitosterol.[9]



3-Epitaraxerol

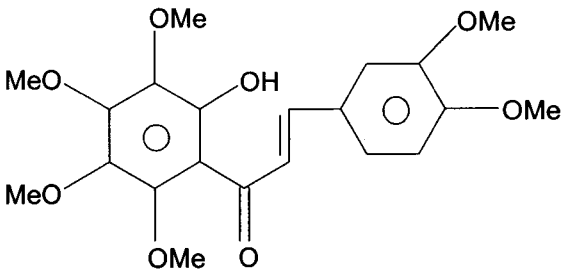
***Macaranga peltata* Muell.**

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



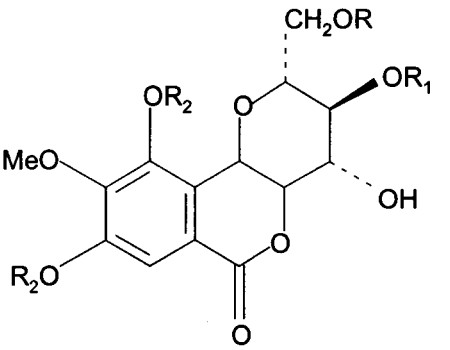
Cyclopeltenyl acetate

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



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

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



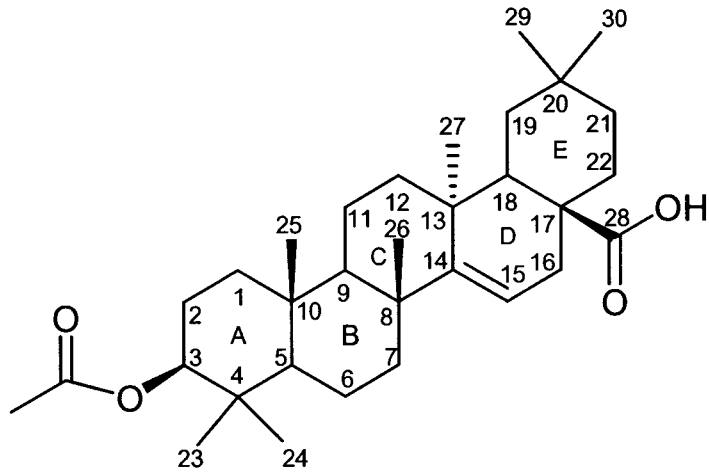
R = R_1 = R_2 = H Bergenin

I R = R_1 = H, R_2 = Me

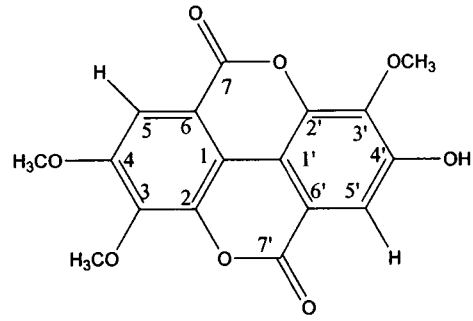
II R = R_2 = Me, R_1 = H

III R = H, R_1 = R_2 = Me

Molikutty[18] isolated 3-acetylaleuritolic acid and 3,4,3'-tri-O-methylellagic acid from the bark of *M. peltata* grown in Kerala.



3-acetylaleuritolic acid



3,4,3'-tri-O-methylellagic acid

1.1.3 Present work

The present investigation was aimed at the isolation and characterisation of compounds present in the barks of *Macaranga peltata,* both from male and female plants. The present work also aims at confirmation of the structure and its stereo aspects of 3-acetylaleuritolic acid earlier isolated

from the same plant[18] using single crystal X-ray analysis. Acid catalysed lactonisation of 3-acetylaleuritolic acid is also focused upon.

1.1.4 Materials and methods

Plant material

The bark of *M. peltata* was collected from Calicut University Campus, Kerala in May 2005 and was authenticated by Dr. A.K. Pradeep, Department of Botany, Calicut University. A voucher specimen of the plant has been deposited in the specially maintained Herbarium of Chemistry Department, Calicut University.

Melting point determination

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

Infrared absorption spectroscopy (IR)

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

Column chromatography (CC)

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

Thin layer chromatography (TLC)

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

Reagents

1. 20% aqueous sulphuric acid (20% H_2SO_4)

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

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

2. Leibermann-Burchard reagent (LB reagent)

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

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

3. Vanillin-sulphuric acid reagent

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

The chromatogram (TLC) was sprayed first with 5% H_2SO_4, followed immediately by 1% ethanolic vanillin. The sprayed plate was then heated to 110°C for 5-10 minutes until maximal visualisation of the spots.

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

4. Anisaldehyde-sulphuric acid reagent (AS reagent)

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

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

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

1.1.5. Extraction, fractionation and isolation of compounds from the petroleum ether extract of the bark of *M. peltata* Muell

Dried and finely powdered barks from male and female plants of *Macaranga peltata* (4 kg each) were extracted separately thrice with petroleum ether (60-80°C, 3 x 7L). The extracts were then concentrated under reduced pressure to about 500 mL of light yellow coloured solution when a white powdery solid separated. It was filtered, washed repeatedly with

petroleum ether, dried and recrystallised from pyridine yielding pure substances MP1F and MP1M from the female plant and male plant respectively.

Table 1.1

Compound isolated from petroleum ether extract

Compound MP1	Extracting solvent	Melting point	Yield
Female Plant(MP1F)	petroleum ether	282^{0}C	2.143g/4Kg
Male plant (MP1M)	petroleum ether	282^{0}C	0.308g/4kg

TLC Analysis of MP1F and MP1M

Both these compounds were purified by column chromatography on silica gel. TLC comparison of the compounds MP1F and MP1M were done using petroleum ether and ethyl acetate mixture in the ratio 6:1 (V/V) and also by using petroleum ether and acetone mixture in the ratio 10:1(V/V). The plates were sprayed using various spray reagents described above. Both of them were found to be the same. (3-acetylaleuritolic acid)

1.1.6 Rearrangement of 3-acetylaleuritolic acid in acid medium

In the earlier work from our department[18] aleuritolic acid was found to lactonise while attempting to oxidise it by using Jones reagent. Therefore it

was thought worthwhile to investigate upon the role of the acid in the lactonisation of acetylaleuritolic acid.

Experimental

3-Acetylaleuritolic acid (100mg each) was dissolved in 50 mL acetone and treated with the following reagents, stirred for 6 hours at about 30° C.

i) 2 mL, 5 mL and 10 mL each of chromic acid (prepared by dissolving 2.3 g CrO_3 in 2.7 mL con. sulphuric acid and diluted to 10 mL using distilled water[50])

ii) 2 mL and 10 mL each of 50% sulphuric acid

iii) 10 mL 50% HCl

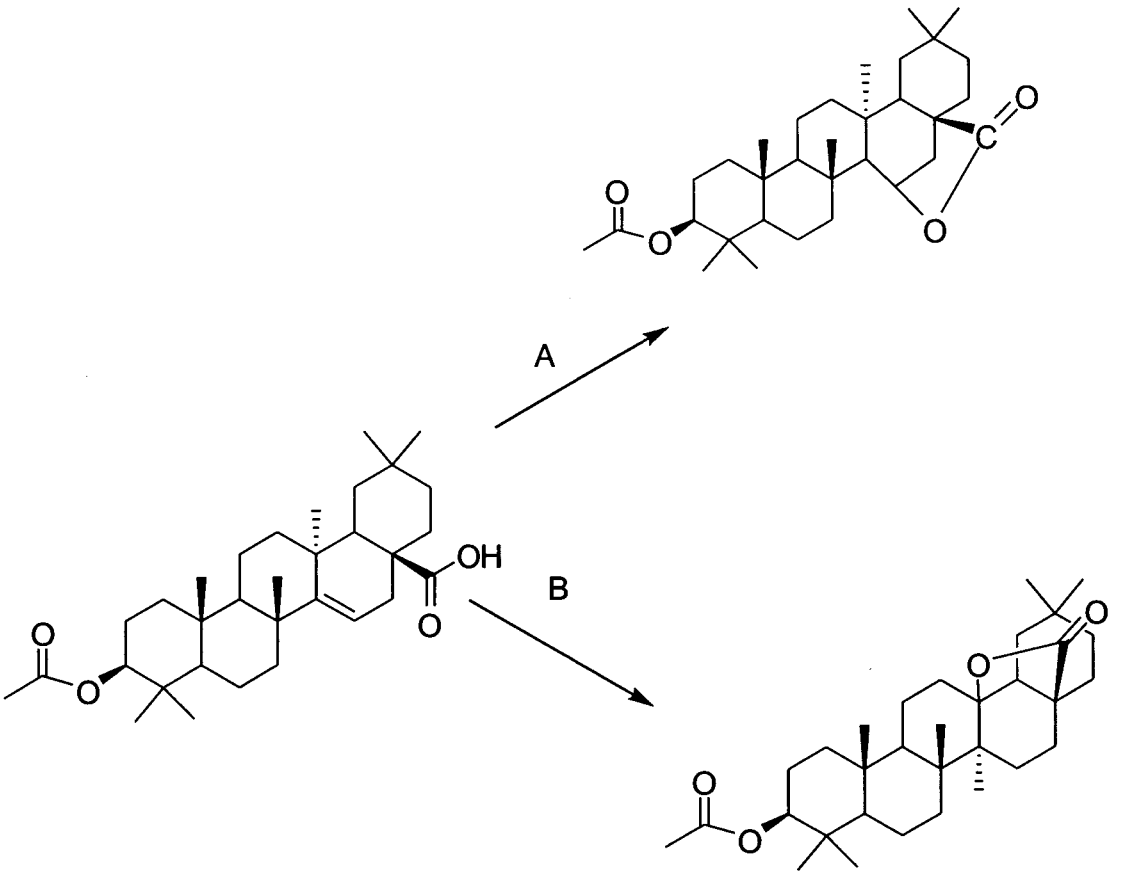
iv) 10 mL CrO_3 aqueous solution (prepared by dissolving 2.3 g CrO_3 in 10 mL distilled water)

v) 5 mL and 10 mL each of 50% phosphoric acid

The reaction mixtures were diluted to 100 mL using distilled water, the compounds precipitated were filtered under suction and washed with distilled water and dried.

In the case of 3-acetylaleuritolic acid there are two possibilities of forming the five membered lactone as shown below. In path B a

rearrangement occurs. The IR spectrum was taken to monitor reaction products obtained under different conditions.



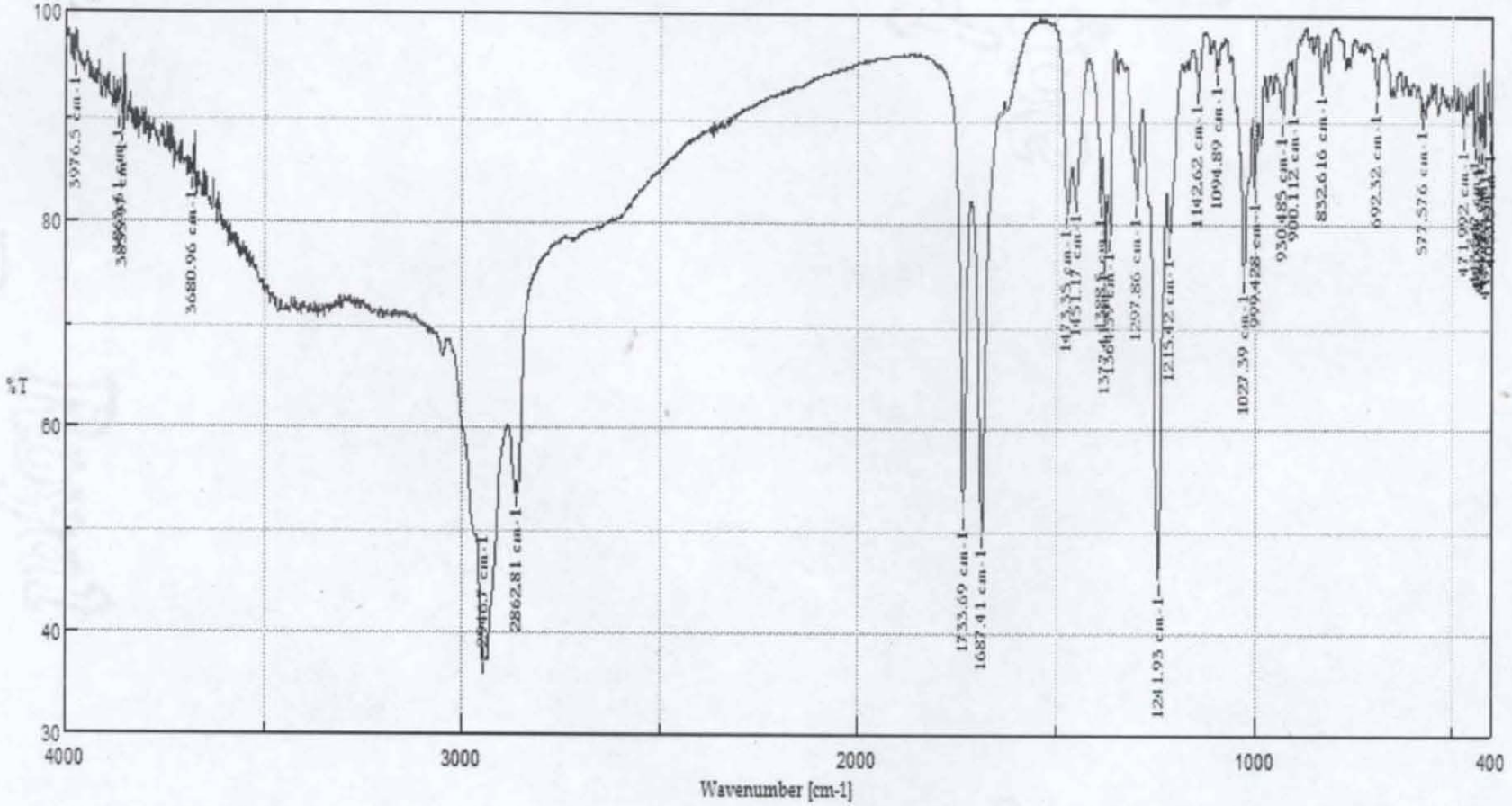


Fig 4. IR Spectrum of 3-acetylaleuritolic acid

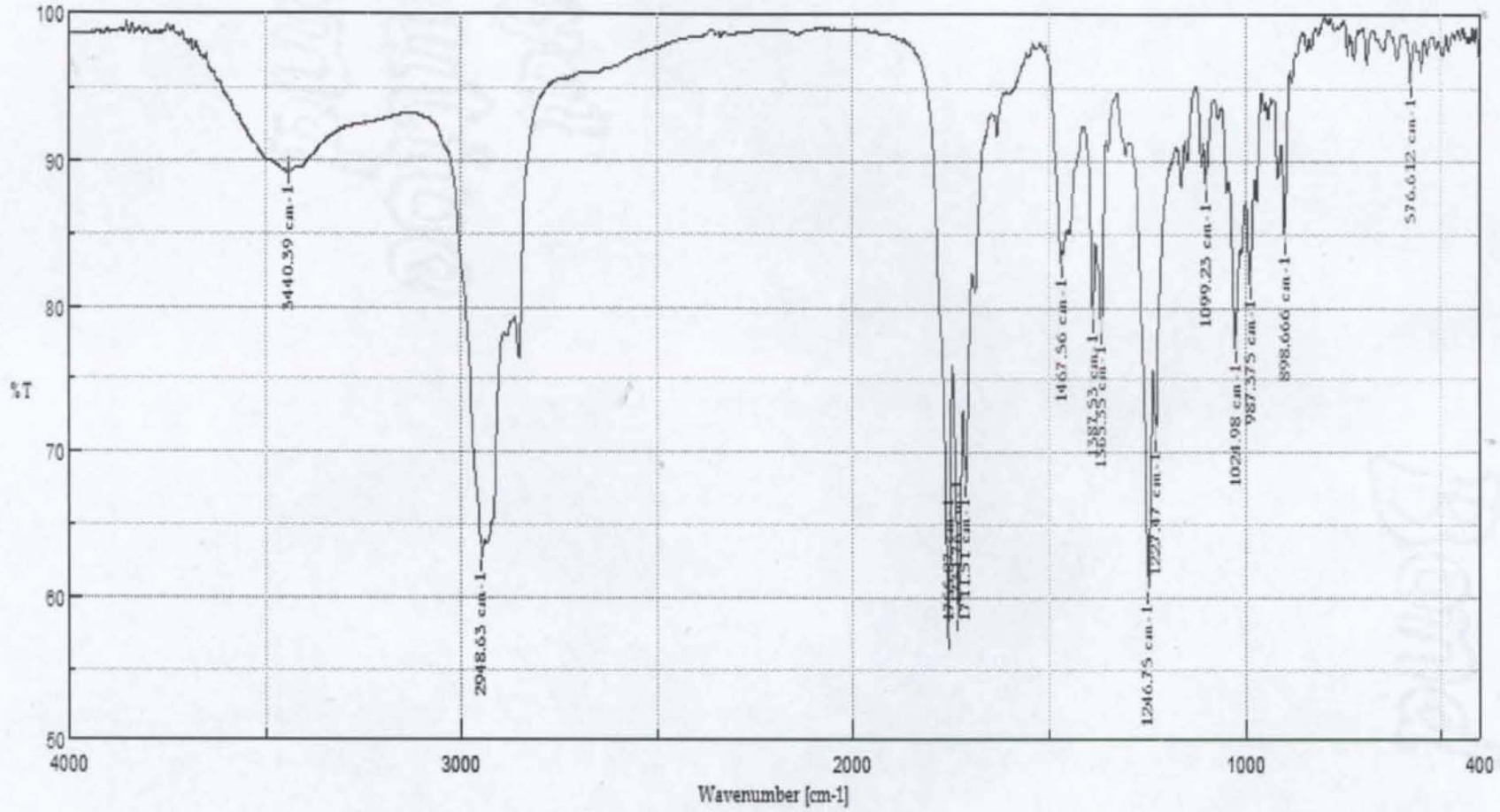


Fig 5. IR Spectrum of Lactonisd 3-acetylaleuritolic acid

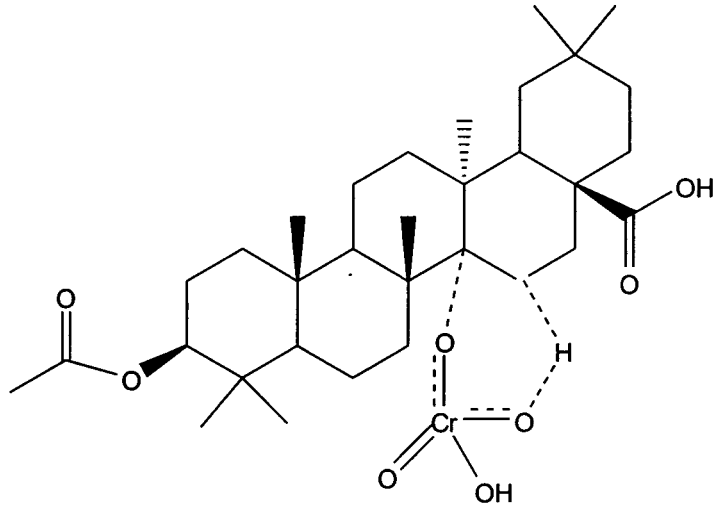
1.1.7 Results and discussion

Characterization of MP1F and MP1M (3-acetylaleuritolic acid)

The compounds were isolated from the petroleum ether extract as colourless needles. They were recrystallised from pyridine and had a melting point of 282°C. They answered Liebermann-Burchard reaction showing a persistent pink colour typical for triterpenes; also they gave a blue colour with vanillin - H_2SO_4 and appeared as a pink spot with anisaldehyde – H_2SO_4 reagent. Bayer's reagent was decolourised indicating the unsaturated nature and produced effervescence with sodium bicarbonate solution. Their IR spectrum exhibited absorption bands for carboxylic acid (3050 cm^{-1}, 1687.41cm^{-1}), ester (1733.69 cm^{-1}), gem dimethyl (1377,1364 cm^{-1}) C=C (1640.5 cm^{-1}) functions and trisubstituted double bond (peak at 830.4 cm^{-1}). On the basis of above facts MP1F and MP1M isolated from male and female plants were concluded to be the same compounds (3-acetylaleuritolic acid) with the support of the works done earlier[18] (MP. 282° C, Lit. 301-302°C[43]). It is also observed from table 1.1 that the female plant yields almost seven times more 3-acetylaleuritolic acid than the male plant and therefore the female plant of *M. peltata* can be regarded as good source of aleuritolic acid which exhibit anti- tumor[46,45] and anti HIV activity[47]

The lactonisation involving the carboxylic acid and a double bond to form a five membered ring is well documented[43] .The lactonisation of 3-

acetylaleuritolic acid to a five membered lactone is observed in all cases except with strong acid such as sulphuric acid and HCl. The extent of conversion was very small for CrO_3 aqueous solution but was fairly good for Jones reagent (mixture of chromic acid and sulphuric acid) and phosphoric acid. If the rearrangement is initiated by the protonation of the double bond, 50% sulphuric acid might have brought up this change. But it did not occur. Therefore a suitable mechanism in which acids like chromic acid and phosphoric acid stabilize the protonated species as shown below was proposed.



This is possible for chromic acid or phosphoric acid but not possible for sulphuric acid or HCl which are strong acids. The methyl group migrates from position 13 to 14 and hydrogen chromate leaves facilitating the lactone formation at position 13. The compound formed showed carbonyl absorption frequency of 1755cm^{-1}, which corresponds to the structure proposed[49]. If the

lactone formed is the one via the path way A, it would give the carbonyl absorption frequency of 1767cm^{-1} [43]. The absorption at 1687.41 cm^{-1} (corresponding to carbonyl absorption of -COOH group of 3-acetylaleuritolic acid) was absent or diminished if there was lactone formation.

The rearrangemet of aleuritolic acid methyl esters to oleanolic acid methyl esters in the presence of a mixture of acetic acid and hydrochloric acid have already been reported[44]. The rearrangement involves the migration of a methyl group from position 13 to 14 and shift of double bond from position 14,15 to 12,13 via the stabilization of carbocation at position 13.

SECTION II:

SINGLE CRYSTEL X-RAY ANALYSIS OF 3-ACETYL ALEURITOLIC ACID ISOLATED FROM *MACARANGA PELTATA*

1.2.1 Introduction

Diffraction of X-rays by crystals

Scattering

When electromagnetic radiation passes through a crystal (or matter in general), the electrons are perturbed by the rapidly oscillating electric field and are set in to oscillation about their nuclei, with a frequency identical to that of the incident radiation. According to electromagnetic theory an oscillating dipole acts as a source of an electromagnetic wave. Thus, each electron in the medium acts as a source of radiation that travels outward with a spherical wave front. In other words, the incident radiation is scattered by the medium with out alteration in its frequency[20]. This is coherent scattering; other kinds of scattering involving frequency changes also occur but are not of interest here.

Interference

Waves scattered from different points interfere with one another, in certain directions the scattered wavelets reinforce one another, and in other

directions they destroy one another. Consider a small volume element '*dr*' containing $\rho(r).dr$ electrons at a vector distance r from an arbitrary origin and let the directions of the incident and scattered waves be indicated by unit vectors S_0 and S, respectively. Comparing the path of the waves scattered at the origin and at 'r' we see that (Fig.1) the latter is longer by $r(S_0\text{-}S)$. Since an increase in path length corresponds to decrease in phase angle $2\pi\Delta/\lambda$, the phase of the wave scattered at r is $2\pi r(S\text{-}S_0)/\lambda$ with respect to the origin. The complex amplitude of the wave scattered at r is proportional to

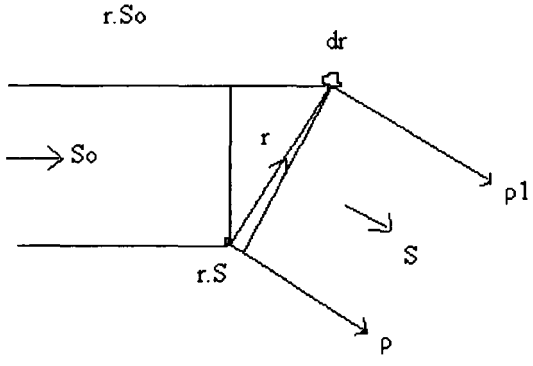


Fig 1.

$\rho(r)e^{[2\pi ir(S\text{-}So)/\lambda]}$ .dr which abbreviated to

$\rho(r)e^{[2\pi irR]}.dr$

where $R = (S\text{-}S_0)/\lambda$

If $\rho(r)$ describes some continuous electron density distribution, the complex amplitude of the superposition of all the wavelets scattered by the distribution is obtained by integration.

$$F(R) = \int 2\pi \rho(r)\, e^{2\pi i r R} dr$$

The vector R is called the scattering vector. The angle between S_0 and S the scattering angle, is usually taken as 2θ, in which case

$$R = 2\sin\theta / \lambda$$

If the wavelength λ is about the same order of magnitude as the distance between scattering points, then interference will occur at measurable scattering angles. That is why we need X-rays ($\lambda \sim 1A^0$) to study the structure of molecules.

The quantity F(R) depends on the electron density distribution and on the scattering vector R and is called structure factor; its absolute value is known as structure amplitude of the scattering in the direction defined by R. $|F(R)|$ is usually expressed as a ratio, the resultant amplitude of the radiation scattered by the distribution $\rho(R)$ to that scattered by a single free electron at the origin.

The scattering from a single point alone is independent of the scattering angle, since no path difference is involved. Therefore point atoms have no physical significance as far as X-ray diffraction is concerned. In

crystal structure analysis it is often useful to analyse the pattern produced by the assemblies of point atoms occupying similar positions in space.

Fourier transforms

The equation $\mathbf{F(R)} = \int \rho(r)\ e^{2\pi i r R} dr$, is an example of Fourier transformation. The structure factor F(R) is called the Fourier transform of the scattering density $\rho(r)$, which can be expressed in turn as the inverse Fourier transform of F(R),

$\rho(r) = \int \mathbf{F(R)}\ e^{-2\pi i r R}\ dR$, is a remarkable reciprocal relationship[20].

If we know the structure we can always calculate scattering pattern. However the reverse calculation is difficult because the scattering pattern gives information only about the absolute value of F(R) but not about its phase. This means that from the scattering pattern we can find out the missing phase information from the qualitative features of the structure or the scattering pattern.

Effect of atomic vibrations

The atoms in a crystal are not at rest but vibrate about their mean positions. This is to make the time-averaged, space-averaged electron density peaks more diffuse. Since the mean vibration amplitude tends to increase with temperature, the reduction of scattering power at large scattering angles becomes more pronounced at high temperature. As first shown by Debye[21]

this reduction in scattering power can be approximated by multiplying f(R) by exponential function $\exp(-B\sin^2\theta/\lambda^2)$ where $B= 8\pi^2\langle u^2\rangle$ where $\langle u^2\rangle$ is the mean square vibrational amplitude known as Debye factor.

Determination of space groups

The determination of unit cell dimensions and space group form an important step in the crystal structure analysis. Certain space groups can be uniquely assigned from the symmetry of diffraction pattern and symmetric absences. In normal scattering the diffraction pattern is always centrosymmetric, whether the structure it self has a centre of inversion or not. Some symmetry elements like screw axis and glide planes acting in this respect as rotational axis and mirror planes respectively. As a result only eleven possible diffraction symmetry can be distinguished; the remaining twenty one crystal classes are assigned to the appropriate Laue class by adding an inversion centre. Although the diffraction pattern is centrosymmetric under normal scattering conditions, the statistical distribution of intensities is different for centrosymmetric and non centrosymmetric crystals. The difference depends in the fact that structure amplitudes are real in former case but complex in the latter case. Centrosymmetric and non centrosymmetric crystals that belong to the same Laue class can thus be distinguished, in principle, by statistical analysis of intensity distribution as proposed by Wilson[22]

1.2.2 Methods of crystal structure analysis

Trial and error analysis

Those early structures were mostly solved by a trial and error procedure; this method considered of postulating model, an atomic arrangement consistent with the cell dimensions, space group, and any prior knowledge about the molecular structure, and checking whether the structure amplitudes calculated for the model were in qualitative agreement with the observed magnitudes for a few selected reflections.

Heavy Atom method

The complete interpretation of the Patterson function is generally extremely difficult for all but the simplest structures. A partial interpretation of the Patterson function became possible by considering the heavy atoms present. This is because the height of the Patterson peak associated with inter atomic vector $r_j - r_i$ is approximately proportional to product of atomic numbers $z_i.z_j$. Peaks corresponding to vectors between heavy atoms stand out prominently.

Consider an organic molecule containing N Carbon (Z=6) atom and one iodine atom Z=53 and suppose there are two such molecule in the unit cell of the crystal with space group $P2_1$. We expect N pairs of peaks corresponding to vectors between symmetry related carbon atoms (weight 6 x

6 = 36) and another 2N (N-1) pairs of peaks corresponding to vectors between atoms not related by symmetry. In addition, we expect 4N pairs of stronger peaks due to I-C vectors (weight 6 x 53 = 318) and finally a single pair of very stronger peaks due to symmetry related I-I interaction (weight 53 x 53 = 2809)

Even when N is quite large, say 50, the I-I peaks should be clearly recognizable. Once they are identified, positions of iodine atoms are known. The 4N pairs of I-C peaks, which are relatively stronger, form the image of the molecule.

The development and increased availability of digital computers during 1950's and early 1960's transformed the heavy atom method into a more or less routine tool by which the molecular structure of complex natural products could be determined by X-ray analysis in many cases faster and more efficiently than the classical methods of degradation . The main bottleneck was often that of preparing a suitable heavy atom derivative and to obtain it in suitable crystal form. The three dimensional density distribution is stored in computer which locates the peaks and selects from a set of possible atomic sites consistent with reasonable bond distances and angles. The postulated structure can be refined, wrong atoms deleted and new ones are inserted by an interactive sequence of successive least-squares calculations and Fourier

synthesis with minimum of human intervention at any stage. Several quite complex heavy atom structures have been solved in this way[23]

Method of Isomorphous Replacement

In the heavy atom method the phase assigned to a given structure amplitude F_H is the phase of heavy atom contribution. The isomorphous replacement method is a variant of this, were the phase angle estimated from the change in $|F_H|$ that occurs when one or more heavy atoms are introduced into the unit cell without appreciable change in the overall structure of the crystal. The heavy atoms may simply replace lighter atoms (eg. Pt for Ni, Br for Cl , Rb for K) or they may be introduced as additional atoms. The latter is important in protein crystallography[24]. The crystals in which atomic positions are same but differ only in the nature of atoms that occupy are called isomorphous. The simple case involves a centrosymmetric structure

Suppose we have two centrosymmetric crystals A and B that differ only in the scattering powers f_A and f_B ($f_B > f_A$) we can express the structure factor of crystals A and B as

$$F_H^A = F_H^R + f_A T_H$$

$$F_H^B = F_H^R + f_B T_H$$

Were F_H^R is the contribution of the atoms common to both structure and T_H is a trigonometric factor that depends only on the positions of the replaceable

atoms. The few atomic positions on which T_H depends can be determined from the Patterson function of the crystal containing the heavier atom (B). The only peaks present in this function corresponds to the vectors among the replaceable atoms and vectors between the common ones; peaks corresponds to vectors among the common atoms are subtracted out, which makes the interpretation much simpler.

Direct Methods

It would be convenient to have methods of deriving phases for structure factors or placing limitations on the possible phases that do not depend on the presence of heavy atoms and do not involve any structural assumptions. This is obviously asking too much because any assignment of phases to Fourier coefficients corresponds to some electron density distribution. Two general criteria that any physically reasonable electron density distribution must satisfy are

(1) The electron density is non negative everywhere.

(2) The electron density consists of discrete, approximately spherical peaks.

If structure amplitudes are known, these criteria are sufficient to restrict the phase angles of the larger Fourier coefficients. The infinity of possible electron-density maps are there by reduced to a small number, and these can be calculated individually and examined for stereochemically

reasonable patterns of peaks. In favourable cases, one or more of calculated distribution will be close enough to the true electron density to allow the approximate atomic positions. The method based on such phase angle restrictions are known as direct methods.

1.2.3 Methods of Crystal structure Refinement

Structure Refinement:-The R factor

The tradition has arisen of using the R factor, sometimes called the reliability index or residual

$$R = \frac{\Sigma \, ||F_o| - |F_c||}{\Sigma |F_o|}$$

Where F_o and F_c are observed and calculated structure factor respectively. The R factor may be useful indicator in deciding whether any particular adjustment to the structural model is an improvement or not, but it is a poor criterion for the correctness of the model. A few large discrepancies between observed and calculated |F| values for individual reflections can be hidden in a low R factor, but they may be enough to indicate that a structure is unacceptable. The R factor depends on the quality of experimental data, for F_o values derived from visual photographic intensity estimates, final R factor in the range 0.1 to 0.15 could be deemed satisfactory; for diffractometer measurements, the value is about 0.07 or less could be expected. Heavy atom contributions alone are enough to produce lower R factors. In non

centerosymmetric equal atom case, the expectation R factor for a completely random structure is 0.59, where as it is 0.83 in the centrosymmetric case[25]

Two main methods for refining a proposed structural model are difference synthesis (F_o-F_c) and the method of least squares. The former involves the step by step adjustment of electron density distribution. The least squares method involves a step-by-step adjustment of calculated |F| values to the observed one.

Molecular structural formulas

Most of the early X-ray analysis of organic crystals was concerned with compounds of known structural formulas. Thus for example, the β-isomers of hexachlorocyclohexane and hexabromocyclohexane were studied in 1926 in an attempt to settle the question of the shape the cyclohexane ring[26]. Only few years earlier the structure of diamond had emerged[27] as one of the first fruits of X-ray analysis, so that Mohr was in a position to interpret the carbon skeleton in aliphatic and alicylic molecules as portions cut out of the diamond structure. He also saw the rapid inversion of the chair form might explain the experimental failure to isolate more than a single isomer of mono substituted cyclohexane derivative and he noted that *cis* and *trans* fused decalins would not be so readily inter convertible and should be isolable. Hendricks and Billeke showed that the crystal of hexachlorocyclohexane is cubic, space group Pa^3 (Th^6), with four molecules per unit cell[26]

Only after heavy-atom methods were developed and applied to pthalocyanins[28] could X-ray analysis be used to elucidate atomic arrangement of complex molecules. By the end of 1940s there was an impressive list of complex natural product molecules whose structures had been determined in this way; cholesterol[29], calciferol[30,31], penicillin[32] and strychnine[33,34] to mention the important ones. Two strychnine analysis, carried out independently and on different derivatives, fully confirmed the molecular structure that had only in 1947 proposed for this important alkaloid [35,36].

Determination of Absolute Configuration

According to Friedel's law X-ray diffraction pattern of a crystal is centrosymmetric, whether the crystal structure itself is centrosymmetric or not. Friedel's law depends on the assumption that phase difference between waves scattered at different points depends only on the path differences, which would imply that any intrinsic phase change connected with the actual scattering must be the same for all scattering centers. This assumption is nearly but not quite correct. Depending upon the energy of incident X-rays some of the atoms may scatter slightly out of phase with the others. For non centrosymmetric crystal structures, this leads to slight difference between the intensities of (hkl) and (h`k`l`) reflections; these would be equal according to Friedl's law. Such intensity deference can be utilized in structure analysis since they help to determine the phases of relevant pair of reflections. More

importantly they provide an experimental basis for defining the absolute reference frame used to describe the structure; for chiral structures this is equivalent to absolute configuration.

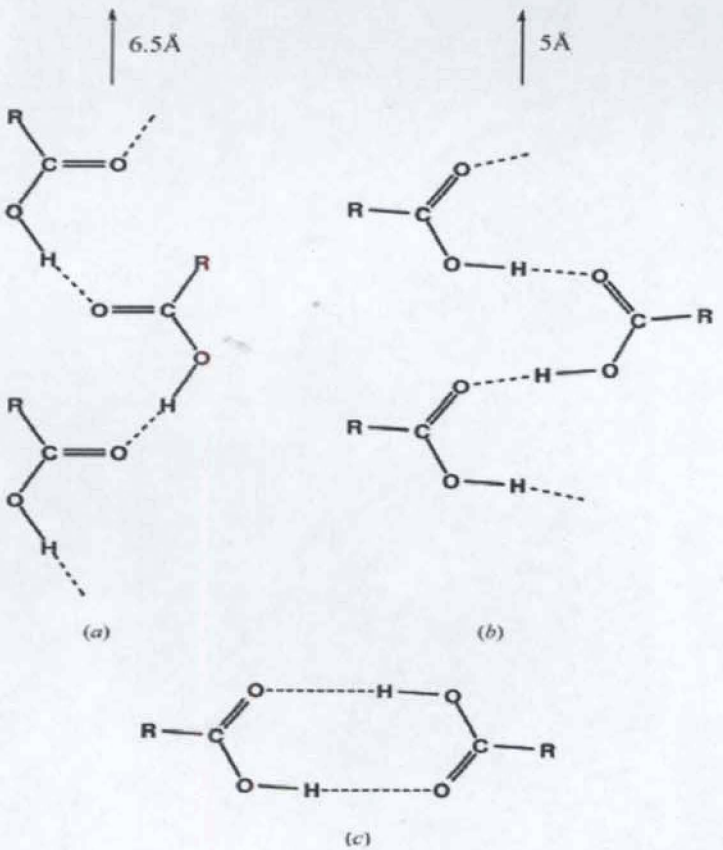
The possibility of utilizing the breakdown of Friedel's law to provide a bridge between macroscopic and molecular chirality was first proposed by T.M Bijvoet in 1949[37]. All the available chemical methods could do was to relate the configurations of many optically active compounds among one another *i.e.*, to establish their configurations relative to the reference compound (+)-glyceraldehyde. In 1950 the X-ray crystallography of NaRb(+) tarterate[38] showed clearly the absolute configuration of (+)tarterate by analyzing the intensity difference between Friedel pairs . Hence the earlier assumption of structure of (+) glyceraldehyde was fortunately correct, though there was equal chance to become wrong[39].

Conformational analysis

X-ray analysis provide information about the preferred conformation of molecules although it has nothing to say about the energy difference between conformations. Energy difference can be derived, in principle, from the measurement of equilibrium concentrations of the relevant conformational isomers. X-ray analysis showed that of the many possible conformations of cyclodecane, one occurred in a wide variety of crystalline derivatives and hence must be somewhat stable than others[40].

H-bond interactions in Carboxylic acids

Hydrogen-bond interactions between carboxylic groups have been so thoroughly investigated[41,42] Two different hydrogen bonding motifs are seen in these types of compounds: a cyclic dimer (Fig. 1c), and a catemer motif that links the carboxylic acids into infinite chains (Figs. 1a and 1b) either through the symmetry of a twofold screw axis or through glide planes. The cyclic dimer is the most abundant hydrogen-bond motif.



1.2.4 Experimental Aspects of x-ray analysis

Single- crystal Diffractometers

A diffractometer is an instrument for measuring the intensities of diffracted beams individually by counting. The number of X-ray photons that arrive at a suitably placed detector. The ancestry of the modern diffractometers goes back to the ionisation spectrometer, the instrument that provided the experimental data for the first crystal structure analysis in 1912. Later photographic recording methods gradually came into general use, since it eliminates the laborious task of recording many reflections.

However, with the need for greater accuracy in intensity measurements and the development of stabilized X-ray tubes and improved radiation defectors, diffractometers began to come into use. In the earlier models the orientation of the crystal and detector for each reflection was changed and adjusted by hand. Modern diffractometers are usually under the control of a computer, which calculates the required orientation of the crystal and counter drives the circles into the correct settings, and measure background and integrated intensities for each reflection in sequence. Several hundred reflections per day can be measured

Modern diffractometers are of the four-circle type. One circle allows the counter to be set at the proper scattering angle (2θ) for each reflection and the other three circles ω, α, φ brings the crystal into correct orientation. The

crystal is attached to a pin on the φ axis and must be brought to the exact centre of the instrument. In general the orientation of the crystal with respect to the diffractometer axis will be unknown at the start, but it can be calculated together with unit cell dimensions. The calculations are done by the computer that is usually linked to the diffractometer.

1.2.5 Present work

Crystal Growth

The crude crystals of MP1F were purified and recrystalised from chloroform. Crystals of large dimension suited for single crystal analysis was grown from distilled pyridine at room temp in a 50 mL conical flask. Colorless needle like crystals (MPX) were separated and washed with pure pyridine under suction. The crystals were dried.

Single crystal X-ray analysis

A large crystal was chosen and observed through a polarized light microscope to examine its single crystallinity (polycrystalline grains can be seen in different colours oriented in different directions against single colour for single crystalline sample). It was then mounted on a special pin using glue. The pin was mounted on the single crystal X-ray diffractometer and screws are adjusted to bring the crystal to the centre of the instrument by

viewing the monitor of the computer attached to the diffractometer. The data collection took eight hours.

Structure determination

Data collection: Bruker AXS APEX2(Bruker, 2004) CCD diffractometer; cell refinement:APEX2/SAINT (Bruker, 2004); data reduction: SAINT/XPREP(Bruker,2004); program(s) used to solve structure SIR92(WINGX); program(s) used to refine structure: SHELXL-97 (Sheldrick, 1997); molecular graphics: ORTEP32(WINGX); molecular packing MERCURY 1.4.1 .

Crystal data and structure refinement

Identification code	MPX
Empirical formula	C33.25 H52 N0.25 O4.38 (The crystal contained solvent molecules used for crystallization)
Formula weight	525.25
Temperature	293(2) K
Wavelength	0.71073 A°
Crystal system, space group	Monoclinic, C2
Unit cell dimensions	a = 37.478(2) A° alpha = 90 deg.
	b = 6.4721(3) A° beta = 120.229(7) deg.
	c = 29.826(2) A° gamma = 90 deg.

Volume	6250.9(6) A^{o3}
Number of molecules per unit cell (Z)	8
Calculated density	1.116 Mg/m^3
Absorption coefficient	0.072 mm^{-1}
F(000)	2306
Crystal size	0.3 x 0.2 x 0.2 mm
Theta range for data collection	0.79 to 22.21 deg.
Limiting indices	-39<=h<=39, -6<=k<=5, -31<=l<=31
Reflections collected / unique	47048 / 7186 [R(int) = 0.0447]
Completeness to theta	= 22.21 99.5 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.98 and 0.94
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	7186 / 1 / 678
Goodness-of-fit on F^2	0.952
Final R indices [I>2sigma(I)]	R1 = 0.0669, wR2 = 0.1911
R indices (all data)	R1 = 0.0880, wR2 = 0.2229
Absolute structure parameter	2(2)
Extinction coefficient	0.0037(5)
Largest diff. peak and hole	1.045 and -0.403 $e.A^{o-3}$

Fourier synthesis indicated the positions of all the non hydrogen atoms. The positions of all the non hydrogen atoms were readily found from a difference synthesis. All positional parameters, anisotropic temperature factors for the hydrogen atoms were refined in the final least-squares calculations giving a final R factor of 0.0669 and goodness of fit S on F^2 = 0.952. The molecular parameters are given in Tables 1.2 to 1.7. The ORTEP diagram of the molecule is given in fig. 2 and the molecular packing in the monoclinic unit cell is given in fig.3. The H-bond parameters were measured using Mercury 1.4.1 software are given in table 1.9. The crystal symmetry equivalent positions are listed in Table 1.10.

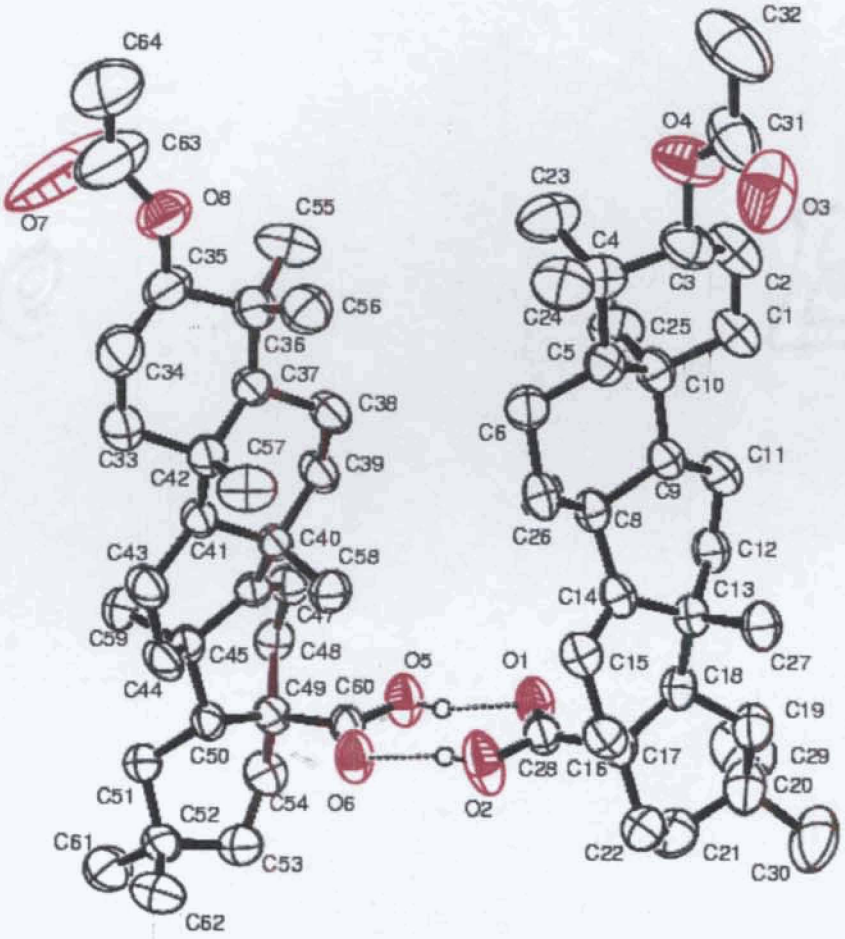


Fig 2. The ORTEP diagram of the molecule

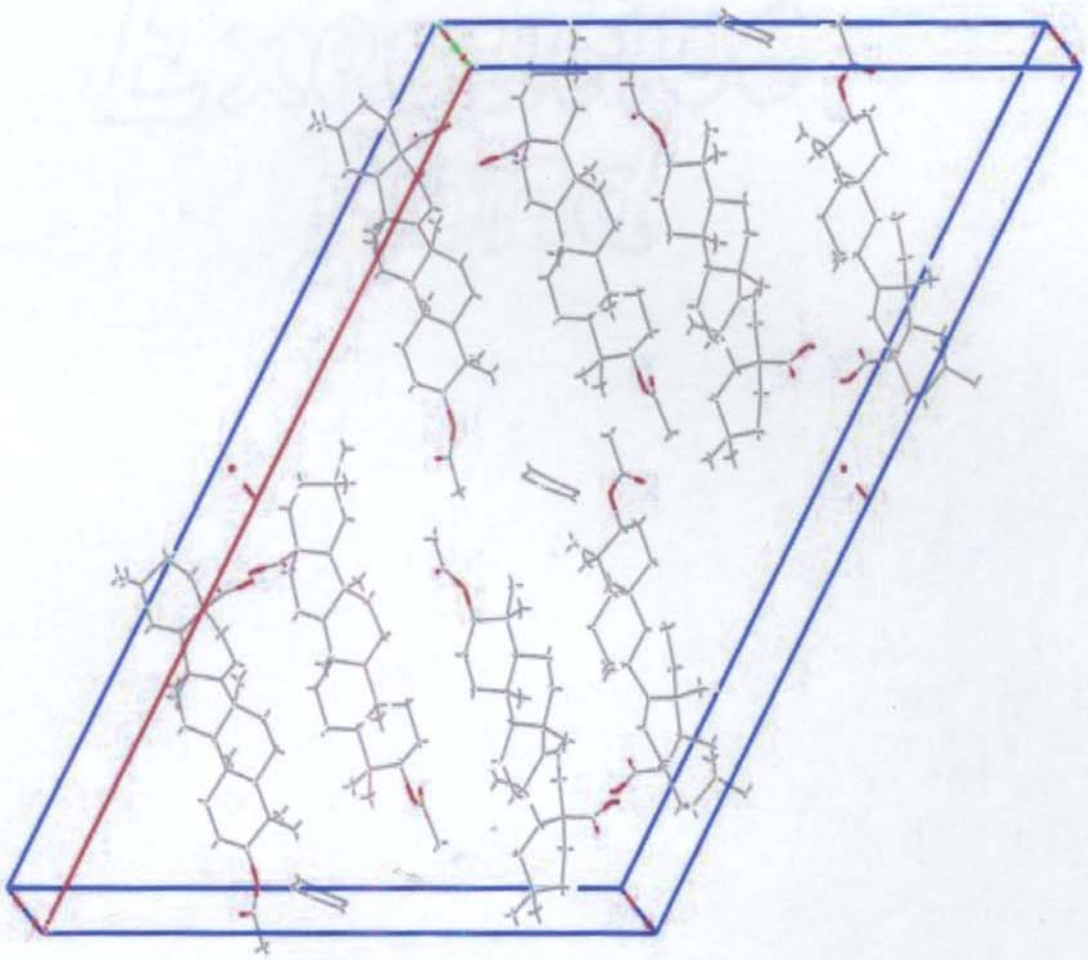


Fig 3. Packing of the molecules in the monoclinic lattice

Table 1.2

Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters (A^{o2} x 10^3) for MPX.

(U(eq) is defined as one third of the trace of the orthogonalized Uij tensor.)

	x	y	z	U(eq)
C(1)	2719(2)	1898(11)	4743(2)	69(2)
C(2)	3134(2)	1560(12)	5212(2)	79(2)
C(3)	3459(2)	2896(11)	5200(2)	69(2)
C(4)	3501(2)	2610(10)	4725(2)	60(1)
C(5)	3060(2)	2908(8)	4235(2)	48(1)
C(6)	3037(2)	2859(10)	3721(2)	56(1)
C(7)	2629(1)	3760(9)	3298(2)	49(1)
C(8)	2245(2)	2671(8)	3244(2)	45(1)
C(9)	2294(1)	2466(8)	3787(2)	43(1)
C(10)	2707(2)	1575(9)	4228(2)	50(1)
C(11)	1907(2)	1508(10)	3767(2)	57(1)
C(12)	1512(2)	1734(9)	3250(2)	52(1)
C(13)	1480(1)	3748(8)	2966(2)	45(1)
C(14)	1856(2)	3996(8)	2901(2)	43(1)
C(15)	1830(2)	5373(8)	2558(2)	50(1)
C(16)	1432(2)	6432(8)	2205(2)	53(1)
C(17)	1088(2)	4854(8)	1987(2)	48(1)
C(18)	1069(1)	3676(9)	2428(2)	49(1)
C(19)	695(2)	4335(11)	2461(2)	64(2)
C(20)	276(2)	3926(12)	1970(2)	76(2)
C(21)	307(2)	4483(12)	1490(2)	77(2)
C(22)	665(2)	5868(10)	1615(2)	68(2)
C(23)	3711(2)	524(12)	4746(3)	88(2)
C(24)	3783(2)	4330(12)	4721(2)	80(2)
C(25)	2754(2)	-748(9)	4160(2)	70(2)
C(26)	2193(2)	572(9)	2964(2)	59(1)
C(27)	1481(2)	5559(9)	3307(2)	58(1)
C(28)	1161(2)	3320(9)	1649(2)	49(1)
C(29)	155(2)	1712(17)	1963(3)	117(3)
C(30)	-54(2)	5296(18)	1984(3)	120(3)
C(31)	4012(2)	3942(18)	6033(3)	90(2)
C(32)	4392(2)	3214(18)	6499(3)	133(4)
C(33)	3763(2)	223(12)	2106(2)	75(2)
C(34)	4185(2)	353(14)	2609(2)	85(2)
C(35)	4213(2)	-1194(11)	2992(2)	67(2)
C(36)	3876(2)	-1063(10)	3139(2)	57(1)
C(37)	3458(2)	-1111(9)	2619(2)	49(1)
C(38)	3077(2)	-1181(10)	2676(2)	57(1)
C(39)	2696(2)	-1800(9)	2173(2)	56(1)

C(40)	2601(1)	-375(7)	1716(2)	41(1)
C(41)	2998(1)	-128(8)	1683(2)	45(1)
C(42)	3398(2)	483(9)	2199(2)	50(1)
C(43)	2931(2)	1201(10)	1220(2)	59(2)
C(44)	2481(2)	1338(9)	778(2)	54(1)
C(45)	2235(1)	-637(8)	695(2)	45(1)
C(46)	2267(1)	-1342(7)	1206(2)	42(1)
C(47)	1997(2)	-2737(8)	1174(2)	49(1)
C(48)	1630(2)	-3304(8)	656(2)	52(1)
C(49)	1449(2)	-1317(8)	357(2)	47(1)
C(50)	1772(1)	-184(8)	267(2)	44(1)
C(51)	1678(2)	-497(9)	-288(2)	54(1)
C(52)	1262(2)	456(9)	-695(2)	57(1)
C(53)	941(2)	96(10)	-527(2)	68(2)
C(54)	1048(2)	-1705(10)	-158(2)	63(2)
C(55)	3907(2)	-2953(11)	3460(2)	84(2)
C(56)	3941(2)	836(11)	3476(2)	72(2)
C(57)	3380(2)	2725(9)	2357(2)	69(2)
C(58)	2423(2)	1661(9)	1794(2)	56(1)
C(59)	2423(2)	-2369(10)	523(2)	61(2)
C(60)	1338(2)	17(9)	689(2)	47(1)
C(61)	1120(2)	-594(13)	-1213(2)	89(2)
C(62)	1312(2)	2756(12)	-731(2)	86(2)
C(63)	4910(3)	-2260(20)	3529(4)	148(5)
C(64)	5319(2)	-1770(20)	3997(3)	145(4)
C(65)	4925(5)	5300(30)	4741(6)	214(8)
C(66)	4942(4)	1830(30)	4739(5)	191(7)
C(67)	4907(6)	3570(50)	4464(7)	235(7)
N(1)	4907(6)	3570(50)	4464(7)	235(7)
O(1)	1090(1)	1497(6)	1611(1)	64(1)
O(2)	1303(2)	4238(7)	1381(2)	89(2)
O(3)	3867(2)	5678(14)	5964(2)	128(3)
O(4)	3851(2)	2502(8)	5664(2)	96(2)
O(5)	1169(1)	-930(6)	920(2)	67(1)
O(6)	1390(1)	1893(6)	725(2)	71(1)
O(7)	4865(3)	-3430(20)	3204(4)	330(10)
O(8)	4615(1)	-1028(8)	3460(2)	82(1)
O(9)	0	2050(60)	0	223(12)
O(10)	0	5760(50)	0	179(9)
O(11)	0	8530(60)	0	242(14)

Table 1.3

Bond lengths [A°] for the sample MPX

	Bond length A°
C(1)-C(2)	1.495(8)
C(1)-C(10)	1.530(7)
C(1)-H(1A)	0.97
C(1)-H(1B)	0.97
C(2)-C(3)	1.507(9)
C(2)-H(2A)	0.97
C(2)-H(2B)	0.97
C(3)-O(4)	1.446(7)
C(3)-C(4)	1.516(8)
C(3)-H(3)	0.98
C(4)-C(24)	1.538(9)
C(4)-C(23)	1.547(9)
C(4)-C(5)	1.572(7)
C(5)-C(6)	1.494(7)
C(5)-C(10)	1.572(7)
C(5)-H(5)	0.98
C(6)-C(7)	1.525(7)
C(6)-H(6A)	0.97
C(6)-H(6B)	0.97
C(7)-C(8)	1.535(7)
C(7)-H(7A)	0.97
C(7)-H(7B)	0.97
C(8)-C(9)	1.542(6)
C(8)-C(26)	1.554(7)
C(8)-C(14)	1.552(7)
C(9)-C(10)	1.552(7)
C(9)-C(11)	1.550(7)
C(9)-H(9)	0.98
C(10)-C(25)	1.539(8)
C(11)-C(12)	1.515(7)
C(11)-H(11A)	0.97
C(11)-H(11B)	0.97
C(12)-C(13)	1.526(7)
C(12)-H(12A)	0.97

	Bond length A°
C(17)-C(18)	1.553(7)
C(17)-C(22)	1.550(8)
C(18)-C(19)	1.512(7)
C(18)-H(18)	0.98
C(19)-C(20)	1.540(8)
C(19)-H(19A)	0.97
C(19)-H(19B)	0.97
C(20)-C(29)	1.499(13)
C(20)-C(30)	1.538(10)
C(20)-C(21)	1.539(9)
C(21)-C(22)	1.495(9)
C(21)-H(21A)	0.97
C(21)-H(21B)	0.97
C(22)-H(22A)	0.97
C(22)-H(22B)	0.97
C(23)-H(23A)	0.96
C(23)-H(23B)	0.96
C(23)-H(23C)	0.96
C(24)-H(24A)	0.96
C(24)-H(24B)	0.96
C(24)-H(24C)	0.96
C(25)-H(25A)	0.96
C(25)-H(25B)	0.96
C(25)-H(25C)	0.96
C(26)-H(26A)	0.96
C(26)-H(26B)	0.96
C(26)-H(26C)	0.96
C(27)-H(27A)	0.96
C(27)-H(27B)	0.96
C(27)-H(27C)	0.96
C(28)-O(1)	1.202(6)
C(28)-O(2)	1.307(6)
C(29)-H(29A)	0.96
C(29)-H(29B)	0.96

C(12)-H(12B)	0.97
C(13)-C(14)	1.525(6)
C(13)-C(27)	1.551(7)
C(13)-C(18)	1.568(7)
C(14)-C(15)	1.322(7)
C(15)-C(16)	1.490(7)
C(15)-H(15)	0.93
C(16)-C(17)	1.511(7)
C(16)-H(16A)	0.97
C(16)-H(16B)	0.97
C(17)-C(28)	1.535(7)
C(33)-C(42)	1.537(7)
C(33)-H(33A)	0.97
C(33)-H(33B)	0.97
C(34)-C(35)	1.482(10)
C(34)-H(34A)	0.97
C(34)-H(34B)	0.97
C(35)-O(8)	1.454(7)
C(35)-C(36)	1.532(8)
C(35)-H(35)	0.98
C(36)-C(55)	1.522(9)
C(36)-C(56)	1.528(8)
C(36)-C(37)	1.556(7)
C(37)-C(38)	1.520(7)
C(37)-C(42)	1.549(7)
C(37)-H(37)	0.98
C(38)-C(39)	1.516(7)
C(38)-H(38A)	0.97
C(38)-H(38B)	0.97
C(39)-C(40)	1.533(7)
C(39)-H(39A)	0.97
C(39)-H(39B)	0.97
C(40)-C(46)	1.535(7)
C(40)-C(41)	1.550(6)
C(40)-C(58)	1.546(7)
C(41)-C(43)	1.536(7)
C(41)-C(42)	1.563(7)
C(41)-H(41)	0.98

C(29)-H(29C)	0.96
C(30)-H(30A)	0.96
C(30)-H(30B)	0.96
C(30)-H(30C)	0.96
C(31)-O(3)	1.220(11)
C(31)-O(4)	1.332(10)
C(31)-C(32)	1.480(11)
C(32)-H(32A)	0.96
C(32)-H(32B)	0.96
C(32)-H(32C)	0.96
C(33)-C(34)	1.538(8)
C(50)-H(50)	0.98
C(51)-C(52)	1.543(7)
C(52)-C(53)	1.535(8)
C(53)-C(54)	1.513(8)
C(53)-H(53A)	0.97
C(53)-H(53B)	0.97
C(54)-H(54A)	0.97
C(54)-H(54B)	0.97
C(55)-H(55A)	0.96
C(55)-H(55B)	0.96
C(55)-H(55C)	0.96
C(56)-H(56A)	0.96
C(56)-H(56B)	0.96
C(56)-H(56C)	0.96
C(57)-H(57A)	0.96
C(57)-H(57B)	0.96
C(57)-H(57C)	0.96
C(58)-H(58A)	0.96
C(58)-H(58B)	0.96
C(58)-H(58C)	0.96
C(59)-H(59A)	0.96
C(59)-H(59B)	0.96
C(59)-H(59C)	0.96
C(60)-O(6)	1.225(6)
C(60)-O(5)	1.304(6)
C(61)-H(61A)	0.96
C(61)-H(61B)	0.96

C(42)-C(57)	1.537(8)
C(43)-C(44)	1.533(7)
C(43)-H(43A)	0.97
C(43)-H(43B)	0.97
C(44)-C(45)	1.522(8)
C(44)-H(44A)	0.97
C(44)-H(44B)	0.97
C(45)-C(46)	1.535(6)
C(45)-C(59)	1.544(7)
C(45)-C(50)	1.579(7)
C(46)-C(47)	1.323(7)
C(47)-C(48)	1.507(7)
C(47)-H(47)	0.93
C(48)-C(49)	1.518(7)
C(48)-H(48A)	0.97
C(48)-H(48B)	0.97
C(49)-C(60)	1.520(7)
C(49)-C(54)	1.533(7)
C(49)-C(50)	1.548(7)
C(50)-C(51)	1.523(6)

C(61)-H(61C)	0.96
C(62)-H(62A)	0.96
C(62)-H(62B)	0.96
C(62)-H(62C)	0.96
C(63)-O(7)	1.173(12)
C(63)-O(8)	1.294(10)
C(63)-C(64)	1.498(12)
C(64)-H(64A)	0.96
C(64)-H(64B)	0.96
C(64)-H(64C)	0.96
C(65)-C(65)#1	1.35(3)
C(65)-C(67)	1.37(2)
C(65)-H(65)	0.93
C(66)-C(67)	1.36(2)
C(66)-C(66)#1	1.39(3)
C(66)-H(66)	0.93
O(2)-H(2)	0.82
O(5)-H(5A)	0.82
O(10)-O(11)	1.79(5)

Table 1.4

Bond Angles (deg.) for MPX

C(2)-C(1)-C(10)	114.1(5)
C(2)-C(1)-H(1A)	108.7
C(10)-C(1)-H(1A)	108.7
C(2)-C(1)-H(1B)	108.7
C(10)-C(1)-H(1B)	108.8
H(1A)-C(1)-H(1B)	107.6
C(1)-C(2)-C(3)	111.6(5)
C(1)-C(2)-H(2A)	109.3
C(3)-C(2)-H(2A)	109.3
C(1)-C(2)-H(2B)	109.3
C(3)-C(2)-H(2B)	109.3
H(2A)-C(2)-H(2B)	107.9
O(4)-C(3)-C(4)	109.8(5)
O(4)-C(3)-C(2)	108.7(5)
C(4)-C(3)-C(2)	114.7(5)
O(4)-C(3)-H(3)	107.8
C(4)-C(3)-H(3)	107.8
C(2)-C(3)-H(3)	107.8
C(3)-C(4)-C(24)	108.4(5)
C(3)-C(4)-C(23)	111.0(5)
C(24)-C(4)-C(23)	107.2(5)
C(3)-C(4)-C(5)	107.2(4)
C(24)-C(4)-C(5)	108.0(5)
C(23)-C(4)-C(5)	114.8(5)
C(6)-C(5)-C(10)	111.2(4)
C(6)-C(5)-C(4)	116.2(4)
C(10)-C(5)-C(4)	115.5(4)
C(6)-C(5)-H(5)	104
C(10)-C(5)-H(5)	104
C(4)-C(5)-H(5)	104.1
C(5)-C(6)-C(7)	110.8(4)
C(5)-C(6)-H(6A)	109.5
C(7)-C(6)-H(6A)	109.5
C(5)-C(6)-H(6B)	109.5
C(7)-C(6)-H(6B)	109.5
H(6A)-C(6)-H(6B)	108.1
C(8)-C(7)-C(6)	114.1(4)

C(10)-C(9)-C(8)	117.5(4)
C(10)-C(9)-C(11)	113.4(4)
C(8)-C(9)-C(11)	112.4(4)
C(10)-C(9)-H(9)	103.8
C(8)-C(9)-H(9)	103.8
C(11)-C(9)-H(9)	103.8
C(1)-C(10)-C(25)	108.6(5)
C(1)-C(10)-C(9)	108.3(4)
C(25)-C(10)-C(9)	112.5(4)
C(1)-C(10)-C(5)	108.2(4)
C(25)-C(10)-C(5)	112.7(4)
C(9)-C(10)-C(5)	106.4(4)
C(12)-C(11)-C(9)	114.5(4)
C(12)-C(11)-H(11A)	108.6
C(9)-C(11)-H(11A)	108.6
C(12)-C(11)-H(11B)	108.6
C(9)-C(11)-H(11B)	108.7
H(11A)-C(11)-H(11B)	107.6
C(11)-C(12)-C(13)	114.3(4)
C(11)-C(12)-H(12A)	108.7
C(13)-C(12)-H(12A)	108.7
C(11)-C(12)-H(12B)	108.7
C(13)-C(12)-H(12B)	108.7
H(12A)-C(12)-H(12B)	107.6
C(14)-C(13)-C(12)	109.9(4)
C(14)-C(13)-C(27)	107.9(4)
C(12)-C(13)-C(27)	108.0(4)
C(14)-C(13)-C(18)	111.6(3)
C(12)-C(13)-C(18)	107.8(4)
C(27)-C(13)-C(18)	111.8(4)
C(15)-C(14)-C(13)	117.1(5)
C(15)-C(14)-C(8)	124.1(4)
C(13)-C(14)-C(8)	118.8(4)
C(14)-C(15)-C(16)	121.1(5)
C(14)-C(15)-H(15)	119.5
C(16)-C(15)-H(15)	119.5
C(15)-C(16)-C(17)	109.0(4)

C(8)-C(7)-H(7A)	108.7
C(6)-C(7)-H(7A)	108.7
C(8)-C(7)-H(7B)	108.7
C(6)-C(7)-H(7B)	108.7
H(7A)-C(7)-H(7B)	107.6
C(7)-C(8)-C(9)	108.6(4)
C(7)-C(8)-C(26)	108.4(4)
C(9)-C(8)-C(26)	113.9(4)
C(7)-C(8)-C(14)	109.7(4)
C(9)-C(8)-C(14)	108.3(4)
C(26)-C(8)-C(14)	107.9(4)
C(19)-C(18)-C(17)	110.8(4)
C(19)-C(18)-C(13)	112.9(4)
C(17)-C(18)-C(13)	114.3(4)
C(19)-C(18)-H(18)	106
C(17)-C(18)-H(18)	106
C(13)-C(18)-H(18)	106
C(20)-C(19)-C(18)	115.2(4)
C(20)-C(19)-H(19A)	108.4
C(18)-C(19)-H(19A)	108.5
C(20)-C(19)-H(19B)	108.5
C(18)-C(19)-H(19B)	108.5
H(19A)-C(19)-H(19B)	107.5
C(29)-C(20)-C(19)	109.5(6)
C(29)-C(20)-C(30)	108.1(7)
C(19)-C(20)-C(30)	109.0(5)
C(29)-C(20)-C(21)	112.0(6)
C(19)-C(20)-C(21)	108.9(5)
C(30)-C(20)-C(21)	109.3(6)
C(22)-C(21)-C(20)	112.8(5)
C(22)-C(21)-H(21A)	109
C(20)-C(21)-H(21A)	109
C(22)-C(21)-H(21B)	109
C(20)-C(21)-H(21B)	109
H(21A)-C(21)-H(21B)	107.8
C(21)-C(22)-C(17)	113.0(5)
C(21)-C(22)-H(22A)	109
C(17)-C(22)-H(22A)	109
C(21)-C(22)-H(22B)	109
C(17)-C(22)-H(22B)	109

C(15)-C(16)-H(16A)	109.9
C(17)-C(16)-H(16A)	109.9
C(15)-C(16)-H(16B)	109.9
C(17)-C(16)-H(16B)	109.9
H(16A)-C(16)-H(16B)	108.3
C(16)-C(17)-C(28)	108.9(4)
C(16)-C(17)-C(18)	111.2(4)
C(28)-C(17)-C(18)	109.8(4)
C(16)-C(17)-C(22)	111.4(5)
C(28)-C(17)-C(22)	105.0(4)
C(18)-C(17)-C(22)	110.3(4)
C(8)-C(26)-H(26B)	109.5
H(26A)-C(26)-H(26B)	109.5
C(8)-C(26)-H(26C)	109.5
H(26A)-C(26)-H(26C)	109.5
H(26B)-C(26)-H(26C)	109.5
C(13)-C(27)-H(27A)	109.5
C(13)-C(27)-H(27B)	109.5
H(27A)-C(27)-H(27B)	109.5
C(13)-C(27)-H(27C)	109.5
H(27A)-C(27)-H(27C)	109.5
H(27B)-C(27)-H(27C)	109.5
O(1)-C(28)-O(2)	121.9(5)
O(1)-C(28)-C(17)	126.3(5)
O(2)-C(28)-C(17)	111.8(5)
C(20)-C(29)-H(29A)	109.6
C(20)-C(29)-H(29B)	109.5
H(29A)-C(29)-H(29B)	109.5
C(20)-C(29)-H(29C)	109.4
H(29A)-C(29)-H(29C)	109.5
H(29B)-C(29)-H(29C)	109.5
C(20)-C(30)-H(30A)	109.5
C(20)-C(30)-H(30B)	109.5
H(30A)-C(30)-H(30B)	109.5
C(20)-C(30)-H(30C)	109.5
H(30A)-C(30)-H(30C)	109.5
H(30B)-C(30)-H(30C)	109.5
O(3)-C(31)-O(4)	121.7(7)
O(3)-C(31)-C(32)	126.1(9)
O(4)-C(31)-C(32)	112.0(10)

H(22A)-C(22)-H(22B)	107.8
C(4)-C(23)-H(23A)	109.5
C(4)-C(23)-H(23B)	109.4
H(23A)-C(23)-H(23B)	109.5
C(4)-C(23)-H(23C)	109.5
H(23A)-C(23)-H(23C)	109.5
H(23B)-C(23)-H(23C)	109.5
C(4)-C(24)-H(24A)	109.4
C(4)-C(24)-H(24B)	109.5
H(24A)-C(24)-H(24B)	109.5
C(4)-C(24)-H(24C)	109.5
H(24A)-C(24)-H(24C)	109.5
H(24B)-C(24)-H(24C)	109.5
C(10)-C(25)-H(25A)	109.5
C(10)-C(25)-H(25B)	109.4
H(25A)-C(25)-H(25B)	109.5
C(10)-C(25)-H(25C)	109.5
H(25A)-C(25)-H(25C)	109.5
H(25B)-C(25)-H(25C)	109.5
C(8)-C(26)-H(26A)	109.5
C(34)-C(35)-C(36)	115.7(5)
O(8)-C(35)-H(35)	107.6
C(34)-C(35)-H(35)	107.6
C(36)-C(35)-H(35)	107.6
C(55)-C(36)-C(35)	108.9(5)
C(55)-C(36)-C(56)	107.2(4)
C(35)-C(36)-C(56)	110.6(5)
C(55)-C(36)-C(37)	109.3(5)
C(35)-C(36)-C(37)	106.0(4)
C(56)-C(36)-C(37)	114.7(5)
C(38)-C(37)-C(42)	110.4(4)
C(38)-C(37)-C(36)	114.8(4)
C(42)-C(37)-C(36)	117.1(4)
C(38)-C(37)-H(37)	104.2
C(42)-C(37)-H(37)	104.2
C(36)-C(37)-H(37)	104.3
C(39)-C(38)-C(37)	111.5(4)
C(39)-C(38)-H(38A)	109.3
C(37)-C(38)-H(38A)	109.3
C(39)-C(38)-H(38B)	109.3

C(31)-C(32)-H(32A)	109.4
C(31)-C(32)-H(32B)	109.6
H(32A)-C(32)-H(32B)	109.5
C(31)-C(32)-H(32C)	109.5
H(32A)-C(32)-H(32C)	109.5
H(32B)-C(32)-H(32C)	109.5
C(34)-C(33)-C(42)	112.9(4)
C(34)-C(33)-H(33A)	109
C(42)-C(33)-H(33A)	109
C(34)-C(33)-H(33B)	108.9
C(42)-C(33)-H(33B)	109
H(33A)-C(33)-H(33B)	107.8
C(35)-C(34)-C(33)	110.8(6)
C(35)-C(34)-H(34A)	109.5
C(33)-C(34)-H(34A)	109.5
C(35)-C(34)-H(34B)	109.5
C(33)-C(34)-H(34B)	109.5
H(34A)-C(34)-H(34B)	108.1
O(8)-C(35)-C(34)	109.2(5)
O(8)-C(35)-C(36)	109.0(4)
C(41)-C(43)-H(43B)	108.6
C(44)-C(43)-H(43B)	108.6
H(43A)-C(43)-H(43B)	107.6
C(45)-C(44)-C(43)	113.9(4)
C(45)-C(44)-H(44A)	108.8
C(43)-C(44)-H(44A)	108.8
C(45)-C(44)-H(44B)	108.8
C(43)-C(44)-H(44B)	108.8
H(44A)-C(44)-H(44B)	107.7
C(44)-C(45)-C(46)	110.5(4)
C(44)-C(45)-C(59)	108.8(4)
C(46)-C(45)-C(59)	107.1(4)
C(44)-C(45)-C(50)	107.8(4)
C(46)-C(45)-C(50)	111.2(4)
C(59)-C(45)-C(50)	111.6(4)
C(47)-C(46)-C(40)	124.2(4)
C(47)-C(46)-C(45)	117.1(4)
C(40)-C(46)-C(45)	118.7(4)
C(46)-C(47)-C(48)	120.7(4)
C(46)-C(47)-H(47)	119.7

C(37)-C(38)-H(38B)	109.3
H(38A)-C(38)-H(38B)	108
C(38)-C(39)-C(40)	113.7(4)
C(38)-C(39)-H(39A)	108.8
C(40)-C(39)-H(39A)	108.8
C(38)-C(39)-H(39B)	108.8
C(40)-C(39)-H(39B)	108.8
H(39A)-C(39)-H(39B)	107.7
C(46)-C(40)-C(39)	110.2(4)
C(46)-C(40)-C(41)	108.0(3)
C(39)-C(40)-C(41)	108.6(4)
C(46)-C(40)-C(58)	107.4(4)
C(39)-C(40)-C(58)	107.7(4)
C(41)-C(40)-C(58)	115.0(4)
C(43)-C(41)-C(40)	112.7(4)
C(43)-C(41)-C(42)	112.7(4)
C(40)-C(41)-C(42)	116.0(4)
C(43)-C(41)-H(41)	104.7
C(40)-C(41)-H(41)	104.7
C(42)-C(41)-H(41)	104.7
C(33)-C(42)-C(57)	109.7(5)
C(33)-C(42)-C(37)	107.0(4)
C(57)-C(42)-C(37)	113.0(4)
C(33)-C(42)-C(41)	107.1(4)
C(57)-C(42)-C(41)	112.0(5)
C(37)-C(42)-C(41)	107.7(4)
C(41)-C(43)-C(44)	114.6(4)
C(41)-C(43)-H(43A)	108.6
C(44)-C(43)-H(43A)	108.6
C(61)-C(52)-C(51)	108.6(5)
C(53)-C(52)-C(51)	109.2(4)
C(54)-C(53)-C(52)	113.0(5)
C(54)-C(53)-H(53A)	109
C(52)-C(53)-H(53A)	109
C(54)-C(53)-H(53B)	109
C(52)-C(53)-H(53B)	109
H(53A)-C(53)-H(53B)	107.8
C(53)-C(54)-C(49)	111.7(5)
C(53)-C(54)-H(54A)	109.3
C(49)-C(54)-H(54A)	109.3

C(48)-C(47)-H(47)	119.6
C(47)-C(48)-C(49)	107.7(4)
C(47)-C(48)-H(48A)	110.2
C(49)-C(48)-H(48A)	110.2
C(47)-C(48)-H(48B)	110.2
C(49)-C(48)-H(48B)	110.2
H(48A)-C(48)-H(48B)	108.5
C(48)-C(49)-C(60)	106.6(4)
C(48)-C(49)-C(54)	111.9(4)
C(60)-C(49)-C(54)	107.1(4)
C(48)-C(49)-C(50)	110.3(4)
C(60)-C(49)-C(50)	109.6(4)
C(54)-C(49)-C(50)	111.2(4)
C(51)-C(50)-C(49)	110.6(4)
C(51)-C(50)-C(45)	114.3(4)
C(49)-C(50)-C(45)	114.1(4)
C(51)-C(50)-H(50)	105.7
C(49)-C(50)-H(50)	105.7
C(45)-C(50)-H(50)	105.7
C(50)-C(51)-C(52)	113.0(4)
C(50)-C(51)-H(51A)	109
C(52)-C(51)-H(51A)	109
C(50)-C(51)-H(51B)	109
C(52)-C(51)-H(51B)	109
H(51A)-C(51)-H(51B)	107.8
C(62)-C(52)-C(61)	111.2(6)
C(62)-C(52)-C(53)	108.5(5)
C(61)-C(52)-C(53)	109.4(5)
C(62)-C(52)-C(51)	109.8(5)
H(59A)-C(59)-H(59B)	109.5
C(45)-C(59)-H(59C)	109.5
H(59A)-C(59)-H(59C)	109.5
H(59B)-C(59)-H(59C)	109.5
O(6)-C(60)-O(5)	121.0(5)
O(6)-C(60)-C(49)	122.6(4)
O(5)-C(60)-C(49)	116.4(5)
C(52)-C(61)-H(61A)	109.5
C(52)-C(61)-H(61B)	109.5
H(61A)-C(61)-H(61B)	109.5
C(52)-C(61)-H(61C)	109.4

C(53)-C(54)-H(54B)	109.3
C(49)-C(54)-H(54B)	109.3
H(54A)-C(54)-H(54B)	107.9
C(36)-C(55)-H(55A)	109.5
C(36)-C(55)-H(55B)	109.5
H(55A)-C(55)-H(55B)	109.5
C(36)-C(55)-H(55C)	109.5
H(55A)-C(55)-H(55C)	109.5
H(55B)-C(55)-H(55C)	109.5
C(36)-C(56)-H(56A)	109.5
C(36)-C(56)-H(56B)	109.5
H(56A)-C(56)-H(56B)	109.5
C(36)-C(56)-H(56C)	109.4
H(56A)-C(56)-H(56C)	109.5
H(56B)-C(56)-H(56C)	109.5
C(42)-C(57)-H(57A)	109.5
C(42)-C(57)-H(57B)	109.5
H(57A)-C(57)-H(57B)	109.5
C(42)-C(57)-H(57C)	109.5
H(57A)-C(57)-H(57C)	109.5
H(57B)-C(57)-H(57C)	109.5
C(40)-C(58)-H(58A)	109.5
C(40)-C(58)-H(58B)	109.5
H(58A)-C(58)-H(58B)	109.5
C(40)-C(58)-H(58C)	109.5
H(58A)-C(58)-H(58C)	109.5
H(58B)-C(58)-H(58C)	109.5
C(45)-C(59)-H(59A)	109.5
C(45)-C(59)-H(59B)	109.5

H(61A)-C(61)-H(61C)	109.5
H(61B)-C(61)-H(61C)	109.5
C(52)-C(62)-H(62A)	109.5
C(52)-C(62)-H(62B)	109.5
H(62A)-C(62)-H(62B)	109.5
C(52)-C(62)-H(62C)	109.5
H(62A)-C(62)-H(62C)	109.5
H(62B)-C(62)-H(62C)	109.5
O(7)-C(63)-O(8)	120.7(8)
O(7)-C(63)-C(64)	124.1(9)
O(8)-C(63)-C(64)	114.2(9)
C(63)-C(64)-H(64A)	109.5
C(63)-C(64)-H(64B)	109.5
H(64A)-C(64)-H(64B)	109.5
C(63)-C(64)-H(64C)	109.4
H(64A)-C(64)-H(64C)	109.5
H(64B)-C(64)-H(64C)	109.5
C(65)#1-C(65)-C(67)	123.7(12)
C(65)#1-C(65)-H(65)	118.1
C(67)-C(65)-H(65)	118.2
C(67)-C(66)-C(66)#1	123.7(12)
C(67)-C(66)-H(66)	118.2
C(66)#1-C(66)-H(66)	118
C(66)-C(67)-C(65)	110.6(17)
C(28)-O(2)-H(2)	109.5
C(31)-O(4)-C(3)	119.1(7)
C(60)-O(5)-H(5A)	109.5
C(63)-O(8)-C(35)	119.8(6)

Symmetry transformations used to generate equivalent atoms: #1 -x+1,y,-z+1

Table 1.5

Anisotropic displacement parameters (A^{o2} x 10^3) for the sample MPX

(The anisotropic displacement factor exponent takes the form:-2 pi^2 [h^2 a^{*2} U11 + ... + 2 h k a* b* U12])

	U11	U22	U33	U23	U13	U12
C(1)	84(4)	80(4)	45(3)	6(3)	34(3)	-3(3)
C(2)	104(5)	83(5)	49(3)	-1(3)	38(3)	-13(4)
C(3)	83(4)	63(4)	45(3)	-5(3)	19(3)	0(3)
C(4)	59(3)	50(3)	59(3)	-2(3)	21(3)	-1(3)
C(5)	61(3)	38(3)	46(3)	-6(2)	28(2)	1(2)
C(6)	50(3)	63(4)	52(3)	-4(3)	25(3)	-3(3)
C(7)	58(3)	54(3)	47(3)	-7(2)	35(2)	-14(3)
C(8)	57(3)	40(3)	46(3)	-5(2)	32(2)	-5(2)
C(9)	54(3)	37(3)	41(2)	-4(2)	27(2)	-5(2)
C(10)	62(3)	44(3)	47(3)	-4(2)	31(3)	-6(3)
C(11)	67(3)	60(4)	49(3)	8(3)	34(3)	-1(3)
C(12)	58(3)	54(3)	56(3)	0(3)	38(3)	-7(3)
C(13)	51(3)	47(3)	44(3)	-5(2)	28(2)	-3(2)
C(14)	56(3)	39(3)	40(2)	-9(2)	28(2)	-11(2)
C(15)	58(3)	45(3)	51(3)	-5(3)	31(3)	-10(3)
C(16)	70(3)	41(3)	51(3)	-1(2)	32(3)	-3(3)
C(17)	53(3)	47(3)	46(3)	-7(2)	27(2)	-2(2)
C(18)	53(3)	50(3)	50(3)	-9(2)	30(2)	-3(2)
C(19)	58(3)	80(4)	57(3)	-12(3)	32(3)	-3(3)
C(20)	66(4)	98(6)	73(4)	-20(4)	42(3)	-9(4)
C(21)	55(3)	97(5)	65(4)	-12(3)	20(3)	1(4)
C(22)	79(4)	63(4)	67(3)	4(3)	41(3)	14(3)
C(23)	80(4)	71(5)	87(4)	-7(4)	21(4)	18(4)
C(24)	72(4)	78(5)	73(4)	-2(3)	23(3)	-13(4)
C(25)	83(4)	42(4)	75(4)	6(3)	33(3)	-1(3)
C(26)	65(3)	52(3)	61(3)	-19(3)	33(3)	-3(3)
C(27)	69(3)	55(3)	54(3)	-15(3)	35(3)	-4(3)
C(28)	60(3)	50(4)	48(3)	0(2)	34(3)	0(3)
C(29)	87(5)	141(8)	100(5)	-13(6)	30(4)	-48(6)
C(30)	61(4)	192(10)	109(6)	-29(6)	44(4)	7(5)
C(31)	75(5)	139(8)	56(4)	-20(5)	34(4)	-34(5)
C(32)	110(6)	202(11)	61(4)	-11(5)	24(4)	-73(7)
C(33)	62(3)	110(6)	61(3)	14(4)	37(3)	8(4)
C(34)	52(3)	137(7)	69(4)	2(4)	31(3)	1(4)

C(35)	49(3)	78(4)	63(3)	-6(3)	20(3)	13(3)
C(36)	61(3)	61(4)	47(3)	-7(3)	25(3)	7(3)
C(37)	59(3)	44(3)	46(3)	3(2)	28(2)	9(3)
C(38)	60(3)	69(4)	43(3)	8(3)	27(3)	-4(3)
C(39)	66(3)	61(4)	49(3)	10(3)	36(3)	-1(3)
C(40)	53(3)	39(3)	39(2)	5(2)	28(2)	6(2)
C(41)	52(3)	46(3)	46(3)	4(2)	30(2)	2(2)
C(42)	55(3)	52(3)	50(3)	1(2)	31(2)	0(3)
C(43)	58(3)	69(4)	54(3)	11(3)	32(3)	-7(3)
C(44)	63(3)	60(4)	49(3)	16(3)	34(3)	-4(3)
C(45)	54(3)	45(3)	46(3)	0(2)	32(2)	1(2)
C(46)	52(3)	32(3)	48(3)	7(2)	29(2)	9(2)
C(47)	65(3)	35(3)	57(3)	8(2)	38(3)	-1(3)
C(48)	63(3)	35(3)	66(3)	-4(3)	39(3)	-3(3)
C(49)	57(3)	41(3)	46(3)	-3(2)	28(2)	-2(2)
C(50)	55(3)	43(3)	40(3)	-2(2)	28(2)	3(2)
C(51)	60(3)	57(4)	48(3)	2(2)	30(3)	10(3)
C(52)	68(3)	60(4)	44(3)	3(3)	29(3)	10(3)
C(53)	67(3)	74(4)	60(3)	7(3)	29(3)	13(3)
C(54)	65(3)	63(4)	56(3)	-9(3)	28(3)	-9(3)
C(55)	91(5)	68(5)	61(4)	16(3)	16(3)	2(4)
C(56)	67(4)	81(5)	59(3)	-10(3)	25(3)	2(3)
C(57)	67(3)	49(4)	72(4)	-2(3)	20(3)	-9(3)
C(58)	60(3)	56(3)	52(3)	-6(3)	28(3)	7(3)
C(59)	73(3)	65(4)	57(3)	-1(3)	42(3)	18(3)
C(60)	60(3)	42(4)	54(3)	3(2)	38(3)	-1(3)
C(61)	90(5)	115(6)	50(3)	-5(4)	27(3)	24(4)
C(62)	94(5)	86(5)	69(4)	26(4)	35(4)	17(4)
C(63)	88(6)	199(12)	105(6)	-48(7)	10(5)	58(7)
C(64)	68(5)	220(13)	114(7)	0(7)	23(5)	17(6)
C(65)	166(13)	143(11)	213(16)	20(10)	5(13)	16(10)
C(66)	125(9)	174(12)	185(13)	-59(11)	11(10)	-20(10)
O(1)	100(3)	42(3)	75(2)	-12(2)	64(2)	-10(2)
O(2)	171(5)	49(3)	100(3)	-9(2)	107(3)	-8(3)
O(3)	89(4)	173(7)	119(4)	-81(5)	51(3)	-32(4)
O(4)	99(3)	92(4)	54(2)	-2(2)	6(2)	-7(3)
O(5)	93(3)	53(2)	86(3)	-7(2)	67(2)	-9(2)
O(6)	115(3)	41(3)	96(3)	2(2)	82(3)	6(2)
O(7)	124(6)	420(20)	259(11)	-192(13)	-41(7)	164(9)
O(8)	57(2)	103(4)	70(3)	-10(2)	22(2)	16(3)

Table 1.6

Hydrogen coordinates (x 10^4) and isotropic displacement parameters (A^{o2} x 10^3) for the sample MPX

	x	y	z	U(eq)
H(1A)	2629	3297	4753	82
H(1B)	2523	961	4759	82
H(2A)	3211	119	5227	95
H(2B)	3121	1871	5521	95
H(3)	3385	4342	5209	83
H(5)	2986	4333	4266	58
H(6A)	3063	1444	3633	67
H(6B)	3265	3648	3740	67
H(7A)	2618	5209	3374	59
H(7B)	2622	3682	2969	59
H(9)	2294	3899	3893	51
H(11A)	1958	50	3850	68
H(11B)	1868	2153	4033	68
H(12A)	1279	1644	3306	63
H(12B)	1492	587	3029	63
H(15)	2063	5687	2538	60
H(16A)	1449	7096	1924	64
H(16B)	1378	7485	2394	64
H(18)	1024	2220	2324	59
H(19A)	701	3618	2750	76
H(19B)	718	5802	2536	76
H(21A)	335	3223	1334	93
H(21B)	54	5162	1237	93
H(22A)	639	7124	1773	81
H(22B)	657	6247	1296	81
H(23A)	3739	390	4445	133
H(23B)	3545	-588	4754	133
H(23C)	3979	477	5053	133
H(24A)	3663	5652	4709	120
H(24B)	3813	4174	4421	120
H(24C)	4049	4236	5029	120
H(25A)	3012	-1230	4443	105
H(25B)	2748	-973	3839	105
H(25C)	2531	-1491	4157	105
H(26A)	1956	-135	2929	88

H(26B)	2435	-261	3164	88
H(26C)	2158	815	2627	88
H(27A)	1248	5431	3356	87
H(27B)	1465	6847	3138	87
H(27C)	1731	5520	3638	87
H(29A)	-105	1453	1654	176
H(29B)	130	1437	2262	176
H(29C)	363	829	1968	176
H(30A)	-316	5074	1675	180
H(30B)	23	6721	2004	180
H(30C)	-76	4946	2282	180
H(32A)	4438	1787	6456	199
H(32B)	4361	3360	6799	199
H(32C)	4623	4022	6546	199
H(33A)	3740	-1105	1943	90
H(33B)	3749	1289	1869	90
H(34A)	4403	111	2531	102
H(34B)	4222	1728	2756	102
H(35)	4196	-2567	2844	80
H(37)	3457	-2450	2465	58
H(38A)	3122	-2162	2946	68
H(38B)	3034	169	2782	68
H(39A)	2460	-1803	2226	67
H(39B)	2732	-3198	2086	67
H(41)	3055	-1517	1604	55
H(43A)	3096	637	1082	70
H(43B)	3030	2587	1343	70
H(44A)	2346	2457	852	65
H(44B)	2477	1676	459	65
H(47)	2034	-3374	1474	59
H(48A)	1426	-4026	706	63
H(48B)	1715	-4202	467	63
H(50)	1729	1293	297	53
H(51A)	1674	-1966	-354	64
H(51B)	1898	119	-325	64
H(53A)	918	1337	-361	82
H(53B)	674	-157	-833	82
H(54A)	1079	-2944	-318	75
H(54B)	824	-1932	-89	75
H(55A)	3867	-4180	3260	125

H(55B)	3698	-2879	3556	125
H(55C)	4174	-2990	3768	125
H(56A)	3928	2061	3286	108
H(56B)	4207	756	3785	108
H(56C)	3730	886	3567	108
H(57A)	3345	3644	2085	104
H(57B)	3633	3051	2670	104
H(57C)	3152	2879	2416	104
H(58A)	2179	1367	1811	84
H(58B)	2354	2572	1507	84
H(58C)	2625	2308	2110	84
H(59A)	2406	-1988	202	91
H(59B)	2274	-3630	477	91
H(59C)	2708	-2564	784	91
H(61A)	1090	-2050	-1180	133
H(61B)	1321	-371	-1318	133
H(61C)	860	-25	-1469	133
H(62A)	1408	3375	-397	129
H(62B)	1050	3348	-977	129
H(62C)	1508	3006	-841	129
H(64A)	5279	-860	4222	217
H(64B)	5494	-1107	3890	217
H(64C)	5447	-3023	4178	217
H(65)	4830	6538	4563	257
H(66)	4883	565	4567	229
H(2)	1338	3381	1204	134
H(5A)	1119	-91	1088	101

Table 1.7

Torsion angles [deg] for the sample MPX.

C(10)-C(1)-C(2)-C(3)	55.6(8)
C(1)-C(2)-C(3)-O(4)	179.6(5)
C(1)-C(2)-C(3)-C(4)	-57.1(8)
O(4)-C(3)-C(4)-C(24)	-67.5(6)
C(2)-C(3)-C(4)-C(24)	169.9(5)
O(4)-C(3)-C(4)-C(23)	50.0(7)
C(2)-C(3)-C(4)-C(23)	-72.7(7)
O(4)-C(3)-C(4)-C(5)	176.2(5)
C(2)-C(3)-C(4)-C(5)	53.5(7)
C(3)-C(4)-C(5)-C(6)	175.2(5)
C(24)-C(4)-C(5)-C(6)	58.6(6)
C(23)-C(4)-C(5)-C(6)	-60.9(7)
C(3)-C(4)-C(5)-C(10)	-51.8(6)
C(24)-C(4)-C(5)-C(10)	-168.4(5)
C(23)-C(4)-C(5)-C(10)	72.0(6)
C(10)-C(5)-C(6)-C(7)	61.3(6)
C(4)-C(5)-C(6)-C(7)	-163.8(5)
C(5)-C(6)-C(7)-C(8)	-57.4(6)
C(6)-C(7)-C(8)-C(9)	48.8(6)
C(6)-C(7)-C(8)-C(26)	-75.4(5)
C(6)-C(7)-C(8)-C(14)	167.0(4)
C(7)-C(8)-C(9)-C(10)	-49.3(6)
C(26)-C(8)-C(9)-C(10)	71.6(6)
C(14)-C(8)-C(9)-C(10)	-168.3(4)
C(7)-C(8)-C(9)-C(11)	176.3(4)
C(26)-C(8)-C(9)-C(11)	-62.8(6)
C(14)-C(8)-C(9)-C(11)	57.3(5)
C(2)-C(1)-C(10)-C(25)	70.8(7)
C(2)-C(1)-C(10)-C(9)	-166.8(5)
C(2)-C(1)-C(10)-C(5)	-51.9(7)
C(8)-C(9)-C(10)-C(1)	169.3(5)
C(11)-C(9)-C(10)-C(1)	-56.7(6)
C(8)-C(9)-C(10)-C(25)	-70.7(6)
C(11)-C(9)-C(10)-C(25)	63.3(6)
C(8)-C(9)-C(10)-C(5)	53.2(5)
C(11)-C(9)-C(10)-C(5)	-172.8(4)
C(6)-C(5)-C(10)-C(1)	-173.5(5)

C(11)-C(12)-C(13)-C(27)	-62.4(5)
C(11)-C(12)-C(13)-C(18)	176.7(4)
C(12)-C(13)-C(14)-C(15)	163.9(4)
C(27)-C(13)-C(14)-C(15)	-78.6(5)
C(18)-C(13)-C(14)-C(15)	44.5(6)
C(12)-C(13)-C(14)-C(8)	-17.3(5)
C(27)-C(13)-C(14)-C(8)	100.2(5)
C(18)-C(13)-C(14)-C(8)	-136.7(4)
C(7)-C(8)-C(14)-C(15)	23.5(6)
C(9)-C(8)-C(14)-C(15)	141.8(5)
C(26)-C(8)-C(14)-C(15)	-94.5(6)
C(7)-C(8)-C(14)-C(13)	-155.2(4)
C(9)-C(8)-C(14)-C(13)	-36.9(6)
C(26)-C(8)-C(14)-C(13)	86.8(5)
C(13)-C(14)-C(15)-C(16)	-7.7(7)
C(8)-C(14)-C(15)-C(16)	173.6(4)
C(14)-C(15)-C(16)-C(17)	-44.5(6)
C(15)-C(16)-C(17)-C(28)	-64.7(5)
C(15)-C(16)-C(17)-C(18)	56.4(5)
C(15)-C(16)-C(17)-C(22)	180.0(4)
C(16)-C(17)-C(18)-C(19)	108.1(5)
C(28)-C(17)-C(18)-C(19)	-131.3(5)
C(22)-C(17)-C(18)-C(19)	-16.1(6)
C(16)-C(17)-C(18)-C(13)	-20.9(6)
C(28)-C(17)-C(18)-C(13)	99.7(5)
C(22)-C(17)-C(18)-C(13)	-145.0(4)
C(14)-C(13)-C(18)-C(19)	-155.6(5)
C(12)-C(13)-C(18)-C(19)	83.7(5)
C(27)-C(13)-C(18)-C(19)	-34.7(6)
C(14)-C(13)-C(18)-C(17)	-27.7(6)
C(12)-C(13)-C(18)-C(17)	-148.4(4)
C(27)-C(13)-C(18)-C(17)	93.1(5)
C(17)-C(18)-C(19)-C(20)	60.9(7)
C(13)-C(18)-C(19)-C(20)	-169.4(5)
C(18)-C(19)-C(20)-C(29)	81.1(7)
C(18)-C(19)-C(20)-C(30)	-160.8(6)
C(18)-C(19)-C(20)-C(21)	-41.6(8)

C(4)-C(5)-C(10)-C(1)	51.3(6)
C(6)-C(5)-C(10)-C(25)	66.4(6)
C(4)-C(5)-C(10)-C(25)	-68.8(6)
C(6)-C(5)-C(10)-C(9)	-57.4(5)
C(4)-C(5)-C(10)-C(9)	167.4(4)
C(10)-C(9)-C(11)-C(12)	-159.2(4)
C(8)-C(9)-C(11)-C(12)	-22.9(7)
C(9)-C(11)-C(12)-C(13)	-34.9(6)
C(11)-C(12)-C(13)-C(14)	55.0(5)
C(11)-C(12)-C(13)-C(27)	-62.4(5)
C(11)-C(12)-C(13)-C(18)	176.7(4)
C(12)-C(13)-C(14)-C(15)	163.9(4)
C(27)-C(13)-C(14)-C(15)	-78.6(5)
C(18)-C(13)-C(14)-C(15)	44.5(6)
C(12)-C(13)-C(14)-C(8)	-17.3(5)
C(27)-C(13)-C(14)-C(8)	100.2(5)
C(18)-C(13)-C(14)-C(8)	-136.7(4)
C(7)-C(8)-C(14)-C(15)	23.5(6)
C(9)-C(8)-C(14)-C(15)	141.8(5)
C(26)-C(8)-C(14)-C(15)	-94.5(6)
C(7)-C(8)-C(14)-C(13)	-155.2(4)
C(9)-C(8)-C(14)-C(13)	-36.9(6)
C(26)-C(8)-C(14)-C(13)	86.8(5)
C(13)-C(14)-C(15)-C(16)	-7.7(7)
C(8)-C(14)-C(15)-C(16)	173.6(4)
C(14)-C(15)-C(16)-C(17)	-44.5(6)
C(15)-C(16)-C(17)-C(28)	-64.7(5)
C(15)-C(16)-C(17)-C(18)	56.4(5)
C(15)-C(16)-C(17)-C(22)	180.0(4)
C(16)-C(17)-C(18)-C(19)	108.1(5)
C(28)-C(17)-C(18)-C(19)	-131.3(5)
C(22)-C(17)-C(18)-C(19)	-16.1(6)
C(16)-C(17)-C(18)-C(13)	-20.9(6)
C(28)-C(17)-C(18)-C(13)	99.7(5)
C(22)-C(17)-C(18)-C(13)	-145.0(4)
C(14)-C(13)-C(18)-C(19)	-155.6(5)
C(12)-C(13)-C(18)-C(19)	83.7(5)
C(27)-C(13)-C(18)-C(19)	-34.7(6)
C(14)-C(13)-C(18)-C(17)	-27.7(6)
C(12)-C(13)-C(18)-C(17)	-148.4(4)

C(29)-C(20)-C(21)-C(22)	-140.2(6)
C(19)-C(20)-C(21)-C(22)	-18.9(8)
C(30)-C(20)-C(21)-C(22)	100.0(7)
C(20)-C(21)-C(22)-C(17)	62.9(7)
C(16)-C(17)-C(22)-C(21)	-166.6(4)
C(28)-C(17)-C(22)-C(21)	75.7(6)
C(18)-C(17)-C(22)-C(21)	-42.5(6)
C(16)-C(17)-C(28)-O(1)	143.5(5)
C(11)-C(12)-C(13)-C(27)	-62.4(5)
C(39)-C(40)-C(46)-C(47)	23.1(6)
C(41)-C(40)-C(46)-C(47)	141.6(5)
C(58)-C(40)-C(46)-C(47)	-93.9(6)
C(39)-C(40)-C(46)-C(45)	-158.0(4)
C(41)-C(40)-C(46)-C(45)	-39.5(6)
C(58)-C(40)-C(46)-C(45)	85.0(5)
C(44)-C(45)-C(46)-C(47)	164.7(4)
C(59)-C(45)-C(46)-C(47)	-77.0(5)
C(50)-C(45)-C(46)-C(47)	45.1(6)
C(44)-C(45)-C(46)-C(40)	-14.3(6)
C(59)-C(45)-C(46)-C(40)	104.0(5)
C(50)-C(45)-C(46)-C(40)	-133.9(4)
C(40)-C(46)-C(47)-C(48)	168.1(4)
C(45)-C(46)-C(47)-C(48)	-10.8(7)
C(46)-C(47)-C(48)-C(49)	-43.7(6)
C(47)-C(48)-C(49)-C(60)	-58.0(5)
C(47)-C(48)-C(49)-C(54)	-174.7(4)
C(47)-C(48)-C(49)-C(50)	61.0(5)
C(48)-C(49)-C(50)-C(51)	102.9(5)
C(60)-C(49)-C(50)-C(51)	-140.1(4)
C(54)-C(49)-C(50)-C(51)	-21.9(6)
C(48)-C(49)-C(50)-C(45)	-27.6(5)
C(60)-C(49)-C(50)-C(45)	89.4(5)
C(54)-C(49)-C(50)-C(45)	-152.4(4)
C(44)-C(45)-C(50)-C(51)	86.9(5)
C(46)-C(45)-C(50)-C(51)	-151.9(4)
C(59)-C(45)-C(50)-C(51)	-32.5(6)
C(44)-C(45)-C(50)-C(49)	-144.5(4)
C(46)-C(45)-C(50)-C(49)	-23.3(6)
C(59)-C(45)-C(50)-C(49)	96.2(5)
C(49)-C(50)-C(51)-C(52)	63.8(6)

C(27)-C(13)-C(18)-C(17)	93.1(5)
C(17)-C(18)-C(19)-C(20)	60.9(7)
C(13)-C(18)-C(19)-C(20)	-169.4(5)
C(18)-C(19)-C(20)-C(29)	81.1(7)
C(18)-C(19)-C(20)-C(30)	-160.8(6)
C(18)-C(19)-C(20)-C(21)	-41.6(8)
C(29)-C(20)-C(21)-C(22)	-140.2(6)
C(19)-C(20)-C(21)-C(22)	-18.9(8)
C(30)-C(20)-C(21)-C(22)	100.0(7)
C(20)-C(21)-C(22)-C(17)	62.9(7)
C(16)-C(17)-C(22)-C(21)	-166.6(4)
C(28)-C(17)-C(22)-C(21)	75.7(6)
C(18)-C(17)-C(22)-C(21)	-42.5(6)
C(16)-C(17)-C(28)-O(1)	143.5(5)
C(18)-C(17)-C(28)-O(1)	21.5(7)
C(22)-C(17)-C(28)-O(1)	-97.1(6)
C(16)-C(17)-C(28)-O(2)	-38.5(6)
C(18)-C(17)-C(28)-O(2)	-160.5(4)
C(65)-C(65)-C(67)-C(66)	18(3)
O(3)-C(31)-O(4)-C(3)	-9.5(11)
C(32)-C(31)-O(4)-C(3)	174.7(6)
C(4)-C(3)-O(4)-C(31)	125.3(6)
C(2)-C(3)-O(4)-C(31)	-108.6(6)

C(45)-C(50)-C(51)-C(52)	-165.8(5)
C(50)-C(51)-C(52)-C(62)	79.6(6)
C(50)-C(51)-C(52)-C(61)	-158.6(5)
C(50)-C(51)-C(52)-C(53)	-39.2(7)
C(62)-C(52)-C(53)-C(54)	-142.2(5)
C(61)-C(52)-C(53)-C(54)	96.3(6)
C(51)-C(52)-C(53)-C(54)	-22.5(7)
C(52)-C(53)-C(54)-C(49)	63.8(6)
C(48)-C(49)-C(54)-C(53)	-162.0(5)
C(60)-C(49)-C(54)-C(53)	81.6(5)
C(50)-C(49)-C(54)-C(53)	-38.2(6)
C(48)-C(49)-C(60)-O(6)	141.3(5)
C(54)-C(49)-C(60)-O(6)	-98.9(6)
C(50)-C(49)-C(60)-O(6)	21.9(7)
C(48)-C(49)-C(60)-O(5)	-41.3(6)
C(54)-C(49)-C(60)-O(5)	78.5(5)
C(50)-C(49)-C(60)-O(5)	-160.7(4)
C(66)#1-C(66)-C(67)-C(65)	-12(3)
O(7)-C(63)-O(8)-C(35)	4(2)
C(64)-C(63)-O(8)-C(35)	173.6(8)
C(34)-C(35)-O(8)-C(63)	-96.8(9)
C(36)-C(35)-O(8)-C(63)	136.0(9)

Symmetry transformations used to generate equivalent atoms: -x+1, y, -z+1

Table 1.8

Comparison of endocyclic torsion angles($\omega_{i,j}$,σ ±0.5-0.8°) about the bond between atoms i and j of cyclohexane rings in 3-acetyl aleuritolic acid molecule in MPX to that of *p*-bromobenzyl 3-acetylaleuritolate[45]

3-acetylaleuritolicacid			*p*-bromobenzyl 3-acetylaleuritolate
Ring A	$\omega_{1,2}$	-55.6	-58.3
	$\omega_{2,3}$	57.1	63.6
	$\omega_{3,4}$	-53.5	-58.7
	$\omega_{4,5}$	51.8	52.1
	$\omega_{5,10}$	-51.3	-49.7
	$\omega_{1,10}$	51.9	51.2
Ring B	$\omega_{5,6}$	-61.3	-63.0
	$\omega_{6,7}$	57.4	57.4
	$\omega_{7,8}$	-48.8	-48.8
	$\omega_{8,9}$	49.3	50.9
	$\omega_{9,10}$	-53.2	-56.8
	$\omega_{5,10}$	57.4	61.0
Ring C	$\omega_{8,9}$	-57.3	-59.2
	$\omega_{9,11}$	22.9	27.7
	$\omega_{11,12}$	34.9	30.3
	$\omega_{12,13}$	-55.0	-53.8
	$\omega_{13,14}$	17.3	19.5
	$\omega_{8,14}$	36.9	35.4
Ring D	$\omega_{13,14}$	-44.5	-42.4
	$\omega_{14,15}$	7.7	8.5
	$\omega_{15,16}$	44.5	42.9
	$\omega_{16,17}$	-56.4	-58.6
	$\omega_{17,18}$	20.9	26.1
	$\omega_{13,18}$	27.7	22.9
Ring E	$\omega_{17,18}$	16.1	20.8
	$\omega_{18,19}$	-60.9	-63.2
	$\omega_{19,20}$	41.6	37.7
	$\omega_{20,21}$	18.9	24.9
	$\omega_{21,22}$	-62.9	-67.3
	$\omega_{17,22}$	42.5	41.2

Table 1.9

Hydrogen bonded geometry (A°,deg.) for the sample MPX

D-H……A	D-H	H…..A	D…..A	D-H…..A
O2-H2…O6	0.820	1.811	2.620	188.97
O5-H5A….O1	0.820	1.917	2.722	166.96

Table 1.10

Symmetry equivalent positions of the crystal lattice

No.	Symmetry op.	Description	Order	Type
1	x, y, z	identity	1	1
2	-x, y,-z	rotation axis(2 fold)	2	2
3	½+x, ½+y ,z	centering vector	1	1
4	-x+½, ½+y,-z	screw axis	2	2

1.2.6 Results and discussion

Single crystal analysis results confirmd the identity of the compound MP1F isolated from *M.peltata* female plant as 3-acetyaleuritolic acid. To the best of our knowledge single crystal study of 3-acetylaleuritolic acid has not been reported earlier. The crystal structure implies that the structure is monoclinic with eight molecules in the unit cell (Fig 3). It is noted from fig. 3 that two molecules of pyridine (medium used for crystal growth) and three molecules of water were also incorporated in the monoclinic unit cell. Because of this the formula given on page 35 as fractional values for atoms. The eight molecule of 3-acetylaleuritolic acid were seen as four pairs bonded by H-bonding through carboxylic acid group. The H-bonded geometry is described as cyclic dimmer, the bond parameters are listed in table 1.9. H-bond parameters are comparable with crystal structure data of carboxylic acids[48]. These strong intermolecular hydrogen bonding is reflected in the low carbonyl absorption frequency (1687.41 cm^{-1}) of the carboxylic acid carbonyl group. The position of the double bond between C(14)-C(15) carbon atoms is characterized by the C=C bond length of 1.322 A^{o} (table 1.3).

Comparison of endocyclic torsion angles($\omega_{i,j}$,σ) about the bond between atoms i and j of cyclohexane rings in 3-acetylaleuritolic acid molecule in MPX solved using Direct Method to that of *p*-bromobenzyl 3-acetylaleuritolate[45] solved using Heavy Atom Method (table 1.7) reveals the

conformal identity of the ring systems. There is some marked deviations in endocyclic torsion angles at ring C: between $\omega_{9,11}$ 22.9° against 27.7° $\omega_{11,12}$ 34.9° against 30.3° and at ring E: $\omega_{17,18}$ 16.1° against 20.8°, $\omega_{19,20}$ 41.6° against 37.7° and $\omega_{20,21}$ 18.9° against 24.9°, which may be due to the presence of the *p*-bromobenzyl substituent at carboxyl group of 3-acetylaleuritolic acid in the earlier work. The conformation of cyclohexane rings is evident from the table of torsion angles (table 1.9), The rings A and B are in chair conformation since all endocyclic dihedral angles are nearly 60°. The C and E rings approximates to twisted boat forms, which is evident from torsion angles and by viewing the structure using Mercury 1.4.1 software. Analysis of corresponding D angles in terms of geometry-related values for boat, twisted boat and 1,3 diplanar forms indicated that this ring has conformation which departs significantly from all three and is of intermediate character, which can be attributed to the double bond present in the ring.

Conclusion

Female plant of *Macaranga peltata* can be regarded as a good source of 3-acetylaleuritolic acid which has been earlier isolated and identified from the seeds of *Phytolacca americana* L. growing in Korea which belongs to a separate family Phytolaccaceae.[51] Aleuritolic acid had also been isolated from the bark of *Aleurites montana* (Euphorbiaceae)[44] in West Bengal.

The single crystal X-ray analysis implies that the structure is monoclinic with eight molecules per unit cell. Comparison of endocyclic torsion angles of cyclohexane rings in 3-acetylaleuritolic acid molecule solved using Direct Method to that of *p*-bromobenzyl 3-acetylaleuritolate[45] solved using Heavy Atom Method reveals the conformal identity of the ring systems.

The rearrangement of 3-acetylaleuritolic acid in acid medium reveals the structural relationship between aleuritolic acid and oleanolic acid. The presence of an acid that can stabilize the transition state is found to be necessary for this rearrangement to occur.

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CHAPTER -II

INVESTIGATION ON *MACARANGA PELTATA* SEED OIL

2.1 Introduction

The use of fats and oils for edible and industrial purposes has been as long as history of mankind itself. Their use in cooking and other purposes in every day life in ancient cultures like the Chinese, the Indian, the Egyptian and the Greco- Roman was just as important as it is in present time. The ancient Aryans in India is known to have used butter fat or ghee for cooking apart from its use in massage oils. There is a description, as old as 1400 BC, regarding the probable use of animal fats for moving larger stones or wooden logs in ancient Egypt. In the ruins of Pompeii, residence of soap was found in a soap boiler which was used to produce soap from potash and fats. Regarding the sources of oils, olives of the Mediterranean, rapeseeds of Europe, sesame seeds of India and soybeans of China are amongst the oldest described by the ancients[1].

For reasons related to both history and climate, there are pronounced geographic patterns of consumption of fats and oils. The ancestors of the present inhabitants of central and northern Europe obtained their edible fats almost exclusively from domestic animals. The food habits and the cuisine

depended on the availability of plastic fats like butter, lard and margarine. In contrast, population pressures in the older civilizations of the Orient and the Mediterranean countries of southern Europe, northern Africa, and the Middle East have long since made extensive raising of livestock impractical, necessitating that the edible oils of these regions be derived primarily from intensively cultivated vegetable crops. In the tropics, conditions are relatively unfavourable for livestock but are well suited to culture of a variety of oil-bearing plants, many of which flourish in the wild state. In contrast to most high-population-density tropical areas, cattle abound in India. Clarified butter or ghee is an important item of Indian cookery, and a hydrogenated shortening called vanaspati is designed to reproduce the coarsely crystalline plastic texture of ghee

More than eighty percent of the world production of fats and oils is used in edible products, and the objective of most processing steps is to convert crude fats of low palatability or undesirable physical form into refined products that meet the regional requirements for food fats. The annual consumption of visible fats—such as lard, butter, shortening, or salad oils that have been separated from the original animal or plant source—ranges from 18 to 25 kilograms per person in various highly industrialized European countries to 23 kilograms per person in the United States. For the world as a whole, the average available supply is 10 kilograms per person; and in many

areas of South America, Africa, and Southeast Asia, the annual consumption is 5 kilograms or less per person[2].

Oils and fats both for edible and industrial purposes may be of either vegetable or animal origin. Among the fats of animal origin there are three categories; they are land animal body fats, milk fats and fats from marine animals (fish, whale etc). Among the fats of vegetable origin there are those which are from annual plants namely the seed fats like ground nuts, sunflower, linseed etc, and those of perennial plants or trees like coconut, oil palm etc. In many cases non oily products are the main product and oil is the byproduct as in the case of cotton seed and soybean.

The oil cakes left after the extraction of oil from seeds and nuts were used as animal feed, poultry feed, fish feed and also for human food. Many oils after extraction are taken without further refining. Some byproducts of oil refining industry like phospholipids or lecithin are used for surface coatings.

Fatty oils can be converted to diesel equivalent, by trans esterification with ethanol, called bio-diesel. Petroleum reserves are depleting rapidly, if the current trends in consumption continues, it will not be go beyond 75 years. The researches in this area are getting momentum, seed oils of plant jatropha has already come into commercial use. Fatty oils from marine algae are also in focus.

2.2 Chemistry of Fats

Chemically, oils and fats are esters of glycerol and fatty acids. The difference between oils and fats is only in their melting points. There are some oils which are liquid in summer and semi-solid or solid in winter, so the term 'fat' is used to indicate both[5].

Oils and fats are triglycerides or triacyl-glycerols. Acyl glycerol and many other compounds which by origin are either fat incorporated or fat soluble substances are called lipids. Lipids which contain the glycerol residue are called glycero-lipids. Lipids which on hydrolysis give fatty alcohol and fatty acid are known as simple lipids (waxes). Fats in which only two acyl groups are substituted are called diacylglycerol and those with just one acyl substituent are called mono acylglycerol. Besides the full and partially substituted acyl glycerol and free fatty acids, it is necessary to consider other components of lipid such as phosphoacylglycerols. This ester can be further esterified. Mono alcohol or glyceride residue form esters of ortho-phosphoric acid, these are also called complex lipids.

There are a number of factors which affect composition of fats and oils in vegetable kingdom, for example climatic condition, soil type in which the plant was grown, geography of the location, maturity of the plant, health of the plant and numerous environmental conditions, specific location of the oil seed within the flower it self[3,4] and most important, the genetic variation in

the plant. For example rapeseeds which generally contain 40-50 percent erucic acid were bred to produce less than 5% of erucic acid[5]. In land animals, fat and oil composition varies according to animal species. There is much difference in composition in pork, beef and lamb fats[6].

2.3 Theories of distribution of fatty acids

A great number of varieties of fats and oils occur in nature. They are mixtures of mixed triglycerides and not of simple triglycerides. In 1927 Hidich and co-workers gave a well known theory known as 'Even distribution'. According to this theory component of fatty acids in natural fat tends to be distributed as broadly as possible among all the triglyceride molecules described in some specific terms[7]. When a fatty acid (A) forms about 35 mol percent or more of the total acid (A+X) in a fat, it will occur at least once $G(AX_2)$ in all the triglyceride molecules. If it forms about 70 mol percent or more percent of total fatty acid (A+X) it will occur at least twice $G(A_2X)$ in any given triglyceride molecule and all the excess of 'A' thus will appear as simple triglyceride GA_3. A minor component fatty acid that forms less than about 1/3 of the total fatty acid will not occur more than once in any triglyceride molecule. Though even distribution theory is applicable to many vegetable oils but fails in oils such as linseed, sunflower, soybean etc.[8-10]. Some investigators[11-14] suggested that animal fats form a random distribution. Gunstone[16] has proposed a theory of acyl group distribution in vegetable fats

based on enzymatic hydrolysis and has related this theory with biosynthesis of triglycerides. According to this view the second hydroxyl group of glycerol is preferentially acylated by C_{18} unsaturated acids. The primary hydroxyl groups of acylglyceride are acylated subsequently by all the remaining fatty acids.

2.4 The fatty acids of nature

In nature the fatty acids occur with an even number of carbon atoms with few exceptions. The fatty acids may be saturated or unsaturated in nature. Most naturally occurring vegetable oils and fats contain only non-conjugated double bonds, which may isomerise to *trans* configuration during extraction and other processing procedures such as oxidation and partial hydrogenation. Although most fatty acids in nature are straight chained, there are also branched chain fatty acids. Besides saturated and unsaturated fatty acids, nature also produces oxygenated functional groups such as hydroxyl, epoxy, keto group etc. Castor oil contains about 85-95% hydroxy fatty acid called ricinoleic acid (12-hydroxyoleic acid). Vernonia oil[15] obtained from *Vernonia anthelmintica* contains about 40% vernolic acid, an epoxydised member (12,13-epoxyoleic acid). The main component of oiticica oil[15] is licanic acid (4-oxo-9,11,13-octadecatrienoic acid) is an example of the keto group containing fatty acid. Exceptionally some fatty acids present in micro organisms are branched[16], may be substituted at one or more position of the carbon chain by one or more methyl or ethyl groups. Oils obtained from

plants of *Thalictrum* genus contain a double bond with *trans* configuration. Some fatty acids in plants contain a cyclopentanoic unsaturated ring at the end of the chain. 'Chaulmoogra' oil extracted from seeds of *Hydnocarpus* contains 11-cyclopent-2-enyl-undecanoic (hydnocarpic), 13-cyclopent-2-enyl-tridecanoic (chaulmoogric) and 13-cyclopent-2-enyltridec-6-enoic (gorlic) acids. All have the (*R*)-(+) stereochemistry at carbon 1 of the cyclopent-2-enyl ring[23]. The existence of cyclopropene three rings in the middle of the fatty acid chain is exemplified by sterculic acid[15,17] (7-(2-heptyl-cycloprop-1-enyl)-heptanoic acid) and dihydrosterculic acid in cotton seed and hemp seed oils. Cyclopropene fatty acids produce unwanted biological effects when ingested by animals[22]. Isano oil contains 18–carbon atom fatty acid with two acetylenic bonds and a double bond (octadeca-17-en-9-11-diynoic acid). Isanolic acid is 8-hyrdoxyoctadeca-17-en-9,11-diynoic acid. [15] .

Essential fatty acids

There are some polyunsaturated fatty acids which are very important for animal health but cannot be synthesized by the animal organism. These poly unsaturated fatty acids are known as essential fatty acids. Linoleic acid is the most important essential fatty acid; its absence in fat causes growth retardation in living animals and skin permeation problems. Linoleic acid is well converted by the enzymes of animal organism via chain elongation and desaturation to dihomo- γ-linolenic acid (20:3 8c,11c,14c) and consequently

to arachidonic acid (20:4.5c,8c,11c.14c) all of which are essential fatty acids. Some essential fatty acids form the building blocks of the physiologically important substance called prostaglandins. These are short lived smooth muscle and vasodepressor regulators formed in animal organism by the enzymatic cyclisation of polyunsaturated fatty acids such as 20:5 and 22:6

2.5 Physical properties of fatty acids

The melting point, boiling point and refractive index increase with increase in chain length while density decreases with increase in chain length. The short chain fatty acids up to 12 carbon atoms can be separated easily by steam distillation. The solubility in water decreases as number of carbon atom increases. Decanoic acid is only very slightly soluble even in boiling water.

Double bonds of the *cis* configuration give bending in the straight chain profile of fatty acid molecule. Double bonds in *trans* configuration fit sterically more smoothly into the zigzag line of the rest of the molecules. *Trans* acid melts at higher temperature than do *cis* ones, which explain the liquid state of the latter at room temperature. The *cis* isomers occur more frequently in nature than *trans* ones.

2.6 Chemical properties

In general, fatty acids are easily transformed by alkalis or metallic oxides to the corresponding salts called soaps. The saturated fatty acids are

not quite inert. Free radicals initiate the oxidation, and halogenation processes which become enhanced at high pressure. Thermal cleavage of the chain and the formation of lower molecular weight scission products at temperatures above 300 °C is quite possible. Heating to high temperature in an inert atmosphere may cause decarboxylation of fatty acids with the formation of hydrocarbons.

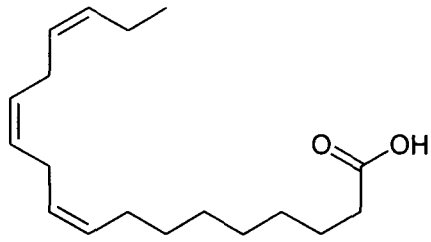
Unsaturated fatty acid may undergo reaction when confronted with oxygen, hydrogen, halogen, inorganic and organic acids, sulphur compounds, heavy metals, heat or radiation. More generally free radial or ion induced process take place. Fatty acids containing more than two isolated double bonds are easily auto oxidised, isomerised or polymerised (eg. drying of oils).

The average degree of unsaturation of fatty acids can be expressed analytically by the iodine value. Difference in stereo configuration can easily be detected by IR spectroscopy. The *trans* acids are characterized by absorption at 970 cm^{-1} corresponding to the out of plane vibration of C-H bond. The refractive index increases with number of double bonds and conjugated character of double bonds.

2.7 Shorthand notation for nomenclature of fatty acids

There are two short hand notation systems, one used by organic and analytical chemists and other used by biochemists. The first system abbreviates fatty acids in the form of 'x:y,zc' where 'x' stands for number of

carbon atoms in fatty acid chain, 'y' stands for the number of double bonds present, 'z' for the position of the double bonds and the 'c' or 't' indicates *cis* or *trans* nature of the double bond. In the second system called ECC system abbreviates fatty acids in the form of, 'x:y, ω m' , were the 'ω' stands for the fact that terminal methyl group of fatty acid chain is taken as C_1 and 'm' for the position of first double bond, for example ,



Linolenic acid

IUPAC Name : Z-9, Z-12, Z-15- octadecatrienoic acid.

Analytical short hand : 18:3, 9c, 12c, 15c

ECC short had : 18:3, ω 3 (6, 9)

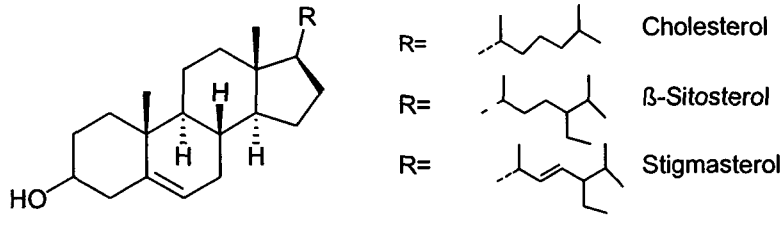
2.8 Minor compounds accompanying lipids

Alcohols

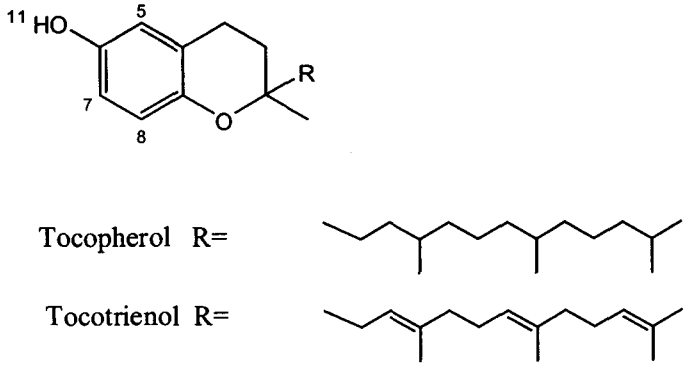
Primarily long carbon chain alcohols can be derived from fatty acid by converting the carboxyl group to hydroxyl methylenic ($-CH_2-OH$) one. Alcohols up to 22 carbon atoms are called fatty alcohols and those of longer chain are called wax alcohols. They are mainly present in the tissue fat of aquatic animals as palmitic, stearic, oleic and linoleic monoesters.

Sterols

Sterols are tetracyclic compounds derived biologically from terpenes. They are fat soluble and therefore found in small quantities in fats and oils. Cholesterol is a common constituent in animal fat such as lard, tallow and butter fat. The hydroxyl group can be free or esterified with a fatty acid. Plant oils contain sterols such as β-sitosterol or stigmasterol. These compounds are present in the unsaponifiable matter of lipid oils. Sterol level varies in the rage 0.05 to 0.6 percent in oils.



Tocopherols



	Substitution	Abbreviation
α-Tocopherol	5,7,8-trimethyl	α-T
α-Tocotrienol	5,7,8-trimethyl	α-T3
γ- Tocotrienol	7,8-dimethyl	γ-T3
δ-Tocotrienol	8-methyl	δ-T3

The tocopherols are widely distributed in nearly all the vegetable oils but not in animal oils and are the most important antioxidant of phenolic nature. All tocopherols are fat soluble and generally occur in their free form, although some fats contain them in a partial esterified from, the phenolic hydroxyl being esterified by a fatty acid. Therefore, in esterified form solubility increases but their anti oxidant activity is totally removed. A homologous series of trocopherols is also known which differ only by the presence of three double bonds in the farnesyl side chain. The amount of tocopherols veries between 30 and 700mg/kg, extremes being 1000-2000 mg/kg as measured in gram, linseed, soybean and cotton seed oils. The standard (100 percent) vitamin E activity is ascribed by agreement with α-tocopherols. The other tocopherols. (β- γ- δ-) thus have less vitamin E activity than α- compound. Tocopherols while protecting the fat, gets oxidised to quinones and tocopherol dimers. Some oxidation products have distinct colour like toco- red (5,6 chromanoquinone) as in heavily oxidized soybean oils.

Waxes

Waxes by definition are esters of long chain aliphatic alcohols and fatty acids, partly of plant and partly of animal origin. The waxes of vegetable oils are mere contaminants originating from seed covers such as barns and husk. Waxes derived form leaves like candelilla and carnauba waxes or from

animal secretions like bee wax or wool wax of sheep also contain among many aliphatic alcohols, free fatty acids, sterols, triterpinic alcohols and aliphatic hydrocarbons which together form the substance popularly known as waxes.

Pigments

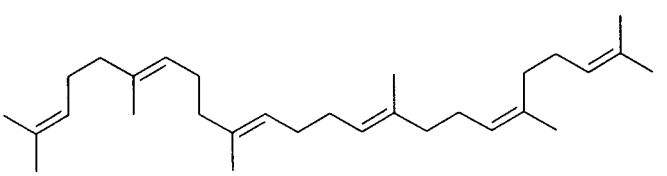
It has been found that colour of different oils and fats is highly influenced by the presence in traces of some oil-soluble pigments like the carotenoids, the chlorophylls and gossypols - characteristic colouring pigments, for example red palm oil, green olive oil and yellow cotton seed oil respectively. The carotenoids are oil-soluble polyisoprenoid hydrocarbon alcohol or carboxylic acid in nature. They contain a considerable number of conjugated double bonds which explain their pronounced colour in the yellow, orange or red origin of visible spectrum. About 70 different carotenoids have been isolated, the most important being α-, β- and γ-carotenes and lycopenes which all have molecular formula $C_{48}H_{56}$. Carotenoids are widely distributed in plant and animal kingdoms, although their concentration in fat is generally low. They are highly oxidisable, yet they provide protective action against radiation damage of the medium in which they are dissolved. The oxidation is a free radical process, causes ultimate destruction of carotenoids and in this way a bleaching of colour take place. Carotenoids are not sensitive towards dilute alkalis but they suffer

considerable damage under acidic conditions and even more by heating above 150 ^{0}C.

The green plant pigments chlorophyll α and β are found mainly in crude olive oil, grape seed oil and also in vegetable oils harvested from unripe seeds like rape seed and soybean. Gossypols, an aldehydic polyphenol are the precursor of the brownish discolouration of cotton seed oil of less than average quality. It is originally of yellowish colour and only by oxidation due to bad conditions during harvesting; it is transformed to the brownish red coloured quinones or dimers with quinonoid structures. Gossypol causes male sterility if orally administered.

Hydrocarbons

The biosynthesis of fatty acids, fatty alcohols, tocopherols, carotenoids and sterols is based on the action of coenzyme-A. One of the most important intermediates is squalene, a polyisoprenoid C_{30} hydrocarbon. Squalene contains six non conjugated double bonds in the molecule. Olive oil contains about 0.5 percent squalene.



squalene

Glycosides

Glycosides are ethers of mostly monosaccharide, tetrosid, pentosid and hexosid sugars with a non-sugar component. The non-carbohydrate part which can be liberated by acids or enzymes from the other linked compound is called aglicone. This is generally the biologically active part providing the specific character of the total oil. Many oil bearing seeds and fat parts contain glycosides the best one being amygdalin, the glycoside of bitter almond. Thioglucosinolates in crucifarae oils are also important glycosides.

Vitamins

The most important fat soluble vitamins which occur in different plant or animal organisms are Vitamin A, D and E. The principal sources of vitamin A or retinol are fish liver oils, egg, liver, butter, cheese and milk. It is a polyisoprene primary alcohol and it represents biologically hydroxylated half of α and β carotenes both of which function as pro-vitamin for retinol. The principal source of vitamin D or calciferol is fish liver oils and it is present in lesser amount in eggs and liver and dairy products. The two important variants are vitamin D2 and D3 which regulate the phosphate absorption in the animal organism. Vitamin E or α – tocopherol is present in liquid vegetable oils, tomatoes and leafy vegetables.

2.9 Present work

Macaranga peltata Muell. is a small tree commonly found in Indian forests.[3] It is found throughout Kerala.[4] It is also found in Bengal, Bihar, Orissa and the Deccan Peninsula, mostly in the hills.[2] The flowering season is January, and seeding in May[2]. Its seeds are black, about 4mm diameter with a hard shell. The kernel is white and oily. The unripe seed is coved by a gummy material. The oil is not used for edible purposes. The seeds are eaten up by crows. This work is aimed at investigating the physical and chemical properties of the seed oil and to find out its composition.

2.10 Materials and Methods

Plant Material

The seeds of *Macaranga peltata* Mull. were collected from Calicut University Campus, Kerala in May 2003 and was authenticated by Dr. A.K. Pradeep, Department of Botany, Calicut University. A voucher specimen of the plant has been deposited in the Herbarium of Chemistry Department, Calicut University.

Column chromatography (CC)

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

UV/Visible spectrometer

The UV/Visible spectrum of the sample (POP) in chloroform was recorded using JASCO V550 spectrometer.

Infrared Spectrometer

The infrared spectrum of the sample (POP) is recorded as liquid film using JASCO FT/IR –4100.

NMR Spectrometer

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

2.11 Experimental

Extraction, fractionation and isolation of oil

The seeds were cleaned by peeling outer skin, and freed from other materials by hand picking. Two kilograms of the seeds were crushed and extracted with petroleum ether (60-80 °C) three times using one liter in each extraction. The petroleum ether extracts were combined and the oil was recovered by the evaporation of petroleum ether under reduced pressure in a

rotary flash vacuum evaporator. The recovered oil (POI) was weighed and found to be about 350g. (oil content = 16%). In order to determine the oil content of the kernels, the seed was broken one by one and separated the shells from kernels. Twenty grams of kernels thus obtained were crushed and extracted using 20 mL petroleum ether (60-80 °C) three times. The petroleum ether extracts were combined which on evaporation under reduced pressure yielded 12.2g oil (POK) (oil content 61%)

Determination of acid value

Acid value gives a measure of the free fatty acids in fat or oil. It is defined as the number of milligrams of potassium hydroxide required to neutralise one gram of fat or oil. The acid value is determined directly by titrating the material in an alcoholic medium with aqueous sodium or potassium hydroxide solution.

The oil was mixed thoroughly before weighing. About 5g of the oil (PO1) was weighed accurately in a 250 mL conical flask. The weight of the oil taken for the test and the strength of the alkali used for the titration was such that the volume of alkali required for the titration does not exceed 10 mL. 100 mL of freshly neutralised hot ethyl alcohol and about 1 mL of 1% phenolphthalein indicator was added. Boiled the mixture for about two minutes and titrated while hot with standard aqueous potassium hydroxide

solution (0.02 N), with vigorous shaking. The calculation of acid value was done using the formula[26]

Acid value = 56.1x Vx N/W

Where

V = Volume of standard KOH solution

N = Normality of standard KOH solution

W = Weight in g. of material taken for the test.

The average value obtained from three experiments was calculated (table 2.1)

Determination of unsaponifiable matter

The material was completely saponified with alcoholic potassium hydroxide solution and extracted with petroleum ether. The petroleum ether extract was washed with aqueous alcohol and then again with water. The petroleum ether extract was evaporated and the residue was weighed. Weight of unsaponifiable matter was the weight of this residue minus the fatty acid present in it, which is determined by titration with sodium hydroxide solution in alcoholic medium.

About 5g of well mixed sample (PO1) was weighed accurately into a round-bottomed flask. 50 mL of alcoholic KOH solution (50%) was added. Boiled under reflux until the saponification was complete. The condenser was

washed with about 10 mL of ethyl alcohol. The mixture was cooled and transferred into a separating funnel. The transfer was completed by washing the flask with ethyl alcohol and then with cold water. Altogether, 50 mL water was added into the separating funnel followed by the addition of 50 mL petroleum ether. Inserted the stopper and was shaken vigorously for one 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 six times, using 50 mL petroleum ether for each extraction.

All the ether extracts were collected in a separating funnel. The combined extract was washed in the funnel three times with 25 mL portions of aqueous alcohol, shaken vigorously and drawn off the alcohol-water layer after each washing. The ether layer was washed again successively with 20 mL portions of water until the water no longer turned pink on the addition of a few drops of phenolphthalein indicator solution. The ether layer was transferred to a conical flask containing a few pieces of porcelain, and evaporated to dryness on a water bath. The last traces of ether were removed by placing the flask in a air-oven at 80 to 90°C for about one hour. The last traces of moisture were removed by adding 5 mL of acetone and evacuated using a vacuum pump at about 50°C for about 15 minutes. Cooled in a desiccator and weighed. The evacuation, cooling and weighing were repeated until a constant weight was obtained.

After weighing the residue was dissolved in warm neutral ethyl alcohol containing a few drops of phenolphthalein indicator solution and titrated with standard sodium hydroxide solution (0.02N). The calculation was done using the following formula[26].

Weight in g. of fatty acid in the extract (as oleic acid) B = 0.282 x V x N

Where

V = Volume of standard sodium hydroxide solution.

N = Normality of standard sodium hydroxide solution

Percent by weight of unsaponifiable matter = 100 (A-B)/W

Where

A = Weight in g. of the residue

W = Weight of the material in g. taken for the test.

The average value obtained from three experiments was calculated (table 2.1).

Determination of iodine value

Iodine value gives a measure of unsaturation in fat or oil. It is defined as the number of milligrams of iodine required to react one gram of fat or oil. The material was treated, in carbon tetrachloride medium, with a known excess of iodine monochloride solution. The excess iodine monochloride was

treated with potassium iodide and the liberated iodine was estimated by titration with sodium thiosulphate solution.

Preparation of iodine monochloride solution

About 5.54g of potassium iodide and 3.5 g. potassium iodate were dissolved in 150 mL of water in one liter standard flask. 150 mL of 10N HCl was added to it and contents were shaken thoroughly. Added about 10 mL of CCl_4, and shaken well and 1% potassium iodide solution was added drop wise, until the orange colour just turned violet, the solution was then diluted to 1000 mL with 5N HCl. The resulting solution was about 0.1N with respect to iodine monochloride in 5N HCl [33].

Standardisation of iodine monochloride solution

25 mL of 0.1N iodine monochloride solution was pipetted into a conical flask and 10 mL of 10% potassium iodide solution was added, shaken well and diluted to 100 mL with distilled water, the liberated iodine was titrated with standard sodium thiosulphate solution(0.1N), using freshly prepared starch solution as indicator[26].

Estimation of Iodine value of the oil

About 5g of the oil was weighed accurately in a clean dry iodine flask to which 25 mL of carbon tetrachloride was added. The weight of the sample was such that there is an excess of 50 to 60% of iodine monochloride solution

over that actually needed. 25 mL of iodine monochloride solution was added, swirled for intimate mixing and allowed to stand for 30 minutes. A blank test simultaneously under similar experimental conditions was carried out. After standing five minutes 15 mL KI solution and 100 mL of water was added, rinsing the stopper also, and titrated the liberated iodine with standard thiosulphate solution (0.1N). The contents of the bottle were continuously swirled until the colour of the solution was straw yellow. 1 mL of starch solution was added and continued the titration until the blue colour disappeared. The calculation was done as follows[26].

$$\text{Iodine value} = 126.9(B - S)N/W$$

Where

B = Volume in mL of standard sodium thiosulphate solution required for the blank

S = Volume in mL of standard sodium thiosulphate solution required for the sample

N = Normality of standard sodium thiosulphate solution

W = Weight in g. of material taken for the test.

The average value obtained from three experiments was calculated (table 2.1).

Determination of saponification value

It is defined as the number of milligrams of potassium hydroxide required to react with one gram of fat or oil The material was saponified by refluxing with a known excess of alcoholic potassium hydroxide solution. The alkali consumed for saponification was determined by titrating the excess alkali with standard hydrochloric acid.

The oil was mixed thoroughly before weighing. About 2.0g of the sample was accurately weighed in a round-bottomed flask. 25 mL of the alcoholic potassium hydroxide solution (4 g KOH dissolved in 2 mL distilled water and made up to 100 mL using rectified spirit) was added and connected the reflux condenser to the flask. The flask was heated on a water- bath for not more than one 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 somewhat, 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 standard hydrochloric acid (0.5N). A blank analysis was conducted at the same time. The saponification value was calculated as follows[26].

$$\text{Saponification value} = 56.1(B - S)N/W$$

Where

B = Volume in mL of standard hydrochloric acid solution required for the blank

S = Volume in mL of standard hydrochloric acid solution required for the sample

N = Normality of standard hydrochloric acid solution

W = Weight in g. of material taken for the test.

The average of three experiments was calculated (table 2.1).

Determination of acetyl value and hydroxyl value

Acetyl value or hydroxyl value gives a measure of number of free hydroxyl groups in the fat or oil. A sample of oil or fat was acetylated by refluxing with acetic anhydride and the excess anhydride was decomposed with water and sodium bicarbonate solution. The saponification value of the washed and dried acetylated oil was determined as before.

About 10g of the material was weighed accurately in a round bottom flask, 20 mL of acetic anhydride was added and boiled the mixture under reflux for about 2 hours. The mixture was poured into a beaker containing 500 mL of water and boiled for 15 minutes. The boiling discontinued, cooled slightly, and removed the water with a siphon. Another 500 mL of water was added and boiled again for 15 minutes. It was then cooled and transferred the contents of the beaker to a separating funnel and rejected the lower layer. The

acetylated sample was washed successively (a) three times with 50 mL of water, (b) twice with 50 mL of sodium bicarbonate solution, and (c) twice with 50 mL of warm water (60 to 70°C). Drained and removed as much of the water as possible, and transferred the acetylated sample to a beaker and added approximately 5 g of anhydrous sodium sulphate, allowed to stand for about one hour with occasional stirring. Filtered through a dry filter paper, the filter paper was removed and kept the filtrate in an oven at 100 to 110°C until it was thoroughly dry. Determined the saponification values of the original material and the acetylated product by the procedure described above. The acetyl and hydroxyl values were calculated as follows[26],

$$\text{Acetyl value} = (S' - S)/(1.000 - 0.00075\ S)$$

$$\text{Hydroxyl value} = (S' - S)/(1.000 - 0.00075\ S')$$

Where

S' = Saponification value after acetylation,

S = Saponification value before acetylation

The average of three experiments was calculated (table 2.1)

Determination of Reichert- Meissl value

Reichert-Meissl value gives a measure of volatile water soluble fatty acids in the oil or fat. The material was saponified by heating with mixture of

glycerol and sodium hydroxide solution and then splitted by treatment with dilute sulphuric acid. The volatile acids in the distillate were filtered out and estimated by titration with standard sodium hydroxide solution.

Weighed accurately 5± 0.01g of the filtered oil into the boiling flask, 20g of glycerol and 2 mL of the concentrated sodium hydroxide solution were added from a burette. The flask and its contents were heated with continuous shaking on a wire gauze over a naked flame until the fat, including any drops adhering to the upper parts of the flask, had been saponified and the liquid became perfectly clear. Overheating during saponification was avoided. Covered the flask with a watch-glass, and allowed the flask to cool a little. Ninety mL of boiling distilled water was added which had been vigorously boiled for about 15 minutes. After thorough mixing, the solution should remain clear. [If the solution is not clear (indicating incomplete saponification) or is darker than light yellow (indicating overheating), repeated the saponification with fresh sample of the oil or fat].

A few pieces of porcelain and 50 mL of dilute sulphuric acid were added, and immediately connected the flask with the distilling apparatus. The flask was placed on an asbestos board so that it fitted snugly into the aperture. After the fatty acids had melted and separated into a clear liquid layer on gentle warming, heated the flask without altering the flame so that 110 mL of liquid distils over in the course of 19 to 21 minutes. The distillation is

considered to begin when the fist drop forms in the still-head. The water flowing in the condenser was kept at a sufficient speed to maintain the temperature of the outgoing water from the condenser between 15°C and 25°C. The distillate was colleted in a graduated flask.

When 110 mL was distilled over, stopped heating the boiling flask and replaced the graduated flask by a measuring cylinder of about 25 mL capacity to catch draining. Closed the graduated flask with the stopper, and placed it in a water-bath at about 15°C for 10 minutes, swirled round the contents of the flask from time to time. The distillate was mixed by closing the flask and inverting it four or five times, filtered through a dry 9cm Whatman No. 4 filter paper. Rejected the first 2 or 3 mL of the filtrate and collected the rest in a dry flask.

100 mL of the filtrate was pipetted out in to a titration flask, added 0.1 mL of phenolphthalein indicator and titrated with standard (0.1N) sodium hydroxide solution until the liquid became slightly pink.

A blank test was carried out without the oil but using the same quantities of reagents and followed the same procedure. During the heating with caustic soda, avoided overheating which will be indicated by the darkening of the solution. The Reichert-Meissl value was calculated as follows[26],

$$\text{Reichert- Meissl value} = (A - B) \times N \times 11$$

Where

A = Volume in mL of standard sodium hydroxide solution required for the test.

B = Volume in mL of standard sodium hydroxide solution required for the blank.

N = Normality of standard sodium hydroxide solution.

The average of two experiments was calculated (table 2.1).

Determination of Polenske value

Polenske value gives a measure of volatile water insoluble fatty acids in the oil or fat. The condenser, the 25 mL cylinder and the receiver used in the Reichert-Meissl value determination were washed into the filter paper through which the distillate was filtered for that determination. After rinsing, the residue on the filter paper was taken up with ethyl alcohol and titrated with sodium hydroxide solution.

After determining the Reicher- Meissl value, detached the still–head and washed the condenser with three successive 15 mL portion of cold distilled water, passed each washing separately through the measuring cylinder, the 110 mL flask, and filter paper. All the washings were discarded.

The insoluble acids were dissolved by three similar washings of the condenser, the measuring cylinder, the 110 mL flask with stopper, and the filter paper with 15 mL portions of ethyl alcohol. The alcoholic washings were combined in a clean flask (The total volume thus amounting to 45 mL), added 0.25 mL of phenolphthalein indicator solution, and titrated with standard (0.1N) sodium hydroxide solution until the solution turned slightly pink[26].

Calculation:

$$\text{Polenske value} = 10 \times V \times N$$

Where

V= Volume in mL of standard sodium hydroxide solution

N = Normality of standard sodium hydroxide solution

The average of two experiments were tabulated (table 2.1).

Determination of peroxide value

The peroxide value is a measure of peroxides contained in the sample of fat expressed as milli-equivalent of peroxide per 1.000g of the martial. The material in an acetic acid chloroform medium was treated with an aqueous solution of potassium iodide. The liberated iodine was titrated with sodium thiosulphate solution.

About 5 g of the sample of oil was weighed accurately in a 250 mL iodine flask, 30 mL of acetic acid-chloroform solution (3:2 v/v) was added. Swirled the flask until the sample was dissolved. 0.5 mL of saturated potassium iodide solution was added. The solution was allowed to stand exactly for one minute, 30 mL of distilled water was added and titrated with 0.1 N standard sodium thiosulphate solution with constant and vigorous shaking. Continued the titration until the yellow colour almost disappeared. Added 0.5 mL starch solution and continued the titration till the blue colour just disappeared[26].

Calculation:

Peroxide value as milli-equavalent per 1.000g. of the sample=S x N x 1000 /W

Where

S = Volume in mL of the sodium thiosulphate solution used up by the sample.

N = Normality of sodium thiosulphate solution

W = Weight in g. of the sample.

The average of three experiments was calculated (table 2.1).

Table 2.1

Acid Value (mg. of KOH/g. oil)	5.8
Saponification value (mg. of KOH/g. oil)	112.7
Iodine value (mg of I_2/g. oil)	24.21
Unsaponifiable matter(% w/w)	32.3
Reichert- Meissl value	0.227
Polenske value	0.319
Acetyl value	40.5
Hydroxyl value	41.8
Peroxide value (m.eq./g. oil)	5.92

Determination of physical properties

Specific Gravity

The specific gravity of the oil sample (PO1) was determined using a specific gravity bottle at a temperature of 30°C using distilled water as standard.

Refractive Index

The refractive index of oil sample (PO1)was measured using Abbe refractometer at a temperature of 30° C using distilled water as the standard.

Melting Point

The melting point of the oil sample (PO1) was determined by open tube capillary-slip method. To sample of oil (PO1) a capillary tube was

inserted. The capillary tube containing the oil was chilled to below 0°C in a freezer. The capillary tube was placed on a thermometer which was placed on a water in a beaker at 0°C. The temperature of the water was raised slowly at a rate of 2°C per minute. Noted the temperature of water when the capillary column raised by the entry of water[26].

Titre Test

The sample of oil (PO1) was saponified with glycerol- caustic potash solution, and the soap was acidified with dilute sulphuric acid to give fatty acids. The fatty acids were recovered using petroleum ether (40-60°C), which on evaporation at reduced pressure yielded fatty acids. About 5g fatty acid sample was taken in a boiling tube and heated well above its melting point in a water bath. A thermometer was inserted in the hot liquid, and placed the boiling tube in an air jacket. The system was allowed to cool slowly. The temperature was noted in every half minute. When fatty acid began to solidify the temperature rises, the maximum temperature noted was the titre value[26].

Viscosity

The viscosity of the oil sample (PO1) was measured at the temperature 30°C in a Ubloid viscometer using distilled water as the standard.

Table 2.2

(Physical properties of PO1)

Specific gravity	0.904
Refractive index	1.421
Melting point	15°C
Titre	39°C
Viscosity	17.3 N s m^{-2}

2.12 Determination of Composition of the oil

Preparation of Fatty acid methyl ester (FAME)

Purification of oil

The lipid oil (PO1) recovered from the seed contains some colouring matter and impurities. It was purified using column chromatography. About 50g of the oil was diluted with 50 mL petroleum ether and poured into a column packed with 200g silica gel using pure petroleum ether, in a column of dimension (4 cm x 100 cm). Elution was carried out using pure petroleum ether, fractions collected between 150 mL and 350 mL was allowed to evaporate, about 43 g. pure colorless oil (POP) was obtained.

Preparation of fatty acid

About 20 g oil (POP) was saponified using 100 mL 10% alcoholic potassium hydroxide solution (10g KOH dissolved in 10 mL water and made

up to 100 mL using aldehyde free rectified spirit) and refluxed for three hours. The absence of oil layer indicated the completeness of saponification of the oil. In order to separate the unsaponifiable matter from potassium salt of fatty acids (soap) the solution was cooled, and transferred into a separating funnel, added 100 mL water into the separating funnel followed by the addition of 50 mL petroleum ether. Inserted the stopper and was shaken vigorously for one 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 six times, using 50 mL petroleum ether for each extraction. Collected all the petroleum ether extract in a separating funnel, washed the combined extract in the funnel three times with 25 mL portions of aqueous alcohol (10% ethanol) solution, shaken vigorously and drawn off the alcohol-water layer after each washing diluted with 100 mL water neutralised with con. hydrochloric acid, the fatty acids precipitated were extracted with petroleum ether (40-60°C), the petroleum ether extract on evaporation yielded about 10g fatty acid (FA). The petroleum ether layer left after aqueous alcohol extraction on evaporation yielded about 6g. unsaponifiable matter (POU).

Preparation of fatty acid methyl ester

About 2g fatty acid (FA) was esterified by using a mixture of methanol, toluene and sulphuric acid in the ratio 88:10:2. The solution was

heated for about one hour at 80°C. The solution was extracted with petroleum ether grade 40-60°C (2 x 30 mL) and dried over anhydrous sodium sulphate. The organic solvent was removed under reduced pressure to yield about 1.5 g fatty acid methyl ester[25] (FAME).

Gas chromatography mass-spectrometry analysis

The GC and GLC methods are widely used for the analysis of fatty acid methyl esters[27-32]. The fatty acid methyl esters sample (FAME) was analyzed using HP 6890 Gas chromatograph fitted with HP 5973 mass selective detector (MSD) and auto sampler; column used was J&W Scientific DB-5 MS 30 m x 0.25 mm, 1D x 0.25 μm film thickness. The carrier gas used was helium at a rate of 1 mL/min (0.14 MPa). Initial GC oven temperature was 70°C held for 4 min and programmed to 300°C at a rate of 10°C/min and kept constant at 300°C for 7 min. The total run time was 34 min. The injection volume was 0.1 μl. EI/MS was taken at 70 eV ionization energy. Scan time was 0.5 sec with 0.1 interscan delay. The library search was carried out using NIST and Wiley GC-MS library. The relative percentage amount of separated compounds was calculated from total ion chromatogram by computerized integrator. The identified components are given in table 2.3.

Table 2.3

GC-MS analysis of the fatty acid methyl ester sample (FAME)

Compound	RT (min)	% abundance
9-Hexadecenoic acid methyl ester	19.022	1.03
Hexadecanoic acid methyl ester	19.272	11.09
8,11-Octadecadienoic acid methyl ester	20.998	18.70
11-Octadecenoic acid methyl ester	21.173	1.52
11-Eicosenoic acid methyl ester	22.726	0.77
13-Docosenoic acid methyl ester	24.440	11.02
Nonadecanoic acid methyl ester	24.601	2.71
Tricosanoic acid methyl ester	26.053	43.87
Hexacosanoic acid methyl ester	26.161	5.81

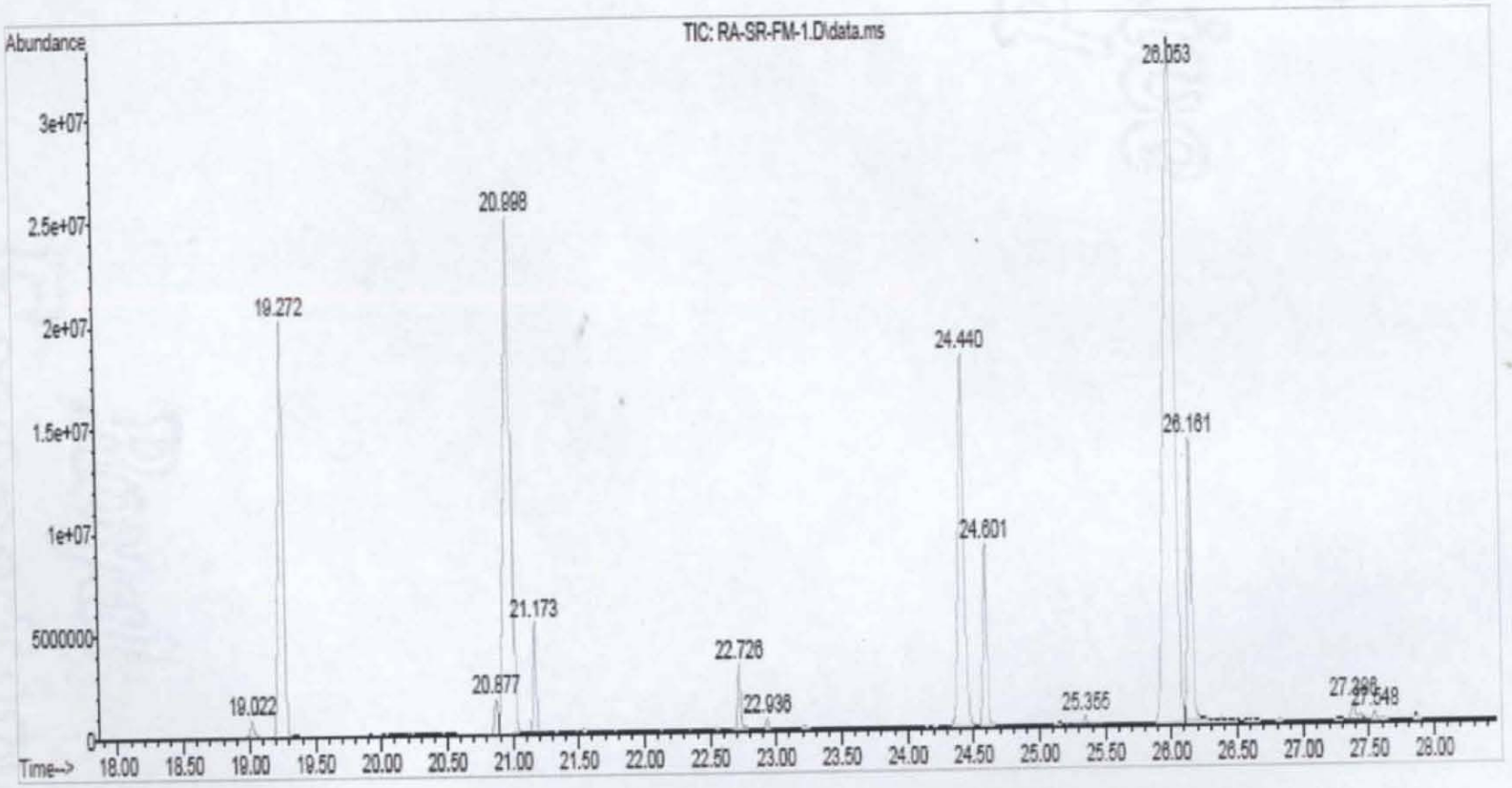


Fig 1. Gas Chromatogram of the sample FAME

2.13 Thin layer chromatographic analysis of unsaponifiable matter (POU)

Thin layer chromatography (TLC)

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

Reagents

1. 20% aqueous sulphuric acid (20% H_2SO_4)

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

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

2. Leibermann-Burchard reagent (LB reagent)

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

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

3. Vanillin-sulphuric acid reagent

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

The chromatogram (TLC) was sprayed first with 5% H_2SO_4, followed immediately by 1% ethanolic vanillin. The sprayed plate was then heated to 110°C for 5-10 minutes until maximal visualisation of the spots.

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

4. Anisaldehyde-sulphuric acid reagent (AS reagent)

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

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

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

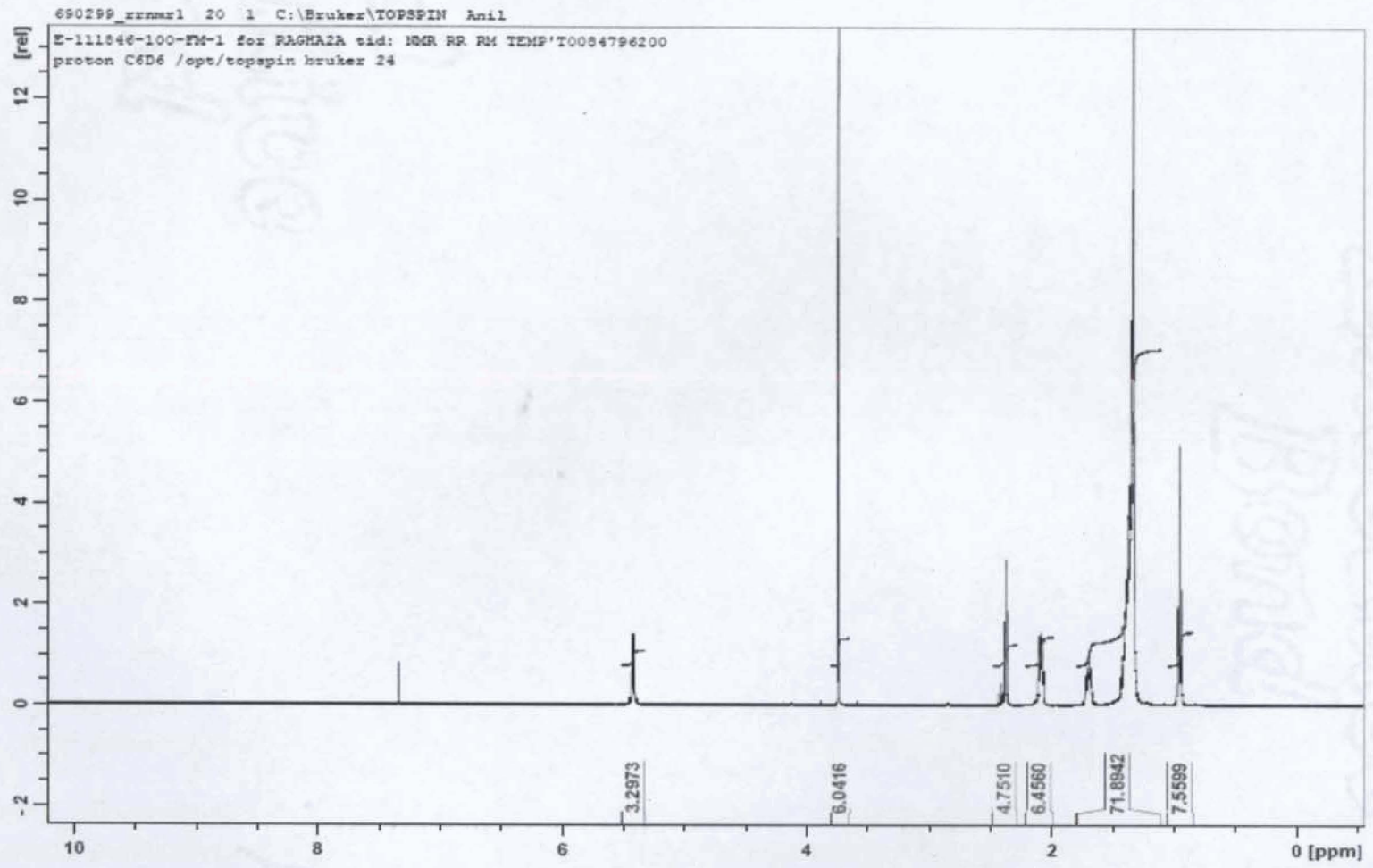


Fig. 2 ^{1}H-NMR spectrum of the sample FAME

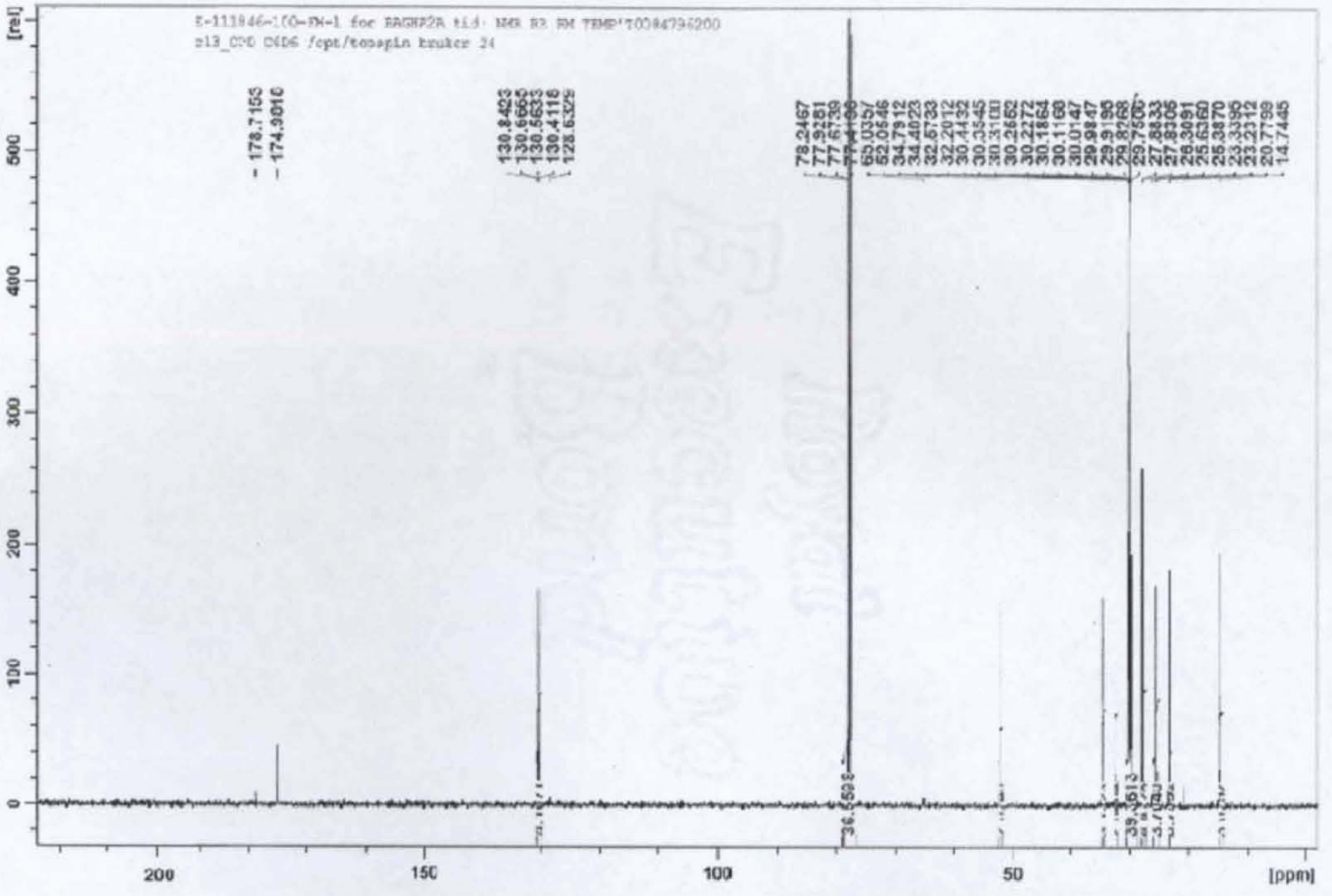


Fig 3. ^{13}C-NMR spectrum of the sample FAME

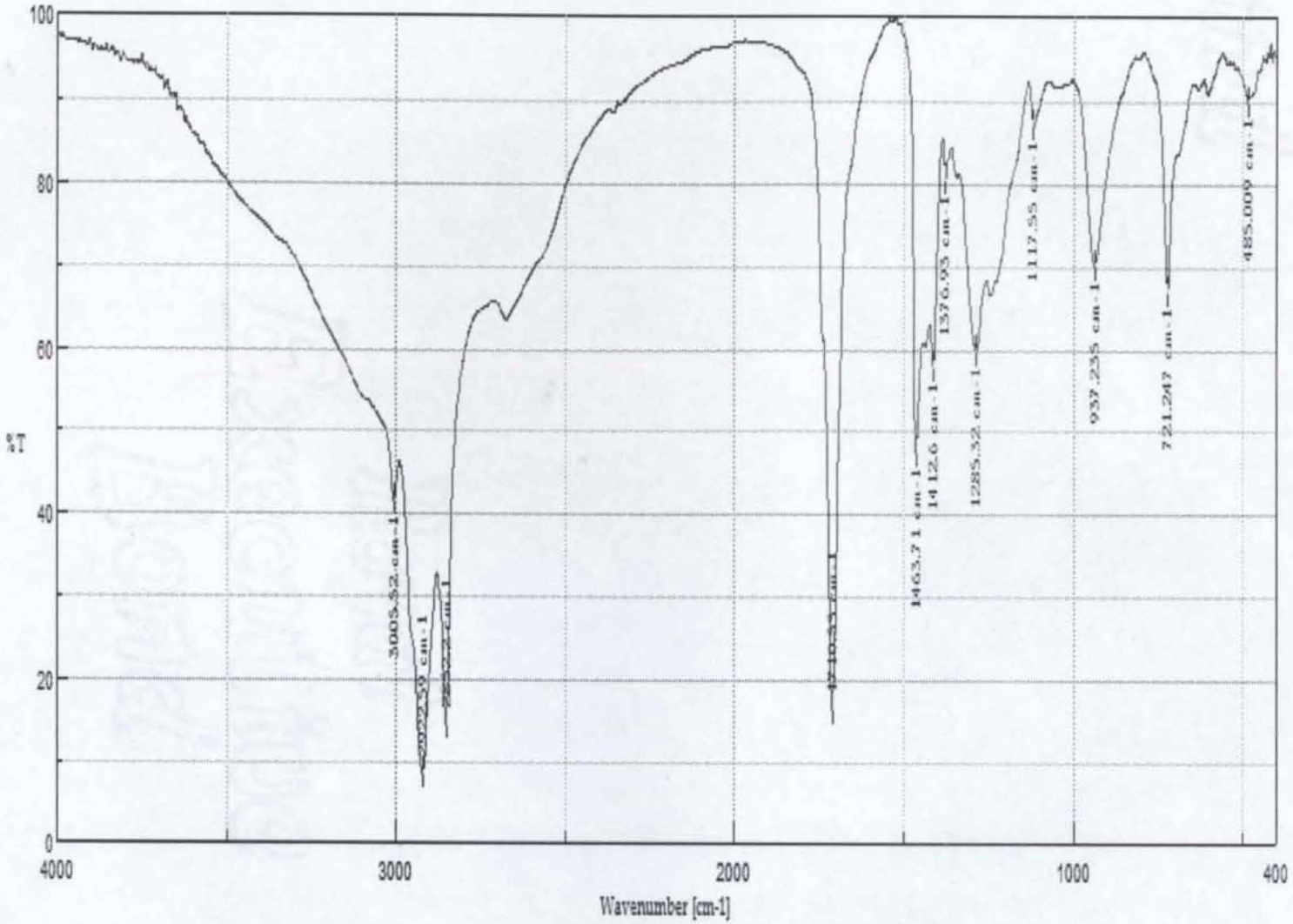


Fig 4 IR spectrum of the sample (POP)

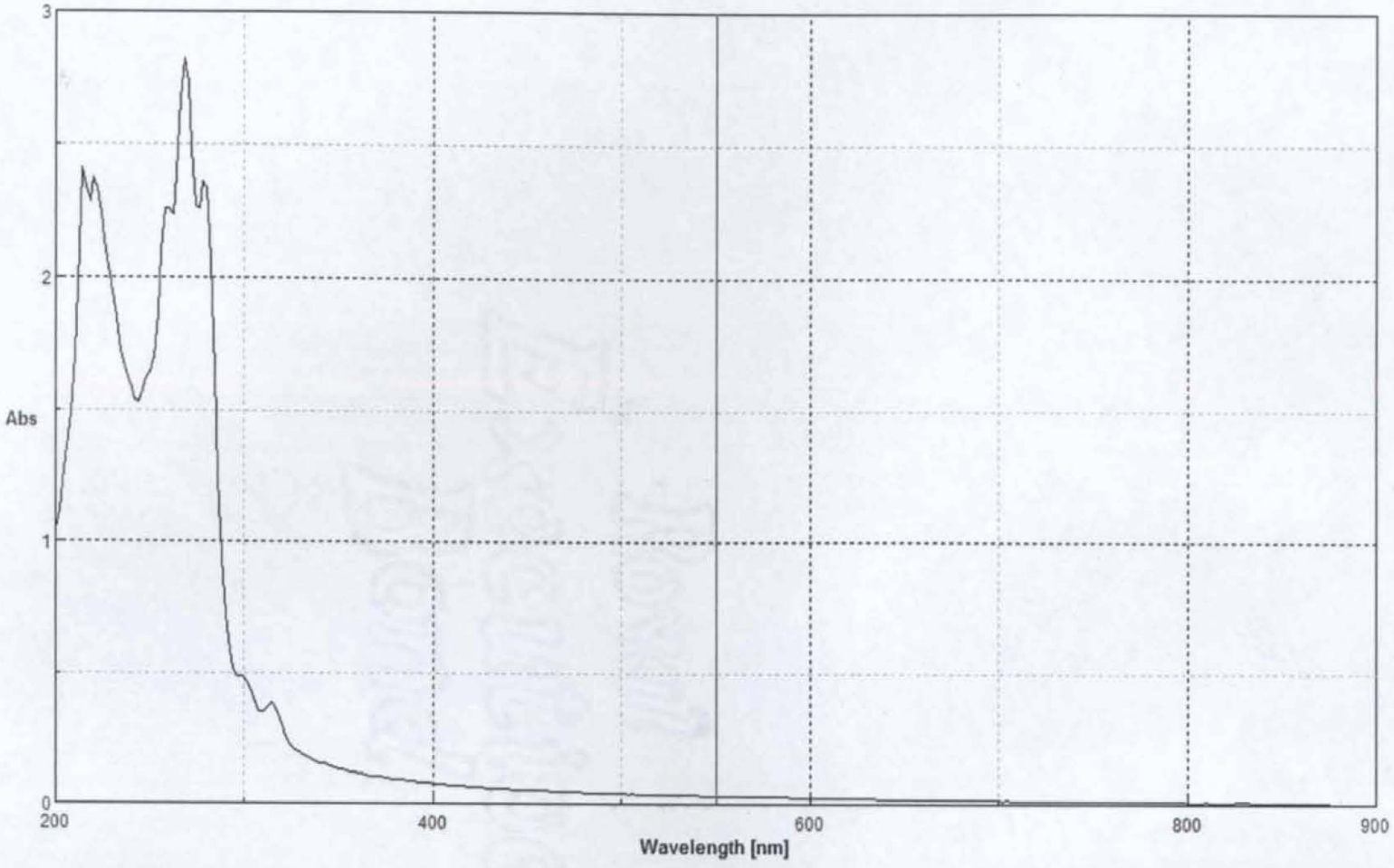


Fig 5. UV spectrum of the sample (POP) λ (in nm) for the absorption peaks are 214,220,260,268,278 and 314

2.14 Results and Discussions

The yield of oil in *Mecaranga peltata* seed is low (16%) when compared to other oil seeds [15]. The oil content of the seed kernels was estimated to be 61% which is comparable with coconut copra[15]. The amount of free fatty acid was quite high The saponification value was low (112.7), which is due to large proportion of unsaponifiable matter (32.3%) and also due to the presence high amounts of high molecular weight fatty acids (table 2.3) like tricosanoic acid (43.8%), 13-docosenoic acid(11.02%) and hexacosanoic acid(5.8%). The low value of saponification implied that the oil is not good enough for soap manufacture. Iodine value was low (24), therefore it comes under the class of non-drying oil. The iodine value was high when compared to palm kernel oil (18) and coconut oil (10) which indicated the presence of some double bonded compounds, which is in agreement with the GC-MS Data. Reichert Meissl value (indicates the soluble volatile fatty acids) and Polenske values (insoluble volatile fatty acids) were very low, indicated that the composition of volatile, low molecular mass fatty acid in the lipid is very low. These values are important for predicting the lathering of soap. Moderate hydroxyl value or acetyl value indicated the presence of monoacyl and diacylglycerols, the GC-MS data did not support the presence of hydroxy fatty acids, the high acid value may be due to the enzymatic hydrolysis of lipids leading to monoacyl and diaceyl glycarols. Peroxide value shows the development of rancidity for the oil stored for one year. The low value refractive index indicated the absence of large number of

double bonds and conjugated double bonds in the oil. Density was comparable with common lipid oils. Viscosity was slightly low when compared with other lipid oils. Melting point of the oil is moderate, which supported the presence of unsaturated fatty acids. The titre test on fatty acid is important for the choice of the oil for making soap. Titre value of 38 ^{O}C is recommended for the oil mixture used for soap manufacture[5]. Oils can be classified to soft oil or hard oil based on the titre value. The analysed sample was neither soft nor hard.

The UV spectrum of the oil sample (POP) had no peak at 233 nm proving the absence of conjugation in fatty acids[17, 25]. The IR spectrum with the absence of band at 965cm^{-1} shows the absence of *trans* double bonds in the fatty acid, which is due to C-H deformation about *trans* double bonds[5]. The H^{1}-NMR spectrum showed the absence of absorption below δ <0.9 ppm. indicating the absence of cylopropanoid fatty acids[23]. C^{13} NMR Data indicated the lack of absorption around δ = 208, showing the absence of keto substituted fatty acids. The GC-MS analysis of fatty acid methyl esters (table 2.3) indicates the presence of long chain fatty acids (C_{23}, C_{22} and C_{26}) as major components; therefore the oil is not suited for the production of Bio-Diesel.

The TLC studies on unsaponifiable matter showed the absence of sterols and triterpenes in the oil.

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CHAPTER -III

PIPER BETLE – COMPOSITION OF LEAF OIL IN THREE VARIETIES FROM KERALA

3.1 Introduction

Essential oils

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

Essential oils have three primary commercial uses; as odorants in perfumes, soaps, detergents and other products; as flavours in baked goods,

candies, soft drinks and many other foods; and as pharmaceuticals, in dental products and many medicines.

Occurrence

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

Depending on the plant family, the essential oil may be elaborated in different specialized secretory structures: in the Apiaceae, the oils are secreted in vittae, in the Lamiaceae they are elaborated in the glandular hairs, in the

Hyperycaceae, in the Myrtaceae and in the Rutaceae they are poured again into lisigenous or schizogenous pockets, in the Pinaceae and in the Asteraceae they are found again in the secretive tissues and so on.[2]

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

History of essential oils

The first records of essential oils come from ancient India, Persia, and Egypt; and both Greece and Rome conducted extensive trade in odoriferous oils and ointments with the countries of the Orient. Most probably these products were extracts prepared by placing flowers, roots, and leaves in fatty oils. They have been found in three thousand years old tombs in the pyramids and early Greek physicians, including Hippocrates, mentioned aromatic plant essences and oil massages for their healing and mood-enhancing qualities. The ancient civilizations of Mesopotamia, more than 5,000 years ago, had machines for obtaining essential oils from plants. The Romans associated

essential oils and their fine aroma with wealth and success. Ayurveda, the world's oldest healing system, has long recommended essential oil massage as a health treatment for many conditions.

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

The development of the modern perfume industry started in France, the world centre of perfumery[3]. 'Perfumes are fragrant substances, generally of complex composition, which gratify the sense of smell'. 'Perfumery is the art of production of perfumes by compounding fragrant substances and in

association with cosmetics'.[6] A few decades ago perfumes were composed primarily from natural materials. Later, isolates obtained from them were also used for compounding perfumes. Today the variety of available natural perfume and flavouring materials is small compared to several thousands of synthetic materials. This apparent competition between natural and synthetic products has been the major cause of development of a large number of perfume and flavouring materials and has introduced into the market several new products for use in perfumery.

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

But as far as flavours are concerned they are often created exclusively from synthetic raw materials; however, certain flavour types cannot be satisfactorily reproduced without the use of an overwhelming proportion of natural oils and extracts with minute additions of safe and harmless, powerful, synthetic flavouring materials.[8]

Essential oils production in India

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

Commercial importance

Essential oils are generally expensive, with prices ranging from several U.S. dollars per kilogram on the low side to several thousand dollars per kilogram. The high price of the natural oils coupled with their limited availability has encouraged a search for substitutes. Great progress has been

made in the synthesis of individual components such as geraniol, citral, linalyl acetate, and the like. These synthetics have been combined with natural oils to extend supplies, and they have also been blended together in an attempt to duplicate the oils themselves. Such reconstituted oils usually lack certain of the odour notes of the natural products, because of the absence of trace ingredients, often unidentified, that may be present in the natural oils. They also tend to have a more "chemical" odour, because of trace impurities in the synthetics that are different from the components of natural oils[3].

3.2 Methods of production of Essential oil

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

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

third process, the vessel containing the plant material on a grid is heated to prevent condensation of steam, so that dry distillation is attained[4].

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

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

Since both enfleurage and maceration are rather expensive processes, some essential-oil specialists have shifted almost completely to using volatile solvents for the recovery of essential oils from plant materials that could not

be processed by steam distillation. Petroleum naphthas, benzene, and alcohol are the primary solvents.

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

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

3.3 Chemical Composition

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

aldehydes, ketones, acids, esters, oxides, lactones, acetals, and phenols are responsible for the characteristic odours and flavours[5].

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

3.4 Essential oils in practice

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

The action of essential oils is carried out particularly:

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Antimicrobial properties

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

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

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

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

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

3.6 Analytical methods

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

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

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

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

Gas chromatography (GC)

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

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

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

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

a. Instrumentation

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

i) Carrier Gas

ii) Sample injection system

iii) The column

iv) One or More Detectors with appropriate read out.

i) Carrier gas

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

ii) Sample injection system

The carrier gas is connected from the gas reservoir to the sample port injector. The sample must be converted into vapour state. The injection port is

heated to a temperature which will ensure rapid vapourisation but not thermal degradation of the solute .The port is designed for instantaneous injection and vaporization of a sample is introduced immediately that the sample are almost always injected by syringe through a silicon rubber diaphragm in the injection port. A solid sample may be dissolved in a suitable solvent and injected as solution. The solute vapour mixes instantaneously with the flowing carrier gas and is swept in to the column.

iii) Columns

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

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

The column container for packed column is usually stainless steel, copper, nickel or glass. Inner diameters may range from 1.6 to 9.5 mm. Length is often 3m. These columns are packed with either a solid substrate (GSC) or a liquid coating on an inert solid support (GLC).

The solid inert support:

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

a) It should be porous and hence have a large surface area.

b) It should be capable of providing good mechanical strength.

c) It should consist of small, uniform and spherical particles.

d) It should be chemically inert at elevated temperatures.

e) It should be readily wetted by the liquid phase to give a uniform coating.

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

The stationary liquid phase:

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

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

ii) It should be thermally stable.

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

iv) It should be chemically inert towards the sample

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

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

iv) Detectors

Any physical property, which varies widely from one gas to another and which can be easily monitored form the basis of the detector. Based on

these physical properties, detectors are of various types. These include thermal conductivity detector (TCD), flame ionisation detector (FID), electron capture detector (ECD), thermionic emission cross section detector (ECD), argon ionisation detector, gas density balance, microwave excited discharge detector etc. Some other main detectors offered by commercial manufactures are helium ionisation, flame thermo couple, piezoelectric absorption detector, argon triode detector, micro detector, and photo-ionisation detectors, sulphur chemiluminescence detector etc.[17].

The FID and TCD are considered universal detectors while PID and ECD are specific detectors.

Thermal conductivity detector (TCD) or Katharometer (KCD)

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

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

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

Flame ionistion Detector(FID)

The ionization detectors are based on the electrical conductivity of gases. So the flame ionization detector is the most widely used and generally applicable detector for gas chromatography. With a burner (H_2-air flame), the effluent from the column is mixed with H_2 and air and then ignited electrically. Most organic compounds, when pyrolysed at the temperature of a H_2/air flame, produce ions and electrons that can conduct electricity through the flame. The resulting current ($\simeq 10^{-12}$A) is then directed into a high impedance operational amplifier for measurement.

FID exhibits a high sensitivity, low noise and is easy to use. The ionization of carbon compounds in a flame is a poorly understood process, although it is observed that the number of ions produced is roughly proportional to the number of reduced carbon atoms in the flame. The FID responds to the no of carbon atoms entering the detector per unit time. It is a mass sensitive, rather than a concentration sensitive device.

b. Basic parameters

b.i. Retention time (t_R)

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

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

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

$t'_R = (t_R - t_M)$

b.iii. Gas hold up time (t_M)

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

b.iv. Retention volume (V_R)

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

Retention volume $V_R = t_R F_c$

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

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

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

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

$$V'_R = V_R - V_M$$

But,

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

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

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

b.2. Retention indices

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

molecules. The n-alkanes are used as the reference compounds because of their stability, ready availability, low cost and wide range of boiling points. The retention time of any analyte is compared with the two n-alkanes which elute nearest to it. The adjusted retention time of the analyte is measured at the same time as those of n-alkanes which elute in front and behind it (containing 'Z' and 'Z+1' carbon atoms respectively) and the retention index of the analyte I is then defined by

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

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

For n-alkanes the term log t'_R (subst)-Log t'_R (n-C_Z) reduces to zero and they have retention indices equal to the number of carbon atom in the molecule multiplied by one hundred. Retention data are generally expressed in terms of Kovats retention indices RI.[18] The indices indicate where compounds will appear on a chromatogram, with respect to straight chain alkanes injected with the sample. By definition the retention index for a normal paraffin is 100 times the number of carbon atoms in the compound regardless of the columns used or the chromatographic conditions.

Gas chromatography - mass spectrometry (GC-MS)

GC-MS is an established technique for the analysis of complex mixtures, holding a prime position in analytical chemistry because of its

combination of sensitivity, wide range of applicability and versatility. The technique is capable of obtaining mass spectra of a few picograms or nanograms of each component. Mass spectrometry is a powerful method for identifying pure substances, but the mass spectra of mixtures are too complicated to be useful. Therefore combined gas chromatography with mass spectrometry provides a very effective tool for the qualitative characterization of complex mixtures by exploiting first the resolving power of GC to obtain the pure component in a flowing stream and then the strength of mass spectrometry to identify those separated compounds. In a mass spectrum the m/z values are plotted against relative abundances, arbitrarily assigning the most abundant ion in the spectrum as 100 percent. This is the base peak in the spectrum.

3.7 *Piper betle*: An Introduction

Piper betle, Linn belongs to the family *Piperaceae.* This plant is cultivated most parts of South India, Bengal, Sri Lanka, Myanmar and Thailand for its leaves. There are about twenty eight varieties found in Kerala, but only a few are commercially important. It is commonly called tambula in sanskrit, betel leaf pepper in english, betel in french, betel pepper in germen, pan-tamboli in hindi, pan in panjabi, tambol in persian, naya valli in telungu, vettilai in tamil and, vettila in malayalam[34].

It is a twinning plant usually planted on a tree support like pepper. In some verities like 'Kuzhikodi' special supports are used for growing the plants. The betel leaves are broadly ovate, about 15cm long and 10cm. broad, and thin, they are acuminate at the apex, unequally cordale at the base, lateral veins well marked 5 to 7 number, curving from the base to apex. Under the microscope, abundant rounded oil cells filled with a brownish secretion are conspicuous. It is tasty warm and aromatic.

The leaves of *Piper betle* are intimately linked with the cultural life of the sub-continent. It is widely cultivated in India with an annual turnover of hundreds of crores of rupees. Fresh betel leaves are very extensively used in India, Malay archipelago as a masticatory and slight stimulant, commonly in conjunction with areca nut, lime, tobacco, catechu, cardamoms and cloves or other spices. The use of lime is to neutralise the phenolics present in betel leaves. The betel leaves thus chewed acts as a gentle stimulant, tonic and digestive. It increases salivary secretion. Generally it is taken early in the morning after meals and at bed time. It sweetens the breath, removing all foulness from the mouth[36]. According to some researchers it acts as an aphrodisiac[27]. The leaves are also known to possess laxative and anthelmintic properties[28,29]. A decoction of leaves is used for healing wounds[29]. The leaf oil has been found to possess antiseptic properties and as a result, has some use in the treatment of respiratory catarrhs. A gargle consisting of the juice or the

essential oil from the leaves mixed in warm water, or the inhalation of leaf oil vapour have been recommended in the treatment of diphtheria[19].

The betel leaf contains an aromatic essential oil which contains a phenol, chaviol, which is a powerful antiseptic, five times more powerful than carbolic acid and twice as strong as euginol. To the "betel phenol" is due to the characteristic odour of the leaves and oil. Medicinally the fresh leaves and the fresh juice of the leaves and the essential oil have aromatic, carminative and astringent properties. The warm leaves smeared with oil form a valuable application to the chest in the case of difficulty of breathing and in coughs, especially those of infancy and childhood. The same application has been recommended in congestion and other affections of liver. A combination of one part of hydrolyzed slaked lime and two parts of the juice of the leaves is very affective for sore throat[34]. The betal leaves warmed by fire and placed in layers over the breast acts as antiglactorrhea, and check the secretion of milk. The juice of leaves is dropped into the ear to relieve earache, dropped in to the eye for pain full eye infections. Internally the juice of leaves with honey is a good expectorant and useful in coughs of children. It is a valuable stomachic and febrifuge. It is given in milk in hysteria; the leaves administered in syrup is useful in general debility. The slander roots with black pepper are used to produce sterility in women. The root is chewed by public singers to improve their voice. Stalk of leaf dipped in caster oil is introduced in to the rectum of the child suffering from constipation and tympanis. In the case of

prolapsusani, the patient is made to sit in medicated bath made of betel leaves and a sufficient amount of water.[34]

Antifungal activity of *P. betle* leaves and of its essential oil has been reported[30]. Chavicol, chavibetol, chavibetol acetate and allylpyrocatechol allylpyrocatechol diacetate isolated form *Piper betle* is reported to have anti fungal properties[30,31]. There is report on its anti HIV protease inhibitory activity from Thailand.[33]

Leaf essential oil of *P. betle* cultivars, Sagar Bangla from Madhya Pradesh, was reported to be highly active against the keratinophilic fungi *Arthroderma benhamiae*, *Microsporum gypseum*, *Trichophyton mentagrophytes*, Ctenomyces serratus, the pathogenic fungi *Aspergillus niger*, *Aspergillus fumigatus, Aspergillus flavus, Aspergillus candidus* and *Aspergillus ochraceus*, and the bacteria *Bacillus subtilis, Bacillus pumilus, Staphylococcus aureus, Salmonella typhi* and *Vibrio cholerae*. The essential oil is more effective against tapeworms (*Taenia solium*) and hookworms (*Bunostomum trigonocephalum*) than the synthetic anthelmintics piperazine phosphate and hexyl resorcinol.[26]

3.8 Present work

The present work aims at comparison of essential oils of three important verities from Kerala. The essential oils of a number of cultivars of

Piper betle have been analysed from Northern parts of India[20-25], and to our knowledge there is no report from south-India.

Materials and Methods

The leaves of *Piper betle* were collected from Tirur, Kerala, India and identified by Dr. Pradeep A.K., Department of Botany, University of Calicut. The voucher specimens have been deposited in the specially maintained herbarium of Calicut University Chemistry Department.

Three hundred grams of the leaves of nadan, selan and kuzikkodi (different varieties) were separately cut into pieces and ground into a paste using an electrical mixer and subjected to steam distillation for 3 hours. The distillates were extracted with diethyl ether (200 mL) and dried using anhydrous sodium sulphate. The dry ether on evaporation yielded 1% each of the oil, based on fresh weight of the leaves.

Olfactoric evaluation

The essential oil of nadan was olfactorically evaluated by professional perfumers. Two hundred micro liter of the essential oil was diluted with dichloro methane and the odor was evaluated after ten seconds by professional perfumers (solvent evaporation time).

GC/MS analysis

The GC-MS analysis were carried out using varian 3400GC fitted with an OPTIC® injector and coupled to a Finnigan ITS40 ion trap mass spectrometer. The coloumn used was an HP ultra2, 50m X 0.2 mm; film thickness 0.33μm; injector temperature 270°C; carrier gas: Helium at 1.6 mL/min; temperature programme from 50°C-270°C at 2°C/min; EI mode 70eV; scan range: 35-450 amu. Component identification were made by comparison with in house reference libraries, commercial libraries and known retention indices. The identified compounds are tabulated in table 3.1.

GC-FID analysis

Quantification by GC-FID was carried out using a HP 5890 GC fitted with an OPTIC® injector and FID detector. Injector temperature: 270°C : Detector temperature 280°C, Column: HP5, 25m X 0.33 mm. Carrier gas : Nitrogen at 0.3 mL/min; temperature programme 30°-280°C at 3° C/min.

Table 3.1

Constituents of *Piper betle* essential oils

Components identified	Nadan (%)	Selan (%)	Kuzhikkodi (%)	RI
trans-Hex-2-enal	0.03	0.2	0.2	849
cis-Hex-3-enol	0.04	0.3	0.04	851
α-Thujene	-	-	0.02	928
α-Pinene	-	-	0.02	936
Sabinene	0.07	-	0.3	976
Myrcene	0.05	-	0.1	990
cis-Hex-3enyl acetate	0.02	0.02	0.02	1005
α –Terpinene	0.05	tr	0.09	1019
para-cymene	0.1	tr	0.09	1026
β-phellandrene	0.1	-	0.2	1032
1,8-Cineole	0.1	-	0.1	1034
γ-Terpinene	0.1	tr	0.2	1061
Guaicol	0.06	0.02	0.07	1091
Linalool	0.4	0.5	0.4	1099
cis-para-Menth-2-en-1-ol	0.1	0.1	0.1	1125
trans-para-Menth-2-en-l-ol	0.1	0.1	0.1	1143
Terpinen-4-ol	6.1	3.6	3.4	1182
para-Cymen-8-ol	0.02	0.01	tr	1187
α-Terpineol	0.2	0.9	0.2	1194
Methyl salicylate	0.07	0.09	0.04	1199
Decanal	0.05	0.1	0.07	1205
Chavicol	0.1	0.02	0.04	1252
Decanol	-	0.07	tr	1270

Safrole	38.1	0.4	35.6	1296
δ-Elemene	0.03	0.1	0.04	1345
α-Cubebene	0.09	0.08	0.08	1358
Eugenol	20.6	58.2	16.2	1362
Chavibetol	0.04	0.04	0.03	1375
α-Copane	0.31	0.2	0.3	1387
β-Bourbonene	0.07	0.2	0.05	1397
β -Elemene	0.2	0.5	0.2	1400
Methyl eugenol	0.8	3.4	0.7	1404
Dodecanal	0.03	0.2	0.04	1408
cis-iso-Eugenol	-	0.02	-	1413
Caryophyllene	1.4	1.4	1.3	1434
Aromadendrene	0.05	0.1	0.05	1454
Humulene	3.1	1.9	2.3	1471
γ-Muurolene	1.1	0.7	0.1	1488
Germacrene-D	1.5	3.5	1.6	1495
β-Selinene	1.7	0.7	1.4	1501
α-Selinene	3.5	1.7	3.6	1510
Eugenyl acetate	3.6	4.8	4.05	1529
Globulol	0.1	0.24	0.09	1607
4-Allyl-1,2-diacetoxybenzene	9.7	3.8	19.2	1649

tr = trace (less than 0.01%)

RI = Retention indices

3.9 Results and Discussion

The essential oils of three different cultivars of *Piper betle* namely, nadan, selan and kuzhikkodi were analysed by GC and GC-MS. Forty, thirty eight and forty three compounds respectively could be identified in them. Safrole was the major component in nadan (38%) and kuzhikkodi (36%) while in selan it was eugenol (58%).

It was interesting to note that both the cultivars nadan and kuzhikkodi contained high concentration of safrole while it was negligible in Selan (Table 1). Eugenol is the major component in selen (58%), while nadan and kuzhikkodi contained 21% and 16% respectively. The content of 4-allyl-1,2-diacetoxybenzene in kuzhikkodi essential oil is high (19%) compared to the earlier reports on other cultivars. Dominating constituents in the three varieties is phenolic derivatives, i.e. in nadan it is 72%, in selan it is 70% and in kuzhikkodi it is 75%. The main compounds in this group are euginol, safrole, eugenyl acetate and 4-allyl-1,2-diacetoxybenzene. Monoterpene content was about 7.6% in nadan, and 5.5% in selan and 5.4% in kuzhikkodi. The main compounds in this group are terpene-4-ol, and sabinene. The sesquiterpene content was about 13.0% in nadan 11.3 % in selan and 11.1 % in kuzhikkodi, represented by humulene, α-selinene, β-selinene and germacrene-D. The essential oil of *piper betle* leaves from Philippines[35] contains chavicol(53%) as the major component along with chavibetol

acetate(15.5%) and caryophyllene(3.9%). The chavicol and chavibetol composition in the three verities from kerala is less than 0.1%. This shows a marked variation of composition among different geographical locations and varieties.

Safrole is a known animal liver carcinogen. Its carcinogenicity correlates well with its DNA adducts formed in rat liver. The effect of safrole on Taiwanese Betal Quid chewing was biologically monitored by studying the metabolism of safrole in the liver. The metabolic products were examined from the urine samples of Betle Quid chewing people and established its carcinogenic effect[32]. Because of its carcinogenicity safrole is not used for flavouring soft drinks, chewing gums and tooth paste. In the light of hepatotoxic and carcinogenic effect of safrole[25] extensive chewing of the leaves of nadan and kuzhikkodi varieties may have health risk. Therefore it would be advisable to breed new varieties like selan with low safrole content.

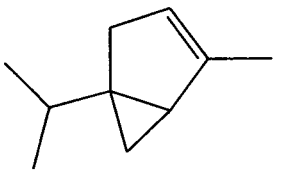
The essential oil of leaves of *piper betle* (nadan) was characterized by professional perfumers as spicy (direction of clove), sour-herbal-pumpkin-like weak fruity notes in the background. The correlation of the chromatographic-spectroscopic data with olefactoric ones allow the conclution that the spicy impression is determined by the identified phenolics (eugenol and its derivatives), and monoterpenic (terpinen-4-ol) and sequiterpenes (humulene)

responsible for the weak fruity odor. Some sesquiterpens like β-selinene contribute to the sour-herbal-pumpkin odour.

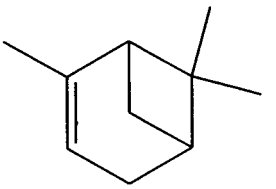
3.10 Conclusion

Safrole was the major component in nadan (38%) and kuzhikkodi (36%) while in selan it was Eugenol (58%). In the light of hepatotoxic and carcinogenic effect of safrole[25] extensive chewing of the leaves of nadan and kuzhikkodi varieties may have health risk. Therefore it would be advisable to breed new varieties like selan with low safrole content.

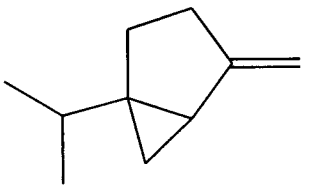
Structures of some of the identified compounds are as follows:



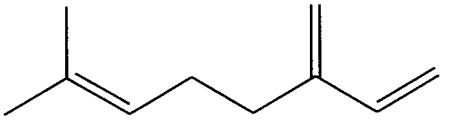
α-Thujene



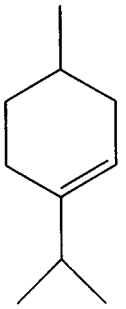
α-Pinene



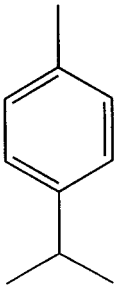
Sabinene



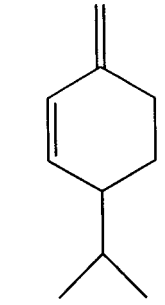
Myrcene



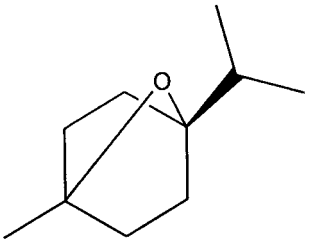
α –Terpinene



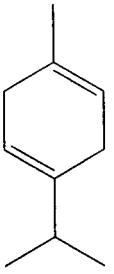
para-cymene



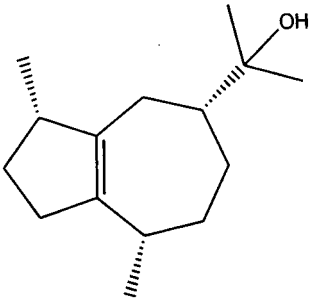
β-phellandrene



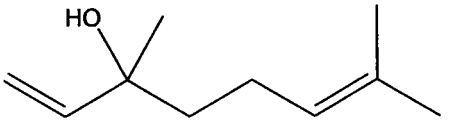
1,8-Cineole



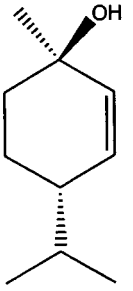
γ-Terpinene



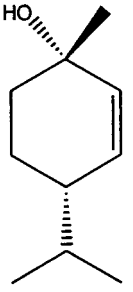
Guaicol



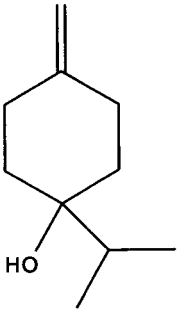
Linalool



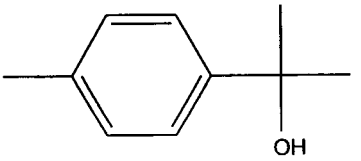
cis-para-Menth-2-en-1-ol



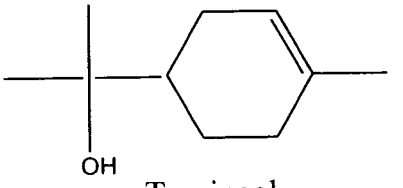
trans-para-Menth-2-en-l-ol



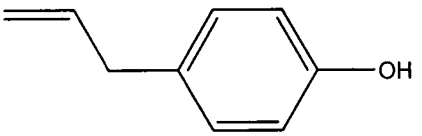
Terpinen-4-ol



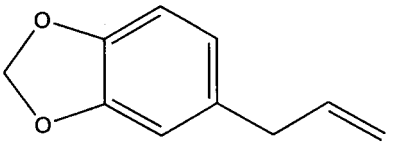
para-Cymen-8-ol



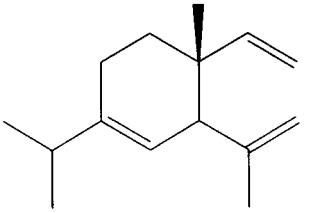
α-Terpineol



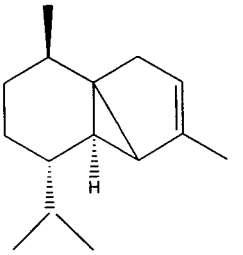
Chavicol



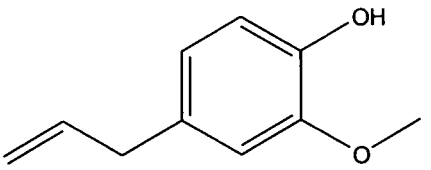
Safrole



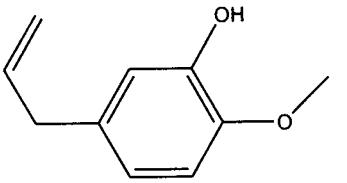
δ-Elemene



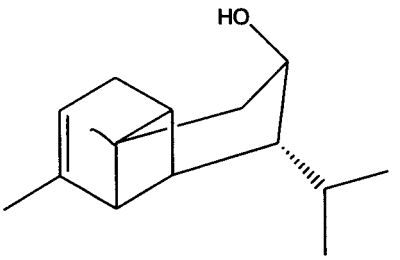
α-Cubebene



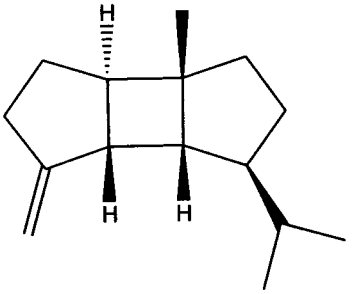
Eugenol



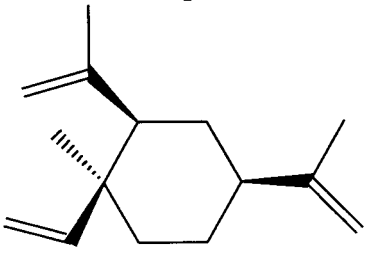
Chavibetol



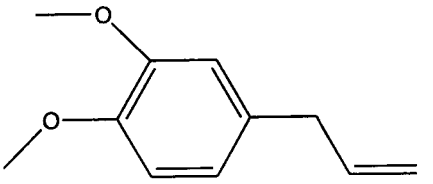
α-Copane



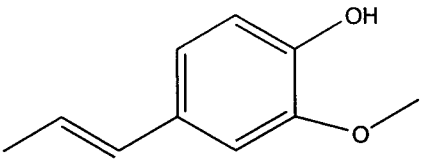
β-Bourbonene



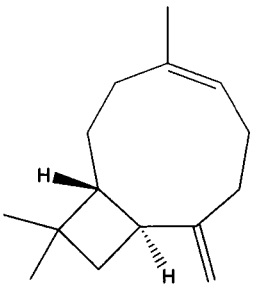
β-Elemene



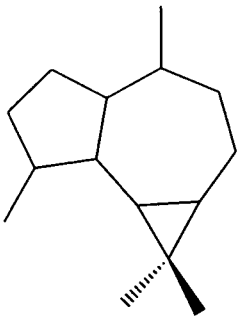
Methyl eugenol



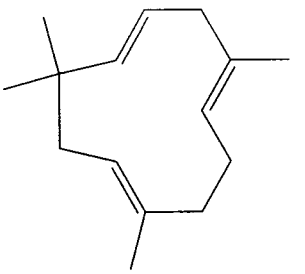
cis-iso-Eugenol



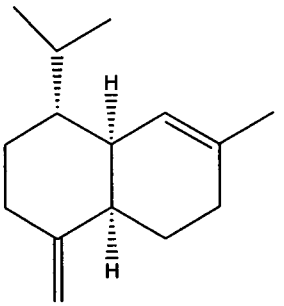
β-Caryophyllene



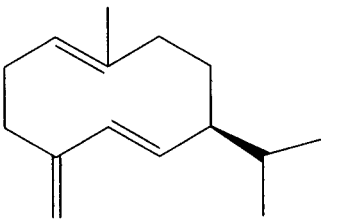
Aromadendrene



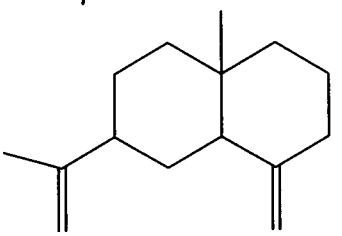
Humulene



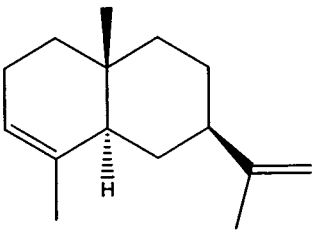
γ-Muurolene



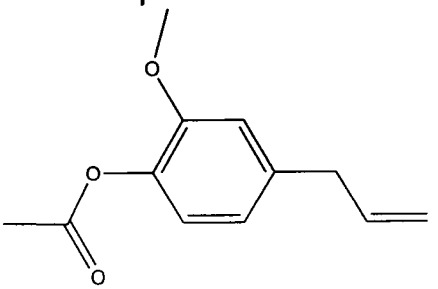
Germacrene-D



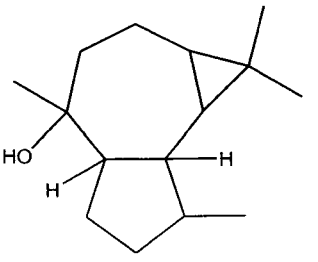
β-Selinene



α-Selinene



Eugenyl acetate



Globulol

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CHAPTER -IV

ESSENTIAL OIL COMPOSITION OF *POGOSTEMON PANICULATUS* AND *HYPTIS CAPITATA* FLOWERS

4.1 Introduction

The genera *hyptis* and *pogostemon* belong to the *Lamiaceae* family[1-4] and many of its reported species are known for their medicinal use as indigenous drugs[5-9]. The two genera are widespread throughout the tropical region[4]. The species *Pogostemon paniculatus* is found only in Indian peninsula and Myanmar[4] and to our knowledge there is no report on the phytochemical investigation on this plant. The plant *Hyptis capitata* has been used in traditional medicine for the treatment of gastro-intestinal disorders, hemorrhoids and asthma[10]. The essential oil of *Hyptis Suaveolnes* is found to possess antimicrobial activity[11]. *P. paniculatus* has been used for the preparation of massage oil in traditional Indian medical system, Ayurveda[12]. In the present work, the essential oil of flowers of both these plants has been examined by GC and GC-MS with a view to finding out the major constituents.

Pogostemon paniculatus Benth. is an erect under-shrub up to 75 cm tall. Leaves up to 8.5x 4 cm, ovate, acuminate, cuneate at base, pubescent with apprised hairs, flowers 0.6 cm long white in terminal spikes, paniculate

spikes; bracts 0.7cm long obliquely ovate and imbricate. Calyx pubescent, teeth lanceolate, nutlets ovoid, smooth. Flowering and fruiting season is between January-March. Habitat, moist deciduous and semi ever green forests and waste lands. It is found is some parts of Kerala. It is a rare and endangered species[19].

Hyptis capitata Jacq is a herb native to tropical America. It is naturalised in western ghats and found throughout Kerala. The flowering season is between September and January. Anuals erect. Leaves opposite petiolate, broadly ovate-oblong, serrate, cuneate, glabrous or minutely peberulous on the nerves beneath. Flowers in globose head; bracts ovate-lanceolate, calyx glabrous, sub-equaly five toothed, teeth erect, sublote corolla slightly two liped; upper lip four lobed. Stamns four, declinate; filaments free; anther cells confluent. Overy four partite; sryle bifed, snutlets four; basal scar small[20].

4.2 Essential oil studies so far reported from *Hyptis* and *Pogostemon* species

The essential oils from leaves and inflorescences of *Hyptis martiusii* Benth were analyzed by GC-MS[14]. The major compounds are Δ-3-carene (22.5%), 1,8-cineole (24.27%), β-caryophyllene (6.15%), and bicyclogermacrene (6.32%), where as the major components in the essential oil of inflorescences of the the plant are Δ-3-carene (13.5%), α-pinene

(5.78%), β-caryophyllene (6.59%), viridiflorene (8.25%), and germacrene-B (5.21%). In another study[15] on *hyptis* genus α -Pinene, myrcene, and β-caryophyllene are the constituents commonly present in species such as *H. suaveolens, H. mutabilis, H. goyazensis.* 1,8-Cineole and β -caryophyllene are the same principal components in the oils of *H. suaueolns* and *H. mutabilis.* A study of essential oil from aerial parts *H. capita* was reported[13] the major components are borneol (16.7%) and piperitone oxide (14.8%).

Pogostemon cablinis is cultivated extensively in Indonesia, Malaysia, China, and Brazil for its essential oil *(patchouli oil),* important to the perfumery industry and in aromatherapy. Thus, the constituents of patchouli oil have frequently been investigated, and the presence of a number of mono and sesquiterpenoids have been reported[16-18], the major compounds are (–)-patchoulol, α-guaiene, α-patchoulene, seychellene, α-bulnesene, norpatchoulenol and pogostol.

4.3 Present work

The objective of the present work was to characterise the volatiles present in the flowers of *Pogostemon paniculatus* and *Hyptis capitata* . To our knowledge no phytochemical investigation is done on *Pogostemon paniculatus.* The essential oil composition of flowers of *Hyptis capitata* is also not reported earlier.

4.4 Experimental

Plant Material

The fresh flowers of plants were collected from Malappuram Dt., Kerala, India, during January 2006. Voucher specimens (no. 75 and 76) of the plants have been deposited in the specially maintained Herbarium of Department of Chemistry at Calicut University.

Isolation of the Volatile Oil

The fresh flowers (500 g each) were steam distilled for 3 hrs. The distillates were extracted with diethyl ether (2 x 100 mL) and dried over anhydrous sodium sulphate. The dry ether on evaporation yielded 600mg (1.3%) and 800 mg (0.4%) of oil based on fresh flowers of *Pogostemon paniculatus* and *Hyptis capitata* respectively.

Gas chromatography (GC)

Quantification by GC-FID was carried out using a HP 5890 GC (Hewlett Packard) instrument fitted with an OPTIC® injector and FID detector. A HP 5 (Hewlett – Packard) Capillary column (25 m x 0.2mm i.d., film thickness 0.33 μm) was used, with nitrogen gas as the carrier gas at a flow rate of 3 mL/min. The column was temperature programmed from 30^0C to 280^0C at a rate of 3^0C/min; injector and the detector temperature; 270^0C and 280^0C respectively. The quantitative data quoted are percentage relative peak areas (RPA).

Gas chromatography – Mass spectrometry (GC-MS)

The GC-MS analysis was carried out using Varian 3400 GC fitted with an OPTIC® injector and coupled to a Finnigan ITS40 ion trap mass spectrometer. The column used was a HP ultra 2 (Hewlett Packard 50m x 0.2 mm i.d., film thickness 0.33 μm); injector temperature programme 50-270^{0}C at 2^{0}C/min; mass spectra, EI mode 70 eV, scan range 35-450 amu. Component identification was made by comparison with in house reference libraries, commercial libraries and known retention indices. The identified compound are listed in table 4.1.

Olfactoric evaluation

The essential oils were olfactorically evaluated by odor trained professional perfumers

Table 4.1

Composition of the essential oil of *H. Capitata* and *P. paniculatus* flowers

Component	H. capitata%	P.paniculatus%	RI
trans-Hex-2-enal	1.1		845
cis-Hex-3-enol	1.7		847
trans-Hex-2-enol	3.0		857
Hexanol	0.6		859
Heptanal	t		896
Benzaldehyde	0.2		961
Heptanol	t		968
Oct-1-en-3-ol	23.3	t	979

Octan-3-ol	0.1		995
1,8-Cinelole	0.7		1036
Benzyl alcohol	0.2		1036
Octanol	1.8		1072
cis-Linalol oxide (furanoid)	1.5		1077
trans-Linalol oxide (furanoid)	0.3		1092
Linalol	13.9	0.2	1102
Nonanal	0.2	t	1107
Oct-1-en-3yl acetate	0.1	t	1113
Phenylethyl alcohol	0.4		1117
Nonanol		t	1172
cis-Linalol oxide (pyranoid)	0.1		1173
trans-Linalol oxide (pyranoid)	0.2		1177
Terpinen-4-ol	0.1		1182
para-Cymen-8-ol	0.1		1189
α-Terpineol	0.1		1195
Oct-1-en-3-yl propionate		0.1	1198
Decanal	0.5	t	1207
cis-Hex-3-enyl-2-methyl butyrate	0.1	t	1234
Hydroquinone	3.8		1269
Decanol	1.6		1272
Oct-3-enyl butyrate		t	1282
Indole		t	1298
Octen-3-yl-2-methylbutyrate		0.5	1325
δ-Elemene		t	1345
Octyl iso-butyrate	0.1		1346
para-Methoxyacetophenone	0.2		1358
Eugenol	0.3		1362
γ-Nonalactone	0.1		1367

α-Copaene	0.3	t	1385
Octyl butyrate	0.2		1391
Bourbonene		0.1	1396
Caryophyllene	0.7	1.8	1432
Octyl-2-methyl butyrate	0.9		1437
Octyl iso-valerate	0.2		1441
Geranylacetone	1.5		1457
cis-β-Farnesene		45.3	1466
Humulene	0.2	0.2	1468
γ-Muurolene	0.9		1488
Germacrene D		4.4	1497
Phenylethyl iso-valerate	0.3		1500
α-Muurolene	0.5		1512
α-Farnesene		0.1	1515
γ-Cadinene	0.7		1528
δ-Cadinene	2.7		1536
Nerolidol	0.6		1571
Farnesene epoxide		41.7	1575

t = trace (<0.1%)

RI = Retention index

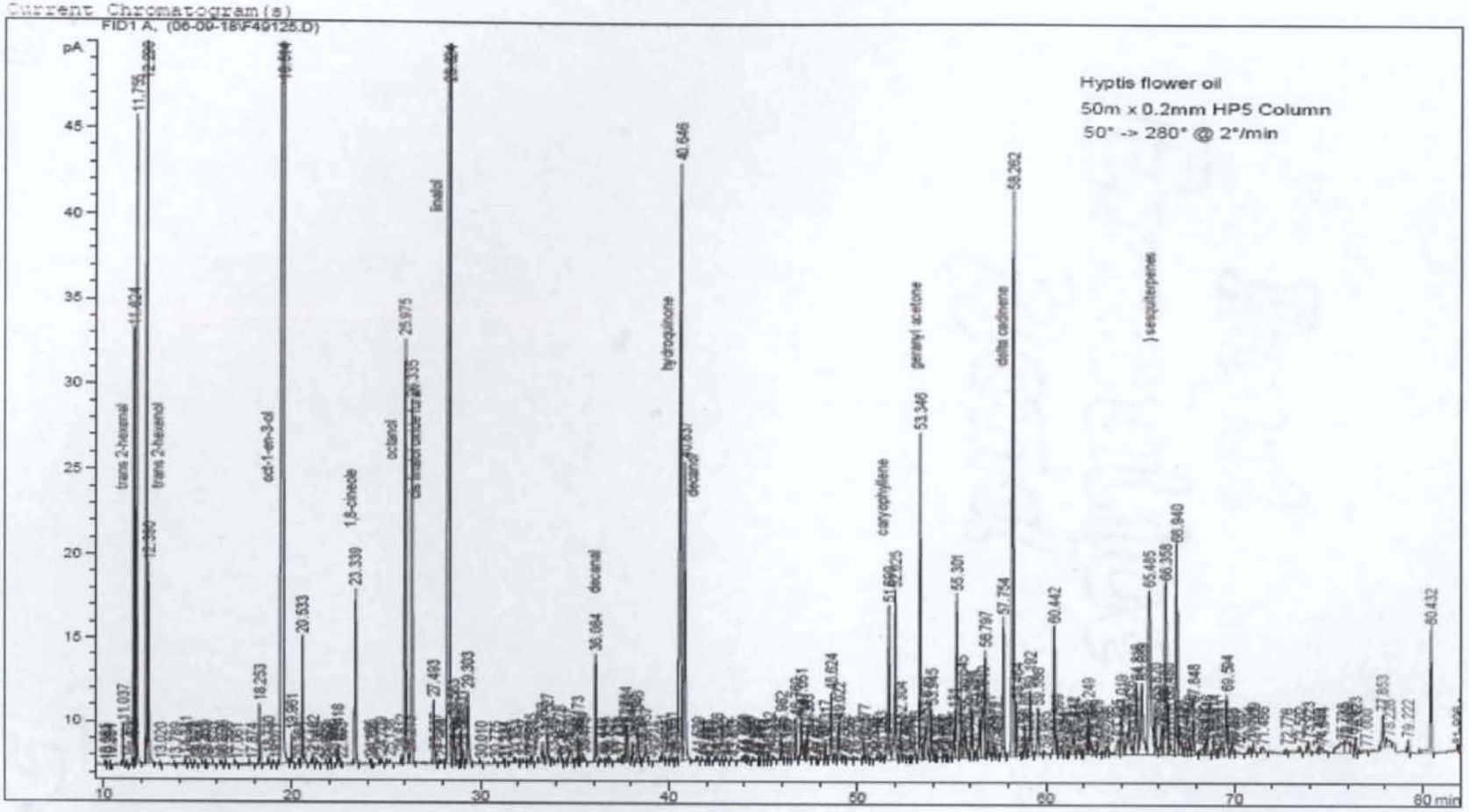


Fig 1. GC-*Hyptis capitata*

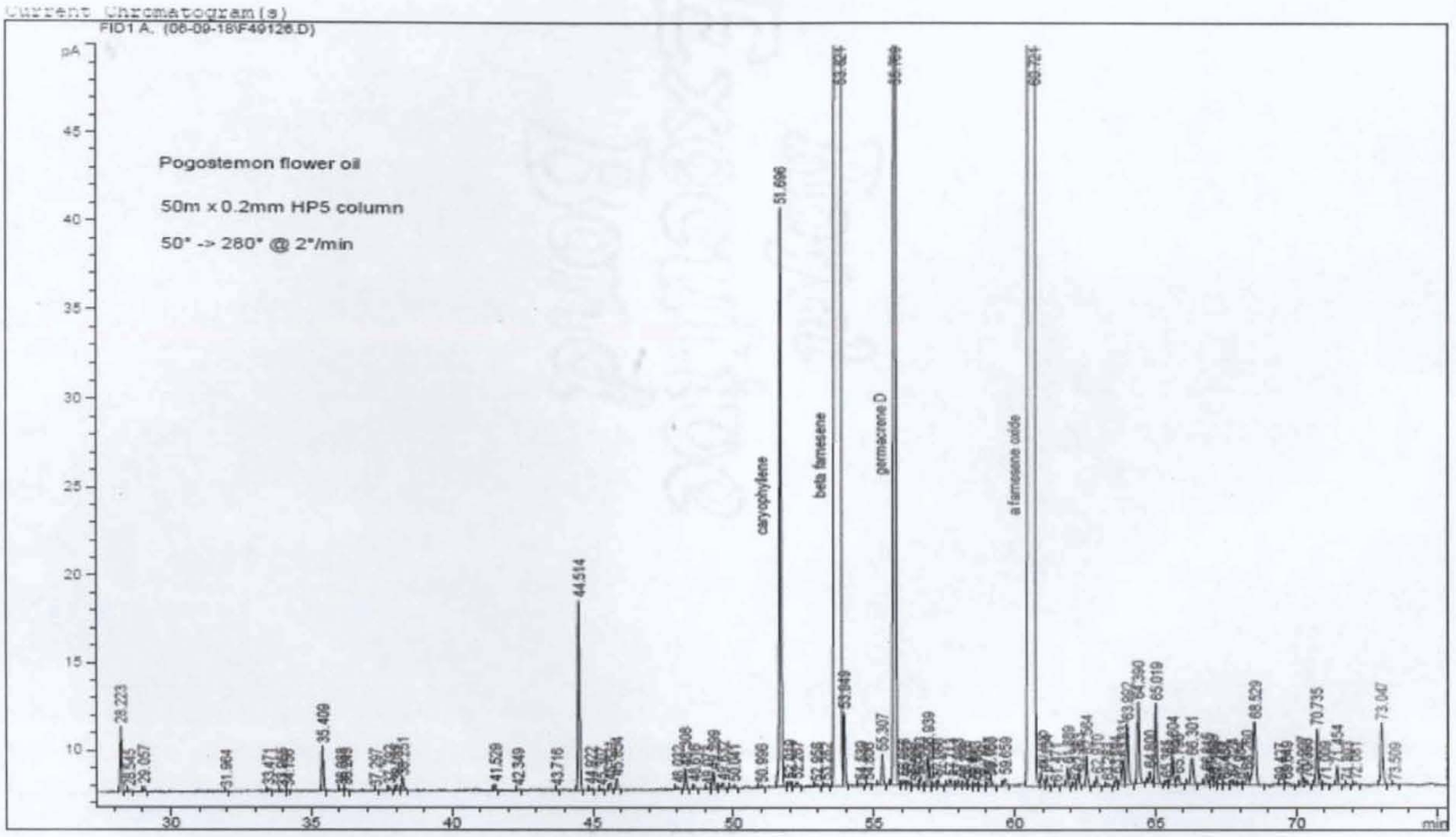


Fig 2. GC-*Pogostemon paniculatus*

4.5 Results and Discussion

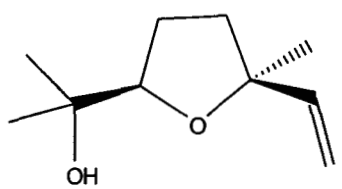
The essential oil of *Pogostemon paniculatus* was yellow in colour and possessed a herbal, woody, earthy odor with seed-like and oatmeal notes with some similarity to patchouli, whereas the essential oil of *Hyptis capitata* is greenish yellow in colour and has herbal, herb-tea like, linalool and comphoraceous / cineole notes. Twenty compounds were identified (table 4.1) in P. paniculatus of which sesquiterpenes were the major class of compounds (93.7%). The major compounds were *cis*-β-farnacene (45.3%) and Farnacene epoxide (41.7%). The only monoterpene found was linalol (0.7%). Non terpenoid compounds constitute only 0.2 percentage. In the essential oil of *Hyptis capitata* flower, forty six compounds were identified of which non terpenoid compounds constitute the major fraction (41.0%). The major compounds were oct-1-en-3ol (23.3%) and linalool (13.8%). In contrast, the earlier reports on the essential oil of *Hyptis capitata* from the aerial parts from the same region showed monoterpenes such as borneol (16.7%) and piperitone oxide (14.8%) as the major constituents[13]. This shows the variation in composition of essential oil from flower and that from aerial parts.

4.6 Conclusion

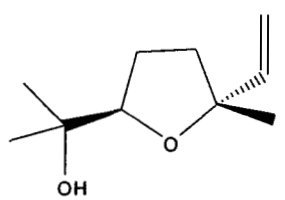
The essential oils represented to 0.3% and 0.4% of the fresh flowers (w/w) of *Pogostemon paniculatus* and *Hyptis capitata* respectively. Twenty compounds representing 94.6% of essential oil of flowers of *Pogostemon*

paniculatus were characterized; *cis*-β-farnacene (45.3%) and farnacene epoxide (41.7%) were found to be the major components. In the essential oil of *Hyptis capitata* 46 compounds representing 66.0% of the oil were identified and the major compounds were oct-1-en-3-ol (23.3%) and linalol (13.8%). The essential oil of *H.capitata* is also noted by the presence of hydroquinone (3.8%), which is a a topoisomerase II poison[21]. Therefore the essential oil of flowers of *H. Capitata* cannot be recommended for perfumery.

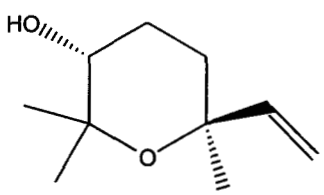
Structures of some of the identified compounds are as follows



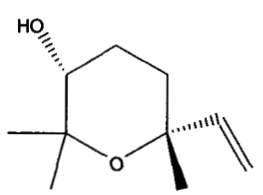
cis-Linalol oxide (furanoid)



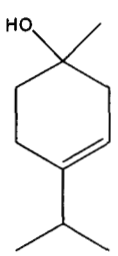
trans-Linalol oxide (furanoid)



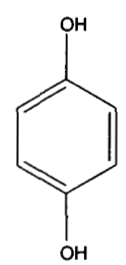
cis-Linalol oxide (pyranoid)



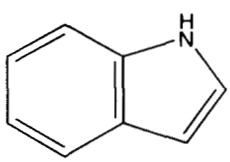
trans-Linalol oxide (pyranoid)



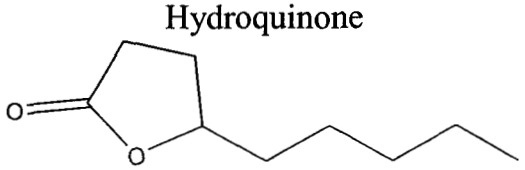
α-Terpineol



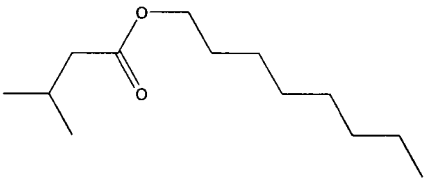
Hydroquinone



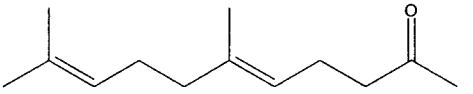
Indole



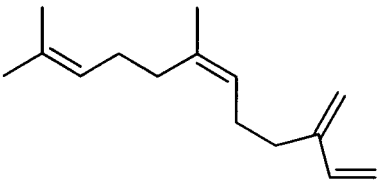
γ-Nonalactone



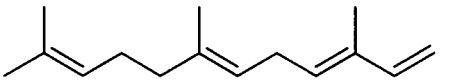
Octyl iso-valerate



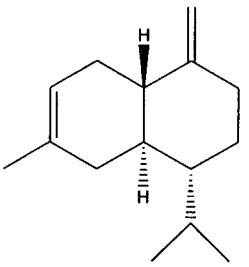
Geranyl acetone



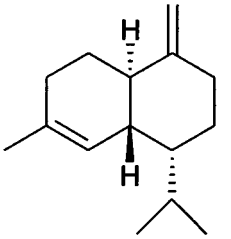
cis-β-Farnesene



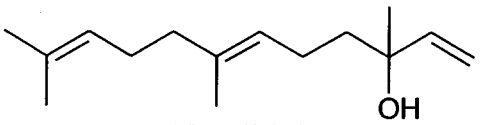
α-Farnesene



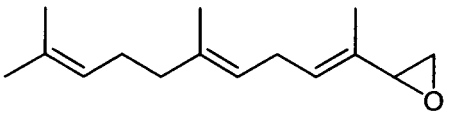
γ-Cadinene



δ-Cadinene



Nerolidol



α-Farnesene epoxide

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CHAPTER -V

SECTION 1:

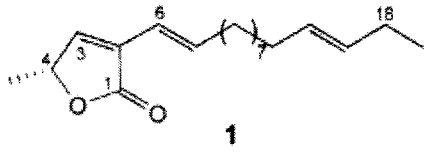
ESSENTIAL OIL COMPOSITION OF *LITSEA LAEVIGATA* FRUITS

5.1.1 Introduction

Litsea laevigata Nees. belongs to the *Lauracea* family. It is found in semi evergreen forests. It is distributed in Idukki, Kannur, Palghat and Wayanad Districts of Kerala[1] and is endemic to south Western Ghats. It is a tree up to 10 m tall, leaves 18 x. 5.6 cm, acuminative, narrowed at base, glabrous and shining above minutely puberulaus banth. Berries are 1.5 cm long, ellipsoid and yellow when ripe. Flowering season is between March and April. The plant is rarely found in semi evergreen forests[2].

5.1.2 Work so far reported on *Litsea* genus

Byung Sun Min et al.[3] isolated two new lactones, litsealactone-A (**1**) and litsealactone-B (**2**) from the leaves of *Litsea japonica*, together with three known lactones, hamabiwalactone-A (**3**), hamabiwalactone-B (**4**), and akolactone-B (**5**)[3] .



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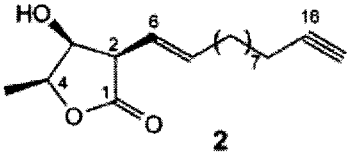

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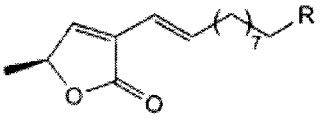
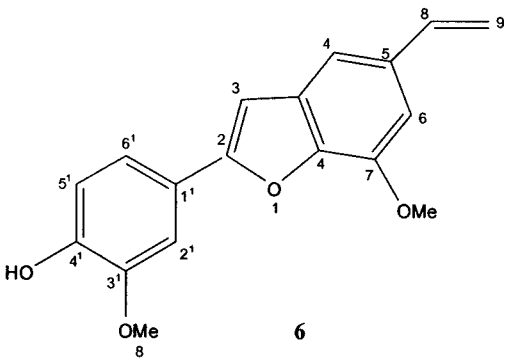


3 R= —C≡CH

4 R= —CH=CH$_2$

5 R= —CH=CH—CH=CH$_2$

Hsing-I Cheng[4], et al. isolated six new compounds from the leaves of *Litsea acutivena*, including one nor-neolignan, dehydroxymethylailanthoidol (**6**), litseakolide-D (**7**), litseakolide-E (**8**), litseakolide-F (**9**), litseakolide-G (**10**), and isolincomolide-D (**11**).



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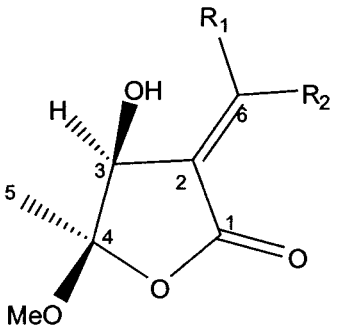
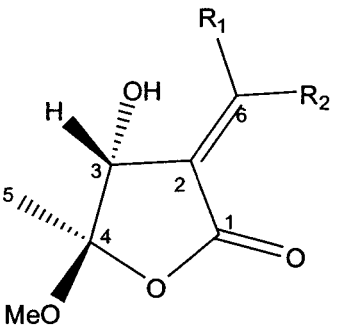
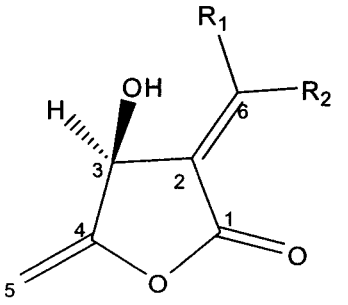


7 $R_1=(CH_2)_8CH_3$, $R_2=H$
8 $R_1=H$, $R_2=(CH_2)_8CH_3$

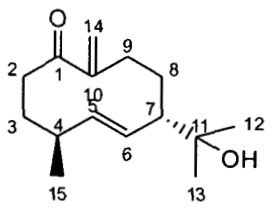


9 $R_1=(CH_2)_8CH_3$, $R_2=H$
10 $R_1=H$, $R_2=(CH_2)_8CH_3$



11 $R_1=H$, $R_2=(CH_2)_{10}CH_3$

Hong-Jie Zhang et al.[5] identified seven new sesquiterpenes, named litseagermacrane (**12**), 7-*epi*-eudesm-4(15)-ene-1R,6R-1α,6α-diol (**13**), 7-*epi*-eudesm-4(15)-ene-1β,6β-diol (**14**), 5-*epi*-eudesm-4(**15**)-ene-1β,6β-diol (**15**), eudesm-4(15)-ene-1 β,6β-diol(**16**) and litseahumulanes-A (**17**) and B (**18**), from leaves and twigs.



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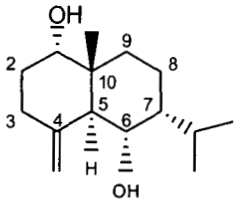

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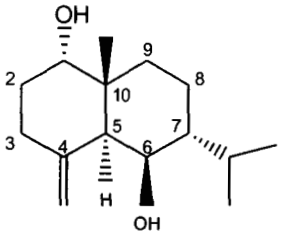

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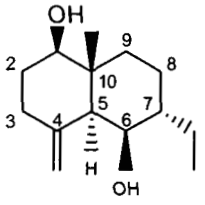

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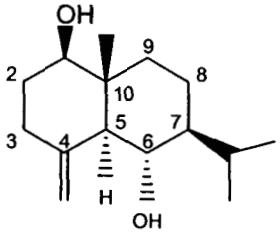

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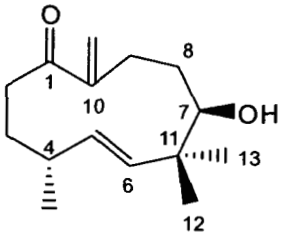

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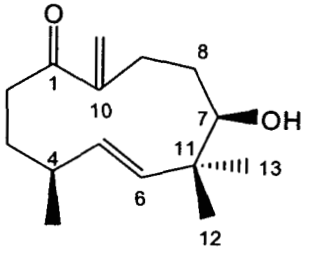

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5.1.3 Present work

The objective of the present work was to characterise the volatiles present in the fruits of *Litsea laevigata* and to evaluate its anti-microbial properties. To the best of our knowledge no phytochemical investigation is reported on this plant.

Experimental

The fresh berries of *Litsea laevigata* were collected from the Munnar forests in May 2007. The plant material was identified by Dr. A.K. Pradeep, Department of Botany, Calicut University, Kerala. A voucher specimen has been deposited in the specially maintained herbarium of the Department of Chemistry at Calicut University.

Essential oil extraction

The fresh fruits (3.5 kg) of *Litsea laevigata* were ground into a paste by means of an electric grinder and was steam distilled for 3 h. The distillate was extracted with diethyl ether (2 x 100 mL) and dried over anhydrous sodium sulphate. After evaporation of the solvent, 12g (0.34% of the fresh weight) of colourless essential oil (LL) was obtained.

Fractionation of essential oil using column chromatography

In order to asses the anti-microbial property of different components, the essential oils were separated into two fractions. A column was packed with 50g silica gel for column chromatography (100-200 mesh) using distilled n-pentane in a column of dimension (3 cm x 50 cm, d x l). About 5g of the essential oil (LL) was added on the column and eluted with 100 mL each of n-pentane, and diethyl ether successively and each 100 mL fractions were collected separately. The pentane fraction on evaporation yielded about 3g of

oil (LLP) and the diethyl ether fraction on evaporation yielded about 1g of oil (LLD).

Olfactoric Evaluations

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

GC and GC-MS analysis

The GC-MS analyses were carried out by using a Shimadzu GC-17A with QP 5050 and the data system compaq-proLinea (class 5k-software), Hewlett-Packard GC-HP 5890 with HP-5970 MSD and PC-Pentium (Böhm co; Chemstation-Software) and Finnigan MAT GCQ with data system Gateway-2000-PS75 (Siemens Co., GCQ-software). An apolar 30 m OV-1-type column (0.32 mm i.d. and 0.25 µm film thickness) and helium as carrier-gas was used. Injector temperature: 250°C; interface heating: 300°C; ion source heating: 200°C, EI-mode; scan range: 41-450 amu. For compound identification Wiley - NBS- and NIST- library spectra (on line) as well as reference MS-spectral data were used.

GC-FID analyses were carried out using a Shimadzu GC-14A with FID and the integrator C-R6A-Chromatopac and a varian GC-3700 with FID

and the integrator C-R1B-Chromatopac (Shimadzu Co.). The same column used for GC-MS was also used for GC-FID. Carrier gas: hydrogen; injector temperature was at 250°C and detector temperature at 320°C; temperature – program: 40°C/5 min to 280°C/5 min with a heating rate of 6°C/min. Quantifications were made by relative % peak-area calculations.

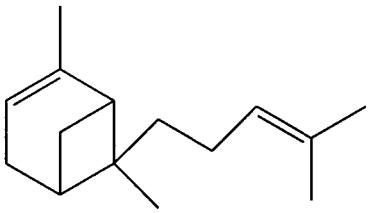
Table 5.1

Constituents of *Litsea laevigata* essential oil and its fractions

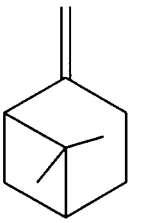
Compound	LL (%)	LLP (%)	LLD (%)
trans-α-Bergamotene	26.7	16.5	3.5
α-Pinene	25	25.3	0.5
β-Pinene	8.2	2.1	0.2
α-Terpineol	5	0.7	13.1
1,8-Cineole	4.6	8.4	26.9
Limonene	4.3	3.1	0.5
α-Copaene	4.1	6.2	0.9
Camphene	4	7.5	0.2
Fenchol	3.5	0.8	11.5
Decanol	2.5	0.7	3.4
Borneol	2.2	0.4	8.5
β-Santalene	1.7	5.1	0.8
α-Farnesene	0.9	1.3	0.8
β-Farnesene	0.8	5.1	0.7
α-Santalol	0.7	1.8	0.4
β-Elemene	0.7	2.1	0.9

α-Fenchene	0.7	1.4	0.3
α-Bulnesene	0.7	0.4	0.4
p-Cymene	0.6	0.4	0.9
Cis-β-Ocimene	0.4	0.2	1.4
β-Bisabolene	0.4	0.3	0.6
β-Myrcene	0.3	0.7	0.2
Pinocarveol	0.3	1	2.1
Caryophyllene	0.3	1.1	1.2
Terpinen-4-ol	0.2	0.1	1.7
Epi-β-Santalene	0.2	2.4	-
Junipene	0.2	0.2	-
Myrtenol	-	1.6	0.4
Verbenol	-	0.9	0.1
Myrtenal	-	0.5	-
Decanoic acid	-	-	4.5
Camphene hydrate	-	-	2.6
Hexadecanol	-	-	1.9
Nonanol	-	-	1.8
Total	99.2	98.2	92.9

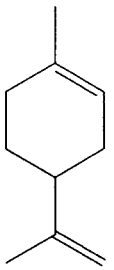
Structures of some of the identified compounds are as follows



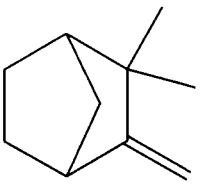
trans-α-Bergamotene



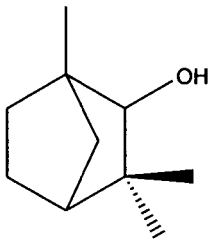
β-Pinene



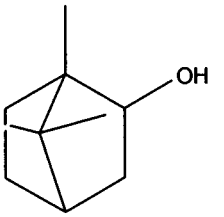
Limonene



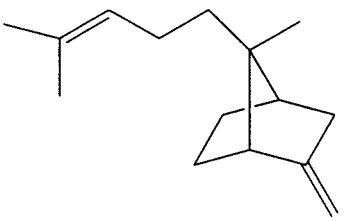
Camphene



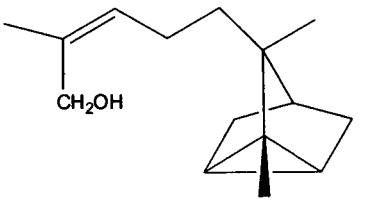
Fenchol



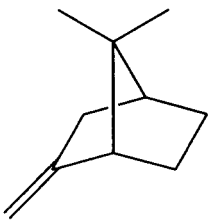
Borneol



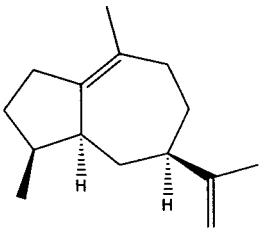
β-Santalene



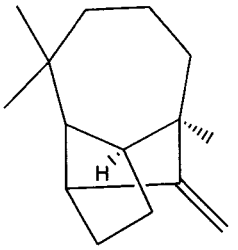
α-Santalol



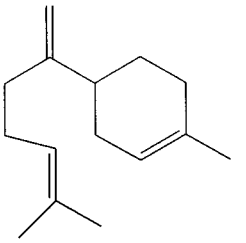
α-Fenchene



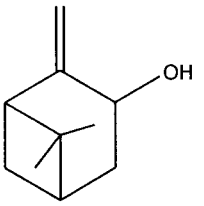
α-Bulnesene



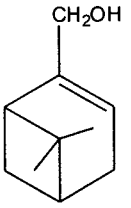
Junipene



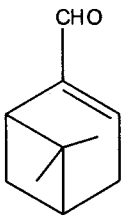
β-Bisabolene



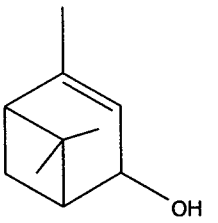
Pinocarveol



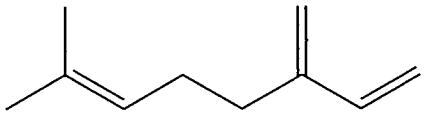
Myrtenol



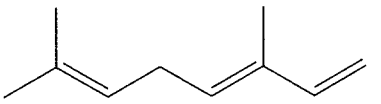
Myrtenal



Verbenol



β-Myrcene



cis-β-Ocimene

5.1.4 Results and Discussion

The essential oil of *Litsea laevigata* (LL) represented 0.34% of the fresh weight of the berries and 99.2 % of the oil was identified by GC and GC-MS. Twenty six compounds were identified (table 5.1) in the essential oil sample LL of which monoterpenes were the major class of compounds (59.3%). The major compounds were α-pinene (25%), β-pinene (8.2%),α-terpineol (5%), fenchol (3.5%), limonene (4.3%) and 1,8-cineole (4.5%). The

percentage of sesquitertpene was about 37.4 % were as nonterpenoid compounds constitute only 2.5%. The important sesquiterpenes are *trans*-α-bergamotene,(26.7%), α-copaene(4.1%) and β-santalene(1.7%).

The essential oil fraction LLP is represented by non polar fraction of the original oil, thirty compounds were identified in this fraction of which monoterpenes were the major class of compounds (54.5%). The percentage of sesquiterpene was about 42.5% were as nonterpenoid compounds constitute only 1.2 %. The essential oil fraction LLD is represented by polar fraction of the original oil, thirty one compounds were identified in this fraction. The important compounds in this fraction are 1,8-cineole(26.9%), α-terpineol (13.1%), fenchol (11.5%) and borneol (8.5%). Both LLP and LLD contained compounds that could not be detected in the original oil. This can be due to the higher concentration of them during fractionation.

The essential oil of *Litsea laevigata* was colourless and possessed soft piney-woody, diffuse earthy, reminding of patchoulene, dry-herbal odour whereas the essential oil of column chromatographic fraction LLP was colourless and has fresh, harsh-terpeny, diffuse citrus-metallic(myrcene-note), soft herbal odour. The essential oil fraction LLD had light yellow colour and had fresh-terpeny, earthy (root-like), mild woody-aldehydic, later patchouli-bulnesene-like, fatty-sour in the background. The piney-woody, earthy and herbal odours can be attributed to the pinenes, terpineol, cineole, α -copaene

and pinocarviol [19,20]. The odour impression of LLP and LLD are comparable but for the harsh-terpeny odour of LLP. This can be attributed to the high α- pinene content of LLP in comparison to LLD. Apart from these aroma compounds this essential oil contains small quantities of santalene and santalone which are sandalwood oil constituents. All these factors make this essential oil valuable in fine perfumery were piney –woody, earthy and herbal odour notes are desirable eg. in shower gels, deodorants etc., moreover the yield of this oil (0.34% of fresh weight) makes it a commercially viable product.

SECTION II:

EVALUATION OF ANTIMICROBIAL PROPERTIES OF *LITSEA LAEVIGATA* FRUIT ESSENTIAL OIL

5.2.1 Introduction to Anti-microbial Studies

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

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

Bacteria

The bacterium is a single-celled organism, that does not have intracellular membrane-bound organelles such as a nucleus, golgi bodies, endoplasmic reticulum or mitochondria. Therefore, the bacterium's essential metabolic and biosynthetic activities must be carried out within the cytoplasm and the cell envelope. Bacteria lack a true nucleus and are classified as prokaryotes. One of the most important cytological features of bacteria is their reaction to a simple staining procedure called the Gram-stain. The procedure involves staining the cells with the dye crystal violet, and a mordant known as Lugol's solution (3:5 I_2/KI) is added to set the stain. The bacteria are next decolourised with alcohol. Finally, the bacteria are

counterstained with safranin. Gram-positive bacteria retain the crystal violet, whereas Gram-negative bacteria, which lose the crystal violet, on counterstaining by safranin appear red in colour.[6] The most plausible explanation for this difference in behaviour lies in the relative differences between the cell walls of the above two types of bacteria.

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

The cell wall of a Gram-negative bacterium is thinner but more complex than that of a Gram-positive bacterium. Only 10 to 20 percent of the cell wall is peptidoglycan; the remainder consists of various polysaccharides, proteins and lipids. Gram-negative bacteria fail to retain the crystal violet-iodine dye during the decolourising procedure partly because of their thin cell walls and partly because of the relatively large quantities of lipoproteins and lipopolysaccharides in the wall.[7]

Modes of action of anti-microbial agents

Microorganisms can be inhibited or killed by various physical and chemical agents. The agents that kill or destroy the organisms are referred as 'cidal' whereas the one that merely halts the growth of the microorganism is called 'static'. If a static agent is removed from a culture, the organism will resume growth, but the effects of cidal agents are irreversible. The manner in which anti-microbial agents inhibit or kill can be attributed to the following kinds of actions.[8]

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

Antibiotics

They are chemical substances produced by certain microorganisms that inhibit or kill other microorganisms. An antibiotic that acts on both Gram-positive and Gram-negative bacteria is called a broad-spectrum antibiotic, whereas narrow-spectrum antibiotics, act on only a specific group of

organisms. The widespread use of antibiotics has increased the number of pathogenic microorganisms that display antibiotic resistance. Drug resistance, may be due to the pre-existing factor in the microorganism or it may be due to some acquired factors. For example, penicillin resistance may result from the production of penicillinase by resistance organisms, which convert penicillin to inactive penicilloic acid.

Fungi

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

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

Fungal cell wall resemble plant cell walls architecturally, but not chemically. Although, cellulose is present in the walls of certain fungi, many fungi have non cellulosic walls. Chitin, a polymer of the glucose derivative, N-acetylglucosamine, is a common constituent of fungal cell walls. The cell wall is composed of cross-linked polysaccharides, proteins and glycoproteins

and it provides the fungus with osmotic stability and rigidity. The polysaccharides, which make up about 80% of the cell wall are polymers of simple sugars and include chitin, glucan, mannan etc. Cellulose and chitosan, two other polysaccharides, make the fungal wall resistant to the degradative effects of acids and alkalies. The fungal cell membrane contains ergosterol and zymosterol.

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

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

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

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

Anti-microbial activity of essential oils

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

Recently carvone, a monoterpene isolated from the essential oil of *Carum carvi* has shown its ability to inhibit the sprouting of potatoes during storage as well as fungicidal activity in protecting tubers from rotting without exhibiting mammalian toxicity.[9]

Essential oil of *Salvia officinalis* has also shown practical potency in enhancing the storage life of some vegetables by protecting them from fungal rottings.[10] There are several reports on the antifungal activity of essential oils. Essential oils from different plant species are known to exhibit various kinds of biological activities including antifungal, anti-microbial, cytostatic, insecticidal, allelopathic, antioxidant and bio regulatory actions.[11]

The volatile oils of black pepper and clove were assessed for antibacterial activity against twenty-five different genera of bacteria. These included animal and plant pathogens and spoilage bacteria.[12] Essential oils extracted from plants such as mentha, *Lavandula officinalis* and from roots and flowers of radish are known to exhibit anti-microbial activity.[13] The oils from onion and garlic exhibit high activity. Pine oil is known to inhibit various species of fungi and has been used in fungicidal preparations.[14, 15]

5.2.2 Anti-microbial activity of *L. laevigata* essential oil samples

The anti-microbial activity of the given samples were carried out by Disk Diffusion Technique[18]. The test microorganisms of *Staphylococcus aureus, Bacillus subtilis, Streptococcus faecalis, Staphylococcus albus* (Gram positive) *Escherichia coli, Pseudomonas aeruginosa, Protieus vulgaris, Klebsiella aerogenes* (Gram negative) and fungi *Candida albicans* and *Aspergillus niger* were obtained from National Chemical Laboratory (NCL) Pune and maintained by periodical sub culturing on Nutrient agar and Sabouraud dextrose medium for bacteria and fungi respectively. The effect produced by the sample was compared by the positive control.

Experimental

Antibacterial activity

The nutrient agar culture medium was prepared by dissolving readymade nutrient agar in distilled water (23 mL/Lit) by heating till it boiled and then sterilised by autoclave at 15 lbs pressure (121°C for 20 min.). The medium was poured into sterile Petri dishes and allowed to solidify and dry. The inoculum was inoculated uniformly over the medium. Within 15 minutes after the plates were inoculated small paper disks impregnated with known amounts of essential oil was applied to the surface of the inoculated plates with sterile forceps. Disks were gently pressed down onto the agar with forceps to ensure complete contact with the agar surface. The spatial arrangements of the disks were not closer than 15 mm to the edges of the plate and far enough apart to prevent overlapping of zones of inhibition. Within 15 minutes after the disks were applied, the plates were inverted and placed in an incubator at 35°C, until bacterial growth was observed. After 16 to 18 hours of incubation, the plates were examined and the diameter of the zone of complete inhibition was measured to the nearest whole millimetre by sliding callipers. Controls were maintained with DMSO and Ciprofloxacin 5μg/disc for bacteria.

Antifungal activity

The readymade Sabouraud medium (Himedia 39g) was dissolved in distilled water (1000mL) and heated to boiling until it dissolved completely. The medium and Petri dishes were autoclaved at pressure of 15 lb/inc^2 for 20 min. The medium was poured into sterile Petri dishes under aseptic conditions in a laminar flow chamber. When the medium in the plates solidified, 0.5mL of (One week old) culture of test organism was inoculated and uniformly spread over the agar surface with a sterile L-shape rod. Solutions were prepared by dissolving the essential oil in DMSO (Dimethyl suplphoxide) and different concentrations were made. After inoculation, cups were scooped out with 6 mm sterile cork borer and the lids of the dishes were replaced. To each cup different concentrations of test solutions were added. Controls were maintained with DMSO and Clotrimazole 5µg/disc for fungi. The treated and the controls were kept in an incubator at room temperature for 24 h to 96 h. Inhibition zones were measured and diameter was calculated in millimetre. Three to four replicates were maintained for each treatment.

The preliminary screening results obtained for the sample LL is tabulated in table 5.2 and the sample LLP and LLD are given in table 5.5. Anti-microbial screening of the essential oil LL at different concentration levels given in table 5.3. The Minimum inhibitory concentration (MIC) is worked out in table 5.4.

Table: 5.2

Preliminary screening of the essential oil LL

Sl.No	Test organism	Diameter of zone of inhibition (mm)	
		Sample (LL)	STD
1	*Staphylococcus aureus*	16	18
2	*Bacillus subtilis*	25	18
3	*Streptococcus faecalis*	22	20
4	*Staphylococcus albus*	35	20
5	*Escherichia coli*	21	17
6	*Pseudomonas aeruginosa*	14	23
7	*Protieus vulgaris*	20	17
8	*Klebsiella aerogenes*	20	23
9	*Candida albicans*	15	13
10	*Aspergillus niger*	19	13

Std- Ciprofloxacin 5µg/disc for bacteria,

Clotrimazole 5µg/disc for fungi

The sample of essential oil was diluted with equal volume of DMSO(50% v/v) was used for the test. The sample volume used in each disk is 50µl.

Table: 5.3

Anti-microbial screening of the essential oil LL at different concentration levels

S.No	Test organism	Diameter of zone of inhibition (mm) at different concentrations			
		A (75%)	B (37.55)	C (18.75%)	D (9.375%)
1	*Staphylococcus aureus*	15	15	9	NI
2	*Staphylococcus albus*	16	16	10	9
3	*Bacillus subtilis*	20	15	8	NI
4	*Escherichia coli*	15	15	14	9
5	*Pseudomonas aeruginosa*	9	9	NI	NI
6	*Klebsiella aerogenes*	14	14	NI	NI
7	*Candida albicans*	12	10	9	8
8	*Aspergillus niger*	21	11	10	8

NI- No inhibitory effect.

The given sample of essential oil was serially diluted with DMSO to get required concentrations.

Table 5.4

Determination of MIC for the given sample LL

Sl. No.	Name of the organism	MIC (%)
1	*Staphylococcus aureus*	18.75
2	*Bacillus subtilis*	18.75
3	*Staphylococcus albus*	9.375
4	*Escherichia coli*	9.375
5	*Pseudomonas aeruginosa*	37.5
6	*Klebsiella aerogenes*	37.5
7	*Candida albicans*	9.375
8	*Aspergillus niger*	9.375

Table 5.5

Anti-microbial screening of the given fractions LLP and LLD

Sl. No	Test Organism	Diameter of zone of inhibition(mm)		
		LLP	LLD	STD
1	*Staphylococcus aureus*	9	16	27
2	*Bacillus subtilis*	11	15	27
3	*Streptococcus faecalis*	26	25	30
4	*Staphylococcus albus*	13	20	34
5	*Escherichia coli*	20	17	30
6	*Pseudomonas aeruginosa*	19	19	35
7	*Protieus vulgaris*	12	17	34
8	*Klebsiella aerogenes*	13	17	30
9	*Candida albicans*	11	12	12
10	*Aspergillus niger*	15	15	25

Std- Ciprofloxacin 5µg/disc for bacteria,

Clotrimazole 5µg/disc for fungi

The sample volume 50 µL

5.2.3 Results and discussion

The result of the anti-microbial screening of the essential oil (LL) is given in table 5.2. All micro-organisms exhibited concentration dependent activity. The oil is very active against gram-positive bacteria such as *Streptococcus albus* and fungi such as *Aspergillus niger*. The essential oil fractions LLP and LLD were less active (table 5.5) against all micro organisms when compared with the original essential oil. This shows a synergic action of molecules in anti-microbial activity. The polar fraction which contains more oxygenated compounds showed slightly higher anti-microbial activity than the nonpolar fraction. This observation is well known. (The order of activity is phenols > aldehydes > alcohols > ketones > ethers > hydrocarbons). The minimum inhibitory concentration (MIC) of the essential oil against different micro-organisms is given in table 5.4. The minimum inhibitory concentration is low for gram positive bacteria such as *Staphylococcus albus* and gram negative bacteria such as *Escherichia coli.* The two fungi C*andida albicans* and *Aspergillus niger* also showed lower MIC. The antimicrobial activity exhibited by the oil is fairly good even though it does not contain phenolics.

The attractive odour of this essential oil along with its promising anti-microbial property makes it a valuable material for a possible therapeutic use.

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