

**PHYTOCHEMICAL INVESTIGATION AND BIOLOGICAL
STUDIES OF SECONDARY METABOLITES FROM SOME
FOLK MEDICINAL PLANTS**

**A thesis submitted to the University of Calicut in partial
fulfillment for the requirement for the degree of**

Doctor of Philosophy

In

Chemistry

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CERTIFICATE

This is to certify that the thesis entitled “Phytochemical Investigation and Biological Studies of Secondary Metabolites from some Folk Medicinal Plants” is an authenticate record of research work carried out by Sri. **Srinivasan G.V.** under my supervision, in partial fulfillment of Degree of *Doctor of Philosophy in Chemistry* of the University of Calicut. It is further certified that this work has not been submitted to any other University/ Institution for the award of Ph.D. degree. His thesis is recommended for acceptance for the award of the Ph.D. degree by the University of Calicut.

Place: Calicut University

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Dr. K. K. Vijayan

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DECLARATION

I hereby declare that the thesis entitled **“phytochemical investigation and biological studies of secondary metabolites from some folk medicinal plants”** submitted to the University of Calicut in partial fulfillment for the requirements for the degree of Doctor of Philosophy in Chemistry is a bonafied record of the work carried out by me in the Dept. of Chemistry, University of Calicut. It is further declare that this work has not been submitted to any other University/ Institution for the award of Ph. D. or any other degrees.

Place: Calicut University

Date:

(Srinivasan. G. V.)

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List of Abbreviations

APCI	Atmospheric Pressure Chemical Ionization
°C	degrees celcius
C18	Octadecyl phase (chromatography column packing)
CC	Column Chromatography
¹³ C-NMR	¹³ Carbon Nuclear Magnetic Resonance
COSY	Correlation Spectroscopy
2D	two Dimensional
d	doublet
δ	chemical shift in parts per million
DEPT	Distortionless enhancement by polarization transfer
DMSO	Dimethyl sulphoxide
eV	electron Volt
EI	Electron Impact ionization
ESIMS	Electrospray Ionization Mass Spectrometry
Fig	Figure
g	gram
GC MS	Gas Chromatography Mass Spectrometry
H	proton
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Heteronuclear Single Quantum Coherence
¹ H NMR	Proton Nuclear Magnetic Resonance
Hz	Hertz
IR	Infra red
J	Coupling constant
LCMS	Liquid Chromatography Mass Spectrometry
LD ₅₀	Lethal Dose 50%
λ _{max}	maximum wavelength
m	multiplet (in NMR)
min	minute(s)
mL	millilitre(s)
m/z	mass-to-charge ratio
μ	micro
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
q	quartet (in NMR)
R _f	Retention factor
RT	Retention Time
s	singlet
t	triplet
TIC	Total ion chromatogram
TLC	Thin Layer Chromatography
UV	Ultraviolet

Introduction

Srinivasan G. V. “phytochemical investigation and biological studies of secondary metabolites from some folk medicinal plants” Thesis. Department of Chemistry, University of Calicut, 2015

INTRODUCTION

1. General Introduction

The chemistry of natural products forms a wide canvas portraying every kind of organic chemistry and going beyond. It acts as a bridge for transferring principles and concepts of chemistry to the biosciences – thus providing molecular basis for biological phenomena. The term natural product is commonly reserved for those organic compounds of natural origin that are unique to one organism or common to a small number of closely related species. In most instances they appear to be non essential to the plant, insect or organism producing them, in marked difference to the other natural organic compounds such as amino acids, carbohydrates, nucleotides, fatty acids and the polymers derived from them.

They function primarily in defense against predators and pathogens, provide reproductive advantage as intra species and inter species attractants and may also act to create competitive advantage as poisons of rival species. For example plant terpenoids are toxins, feeding deterrents to herbivores, attractants to pollinators and many possess pharmacological activity. Phenolic compounds play important roles in plants. Tannins and flavonoids serve as defenses against herbivores and pathogens. Many flavonoids are important attractants for pollinators and seed dispersers. Some phenolic compounds have allelopathic activity and may adversely influence the growth of neighbouring plants. Throughout evolution, plants have developed defenses against herbivory and microbial attack and produced other natural products to foster competitiveness^{1,2}.

Throughout the history plants have been used as the primary source of food, timber, fuel and medicine, among other purposes. Plants are one of the most important sources for the development of new drugs due to their amazing ability to produce a vast structural array of chemical components, of which only relatively few have been studied. Today plants, plant extracts and plant-derived components are being used in a multitude of herbal remedies and phytopharmaceuticals and also as flavouring agents, biopesticides etc.

A WHO survey (in the year 2000) reveals that 47% of some 300 million new prescriptions contained one or more active ingredients of drugs as drugs of natural origin. About 70% of the world population, especially from third world countries relies chiefly on traditional medicines for their primary health care needs and major part of traditional therapy involves the use of plant extracts. There are about 120 distinct chemical substances derived from plants that can be considered as important drugs currently in use. They are used in 62 therapeutic categories. Many well-known drugs have also been isolated from plants including artemisinin from *Artemisia annua*, paclitaxel from *Taxus brevifolia*, vinblastine and vincristine from *Catharanthus roseus* and galanthamine from the Amaryllidaceae family which forms the drugs of choice for the treatment of various cancers^{3,4}.

In the biosynthesis of secondary metabolites there exist four principle building blocks: acetate, mevalonate, shikimate and amino acids. However, most of these intermediates are also used for the production of primary metabolites such as the fatty acids, nucleosides and polypeptides. A theory therefore has been put forward which suggests that the secondary metabolism arose as a way of using the acetate, shikimate and amino acids that were excess to requirements ('shunt metabolites').

It was proposed that changes in genetic make up of an organism (mutations), whether they are natural, chemical mistakes or exposure to chemicals, viruses or

radiation have lead to the increased production of the key intermediates. When these compounds are able to provide useful biological activities that increase the chances of survival for an organism, they are more likely to be passed on through the generations¹.

2. Medicinal plants - Indian Wealth and Heritage

India is a varietal emporium of medicinal plants and is one of the richest countries in the world as regards to genetic resources of medicinal plants. All known types of agro climatic and ecologic conditions are met within India. The biogeographic position of India is unique which makes India rich in all the three levels of biodiversity such as species diversity, genetic diversity and habitat diversity⁵. A survey conducted by the All India Coordinated Research Project on Ethnobiology (AICRPE) during the last decade recorded over 8000 species of wild plants used by the tribals and other traditional communities in India for treating various health problems⁶. The Indian subcontinent, with the history of one of the oldest civilization, harbors many traditional health care systems. One of the ancient classics, “Charaka Samhita” is the oldest text available on the complete treatment of diseases which specifies the use of hundreds of herbs in the complete treatment of diseases⁷. The Ayurveda, whose history goes back to 500 B.C., is one of the ancient health care systems, which is a potential source information of indigenous drugs and formulations based on medicinal plants. A large number of such herbs are mentioned in “Bhavprakasha”⁸ as well as "Aryavaidhya Kalanidhi"⁹. “Indian Materia Medica” also gives description of a large number of medicinal plants for the treatment of various diseases¹⁰.

In the rural areas, 75% of the population is dependent on herbal medicines for healthcare. In the last few decades, herbal medicine has been found to have some

impressive credentials. In India, over 2600 plant species have been considered useful in the traditional system of medicine like Ayurveda, Unani, Siddha and Home remedies¹¹. Number of herbal drugs and their compositions are recommended for combating human ailments in the ancient texts as well as in modern medicine¹².

3. Plants as sources of important Drugs

The knowledge of plants began with use and ends in use. In prehistoric age, man started to use plants growing around in various ways as diet and drugs. When the concepts were developed on later stage, their possible mode of action and properties were defined scientifically. The object of this scientific knowledge was to enable physician to make judicious use in a more effective way. Looking to the prime importance of use (prayoga), Charaka has said that “real expert of medicinal plants is he who is well versed in their use besides their names and forms”. Charaka Samhitha, a classic work of Charaka (1000 B. C.) says “the proper use makes a good remedy even out of poison while a good medicinal plant acts as poison if used improperly”. He also opined that the best among physicians is the one who knows the administration of medicinal plants and their rational use in different treatment protocols¹³.

The significance of traditional medicine in the discovery of biologically active chemical compounds from natural sources is very important. Among some of the earliest successes in developing drugs from natural products, one can mention the isolation of the antimalarial drugs such as the cinchona tree alkaloids, pain relievers such as the morphine alkaloids and the development of aspirin. Quinine (**I**), originally isolated from the bark of cinchona trees, *Cinchona succirubra*, was one of the principal antimalarial drug. Morphine (**II**), the major alkaloid of

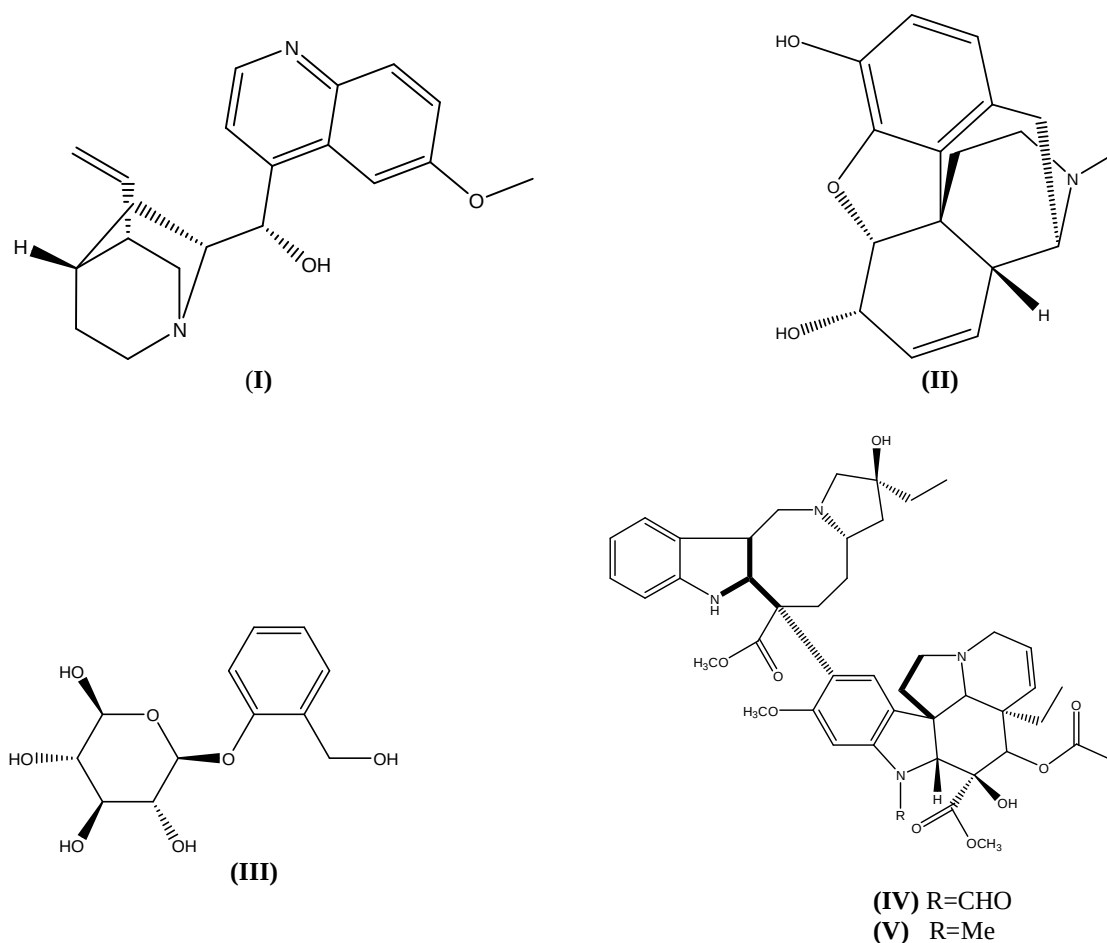
Papaver somniferum was first isolated between 1803/06 and was widely used for pain relief. It is being used in modern medicine as a third line analgesic despite its addict forming characteristic¹⁴. The Egyptian pharmaceutical record, “Ebers papyrus”, indicates the use of willow leaves as an antipyretic agent. Following on this knowledge, chemists began to isolate the compounds responsible for the remedy and salicin (**III**) was isolated from the bark of the white willow, *Salix alba*, in 1825-26. It was subsequently converted to salicylic acid via hydrolysis and oxidation and proved potent as an antipyretic that was manufactured and used worldwide. To overcome the severe gastrointestinal toxicity of salicylic acid, it was converted into acetylsalicylic acid (ASA) via acetylation and started to be marketed under the trade name aspirin in 1899. Aspirin is still the most widely used analgesic and antipyretic drug in the world¹⁵.

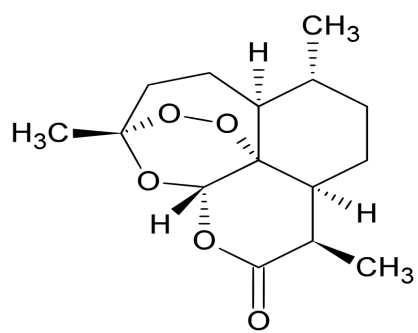
More recently, the vinca alkaloids, vinblastine (**IV**) and vincristine (**V**) were isolated as antineoplastic agents from *Catharanthus roseus* and subsequently derivatized to vinorelbine and vindesine. These drugs are currently used for the cancer treatment¹⁴. Similarly, a potent antimalarial drug, artemisinin (**VI**), a sesquiterpenoid endoperoxide was isolated from *Artemisia annua* as a remedy against the multidrug resistant strains of *Plasmodium*. This plant is long been used as an antimalarial drug in the traditional Chinese medicine (TCM). Using the basic structure of artemisinin, semisynthetic compounds were synthesized with the aim of optimizing the pharmacology of the principal molecule leading to the identification of artemether and dihydroartemisinin as potent antimalarial agents that are now in a widespread use around the world¹⁴.

Rouvolfia serpentina, which has now attained world wide popularity, finds mention in ancient Hindu scriptures as well as in the monumental works of Charaka. The plant was mentioned as useful antidote for snake bites and insect stings. The great importance of this drug, reserpine (**VII**) have been realized also in

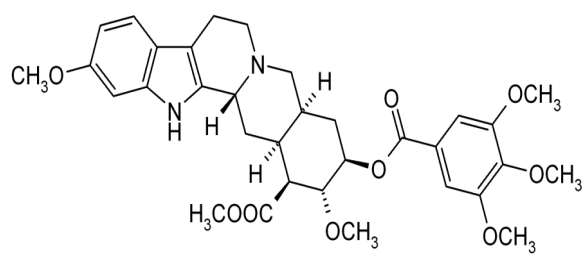
modern therapy. From Ma Huang (*Ephedra spp*) of China, Ephedrine (**VIII**) was isolated in 1887 and later introduced as a drug in 1925. Some of the important plant drugs are atropine (**IX**) from *Atropa belladonna*, codeine (**X**) from *Papaver somniferum*, etc. The structures of these compounds are depicted in Fig 1.

These few accounts underscore not only the potential of natural products as a source of drugs and the solid link between the folk medicine and drug development but also the necessity of natural products research.

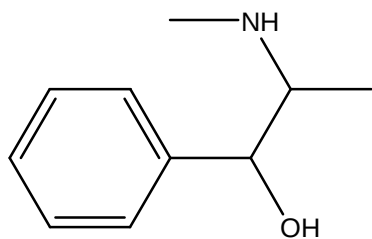




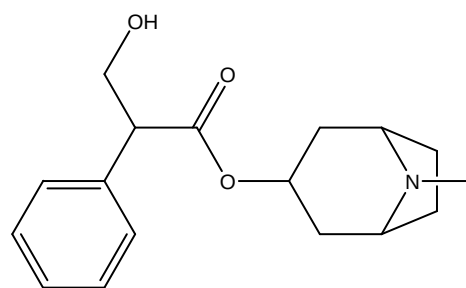
(VI)



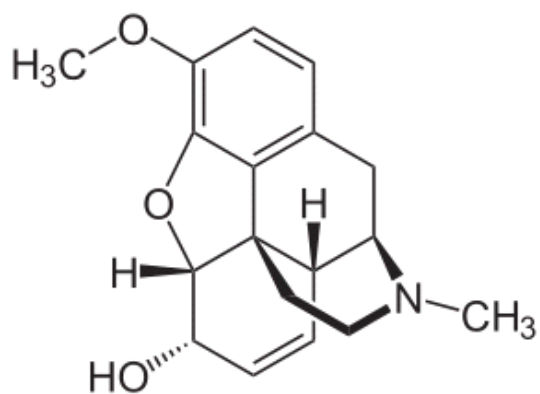
(VII)



(VIII)



(IX)



(X)

Fig 1 Structures of some natural products of plant origin

4. Folk Medicinal Plants

Folk medicine is an indigenous healing practices used to treat injuries and diseases. This comprises knowledge systems that developed over generations in different societies that have been passed down through families before the advent of modern medical system. The folk medical practitioners mainly use herbal preparations such as juices decoctions of medicinal plants for the healing action. Folk medicine may also be referred to as traditional medicine, alternative medicine, indigenous medicine, complementary medicine, or natural medicine^{16,17}.

All cultures and societies have knowledge best described as folk medicine. Folk medicine often coexists with formalized, education based and institutionalized systems of healing such as Allopathic system or Great traditional medicine systems like Ayurveda, Unani and Chinese medicine. But they are distinguishable from formalized or institutionalized healing systems. Some examples of strong informal and to some degree institutionalized folk medicine traditions are: Traditional Korean medicine, Arabic indigenous medicine (source of Unani medicine, along with Ancient Greek medicine), Haitian folk medicine, Various African herbal folk remedies, Celtic traditional medicine (in part practiced by the Irish medical families), Japanese Kampō medicine, Traditional Aboriginal Bush medicine and Georgian folk medicine¹⁶.

In ancient scriptures like the Vedas, Samhitas and the Puranas and in tribal oral tradition, a vast data on medicinal plants of India is available. Here the folklore medicine is taken to mean the knowledge and practice that have survived through only traditions in certain human societies, particularly among the primitive and rural societies. The side effects and limitations of modern medicine forces us to

accept and appreciate the utility of folk medicine in present era^{18,19}. The details of folk uses of selected Indian medicinal plants are given below²⁰.

Acacia catechu (Khadir) - Kols of Uttar Pradesh use its leaves for blood dysentery.

Ailanthus excelsa (Aralu) - Inhabitants of Saurashtra district in Gujarat use its leaves for rheumatism.

Argemone mexicana (Swarnakshiri) - Korku tribes of Akola district in Maharashtra use its roots for piles. Roots are burnt and fermentation is done. Bhils, dhanka, dubada and nayaka tribes of Khedbrahma region in north Gujarat use the leaf juice for syphilis.

Bauhinia variegata (Kanchanar) - Inhabitants of Garhwal Himalaya use its bark for malaria.

Berberis asiatica (Daruharidra) - Inhabitants of Assam use its roots for the treatment of bleeding piles.

Betula utilis (Bhoj patra) - Johari tribes of Uttar Pradesh (UP) use its bark for jaundice and ear ache.

Boerhaavia diffusa (Punarnava) - Inhabitants of Garhwal Himalaya use its roots for piles. Bhils of Jhabua district in Madhya Pradesh use its roots paste to cure blood dysentery.

Butea monosperma (Palash) - Andh, Bhil, Gond, Halba, Korku and Malhar tribes of Kandala region in Gujarat use its flower for urinary complaints.

Calotropis gigantea (Arka) - Bhil, agaria, dhodia, dubla and khaknar tribes of Dahanu forest in Maharashtra use its root extract to kill guinea worms.

Cassia tora (Chakramarda) - Buksa of Bijnor district in UP eat the young leaves for rheumatic pain.

Cissampelos pareira (Patha) - Local people of Assam use its leaves for jaundice. Kol, kutia kondh and soura tribes of Orissa use its root paste for leprosy.

Clitoria ternatea (Aparajita) - Inhabitants of Dhasan valley in Bundelkhand region of UP apply the powdered root externally for the treatment of goiter and leprosy.

Curcuma aromatica (Vana haridra) - Khasi and gangte tribes of Meghalaya use internally its rhizome paste with water to kill intestinal worms.

Cuscuta reflexa (Amar bel) - Tharu of Kheri district of UP use the paste of plant for treatment of swelling of testicles. It is also used to check fall of hair.

Datura metel (Datura) - Inhabitants of Rajasthan and West Bengal use *Dhatura* for leprosy.

Ficus religiosa (Ashwatha) - Rabha tribes of West Bengal use its bark for haematuria. Inhabitants of Jammu & Kashmir give the extract of its bark to relieve whooping cough.

5. Natural Product Analysis over the Last Decades

Since antiquity, man has strived to study and analyse the products of the natural world around him. The study of natural products had a number of rewards. It has led to the discovery of a variety of useful compounds that finds application in diverse applications that benefits the quality of human life. The most important areas of application of natural products are in the treatment of human and veterinary ailments. Currently, around 119 chemical substances derived from 90 plant species are considered important drugs that are in use in one or more countries¹⁴.

The plants were the source of most therapeutics. Up to the twentieth century, the main method used for their quality control was the observation of characteristic anatomical elements of plants under the microscope, while chemical analysis of constituents played a minor role. As the importance of the contribution of the

individual natural products to the pharmacological and biological activities of the organism was understood, the demand for more suitable qualitative and quantitative analytical techniques has been realized. At first, adequate methods did not exist for the analysis of the very complex mixtures like plant extracts. It was not until the 1950's that the breakthrough in the analysis of natural products was provided by the application of chromatographic separation methods and spectroscopic identification and structure elucidation techniques. These have been progressively improved over the last few decades, utilizing very efficient chemical, physical and biological and most importantly physicochemical (Spectroscopic) methods are now available ²¹. The other innovation has been the progressive reduction in sample size necessary for the analysis. The general term "separation techniques" refers to various forms of chromatography. The choice of analytical methods depends on the chemical class of the analytes in question. An example is given by the flavonoids, for which a recent review article has been published²². The different techniques which can be used for their analysis are shown in Fig 2 and these can be applied to many classes of natural products. Extraction and sample preparation are also necessary (Fig 2).

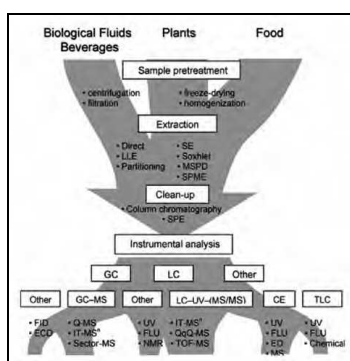


Fig 2 Strategies for the analysis of flavonoids in biological fluids, beverages, plants and food. Abbreviations: LLE, liquid-liquid extraction; SE, solvent extraction; MSPD, matrix solid-phase extraction; SPME, solid-phase micro-extraction; SPE, solid-phase extraction; GC, gas chromatography; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; CE, capillary electrophoresis; TLC, thin layer chromatography; FID, flame ionisation detection; ECD, electron capture detection; Q, quadrupole; QqQ, triple-quadrupole; IT, ion-trap; FLU, fluorescence; NMR, nuclear magnetic resonance; TOF, time-of-flight and ED, electrochemical detection

Bioactivity guided fractionation of crude extracts is the approach adopted in the search for bioactive constituents from plants or from other natural sources²³. By following this procedure alone, there is a risk of unnecessarily isolating known compounds. Furthermore, potentially lead compounds which are not active in the given bioassays may simply be missed. The rediscovery of known compounds poses a significant challenge in natural product research and calls for ingenious dereplication strategies, i.e., ways of avoiding the time-consuming isolation of known constituents. This can be achieved by the application of sophisticated techniques such as LC MS or LC UV and LC NMR at the earliest stage of separation²⁴. These analyses are valuable to detect compounds with interesting structural features and to target their isolation.

Suitable bioanalytical methods for the detection of natural products in biological matrices, their structural characterization and the quantitative analysis of their metabolites are needed. The analytical methods for natural products should be incomplete without a mention of biological or pharmacological tests. These biological screening methods are an essential complement to the chemical screening methods. In order to obtain results fast and with the minimum of outlay in terms of expense and material, simply so-called “bench top bioassays” can be used²⁴. Some of these are listed in Table 1.

Table 1 Some simple bench top bioassays

Antifungal: *Cladosporium cucumerinum*, *Candida albicans*

Antibacterial: *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*

Molluscicide: *Biomphalaria glabrata*, *Bulinus globosus*

Piscicide: *Oryza latipes*, *Tilapia sp.*

Larvicide: *Aedes aegypti*, *Anopheles sp.*, *Culex sp.*

Spermicide: human spermatozooids

Insecticide: *Diabrotica* sp., *Aphis* sp.

Antifeedant: *Spodoptera* sp., *Epilachna varivestis*

Antioxidant and free-radical scavengers

Germination inhibitors

6. Aim and Objective of the present work

The objective of the present work is the isolation, identification, chemical and biological studies of the chemical compounds and extraction of the essential oils and their antimicrobial activities from the following folk medicinal plants.

- i. *Biophytum reinwardtii* (Zucc.) Klotzsch.
- ii. *Salacia fruticosa* Heyne ex Laws.
- iii. *Ixora coccinea* L.
- iv. *Costus pictus* D. Don
- v. *Leea indica* (Burm. f.) Merr.

These five plants have been chosen for the present work as they are extensively used in folk medicine for the treatment of array of diseases and only limited phytochemical works have been reported in these plants. For example the leaves and roots of *B. reinwardtii* are used for the treatment of fever, for chest complaints, convulsions, cramps and inflammatory tumours. The leaves are diuretic and possess astringent and antiseptic properties. In Bihar, the leaves and roots are given for insomnia, fever, gonorrhoea and lithiasis. In Philippines, the powdered seeds are used as a vulnerary; along with butter applied to abscesses to promote suppuration. The root, bark and stem of *S. fruticosa* is used in Ayurveda and folk medicinal practices for the treatment of diabetes type II. *S. fruticosa* is endemic to this region of Western Ghat and no phytochemical investigation has been carried out in this plant.

The roots of *I. coccinea* are reported to possess astringent and antiseptic properties and are used in headache and applied to sores and chronic ulcers in folk medicine. The root is made into a paste with coconut milk and applied to ulcers, wounds and skin eruptions, especially in children. The Korku tribes of Maharashtra use the roots of this plant along with long pepper and little water to treat acute dysentery. The people in Kerala used to consume the fresh raw leaves of *C. pictus* for its antidiabetic properties. In Malaysia, the whole plant of *L. indica* is used traditionally for headache, body pain and skin complaints.

7. Abstract of the present work

The research work carried out has been presented in 5 chapters in the thesis.

- i. The Chapter-1 is divided into 2 sections. Section-1.1 describes the isolation and structural elucidation of a new flavonoid diglycoside namely apigenin-4',5-O- β -D-diglucoside (BR-1) from the whole plant of *Biophytum reinwardtii*. The structure of this compound was identified by chemical and spectroscopic techniques viz., UV, IR, ^1H & ^{13}C NMR, 2D NMR & MS techniques. The antioxidant activity of the compound BR-1 viz., DPPH free radical scavenging activity and modified ferric reducing/antioxidant power (FRAP) assay were also investigated.

The section-1.2 describes the anticancer activities of BR-1 against HeLa, human cervical cancer cell lines and HT 29, human colon cancer cell lines by MTT assay. BR-1 showed significant activity against HeLa cell lines and moderate activity against HT 29 cell lines.

- ii. In Chapter-2, the isolation and structural elucidation of mangiferin (2- β -D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthen-9-one) from the roots of *Salacia fruticosa* Heyne ex Laws is described. It was characterized on the basis of chemical and spectroscopic techniques (UV, IR, ^1H & ^{13}C NMR, ESIMS and APCIMS). Mangiferin has been reported for the first time from this species. In order to determine the mangiferin content in the aqueous methanol extract of the roots of *S. fruticosa*, quantitative analytical methods by means of HPLC and HPTLC were developed. The antioxidant potential of 80% aqueous methanol extract of *S. fruticosa* was evaluated viz., free radical scavenging activity by 2,2'-diphenyl-1-picrylhydrazyl (DPPH) reduction.
- iii. In Chapter-3, the identification of chemical compounds in the essential oil extracted from the roots of *Ixora coccinea* L. and the chemical compounds present in its cold hexane extract by GC-MS technique is described. Twenty seven compounds were identified in the essential oil that constitutes 98.50% of the total essential oil. The main sesquiterpenes identified were β -sesquiphellandrene (17.83%), *ar*-curcumene (2.77%), *E*- α -bergamotene (2.07%), α -zingiberene (1.92%), caryophyllene oxide (1.88%) and δ -nerolidol (1.44%). The antimicrobial activity of the essential oil was evaluated against three Gram-positive and two Gram-negative bacteria and five pathogenic fungi by disc diffusion method. The results showed that the oil exhibited good antibacterial and moderate antifungal activities.
- iv. The Chapter-4 is divided into 2 sections. Section-4.1 describes the extraction of essential oil/hydrodistillate from the different plant parts of *Costus pictus* viz., leaves, stem, flowers, root & rhizome; identification of the chemical compounds present in them by GC-MS analysis and the

screening of antimicrobial activity of the essential oils/hydrodistillates by disc diffusion method. Section-4.2 describes the isolation, identification and quantification of diosgenin from the root of *C. pictus*.

- v. Chapter-5 is divided into 3 sections viz., section-5.1 describes the phytochemical works on *Leea indica* leaves & root, section-5.2 describes the phytochemical works on *L. indica* flowers and section-5.3 describes the phytochemical works on *L. indica* fruits.

Section-5.1 describes the isolation and identification of gallic acid, ursolic acid, lupeol, β -sitosterol and ethyl palmitate from the root of *L. indica* (Burm. f.) Merr. Gallic acid, lupeol, β -sitosterol and ursolic acid were also identified from the leaves of *L. indica*. This seems to be the first report of the presence of gallic acid & other compounds in the roots and leaves of *L. indica*.

The antimicrobial activity of the petroleum ether and methanol extracts of leaves and root of *L. indica* was evaluated against three Gram positive and two Gram-negative bacteria and five pathogenic fungi by disc diffusion method. The results showed that the plant exhibits good antibacterial and moderate antifungal activities. The phenolic content in the leaves and root of *L. indica* were also estimated and compared by adopting standard procedures.

Section-5.2 describes the identification of the chemical compounds present in the essential oil extracted from the flowers of *L. indica* (collected from 2 different accessions) by GC-MS technique. More than 95% of the oil consisted of the esters of phthalic acid. Dibutylphthalates were identified as the major components in the essential oil. Monobutyl carbonotrithioate,

a sulphur compound was also identified in trace amount (0.01%). The essential oil showed moderate antibacterial activity against three Gram positive and two Gram negative bacteria and five pathogenic fungi.

Section-5.3 describes the identification of gallic acid, β -sitosterol and ursolic acid from the fruits of *L. indica* by directly doing co-TLC of the fruit extract with these standards. The preliminary phytochemical screening of the 80% aq. MeOH extract of the fruit revealed the presence of the following compounds: triterpenes, sterols, flavonoids, phenolic compounds, saponins, glycosides etc. Since the fruit is edible, it would be desirable to study its phytochemical profile. The characterization of still unexplored constituents in the fruits of *L. indica* remains open to future investigation.

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Chapter 1

Srinivasan G. V. “phytochemical investigation and biological studies of secondary metabolites from some folk medicinal plants” Thesis. Department of Chemistry, University of Calicut, 2015



Biophytum reinwardtii (zucc.) klotzsch.

CHAPTER – 1

ISOLATION & IDENTIFICATION OF A NEW APIGENIN

DIGLYCOSIDE FROM BIOPHYTUM REINWARDTII

(ZUCC.) KLOTZSCH., ITS ANTIOXIDANT AND

ANTICANCER STUDIES

INTRODUCTION

Biophytum DC. is a large genus of herbaceous plants belonging to the family Oxalidaceae with seventy species distributed in pantropical regions including twelve species from India. *Biophytum reinwardtii* (Zucc.) Klotzsch. is a common plant seen during the rainy season throughout the warm parts of India. It is distributed from Kumaun to Arunachal Pradesh, upto an altitude of 1800 m and extending into peninsular India¹. It is also distributed in Malaysia and China. It is a member of *Dasapushpa* group of plants. Its vernacular names are Lajjaluka/Jalapushpam in Sanskrit; Mukkutti in Malayalam and Tamil; Lajalu in Hindi and Jhalai in Bengali.

The leaves and roots of *B. reinwardtii* are used for fever. The plant is reported to possess tonic and stimulant properties, used for chest complaints, convulsions,

cramps, inflammatory tumours and its ash for stomachache. The whole plant is dried, powdered and given internally to cattle to stop excess salivation. The leaves are diuretic and possess astringent and antiseptic properties. The pounded and bruised leaves or their juice is used in dressing burns and convulsions. Leaf paste is applied to wounds and cuts and leaf decoction given for asthma and phthisis. The mature leaves contain insulin like principle and are recommended for diabetes. Saline extract of the leaves showed hypoglycaemic activity in rabbits. The whole plant is used as a sedative, for removing dandruff and as hair tonic. In Bihar, the leaves and roots are given for insomnia, fever, gonorrhoea and lithiasis. In Philippines, the powdered seeds are used as a vulnerary; along with butter applied to abscesses to promote suppuration^{2,3}.

B. reinwardtii and *B. sensitivum* grow sympatrically and thus are mixed in drug collection and both are used in the same way³. They are used in a number of Ayurvedic formulations like *Chemparuthyadi tailam*⁴.

The aqueous extract of the fresh leaves of the plant is reported to have antimicrobial activity against *Mycobacterium tuberculosis*. The aqueous and alcoholic extracts of the plant showed antifungal activity against *Aspergillus niger* and antibacterial activity against *Staphylococcus aureus*. Both the extracts were inactive against *E. coli*⁵.

(I) Morphological description of *B. reinwardtii*

It is a variable hairy annual, 5-15 cm in height with pinnate leaves 3-12 cm long, leaflets 3-12 pairs, oblong, yellow dimorphic flowers and globose capsules approximately 0.35 cm long^{2,6}.

(II) Abstract of the present work

The Chapter-1 is divided into 2 sections. Section-1.1 describes the isolation and structural elucidation of a new flavonoid diglycoside namely apigenin-4',5-O- β -D-diglucoside (BR-1) from the whole plant of *B. reinwardtii*. The antioxidant activity of the compound BR-1 viz., DPPH free radical scavenging activity and modified ferric reducing/antioxidant power (FRAP) assay were also investigated. The section-1.2 describes the anticancer activities of BR-1 against HeLa, human cervical cancer cell lines and HT 29, human colon cancer cell lines by MTT assay.

REVIEW OF LITERATURE

Only very limited phytochemical studies have been reported so far in *B. reinwardtii* (Zucc.) Klotzsch. Ethyl palmitate, lupeol, β -sitosterol and linoleic acid has been isolated from the whole plant of *B. reinwardtii*⁷. Chlorophyll, sugar, proteins and the non-enzymic antioxidants such as flavonoids, tannins and phenols present in the plant were also reported. The total chlorophyll content in the *B. reinwardtii* was reported to be 1.16 mg/g of plant tissue. The protein and sugar content in the plant was reported to be 7.34% and 18% respectively. The amount of non-enzymic antioxidants such as phenols, flavonoids and tannins were reported to be 9.24%, 4.64% and 3.77% respectively⁷. The methanol and 80% aqueous methanol extracts of *B. reinwardtii* exhibited poor DPPH free radical scavenging activity⁷.

A few phytochemical and pharmacological works were published from its closely related species *B. sensitivum* (L.) DC. and are given as follows. Amentoflavone (I3', II8-biapigenin) was reported from the roots of *B. sensitivum*. It has been

proved to be a selective inhibitor of cyclooxygenase COX-1 catalyzed prostaglandin biosynthesis when tested *in vitro* with an IC_{50} value of 12.4 μ M. Doses of up to 37 μ M showed only a slight inhibition in the corresponding COX-2 assay. The quantification of amentoflavone was carried out by reversed phase HPLC in methanol and aqueous extracts of the roots, stems and leaves. The highest amount of amentoflavone was detected in methanol extract of roots and stems (0.26-0.35%), while considerably lower amount was detected in the corresponding water extract⁸. Two biflavones, cupressuflavone and amentoflavone, three flavonoids, luteolin 7-methyl ether, isoorientin and 3'-methoxyluteolin 7-O-glucoside, as well as two acids, 4-caffeoylquinic acid and 5-caffeoylquinic acid were reported from the aerial parts of *B. sensitivum*⁹.

Essential oil was extracted from the whole plant of *B. sensitivum* by steam distillation and identified the chemical constituents by GC-MS analysis. Few terpenoid glycosides and phenolic compounds were also reported¹⁰.

Amentoflavone isolated from *B. sensitivum* is reported to inhibit the process of angiogenesis in mice by *in vivo* studies¹¹.

Immunostimulatory and antitumour activity¹², anti-inflammatory activity¹³, Immunomodulatory activity¹⁴, apoptotic effect¹⁵ of *B. sensitivum* has been reported.

Alcoholic extract of *B. sensitivum* was studied for its immunomodulatory and antitumour activities. The extract was 100% toxic at a concentration of 0.5 mg/mL to Dalton's lymphoma ascites (DLA) and Ehrlich ascites carcinoma (EAC) cells. *B. sensitivum* extract was also found to be cytotoxic towards L929 cells in culture at a concentration of 0.1 mg/mL. Administration of *B. sensitivum* extract (500 μ g/dose/animal) could inhibit the solid tumour development in mice induced with

DLA cells and increase the lifespan of mice bearing Ehrlich ascites carcinoma tumours by 93.3%. *B. sensitivum* treatment significantly ($p < 0.001$) reduced the tumour cell glutathione (GSH) levels as well as serum gamma glutamyl transpeptidase (GGT) and nitric oxide (NO) levels in ascites tumour bearing animals. The total WBC count was also increased to 14,087 cells/mm on the 12th day in BALB/c mice. The number of plaque forming cells also enhanced significantly ($p < 0.001$) and bone marrow cellularity and beta-esterase positive cells were also increased by the administration of *B. sensitivum* extract¹⁶.

An ethanolic extract of *B. sensitivum* was evaluated for its antioxidant potential *in vitro* and *in vivo*. *B. sensitivum* was found to scavenge superoxide radicals generated by the photoreduction of riboflavin and hydroxyl radicals generated by the Fenton reaction and inhibited *in vitro* lipid peroxidation at concentrations of 50, 95 and 20 $\mu\text{g/mL}$ (IC_{50}) respectively. The drug also scavenged nitric oxide ($\text{IC}_{50} = 100 \mu\text{g/mL}$). The extract also induced the dose-dependant scavenging of nitric oxide in culture. Intraperitoneal administration of *Biophytum* extract inhibited superoxide generation in macrophages *in vivo*. The administration of *B. sensitivum* to mice significantly increased the catalase activity. The extract produced a significant increase in glutathione levels in blood and liver. The levels of glutathione-S transferase and glutathione reductase increased and those of glutathione peroxidase decreased after administering the *Biophytum* extract. The results of this study indicate that *B. sensitivum* has significant antioxidant activity both *in vitro* and *in vivo*¹⁷.

The leaf extract of *B. sensitivum* has been reported to have hypoglycaemic effect on alloxan diabetic rabbits^{18,19} and have good antibacterial activity²⁰.

The antiinflammatory activity of aqueous and methanol extracts of aerial parts, an aqueous extract of roots as well as ultrafiltration fractions of a methanol extract of

roots of *B. sensitivum* were evaluated in the carrageenin induced rat paw oedema model. All the extracts except the methanol extract of aerial parts exhibited antiinflammatory activity. The inhibition of oedema was found to be maximum with aqueous extracts²¹.

A pectic substance, a bioactive polysaccharide, has been isolated from the aq. extract of the aerial part of *B. petersianum* Klotzsch. This pectic substance has reported to contain both rhamnogalacturonan and xylogalacturonan regions²².

SECTION - 1.1

ISOLATION & IDENTIFICATION OF A NEW APIGENIN DIGLYCOSIDE FROM BIOPHYTUM REINWARDTII (ZUCC.) KLOTZSCH. AND ITS ANTIOXIDANT STUDIES

MATERIALS AND METHODS

(I) Plant material

The whole plant of *Biophytum reinwardtii* was collected from Kozhikode, Kerala, India during September 2009 and was authenticated by Dr. P. S. Udayan, Professor, Dept of Botany, Sri Krishna College, Guruvayoor. A voucher specimen was deposited at the herbarium of the University of Calicut for future references. The collected material was then washed with tap water, dried in the shade and coarsely powdered in the mixer grinder.



Fig 1.1.1 *Biophytum reinwardtii* whole plant

(II) Reagents and instrumentation

Solvents used were of analytical/ HPLC grade (Merck). DPPH (2,2'-diphenyl-1-picrylhydrazyl) was purchased from Sigma Aldrich. BHA (3-t-butyl-4-hydroxyanisole), ascorbic acid, gallic acid and TPTZ (2,4,6-tripyridyl-s-triazine) were purchased from Merck. All other reagents were of analytical grade. Silica gel (Merck, 60-120 mesh) was used for column chromatography. Precoated silica gel 60 F₂₅₄ plates (Merck) of uniform thickness 0.2 mm were used for TLC analysis. The melting point was determined using a GUNF melting point apparatus and are uncorrected. Optical rotation was measured on a Perkin Elmer 241 polarimeter. HPLC system (Shimadzu, LC-20AD) equipped with diode array detector (SPD-M 20A) and SIL-20A HT autosampler was used for the HPLC analysis.

UV and IR spectra were measured on a Shimadzu UV-1700 and on a Shimadzu FTIR-8400S spectrophotometer respectively. ¹H and ¹³C NMR spectra were obtained on a Supercon, Bruker NMR spectrometer operating at 300 MHz for δ_H and 75 MHz for δ_C in DMSO-d₆ using TMS as internal standard. 2D NMR experiments were carried out on a Bruker NMR spectrometer operating at 500 MHz in DMSO-d₆. ESIMS spectrum was recorded in Waters TQD LC MSMS system using the following parameters: Capillary voltage: 3 kV, cone: 30 V, extractor: 3 V, source temp: 150 °C, dissolution temp: 350 °C, dissolution gas (N₂) flow: 650 L h⁻¹, collision gas (Ar) flow: 0.1 mL min⁻¹.

The elemental analysis has performed in Thermo Finnigan FLASH EA 1112 series CHNS analyzer. GC-MS analysis was carried out in Perkin Elmer Clarus 680 GC and Clarus 600 T MS system. EIMS spectrum recorded at 70 eV and NIST library was used for the library search.

RESULTS & DISCUSSION

(I) Isolation and Structural elucidation of BR-1

A 80% aq. methanol extract of *B. reinwardtii* was fractionated with petroleum ether, ethyl acetate and n-butanol in that order. The butanol extract was subjected to column chromatography over silica gel followed by preparative TLC to yield a pure compound BR-1 (Fig 1.1.2). This was checked for homogeneity by HPLC which afforded RT of 7.12 min (Fig 1.1.3).



Fig 1.1.2 Photo of the isolated compound BR-1

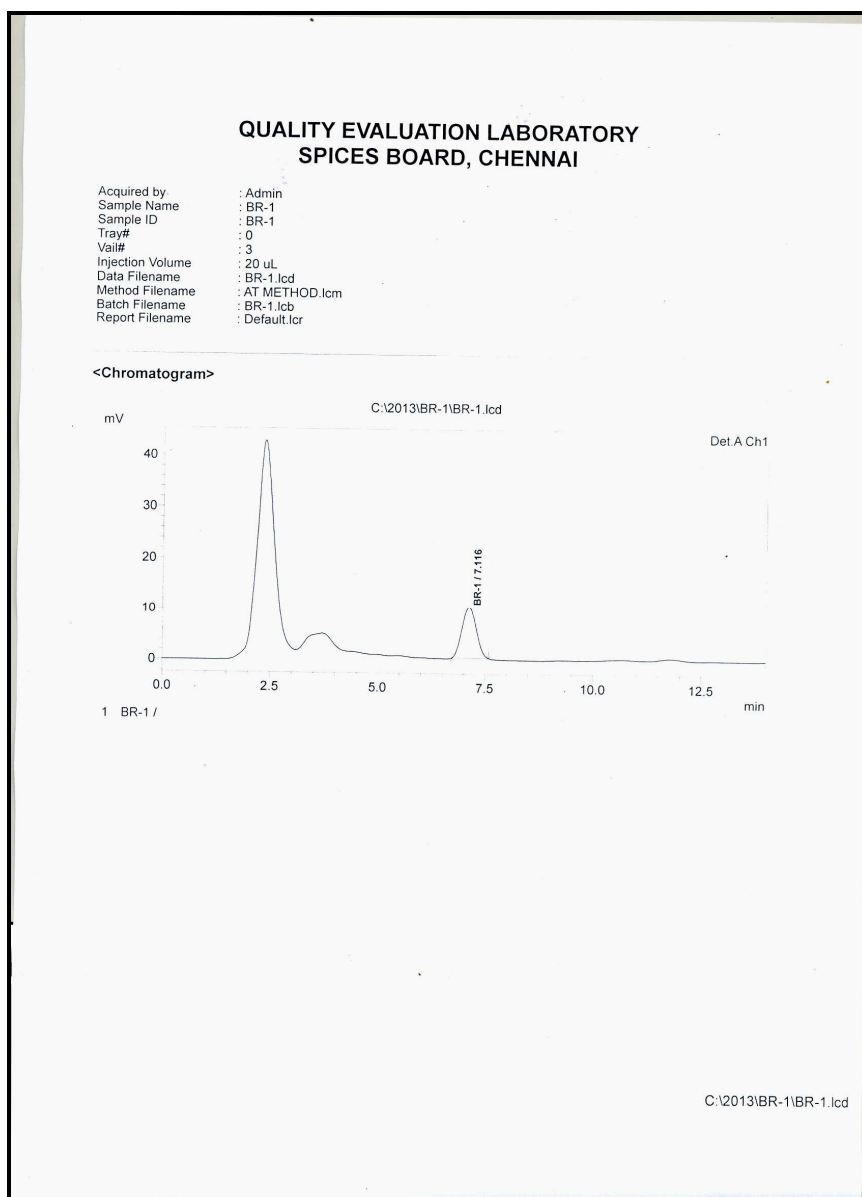


Fig 1.1.3 HPLC Chromatogram of BR-1

The compound BR-1 was assigned the molecular formula $C_{27}H_{30}O_{15}$ by analysis of ESIMS (+ve & -ve modes). BR-1 showed a quasi molecular ion peak at m/z 595.3274 $[M+H]^+$ and 593.2058 $[M-H]^-$ in the ESIMS. CHN analysis of BR-1 also agrees with the assigned molecular formula: (Found: C, 54.3%; H, 5.15%. $C_{27}H_{30}O_{15}$ requires: C, 54.55%; H, 5.05%). UV-Visible spectrum of BR-1 exhibited maximum absorption at 271 and 332 nm with a characteristic flavonoid pattern

indicates the presence of a flavonoid nucleus in the molecule. The IR spectrum presents characteristic absorption bands at 3405 cm^{-1} for OH group and 1654 cm^{-1} for α,β -unsaturated carbonyl group and absorption at 1618 cm^{-1} for C=C group in the molecule.

(I. i) NMR spectral studies of BR-1

The ^1H NMR spectrum (Table 1.1.1) taken in DMSO-d_6 , exhibited a characteristic signal at δ 13.52 corresponding to free phenolic group. The aromatic protons exhibited at δ 7.96 (2H, d, $J = 9.2\text{ Hz}$) for H-2' and H-6' protons and at δ 6.94 (2H, d, $J = 8.6\text{ Hz}$) for H-3' and H-5' protons. The other AX coupling system at δ 6.73 (1H, d, $J = 2.0\text{ Hz}$) and δ 6.84 (1H, d, $J = 2.0\text{ Hz}$) was assigned to H-6 and H-8 protons respectively. These ^1H NMR δ values mostly corresponds with flavonoid proton shifts. The spectrum also supported the presence of two glucose moieties with the anomeric proton signals at δ 5.15 ($J = 7\text{ Hz}$) and at δ 5.03 ($J = 7\text{ Hz}$). The other proton signals of glucose moieties are also present at δ 3.18 (H-2''), 3.50 (H-3''), 3.38 (H-4''), 3.22 (H-5''), 3.60 & 3.80 (H-6''), 3.25 (H-2'''), 3.38 (H-3'''), 4.60 (H-4'''), 3.90 (H-5'''), 3.40 & 3.80 (H-6'''). This confirms the presence of two glucose moieties in the molecule. The ^1H NMR δ values mostly corresponds with flavonoid aglycon proton shifts (see Appendix-III). These data indicates that the molecule is a hydroxy flavonoid diglycoside. For further confirmations the ^{13}C and 2D NMR studies are explained below.

The ^{13}C NMR (Table 1.1.1) spectrum gave 27 carbon signals which indicated the presence of 15 carbon signals due to the flavonoid skeleton. The DEPT spectrum indicates the presence of two CH_2 carbons, eight quaternary carbons and 17 CH carbon atoms in the compound (see Appendix-III). On comparison, ^1H and ^{13}C NMR spectral data of the aglycone moiety matches well with those reported for apigenin in the literature²³.

^1H - ^1H COSY spectra showed cross peaks between H-2' (δ 7.96) and H-3' (δ 6.94); H-6 (δ 6.73) and H-8 (δ 6.84) indicating that they are adjacent to each other (Scheme 1.1.3). HMBC spectra showed the long range correlations between signals at δ 161.45 (C-4') and the protons at δ 5.03 (H-1''), δ 6.94 (H-5' & 3') and δ 7.96 (H-2' & 6') confirming that the glycosylation was at C-4'. Further correlations were showed between the signal at δ 164.25 (C-5) and the anomeric proton at δ 5.15 (H-1''') and the proton at δ 6.73 (H-6) (Scheme 1.1.1 & Scheme 1.1.2). If the glycosylation is at C-7 OH, the correlation would have been obtained between C-7 (156.52) with C-8 (6.84, H-8) and C-6 (6.73, H-6). This observation confirms that the second glycosylation is at C-5. The other correlations in HMBC spectrum are given in Table 1.1.1.

Table 1.1.1 ^1H and ^{13}C -NMR spectral data of BR-1 in DMSO- d_6

Carbon position	$\delta^{13}\text{C}$	$\delta^1\text{H}$	HMBC
1			
2	162.53		H-3, 2', 6'
3	103.26	6.7 (1H, s)	
4	182.17		H-3
5	164.25		H-6, 1'''
6	104.24	6.73 (1H, d, J = 2 Hz)	
7	156.52		H-6, 8
8	94.00	6.84 (1H, d, J = 2 Hz)	
9	159.41		H-8
10	106.00		H-3, 6, 8

7-OH		13.52 (1H, s)	
1'	121.90		H-3, 2', 3', 5', 6'
2'	128.68	7.96 (1H, d, J = 9.2 Hz)	
3'	115.08	6.94 (1H, d, J = 8.6 Hz)	
4'	161.45		H-2', 3', 5', 6', 1''
5'	115.02	6.94 (1H, d, J = 8.6 Hz)	
6'	128.68	7.96 (1H, d, J = 9.2 Hz)	
1''	101.26	5.03 (1H, d, J = 7 Hz)	
2''	80.99	3.18 (1H, m)	
3''	77.29	3.50 (1H, m)	
4''	73.85	3.38 (1H, m)	
5''	70.91	3.22 (1H, m)	
6''	60.87	3.60 (1H, dd, J = 10.2, 2 Hz)	
		3.80 (1H, dd, J = 11.8, 4.2 Hz)	
1'''	101.26	5.15 (1H, d, J = 7 Hz)	
2'''	78.97	3.25 (1H, m)	
3'''	75.81	3.38 (1H, m)	
4'''	72.71	4.60 (1H, m)	
5'''	69.55	3.90 (1H, m)	
6'''	60.37	3.40 (1H, dd, J = 10.2, 2 Hz)	
		3.80 (1H, dd, J = 11.8, 4.2 Hz)	

On acid hydrolysis, BR-1 yielded only D-glucose which was identified by comparison with an authentic sample by TLC and optical rotation $[\alpha]_D^{22}$: 51° (H_2O , c 0.5). Its absolute configuration was determined by GC-MS analysis of their TMSi derivative. D-glucose was identified by GC-MS of the pertrimethylsilylated methylglucoside. Furthermore HMBC spectrum disclosed long range correlations between the anomeric proton H-1'' (δ 5.03, d, J = 7 Hz) and C-4' (δ 161.45) and also between H-1''' (δ 5.15, d, J = 7 Hz) and C-5 (δ 164.25) which confirmed the situation of β -glucopyranosyl moiety at C-4' and C-5 of apigenin. With the interpretation of 1H NMR, ^{13}C NMR & other 2D NMR spectra (Table 1.1.1), all protons and carbons were assigned.

The above information suggests that BR-1 is 5-((2S,3S,4S,5S)-tetrahydro-3,4,5-trihydroxy-6-(hydroxymethyl)-2H-pyran-2-yloxy) -2-(4-((2S,3S,4S,5S)-tetrahydro-3,4,5-trihydroxy-6-(hydroxymethyl)-2H-pyran-2-yloxy)phenyl)-7-hydroxy-4H-chromen-4-one named apigenin-4',5-O- β -D-diglucoside. This diglycoside is not previously reported from plant sources. The structure of apigenin-4',5-O- β -D-diglucoside is shown in Fig 1.1.4. All the spectra of BR-1 are given in Appendix – III.

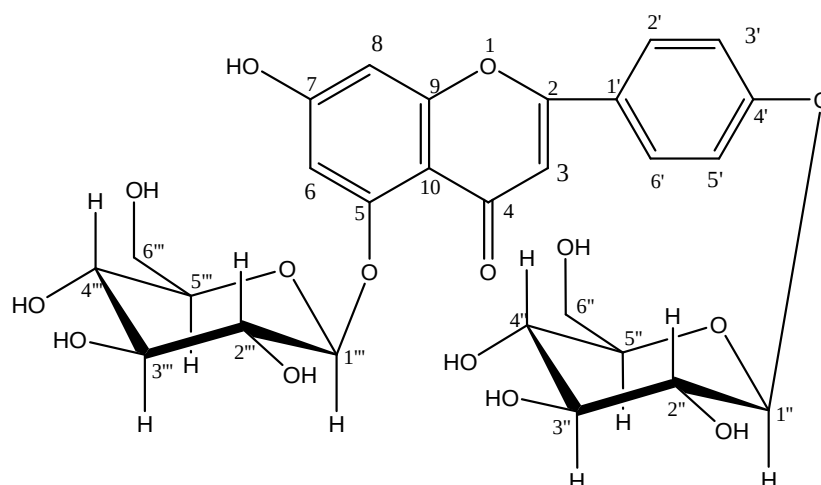
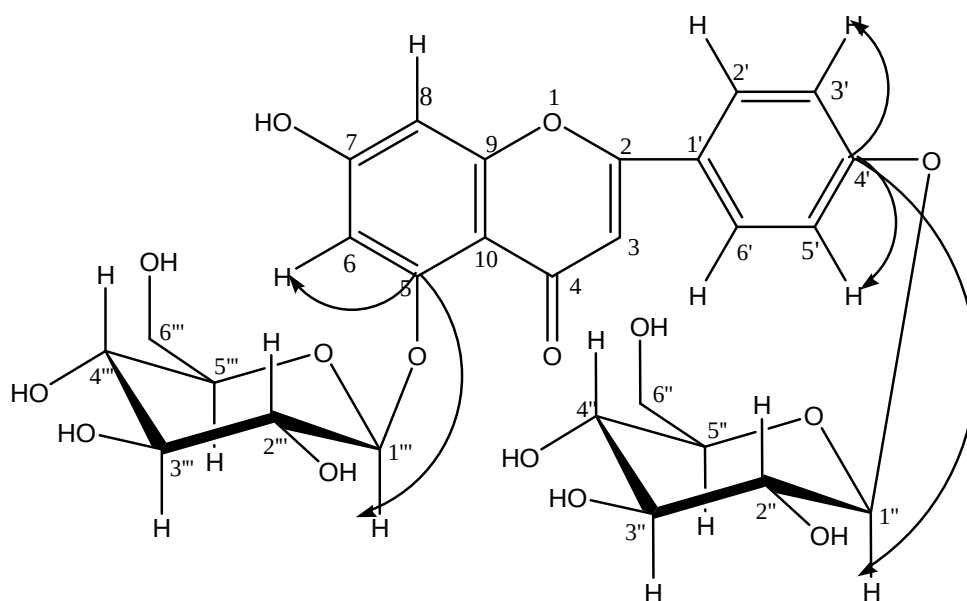
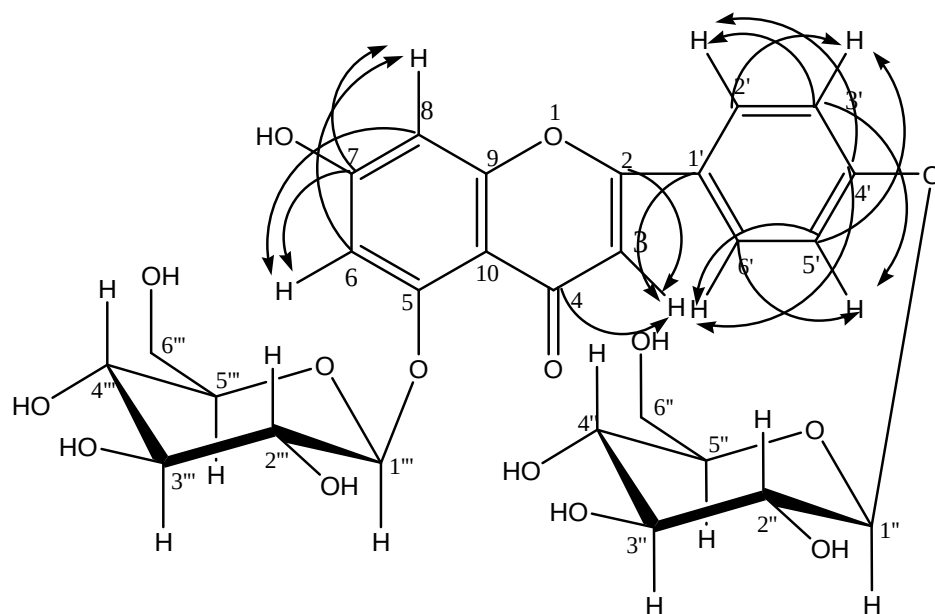


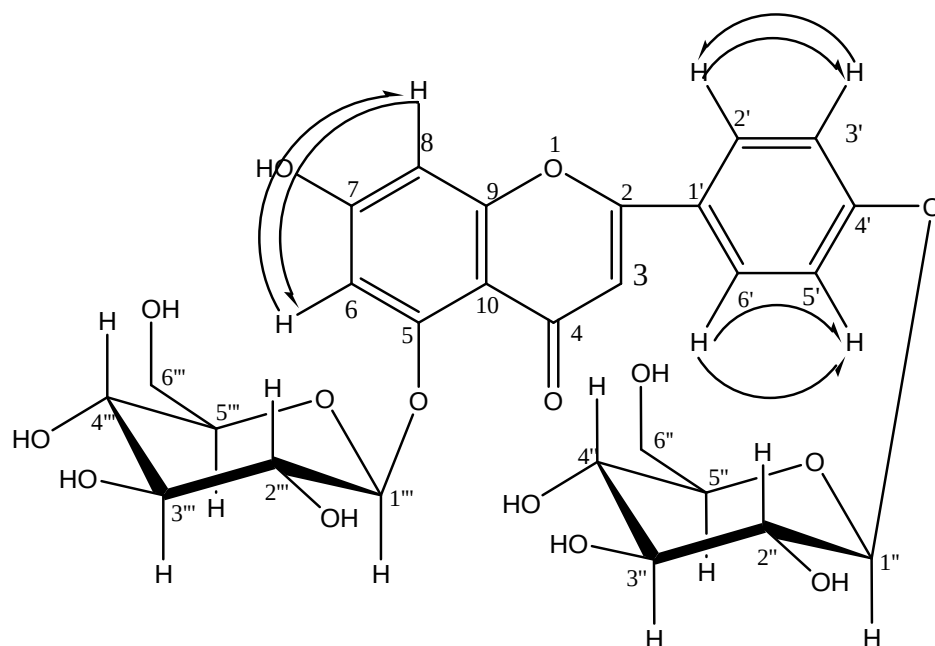
Fig 1.1.4 Structure of apigenin-4',5-O- β -D-diglucoside ($C_{27}H_{30}O_{15}$)



**Scheme 1.1.1 HMBC Correlation showing Connectivity of aglycone C
with anomeric H of glucose moieties**



**Scheme 1.1.2 HMBC Correlation showing Connectivity of aglycone C
with H**



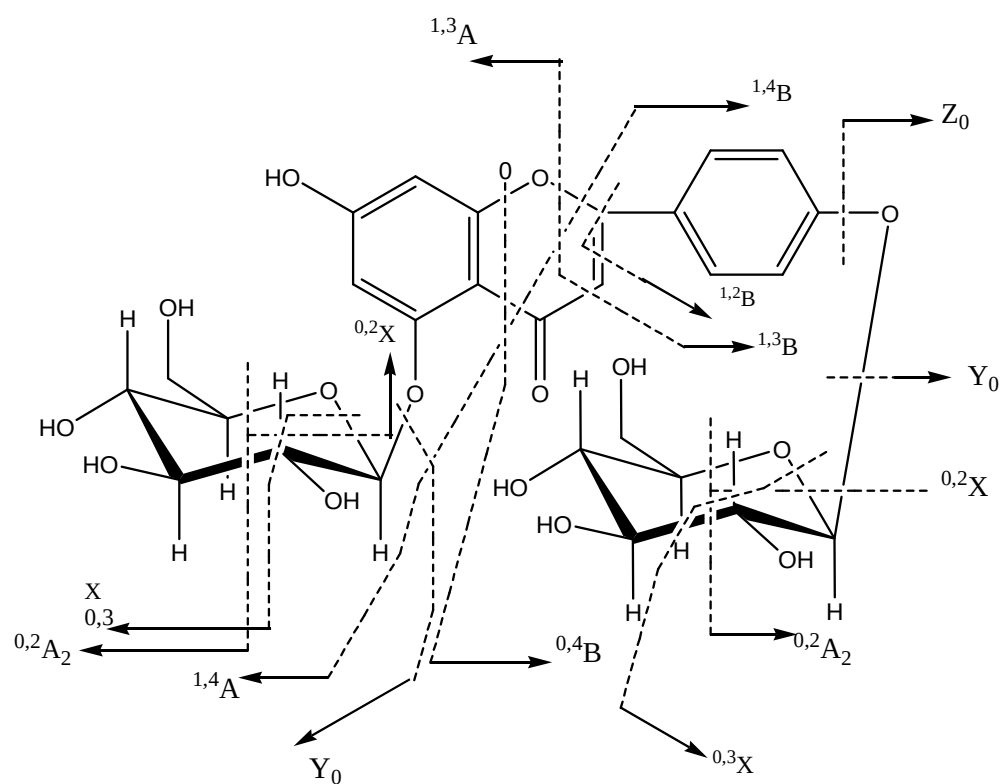
Scheme 1.1.3 H-H Correlation between H of aglycone

(I.ii) Mass spectral studies of BR-1

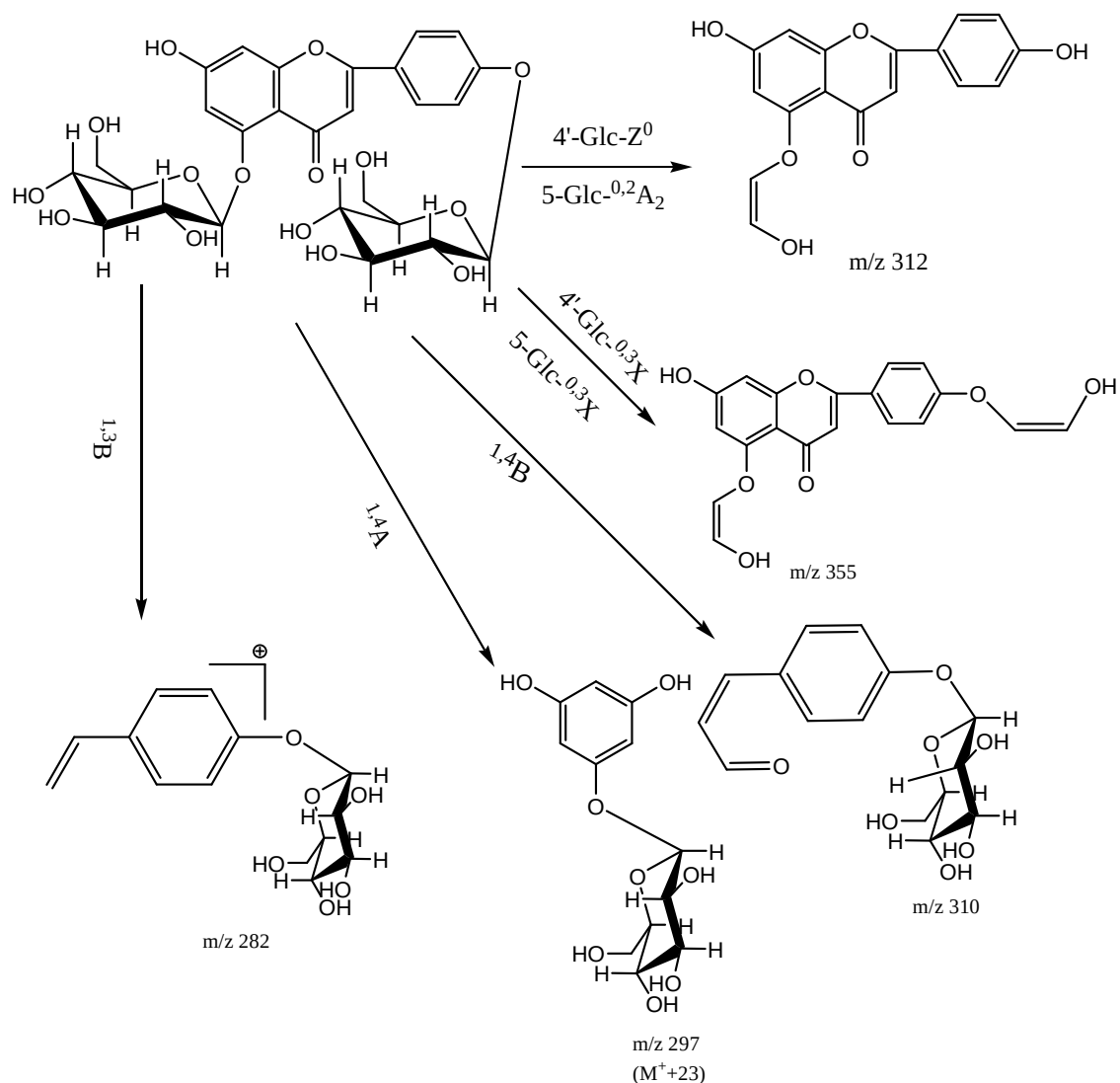
The mass spectral characterization of BR-1 was carried out by +ve and –ve ion ESI-MSMS spectral analysis. The molecular ion is observed at m/z value of 595 $(M+H)^+$ in +ve mode and m/z value of 593 $(M-H)^-$ in –ve mode indicating that the molecular wt is 594. A peak at m/z 577 $[M+H-H_2O]^+$ indicates loss of water molecule from the molecular ion, a pattern usually exhibited by flavonoids. The pattern of degradation is represented in the scheme 1.1.4. This is based on the ESI-MS (positive & negative ion) degradation pattern of flavonoids and flavonoid glycosides published earlier^{33,34,35}.

The prominent daughter ions formed in the MSMS spectra of the compound BR-1

gave major ions with the m/z values 355, 312, 310, 297 and 282 apart from the molecular ion. The possible mechanism of formation of the fragment ions and the structures of the ions are depicted in the scheme 1.1.5 below. These are characteristic of flavonoid diglucoside and thus the compound BR-1 can be assigned the structure **apigenin-4', 5-O- β -D-diglucoside**.



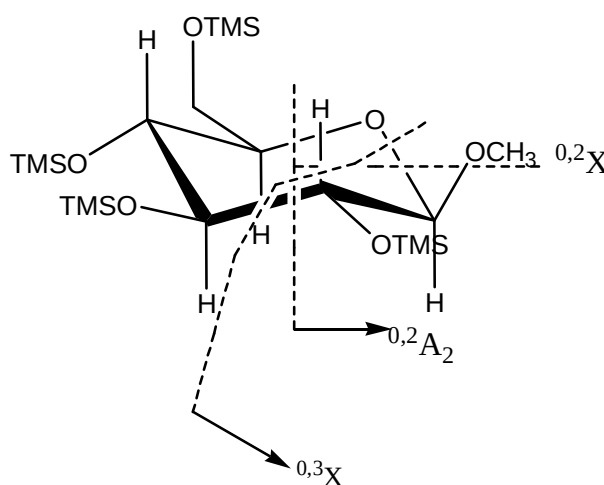
Scheme 1.1.4 MS degradation pattern of BR-1



Scheme 1.1.5 MS degradation pattern of BR-1 and formation of daughter ions

(I.iii) Mass spectral studies of Trimethylsilylated glucose

The compound BR-1 on hydrolysis with methanolic hydrochloric acid and subsequent trimethyl silylation and MS analysis gave a degradation pattern with ions of m/z values characteristic of methyl-2,3,4,6-tetra-O-trimethylsilyl- β -D-



Scheme 1.1.6 MS degradation pattern of trimethylsilylated glucose

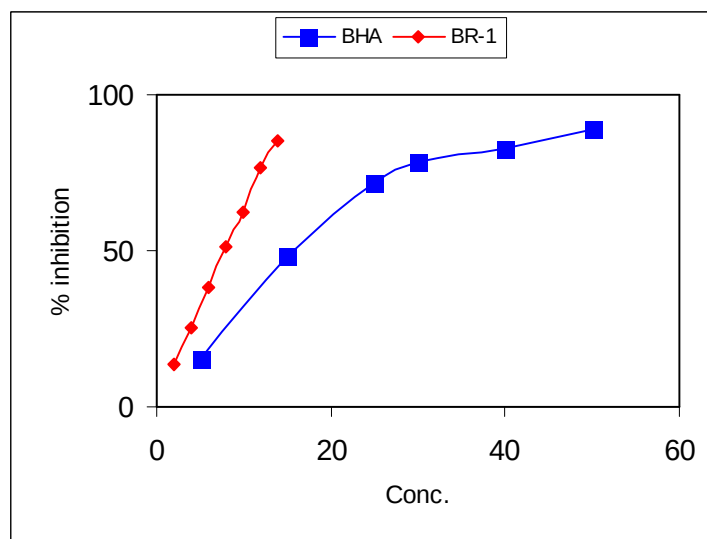
(II) Antioxidant studies

The antioxidant activity of BR-1 was evaluated by *in vitro* DPPH free radical scavenging activity and FRAP assay. The results obtained in the present study demonstrate that BR-1 showed good antioxidant properties in DPPH & FRAP assay. BR-1 showed a concentration dependent antiradical activity by inhibiting DPPH radical with an EC₅₀ value of 7.85 ± 0.32 µg/mL, a better activity than BHA (EC₅₀: 16.30 ± 0.20 µg/mL), which was used as the positive control (Table 1.1.2, Fig 1.1.5). The lower the EC₅₀, the higher the antioxidant level.

Table 1.1.2 DPPH free radical scavenging activity of std BHA and BR-1

BHA			BR-1		
Conc. ($\mu\text{g/mL}$)	% Inhibition	^a EC ₅₀ ($\mu\text{g/mL}$)	Conc. ($\mu\text{g/mL}$)	% Inhibition	^a EC ₅₀ ($\mu\text{g/mL}$)
5	15.65 $\pm$ 0.11	16.30 $\pm$ 0.20	2	13.29 $\pm$ 0.18	7.85 $\pm$ 0.32
15	48.23 $\pm$ 0.18		4	25.06 $\pm$ 0.23	
25	71.65 $\pm$ 0.14		6	38.51 $\pm$ 0.44	
30	78.35 $\pm$ 0.23		8	51.01 $\pm$ 0.32	
40	82.55 $\pm$ 0.35		10	62.49 $\pm$ 0.46	
50	88.69 $\pm$ 0.42		12	76.63 $\pm$ 0.58	
			14	85.36 $\pm$ 0.61	

^aEC₅₀ = Concentration of antioxidant required to reduce by 50% the initial conc. of DPPH
 n = 4, Mean $\pm$ SD

**Fig 1.1.5 Plot of DPPH free radical scavenging activity of BHA and BR-1**

Similarly in FRAP assay BR-1 exhibited good ferric reducing ability comparable with ascorbic acid but lower than gallic acid (Table 1.1.3, Fig 1.1.6). Ascorbic acid and gallic acid are used as reference standards in antioxidant studies. EC_1 values of these antioxidants are in the order BR-1>ascorbic acid>gallic acid, the higher the EC_1 values, expressed as $\mu\text{mol/L}$, the lower the antioxidant activity²⁴.

Table 1.1.3 FRAP assay of std antioxidants and BR-1

	Gallic acid	Ascorbic acid	BR-1
^a EC_1	183 ± 5	396 ± 7	410 ± 8
($\mu\text{mol/L}$)			

^a EC_1 = Concentration of antioxidant having a ferric reducing ability equivalent to that of 1 mmol/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$.

n = 3, Mean $\pm$ SD

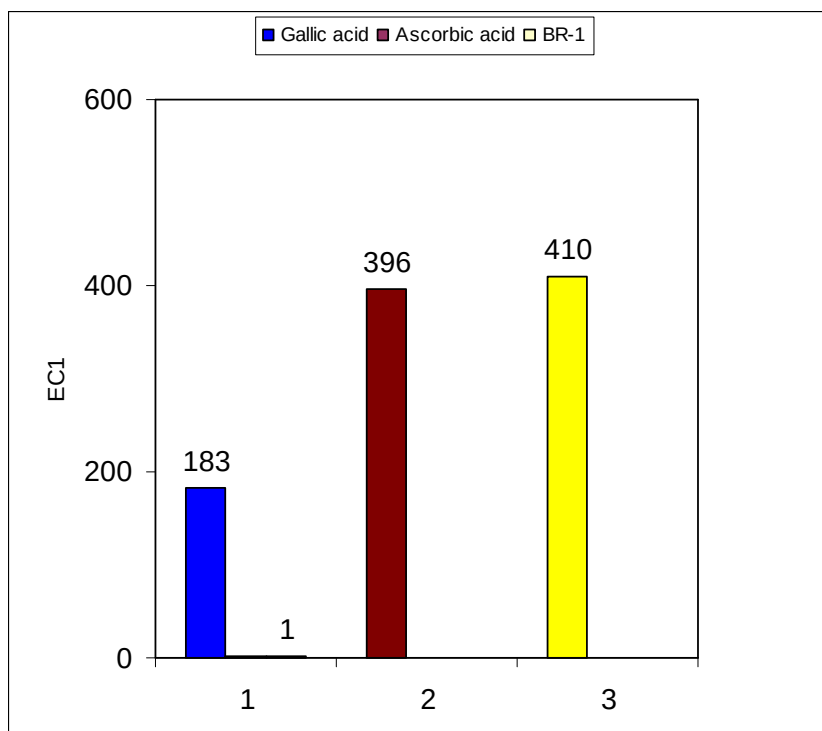


Fig 1.1.6 Plot of FRAP assay of gallic acid, ascorbic acid and BR-1

EXPERIMENTAL

(I) Extraction and Isolation of BR-1

1.2 kg of the dried and coarsely powdered plant material was extracted with 80% aq. methanol (10 L) in a Soxhlet extractor for 12 h. It was filtered and concentrated under suction in a rotary flash evaporator to obtain about 400 g of the crude green coloured extract. It was then fractionated with petroleum ether (b.p. 60-80 °C) followed by ethyl acetate. The red coloured aqueous methanol extract thus obtained was again fractionated with n-butanol (2 L). The butanol was removed from the extract under suction in a rotary flash evaporator to obtain 150 g of the solid mass. It was subjected to column chromatography on a column (100 cm x 4.5 cm) filled with silica gel (200-400 mesh). Before filling up into the column, silica gel was activated at 110° C for 1 h in a hot air oven. The column was eluted with acetone and different combination of acetone and methanol. Aliquots of 20 mL fractions were collected in different test tubes and monitored by TLC (7.5 x 2.5 cm). Identical fractions were pooled together. The fraction eluted with acetone-MeOH mixture (10:90), yielded a single spot in TLC (acetone-CHCl₃-H₂O-MeOH-2-propanol, 5:0.6:2.5:2:1, R_f 0.34). The fractions 33-61 (580 mL) were combined, concentrated in rotary flash evaporator and subjected to preparative TLC on pre coated silica gel 60 F₂₅₄ plates using the above mentioned solvent system to afford yellow coloured compound, BR-1. It was then recrystallized from MeOH-water 1:1 (v/v) to obtain pure yellow amorphous compound BR-1 (110 mg).

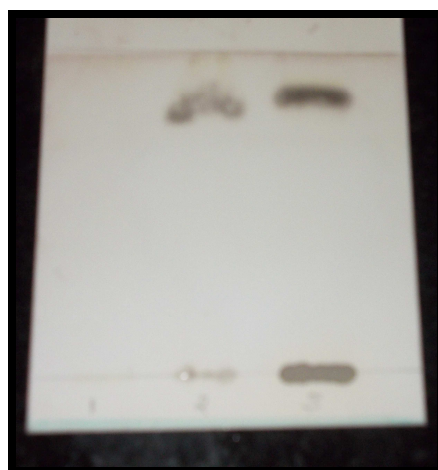
(II) High Performance Liquid Chromatography

BR-1 was dissolved in 1:1 DMSO-MeOH solvent system and 20 µL of BR-1 was injected into the HPLC system. Reversed phase (RP) Sunfire C18 column (100 x

4.6 mm, 5 μ m) was kept at 40 °C in a column oven. The compound was eluted with the isocratic mobile phase 45:55 MeOH-H₂O (v/v). The flow rate was set at 1 mL min⁻¹. The diode array detector was set at a wavelength of 332 nm. BR-1 was eluted at RT of 7.12 min.

(III) BR-1 - Apigenin-4',5-O- β -D-diglucoside

Yellow amorphous powder, melting at 232 °C with charring. $[\alpha]_D^{22}$: 82° (DMSO, c 0.01). UV $\lambda_{\max}$ nm: 271, 332 in DMSO; IR (KBr) $\bar{\nu}_{\max}$ cm⁻¹: 3405, 2925, 2854, 1654, 1618, 1457, 1189, 1093, 923; ESIMS (- ve mode): m/z 593 [M-H]⁻; ESIMS (+ ve mode): m/z 595 [M+H]⁺. The ¹H and ¹³C NMR spectral data are given in Table 1.1.1. BR-1 was subjected to the acid hydrolysis by refluxing 25 mg of it with 5 mL of 2 N HCl for 3 h. It was then analyzed by pre-coated silica gel TLC in CH₂Cl₂-CH₃OH-CH₃COOH-H₂O (5:5:2.5:1) solvent system. After spraying the developed plate with 10% ethanolic H₂SO₄ and heating at 110 °C in the hot air oven for 5 min, only a dark spot corresponding to glucose at R_f 0.87 (Fig 1.1.7) was obtained.



**Fig 1.1.7 Track 1: Unhydrolyzed BR-1; Track 2: BR-1 hydrolyzed;
Track 3: Std glucose**

(IV) Configuration of the glucose

Glucose was extracted from the hydrolysate by silica gel TLC in the above described solvent system. It was dissolved in pyridine and analyzed as their TMSi methylglucoside obtained after methanolysis (0.5 N HCl in MeOH for 24 h, 70 °C) and trimethylsilylation²⁵. The configuration of the glucose was established by capillary GC and GC-MS²⁶.

(V) Methanolysis, trimethylsilylation and GC MS analysis

The dried sugar (10 mg) was dissolved in 0.5 mL of methanolic 0.5 N HCl. N₂ was bubbled through the solution for 30 s. After methanolysis for 24 h at 70 °C, the acid solution was neutralized by the addition of silver carbonate. This mixture was kept at room temperature for 24 h. The precipitate was centrifuged and the supernatant collected. The residue was washed twice with 0.5 mL of methanol. The pooled supernatants were evaporated under reduced pressure at 35 °C. The residue was dried for 12 h in a desiccator. Finally, the sample was trimethylsilylated with 0.1 mL of silylating agent (pyridine - hexamethyldisilazane - trimethylchlorosilane, 5:1:1, by vol) for 30 min at room temperature.

GC-MS analysis was performed by the splitless injection of 1 µL of the sample on a Perkin Elmer gas chromatograph fitted with cross bonded 5% diphenyl-95% dimethyl polysiloxane Elite-5 capillary column (30 m x 0.25 mm x 0.25 µm coating thickness), coupled with a mass detector. GC-MS operating conditions are as follows: injector temperature 200 °C, transfer line 220 °C, detector temperature 220 °C. The oven temperature was kept at 175 °C, carrier gas: helium at a flow rate of 1 mL min⁻¹, mass spectra: electron impact at 70 eV, ion source temperature: 250 °C. The components were identified by NIST database matching (Fig 1.1.8 & 1.1.9).

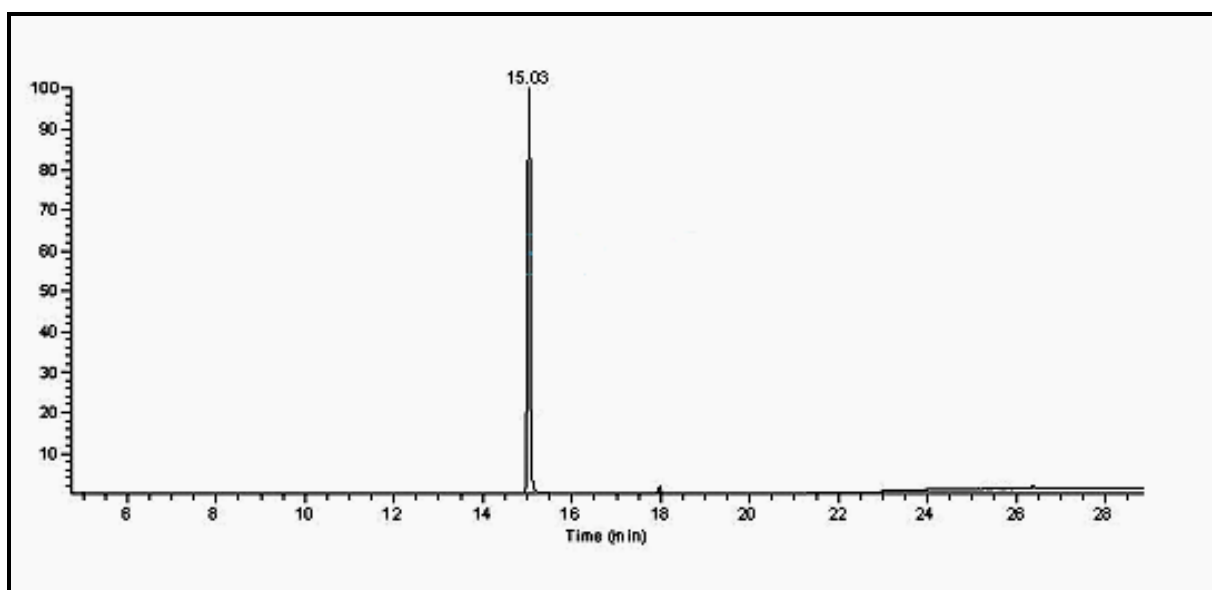


Fig 1.1.8 GC-MS chromatogram of D-glucose trimethylsilyl ether

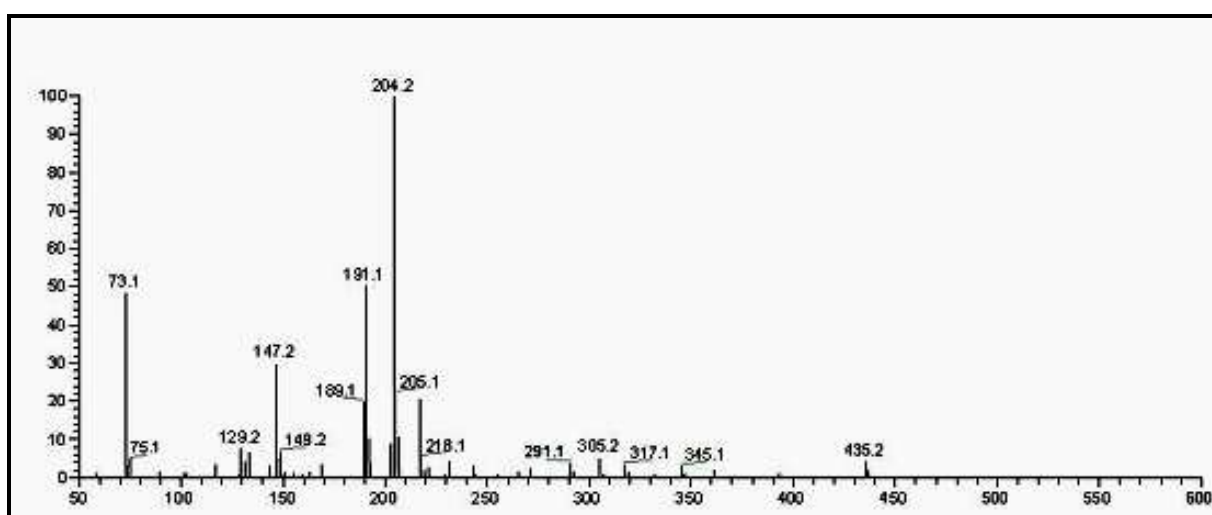


Fig 1.1.9 EI mass spectrum of D-glucose trimethylsilyl ether

(VI) Antioxidant studies – DPPH free radical scavenging activity

Stock solutions containing 25 µg/mL of BR-1 and 50 µg/mL of BHA was prepared in methanol. A stock solution of DPPH (1.3 mg/mL methanol) was prepared such that 75 µL of it in 3 mL methanol gave an initial absorbance of 0.9. Antioxidant activity was measured by a decrease in the absorbance at 516 nm of methanolic solution of coloured DPPH brought about by the compound BR-1^{27,28,29}. Decrease in the absorbance of BR-1 at different concentrations was noted after 15 min. EC₅₀ was calculated from % inhibition. BHA was used as the positive control.

(VII) Antioxidant studies – FRAP assay

The antioxidant capacity of the BR-1 was estimated using standard protocol^{30,31,32} with some modifications. Briefly 900 µL of FRAP reagent, prepared freshly and warmed at 37 °C was mixed with 90 µL of distilled water and 30 µL of the BR-1. The final dilution of the test sample in the reaction mixture was 1/34. The FRAP reagent contained 2.5 mL of 10 mmol/L of TPTZ solution in 40 mmol/L HCl, 2.5 mL of 20 mmol/L FeCl₃.6H₂O and 25 mL of 300 mmol/L acetate buffer (3.1 g of CH₃COONa in 1 L of distilled water) having pH 3.6. The absorbance was measured at 4 min at 595 nm using a UV-vis spectrophotometer. Aqueous solution of known Fe (II) concentrations in the range of 100-2000 µmol/L (FeSO₄.7H₂O) was used for calibration. The parameter equivalent concentration 1 or EC₁ was defined as the concentration of antioxidant having a ferric-TPTZ reducing ability equivalent to that of 1 mmol/L FeSO₄.7H₂O. EC₁ was calculated as the concentration of the antioxidant giving an absorbance increase in the FRAP assay equivalent to theoretical absorbance value of a 1 mmol/L concentration of Fe (II) solution. Water is used as reagent blank and gallic and ascorbic acids were used as positive controls.

CONCLUSIONS

During the process of metabolism the unwanted molecules are produced as the by-product of the aerobic process within mitochondria, which are known as free radicals; they are highly reactive in nature. They contain unpaired electron and have short life. Free radical damages the membrane and there by changes the receptors alignment and ion channels. They also attack the sulphur containing enzymes resulting in their inactivation, cross-linking and denaturation. Nucleic acids are also affected by free radicals. Ultimate result is the destruction of endothelial cells of blood vessels, macrophage invasion, suppressed immune response, inflammatory response, destruction of lung tissue, hastening of ageing process and also impotency and infertility. Free radicals are involved in more than 50 diseases. Day to day researches is adding more number of diseases to this list. Alzheimer's disease, coronary heart diseases, cancer, rheumatoid arthritis, malignancy, immune deficiencies etc are examples of such diseases.

Antioxidants are scavengers of free radicals, which protects the organism against free radicals. Antioxidants play very important role in protecting the body from the harmful effects of free radicals. Many medicinal plants are potent sources of antioxidants. Several plant extracts and different classes of phytochemicals have been shown to have antioxidant activities. Increased intakes of dietary antioxidants may help to maintain an adequate antioxidant status in the living organism. Increased intakes of fruits and vegetables have been related to a reduction of the risk of cardiovascular disease and certain types of cancer. Dietary antioxidants other than vitamins such as flavonoids have been shown to be a major dietary factor responsible for such protective effects.

In the present study, apigenin-4',5-O- β -D-diglucoside, a novel flavonoid diglycoside has been isolated from the whole plant of *Biophytum reinwardtii*. This

compound has been screened for the antioxidant activity viz. DPPH free radical scavenging activity and FRAP assay. From these two assays, it has been found that apigenin-4',5-O- β -D-diglucoside exhibits good antioxidant properties. The plants containing such radical scavengers are well known for their therapeutic activity. The above results suggest that, this compound due to its good antioxidant properties may have good potential to act as anti cancer agent.

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SECTION - 1.2

EVALUATION OF ANTICANCER ACIVITY OF APIGENIN-4',5-O- β -D-DIGLUCOSIDE BY MTT ASSAY

INTRODUCTION

Cancer is a dreadful disease, which features uncontrollable cellular growth, local tissue invasion and metastasis and cause several deaths per year. In the case of modern therapy, there are serious side effects to chemotherapy, surgical and radiation treatment protocols. Hence current pharmacological research is focused on Indian traditional system of medicine, which is natural and safe without any side effects. Use of herbs has been authorized as an alternative form of medicine with less toxicity and safe for patients. The phytochemicals such as alkaloids, flavonoids and others play a major role in fight against cancer cells³³.

The present study was designed to evaluate cytotoxicity of apigenin-4',5-O- β -D-diglucoside (**BR-1**) in two cancer cell lines viz., HeLa, human cervical cancer cell lines and HT 29, human colon cancer cell lines. Different concentrations of **BR-1** such as 6.25, 12.5, 25, 50 and 100 μ g/mL were tested for cytotoxicity by MTT assay. DMSO was used as the control. Based on my previous research findings that **BR-1** possesses good antioxidant activity, the study was extended to test the anticancer potential of this compound.

MATERIALS AND METHODS

(I) Apigenin-4',5-O- β -D-diglucoside (BR-1)

BR-1 isolated from *Biophytum reinwardtii* as described in section 1.1 was used for this anticancer study. The different concentrations of **BR-1** were prepared in DMSO.

(II) Cell lines & cultures used

The two cancer cell lines viz., HeLa, human cervical cancer cell lines and HT 29, human colon cancer cell lines were purchased from National Centre for Cell Sciences (NCCS), Pune, India. The cells were maintained in Dulbecco's Modified Eagles Media (Hi Media) supplemented with 10% Foetal Bovine Serum (FBS-Invitrogen) and grown to confluency at 37 °C in 5% CO₂ in a humidified atmosphere in a CO₂ incubator (NBS, EPPENDORF, GERMANY). The cells were trypsinized [500 μ L of 0.025% Trypsin in PBS/ 0.5 mmol EDTA solution (Hi Media)] for 2 minutes and passaged to T flasks in complete aseptic conditions. Extracts were added to grown cells at a final concentration of 6.25, 12.5, 25, 50 and 100 μ g/mL from a stock of 20 mg/mL and incubated for 24 hours. The untreated cell lines (DMSO) were used as the control. The % difference in viability was determined by standard MTT assay after 24 hours of incubation. MTT and DMSO were purchased from S.D. Fine Chemicals, Mumbai.

RESULTS & DISCUSSION

The present study suggests significant toxicity of apigenin-4',5-O- β -D-digluconide (**BR-1**) against HeLa cancer cell lines and moderate toxicity against HT 29 cell lines. MTT, a pale yellow substrate that produced a dark blue formazan product when incubated with live cells. From MTT assay, after treatment with various concentrations of **BR-1**, cell viability and percentage toxicity were compared with untreated cells (DMSO).

Cell counting and cell viability tests confirmed anticancer activity in both cell lines. The decrease in cell viability and increase in cytotoxicity by **BR-1** was observed on HeLa and HT 29 cell lines in a dose dependent manner. The photographs of anticancer activity of **BR-1** at various concentrations against HeLa and HT 29 cell lines are given in Fig 1.2.1 and Fig 1.2.2.

Concentration required for a 50% inhibition of viability (IC_{50}) was determined graphically (Fig 1.2.3). Graphs were plotted using % of cell viability against the conc. of the sample. **BR-1** exhibited cytotoxicity towards HeLa and HT 29 cell lines with IC_{50} values of 24.77 μ g/mL and 106.76 μ g/mL respectively (Table 1.2.1).

In the present study, **BR-1** is found to be effective against both cervical and colon cancer cell lines. This is in support with its good antioxidant properties. **BR-1** did not show cytotoxic effect towards normal cell lines. Further antiproliferative activity of total flavonoids and alkaloids isolated from different plants were reported earlier³⁴. The association between flavonoids and reduced cancer risk has been reported which showed that a decrease in cancer risk with consumption of vegetables and fruits rich in flavonoids³⁵.

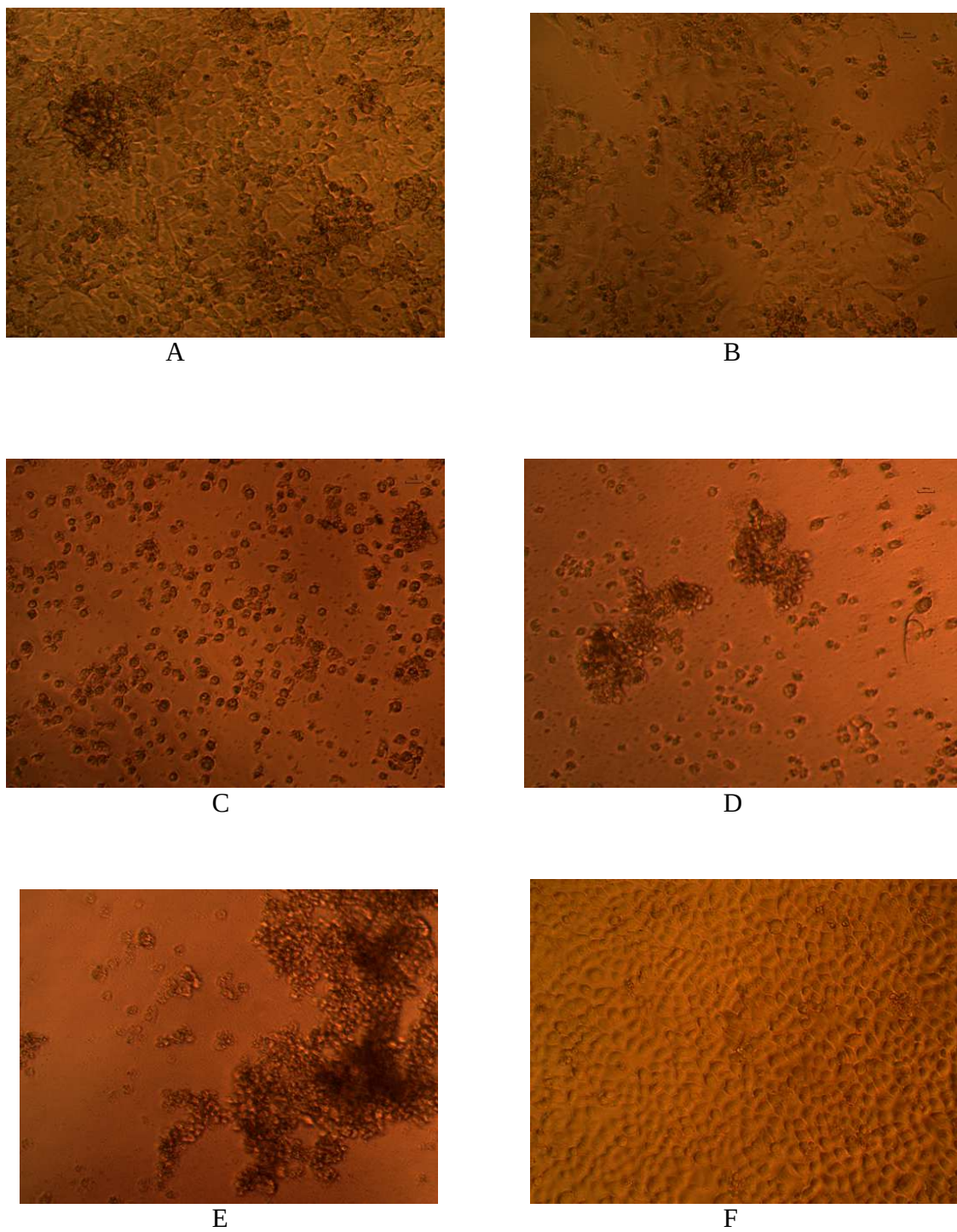


Fig 1.2.1 Cytotoxicity of BR-1 on HeLa cell lines at different concs.
A) 6.25 µg/mL, B) 12.5 µg/mL, C) 25 µg/mL, D) 50 µg/mL, E) 100 µg/mL and F)
Control

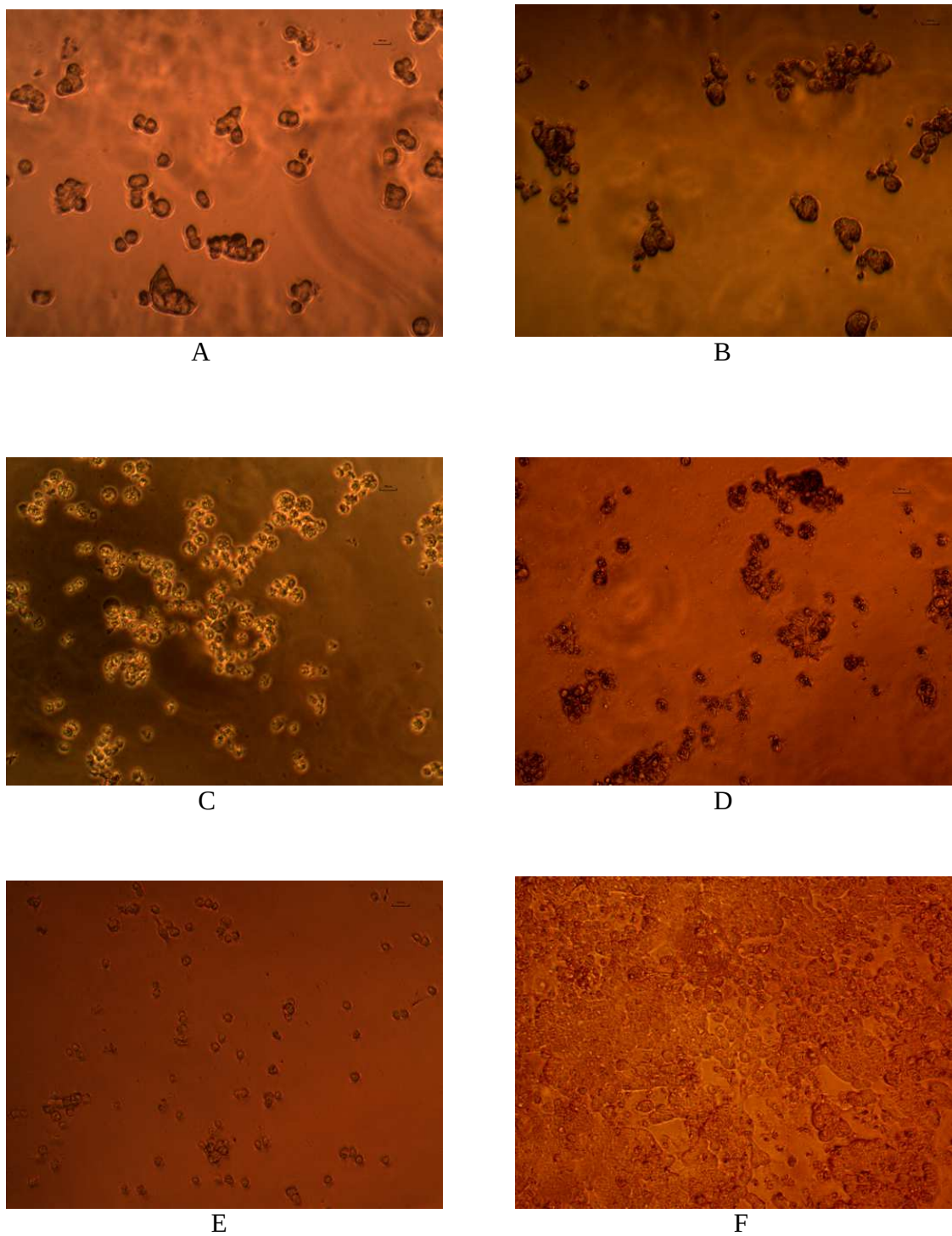


Fig 1.2.2 Cytotoxicity of BR-1 on HT 29 cell lines at different concs.

**A) 6.25 µg/mL, B) 12.5 µg/mL, C) 25 µg/mL, D) 50 µg/mL, E) 100 µg/mL and F)
Control**

The aglycone, apigenin, a common dietary flavonoid abundantly present in fruits and vegetables is reported to have promising biological effects such as prevention and therapy of prostate cancer, suppression of tumourigenesis and angiogenesis in melanoma³⁶ and breast, skin and colon carcinomas³⁷. It has been shown to exhibit antitumoural effects in leukemia cells by induction of apoptosis through activation of caspases, inhibition of fatty acid synthase and topoisomerase and modulation of Bax and Bcl-2 expression³⁸ and reported to have antileukemic effects³⁹.

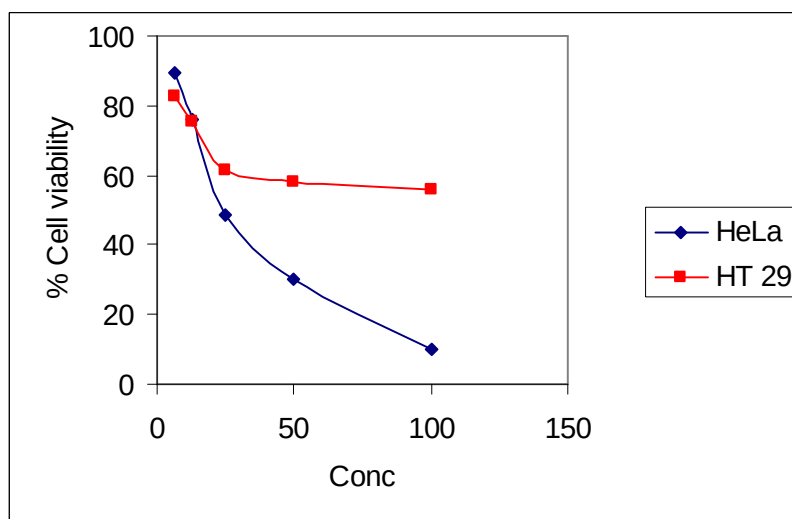


Fig 1.2.3 MTT assay for cytotoxicity and cell viability

Table 1.2.1 MTT assay for cytotoxicity and cell viability

Sample Conc. ($\mu\text{g/mL}$)	HeLa Cell lines			HT 29 Cell lines		
	A_{540}	% cell	$^a\text{IC}_{50}$	A_{540}	% cell	$^a\text{IC}_{50}$
		viability	($\mu\text{g/mL}$)		viability	($\mu\text{g/mL}$)
Control	0.9995			0.7012		
6.25	0.8907	89.11456		0.5806	82.80091	
12.5	0.7615	76.18809		0.5272	75.1854	
25	0.4864	48.66433	24.77	0.4321	61.62293	106.76
50	0.2988	29.89495		0.4081	58.20023	
100	0.102	10.2051		0.3922	55.93269	

$^a\text{IC}_{50}$ = Concentration of the sample required for 50% inhibition of cell viability

EXPERIMENTAL

(I) *In vitro* assay for cytotoxicity (MTT assay) using BR-1

The anticancer activity of **BR-1** was determined by MTT assay^{40,41} MTT is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble dark purple coloured formazan product. The cells are

then solubilised with an organic solvent DMSO and the released, solubilised formazan product was measured at 540 nm. Since the reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells.

MTT was dissolved in phosphate buffered saline (PBS) at 5 mg/mL. The cells (1×10^5 /well) were plated in 1 mL of medium/well in 24- well plates. After 48 hours incubation, the cell reached the confluence. Then the cells were incubated in the presence of various concentrations of **BR-1** in DMSO for 24 h at 37 °C. After removal of the sample solution and washing with PBS 200 μ L/well (pH 7.4), stock MTT solution (10 μ L per 100 μ L medium) was added to all wells of an assay and plates were incubated at 37 °C for 4 h. After 4 h, MTT was removed by washing with PBS and 200 μ L of DMSO was added to the culture. Incubation was done at room temperature for 30 minutes until the cell got lysed and the colour was obtained. The solution was transferred to centrifuge tubes and centrifuged at top speed for 2 minutes to precipitate cell debris. Optical density was read at 540 nm using DMSO as blank in a micro plate reader (ELISASCAN, ERBA).

The concentrations of the **BR-1** showing complete cytotoxic effect were recorded. Viable cells were determined by the absorbance at 540 nm. Measurements were performed and concentration required for 50% inhibition of viability (IC_{50}) was determined graphically. The minimum concentration non toxic in Vero but completely inhibiting the cancer cells (HeLa or HT 29) is considered as the effective drug concentration. The effect of the samples on the proliferation of the cell lines HeLa and HT 29 were expressed as the % cell viability using the following formula:

$$\% \text{ cell viability} = \frac{\text{Absorbance at 540 (A}_{540}) \text{ of treated cells} \times 100}{A_{540} \text{ of control cells}}$$

CONCLUSIONS

Apigenin-4',5-O- β -D-diglucoside possesses good anticancer activity in line with its antioxidant activity. It is very effective against HeLa and moderately cytotoxic to HT 29 cancer cell lines. Hence apigenin-4',5-O- β -D-diglucoside has the potential to be developed as a drug for cervical cancer treatment. Further detailed *in vivo* studies are required before the use of apigenin-4',5-O- β -D-diglucoside as an anti cancer drug.

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Chapter 2

Srinivasan G. V. “phytochemical investigation and biological studies of secondary metabolites from some folk medicinal plants” Thesis. Department of Chemistry, University of Calicut, 2015



***Salacia fruticosa* Heyne ex Laws.**

CHAPTER – 2

ISOLATION, IDENTIFICATION, QUANTIFICATION & ANTIOXIDANT STUDIES OF MANGIFERIN FROM THE ROOTS OF SALACIA FRUTICOSA HEYNE EX LAWS.

INTRODUCTION

Salacia belongs to the Hippocrateaceae (Celastraceae) family is a large genus of about 200 species distributed in Tropical America, Africa and Asia. About 21 species are reported in India¹. This genus consisting mainly of climbing or creeping shrubs or rarely small trees is known to contain anthocyanidines, catechins, phenolic acids, quinones, friedooleananes, quinonemethide and related triterpenoids (celastrols), mangiferin, gutta-percha and dulcitol. *Salacia fruticosa* Heyne ex Laws. is a climbing shrub, endemic to the Western Ghats (in semi evergreen to evergreen forests, up to 1300 m) and its distribution is restricted to the states of Maharashtra, Goa, Karnataka, Tamil Nadu and Kerala of Indian sub continent. Its vernacular names are Vairi/Pitika in Sanskrit; Ekanayakam/Korandi/Ponkorandi in Malayalam and Ponkoranti in Tamil. The root of Pitika is an important ingredient of Ayurvedic formulations like

*Paranthyadi Taila, Nisakatakadi Kashaya, Neerooryadigudika, Valiya Marmagudika, Narayana Gula, Amrithadi Choorna etc*².

The roots of *S. fruticosa* are acrid, bitter, thermogenic, urinary, astringent, anodyne, anti-inflammatory, depurative, emmenagogue, vulnerary, liver tonic and stomachic. They are useful in vitiated conditions of vata, diabetes, haemorrhoids, inflammations, leucorrhoea, leprosy, skin diseases, amenorrhoea, dysmenorrhoea, wounds, ulcers, hyperhidrosis, hepatopathy, dyspepsia, flatulence, colic and spermatorrhoea³.

Salacia reticulata Wight, also known as Pitika in Sanskrit is a reputed remedy for diabetes in Ayurvedic practice. Its distribution is restricted to India and Sri Lanka. Due to the acute scarcity of this species, other species belonging to the same genus are being used in its place. *S. reticulata* roots are useful in vitiated conditions of vata, diabetes, haemorrhoids, inflammations, leucorrhoea, leprosy, skin diseases, amenorrhoea, wounds, ulcers, colic and spermatorrhoea⁴.

In traditional medicine of Sri Lanka, an aqueous extract of *S. reticulata* root is used in the treatment of diabetes. Its oral hypoglycaemic activity also has been demonstrated in experimental rats⁵. Salacinol is one of the active principles in the aqueous extract of *S. reticulata*, which are traditionally used in India and Sri Lanka for the treatment of Type II diabetes. Mangiferin has been reported from the root bark of *S. reticulata*⁶. Aldose reductase and alpha glucosidase inhibitors salacinol and kotalanol were isolated from the roots of *S. reticulata*. These are the antidiabetic principles found in the aqueous extract of *S. reticulata*^{7,8}.

Roots and stems of *S. oblonga* are being used in the treatment of diabetes in the Ayurvedic system of Indian traditional medicine⁵. It also possess anti obese properties. The ethyl acetate extracts of leaves and stems of *S. beddomei* were

reported to possess antibacterial activity⁹. Antidiabetic activity of roots of *S. macrosperma* has been reported¹⁰. Furthermore, several plants from the *Salacia* genus (eg. *S. prinoidea*, *S. fruticosa* and *S. reticulata*) have been used for many years in the Ayurvedic system to treat diabetic conditions⁴.

There is a considerable interest in the antioxidant and free radical scavenging properties of medicinal plants and compounds isolated from them¹¹. For example polyphenols are a broad family of naturally occurring physiologically active compounds. Xanthones are closely related to the polyphenols. They have a remarkable effect on cardiovascular system, are antibiotic, antiviral and anti-inflammatory. They include some of the most powerful natural antioxidants. It is desirable to search for natural products that can be used to prevent the free radical initiation or the progression of a number of diseases. Free radical damages can be considered as one of the major causative factor involved in many diseases, including inflammation and dementia¹².

It is well established that antioxidants prevent injury to blood vessel membranes, thereby optimizing blood flow to the heart and brain. They defend against cancer-causing DNA damages and thus help lowering the risk of cancer, cardiovascular and various mental illnesses including Alzheimer's diseases¹³. The reactive oxygen species such as O_2^- and OH play a prominent role in the stimulation, propagation and maintenance of both acute and chronic inflammatory processes as well as pain causing tissue damages. These adverse effects due to pain and excessive inflammation has been shown to be reduced by the use of suitable antioxidants either by preventing the formation of oxygen free radicals or by scavenging them before they react with sites such as unsaturated lipids in the cell membrane^{14,15}.

(I) Morphological description of *S. fruticosa*

S. fruticosa is a Climbing shrub with rough branchlets. Leaves are ovulate to elliptic- lanceolate (5-12 x 2.5-5 cm), apex acute, base cuneate, chartaceous and margin serrate. Flowers are pale yellow, many inflorescence; cymes branched and 1.5 cm long. Calyx lobes deltoid and minutely ciliate. Petals orbicular, 2 mm across margin, white Stamens, 3 filaments recurved, ovary rounded, partially immersed in the disc, 3- loculed; stigma capitate. Fruit, a berry, globose or sub globose and smooth (2.5 x 2 cm). Seeds are angular (1.5 x 1 cm).

(II) Abstract of the present work

In the present work, mangiferin (Fig 2.1), 2- β -D-glucopyranosyl-1,3,6,7-tetrahydroxy-9*H*-xanthen-9-one, a xanthone glycoside has been isolated for the first time from the roots of *S. fruticosa*. Mangiferin is present in large amounts in the roots and obtained directly from the aq. Methanolic extract. HPLC and HPTLC methods were developed for the quantification of mangiferin in the 80% aqueous methanol extract of *S. fruticosa* roots. The antioxidant activity of 80% aqueous methanol extract of the root was also studied by *in vitro* DPPH method.

REVIEW OF LITERATURE

Only very limited phytochemical studies have been reported so far in *Salacia fruticosa* Heyne ex Laws. From the root bark of *S. fruticosa* β -sitosterol, α -amyrin, friedelan-3-one (friedelin), friedel-1-ene-3-one, friedelan-3-one-29-al and friedelan-3-one-29-ol have been reported¹⁶. Some literature reviews of other species of *Salacia* are given below.

Salacinol, a potent antidiabetic principle with unique thiosugar sulfonium sulfate structure has been isolated from *S. reticulata*⁷. Two quinonemethide triterpenoids (celastroloids), isoiguesterinol and 30-hydroxypristimerin, along with salacenonal, several known celastroloids and friedo-oleanane triterpenoids has been isolated from the outer root bark of *S. reticulata*¹⁷. An alpha glucosidase inhibitor kotalanol has been isolated from *S. reticulata*, which has an action similar to that of acarbose⁸. Three active components salacinol, kotalanol and mangiferin have been isolated from the extracts of *S. oblonga* and *S. reticulata*⁷. Mangiferin, gutta-percha, sitosterol, pristimerin, epikokoondiol, salacenonal and salaciquinone has been isolated from the root bark of *S. reticulata* and iguesterin, pristimerin and epi-kokoondiol from its stem bark^{18,19,20}.

Friedelane-type triterpenes, salasones D and E, norfriedelane-type triterpene, salaquinone B and a polyacylated eudesmane- type sesquiterpene, salasol B have been isolated from the stems of *S. chinensis* Linn²¹.

Four stilbenes- Lehmachols A-D have been isolated from the bark of *S. lehmbachii*, collected in Papua New Guinea²².

Triterpene, 1 β , 15 α -dihydroxy-friedelan-3-one has been isolated from the stem bark of *S. beddomei*²³. Two lupane triterpenoids, 20,29-epoxysalacianone (20,29-epoxylupane-3, 21-dione) and 6 β -hydroxysalacianone [6 β -hydroxylup-20(29)-ene-3, 21-dione] have been isolated from the hexane and ethyl acetate extracts of the stem bark of *S. beddomei*, together with the known compounds 3 β , 28-dihydroxylup-20 (29)-ene (betulin) and 2 α , 3 β -dihydroxylup-20(29)-ene²⁴. The lupine compounds namely lup-20 (29)-en-3-one, friedelan-3-one, 15 α -hydroxyfriedelan-3-one, β -sitosterol, 15 α -hydroxyfriedelane-1, 3-dione and pristimerin, salacianone and salacianol have been isolated from the stem bark of *S.*

*beddomei*²⁵. The ethyl acetate extracts of the leaves and stems of *S. beddomei* shows excellent antibacterial activity⁹.

The norfriedelene-1,3-dione has been isolated from the root bark of *S. oblonga*²⁶. The water-soluble portion of the methanolic extract of *S. oblonga* inhibits α -glucosidase and two of the active ingredients—salacinol and kotalanol—have been isolated from that fraction²⁷. *S. oblonga* improves cardiac fibrosis and inhibits postprandial hyperglycemia in obese Zucker rats²⁸. *S. oblonga* root improves cardiac lipid metabolism in Zucker diabetic fatty rats²⁹.

Since the plant *S. fruticosa* is endemic to this region of Western Ghat and no phytochemical investigation has been carried out, I've undertaken this phytochemical study.

MATERIALS AND METHODS

(I) Plant material

The roots of *S. fruticosa* were collected from Koorachundu, 40 Km from Kozhikode, Kerala, India in May 2007 and were authenticated by Dr. P. S. Udayan, Professor, Dept of Botany, Sri Krishna College, Guruvayoor. A voucher specimen was deposited at the herbarium of the University of Calicut for future references. The roots were washed with tap water, dried in the shade and coarsely powdered.

(II) Reagents and instrumentation

Solvents used were of analytical/ HPLC grade (E. Merck). DPPH was purchased from Sigma Aldrich. BHA was purchased from E. Merck. Precoated silica gel 60

F₂₅₄ plates (E. Merck) of uniform thickness 0.2 mm were used for HPTLC analysis. The Shimadzu UV-1700 model with a double beam spectrophotometer was used for UV (both absorbance and spectrum) analysis. IR spectrum was recorded in Shimadzu FTIR-8400S spectrophotometer using 8000 DRS reflectance attachment. Integration was done using Kubelka Munk conversion. ¹H and ¹³C NMR spectra were recorded in Supercon, Bruker (Germany) at 200 and 50 MHz respectively using TMS as internal standard. APCI-MS and ESIMS spectra were recorded in LCMS-2010A Shimadzu spectrometer.

(III) HPLC instrument

The Shimadzu (Japan) HPLC system consisted of LC-10AT VP pump, SPD M10A VP photodiode array detector, SCL-10A system controller, CLASS-VP 6.12 SP5 integration software and a Rheodyne model 7725 i syringe-loading sample injector fitted with a 20 µL injection loop, was used for the analysis. Baseline resolution of mangiferin was obtained at 26 ± 2 °C using a Phenomenex Luna C₁₈ column (250 x 4.6 mm i.d; 5 µm) and Phenomenex guard column (4 x 2 mm i.d; 5µm). HPLC method was validated by performing the sensitivity, linearity and recovery studies.

(IV) HPTLC instrument

A Camag HPTLC system (Switzerland) equipped with a sample applicator Linomat V, twin trough linear developing chamber, TLC scanner III attached with integration software CATS 4.06 and precoated silica gel 60 F₂₅₄ plates (E. Merck) were used. The mobile phase used was ethyl acetate-methanol-water (20:2:1 v/v). The stationary phase, mobile phase, scanning wavelength and mode of detection (by densitometry) for the chromatographic method was optimized. HPTLC method was validated by performing the sensitivity, linearity and recovery studies.

RESULTS & DISCUSSION

(I) Structural elucidation of SF-1

The isolated compound (SF-1) from 80% aqueous methanol extract from *S. fruticosa* root gave green colour with 10% MeOH/FeCl₃ and deep red colour with Fast Blue Salt (FBS) reagents. The intensity of yellow fluorescence on exposure to UV 365 nm increase after spraying with 10% EtOH/KOH reagent and also on exposing it to NH₃ vapours. These observations are characteristics of typical flavonoid compound/compounds.

(II) Spectral data

The spectral data of SF-1 are as follows:

UV λ_{max} (nm): 263 (band-II), 322, 373 (band-I) in DMSO; 289, 325, 360, 406 in (DMSO + NaOMe); 270, 332, 393 in (DMSO + AlCl₃); 264, 322, 370 in (DMSO + AlCl₃ + HCl); 273, 333, 396 in (DMSO + NaOAc); IR (KBr) $\bar{\nu}$ cm⁻¹: 3369, 3184, 2940, 1652, 1622, 1259; APCIMS (+ ve mode): m/z 423 [M+H]⁺, 405, 303, 279, 193; APCIMS (-ve mode): 421 [M-H]⁻, 331, 301, 191; ESIMS (- ve mode): m/z 421 [M-H]⁻. The ¹H and ¹³C NMR spectral data of SF-1 are given in Table 2.1. All the spectra of SF-1 are given in Appendix IV.

Table 2.1 ^1H and ^{13}C NMR spectral data of SF-1

Carbon No.	^{13}C NMR (δ in ppm)	^1H NMR [δ in ppm, multiplet, J (Hz)]
1	161.65	-
2	108.41	-
3	163.59	-
4	93.91	6.35, s
4a	156.65	-
5	102.65	6.82, s
6	153.86	-
7	143.58	-
8	106.98	7.43, s
8a	112.17	-
9	179.42	-
9a	101.77	-
10a	151.13	-
1'	80.97	4.80, d, 9.6
2'	73.63	4.33, t, 9.0
3'	71.17	4.01, m
4'	70.35	3.78, m
5'	78.84	3.73, m
6'	61.38	^a 3.64, dd, 11.2, 2.9 ^b 3.58, dd, 11.2, 5.4
1 (OH)	-	13.78, s
3, 6, 7 (OH)	-	9.94, broad s
2', 3', 4', 6' (OH)	-	3.32, s

UV-Visible spectrum of SF-1 exhibited maximum absorption at 263, 322 and 373 nm indicating it to be a xanthone. In the UV spectrum, recorded with the shift reagent AlCl_3 , bathochromic shifts in all absorption bands were observed from 263 to 270, 322 to 332 and 373 to 393 nm and the latter of these shifts is characteristic for the presence of chelating hydroxyls at the positions C-1 or C-8 of the molecule. Additionally, a bathochromic shift appeared in the UV absorption band from 322 to 333 nm, when recorded the UV spectrum using sodium acetate as shift reagent, which is characteristic for the presence of moderately acidic hydroxyl groups at positions C-3 or C-6. The UV spectrum, recorded in sodium acetate showed a pronounced bathochromic shift of the longest wavelength maximum ($\lambda = 373$ to 396 nm), indicating a free 3-OH in the Xanthone molecule.

The IR spectrum exhibits characteristic absorption bands at 3369 cm^{-1} for OH group, 1652 cm^{-1} for α, β -unsaturated carbonyl group and 1622 cm^{-1} for C=C group.

The compound SF-1 showed a quasi molecular ion peak at m/z 423 $[\text{M}+\text{H}]^+$ in the APCIMS (+ve mode), m/z 421 in the APCIMS and ESIMS (-ve mode) which suggested an elemental composition of $\text{C}_{19}\text{H}_{18}\text{O}_{11}$.

The ^1H NMR of SF-1 revealed the presence of three downfield aromatic singlet signals at δ 6.35 (1H, H-4), 6.82 (1H, H-5) and 7.43 (1H, H-8). Furthermore, the characteristic chemical shifts of the sugar moiety were observed at δ 4.80 (1H, d, $J = 9.6\text{ Hz}$, H-1'), 4.33 (1H, t, $J = 9.0\text{ Hz}$, H-2'), 4.01 (1H, m, H-3'), 3.78 (1H, m, H-4'), 3.73 (1H, m, H-5'), 3.64 (1H, dd, $J = 11.2, 2.9$, H-6'a) and 3.58 (1H, dd, $J = 11.2, 5.4$, H-6'b).

^{13}C NMR revealed 19 carbon atoms in the molecule. The aglycone had 13 carbons, including 10 quaternary aromatic carbons at δ 161.65 (C-1), 108.41 (C-2), 163.59 (C-3), 156.65 (C-4a), 153.86 (C-6), 143.58 (C-7), 112.17 (C-8a), 179.42 (C-9),

101.77 (C-9a) and 151.13 (C-10a) and 3 methines at δ 93.91 (C-4), 102.65 (C-5) and 106.98 (C-8).

The anomeric carbon at δ 80.97 (C-1'), suggested the C-glycoside moiety. In addition the other shifts of the sugar carbons appeared at δ 73.63 (C-2'), 71.17 (C-3'), 70.35 (C-4'), 78.84 (C-5') and 61.38 (C-6'). The location of the OH at C-1 was based on the downfield singlet proton at δ 13.78, hydrogen bonded with the carbonyl. The location of the protons at C-4, C-5 and C-8 was based on their singlet form in ^1H NMR. From the δ values of ^1H and ^{13}C NMR spectra, which are suggestive of a xanthone molecule with a C-glycosidic link.

From the above mentioned spectroscopic data, the compound SF-1 was identified as **Mangiferin**. The ^1H and ^{13}C NMR spectra of the isolated mangiferin agree well with those reported in the literatures^{30,31,32}. The structure of Mangiferin is as follows (Fig 2.1).

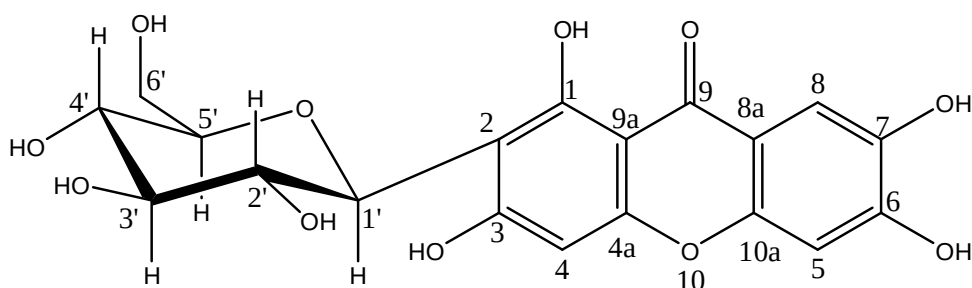


Fig 2.1 Structure of Mangiferin ($\text{C}_{19}\text{H}_{18}\text{O}_{11}$)

(III) Quantification of Mangiferin – by HPLC & HPTLC techniques

The standard calibration curve of Mangiferin was plotted using standard solutions at concentrations of 5, 10, 20, 30, 40, 50, 60, 80, 100 and 200 $\mu\text{g/mL}$. The extract was injected in HPLC system and run the system with mobile phase acetonitrile-

H₂O containing 0.1% ortho phosphoric acid (25:75 v/v) at a flow rate of 0.8 mL/min and the detection was done at 261 nm. Using the response from the system, mangiferin was quantified. The amount of mangiferin present in the dried root of *S. fruticosa* was found to be 5.80% w/w. The HPLC chromatogram of the std Mangiferin and the extract are given below (Fig 2.2 & 2.3).

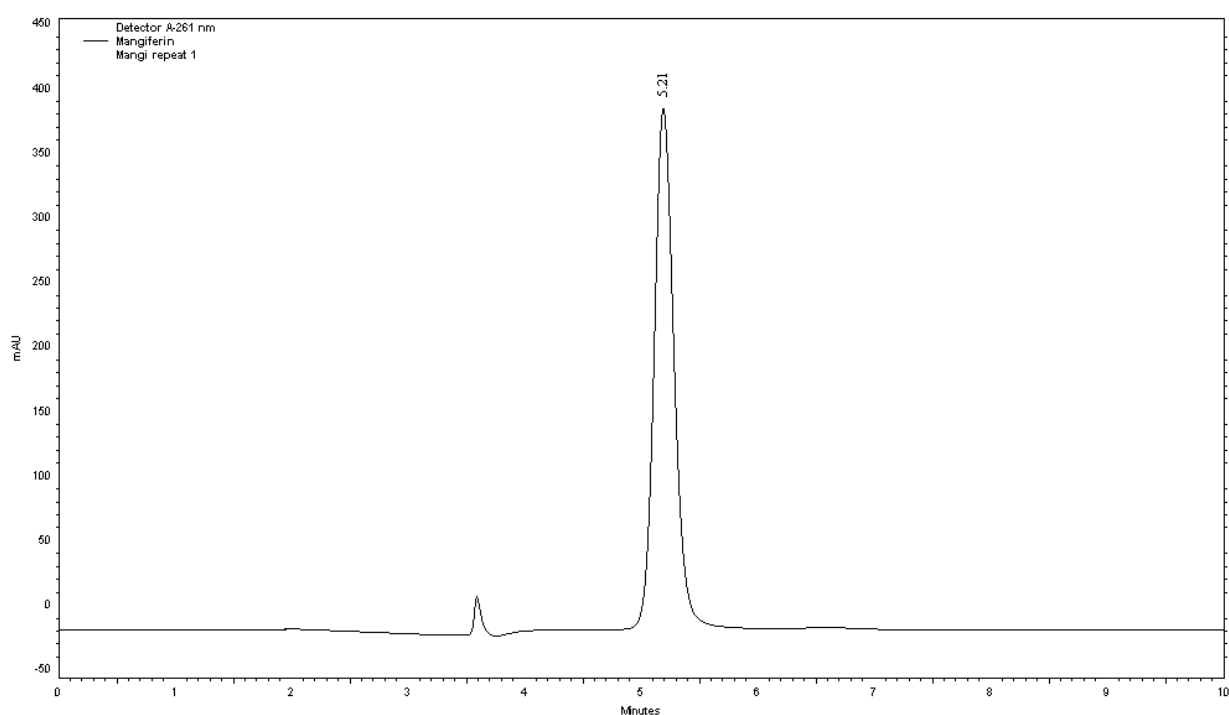


Fig 2.2 HPLC chromatogram of standard mangiferin

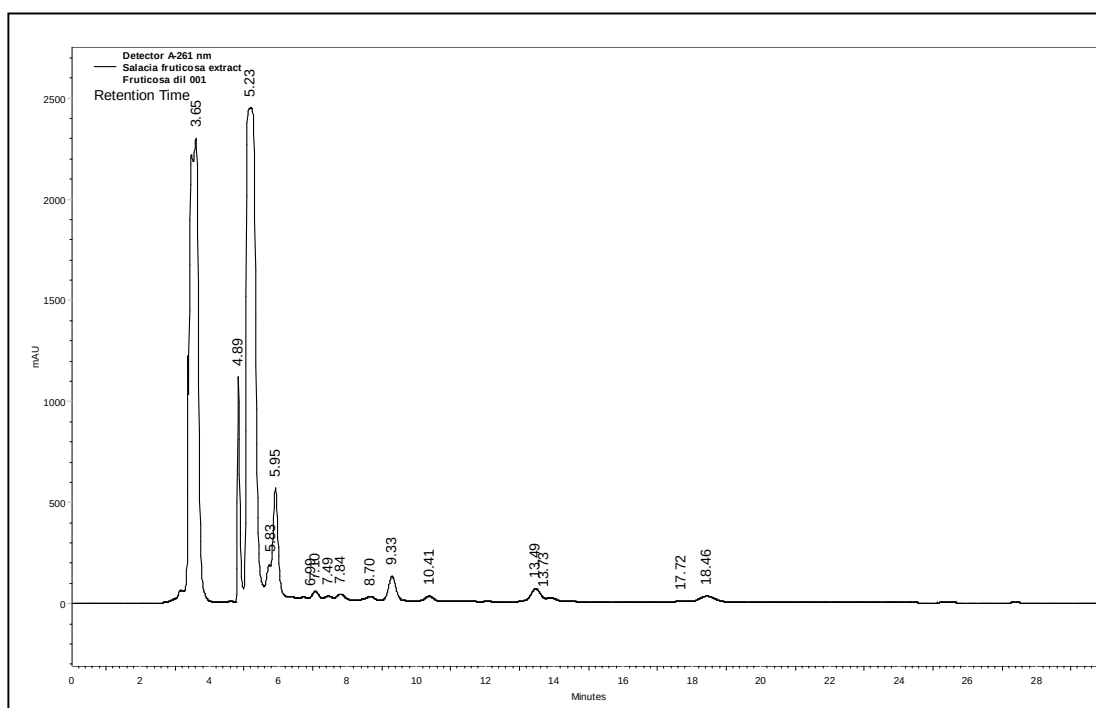


Fig 2.3 HPLC chromatogram of *S. fruticosa* extract

Similarly mangiferin was also quantified by using HPTLC method. The amount of mangiferin present in the dried roots was found to be 6.34% w/w. The view of developed TLC plates (Vis light, UV 254 nm & UV 365 nm) showing mangiferin is given in Fig 2.4. The chromatogram of the standard and the extract are given below (Fig 2.5 & 2.6)

The quantification of Mangiferin has been reported previously from two of the species of *Salacia* namely *Salacia oblonga* and *Salacia roxburghii* and are 0.174% and 0.319% respectively³³.

From the literatures, it was found that the highest amount of mangiferin was reported from the bark of *Hindi sinnara* variety of *Mangifera indica* (11.80%)³⁴. Now to the best of my knowledge the mangiferin content in the roots of *S. fruticosa* seems to be the second highest.

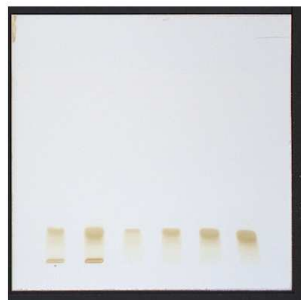


Fig 2.4.1 View of TLC plate in Visible light

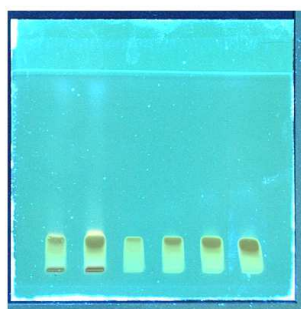


Fig 2.4.2 View of TLC plate in UV 365 nm

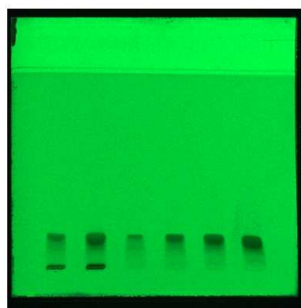


Fig 2.4.3 View of TLC plate in UV 254 nm

Fig 2.4 TLC plates showing mangiferin spots

Track 1 & 2 extract,

Track 3-6 Mangiferin

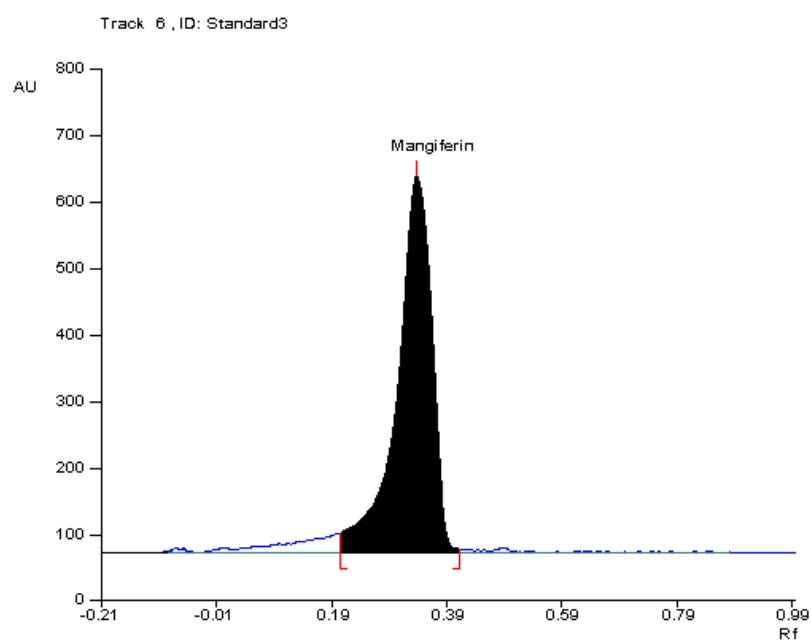


Fig 2.5 HPTLC chromatogram of Mangiferin

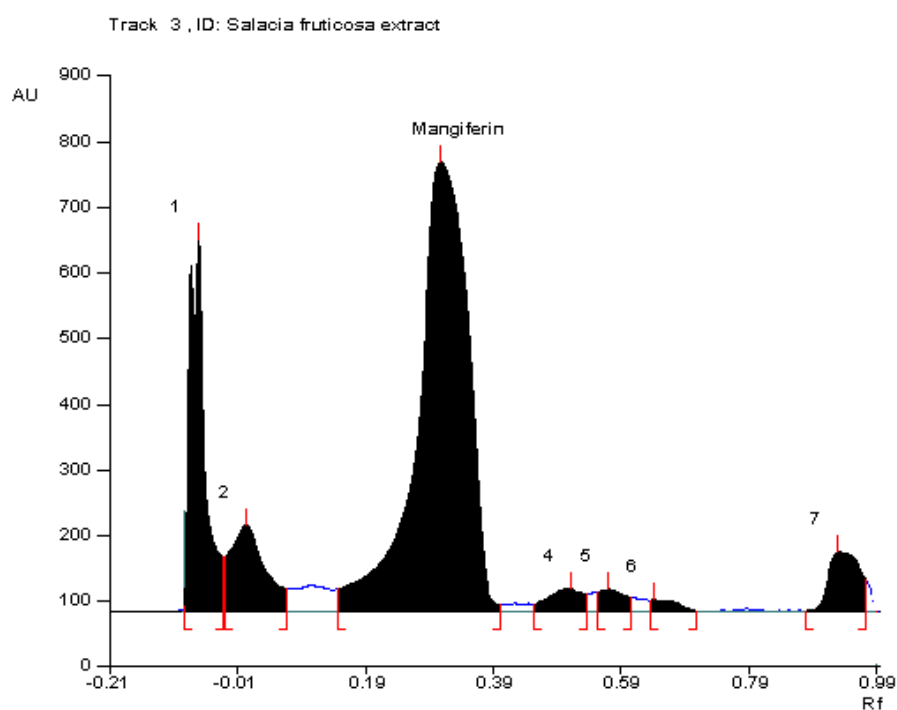


Fig 2.6 HPTLC chromatogram of *S. fruticosa* extract

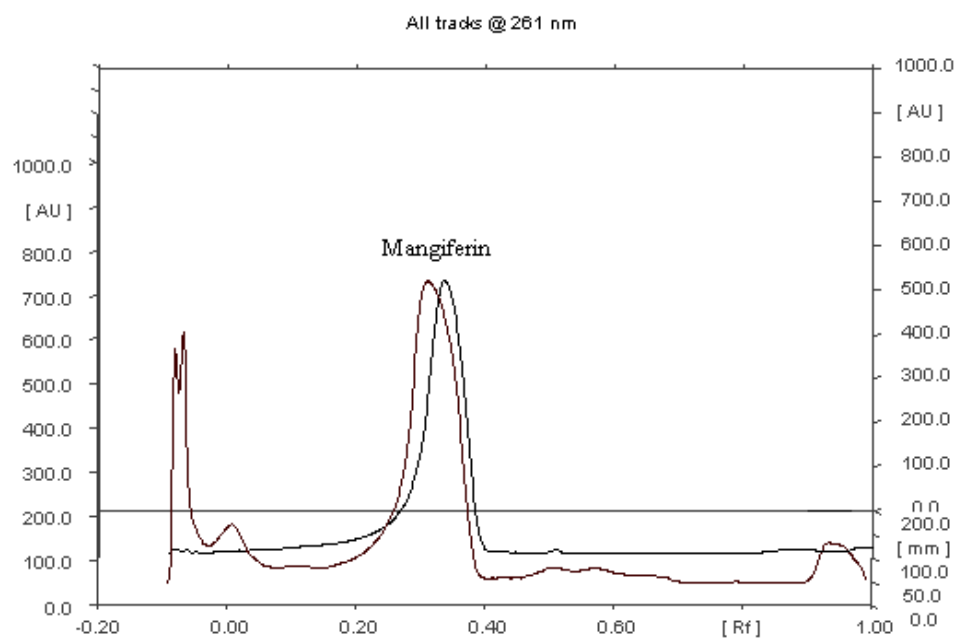


Fig 2.7 Densitometric plot of the extract and std. mangiferin

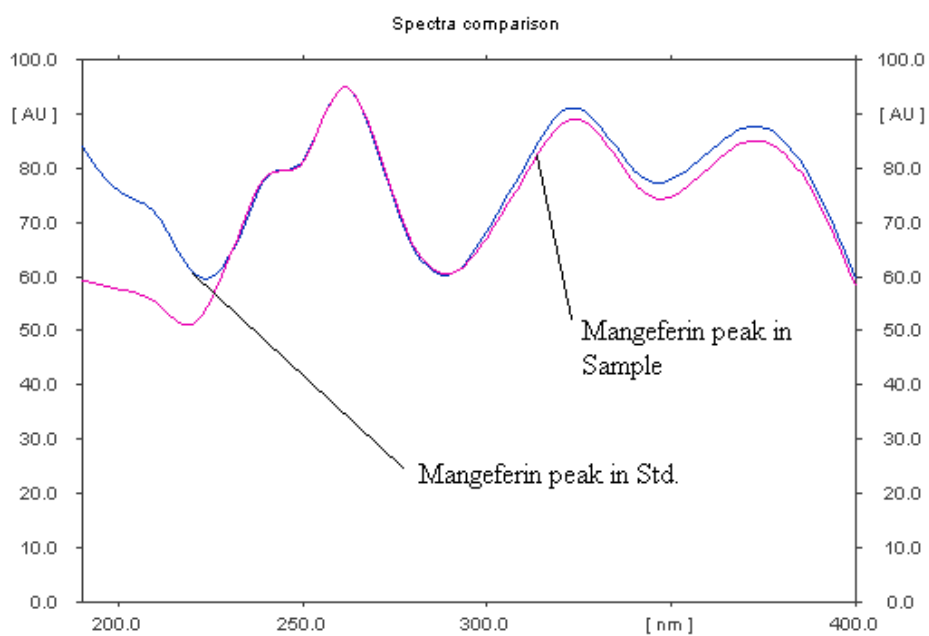


Fig 2.8 Spectral comparison of Mangiferin peak in sample and standard

(IV) Antioxidant studies

The antioxidant activity of 80% aqueous methanolic extract of the roots of *S. fruticosa* was evaluated by *in vitro* DPPH radical scavenging activity. The results obtained in the present study demonstrate that methanolic extract of the roots of *S. fruticosa* has good antioxidant properties.

The root extract of *S. fruticosa* showed promising antioxidant properties with DPPH assay. The methanol extract showed a concentration dependent antiradical activity by inhibiting DPPH radical, with an EC₅₀ value of 6.9 µg/mL. The methanol extract of the roots of *S. fruticosa* showed a better activity than BHA (EC₅₀ = 16.5 µg/mL), which was used as the positive control. (Table 2.2, Fig 2.9).

$$\% \text{ Inhibition of DPPH radical} = \frac{\text{Abs}_{\text{extract absent}} - \text{Abs}_{\text{extract present}}}{\text{Abs}_{\text{extract absent}}} \times 100$$

The present study which attributes higher free radical scavenging activity for the roots of *S. fruticosa* and it could be directly correlated with the higher amount of mangiferin present in it, which has already been shown to have strong antioxidant activity. EC₅₀ of mangiferin was reported to be of 5.80 ± 0.96 µg/mL³⁵.

The role of free radical oxidative stress in various disease conditions has been well established. Superoxide radical, hydroxyl radical, peroxy radical and singlet oxygen are some of the important reactive species that cause damage to the biological system³⁶. Certain plants exhibit efficient antioxidant properties due to their phenolic constituents. The plant drugs containing radical scavengers are well known for their therapeutic activity^{37,38}. The free radical scavenging property may be one of the mechanisms by which these drugs are effective in tradition medicine.

Table 2.2 Antioxidant activity of standard BHA and 80% aq methanol extract of *S. fruticosa* root

Std. BHA			<i>S. fruticosa</i> root		
Conc. ($\mu\text{g/mL}$)	% Inhibition ^a	EC ₅₀ ($\mu\text{g/mL}$)	Conc. ($\mu\text{g/mL}$)	% inhibition ^a	EC ₅₀ ($\mu\text{g/mL}$)
5	15.78 $\pm$ 0.10		1.75	24.29 $\pm$ 1.53	
15	47.26 $\pm$ 0.18		3.51	28.90 $\pm$ 3.67	
25	70.45 $\pm$ 0.09		5.26	39.91 $\pm$ 2.46	
30	76.65 $\pm$ 0.33	16.50	7.02	51.01 $\pm$ 4.72	6.90
40	81.41 $\pm$ 0.24		8.77	53.49 $\pm$ 5.11	
50	85.65 $\pm$ 0.43		10.52	58.18 $\pm$ 5.01	
			14.03	72.36 $\pm$ 6.48	

^aEC₅₀ = Concentration of antioxidant required to reduce by 50% the initial conc. of DPPH

n = 4, ^aMean $\pm$ SD

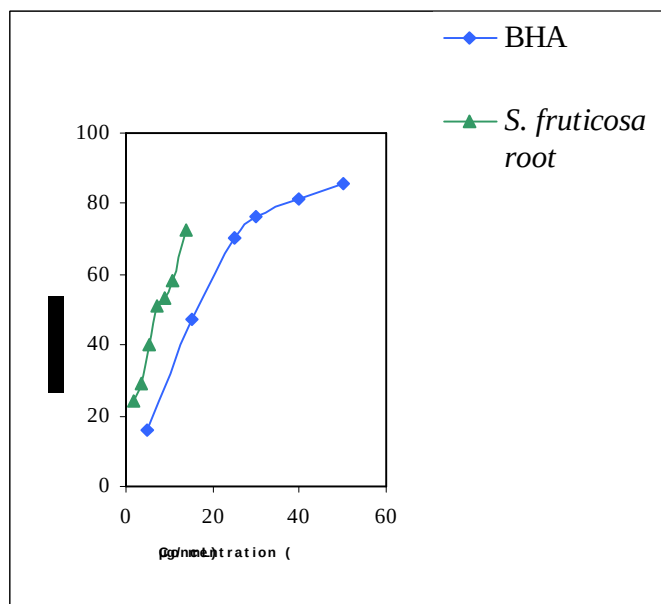


Fig 2.9 Plot of antioxidant activity of BHA and *S. fruticosa* extract

EXPERIMENTAL

(I) Isolation of mangiferin

500 g of the powdered root was extracted with 1.5 L 80% aqueous methanol in a Soxhlet extractor for 8 hours at 65 °C. It was filtered and concentrated to 75 mL under suction in a rotary flash evaporator. The extract was kept for a period of 12 h at room temperature without disturbing. The yellow coloured compound (SF-1) separated out at the bottom was filtered and washed with 10 mL portions of methanol (x 4). It was then recrystallized from dioxane-water 1:1 (v/v) to obtain yellow crystals having (yield: 6.2 g) m.p. 271-275 °C.

(II) Quantification of Mangiferin – HPLC & HPTLC methods

(i) Preparation of standard solution of the extract and mangiferin for HPLC and HPTLC analyses

1.3 g of the powdered root of *S. fruticosa* extracted with 100 mL 80 % aqueous MeOH in a soxhlet extractor for 8 h. The solvent was removed under suction. The residue was redissolved in 80% MeOH and made up to the mark in a 10 mL volumetric flask so that 1 mL of standard solution contains 3.5085 mg of the extract. While making up 0.5 mL DMSO was added in order to ensure the complete solubility of mangiferin in the extract. It was then filtered through 0.20 µm nylon-6, 6 membrane. This solution was used for HPLC and HPTLC analyses.

A std. solution containing 1.1 mg/mL of isolated mangiferin was prepared in 80% methanol. While making 5 drops of DMSO was added to ensure the solubility of mangiferin.

(ii) HPLC analysis

Baseline resolution of mangiferin was obtained at 26 ± 2 °C using a Phenomenex Luna C-18 column using the solvent system acetonitrile- H₂O containing 0.1% ortho phosphoric acid (25:75 v/v). The mobile phase was passed through 0.45 µm PVDF filter and degassed before use. The flow rate was kept constant at 0.8 mL/min and the detection was done at 261 nm. For calibration, standard solutions of mangiferin were prepared at concentrations of 5, 10, 20, 30, 40, 50, 60, 80, 100, and 200 µg/mL using methanol as solvent. The standard solution was injected in triplicate and the average detector response was measured. The root extracts were assayed in triplicate, peak areas corresponding to mangiferin (Retention time: 5.23 min) were compared with the calibration curve and thus the amount of mangiferin was determined. The amount of mangiferin present in the root is 5.80% w/w (dry weight basis).

The developed HPLC method was validated for specificity, linearity, precision and accuracy. The limit of quantification for mangiferin was 0.05 µg/mL. A correlation coefficient of 0.987 indicates good linearity between the concentration and the area. The recovery studies were also carried out and the recovery value of mangiferin by this method was $97.1 \pm 0.6\%$. The proposed method is simple, accurate and reliable for quantitative monitoring of mangiferin in *S. fruticosa*.

(iii) HPTLC analysis

A 1.1 mg/mL solution of mangiferin and 3.5 mg/mL solution of methanol extract were used for HPTLC analysis. Methanolic extract of *S. fruticosa* (2, 4 and 6 µL) and standard solution of mangiferin (1, 2, 4, 6, 8 and 10 µL) were applied on precoated TLC plate (20 x 10 cm) using Linomat V applicator with a band width of 6 mm and the distance between the tracks was 5 mm. The applied plates were

developed in a solvent system of ethyl acetate–methanol–water (20:2:1 v/v) in a Camag twin trough developing chamber for 15 minutes. The developed plates were observed under UV and visible lights and then scanned densitometrically at 261 nm in order to identify the spots (Fig 2.7 & 2.8) of active ingredients (R_f of mangiferin 0.34). The amount of mangiferin was determined using the calibration curve plotted between the concentrations and the area of the peaks for the standard. The amount of mangiferin was found to be 6.34 % (w/w).

Calibration curve of standard mangiferin was examined by using 1 mg/mL working solution prepared from stock solution. These solutions were applied to the HPTLC plate at different concentrations and developed to a distance of 8 cm. The concentration of mangiferin was then plotted against the area under curve of the respective peaks of the chromatograms developed. The same data were also subjected to statistical analysis using linear regression analysis and the correlation coefficient was determined. A correlation coefficient of 0.977 indicates good linearity between the concentration and the area. The recovery studies were also carried out and the recovery value of mangiferin by this method was 96.4 ± 1.2 %.

(III) Antioxidant studies

10 g of the powdered root of the *S. fruticosa* was extracted with 80% aqueous methanol in a Soxhlet apparatus for 8 h at 65 °C. The solvent was then removed under suction below 65 °C. A stock solution containing 17.54 µg/mL of the extract was prepared in methanol. A stock solution containing 50 µg/mL of BHA was also prepared in methanol. A stock solution of DPPH (1.3 mg/mL methanol) was prepared such that 75 µL of it in 3 mL methanol gave an initial absorbance of 0.9. These stock solutions were used to measure the antioxidant activity. Antioxidant activity was measured by a decrease in the absorbance at 516 nm of methanolic solution of coloured DPPH brought about by the sample^{39,40}. Decrease in the

absorbance in the presence of 80% methanol extracts at different concentrations was noted after 15 min. EC_{50} was calculated from % inhibition. BHA was used as the positive control.

CONCLUSIONS

Mangiferin has been isolated for the first time from the roots of *S. fruticosa*. It was characterized using different spectroscopic techniques. The amount of mangiferin present in the dried roots was estimated by HPLC (5.80%) and HPTLC (6.34%) techniques. The developed HPLC & HPTLC methods were found to be accurate, precise and sensitive and can be used for the routine quality control analysis and quantitative determination of Mangiferin in the roots of *S. fruticosa*. The appreciable amount of mangiferin present in the roots of *S. fruticosa* is noteworthy. The antioxidant activity of the 80% aqueous methanol extract was also measured by *in vitro* DPPH assay. The roots exhibited very good antioxidant activity ($EC_{50}=6.9 \mu\text{g/mL}$), two times higher than that of BHA ($EC_{50}=16.5 \mu\text{g/mL}$). The higher antioxidant activity exhibited by the roots of *Salacia fruticosa* is attributed to its higher mangiferin content.

Mangiferin has showed to have various biological activities. Mangiferin is useful in medicine for the treatment of viral diseases of skin and mucous membranes⁴¹. Mangiferin possess anti-inflammatory, antihepatotoxic and antiviral activities. It also stimulates the central nervous system. It is widely distributed in the following families: Anacardiaceae, Malpighiaceae, Guttiferae, Iridaceae, Liliaceae, Gentianaceae, Leguminosae, Flacourtiaceae, Convulvulaceae, Celastraceae and Sapotaceae and in the fern *Athyrium mesosorum* (Polypodiaceae). It occurs in the

above plants along with isomangiferin (where the C-glucose is at the 4th position) and in O-glycosidic combination⁴². Mangiferin was found to inhibit sucrase, isomaltase and aldose reductase from rat with IC₅₀ values of 87, 216 and 1.4 µg/mL respectively. The inhibitory activities of mangiferin are competitive for sucrase and isomaltase with inhibition constant (K_i) 55 and 70 µg/mL respectively⁴³. The present investigation supports and scientifically validates the traditional use of the roots of *S. fruticosa* for the treatment of diabetes.

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Chapter 3

Srinivasan G. V. “phytochemical investigation and biological studies of secondary metabolites from some folk medicinal plants” Thesis. Department of Chemistry, University of Calicut, 2015



Ixora coccinea L.

CHAPTER – 3

IDENTIFICATION OF CHEMICAL COMPOUNDS IN THE ESSENTIAL OIL FROM THE ROOTS OF IXORA COCCINEA L. AND ITS ANTIMICROBIAL STUDIES

INTRODUCTION

Ixora coccinea L. and other species of *Ixora* are shrubby members of the family Rubiaceae. They are distributed in the tropical and subtropical regions of the world. About 30 species occur in India. *I. coccinea* is a small evergreen shrub. It is a traditional medicinal plant, cultivated as well as seen in the wild throughout India¹. It has vernacular names in almost all the languages. It is called ‘Jungle Geranium’ and ‘Flame of the Woods’ in English. In Sanskrit, it is called Bandhuka/Raktaka; in Malayalam as Thetti/Techi; in Tamil as Tachi/Sinduram/Sedaram etc².

The roots of *I. coccinea* are reported to possess astringent and antiseptic properties and are used in headache and applied to sores and chronic ulcers in folk medicine. These are also reported to stimulate gastric and bile secretion to provide relief in abdominal pain^{3,4}. The roots exude a yellow juice and give out a disagreeable

rancid odour. They are reported to possess sedative and stomachic properties and are used in hiccups, fever, gonorrhoea and dysentery^{3,2}. The root is made into a paste with coconut milk and applied to ulcers, wounds and skin eruptions, especially in children. Even the discolouration caused by using silver ornaments will disappear on using this paste. The roots are used for wound healing in case of viper bites² and as ointments to treat scabies⁵. The Korku tribes of Maharashtra use the roots of this plant along with long pepper and little water to treat acute dysentery⁶.

The leaves of *I. coccinea* are used to treat diarrhoea³. The flowers are astringent, bitter, sweet, carminative and digestive⁷ and are used in the treatment of leucorrhoea, dysmenorrhoea, haemoptysis, catarrhal bronchitis and dysentery^{1,8}. It is also reportedly used to treat cholera⁹. The flowers of *I. coccinea* along with *Nigella sativa* seeds are used to prepare medicated oil to treat scabies especially in children. A paste of the fruit with *Rubia cordifolia* and coconut oil is used for dermatitis. A decoction of the flowers or the bark is used as a lotion for eye troubles and for sores and ulcers⁸. The flowers have psychosomatic properties. They are used in psychotherapy. A preparation using *I. coccinea* roots and flowers, turmeric, *Oxalis minima* leaves and *Jasminum grandiflorum* is used to treat stomach ailments².

The whole plant extract of *I. coccinea* is used as an astringent and in the treatment of dysentery and catarrhal tuberculosis¹⁰.

I. coccinea is extensively used in the preparation of a number of Ayurvedic formulations. It is the source of the Ayurvedic drug 'Paranthi'. Sahasrayoga describes a recipe including flowers and roots of this plant, turmeric and *Ocimum sanctum* prepared in ghee to treat gastric ulcer. The plant is used in the preparation of *Ashtachoornam*, where its role is to maintain normal blood pressure. It is also

used in the treatment of diabetes, especially gangrene formation⁵. The root is an important ingredient in the formulations like *Paranthyadi Taila*, *Nisadi Kashaya*, *Khadirasaradi Ghritha*, *Abhrabhasma* etc and the flowers are used in the preparation of *Chemparuthyadi Taila*¹¹.

(I) Description of the plant

I. coccinea is a shrub or small tree, occurring along the Western coast, widely grown in gardens throughout India and other tropical countries as an ornamental plant. The leaves are opposite, sessile or nearly so, elliptic, ovate or obovate, 2-4 inches long, apiculate, obtuse or mucronate, coriaceous, glaucous; flowers tubular in dense terminal corymbose cymes, numerous, bright scarlet red, sometimes yellow, pink or orange yellow; fruits fleshy berries, reddish, globose, about 0.25 inches in diameter, crowned with persistent calyx, purple when ripe; seeds concave. The fruits are edible and the flowers are used as flavouring agent. Based on the flower colour and the length of corolla tube, the species was divided into three varieties namely *coccinea*, *bandhuca* and *lutea*².

(II) Abstract of the present work

The literature survey revealed that the composition of the essential oil from *I. coccinea* roots and its antimicrobial activity has not been investigated in detail. In the present work, a number of compounds has been identified in addition to the already reported ones from the root essential oil. The antibacterial and antifungal activities of the root essential oil were also investigated by disc diffusion method. The chemical compounds present in the cold hexane extract of *I. coccinea* roots were also identified by GC MS technique.

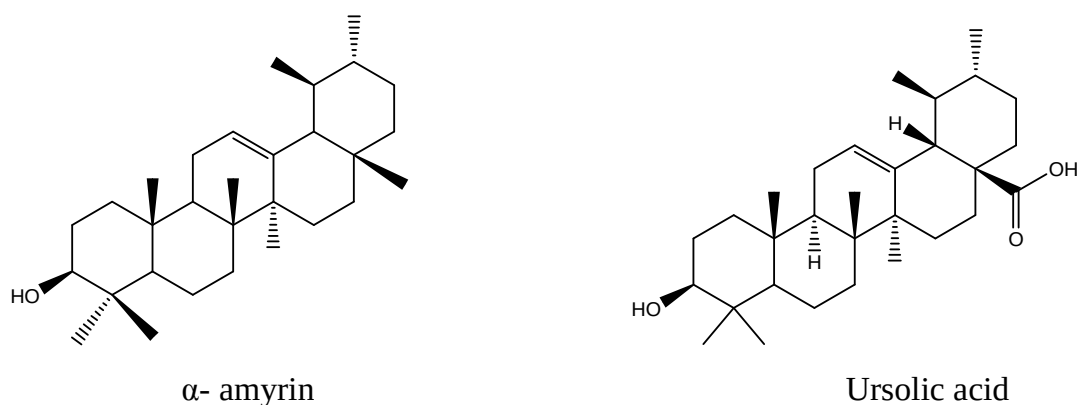
REVIEW OF LITERATURE

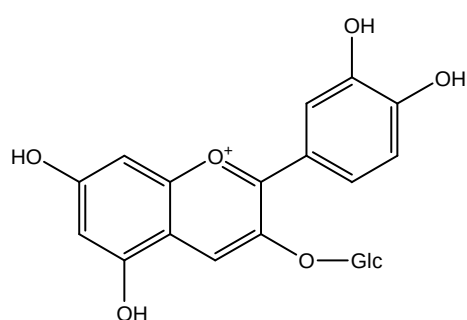
A number of chemical compounds have been isolated from different parts of *I. coccinea*. The roots were reported to contain acrid aromatic oil and fatty acids¹². Palmitic, stearic, oleic, linoleic, myristic and Δ -9,11-octadecadienoic acids^{13,14}, α -amyrin and β -sitosterol were reported from the roots¹⁵. Tannins were also reported from the roots of *I. coccinea*¹⁶. Mannitol, myristic and Δ -9,11-octadecadienoic acids have been reported from the root bark of *I. coccinea*¹³.

From the flowers of *I. coccinea*, quercetrin¹³, monoglycosides of cyanidin and delphinidin¹⁷, rutin, kaempferol-3-rutinoside, leucocyanidin glycoside^{18,19}, *d*-mannitol¹⁹ and ursolic acid²⁰ were reported. The anthocyanins were also reported in the *I. coccinea* flowers¹⁸.

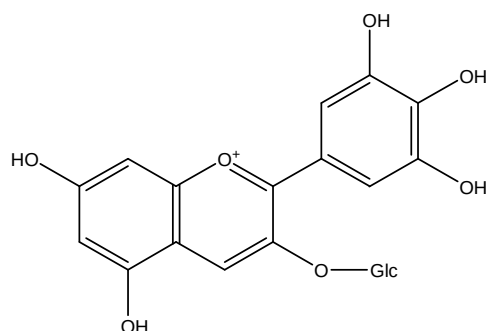
Rutin and lupeol have been reported from the leaves of *I. coccinea*²¹. *D*-mannitol was also reported from the stem bark of *I. coccinea*¹⁹.

The structures of some of the compounds isolated from *I. coccinea* are presented in Fig 3.1.

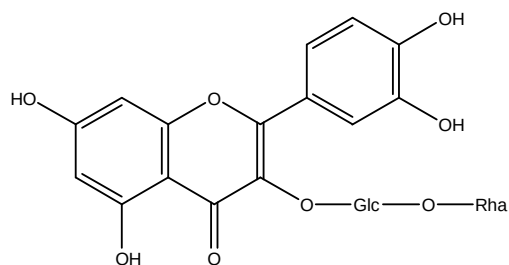




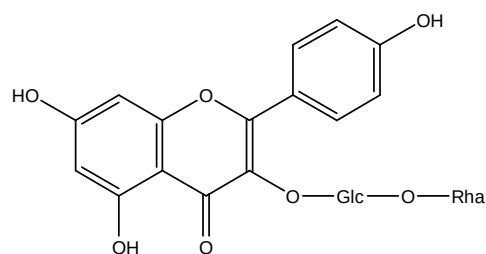
cyanidin-3-glucoside



delphinidin-3-glucoside



Rutin



kaempferol-3-rutinoside

Fig 3.1 structure of some compounds isolated from *I. coccinea*

A no. of pharmacological activities was reported for this plant. Central Nervous System (CNS) depressant activity was reported for *I. coccinea* whole plant excluding root in mice as revealed by its effects on behaviour, induced pyrexia, barbiturate induced hypnosis and rotarod performance. The CNS depressant activity was found to be of low magnitude. The LD₅₀ of the extract was 750 mg/Kg²². The extract also had a semen coagulant effect. Fractions obtained from the ethyl acetate extract of the whole plant of *I. coccinea* were subjected to the micronucleus test and found to be antimutagenic against a known carcinogen, 4-nitroquinoline in two *Bacillus subtilis* strains namely H₁₇-Rec⁺ and M₄₅-Rec⁺²³.

Lupeol isolated from the leaves of *I. coccinea* showed marked anti-inflammatory activity in the carrageenan induced paw oedema model in albino rats and antimitotic activity in cytotoxic studies²¹.

I. coccinea roots showed anti-inflammatory activity comparable to indomethacin²⁴. Petroleum ether extracts of *I. coccinea* roots is used against the phytopathogen, *Helminthosporium sativum*²⁵.

Antimicrobial principles have been reported from the 50% ethanolic extract of the flowers of *I. coccinea*²⁶. The therapeutic value of the alcoholic extracts of this plant against prokaryotic infection is very high²⁷. An aqueous extract of the flowers of *I. coccinea* was shown to be antimitotic to onion root tip cells²⁸. Volatile oil distilled from the flowers of *I. coccinea* was shown to possess antibacterial activity against *Bacillus subtilis*¹⁴. Ethanol extract of *I. coccinea* flowers was studied for its effect on wound healing, using a dead space wound model in rats. Significant increase in granuloma tissue weight, tensile strength, hydroxyproline and glycosaminoglycan content was observed. The prohealing action seemed to be due to increased collagen deposition as well as alignment and maturation. The drug induced a hypertrophic effect on the thymus gland but had no effect on the adrenals²⁹.

The use of *I. coccinea* flowers for a variety of skin disorders and for wound healing is interesting. The presence of a variety of flavonoids may very well contribute to this property of the extract. The present finding that *I. coccinea* hastened normal healing opens up a new era to explore the possible interactions between steroids and other drugs in influencing wound healing in a beneficial manner, especially in situations like anti cancer agent induced suppression of healing. The anticancer activity and cytotoxic and antitumour principles from *I. coccinea* flowers were reported. The intraperitoneal administration of 200 mg/Kg

of the active fraction of *I. coccinea* flowers increased the life span of DLA and EAC ascitic tumour bearing mice by 113 and 68% respectively. The active fraction showed less activity against solid tumours. It showed 50% cytotoxicity to DLA, EAC and Sarcoma - 180 cells *in vitro* at concentrations of 18, 60 and 25 µg/mL respectively. It was not toxic to normal lymphocytes, whereas it was toxic to transformed lymphocytes. The active fraction inhibited tritiated thymidine incorporation into cellular DNA^{30,31,32}.

The active fraction from *I. coccinea* flowers showed chemoprotective effects on cyclophosphamide (CYP) induced toxicity by increasing the life span of treated mice. It prevents body weight loss and maintaining near normal leucocyte and haemoglobin levels, compared with CYP treated controls. Decreased serum glutamate pyruvate transaminase and serum alkaline phosphatase levels in the *I. coccinea* treated groups indicated protection against hepatotoxicity. The active fraction was identified by phytochemical methods ursolic acid, a triterpenoid³³.

MATERIALS AND METHODS

(I) Plant material

The roots of *I. coccinea* were collected from Kottakamilzhthi Kunnu (Latitude: 104°3'; Longitude: 76°12' and Altitude: 120 m above sea level), 30 km from Thrissur District, Kerala, India in June 2008 and duly identified by Dr. P. S. Udayan, Professor, Dept of Botany, Sri Krishna College, Guruvayoor, Kerala. A voucher specimen was deposited at the herbarium of the University of Calicut for future references. The roots were washed with water, cut into small pieces and grinded in a mixer grinder.



Fig 3.2 *Ixora coccinea* L. Roots

(II) GC-MS analysis

GC-MS analysis was performed by the split injection (1:40) of 0.1 μL of the oil in hexane and hexane extract on a Shimadzu GCMS QP2010 (Japan) gas chromatograph fitted with cross bonded polyethylene glycol RTX-WAX (MS) capillary column (30 m x 0.25 mm x 0.25 μm coating thickness), coupled with a mass detector. GC-MS operating conditions are as follows: injector temperature 250 $^{\circ}\text{C}$, transfer line 250 $^{\circ}\text{C}$, detector temperature 220 $^{\circ}\text{C}$, oven temperature programme: 60 $^{\circ}\text{C}$ hold for 5 min, 110 $^{\circ}\text{C}$ with ramping 5 $^{\circ}\text{C min}^{-1}$, 200 $^{\circ}\text{C}$ with ramping 3 $^{\circ}\text{C min}^{-1}$, 220 $^{\circ}\text{C}$ with ramping 5 $^{\circ}\text{C min}^{-1}$, hold 220 $^{\circ}\text{C}$ for 15 min, carrier gas: helium at a flow rate of 1.67 mL min^{-1} , mass spectra: electron impact (EI+) at 70 eV, ion source temperature: 220 $^{\circ}\text{C}$. Individual components were identified by Wiley 139.LIB and NIST 05.LIB database matching. The percentage composition was determined by area normalization (from TIC). TIC of the essential oil/hexane extract of *I. coccinea* root are given in Appendix V.

RESULTS AND DISCUSSION

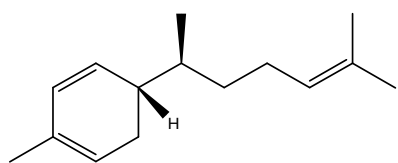
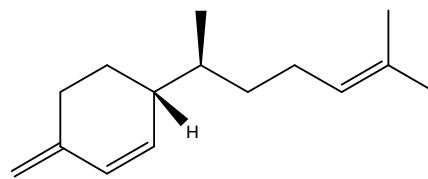
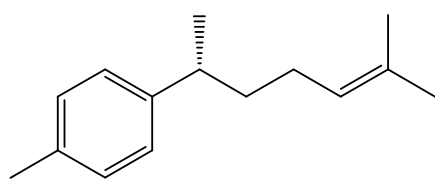
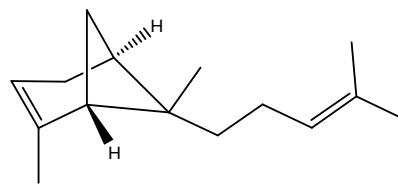
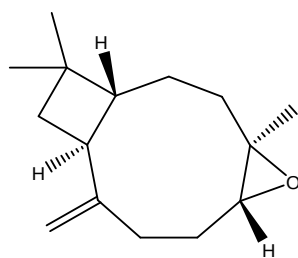
The essential oil obtained by hydrodistillation of roots of *I. coccinea* gave a yield of 0.05% v/w on fresh plant material basis. The isolated oil was a yellowish liquid with a strong aromatic fragrance. Table 3.1 shows its chemical composition. Twenty seven compounds were identified by comparison of the mass spectra of each GC component with those of the standards. Sesquiterpenes (29.93%) and fatty acids (51.26%) predominated, with the most abundant fatty acids being dodecanoic acid (21.14%) followed by octanoic acid (11.03%). The major sesquiterpenes identified include β -sesquiphellandrene (17.83%), *ar*-curcumene (2.77%), *E*- α -bergamotene (2.07%), α -zingiberene (1.92%), caryophyllene oxide (1.88%) and δ -nerolidol (1.44%). These main components comprised 60.08% of the essential oil. The fatty acids were reported previously from the roots of *I. coccinea*^{12,13,14}.

A number of constituents which were not reported previously in *I. coccinea* root essential oil were identified now. These included propylisocyanate, α -farnesene, β -farnesene, α -zingiberene, 2,4-decadienal, β -sesquiphellandrene, *ar*-curcumene, nortricyclene, *E*- α -bergamotene, caryophyllene oxide, δ -nerolidol, 1-methylbutyl nitrite, di-*n*-octyl disulphide, prop-1-enyl hydrogen carbonate, 5-octen-1-ol, 9,12,15-octadecatrien-1-ol, 1,8-nonadiene and 3,9-dodecadiyne. The Structures of some of the identified compounds from the essential oil of *I. coccinea* root are shown in Fig 3.3.

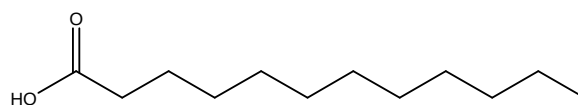
The compounds present in the cold hexane extract of the root of *I. coccinea* were identified by GC MS analysis. The major compounds identified were (RT in min) cyclopentaneundecanoic acid (5.25), nonanoic acid (10.63), azelaic acid (12.66), cyclohexanepropanoic acid (14.88) and 7-nonenoic acid (17.3).

Table 3.1 Chemical composition of essential oil of *Ixora coccinea* root

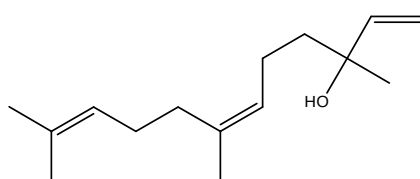
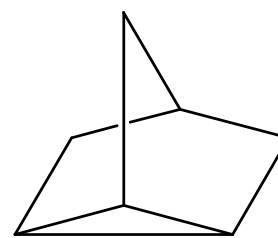
Sr No.	Retention time (min)	Compound identified	Concentration (%)
1	9.41	propylisocyanate	0.44
2	18.94	α -farnesene	0.99
3	21.39	β -farnesene	1.03
4	23.71	α -zingiberene	1.92
5	24.16	Δ -9,11-octadecadienoic acid	0.07
6	24.26	2,4-decadienal	0.43
7	24.68	β -sesquiphellandrene	17.83
8	24.76	ar-curcumene	2.77
9	25.54	Nortricyclene	0.50
10	25.72	<i>E</i> - α -bergamotene	2.07
11	29.91	Linoleic acid	2.12
12	31.24	Caryophyllene oxide	1.88
13	33.73	δ -nerolidol	1.44
14	34.02	Octanoic acid	11.03
15	39.19	1-methylbutyl nitrite	0.12
16	40.73	Decanoic acid	3.99
17	42.26	Di-n-octyl disulphide	0.13
18	45.23	Prop-1-enyl hydrogen carbonate	0.09
19	46.93	Dodecanoic acid	21.14
20	48.22	5-octen-1-ol	0.39
21	51.59	Oleic acid	1.46
22	52.19	Tetradecanoic acid	3.12
23	52.63	9,12,15-octadecatrien-1-ol	7.47
24	54.04	1,8-nonadiene	0.15
25	57.08	Stearic acid	0.62
26	58.51	3,9-dodecadiyne	7.59
27	59.99	Hexadecanoic acid	7.71
	-	Total Isolate	98.50
	-	Unknown	1.50

*α*-zingiberene*β*-sesquiphellandrene*ar*-curcumene*E-α*-bergamotene

Caryophyllene oxide



Dodecanoic acid

*δ*-nerolidol

Nortricyclene

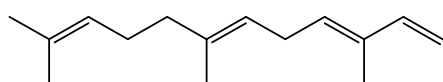
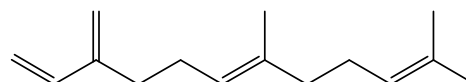
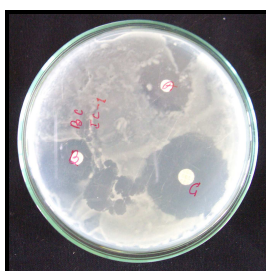
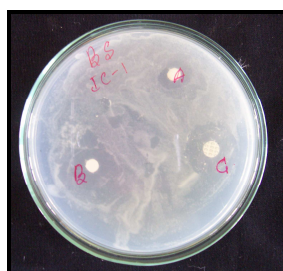
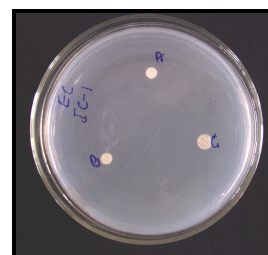
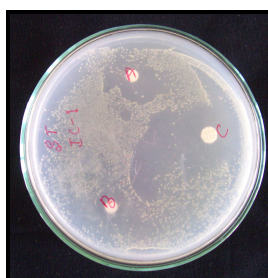
*α*-farnesene*β*-farnesene

Fig 3.3 Structure of some of the identified compounds from the essential oil of *I. coccinea* root

The antibacterial and antifungal activities of the *I. coccinea* root essential oil have been assayed at concentrations of 1% and 2% against strains of both Gram-positive and Gram-negative bacteria and pathogenic fungi. The susceptibility testing was carried out by measuring the inhibitory zone diameters on nutrient agar, with conventional disc diffusion method and the inhibitory zone diameters were read and rounded off to the nearest whole number (mm) for analysis. The inhibitory effects of the essential oil against these microorganisms are given in Tables 3.2 and 3.3.

Plate 3.4.1 *Bacillus cereus*Plate 3.4.2 *Bacillus subtilis*Plate 3.4.3 *Escherichia coli*Plate 3.4.4 *Salmonella typhimurium*Plate 3.4.5 *Staphylococcus aureus*

A = 1% test solution in DMSO
B = 2% test solution in DMSO
C = 0.1% Cephatoxime in DMSO

Fig 3.4 Antibacterial activities of essential oil of *I. coccinea* root against different strains

Table 3.2 *In vitro* antibacterial activity of essential oil of *Ixora coccinea* root

Tested bacteria	Diameter of inhibition zone (mm) ^a		
	Essential oil ^b		Cephatoxime ^b
	1%	2%	0.1%
<i>Bacillus cereus</i>	12±0.04	15±0.05	35±0.08
<i>Bacillus subtilis</i>	10±0.01	15±0.02	26±0.04
<i>Escherichia coli</i>	10±0.05	10±0.05	35±0.07
<i>Salmonella typhimurium</i>	15±0.08	15±0.07	30±0.08
<i>Staphylococcus aureus</i>	12±0.05	10±0.02	40±0.06

^aValues are the mean of three replicates

^bStandard and test solutions prepared in DMSO

Control DMSO = No activity against tested microorganisms

Plate 3.5.1 *Aspergillus niger*Plate 3.5.2 *Fusarium oxysporum*Plate 3.5.3 *Aspergillus flavus*

A = 1% test solution in DMSO

B = 2% test solution in DMSO

C = 0.2% Bavistin in DMSO

Fig 3.5 Antifungal activities of essential oil of *Ixora coccinea* root against different strains

Table 3.3 *In vitro* antifungal activity of essential oil of *Ixora coccinea* root

Tested pathogenic fungi	Diameter of inhibition zone (mm) ^a		
	Essential oil ^b		Bavistin ^b
	1%	2%	0.2%
<i>Alternaria alternata</i>	-	-	-
<i>Aspergillus flavus</i>	8±0.04	10±0.06	40±0.04
<i>Aspergillus niger</i>	-	20±0.06	30±0.02
<i>Fusarium oxysporum</i>	-	8±0.03	25±0.03
<i>Penicillium notatum</i>	-	-	35±0.06

^aValues are the mean of three replicates

^bStandard and test solutions prepared in DMSO

Control DMSO = No activity against tested microorganisms

The screening results indicate that the essential oil showed good antibacterial activity against both Gram-negative bacterial strains *Escherichia coli* (MTCC-2125) and *Salmonella typhimurium* (MTCC-1357) and Gram-positive bacterial strains *Bacillus subtilis* (MTCC-1789), *B. cereus* (MTCC-1272) and *Staphylococcus aureus* (MTCC-3160). Similarly, the screening results against the pathogenic fungi indicated that the essential oil showed moderate antifungal activity against *Aspergillus flavus* (MTCC-3306), *A. niger* (MTCC-2208) and *Fusarium oxysporum* (MTCC-1755) and no activity against *Alternaria alternata* (MTCC-1362) and *Penicillium notatum* (MTCC-2647).

In the present study, we may attribute the antibacterial and antifungal activities of the root essential oil of *I. coccinea* are due to the higher percentage of various sesquiterpenes in it. The antifungal activity of caryophyllene oxide has been well established, which constitutes 1.88% of essential oil³⁶. Antimicrobial activities of essential oils are well documented, which are found application as antibacterial, antifungal or antiviral agents for food preservatives, in clinical microbiology or in pharmaceutical preparations^{37,38}. According to literatures, these components exert antimicrobial effects through the disruption of bacterial or fungal membrane integrity and the inhibition of respiration and ion transport processes. With the alarming incidence of antibiotic resistance in microorganisms of medical importance, there is increasing interest in plants as sources of agents for the treatment of infectious diseases^{39,40,41,42,43}. Additionally, the main advantage of naturally occurring antimicrobial agents is that they do not enhance resistance among microorganisms, a phenomenon commonly encountered with the use of synthetic antibiotics⁴⁴.

EXPERIMENTAL

(I) Isolation of the essential oil

The essential oil was obtained by hydrodistillation of the root (900 g) in a Clevenger apparatus (using 3L water) for 3 h. The essential oil obtained was dissolved in diethyl ether (10 mL). The organic phase was dried over anhydrous sodium sulphate and filtered. After removal of the solvent, yellow transparent oil (0.05% v/w) with pleasant odour was obtained which was stored in refrigerator at 4 °C until analyzed.

(II) n-hexane extract of *I. coccinea* root

The dried and coarsely powdered root of *I. coccinea* (50 g) was soaked in 300 mL of n-hexane in room temp for 24 h with intermittent shaking. It was then filtered and hexane was removed under suction. The residue obtained was subjected to GC MS analysis to identify the components present it.

(III) Antimicrobial assay

Test microorganisms employed for *in vitro* antimicrobial assay were obtained from P. G. Centre, Department of Microbiology, Kuvempu University, Dhavangare, Karnataka, India. The antimicrobial assay of the essential oil was performed by standard filter paper disc diffusion technique^{34,35}. A total of three Gram-positive, two Gram-negative bacteria (Fig 3.4) and five pathogenic fungi (Fig 3.5) were used for this antimicrobial screening. The test solution at two different concentrations (1% and 2%) was prepared by dissolving the essential oil in Dimethyl sulphoxide (DMSO). Cephatoxime (0.1%) and bavistin (0.2%) were used as standards for antibacterial and antifungal activities respectively. A blank disc impregnated with DMSO followed by drying off was used as a negative control.

Briefly, the test discs, standard discs and blank discs were placed in the Petri dishes that contain the specific bacterial or fungal culture and then left in a refrigerator at 4 °C for 12-18 h in order to diffuse the material from the discs to the surrounding media. The Petri dishes were then incubated at 37 °C for overnight to allow the bacterial growth and 48-72 h for fungal growth. The antibacterial and antifungal activities of the essential oil were then determined by measuring the respective zones of inhibition in mm.

CONCLUSIONS

I. coccinea is regarded as a valuable plant source in both traditional medicine and modern drug development. The various compounds identified from the root essential of *I. coccinea* are reported to possess a wide range of pharmacological activities. The results from the present study offer a scientific proof for the traditional use of *I. coccinea* roots. The essential oil from the root was found effective against some bacterial and fungal pathogens which are associated with fever, skin diseases, dysentery and respiratory problems. The antimicrobial activity results presented here demonstrate that this plant essential oil has a commercial potential. The antimicrobial activity shown by the oil may be due to a synergistic effect of various components present in it. It is usually found that such combined effect of various components is responsible for therapeutic activities of the plant extracts. However, this needs further investigation.

Though the existing chemical literature on this important medicinal plant is impressive, the characterization of still unexplored constituents remain open to future investigation.

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Chapter 4

Srinivasan G. V. “phytochemical investigation and biological studies of secondary metabolites from some folk medicinal plants” Thesis. Department of Chemistry, University of Calicut, 2015



***Costus pictus* D. Don**

CHAPTER – 4

***IDENTIFICATION OF CHEMICAL COMPOUNDS IN THE
ESSENTIAL OIL FROM THE COSTUS PICTUS D. DON
PLANT PARTS, ITS ANTIMICROBIAL STUDIES &
ISOLATION AND QUANTIFICATION OF DIOSGENIN
FROM ITS ROOT***

INTRODUCTION

The genus *Costus* Linn. belongs to the family Costaceae. On the basis of the presence of spirally arranged leaves and rhizomes being free from aromatic essential oils, it has been separated from family Zingiberaceae. More than 100 species of the genus are distributed in the tropics all over the world. *Costus pictus* D. Don syn. *Costus mexicanus* (DC.) Greene is commonly known as Spiral ginger, Stepladder or Insulin plant. It is a plant originated in Mexico and introduced from there to India very recently. In India it is grown in gardens as ornamental plant

especially in Kerala state. The major attraction of this plant is its stem with spiral leaves and light airy and tissue paper like flowers. Red painted stem enhances the beauty of the glossy linear leaves and strongly spiralling canes¹.

The people in Kerala used to consume the fresh raw leaves for its anti-diabetic properties. The hypoglycaemic activity of *C. pictus* is mainly because of secondary metabolites present in it¹. Despite some preliminary studies the detailed phytochemical investigation of this species has not been reported so far.

Costus speciosus (Koenig ex Retz) Smith, the species similar to *C. pictus* is commonly known as Channakkoova in Malayalam and Keu in Hindi. The leaves of *C. speciosus* are less fleshy and have an acrid taste. Its rhizomes are cooked and eaten, the roots are used as tonic and anthelmintic and the leaves for curing diabetes¹.

(I) Description of the plant

The *C. pictus* plant grows up to 2–3 m and spread 1.5–2 m. The flowers of the plant are in a terminal cone, yellow in colour with an orange red tip. The flowers last for 3–4 days. The flowers are displayed in a dramatic form high above the leaves. While the flowers do not produce any aroma, they do make a beautiful effect sitting atop of the tall spiralling stems. The propagation of the plant is carried out through stem cuttings and also from rhizomes. Like *C. speciosus*, the leaves of this species are less fleshy and have an acrid taste. Its rhizomes are cooked and eaten. People of Kerala eat the leaves for curing diabetes. The roots are used as tonic and anthelmintic¹.

(II) Abstract of the present work

The Chapter-4 is divided into 2 sections. Section-4.1 describes the extraction of essential oil/hydrodistillate from the different plant parts of *C. pictus*, identification of the chemical compounds present in them by GC-MS analysis and the screening of antimicrobial activity of the essential oils by disc diffusion method. Section-4.2 describes the isolation, identification and quantification of diosgenin from the root of *C. pictus*.

The literature survey revealed that only very few phytochemical works has been reported in this plant. Even though there are few reports on the chemical compounds from the essential oil of *C. pictus*, it has not covered all the plant parts. Moreover their antibacterial studies have been limited to the leaf essential oil. Hence in the present work, all the plant parts – root, rhizome, leaf, stem and flower of *C. pictus* has been taken and their antifungal activities has been studied for the first time in addition to their antibacterial activities. A number of compounds has bee identified by GC-MS analysis in addition to the already reported ones from the different plant parts namely stem, leaf and flower essential oil of *C. pictus*. The antibacterial and antifungal activities of essential oil from all plant parts were also investigated by disc diffusion method and a made it a comprehensive work.

Diosgenin, a steroid saponin has been isolated for the first time from the root of *C. pictus*. It was identified by m.p., mixed m.p & co-TLC with the reference standard. The quantification of diosgenin in root of *C. pictus* was also carried out by HPLC method.

REVIEW OF LITERATURE

Costus pictus is well known for its antidiabetic properties¹. The antidiabetic effect of the methanol extract of *C. pictus* leaves has been reported². The earlier studies of the plant extracts on alloxan induced diabetic rats have shown the prevention of diabetes and related complications. The methanolic leaf extract of *C. pictus* is used to lower blood glucose level in alloxan induced diabetic rats³. The powdered leaves of *C. pictus* are known to possess therapeutic effect. When supplemented to streptozotocin induced diabetic rats, it is found to reduce blood glucose level by 21% after 15 days of supplementation⁴.

The antioxidant and anticancer properties of the bark of *C. pictus* has been reported. The chloroform fraction of the bark of *C. pictus* possesses highest antioxidant activity. Chloroform and methanol extracts of *C. pictus* has been reported to possess potent anticancer properties against HT29 and A549 cells⁵.

The chloroform extract of *C. pictus* leaf has been reported to possess superoxide radical scavenging activity, inhibition of lipid peroxidation and potent antioxidant activity *in vitro*⁶.

The antibacterial activities of the aqueous and methanol extracts of the leaf, root, stem and flower of *C. pictus* has been reported. The antioxidant activity of these different extracts was evaluated by DPPH free radical scavenging activity and in all case methanol extract was reported to have highest antioxidant activity. The total phenolic content in the leaf, root, stem and flower of *C. pictus* has been reported and ranged from 148 to 233 µg of gallic acid/g of plant extracts. Also the flavonoid content has been reported varies from 370 to 930 µg of quercetin/g of plant extracts⁷.

Jayasri et al. estimated the presence of trace elements in *C. pictus* and reported that the leaves and rhizomes contain appreciable amounts of various elements such as K, Ca, Cr, Mn, Cu and Zn. These trace elements may be responsible for potentiating insulin action⁸. *C. pictus* leaf has been reported to contain various phytochemicals like alkaloids, glycosides, carbohydrates, saponins, proteins and phenols^{9,10}. Shilpa et al. isolated methyl tetracosanate from bioactivity guided purification of methanol extract of *C. pictus* leaves which showed an optimum glucose uptake at 1 ng/mL in 3T3-L1 adipocytes¹¹.

Beena and Reddy analyzed the volatile constituents of *C. pictus* stems, leaves and rhizomes and palmitic acid was found to be the major component in the stem, leaf and rhizome¹². The essential oils of the stems, leaves and rhizomes of *C. pictus* from southern India were reported. The essential oil of *C. pictus* stem is rich in hexadecanoic acid (28.3%) followed by 9, 12-octadecadienoic acid (18.33%), linalyl propanoate (6.03%), dodecanoic acid (5.62%), tetradecanoic acid (4.82%), α -eudesmol (3.55%), γ -eudesmol (3.21%) and 4-ethoxy phenol (3.06%). The leaf essential oil contains hexadecanoic acid (24.51%), 2-pentanol (22.48%), β -ionone (8.69%), α -ionone (8.01%), farnesyl acetone (7.04%) and dodecanoic acid (3.96%). Hexadecanoic acid (25.26%), dodecanoic acid (16.56%), tetradecanoic acid (10.20%), linalool (8.48%), 9, 12-octadecadienoic acid (7.74%) and α -terpineol (4.44%) are the main constituents of the rhizome essential oil¹².

Shiny et al. isolated a glycosidic compound from the methanol extract of *C. pictus* leaf which was similar to β -L-Arabinopyranose methyl glycoside and suggested that it might be the inducer molecule of its antidiabetic property¹³.

The antibacterial property of the *C. pictus* leaf essential oil was checked against ten pathogenic bacteria namely *Bacillus cereus*, *Enterobacter faecalis*, *Salmonella paratyphi*, *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus faecalis*,

Proteus vulgaris, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Serratia marcescens* and they showed varying degrees of antibacterial activity on the microorganisms tested¹⁴.

A number of phytochemical and pharmacological works were reported from its closely related species, *Costus speciosus* and are given as follows. The important chemical constituents reported from *C. speciosus* are Diosgenin, glyceollins II and III, tigogenin, α -amyrin stearate, β -amyrin, lupeol etc¹⁵. The leaves were reported to contain the antifungal compounds. Palmitates from the leaves and many aliphatic esters from its rhizomes, seeds and roots were reported. Seeds in addition contain α -tocopherol. The rhizomes are rich in starch and fibre¹⁵.

Dasgupta et al. reported diosgenin and tigogenin in the rhizomes of *C. speciosus*¹⁶. Sarin et al. determined the diosgenin content in the rhizome of *C. speciosus*¹⁷.

Gupta et al. isolated and characterized new compounds viz, 5 α -stigmast-9,11-en-3 β -ol, 24-hydroxy hentriacontan-27-one and 24-hydroxytriacontan-26-one from the root of *C. speciosus*¹⁸. Gupta et al. also reported new compounds such as 14-oxotricosanoic acid, 14-oxooctacosanoic acid etc. from the rhizome of *C. speciosus*^{19,20}. De Kraker et al. isolated Germacrenes from fresh *costus* roots²¹.

Singh et al. reported eight new saponins namely consticoside A, B, C, D, E, F, G and H from the seeds²². Mahmood et. al isolated and characterized three new quinones and α -tocopherol from the seeds of *C. speciosus*^{23,24}.

Six compounds were isolated from the rhizome of *C. speciosus* and elucidated as diosgenin, prosapogenin B of dioscin, diosgenone, cycloartanol, 25-en-cycloartenol and octacosanoic acid²⁵. Four compounds were isolated from the

rhizome of another related species *C. tonkinensis* and elucidated as tetracosanoic acid, succinic acid, β -sitosterol and daucosterin²⁵.

C. speciosus has been reported for the pharmacological activities like antifungal, anti-inflammatory, astringent, stimulant, depurative, anthelmintic, antidiabetic, hyperglycemic etc¹⁵.

SECTION - 4.1

IDENTIFICATION OF CHEMICAL COMPOUNDS IN THE ESSENTIAL OIL FROM THE COSTUS PICTUS D. DON PLANT PARTS & ITS ANTIMICROBIAL STUDIES

MATERIALS AND METHODS

(I) Plant material

The root, rhizome, stem, leaf and flower of *Costus pictus* were collected from Kozhikode District, Kerala, India during May 2014 and duly identified by Dr. P. S. Udayan, Professor, Dept of Botany, Sri Krishna College, Guruvayoor, Kerala. A voucher specimen was deposited at the herbarium of the University of Calicut for future references. The root, rhizome, stem and leaf were washed with water, cut into small pieces and grinded in a mixer grinder. The flowers were also washed with water and grinded in a mixer grinder.



Fig 4.1.1 *Costus pictus* – roots & rhizomes

(II) GC-MS analysis

GC-MS analysis was performed by the split injection (1:10) of 1.0 μL of the essential oil in hexane on a Perkin Elmer GC (Clarus 680) fitted with Elite 5 cross bonded 5% diphenyl-95% dimethyl polysiloxane capillary column (30 m x 0.25 mm x 0.25 μm coating thickness), coupled with a mass detector (Clarus 600 T) and Turbo Mass version 5.4.2 software. GC-MS operating conditions are as follows: injector temperature: 250 $^{\circ}\text{C}$ for all plant parts; transfer line temperature: 280 $^{\circ}\text{C}$ for all plant parts; detector temperature: 280 $^{\circ}\text{C}$ (for flower & stem), 300 $^{\circ}\text{C}$ (for leaf, root & rhizome).

Oven temperature programme: (i) for flower: 100 $^{\circ}\text{C}$ hold for 5 min, 250 $^{\circ}\text{C}$ with ramping 5 $^{\circ}\text{C min}^{-1}$, hold 250 $^{\circ}\text{C}$ for 10 min. (ii) for leaf: 150 $^{\circ}\text{C}$ hold for 10 min, 250 $^{\circ}\text{C}$ with ramping 3 $^{\circ}\text{C min}^{-1}$, 275 $^{\circ}\text{C}$ with ramping 1 $^{\circ}\text{C min}^{-1}$, hold 275 $^{\circ}\text{C}$ for 5 min. (iii) for stem: 60 $^{\circ}\text{C}$ hold for 5 min, 110 $^{\circ}\text{C}$ with ramping 5 $^{\circ}\text{C min}^{-1}$, 220 $^{\circ}\text{C}$ with ramping 3 $^{\circ}\text{C min}^{-1}$, 250 $^{\circ}\text{C}$ with ramping 5 $^{\circ}\text{C min}^{-1}$, hold 250 $^{\circ}\text{C}$ for 5 min. (iv) for root & rhizome: 100 $^{\circ}\text{C}$ hold for 5 min, 280 $^{\circ}\text{C}$ with ramping 10 $^{\circ}\text{C min}^{-1}$, hold 280 $^{\circ}\text{C}$ for 10 min.

Carrier gas: helium at a flow rate of 1.0 mL min^{-1} , mass spectra: electron impact (EI+) at 70 eV, ion source temperature: 250 $^{\circ}\text{C}$ (for flower & stem), 280 $^{\circ}\text{C}$ (for leaf, root & rhizome). Individual components were identified by NIST database

matching. The percentage composition was determined by area normalization from TIC. TIC of the essential oil of different plant parts of *C. pictus* are given in Appendix VI.

RESULTS AND DISCUSSION

The essential oil/ hydrodistillate obtained by the hydrodistillation of flower, leaves, stem, rhizome and root of *C. pictus* gave a yield of 0.06% v/w, 0.02% v/w, 0.10% v/w, 0.03% v/w and 0.05% v/w respectively on fresh plant material basis. Table 4.1.1 shows the chemical composition of the essential oil/ hydrodistillate from the different plant parts of *C. pictus*. The chemical compounds were identified by comparison of the mass spectra of each GC component with those of the standards available by library search. Palmitic acid (n-hexadecanoic acid) was found to be the major constituent in all the five hydrodistillate together with other fatty acids, hydrocarbons and mono, di and sesquiterpenes.

The hydrodistillate from the flowers of *C. pictus* contains mono and sesquiterpenes such as thymol (5.23%), nerolidol (0.91%), α -farnesene (0.67%), caryophyllene (0.28%) and trans-Z- α -bisabolene epoxide (0.22%) together with other compounds. The leaf hydrodistillate contains β -sitosterol acetate (0.18%), diterpenes such as phytol (2.16%), isophytol (1.64%) and geranyl geraniol (1.21%) together with other compounds. The stem hydrodistillate contains 5.66% of phytol together with other compounds. The hydrodistillate from the rhizome of *C. pictus* contains monoterpenes such as thymol (0.66%) and β -myrcene (0.08%) together with other compounds. The hydrodistillate from the root of *C. pictus* contains mainly fatty acids and long chain hydrocarbons. The chemical compounds identified from the hydrodistillate of all plant parts of *C. pictus* are given in Table 4.1.1.

Table 4.1.1 Chemical compounds identified in the hydrodistillate of *Costus pictus* plant parts

Sr.no	Name of the compound	Flower (RT min, Conc %)	Leaf (RT min, Conc %)	Stem (RT min, Conc %)	Rhizome (RT min, Conc %)	Root (RT min, Conc %)
1	Thymol	10.06, 5.23	-	-	8.19, 0.66	-
2	β -caryophyllene	13.22, 0.28	-	-	-	-
3	Trans-Z- α -bis-abolene epoxide	15.42, 0.22	-	-	-	-
4	α -farnesene	16.70, 0.67	-	-	-	-
5	δ -nerolidol	24.28, 0.91	-	-	-	-
6	Homopiperazine	20.22, 0.52	-	-	-	-
7	Geranyl geraniol	-	27.27, 1.21	-	-	-
8	Isophytol	-	28.11, 1.64	-	-	-
9	Phytol	-	31.53, 2.16	27.14, 5.66	-	-
10	β -myrcene	-	-	-	7.23, 0.08	-
11	Kaur-16-ene	-	-	-	-	16.96, 11.04
12	Thunbergol	-	-	-	-	19.41, 4.24
13	β -sitosterol acetate	-	21.74, 0.18	-	-	-
14	n-decanoic acid	12.12, 0.26	-	-	-	-
15	Tetradecane	12.67, 0.51	22.66, 0.13	-	9.98, 1.87	-
16	Dodecanoic acid	17.25, 0.80	17.73, 0.11*	-	12.37, 0.14*	-
17	Tetradecanoic acid	21.54, 1.71	24.46, 1.55	-	14.76, 1.72*	14.67, 0.52
18	E-7-octadecene	22.89, 0.06	-	-	-	-

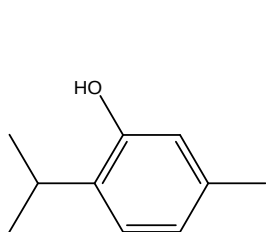
19	Pentadecanoic acid	23.58, 1.85	26.70, 0.68	-	16.83, 2.33	-
20	5-methyl heneicosane	24.10, 0.05	-	-	-	-
21	Heptacosanoic acid methyl ester	24.55, 0.03	-	-	-	-
22	n-hexadecanoic acid	26.55, 36.82	30.12, 43.50*	23.14, 46.86*	17.04, 46.23*	16.85, 38.17
23	Eicosanoic acid	27.50, 0.88	25.35, 1.24	15.17, 2.84	15.38, 0.02	15.75, 0.65
24	1-octadecyne	27.78, 0.97	-	-	-	12.95, 0.12
25	Octacosane	27.87, 0.72	40.67, 0.15	-	19.90, 2.58	17.16, 0.20
26	Tritetracontane	27.93, 0.04	34.78, 0.03	-	-	-
27	9,12-octadecadienoic acid	29.51, 28.68	-	28.30, 18.94*	18.58, 14.03	-
28	Octadecanoic acid	29.69, 0.35	-	-	-	-
29	17-pentatriacontene	30.93, 0.24	25.79, 0.04	26.92, 0.06	-	-
30	Hexacosane	31.61, 5.64	-	-	-	-
31	Hexatriacontane	33.07, 2.15	38.79, 0.12	29.97, 0.48	12.80, 0.09	15.11, 0.21
32	1-octacosanol	34.19, 1.28	-	-	-	-
33	Tetratetracontane	34.74, 3.22	-	32.86, 0.14	-	16.17, 0.04
34	1-heneicosanol	37.62, 0.50	-	-	-	-
35	1-decosene	37.78, 0.21	-	-	-	-
36	1-tetracosanol	38.02, 0.43	-	-	-	-
37	Tetracosane	38.18, 0.55	-	-	14.0, 0.02	-

38	2-aminonona- decane	-	14.68, 0.05	-	-	-
39	Dotriacontane	-	15.43, 0.94	23.67, 1.08	16.18, 0.01	19.02, 0.11
40	Hexadecane	-	18.35, 0.09	-	-	9.97, 5.83
41	Hexadecyloxira- ne	-	26.25, 0.02	21.71, 0.52	-	-
42	2-hexyl-1- decanol	-	26.36, 0.76	-	-	-
43	Z-5,17- octadecadien-1- ol acetate	-	32.86, 19.89	-	-	-
44	1-pentacontanol	-	33.01, 0.48	-	-	-
45	Pentatriacontane	-	35.97, 0.06	-	-	-
46	n- butylmyristate	-	-	18.88, 0.26	-	-
47	2-methyl- octadecane	-	-	20.14, 0.08	-	-
48	9,12- tetradecadien-1- ol	-	-	28.43, 6.32	-	-
49	1-hexacosene	-	-	28.95, 0.75	-	-
50	14-hepta- decenal	-	-	-	12.96, 0.03	-
51	Octadecanal	-	-	-	14.18, 0.89	-
52	Heneicosane	-	-	-	15.13, 0.07	13.99, 0.08
53	Oleic acid	-	-	-	15.46, 0.02	-
54	Heptadecanoic acid	-	-	-	17.79, 0.07	-
55	9,17-	-	-	-	18.61, 4.65	-

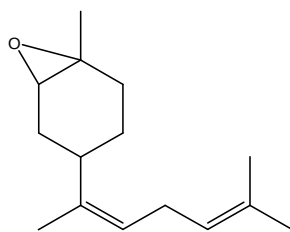
	octadecadienal					
56	Heptacosane	-	-	-	20.73, 0.03	18.11, 0.02
57	11-tridecen-1-ol	-	-	-	-	10.11, 0.02
58	2-heptadecenal	-	-	-	-	13.72, 0.10
59	Hexadecanal	-	-	-	-	14.17, 3.96
60	Octadecanol	-	-	-	-	15.30, 0.02
61	Tetradecanal	-	-	-	-	16.36, 0.01
62	9-octadecenal	-	-	-	-	18.48, 0.17
	Total isolate	95.78	75.62	84.01	75.54	65.51
	Unidentified	4.22	24.38	15.99	24.46	34.49

* Previously reported compounds

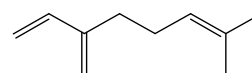
The structure of some of the identified compounds from the hydrodistillate of *C. pictus* plant parts are shown in Fig 4.1.2.



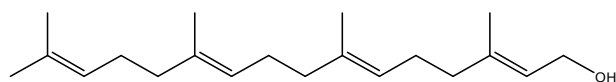
Thymol



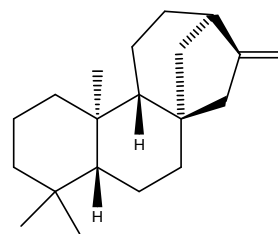
Trans-Z- α -bisabolene epoxide



β -myrcene



Geranyl geraniol



Kaur-16-ene

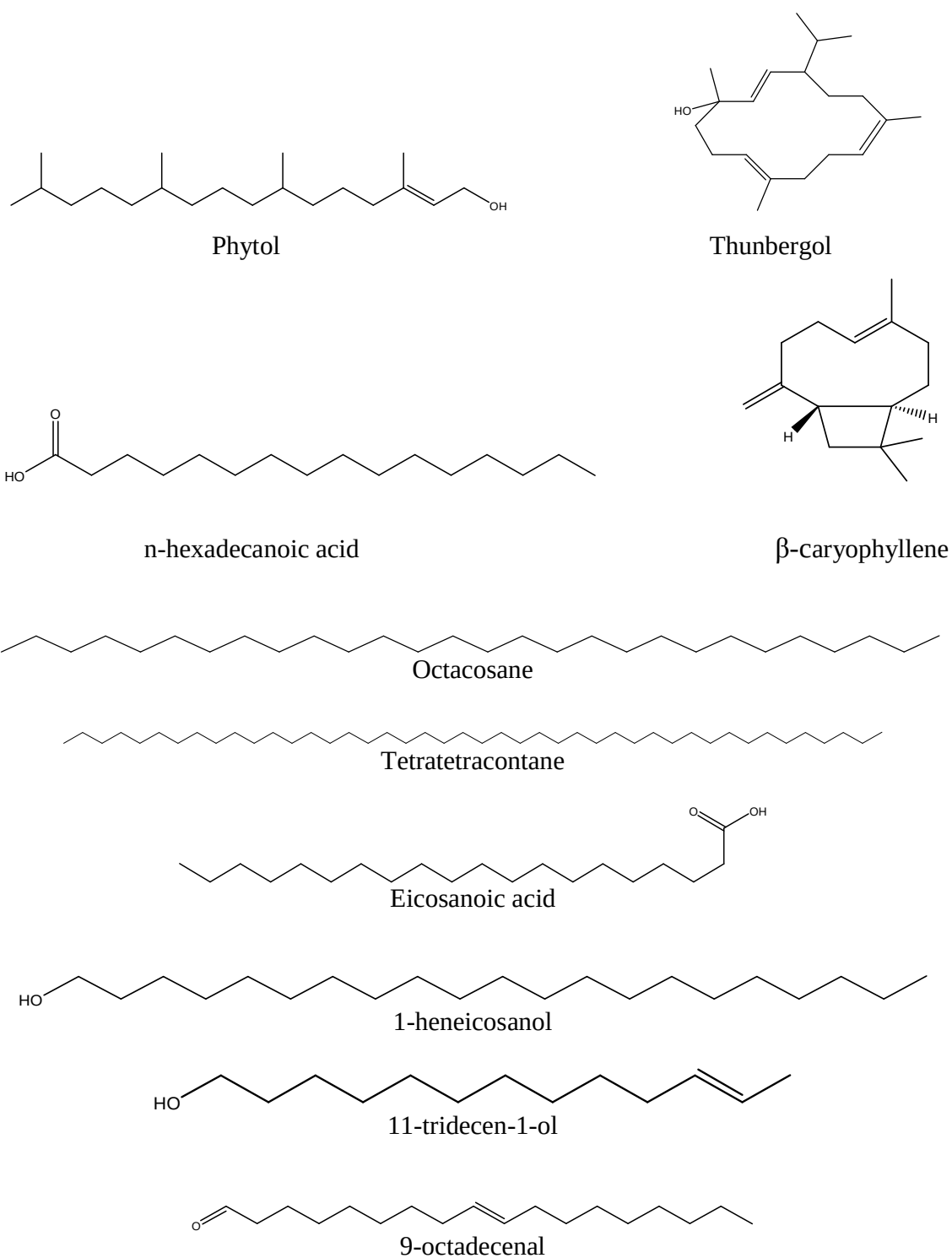
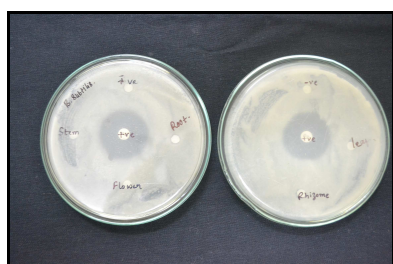


Fig 4.1.2 Structure of some of the identified compounds from the hydrodistillate of *C. pictus* plant parts

Phytol, a diterpene alcohol is used in the fragrance industry. It is also used in the cosmetics, shampoos, toilet soaps, household cleaners and detergents. Phytol can be used as a precursor for the manufacture of synthetic forms of Vitamins E and K1. β -myrcene and δ -nerolidol are also used in the perfumery. Gernayl geraniol is an intermediate in the biosynthesis of vitamins E and K. It is pheromone for a variety of insects and a potent inhibitor of *Mycobacterium tuberculosis*. Trans-Z- α -bisabolene epoxide and α -farnesene are also act as pheromone.

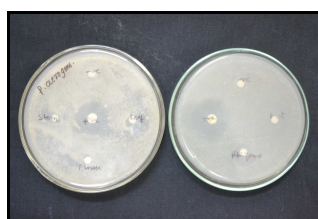
The antibacterial and antifungal activities of the hydrodistillate of *C. pictus* plant parts viz., flower, leaves, stem, rhizome and root have been assayed at concentration of 2% against strains of both Gram positive and Gram negative bacteria and pathogenic fungi. The susceptibility testing was carried out by measuring the inhibitory zone diameters on nutrient agar, with conventional disc diffusion method and the inhibitory zone diameters were read and rounded off to the nearest whole number (mm) for analysis. The inhibitory effects of the hydrodistillate against these microorganisms are given in Tables 4.1.2 and 4.1.3.



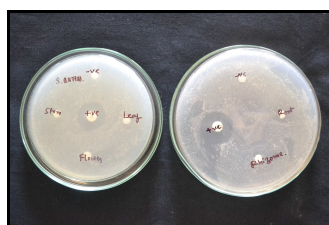
Bacillus subtilis



Escherichia coli



Pseudomonas aeruginosa



Staphylococcus aureus



Salmonella typhimurium

Fig 4.1.3 Antibacterial activities of hydrodistillate of *C. pictus* plant parts against different strains

Table 4.1.2 *In vitro* antibacterial activity of essential oil of *C. pictus* plant parts

Sample ^b	<i>Escherichia coli</i>	<i>Bacillus subtilis</i>	<i>Pseudomonas aeruginosa</i>	<i>Staphylococcus aureus</i>	<i>Salmonella typhimurium</i>
	Diameter of Inhibition zone (mm) ^a				
Positive Control (Streptomycin)	20 ± 0.05	25 ± 0.06	20 ± 0.03	15 ± 0.06	25 ± 0.08
Negative Control (Hexane)	-	-	-	-	-
Leaf hydrodistillate	5 ± 0.02	-	5 ± 0.06	6 ± 0.02	-
Stem hydrodistillate	5 ± 0.02	-	4 ± 0.02	8 ± 0.05	-
Flower hydrodistillate	5 ± 0.04	6 ± 0.03	6 ± 0.05	6 ± 0.05	-
Root hydrodistillate	-	5 ± 0.01	8 ± 0.04	10 ± 0.08	6 ± 0.03
Rhizome hydrodistillate	-	6 ± 0.03	15 ± 0.09	7 ± 0.04	-

^aValues are the mean of three replicates

^bStandard and test solutions prepared in hexane

The screening results indicate that the leaf and stem hydrodistillates showed moderate antibacterial activity against both Gram negative bacterial strains *Escherichia coli* (MTCC-2125), *Pseudomonas aeruginosa* (MTCC-1688) and Gram positive bacterial strain *Staphylococcus aureus* (MTCC-3160) and no activity against Gram negative bacterial strain *Salmonella typhimurium* (MTCC-1357) and Gram positive bacterial strain *Bacillus subtilis* (MTCC-1789). The flower hydrodistillate showed moderate antibacterial activity against all the four bacterial strains except *S. typhimurium*. The root hydrodistillate showed good antibacterial activity against *S. aureus*; moderate antibacterial activity against *B. subtilis*, *P. aeruginosa* and *S. typhimurium* and no activity against *E. coli*. The

rhizome hydrodistillate showed good antibacterial activity against *P. aeruginosa*; moderate antibacterial activity against *B. subtilis* and *S. aureus* and no activity against *E. coli* and *S. typhimurium*.

E. coli strain was highly susceptible with MIC to 13.5 ± 0.3 mg/mL with leaf, stem and flower hydrodistillates. *B. subtilis* strain was highly susceptible with MIC to 11 ± 0.6 mg/mL with flower, root and rhizome hydrodistillates. *P. aeruginosa* was susceptible with MIC to 14.2 mg/mL with stem hydrodistillate; 13 ± 0.5 mg/mL with leaf and flower hydrodistillates; 8.39 mg/mL with root hydrodistillate and 2.6 mg/mL with rhizome hydrodistillate. *S. aureus* was susceptible with MIC to 12.8 ± 0.2 mg/mL with flower and leaf hydrodistillates; 8.65 ± 0.7 mg/mL with stem and rhizome hydrodistillates and 4.7 mg/mL with root hydrodistillate. *S. typhimurium* was susceptible with MIC to 14 mg/mL with root hydrodistillate.

Similarly, the screening results against the pathogenic fungi indicated that only root and leaf hydrodistillates showed moderate antifungal activity against *Aspergillus tamarii* (MTCC-8841) and *Rhizopus stolonifer* (MTCC-2591) respectively and showed no activity against other pathogenic fungi tested namely *Fusarium oxysporum* (MTCC-4118), *Cladosporium cladosporioides* (MTCC-3507) and *Nigrospora oryzae* (MTCC-8465). The hydrodistillate from other plant parts showed no activity against these five tested fungi.

Aspergillus tamarii strain was highly susceptible with MIC to 11.5 ± 0.3 mg/mL with root hydrodistillate. *Rhizopus stolonifer* strain was highly susceptible with MIC to 13 ± 0.6 mg/mL with leaf hydrodistillate.

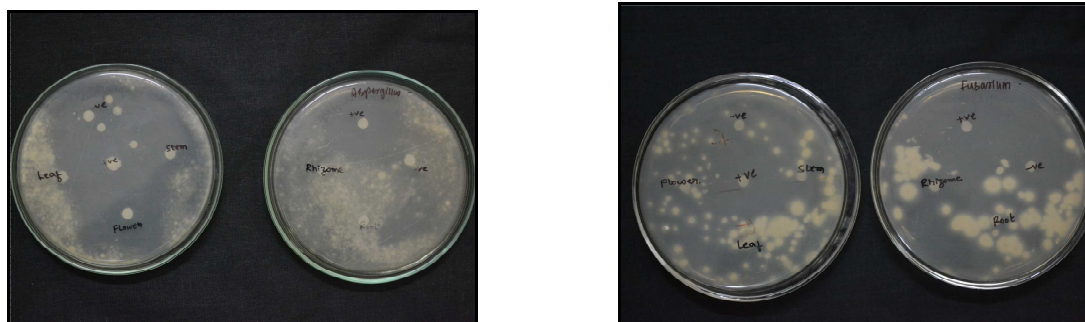
*Aspergillus tamari**Fusarium oxysporum*

Fig 4.1.4 Antifungal activities of hydrodistillate of *C. pictus* plant parts against different strains

Table 4.1.3 *In vitro* antifungal activity of hydrodistillate of *C. pictus* plant parts

Sample ^b	<i>Fusarium oxysporum</i>	<i>Aspergillus tamarii</i>	<i>Rhizopus stolonifer</i>	<i>Cladosporium cladosporioides</i>	<i>Nigrospora oryzae</i>
	Diameter of Inhibition zone (mm) ^a				
Positive Control (Bavistin)	30 ± 0.09	36 ± 1.0	40 ± 0.07	38 ± 0.06	52 ± 1.2
Negative Control (Hexane)	-	-	-	-	-
Leaf hydrodistillate	-	-	5 ± 0.03	-	-
Stem hydrodistillate	-	-	-	-	-
Flower hydrodistillate	-	-	-	-	-
Root hydrodistillate	-	7 ± 0.05	-	-	-
Rhizome hydrodistillate	-	-	-	-	-

^aValues are the mean of three replicates

^bStandard and test solutions prepared in hexane

In the present study, we may attribute the antibacterial and antifungal activities of the hydrodistillate of *C. pictus* plant parts are due to the higher percentage of various mono, di and sesquiterpenes present in it. The antifungal activity of sesquiterpenes such as caryophyllene oxide has been well established²⁶. The antimicrobial activities of essential oils are well documented, which are found application as antibacterial, antifungal or antiviral agents for food preservatives, in clinical microbiology or in pharmaceutical preparations^{27,28}. According to literatures, these components exert antimicrobial effects through the disruption of bacterial or fungal membrane integrity and the inhibition of respiration and ion transport processes. With the alarming incidence of antibiotic resistance in microorganisms of medical importance, there is increasing interest in plants as sources of agents for the treatment of infectious diseases^{29,30,31,32,33}. Additionally, the main advantage of naturally occurring antimicrobial agents is that they do not enhance resistance among microorganisms, a phenomenon commonly encountered with the use of synthetic antibiotics³⁴.

The antimicrobial properties of the mono, di and sesquiterpenes isolated from different plants were reported earlier. Trans-Z- α -bisabolene epoxide, thymol, β -caryophyllene etc are reported to have good antibacterial and antifungal properties. Essential oil with phenols such as thymol, eugenol, carvacrol etc as main compounds expresses the highest and broadest activity against both bacteria and fungi. The highest activity of the phenols is explained by the acidic character of the -OH group forming hydrogen bond with an enzyme active centre. The antimicrobial activity of hydrocarbons is lower than that of oxygenated essential oil components^{35,36,37,38}.

Thymol has been reported to show antibacterial activity against bacterial strains including *Aeromonas hydrophila* and *Staphylococcus aureus*. The antibacterial

activity is caused by inhibiting growth and lactate production and by decreasing cellular glucose uptake³⁵. The antifungal nature of thymol is caused by thymol's ability to alter the hyphal morphology and cause hyphal aggregates, resulting in reduced hyphal diameters and lyses of hyphal wall³⁹.

EXPERIMENTAL

(I) Isolation of the essential oil

The essential oil/hydrodistillate was obtained by the hydrodistillation of the rhizome (2 Kg), root (300 g), leaf (1.5 Kg), stem (1.8 Kg) and flower (100 g) of *C. pictus* separately in a Clevenger apparatus for 3 h. The hydrodistillate obtained was dissolved in diethyl ether (10 mL). The organic phase was dried over anhydrous sodium sulphate and filtered. After removal of the solvent, yellow transparent oil with pleasant odour in the case of leaf (0.02% v/w), stem (0.10% v/w) and root (0.05% v/w) and colourless oil with pleasant odour in the case of rhizome (0.03% v/w) and flower (0.06% w/w) was obtained which was stored in refrigerator at 4 °C until analyzed.

(II) Antimicrobial assay

Test microorganisms employed for *in vitro* antibacterial assay were obtained from Department of Microbiology, Kuvempu University, Shankarghatta, Shimoga, Karnataka and antifungal assay were obtained from Department of Microbiology, University of Mysore, Manasagangothri, Mysore, Karnataka. The antimicrobial assay of the hydrodistillate was performed by standard filter paper disc diffusion technique^{40,41}. A total of two Gram positive, three Gram negative bacteria (Fig 4.1.3) and five pathogenic fungi (Fig 4.1.4) were used for this antimicrobial

screening. The test solution at a concentration of 2% was prepared by dissolving the essential oil in hexane and Minimum Inhibitory Concentration (MIC) was reported. 0.1% streptomycin (Hi Media) and bavistin (0.2%) were used as standards for antibacterial and antifungal activities respectively. A blank disc impregnated with hexane followed by drying off was used as a negative control.

Briefly, the test discs, standard discs and blank discs were placed in the Petri dishes that contain the specific bacterial or fungal culture and then left in a refrigerator at 4 °C for 12-18 h in order to diffuse the material from the discs to the surrounding media. The Petri dishes were then incubated at 37 °C for overnight to allow the bacterial growth and 48-72 h for fungal growth. The antibacterial and antifungal activities of the hydrodistillate were then determined by measuring the respective zones of inhibition in mm.

CONCLUSIONS

The present investigation has justified the folklore use of *Costus pictus*. The potential for developing antimicrobial drug through screening of higher plants is rewarding as it will lead to the development of a phytomedicine to act against microbes. The plant based antimicrobials have enormous therapeutic potential as they can serve the purpose without any side effects that are often associated with synthetic compounds.

C. pictus is regarded as a valuable plant source in folklore and modern drug development. The various compounds identified from the hydrodistillate of the plant parts of *C. pictus* are reported to possess a wide range of pharmacological activities.

The hydrodistillates from *C. pictus* were found effective against some bacterial and fungal pathogens which are associated with fever, skin diseases, dysentery and respiratory problems. The antimicrobial activity results presented here demonstrate that this plant hydrodistillate has a commercial potential. The antimicrobial activity shown by the hydrodistillate may be due to a synergistic effect of various components present in it. It is usually found that such combined effect of various components is responsible for therapeutic activities of the plant extracts.

Eventhough the hydrodistillate from the different parts of *C. pictus* shows promising results for antimicrobial activity against various bacterial and fungal strains, the higher palmitic acid (n-hexadecanoic acid) content in them is worrisome. The hydrodistillates from the flowers, leaves, stem, rhizome and root of *C. pictus* contained palmitic acid as the major constituent with concs. 36.82%, 43.5%, 46.86%, 46.23% and 38.17% respectively. Palmitic acid increases Low Density Lipoprotein (LDL) cholesterol levels and raise the LDL to HDL ratio in healthy adults. High levels of LDL cholesterol increase the risk for coronary heart diseases. Consumption of foods rich in saturated fatty acids and cholesterol has long been recognized as an important precursor for the development of coronary heart diseases and saturated fatty acids elevate LDL concentration in the blood. Moreover palmitic acid induces degeneration of myofibrils in rat adult cardiomyocytes. Exposure of adult rat cardiomyocytes to palmitic acid for 18 hours has shown destroyed both the contractile elements and the cytoskeleton. The deleterious effect of palmitic acid on myofibrils preceded DNA fragmentation and was detectable 3 h after administration of palmitic acid. Palmitic acid, being the major component of the hydrodistillate from the different parts of *C. pictus*, the constant use of especially *C. pictus* leaves for diabetic treatment may cause serious health hazards and it must be avoided.

SECTION - 4.2

ISOLATION, IDENTIFICATION & QUANTIFICATION OF DIOSGENIN FROM THE ROOT OF COSTUS PICTUS D. DON

MATERIALS AND METHODS

(II) Plant material

The root of *C. pictus* collected as in section 4.1 was used for this phytochemical study. The roots were washed with tap water, dried in the shade and coarsely powdered in a mixer grinder.

(II) Chemicals and Reagents used

Solvents such as methanol, petroleum ether, ethyl acetate etc used were of analytical/ HPLC grade (E. Merck). Precoated silica gel 60 F₂₅₄ plates (E. Merck) of uniform thickness 0.2 mm were used for TLC. HPLC water was obtained from Millipore water purification system. The standard diosgenin was purchased from Sigma Aldrich. Antimony trichloride was purchased from S. D. Fine Chemicals, Mumbai.

(III) HPLC instrument

The Shimadzu (Japan) HPLC system consisted of LC-10AT VP pump, SPD M10A VP photodiode array detector, SCL-10A system controller, CLASS-VP 6.12 SP5 integration software and a Rheodyne model 7725 i syringe-loading sample injector fitted with a 20 μ L injection loop, was used for the analysis. Baseline resolution of diosgenin was obtained at 26 ± 2 °C using a Phenomenex Luna RP C₁₈ column (250 x 4.6 mm i.d; 5 μ m) and Phenomenex guard column (4 x 2 mm i.d; 5 μ m). HPLC method was validated by performing the sensitivity, linearity and recovery studies.

RESULTS & DISCUSSION

(I) Isolation and identification of diosgenin from the root of *Costus pictus*

The preliminary phytochemical investigation of the root of *C. pictus* revealed the presence of steroid and saponin compounds among other secondary metabolites. In line with the presence of diosgenin in the root of *Costus speciosus* (a closely related species), an attempt has been made to check the presence of diosgenin in the root of *C. pictus*. The TLC profile of the 80% aq. methanol extract (acidified with HCl) gave a greenish blue spot corresponding to standard diosgenin at R_f 0.38 on spraying with 10% antimony trichloride reagent in CHCl₃ (Fig 4.2.1). Diosgenin was successfully isolated from the root of *C. pictus* by adopting the reported method⁴². The diosgenin (Fig 4.2.2) was confirmed by its m.p. (205-207 °C), mixed m.p. and co-TLC with the authentic standard.

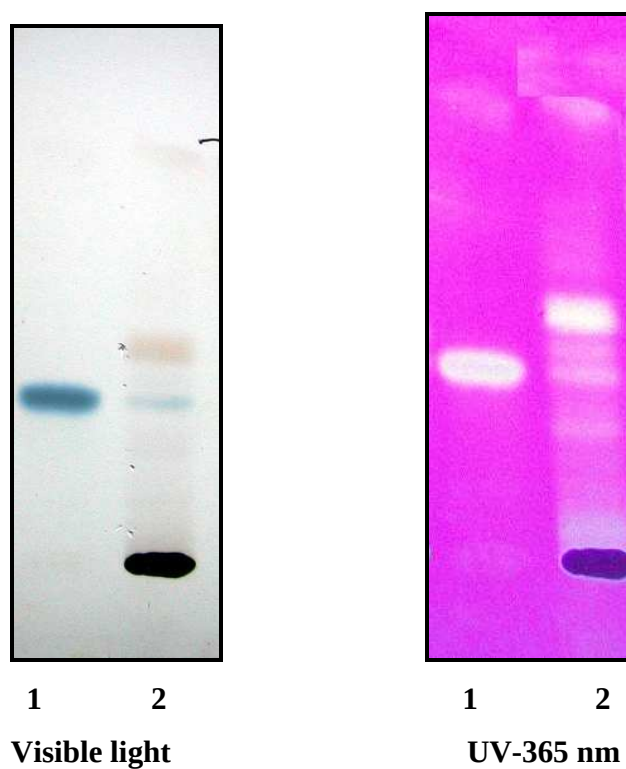


Fig 4.2.1 TLC Plate dipped in 10% antimony trichloride reagent
Track 1. Standard diosgenin
Track 2. Methanolic extract of *C. pictus* roots

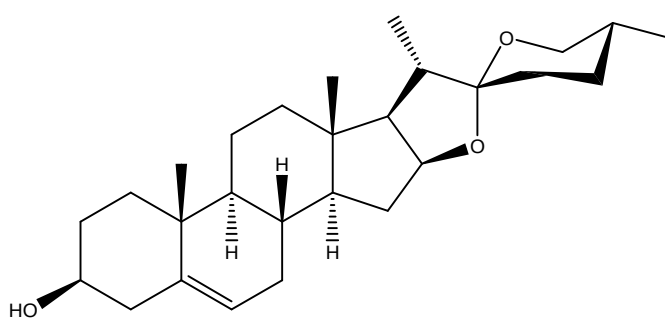


Fig 4.2.2 Structure of Diosgenin (C₁₉H₁₈O₁₁)

(II) Quantification of diosgenin by HPLC method

HPLC method was developed for the quantification of diosgenin in the *C. pictus* roots. 1 mg/mL solution of standard diosgenin was prepared. From the stock solution different concentrations viz. 50, 100, 200, 300, 400 and 500 µg/mL were prepared and calibration curve was plotted to check the linearity. 1 mg/mL solution of methanolic extract of *C. pictus* root was used as the test sample. The mobile phase used was 70:30 v/v acetonitrile: water (containing 0.1% HCOOH) as at a flow rate of 1.0 mL/min under isocratic conditions. The chromatogram was viewed at a wave length of 203 nm. Diosgenin was eluted at RT of 3.91 min.

Calibration curve was plotted to check the linearity range and diosgenin content was found to be 1.32% w/w in the dried roots of *C. pictus*. The HPLC chromatogram of the std diosgenin and the methanol extract of the root of *C. pictus* are given below (Fig 4.2.3 & 4.2.4).

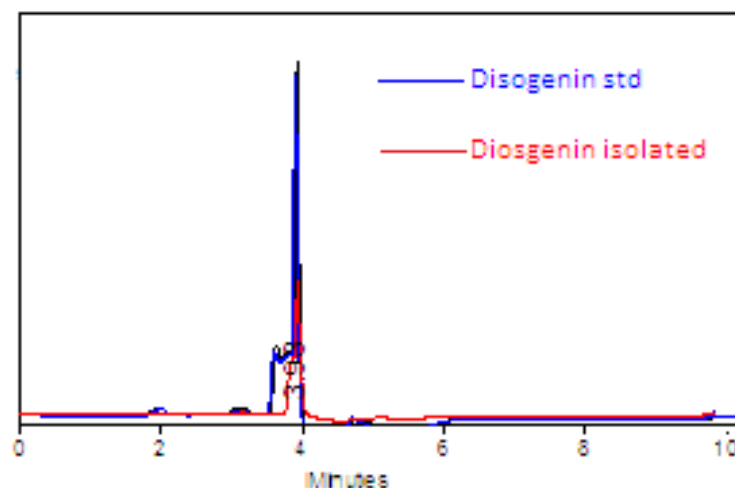


Fig 4.2.3 HPLC chromatogram of standard diosgenin

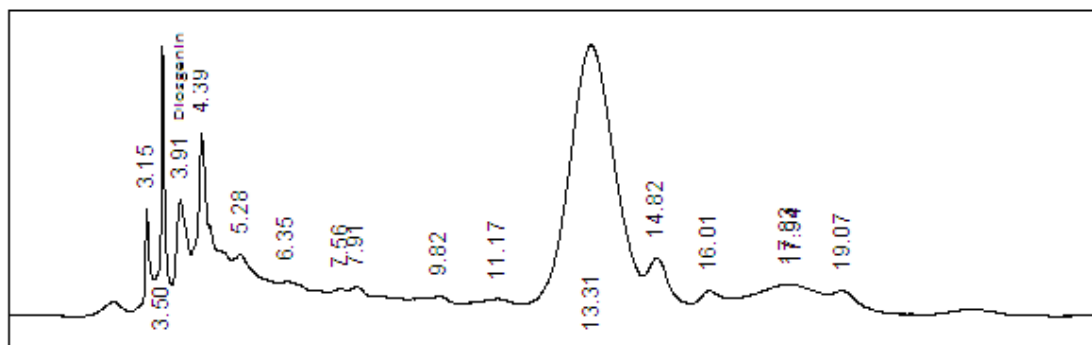


Fig 4.2.4 HPLC chromatogram of *Costus pictus* root extract

EXPERIMENTAL

(I) Isolation and identification of diosgenin

200 g of the coarsely powdered roots of *C. pictus* was completely defatted with petroleum ether (b.p. 60-80 °C) in a Soxhlet apparatus. The dried defatted marc was extracted with MeOH (1 L) in a Soxhlet apparatus for 12 h. MeOH extract was then evaporated to dryness in a rotary evaporator under vacuum at 50 °C. About 200 mL of 4N HCl was added to the residue, heated to hydrolyse on a water bath for 30 min. It was cooled, filtered and the aq. layer was partitioned with 50 mL of CHCl₃. The lower chloroform layer was collected. This was repeated 6 times. The combined chloroform extract was evaporated to about 25 mL in a rotary evaporator and allowed to cool. On cooling, colourless solid was separated out. It was recrystallized from hot methanol which yielded crystalline diosgenin (16 mg) with m. p. (205-207 °C). The diosgenin was confirmed by m. p., mixed m. p. and co-TLC with the authentic standard.

The Thin Layer Chromatogram of the plant extract was developed using the solvent system toluene: ethyl acetate: formic acid (5:2:1). The analyte detection was done under visible and UV light. The developed plates were derivatised using 10% SbCl_3 reagent in CHCl_3 .

(II) Quantification of diosgenin by HPLC method

(i) Preparation of standard solution of the extract and diosgenin for HPLC analysis

10 g of the powdered root of *C. pictus* was extracted with 100 mL 80% aqueous MeOH (acidified with 4N HCl) in a soxhlet extractor for 8 h. The solvent was removed under suction. The residue was redissolved in MeOH and made up to the mark in a 25 mL volumetric flask with MeOH so that 1 mL of standard solution contains 1 mg of the extract. It was then filtered through 0.20 μm nylon-6, 6 membrane. This solution was used for the HPLC analysis.

A stock solution containing 1 mg/mL of standard diosgenin was prepared in methanol. The working standards were prepared by suitably diluting the stock solution with methanol.

(ii) HPLC analysis

Baseline resolution of diosgenin was obtained at 25 ± 2 °C using a Phenomenex Luna C-18 column using the solvent system 70:30 v/v acetonitrile: water (containing 0.1% HCOOH). The mobile phase was passed through 0.45 μm PVDF filter and degassed before use. The flow rate was kept constant at 1.0 mL/min and the detection was done at 203 nm. For calibration, standard solutions of diosgenin were prepared at concentrations of 50, 100, 200, 300, 400 and 500 $\mu\text{g/mL}$ using

methanol as solvent. The standard solution (20 μ L) was injected in triplicate and the average detector response was measured. The root extracts were assayed in triplicate, peak areas corresponding to diosgenin (RT: 3.91 min) were compared with the calibration curve and thus the amount of diosgenin was determined. The amount of diosgenin present in the root was found to be 1.32% w/w (dry weight basis).

The developed HPLC method was validated for specificity, linearity, precision and accuracy. The limit of quantification for diosgenin was 0.1 μ g/mL. A correlation coefficient of 0.998 indicates good linearity between the concentration and the area. The recovery studies were also carried out and the recovery value of diosgenin by this method was $83.1 \pm 0.5\%$. The proposed method is simple, accurate and reliable for quantitative monitoring of diosgenin in the root *C. pictus*.

CONCLUSIONS

Diosgenin, a steroid saponin has been reported for the first time from the roots of *C. pictus*. Diosgenin is a major bioactive constituent of various edible pulses and roots. It is well characterized in the seeds of fenugreek (*Trigonella foenum graecum* Linn.) as well as in the root tubers of wild yams (*Dioscorea villosa* Linn.). Diosgenin is structurally similar to cholesterol and other steroids. Since its discovery, diosgenin is the single main precursor in the manufacture of synthetic steroids in the pharmaceutical industry. Diosgenin is used for the commercial synthesis of cortisone, pregnenolone, progesterone and other steroid products⁴³. Diosgenin is reported to have antitumour, hypocholesterolemic, antidiabetic, antioxidant, antiobesity, antiinflammatory etc activities⁴⁴.

In the present scenario, China and Mexico are the top two countries with the richest yam resource in the world, the yield of diosgenin accounts for 67% of world production. *C. speciosus*, a closely related species of *C. pictus* is otherwise known as the 'Indian source of diosgenin'. The outcome of the present study is that, the root of *C. pictus* contains diosgenin. Hence the root of *C. pictus* can also be used an important raw material for the production of diosgenin along with *C. speciosus* (upto 3.37% of diosgenin has been reported from its rhizome). In order to find out for getting maximum yield of diosgenin from the root of *C. pictus*, different factors which may contribute the production of diosgenin such as geo climatical conditions, harvesting time, age of the plant etc has to be investigated in detail.

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Chapter 5

Srinivasan G. V. “phytochemical investigation and biological studies of secondary metabolites from some folk medicinal plants” Thesis. Department of Chemistry, University of Calicut, 2015



Leea indica (Burm. f.) Merr.

CHAPTER – 5

ISOLATION & IDENTIFICATION OF CHEMICAL COMPOUNDS FROM LEEA INDICA (BURM. F.) MERR. PLANT PARTS AND ITS ANTIMICROBIAL STUDIES

INTRODUCTION

Leea indica (Leeaceae) is widely spread in the forests of tropical and subtropical India from the Himalayas as far west as Kumaon and Southwards to the Peninsula. It is a perennial shrub with stout, soft wooded and glabrous stems. The leaves and root of *L. indica* are reportedly of medicinal value. Its flowers are greenish –white in large, trichotomus, divaricated cymes on short peduncles which flower during June- August. The leaves are useful for the treatment of diabetes and the ointment prepared from roasted leaves relieves vertigo^{1,2,3}.

Its root is reported to possess sudorific, antidiarrhoeal, antidysenteric and antispasmodic activities. According to ethnobotanical information, the roots are used for the treatment of allergy, skin diseases and ear troubles while leaf is effective against snake bite^{1,4}. The traditional healers of Bilaspur region of

Chattisgarh of Indian continent reported that a typical insect ingest this herb in wild. The healers use this insect as ‘medicinal insect’ in treatment of common fever. The insect is actually used in the form decoction⁵.

In Malaysia, the whole plant is used traditionally for headache, body pain and skin complaints^{6,7}. Its vernacular names are Karkatajihva in Sanskrit; Kutanaluku/Naluku/Nallu in Malayalam; Ottanali in Tamil; Kurkurjiwah in Hindi etc. The root of *Leea indica* are used in a number of Ayurvedic formulations like *Nagaradi Taila*⁸.

(I) Morphological description of *Leea indica*

Leea indica is a shrub or small tree with stout, soft wooded, glabrous stem, with tough branches and sometimes developing aerial roots. Leaves twice or thrice pinnate, leaflets 3-7 variable in size and shape, large, oblong or lanceolate, acuminate, coarsely serrate. Stipules large. Flowers greenish-white, in large, trichotomus, divaricate cymes on short peduncles. Fruits berries, black or dark blue, succulent, depressed, 4-6 furrowed, 4-6 seeded. Flowering and fruiting from June-December¹.

(II) Abstract of the present work

The Chapter-5 is divided into 3 sections viz., section-5.1 describes the phytochemical works on *Leea indica* leaves & root; section-5.2 describes the phytochemical works on *L. indica* flowers and section-5.3 describes the phytochemical works on *L. indica* fruits.

The Section-5.1 describes the isolation and identification of gallic acid, ursolic acid, lupeol, β -sitosterol and ethyl palmitate from the root of *L. indica*. Gallic acid, lupeol, β -sitosterol and ursolic acid were also identified from the leaves of *L. indica*. The antimicrobial activity of the petroleum ether and methanol extracts of leaves and root of *L. indica* was evaluated against three Gram positive and two Gram-negative bacteria and three pathogenic fungi by disc diffusion method. The phenolic content in the leaves and root of *L. indica* were also estimated and compared by adopting standard procedures.

The Section-5.2 describes the identification of the chemical compounds present in the essential oil extracted from the flowers of *L. indica* (collected from 2 different accessions) by GC-MS technique. The antimicrobial activity of the flower essential oil of *L. indica* was evaluated against three Gram positive and two Gram-negative bacteria and three pathogenic fungi by disc diffusion method.

The Section-5.3 describes the identification of gallic acid, β -sitosterol and ursolic acid from the fruits of *L. indica* by directly doing co-TLC of the fruit extract with these standards. The preliminary phytochemical screening of the 80% aq. MeOH extract of the fruit revealed the presence of the following compounds: triterpenes, sterols, flavonoids, phenolic compounds, saponins, glycosides etc.

REVIEW OF LITERATURE

Very few phytochemical studies have been reported in *L. indica*. Twenty-three known chemical compounds were identified in the leaves of *L. indica* (Burm. f.) Merr. by GC-MS analysis. The identified compounds include eleven hydrocarbons, phthalic acid, palmitic acid, 1-eicosanol, solanesol, farnesol and

three phthalic acid esters⁹. The methanol extract of *L. indica* showed good antioxidant and nitric oxide inhibitory activities. The antioxidant activity of the methanol extract of the whole plant of *L. indica* using FTC and TBA methods was higher than that of quercetin and tocopherol but lower than that of 2,6-di-*t*-butyl-4-methylphenol (BHT). Similarly the methanol extract of *L. indica* showed strong DPPH radical scavenging activity (IC₅₀: 25 µg mL⁻¹) compared with standards vitamin C (IC₅₀: 9 µg mL⁻¹), quercetin (IC₅₀: 11 µg mL⁻¹) and BHT (IC₅₀: 12.5 µg mL⁻¹)¹⁰. α -tocopherol, β -amyrin and D-glucopyranoside have been reported recently from the whole plant of *L. indica*¹¹.

A number of chemical compounds have been reported from the plants belonging to the genus *Leea*. Leeaoside, a new megastigmane diglycoside, was isolated along with four known compounds; benzyl-*O*- α -L-rhamnopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranoside, quercetin-3-*O*- α -L-rhamnopyranoside, myricetin-3-*O*- α -L-rhamnopyranoside and citroside A from the leaves of *L. thorelli*¹².

Quercitrin-3'-sulphate¹³, antioxidant flavonoids and phenolics¹⁴ were isolated from the leaves of *L. guinensis*. Sixty nine compounds were identified from the essential oil of the leaves and wood of *L. guinensis* by GC-MS analysis¹⁵. The aqueous extract of the leaves of *L. guinensis* has been reported to possess anti-edematogenic activity¹⁶.

The chemical composition of essential oil from the leaves of *L. longifolia* Merr. has been established¹⁷.

The crude methanolic extracts of the leaves, stem and root bark of *L. tetramera* was reported to possess antibacterial activity¹⁸.

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SECTION - 5.1

COMPARISON OF ANTIMICROBIAL ACTIVITY AND TOTAL PHENOLS OF THE LEAVES AND ROOT OF *LEEA* *INDICA* AND ISOLATION OF COMPOUNDS FROM ITS LEAVES AND ROOT

MATERIALS AND METHODS

(I) Plant material

The leaves and root of *L. indica* were collected from Calicut University Campus, Kerala, India (leaves in March 2005 and root in May 2008) and authenticated by Dr. A. K. Pradeep, Department of Botany, University of Calicut. A voucher specimen No. 106601(CALI) has been deposited in the Herbarium of this University for future references. The collected leaves and roots were then washed with tap water, shade dried and coarsely powdered in a mixer grinder. These coarsely powdered materials were used for the phytochemical investigation.



L. indica leaves



L. indica root

(II) Reagents and instrumentation

All the solvents used were of Guaranteed Reagent quality (Merck, India). Precoated silica gel 60 F₂₅₄ plates of thickness 0.2 mm (Merck, Germany) were used for TLC. The spots were visualized by spraying with anisaldehyde-sulphuric acid (AS) reagent followed by heating at 110 °C for 5 min and also by using 10% MeOH/FeCl₃ reagent. The standard samples were purchased from Sigma Aldrich (USA).

UV spectra were recorded on Shimadzu UV-1700 model (Japan) double beam spectrophotometer and IR spectra on a Perkin - Elmer 377 spectrometer (USA) as KBr pellet. ¹H and ¹³C NMR spectra were recorded in DMSO-d₆ at 300 and 75 MHz respectively on a Bruker Spectrospin NMR instrument (Germany) using TMS as internal standard. EI-MS spectrum was scanned at 70 eV on a JEOL JMS600 instrument (Japan) and FAB-MS on a JEOL JMS600 instrument using 3-nitrobenzyl alcohol as matrix. CHN analysis was conducted using VarioEL III CHNS instrument (Germany).

RESULTS & DISCUSSION

(I) Identification of chemical compounds from the leaves of *L. indica*

The PE extract of the leaves of *L. indica* on column chromatography yielded two fractions. The compounds present in these two fractions were identified by using GC MS analysis and are as follows: n-octadecane, n-eicosane, n-tricosane, n-tetracosane, n-tetratetracontane, 17-pentatriacontene, n-heptacosane, n-tetratriacontane, n-tritetracontane, lycopersen, n-heptadecane, farnesol, solanesol, 1-eicosanol, palmitic acid and phthalic acid. Similarly D1 obtained from the butanol extract was subjected to GC MS analysis. Three compounds were identified from this fraction - di-n-butyl phthalate, butyl-2-ethylhexyl phthalate and isooctyl phthalate.

From the fraction D₂, a pure reddish brown solid was obtained which was identified as n-butylgallate (C₁₁H₁₄O₅, 226.22) from the spectral data. The structure of n-butylgallate is shown in Fig 5.1.1 along with the δ values of the ¹H and ¹³C nuclei. UV, IR, ¹H NMR, ¹³C NMR and mass spectrometry data of D₂ is as follows. All the Spectra of D₂ are given in Appendix – VII.

UV (λ_{max} nm, CH₃OH): 221.51, 276.47; IR ($\bar{\nu}$ cm⁻¹, KBr): 3493, 3329 (O–H str.), 2962 (C–H str.), 1687 (C=O str.); ¹H NMR (δ ppm, 300 MHz, DMSO-d₆, TMS): 0.92 (t, J = 7.5 Hz, 3H, CH₃), 1.43 (sextet, J = 7.2 and 7.5 Hz, 2H, CH₂), 1.58 (quintet, J = 6.3 and 7.8 Hz, 2H, CH₂), 4.15 (t, J = 6.0 Hz, 2H, CH₂), 6.90 (s, 2H, ArH), 8.90 (s, 1H, OH), 9.25 (s, 2H, OH); ¹³C NMR (δ ppm, 75 MHz, DMSO-d₆, TMS): 13.55, 18.77, 30.34, 63.55, 108.45, 120.59, 137.21, 144.43, 166.80; EIMS:

m/z 226 (M^+ , 100%), 211, 209, 181, 133, 75; FABMS: m/z 227 ($M+1$), 171 (100%), 153, 149, 138, 125, 102, 89, 79, 57. Elemental analysis: Anal. found (%): C, 57.99; H, 7.01; O, 35. Calculated for $C_{11}H_{14}O_5$: C, 58.41; H, 6.19; O, 35.40.

During 1H NMR analysis by the addition of D_2O , peaks at δ 8.90 and 9.25 ppm disappeared, which clearly indicate that both protons belong to OH group.

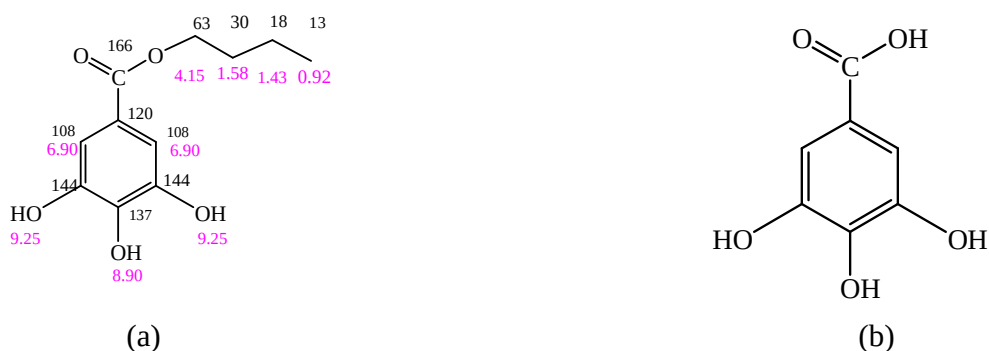


Fig 5.1.1 Structure of (a) n-butylgallate and (b) gallic acid

The preliminary phytochemical screening of the 80% aq. methanol extract of the leaves revealed the presence of some triterpenes and steroids. In order to identify these compounds co-TLC was carried out with a number of standards. Thus, two triterpenes, lupeol ($R_f = 0.85$) and ursolic acid ($R_f = 0.28$) and one sterol, β -sitosterol ($R_f = 0.58$) were identified from the methanol extract by co-TLC against authentic samples (Fig 5.1.2).

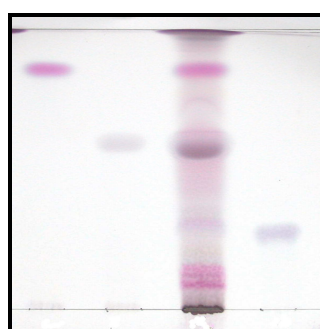
The presence of gallic acid ($R_f = 0.49$) (Fig 5.1.1b) in the leaves of *L. indica* was unambiguously confirmed by co-TLC against an authentic sample (Fig 5.1.3). The acidification of the methanol extract led to a precipitate of gallic acid which dissolved in butanol. This gallic acid gets esterified into butylgallate under hot

conditions while concentrating the butanol extract. The butylgallate was isolated using a silica gel column and identified by spectroscopic techniques.

In order to confirm this, the methanol extract was subjected to co-TLC with isolated butylgallate and a standard of gallic acid using a toluene-ethyl acetate-formic acid-methanol (30:30:9:1.25) solvent system. After spraying the TLC plate with 10% MeOH/FeCl₃ reagent, only the green spot of gallic acid was matching with the one present in the extract ($R_f = 0.49$). There is no corresponding spot in the methanol extract for n-butylgallate ($R_f = 0.74$). Therefore it can be stated that the leaves of *L. indica* contain gallic acid in the free state and not as its butyl ester.

(II) Identification of β -sitosterol, lupeol, ursolic acid and gallic acid from the leaves of *L. indica* by co-TLC with standards

A co-TLC analysis was carried out with the 80% aq. methanol extract of the leaves on a precoated silica gel 60 F₂₅₄ plate. For this analysis 10 μ L of the extract and standard solutions were applied on the precoated plate (5 x 10 cm) and developed to a distance of 8 cm. Lupeol, β -sitosterol and ursolic acid in the methanol extract were identified by co-TLC against standard samples using PE-ethyl acetate (4:1) solvent system. The presence of gallic acid in the methanol extract was also identified by spotting against standard sample using 30:30:9:1.25 toluene-ethyl acetate (EA)-formic acid (FA)-methanol solvent system. The structures of the identified chemical compounds are given in fig 5.1.4.



Track: 1-Std. lupeol
Track: 2-Std. β -sitosterol
Track: 3-MeOH extract
Track: 4-Std. ursolic acid

Solvent system: 4:1 PE-EA
Visualization: AS reagent in Vis.

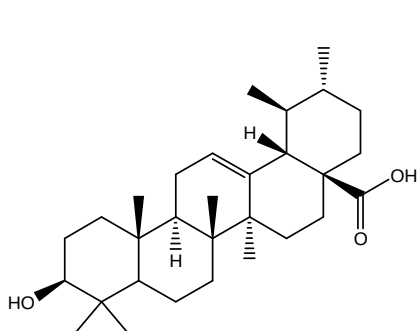
Fig 5.1.2 Co-TLC of MeOH extract with lupeol, β -sitosterol and ursolic acid



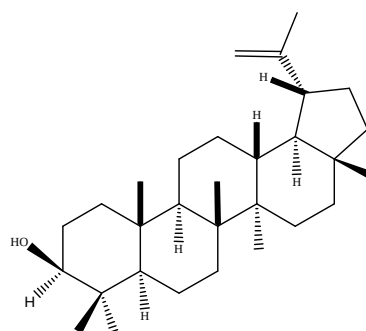
Track: 1-MeOH extract
Track: 2-Std. gallic acid

Solvent system: 30:30:9:1.25
Toluene-EA-FA-MeOH
Visualization: 10%
MeOH/ FeCl_3

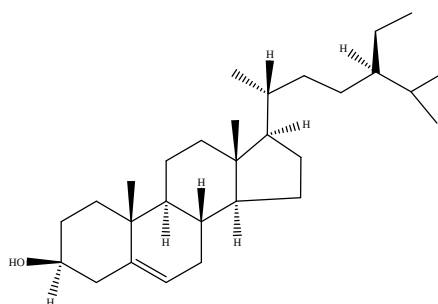
Fig 5.1.3 Co-TLC of MeOH extract with gallic acid



Ursolic acid



Lupeol



β -sitosterol

Fig 5.1.4 structures of some identified compounds from the leaves of *L. indica*

(III) Identification of chemical compounds from the root of *L. indica*

The PE extract of the root of *L. indica* on column chromatography yielded ethyl palmitate, lupeol, β -sitosteol and ursolic acid. From the methanol extract, gallic acid was obtained which was identified from the spectral data and co-TLC with the standards.

(IV) Comparison of antimicrobial activities and total phenolic content of the leaves and the root of *L. indica*

Petroleum ether extract of *L. indica* leaves showed good activity against Gram positive bacterial strains *Bacillus subtilis* and *Staphylococcus aureus*, moderate activity against Gram negative bacterial strains *Escherichia coli* (Fig 5.1.5) and *Salmonella typhimurium* and no activity against Gram positive bacterial strain *Bacillus cereus*. Similarly methanol extract of leaves showed moderate activity against *Escherichia coli* and *Staphylococcus aureus* and no activity against *Bacillus cereus*, *Bacillus subtilis* and *Salmonella typhimurium* strains.

The petroleum ether extract of root showed poor activity against *Escherichia coli* while the methanol extract showed moderate activity against *Escherichia coli* and poor activity against *Staphylococcus aureus* strains. Both the extracts showed no activity against *Bacillus cereus*, *Bacillus subtilis* and *Salmonella typhimurium* strains. The results are summarized in table 5.1.1.

Similarly the screening results against some pathogenic fungi indicate that the petroleum ether extract of the leaf and root showed good antifungal activity

against, *Aspergillus flavus*, moderate activity against *Aspergillus niger* and no activity against *Fusarium moneliformae*, *Penicillium notatum* and *Alternaria alternata* strains. The methanol extract of the leaf showed moderate antifungal activity against *Aspergillus niger* and *Fusarium moneliformae* and no activity against *Aspergillus flavus*, *Penicillium notatum* and *Alternaria alternata* strains. While methanol extract of the root showed poor activity against *Aspergillus niger* and *Aspergillus flavus* and no activity against other tested strains. The results are summarized in table 5.1.2.

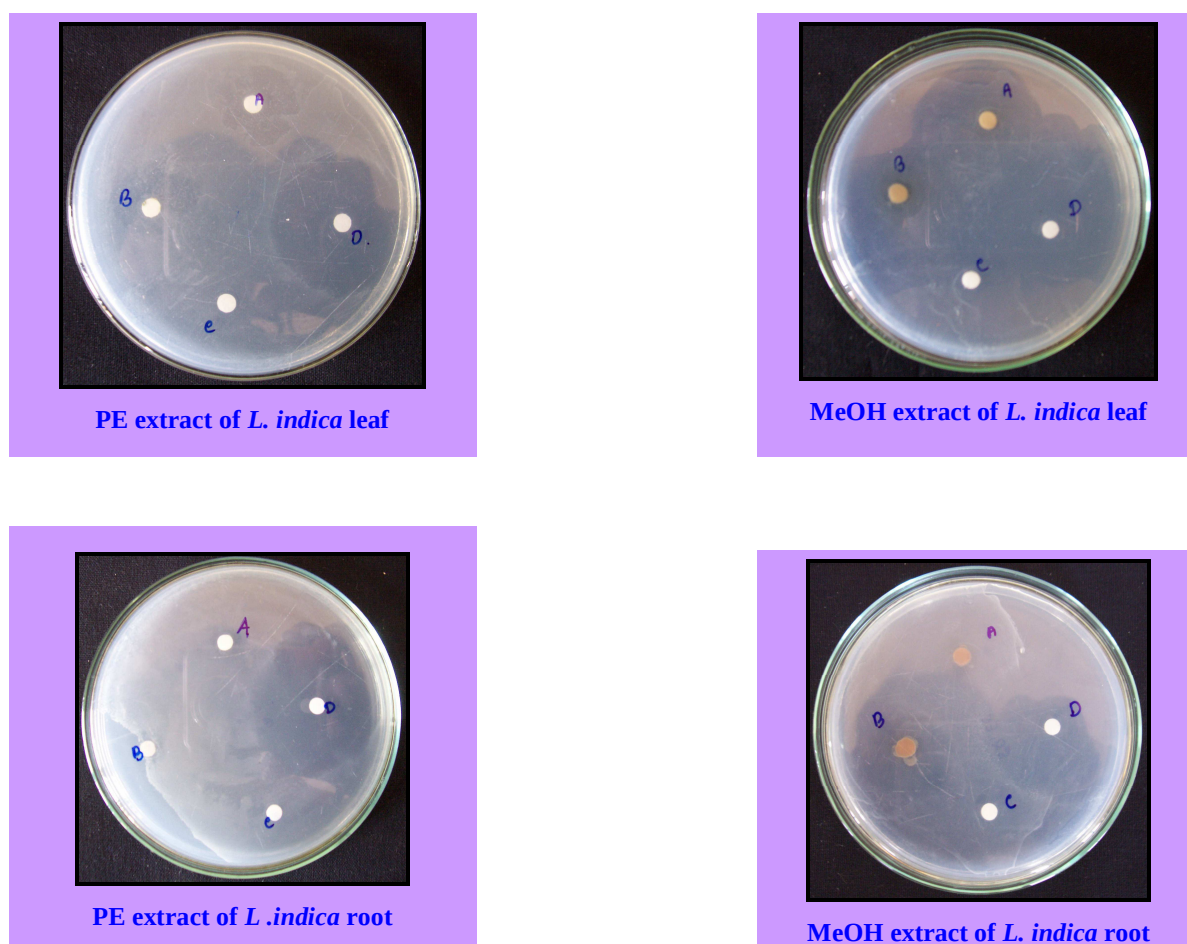


Fig. 5.1.5 Zone inhibition of (A) 1% solution of the extract; (B) 2% solution of the extract; (C) negative control; (D) positive control against *Escherichia coli*

Table 5.1.1 Antibacterial activity of Petroleum ether (PE) and methanol (MeOH) extracts of *L. indica* leaf and root

Tested bacteria	Diameter of inhibition zone (mm) ^a								
	L. indica leaf				L. indica root				Cephatoxime 0.1%
	PE extract		MeOH extract		PE extract		MeOH extract		
	1%	2%	1%	2%	1%	2%	1%	2%	
Bacillus cereus	-	-	-	-	-	-	-	-	8
Bacillus subtilis	10	12	-	-	-	-	-	-	12
Escherichia coli	8	10	6	7	2	3	10	20	34
Salmonella typhimurium	12	12	-	-	-	-	-	-	36
Staphylococcus aureus	10	10	5	9	-	-	4	9	14

^a Values are the mean of three replicates

Table 5.1.2 Antifungal activity of Petroleum ether (PE) and methanol (MeOH) extracts of *L. indica* leaf and root

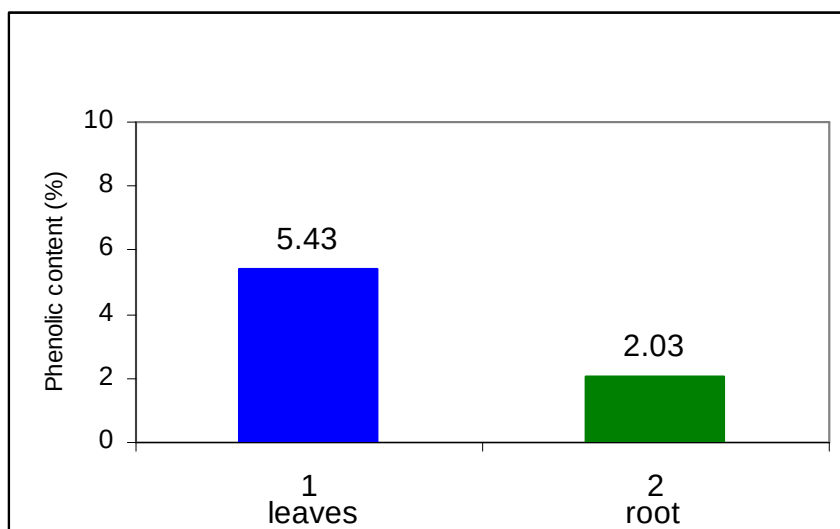
Tested pathogenic fungi	Diameter of inhibition zone (mm) ^a								
	<i>L. indica</i> leaf				<i>L. indica</i> root				Bavistin carbendazim 0.1%
	PE extract		MeOH extract		PE extract		MeOH extract		
	3%	4%	3%	4%	3%	4%	3%	4%	
<i>Alternaria alternata</i>	-	-	-	-	-	-	-	-	14
<i>Aspergillus flavus</i>	7	8	-	-	5	6	3	3	11
<i>Aspergillus niger</i>	1	3	2	5	3	2	1	2	20
<i>Fusarium moneliformae</i>	-	-	4	4	-	-	-	-	9
<i>Penicillium notatum</i>	-	-	-	-	-	-	-	-	14

^a Values are the mean of three replicates

The phenolic content in the leaves and root of *L. indica* were estimated by the method of Singleton and Rossi (Table 5.1.3, Fig 5.1.6). The leaves are found to have a higher phenolic content (5.43%) when compared to its root (2.03%).

Table 5.1.3 Estimation of total phenols in the leaf and root of *L. indica*

Wt. of std. gallic acid (μg)	Absorbance	MeOH leaf extract				MeOH root extract			
		Wt. (μg)	Absorba- nce	Phenolic content (%)	Mean phenolic content (%)	Wt. (μg)	Absorba- nce	Phenolic content (%)	Mean phenolic content (%)
50	0.1820	1000	0.2051	5.5	5.43	2000	0.1596	2.15	2.03
100	0.4031	2000	0.4366	5.5		4000	0.3157	2.0	
150	0.5903	3000	0.6210	5.33		6000	0.4620	2.0	
200	0.8014	4000	0.8435	5.25		8000	0.6233	2.0	
250	0.9987	4500	0.9976	5.56		9000	0.7155	2.0	

**Fig 5.1.6 Plot of total phenols in the leaf and root of *L. indica***

EXPERIMENTAL

(I) Isolation of chemical compounds from the leaves of *L. indica*

The powdered material (2.7 Kg) was extracted with methanol (5×3 L) in a Soxhlet apparatus for 12 h. The combined extract was concentrated to 700 mL in a Buchi Rotary evaporator (India) at 60 °C. It was then fractionated using petroleum ether (60-80 °C) and 1-butanol. The PE fraction was subjected to column chromatography and collected two fractions.

The residual methanol extract (85 g) after fractionation with PE was shaken with 2M HCl and 1-butanol (1:10) for 5 min to obtain the 1-butanol fraction which was concentrated under reduced pressure to get 30 g of a reddish brown mass. A column chromatographic separation of this residue on a silica gel (60-120 mesh) column using CHCl₃ and CHCl₃–acetone mixtures as the eluant. The fraction collected by elution with CHCl₃ alone afforded a reddish brown liquid D₁ (0.08 g) and 5:2 CHCl₃-acetone mixture yielded D₂ as a pure solid, which was further recrystallised from hot methanol to obtain reddish brown crystals having m.p. at 120-123 °C (0.15 g). This compound was identified as gallic acid by spectroscopic techniques.

(II) Isolation and characterization of ethyl palmitate, lupeol, β -sitosterol and ursolic acid from the root of *L. indica*

About 600 g of the powdered root was extracted with methanol (2×5 L) for 12 h in a Soxhlet extractor. The combined extract was filtered and concentrated in a rotary

flash evaporator to 500 mL. It was then fractionated with petroleum ether (PE; b.p. 60-80 °C). The solvent was removed from the PE extract to obtain 16 g of the residue. Solvent was also removed from the fractionated methanol extract to obtain 25 g of the residue. The PE extract was then loaded into a column packed with silica gel (60-120 mesh; 200 g). The column was eluted with hexane, different ratios of hexane and ethyl acetate and then with ethyl acetate alone. Every 50 mL fractions were collected and similar fractions were identified with TLC and pooled together. PE-ethyl acetate (4:1) solvent system was used for doing TLC and anisaldehyde-sulphuric acid (AS) reagent for derivatization.

The fractions 1-8 (hexane) gave a blue spot at R_f 0.94 on derivatization. The concentrated volume was allowed to evaporate slowly at the room temperature when a colourless waxy solid was obtained (m.p. 26-28 °C). The compound was identified as ethyl palmitate (C_1) by co-TLC and mixed m.p. with its standard. The fractions 17-23 (95:5 hexane-ethyl acetate) gave a pink spot at R_f 0.85. A colourless amorphous solid was obtained on removing the solvent, which on repeated crystallization from ethanol, colourless needles (28 mg; m.p. 214-216 °C) was obtained. It was identified as lupeol (C_2) by co-TLC, mixed m.p. and comparison of UV and IR spectra with its standard.

The fractions 29-37 (90:6 hexane-ethyl acetate) were found to consist of a mixture of compounds. These compounds were separated by preparative TLC using PE-ethyl acetate (4:1) solvent system. The compounds at R_f values 0.58 (C_3) and 0.28 (C_4) gave blue and pale pink colours respectively on derivatization with AS reagent. C_3 on crystallization from ethanol gave colourless crystalline compound (15 mg; m.p. 140-141 °C). C_3 was identified as β -sitosterol by co-TLC, mixed m.p. and comparison of UV and IR spectra with its standard. C_4 was obtained as

colourless crystal (18 mg; m.p. 284-286 °C) and was identified as ursolic acid by co-TLC, mixed m.p. and comparison of UV and IR spectra with its standard.

(III) Isolation of gallic acid from methanol fraction of the root of *L. indica*

A column chromatographic separation of methanol residue on a silica gel (60-120 mesh) column was done using CHCl₃ and CHCl₃-acetone mixtures as the eluant. The fraction collected by elution with 50:30 CHCl₃-acetone mixture yielded a colourless solid, which was further recrystallised from hot methanol to obtain colourless crystals having m.p. at 250-253 °C (40 mg). The isolated compound was identified as gallic acid by co-TLC, mixed m.p. and from its UV, IR, ¹H and ¹³C spectra and mass spectrometry.

(IV) Identification of lupeol, β -sitosterol, ursolic acid and gallic acid from the root of *L. indica* by co-TLC with standards

A co-TLC analysis was carried out with the isolated compounds C1 to C4 of the root on a pre coated silica gel 60 F₂₅₄ plate. For this analysis, 10 μ L of the C1 to C3 and standard solutions in methanol were applied on the pre coated plate (5 x 10 cm) and developed to a distance of 8 cm. Lupeol, β -sitosterol and ursolic acid were identified by co-TLC against standards using PE-ethyl acetate (4:1) solvent system. The presence of gallic acid was confirmed by spotting the C4 against standard and developed TLC plate using 30:30:9:1.25 toluene-ethyl acetate (EA)-formic acid (FA)-methanol solvent system.

(V) Antimicrobial assay

For antimicrobial studies, the powdered material, 50 g each of the leaves and root of *L. indica* were extracted successively with petroleum ether (60-80 °C) and methanol in a Soxhlet extractor for 12 h. The solvents were removed under suction to yield 1.70% (w/w) of petroleum ether extract and 4.65% (w/w) of methanol extract.

Test microorganisms employed for *in vitro* antimicrobial assay were obtained from P. G. Centre, Department of Microbiology, Kuvempu University, Dhavangare, Karnataka, India. The antimicrobial assay of the essential oil was performed by standard filter paper disc diffusion technique^{19,20}. A total of three Gram positive, two Gram negative bacteria and five pathogenic fungi were used for this antimicrobial screening. The test solutions were prepared by dissolving the extracts in DMSO. For antibacterial studies, 0.5% and 1% of the test solution was used and 3% and 4% of the test solution was employed for the antifungal studies. To compare the antibacterial and antifungal activities, cephatoxime (0.1%) and bavistin carbendazim (0.1%) were used as standard antibiotics respectively. As negative control, a blank disc impregnated with DMSO followed by drying off was used.

Briefly, the test discs, standard discs and blank discs were placed in a petridish with a particular bacteria or fungi and then left in a refrigerator at 4 °C for 12-18 h in order to diffuse the material from the discs to the surrounding media. The petridishes were then incubated at 37 °C for overnight to allow the bacterial growth and 48-72 h for fungal growth. The antibacterial and antifungal activities of the extracts were then determined by measuring the respective zones of inhibition in mm.

(VI) Estimation of total phenolic content

1 mg/mL stock solution of the leaf and root methanol extract of the plant was prepared in methanol. From the stock solution, 0.2 mL of the extract was taken into a 25 mL standard flask and 10 mL of water and 1.5 mL of the Folin-Ciocalteu's phenol reagent were added to it. The mixture was kept for 5 minutes and then 4 mL of 20% Na₂CO₃ solution was added and made upto 25 mL with double distilled water. The mixture was kept for 30 minutes and the absorbance of blue color developed was read at 765 nm. Percentage of total phenolics was calculated from the calibration curve of standard gallic acid plotted by using the above procedure and total phenolics were expressed as percentage gallic acid²¹.

CONCLUSIONS

The present investigation has helped in identifying the compounds present in the leaves and root of *Leea indica*, a hitherto uninvestigated species. Among the identified compounds, gallic acid is reported to have good antifungal and antiviral properties. Gallic acid is also an antioxidant compound and protects the cell against oxidative damage. The leaves of *L. indica* contains a higher percentage of gallic acid (5.43%) compared to its root (2.03%).

The presence of a large number of long-chain hydrocarbons reported in the leaves of *L. indica* is typical in the leaves of tropical plants which to some extent lower the rate of transpiration. Strong antioxidant activity has been reported for the methanol extract of the leaves of *L. indica*. The strong antioxidant activity of the leaves of *L. indica* can be attributed due to the presence of gallic acid, a well known antioxidant compound in its leaves.

The petroleum ether and methanol extracts of the leaves and root of *L. indica* was found effective against some bacterial and fungal strains which are associated with fever, skin diseases, dysentery and respiratory problems. The antimicrobial activity results presented here demonstrate that this plant has a commercial potential. The antimicrobial activity shown by the petroleum ether extract of the leaves and root of *L. indica* may be due to a synergetic effect of various components present in it. A number of compounds reported from the petroleum ether extract of *L. indica* leaves had been found to possess antimicrobial activities. Further investigations are needed to ascertain the compounds present in the petroleum ether extract of the root working against microbial strains. Gallic acid present in the methanol extracts of the leaves and root play a pivotal role in their antimicrobial activities.

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SECTION - 5.2

**CHEMICAL COMPOSITION AND ANTIMICROBIAL
ACTIVITY OF THE ESSENTIAL OIL OF *LEEA INDICA*
(*BURM. F.*) MERR. FLOWERS**

MATERIALS AND METHODS**(III) Plant material**

The flowers of *Leea indica* were collected from Dhoni Forest, 16 Km from Palakkad District, Kerala, India (accession 1, A1) and from Calicut University Campus, Kerala, India (accession 2, A2) in August 2007 (325 and 200 g respectively) and duly identified by Dr. A. K. Pradeep, Department of Botany, University of Calicut.

(II) GC-MS analysis

GC-MS analysis was performed by the split injection (1:40) of 0.1 µL of the oil in hexane on a Shimadzu GCMS-QP2010 (Japan) gas chromatograph fitted with cross banded 5% diphenyl 95% dimethyl polysiloxane RTX5 (MS) capillary column (30 m x 0.25 mm x 0.25 µm coating thickness), coupled with a mass

detector. GC-MS operating conditions were as follows: injector temperature 250 °C, transfer line 250 °C, Detector temperature 220 °C, oven temperature programme: 60 °C hold for 5 min, 110 °C with ramping 5 °C min⁻¹, 200 °C with ramping 3 °C min⁻¹, 220 °C with ramping 5 °C min⁻¹, hold 220 °C for 5 min. carrier gas: helium at 1.67 mL min⁻¹, mass spectra: electron impact (EI⁺) at 70 eV, ion source temperature: 220 °C. Individual components were identified by Wiley139.LIB and NIST05.LIB database matching. The percentage composition was determined by area normalization. TIC of the essential oil of *L. indica* flowers collected from two accessions are given in Appendix VII.

RESULTS AND DISCUSSION

The compounds present in the essential oil of the flowers of *L. indica* collected from two different accessions were identified and compared by GC-MS analysis. The major constituents present in the flowers were identified to be phthalic acid esters (96.93% in A1 and 95.78% in A2). 3H-pyrazole and monobutyl carbonotrithioate were identified only in A1 while bis-(2-pentyl)phthalate and 5-decyne were detected only in A2. The percentage of other compounds differs slightly in the two accessions (Table 5.2.1). The percentage of diisobutylphthalate, the major compound from the essential oil of both accessions was found to be more or less same (> 75%).

Phthalates are used as plasticizers in the production of polyvinyl chloride and other plastics. It has been demonstrated that phthalates are widely distributed in the environment but that their levels are low because they are subject to relatively rapid photochemical and biological degradation²⁴.

Table 5.2.1 Chemical composition of the essential oil of *L. indica* flower

Sr. No.	Retention time (min)	Chemical compounds identified	
		Accession 1 (Conc %)	Accession 2 (Conc %)
1	2.489	Homobenzvalene (0.07)	Homobenzvalene (0.02)
2	11.611	Guaiacol (0.03)	Guaiacol (0.01)
3	17.868	1,4-Benzenediol (0.49)	1,4-Benzenediol (0.66)
4	18.189	Anethole (0.07)	Anethole (0.05)
5	20.459	3,5-Heptadien-2-one (0.08)	3,5-Heptadien-2-one (0.12)
6	29.707	3H-pyrazole (0.03)	-
7	40.042	Di-isobutylphthalate (79.00)	Di-isobutylphthalate (75.64)
8	40.166	Monobutyl carbonotrithioate (0.01)	-
9	41.251	Di-n-butylphthalate (7.18)	Di-n-butylphthalate (7.48)
10	41.812	Isobutyl-2-pentylphthalate (0.45)	Isobutyl-2-pentylphthalate (0.58)
11	42.807	n-Butylisobutylphthalate (6.11)	n-Butylisobutylphthalate (7.87)
12	42.960	n-Butyl-2-ethylhexylphthalate (0.40)	Butyl-2-ethylhexylphthalate (0.40)
13	43.439	n-Butylisohexylphthalate (3.67)	Butylisohexylphthalate (3.71)
14	44.044	-	Bis(2-pentyl)phthalate (0.06)
15	44.106	n-Pentylphthalate (0.12)	n-Pentylphthalate (0.04)
16	47.211	Phytol (0.03)	Phytol (0.10)
17	50.480	-	5-Decyne (0.04)
-	-	Unidentified (2.26)	Unidentified (3.22)

There are only fewer reports of the occurrence of the phthalates from plants. Di-isooctyl phthalate has been reported from *Limonium bicolor*²⁵ and *Dracaena cochinchinensis* (Lour.) S.C. Chen²⁶. Butyl and isobutyl phthalates were reported from *Dracaena cochinchinensis* (Lour.) S.C. Chen⁵. Di-(2-ethyl)hexylphthalate has been

isolated from the leaves of *Cassia auriculata*²⁷. Bis(2-ethylhexyl)phthalate was reported from the roots of *Euphorbia hylonoma*²⁸. Bis(2-methylheptyl)phthalate has been isolated from the aerial parts of *Hypericum hyssopifolium* L²⁹. Diethyl phthalate has been reported from the essential oil from the skin of water caltrop³⁰. Diethyl phthalate and derivatives of phthalic acids were isolated from the root exudates of barnyard grass *Echinochloa crusgalli*³¹.

Phthalates are reported to have antimicrobial and other pharmacological activities. So far phthalic acid has been known to be produced by *Gibberella fujikuroi*³². Bis-(ethylhexyl)phthalate reported from *Streptomyces bangladeshiensis* shows antimicrobial activity against Gram positive bacteria and some pathogenic fungi³³. Bis(2-methylheptyl)phthalate isolated from *Pongamia pinnata* leaves exhibit antiviral activity against White Spot Syndrome Virus of *Penaeus monodon Fabricius*³⁴. Di(2-ethylhexyl)phthalate isolated from *Alchornea cordifolia* reported to lower anti-inflammatory activity³⁵. Di-isooctylphthalate isolated from *Nigella glandulifera* Freyn. was identified as inhibiting melanogenesis³⁶.

Phthalates are also reported to affect the human beings. Di-2-ethylhexylphthalate (DEHP), a commonly used plasticizer is harmful to human health³⁷. Di-2-ethylhexylphthalate, di-n-hexylphthalate and di-n-octylphthalate were reportedly affect the functioning of liver, kidney and thyroid³⁸. Di(2-ethylhexyl)phthalate, was also hepatocarcinogenic in rats³⁹.

The antibacterial and antifungal activities of the *L. indica* flower essential oil from accession 1 have been assayed at concentrations of 0.5% to 4% against strains of both Gram positive and Gram negative bacteria and pathogenic fungi (Fig. 5.2.2). The susceptibility testing was carried out by measuring the inhibitory zone diameters on nutrient agar, with conventional paper disc method and the inhibitory zone diameters were read and rounded off to the nearest whole number (mm) for

analysis. The inhibitory effects of the essential oil against these microorganisms are given in Tables 5.2.2 and 5.2.3.

The screening results indicate that the essential oil showed good antibacterial activity against Gram negative bacterial strains *Escherichia coli* and *Salmonella typhimurium* and moderate activity against Gram positive bacterial strains *Bacillus subtilis*, *Bacillus cereus* and *Staphylococcus aureus*.

Similarly the screening results against some pathogenic fungi indicate that the essential oil showed good antifungal activity against *Penicillium notatum*, moderate activity against *Aspergillus niger* and *Fusarium moneliformae* and no activity against *Aspergillus flavus* and *Alternaria alternata* strains.

Phthalates are reported to have good antimicrobial activities. In the present study, the antibacterial and antifungal activities of the essential oil of *L. indica* flowers is due to the higher percentage of phthalates in it. Guaiacol, anethole and 3H-pyrazole are reported to have antibacterial/antifungal activity. The presence of these compounds increases the antimicrobial potential of the essential oil of *L. indica* flowers.

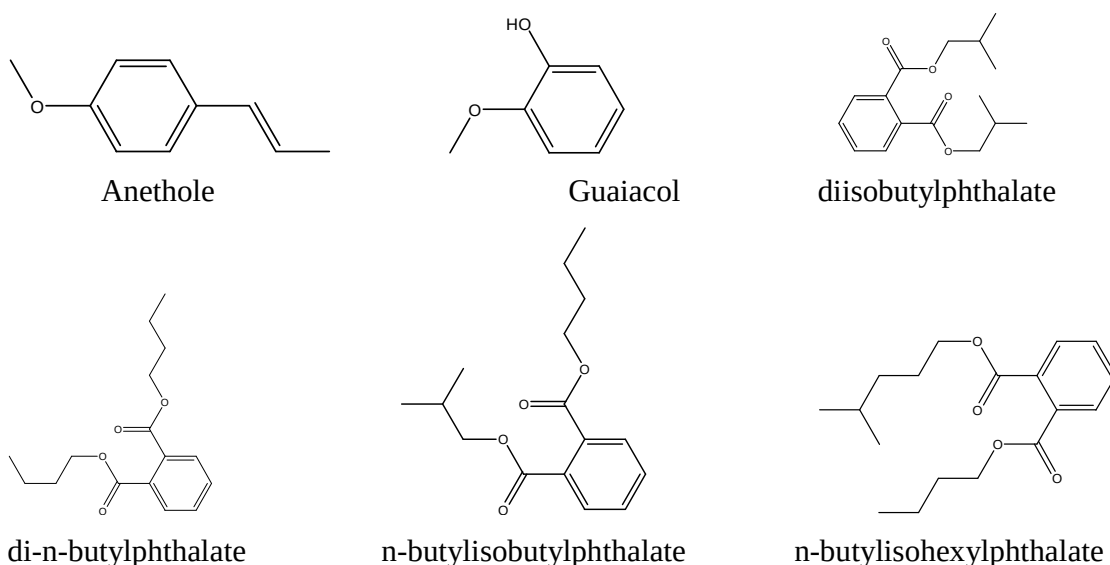
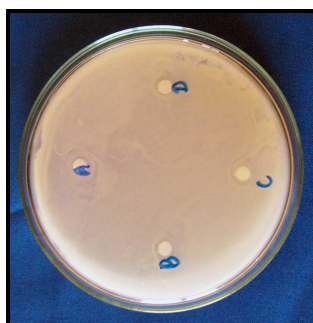
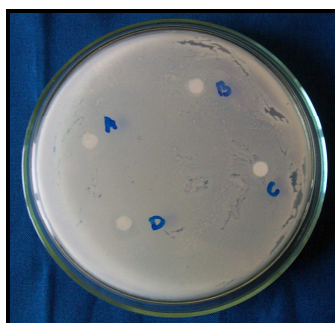
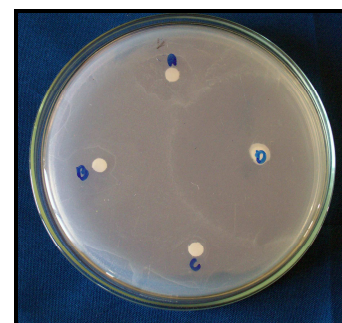
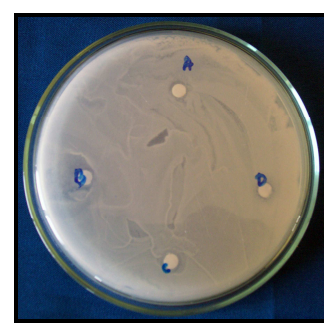
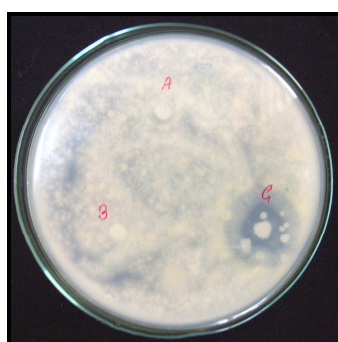
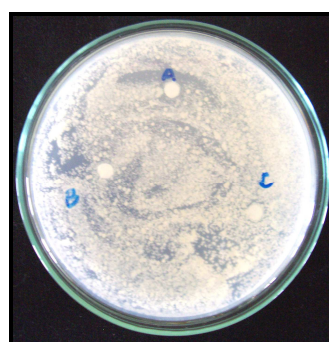
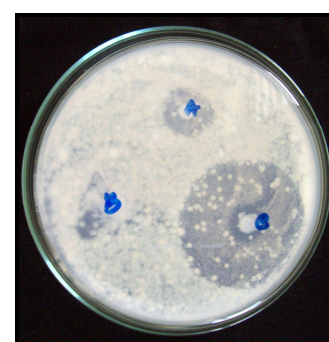


Fig 5.2.1 Structure of some of the identified compounds from the essential oil of *L. indica* flowers

*Bacillus cereus**Bacillus subtilis**Escherichia coli**Salmonella typhimurium**Staphylococcus aureus*

A = 0.5% solution
B = 1% solution
C = -ve control
D = +ve control

*Aspergillus niger**Fusarium moneliformae**Penicillium notatum*

A = 3% solution
B = 4% solution
C = +ve control
D = -ve control

Fig 5.2.2 Antibacterial and antifungal activities of essential oil against different strains

Table 5.2.2 *In vitro* antibacterial activity of essential oil of *L. indica* flower

Tested bacteria	Diameter of inhibition zone (mm) ^a		
	Essential oil		Cephatoxime
	0.5%	1%	0.1%
<i>Bacillus cereus</i>	6	7	8
<i>Bacillus subtilis</i>	-	6	9
<i>Escherichia coli</i>	10	10	25
<i>Salmonella typhimurium</i>	8	11	23
<i>Staphylococcus aureus</i>	5	6	10

^aValues are the mean of three replicates**Table 5.2.3 *In vitro* antifungal activity of essential oil of *L. indica* flower**

Tested pathogenic fungi	Diameter of inhibition zone (mm) ^a		
	Essential oil		Bavistin carbendazim
	3%	4%	0.1%
<i>Alternaria alternata</i>	-	-	16
<i>Aspergillus flavus</i>	-	-	9
<i>Aspergillus niger</i>	8	7	20
<i>Fusarium moneliformae</i>	6	5	7
<i>Penicillium notatum</i>	18	21	40

^aValues are the mean of three replicates

EXPERIMENTAL

(I) Isolation of the essential oil

The essential oil was obtained by hydrodistillation of the flowers of *L. indica* in a Clevenger apparatus for 3 h. The essential oil was reextracted with diethyl ether and dried over anhydrous Na₂SO₄. The light yellow transparent oil (0.18% v/w from A1 and 0.13% v/w from A2) has a bitter odour and stored in a refrigerator at 4 °C until analyzed.

(II) Antimicrobial assay

Test microorganisms employed for *in vitro* antimicrobial assay were obtained from P. G. Centre, Department of Microbiology, Kuvempu University, Dhavangare, Karnataka, India. The antimicrobial assay of the essential oil was performed by standard filter paper disc diffusion technique^{22,23}. A total of three Gram positive, two Gram negative bacteria (Table 5.2.2) and five pathogenic fungi (Table 5.2.3) were used for this antimicrobial screening. The test solution was prepared by dissolving the essential oil from A1 in DMSO. For antibacterial studies, 0.5% and 1% of the test solution was used and 3% and 4% of the test solution was employed for the antifungal studies. To compare the antibacterial and antifungal activities, cephatoxime (0.1%) and bavistin carbendazim (0.1%) were used as standard antibiotics respectively. As a negative control, a blank disc impregnated with DMSO followed by drying off was used.

Briefly, the test discs, standard discs and blank discs were placed in a petridish with a particular bacteria or fungi and then left in a refrigerator at 4 °C for 12-18 h

in order to diffuse the material from the discs to the surrounding media. The petridishes were then incubated at 37 °C for overnight to allow the bacterial growth and 48-72 h for fungal growth. The antibacterial and antifungal activities of the essential oil were then determined by measuring the respective zones of inhibition in mm.

CONCLUSIONS

The results from the present study offer a scientific proof for the traditional use of the *L. indica* flowers. The essential oil from the flowers was found effective against some bacterial and fungal strains which are associated with fever, skin diseases, dysentery and respiratory problems. The antibacterial/ antifungal activity shown by the oil may be due to a synergetic effect of various components present in it. It is usually found that such combined effect of various components is responsible for therapeutic activities of the plant extracts. However this needs further investigation and remain open to future investigation.

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SECTION - 5.3

PHYTOCHEMICAL STUDIES OF THE FRUITS OF LEEA

INDICA

MATERIALS AND METHODS

(II) Plant material

The fully ripened fruits of *L. indica* were collected from Calicut University Campus, Kerala, India. It is dried in sunlight and coarsely powdered in a mixer grinder.



Fig 5.3.1 *L. indica* dried fruits

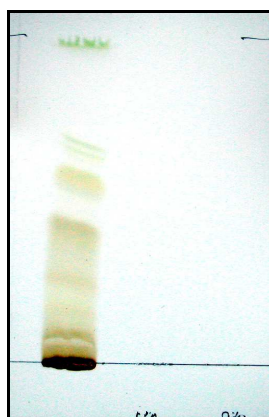
RESULTS & DISCUSSION

(I) Identification of chemical compounds from the fruits of *L. indica*

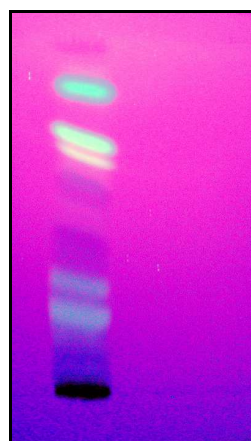
The preliminary phytochemical screening of the 80% aq. MeOH extract of the dried fruits of *L. indica* revealed the presence of the following compounds: terpenoids, sterols, flavonoids, phenolic compounds, saponins, tannins, glycosides etc. Further detailed study has to be carried out to isolate and identify the chemical compounds present in the fruits of *L. indica*.

The TLC profile of PE (b.p. 60-80 °C) extract of the dried fruits of *L. indica* was found to contain ursolic acid (R_f 0.66), β -sitosterol (R_f 0.72) among other terpenoid compounds. The TLC plate was developed using 9:1 Toluene-Ethyl acetate solvent system (Fig 5.3.2). It was then sprayed with Anisaldehyde- H_2SO_4 spray reagent and heated at 110 °C.

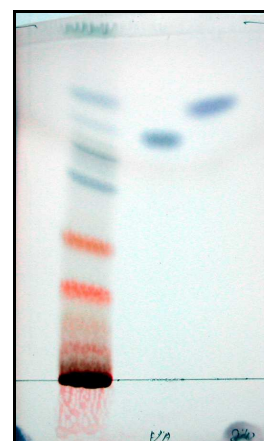
The methanol extract of the dried fruits of *L. indica* was subjected to co-TLC with gallic acid. The TLC plate was developed with 5:3:1:0.5 Toluene-Acetone-Methanol-Formic acid solvent system and sprayed with 10% MeOH/ $FeCl_3$ reagent. The spot at R_f 0.42 corresponding to gallic acid was obtained. A no. of other phenolic compounds were also detected in the TLC plate (fig 5.3.3).



VIS



UV 365 nm



AS reagent in Vis.

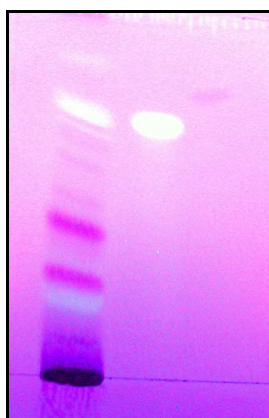
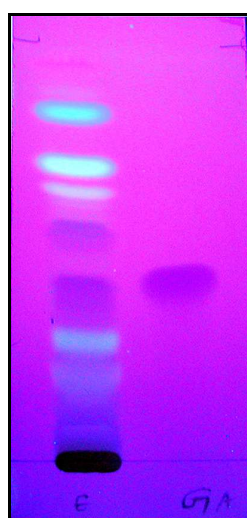
AS reagent in UV
365 nm

Fig 5.3.2 TLC profile of PE extract
Solvent system: 9:1 Toluene-Ethyl acetate
Track 1: Extract; Track 2: Ursolic acid; Track 3: β -sitosterol



VIS



UV 365 nm

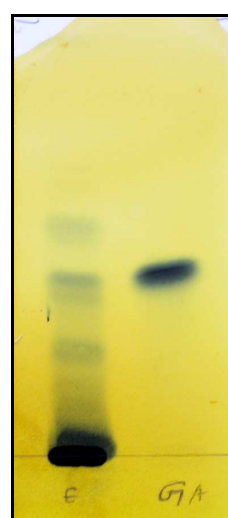
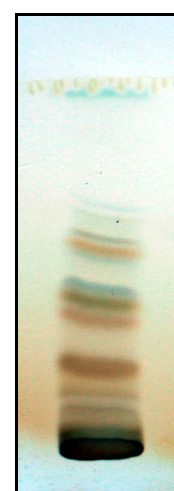
 FeCl_3 reagentExposed I_2
vapours

Fig 5.3.3 TLC profile of methanol extract
Solvent system: 5:3:1:0.5 Toluene-Acetone-Methanol-Formic acid
Track 1: Extract; Track 2: Gallic acid

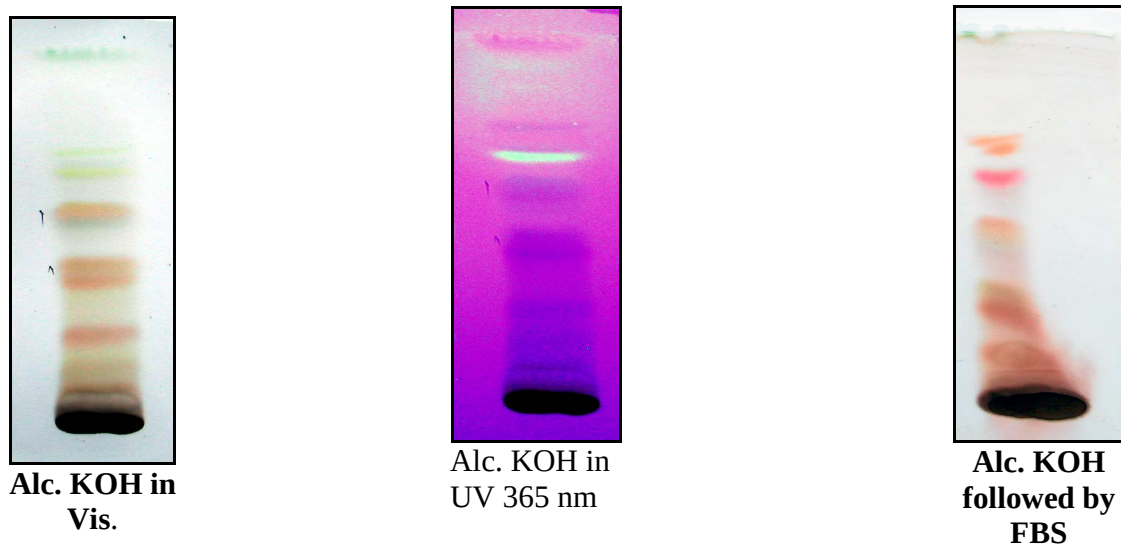


Fig 5.3.4 TLC profile of methanol extract
Solvent system: 5:3:1:0.5 Toluene-Acetone-Methanol-Formic acid

EXPERIMENTAL

(I) Preliminary phytochemical screening

50 g of the dried fruits of *L. indica* was extracted with 80% aq. methanol. The methanol extracts was concentrated under suction in a rotary flash evaporator and subjected to preliminary phytochemical screening for the detection of the major class of compounds^{40,41}. The details of the tests are as follows:

1. Test for carbohydrates:

A. Molisch's test: Dissolved small quantity (300 mg) of the extract separately in 4 mL distilled water and filtered. The filtrate was subjected to Molisch's test. Violet ring was obtained. This indicates the presence of carbohydrates.

B. Fehling's test: Dissolved small quantity of the extract in water and treated with Fehling's solution. Reddish brown colour obtained. This indicates the presence of carbohydrates.

2. Test for phenols:

Phosphomolybdic acid test: The methanolic extract was spotted on a filter paper. A drop of phosphomolybdic acid reagent was added to the spot and was exposed to ammonia vapours. Blue coloration of the spot was observed. This indicates the presence of phenols.

3. Test for flavonoids:

A. Shinoda test: To 2-3 mL of methanolic extract, a piece of magnesium ribbon and 1 mL of conc. HCl were added. Red colour was obtained, which indicate the presence of flavonoids.

B. Lead acetate test: To 5 mL of the extract, added 1 mL of lead acetate solution. Flocculent white precipitate was obtained, which indicate the presence of flavonoids.

4. Test for tannins:

Braemer's test: To 2-3 mL of methanolic extract, 10% alcoholic FeCl₃ solution was added. Dark blue colour was obtained, which indicate the presence of tannins.

5. Test for steroids/ terpenoids:

Liebermann-Burchard test: To 1 mL of methanolic extract, 1 mL of CHCl₃, 2-3 mL of acetic anhydride and 1 to 2 drops of concentrated H₂SO₄ were added. Red coloration of the solution, which indicates the presence of terpenoids.

6. Test for alkaloids:

A. Dragendorff's Test: A drop of methanolic extract was spotted on a small piece of precoated TLC plate and the plate was sprayed with Dragendorff's reagent. No orange coloration, which indicates the absence of alkaloids.

B. Hager's test: The extract was treated with few mL of Hager's reagent. No yellow precipitate, which indicates the absence of alkaloids.

C. Wagner's test: The extract was treated with few mL of Wagner's reagent. No reddish brown precipitate, which indicates the absence of alkaloids.

7. Test for glycosides:

Legal's test: Dissolved the extract (0.1 g) in pyridine (2 mL), added sodium nitroprusside solution (2 mL) and made alkaline with NaOH solution. The red colour solution, which indicates the presence of glycosides.

8. Test for saponins:

Foam test: Diluted 1 mL of methanolic extract separately with distilled water to 20 mL and shaken in a graduated cylinder for 15 minutes. Formation of foam indicates the presence of saponins.

9. Test for anthraquinones:

Borntrager's test: About 50 mg of methanolic extract was heated with 10% FeCl_3 solution and 1 mL of conc. HCl. The extract was cooled, filtered and the filtrate was shaken with diethyl ether. The ether extract was further extracted with strong NH_3 . No Pink or deep red coloration of aqueous layer, which indicates the absence of anthraquinones.

10. Test for amino acids:

Ninhydrin test: Dissolved a small quantity of methanolic extract in a few mL of water and added ninhydrin in acetone. Purple colour was obtained which indicates the presence of amino acids.

(II) TLC fingerprint profiling

(a) Extraction:

About 50 g of the powdered material was extracted with 300 mL petroleum ether in a Soxhlet extractor for 8 hours below 60 °C. The Marc dried and further sequentially extracted with methanol. The individual extracts were concentrated in a rotary flash evaporator under reduced pressure. The residues obtained were redissolved in the respective solvents and made up to 10 mL in volumetric flasks. These solutions were used for TLC fingerprint profiling.

(b) Chromatographic conditions:

Chamber saturation: Developing chamber saturated with the solvent system.

Stationary phase: Pre-coated silica gel 60 F₂₅₄ plates.

(c) Solvent systems:

1. Toluene - Ethyl acetate (9:1) for PE extract
2. Toluene-Acetone-Methanol-Formic acid (5:3:1:0.5) for methanol extract

(d) Detection:

By visualization under visible light, UV 365 nm, in iodine chamber and by derivatization with Anisaldehyde - Sulphuric acid reagent, 10% EtOH/KOH, 10% EtOH/H₂SO₄ reagent and NH₃ vapours.

(e) Procedure:

10 µL of both the extracts were used for spotting. TLC plates were then developed with the above mentioned solvent systems. The TLC profiles of two extracts derivatized with different spray reagents.

CONCLUSIONS

The presence of gallic acid, ursolic acid and β -sitosterol were identified in the fruits of *L. indica*. The preliminary phytochemical screening of the 80% aq. MeOH extract of the dried fruits of *L. indica* revealed the presence terpenoids, sterols, flavonoids, phenolic compounds etc. TLC fingerprint profiles of the extracts of the fruits of *L. indica* revealed the presence of a no. of steroid, flavonoid and triterpenoids. Further detailed study has to be carried out to isolate and identify the chemical compounds present in the fruits of *L. indica*. Though the present phytochemical investigation of the fruits of *L. indica* is impressive, the characterization of still unexplored constituents remain open to future investigation. Since the fruit is edible, it would be desirable to investigate its phytochemical profile in detail. It seems likely that a number of compounds from fruits of *L. indica* may provide interesting leads for pharmacological evaluation.

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Appendix

Srinivasan G. V. “phytochemical investigation and biological studies of secondary metabolites from some folk medicinal plants” Thesis. Department of Chemistry, University of Calicut, 2015

Appendix I

List of Published papers

1. Srinivasan G.V., Ranjith C., Vijayan K.K. Identification of chemical compounds from the leaves of *Leea indica*. *Acta Pharmaceutica*. 2008, 58: 207-214.
2. Srinivasan G.V., Sadashiva C.T., George N., Balachandran I. Chemical compounds in *Biophytum reinwardtii* and their antioxidant potential. *Journal of Tropical Medicinal Plants*. 2008, 9(2): 338-342.
3. Srinivasan G.V., Sharanapa P., Leela N.K., Sadashiva C.T., Vijayan K.K. Chemical composition and antimicrobial activity of the essential oil of *Leea indica* (Burm. f.) Merr. flowers. *Natural Product Radiance*. 2009, 8(5): 488-493.
4. Srinivasan G.V., Ravi S., Tushar K.V., Balachandran I. Quantitative determination of mangiferin from the roots of *Salacia fruticosa* and the antioxidant studies. *Journal of Tropical Medicinal Plants*. 2009, 10(2): 213-218.
5. Srinivasan G.V., Sharanapa P., Vijayan K.K. Comparison of antimicrobial activity and total phenols of the leaves and root of *Leea indica* and isolation of compounds from its root. *International Journal of Biomedical Research and Analysis*. 2010, 1(2): 92-95.
6. Srinivasan G.V., Sharanapa P., Udayan P.S., Leela N.K., Vijayan K.K. Chemical composition and antimicrobial activity of the essential oil of *Ixora coccinea* L. root. *Journal of Medicinal and Aromatic Plant Sciences*. 2010, 32: 27-30.

Appendix II

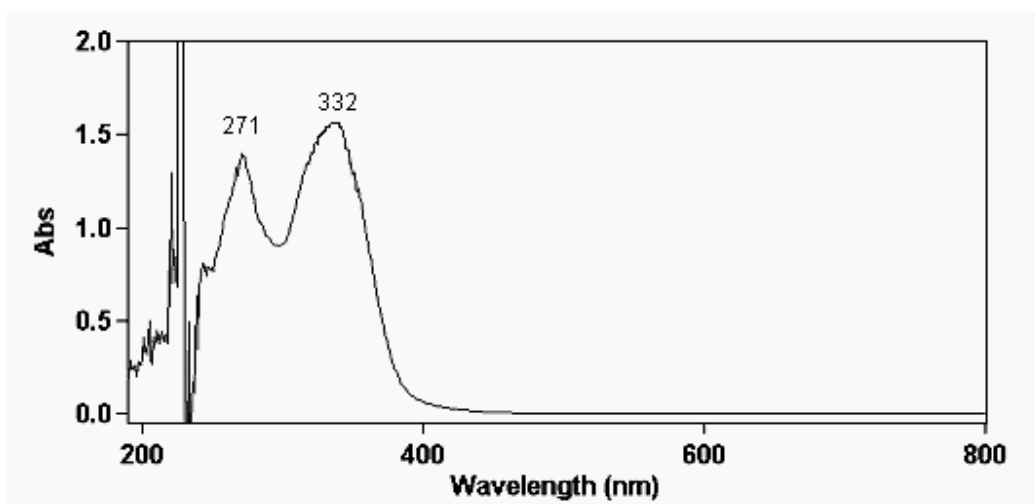
List of Communicated papers

1. Srinivasan G.V., Vijayan K.K., Ravi S. A New Flavonoid Diglycoside from *Biophytum reinwardtii* (Zucc.) Klotzsch., and Evaluation of its Antioxidant and Anticancer Activities. Communicated to Natural Product Communications.
2. Srinivasan G.V., Vijayan K.K., Sharanappa P., Jagadeesh K.S. Identification of chemical compounds in the essential oil from *Costus pictus* D. Don plant parts, its antimicrobial studies & isolation and quantification of Diosgenin from its root. Communicated to Records of Natural Products.

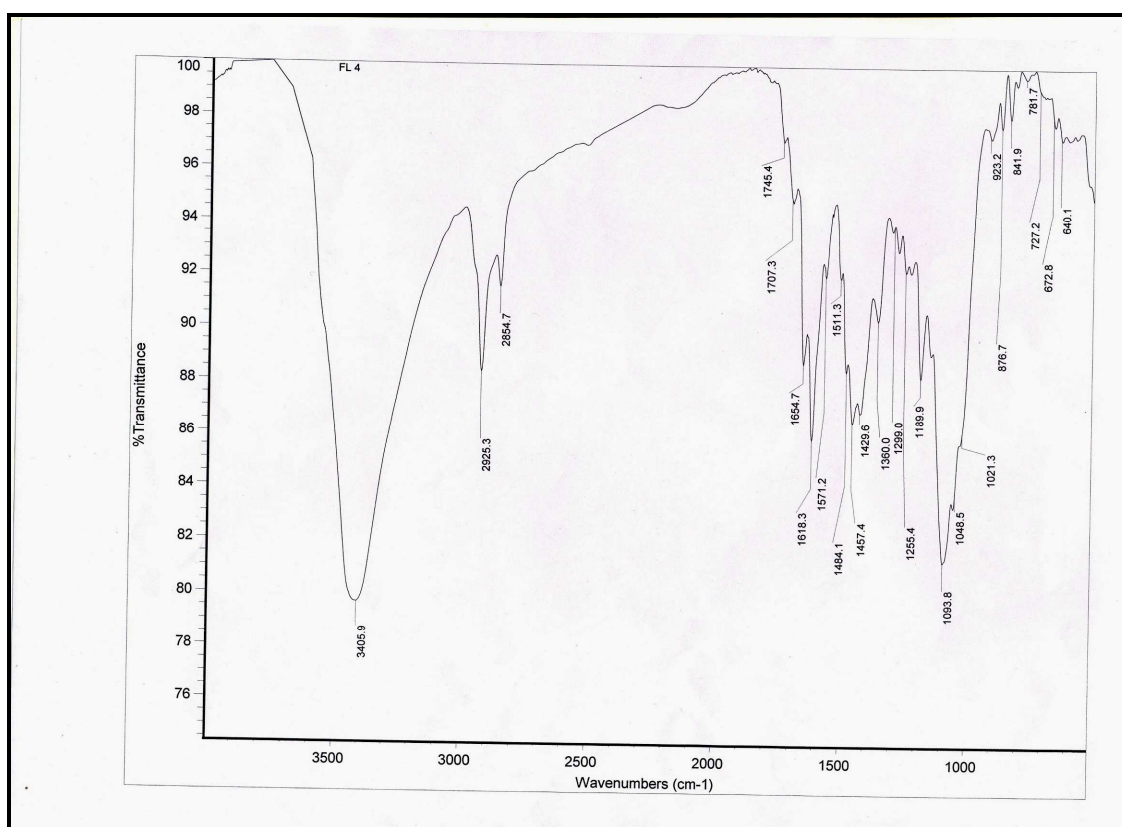
Appendix III

Spectra of apigenin-4',5-O- β -D-diglucoside (BR-1)

isolated from the whole plant of Biophytum reinwardtii



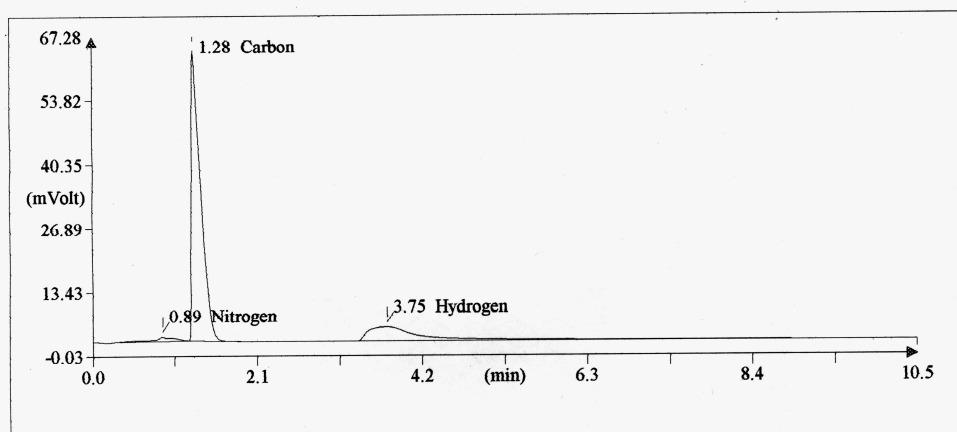
UV-Vis spectrum of BR-1

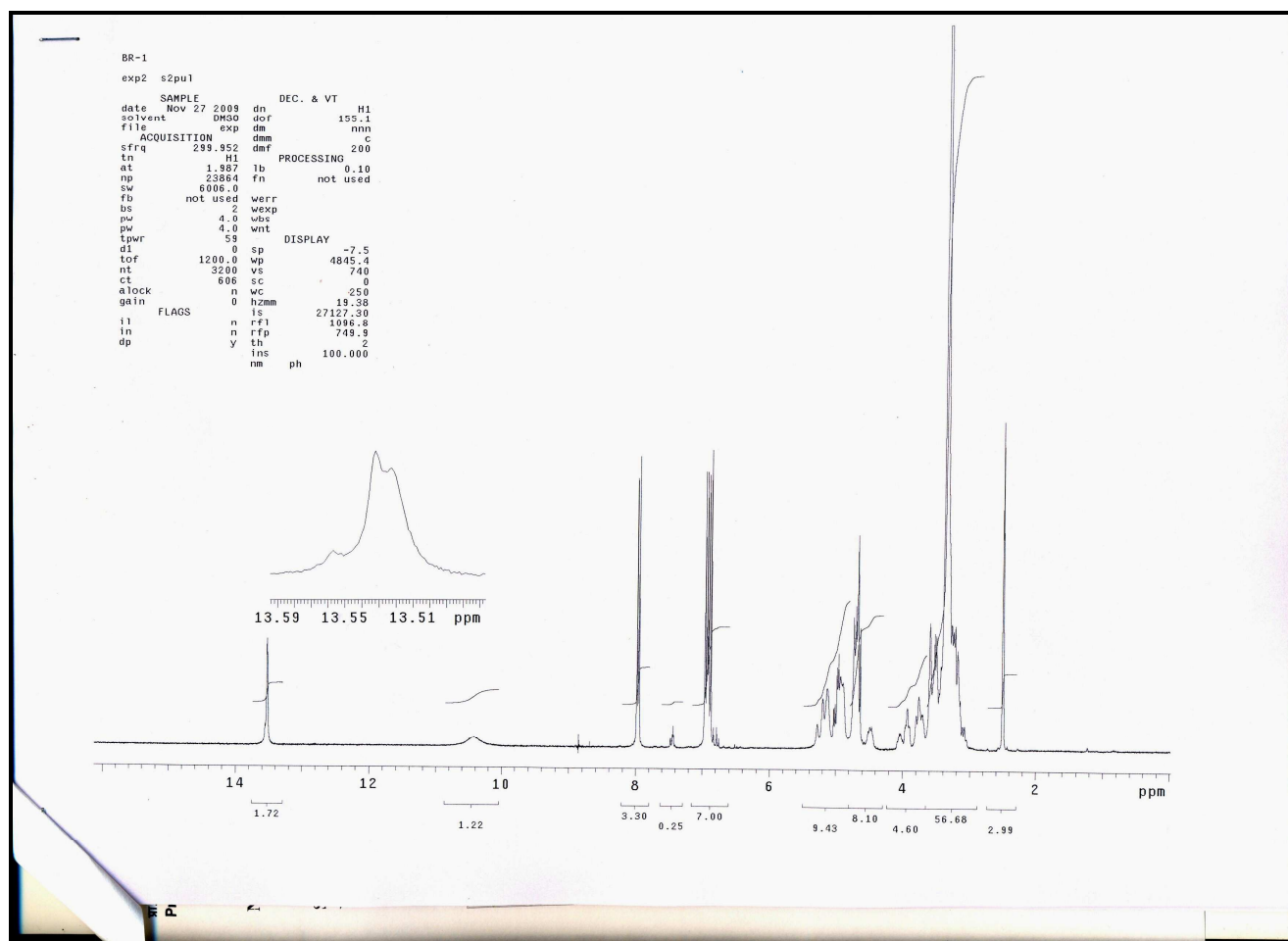


IR spectrum of BR-1

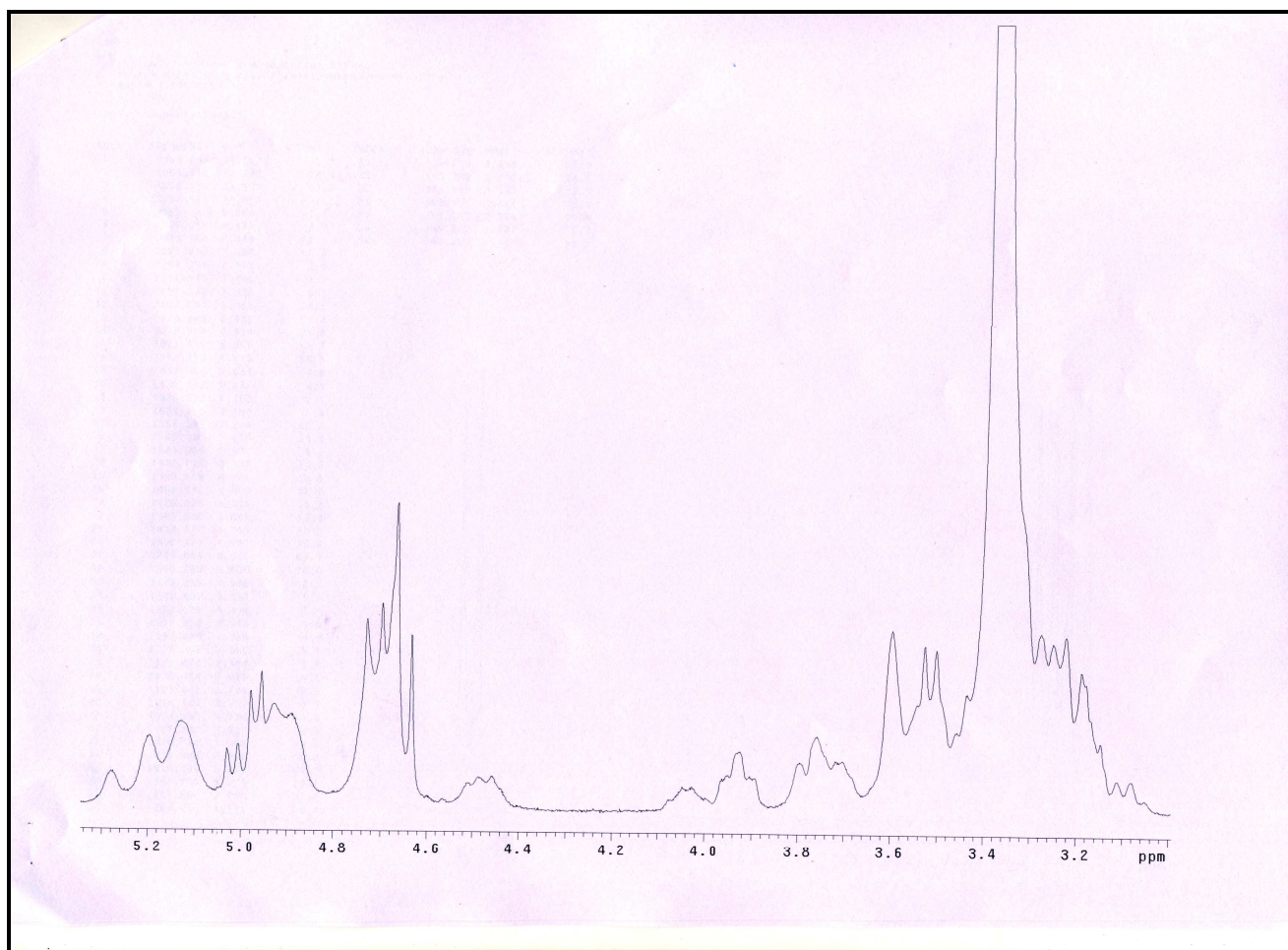
Title: SAIF-IIT, Powai, Mumbai

Operator ID: SMD
Company name: ThermoFinnigan
Method filename: C:\Eager 300 for EA1112\data\Sys_data_example\chn-2009\30-11-2009-CHNS.mth
Method name: NCHS
Analysed: 11/30/2009 13:37
Printed: 12-01-2009 10:34
Elemental Analyser method:
Sampler method:
Sample ID: 30-11-2009-012-BR-1-CHN-144 (# 12)
Analysis type: UnkNown
Chromatogram filename: 30-11-2009-012-BR-1-CHN-144.dat
Calibration method: Least Squares to Linear fit
Sample weight: 2.172
Protein factor: 6.25

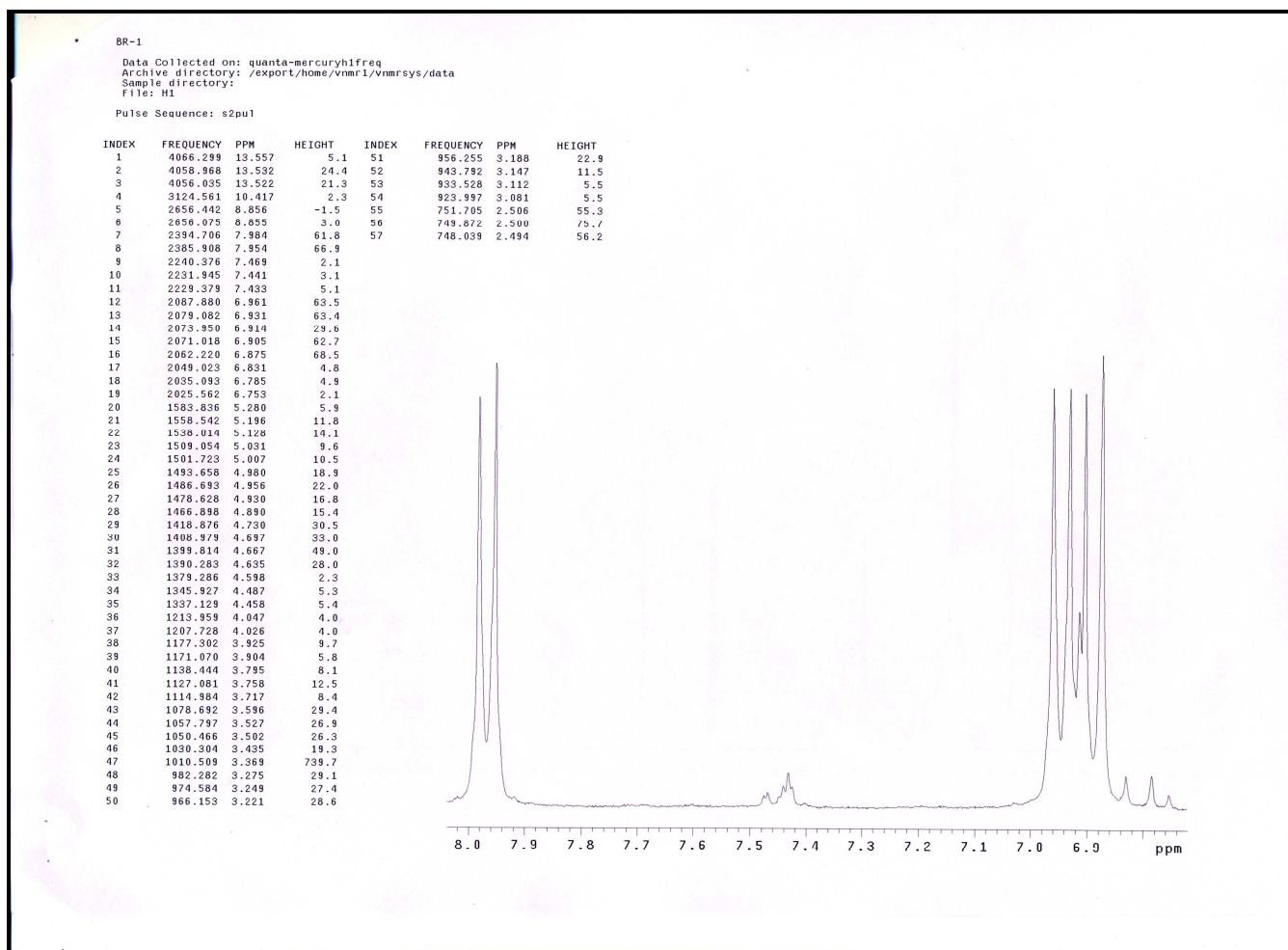
**Elemental analysis of BR-1**

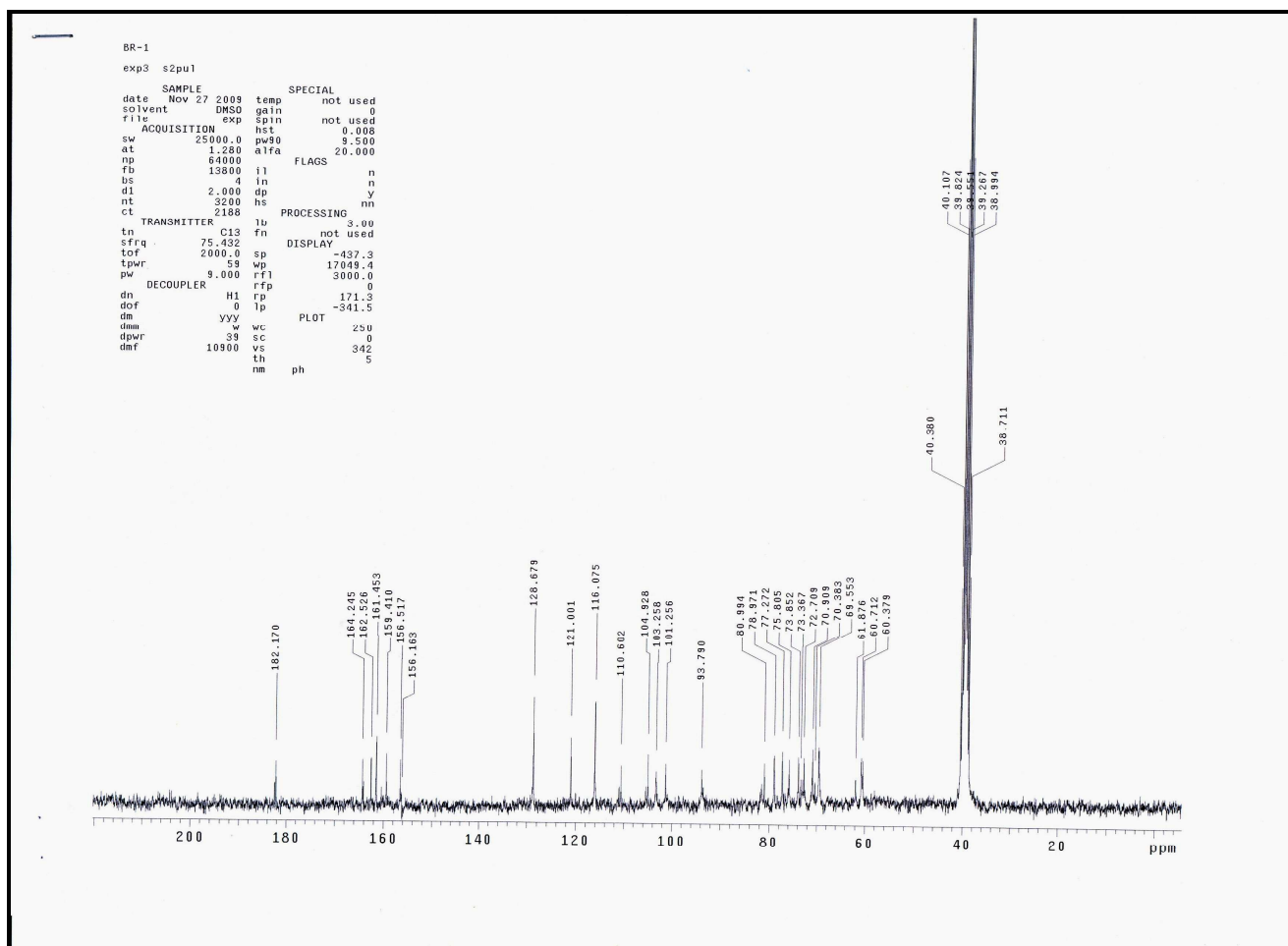


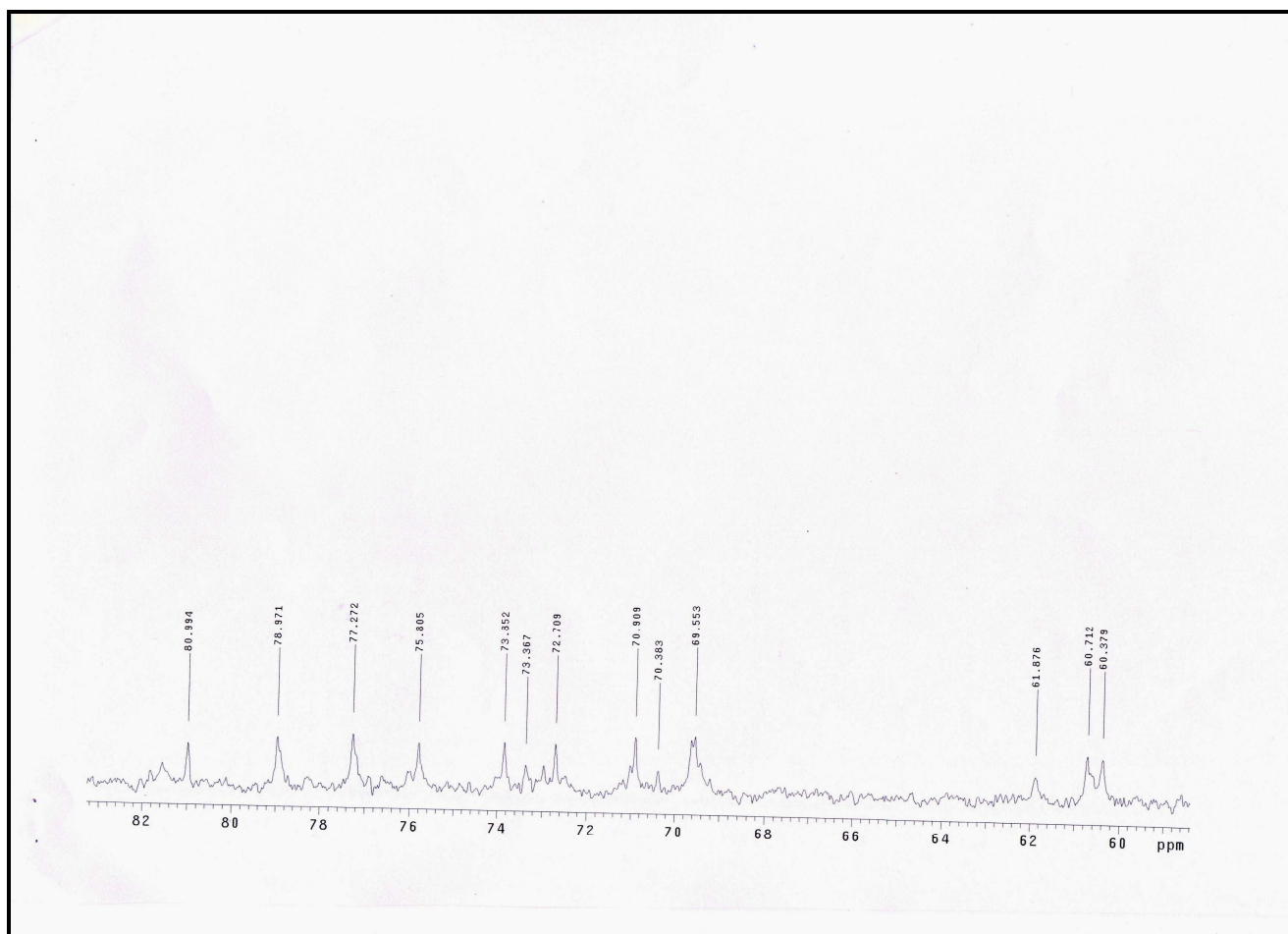
¹H NMR spectrum of BR-1



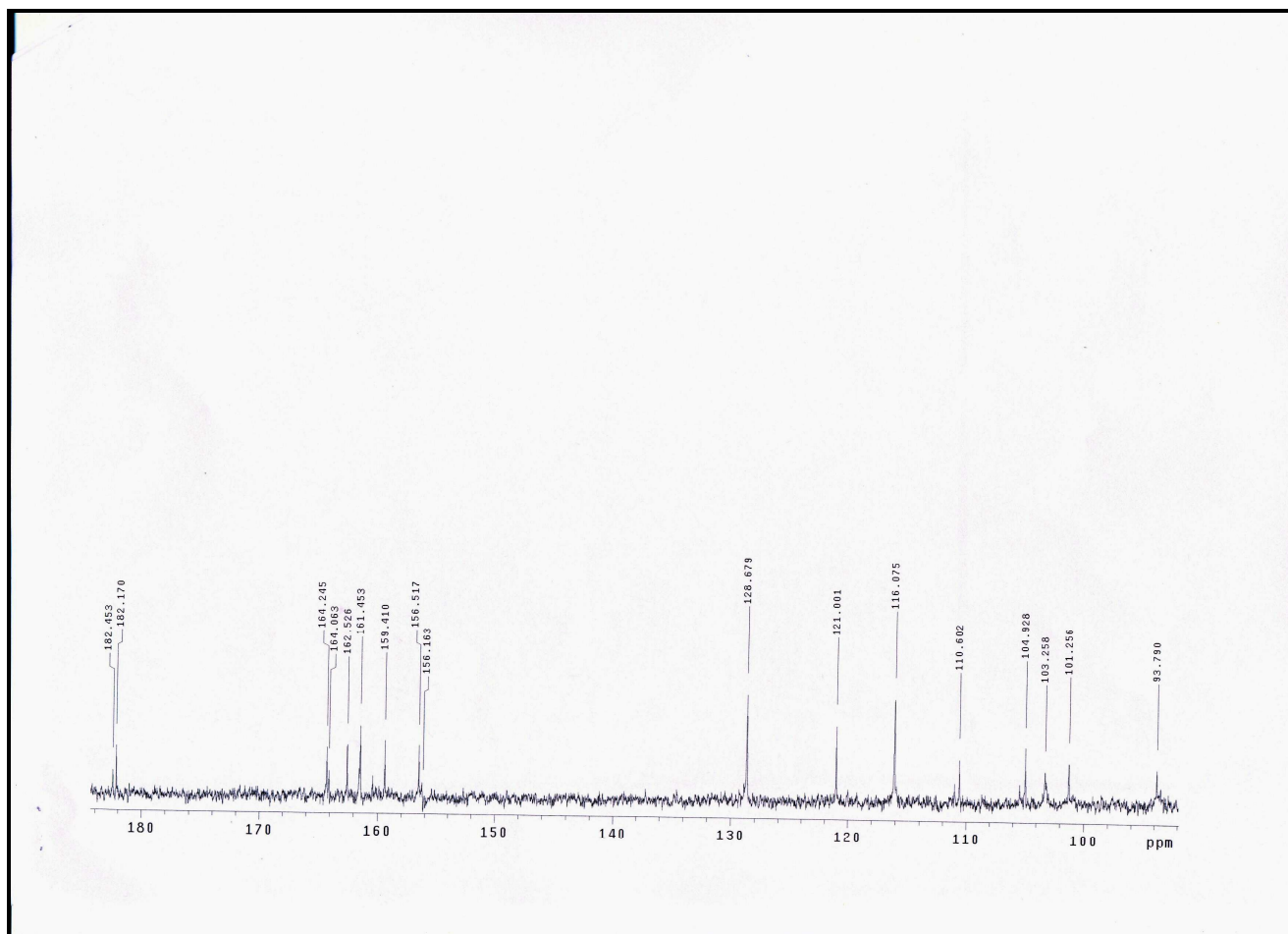
^1H NMR spectrum of BR-1

¹H NMR spectrum of BR-1

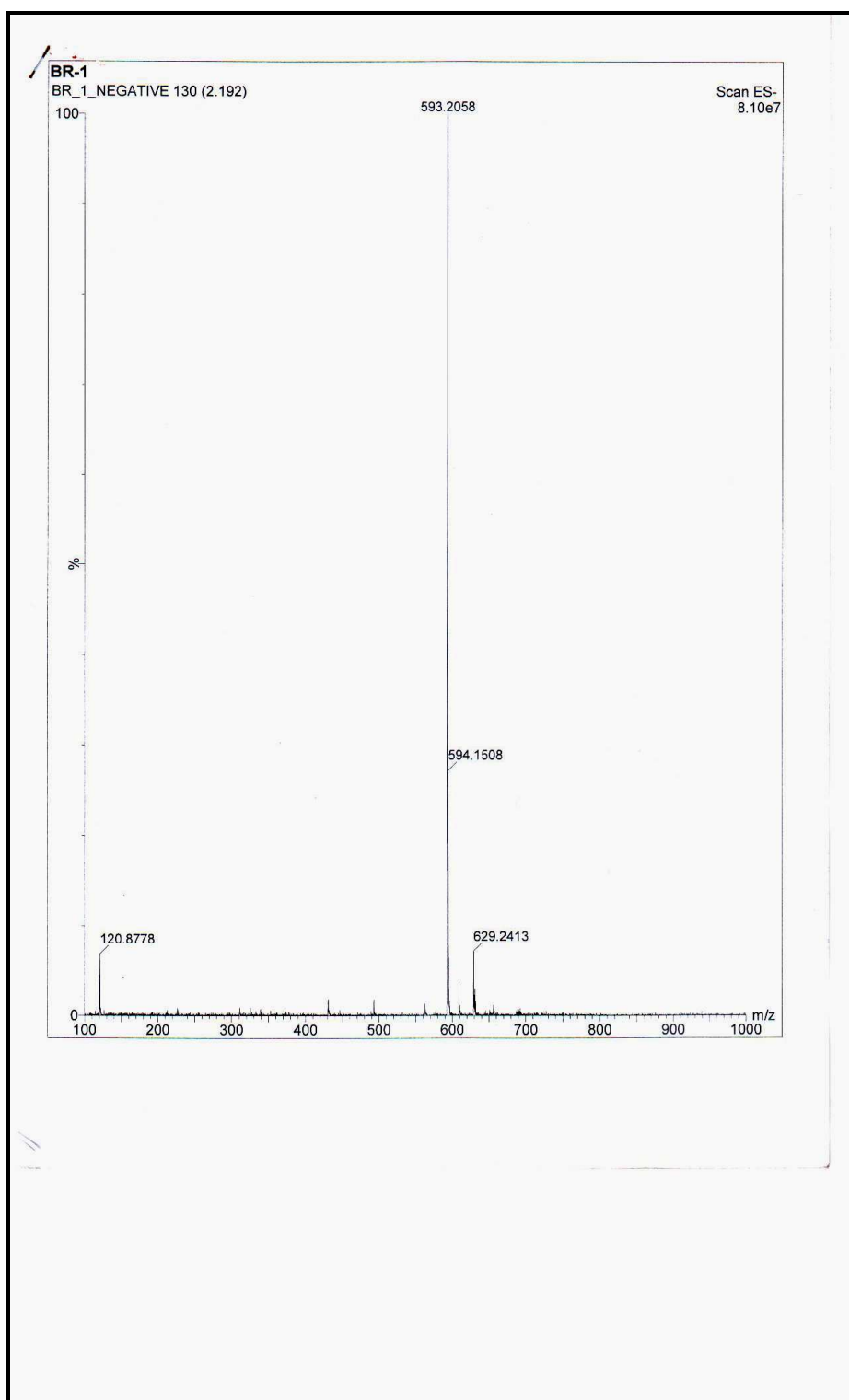
¹³C NMR spectrum of BR-1



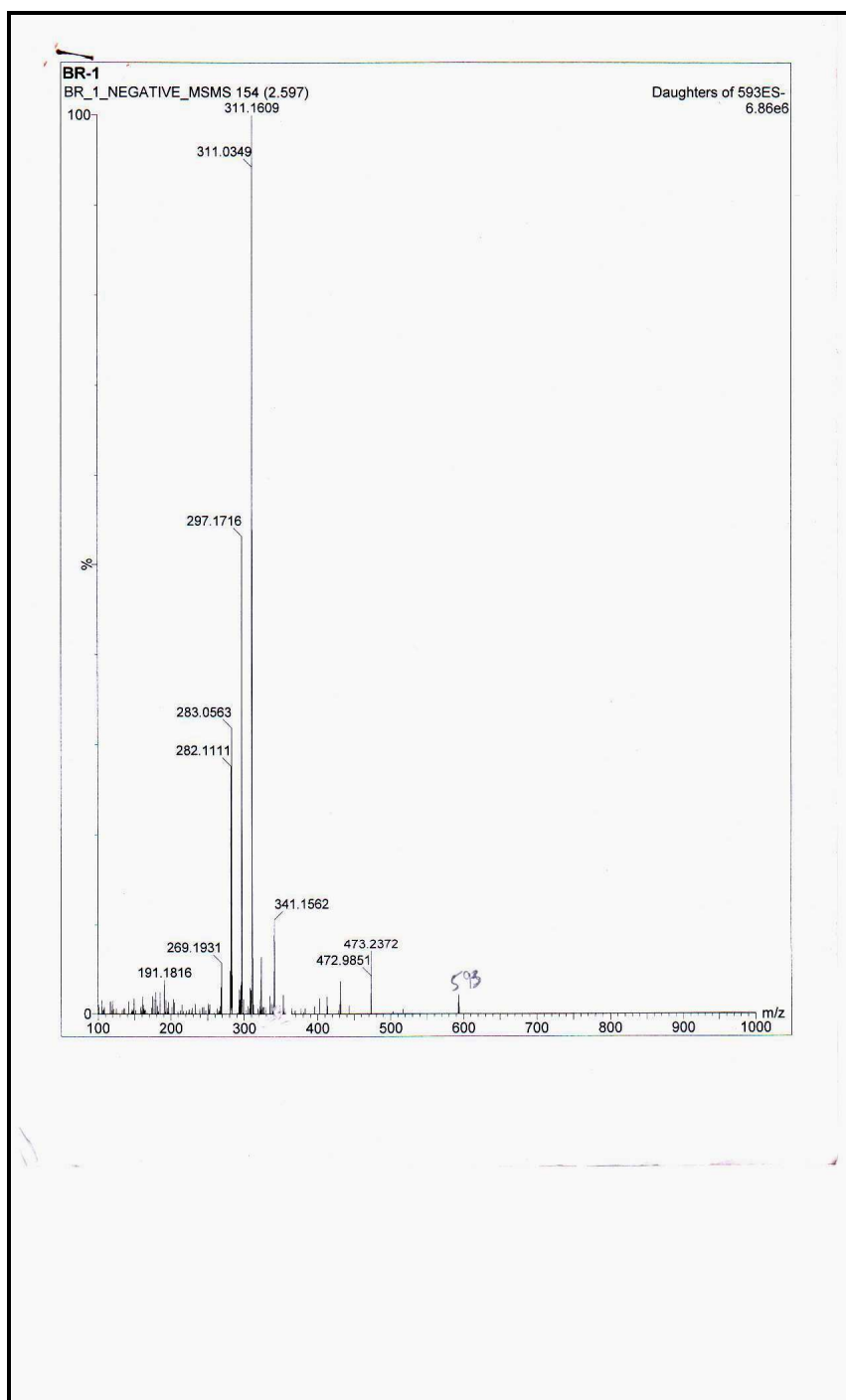
^{13}C NMR spectrum of BR-1



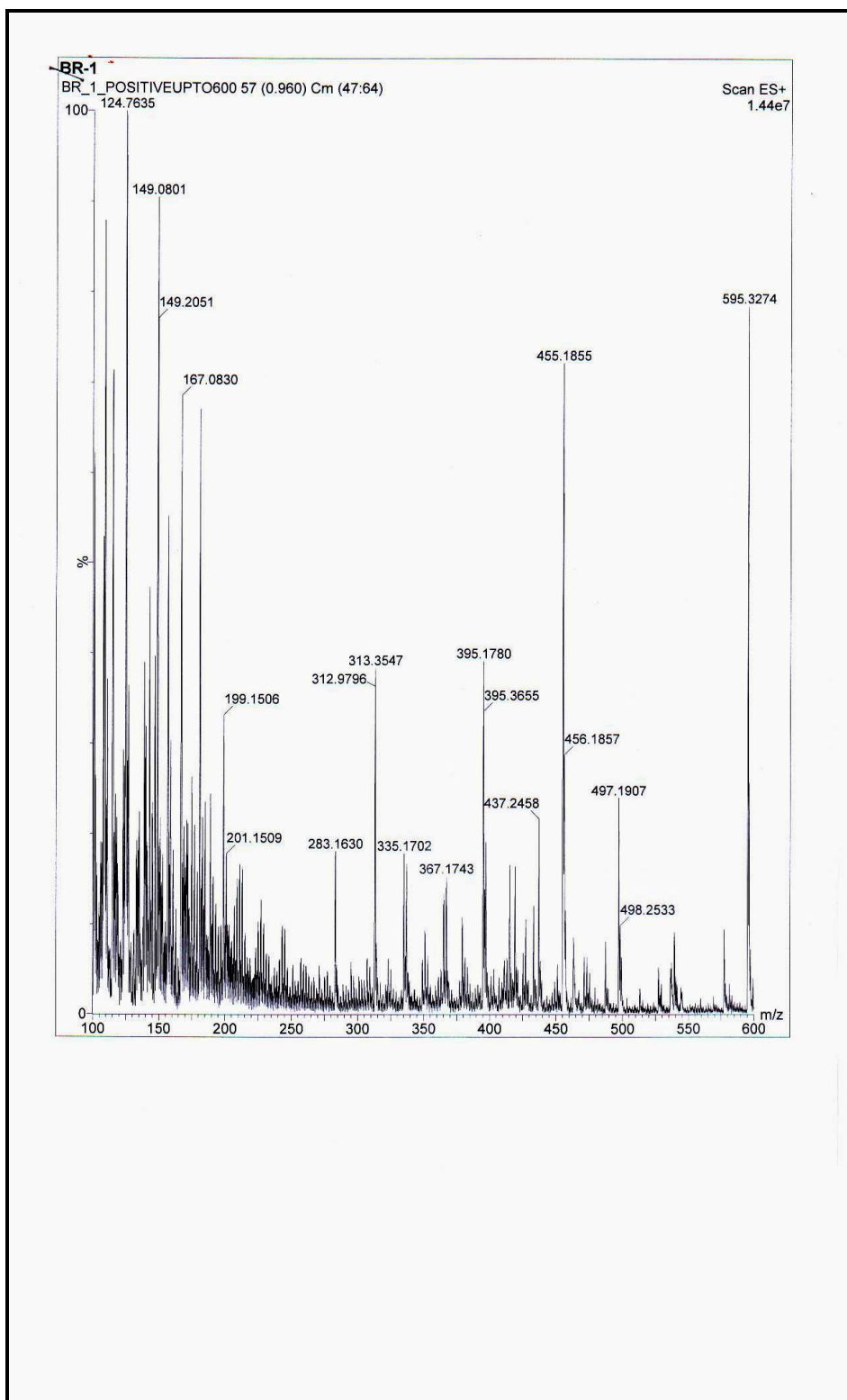
^{13}C NMR spectrum of BR-1



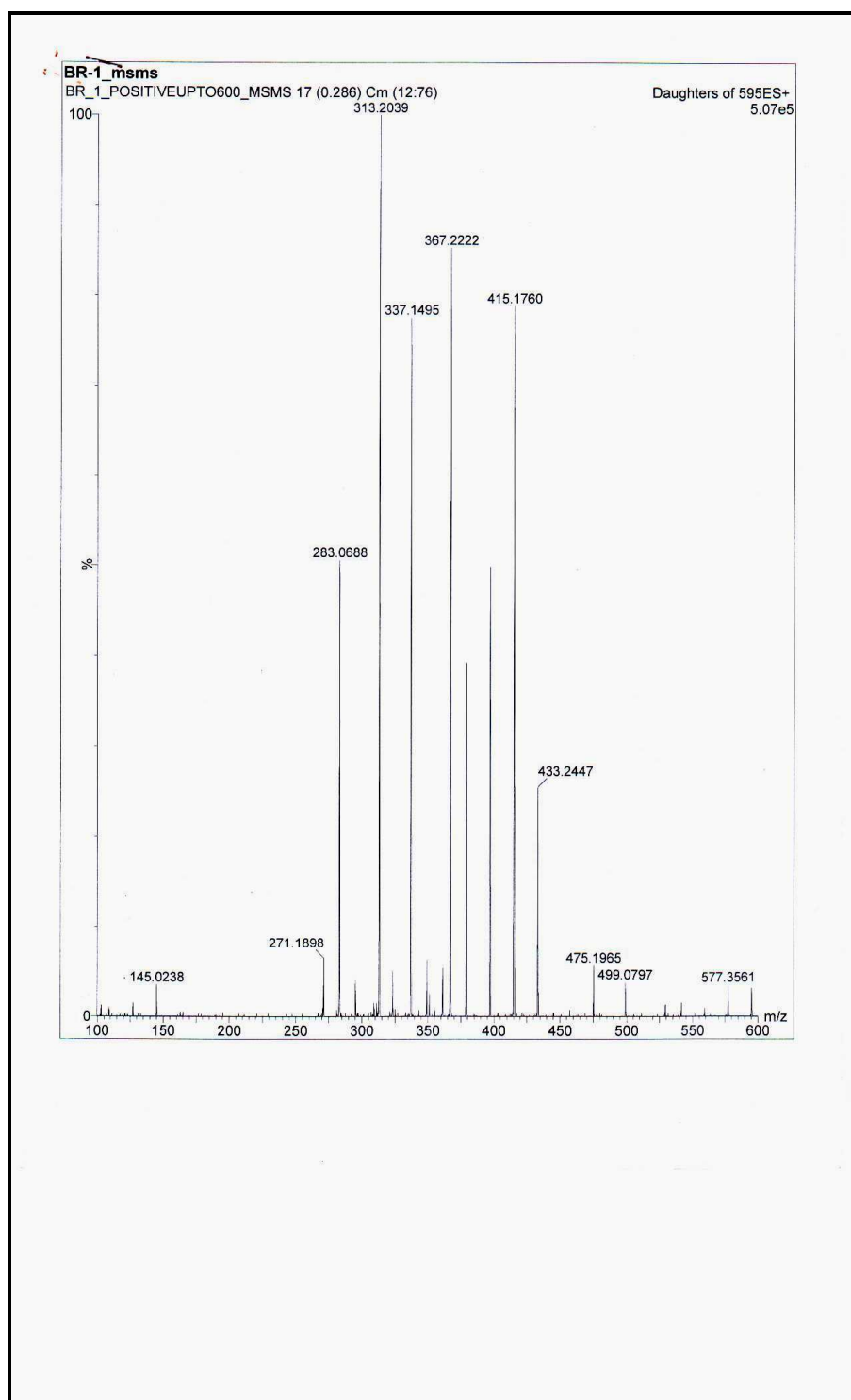
ESI MS spectrum of BR-1 (– ve mode)



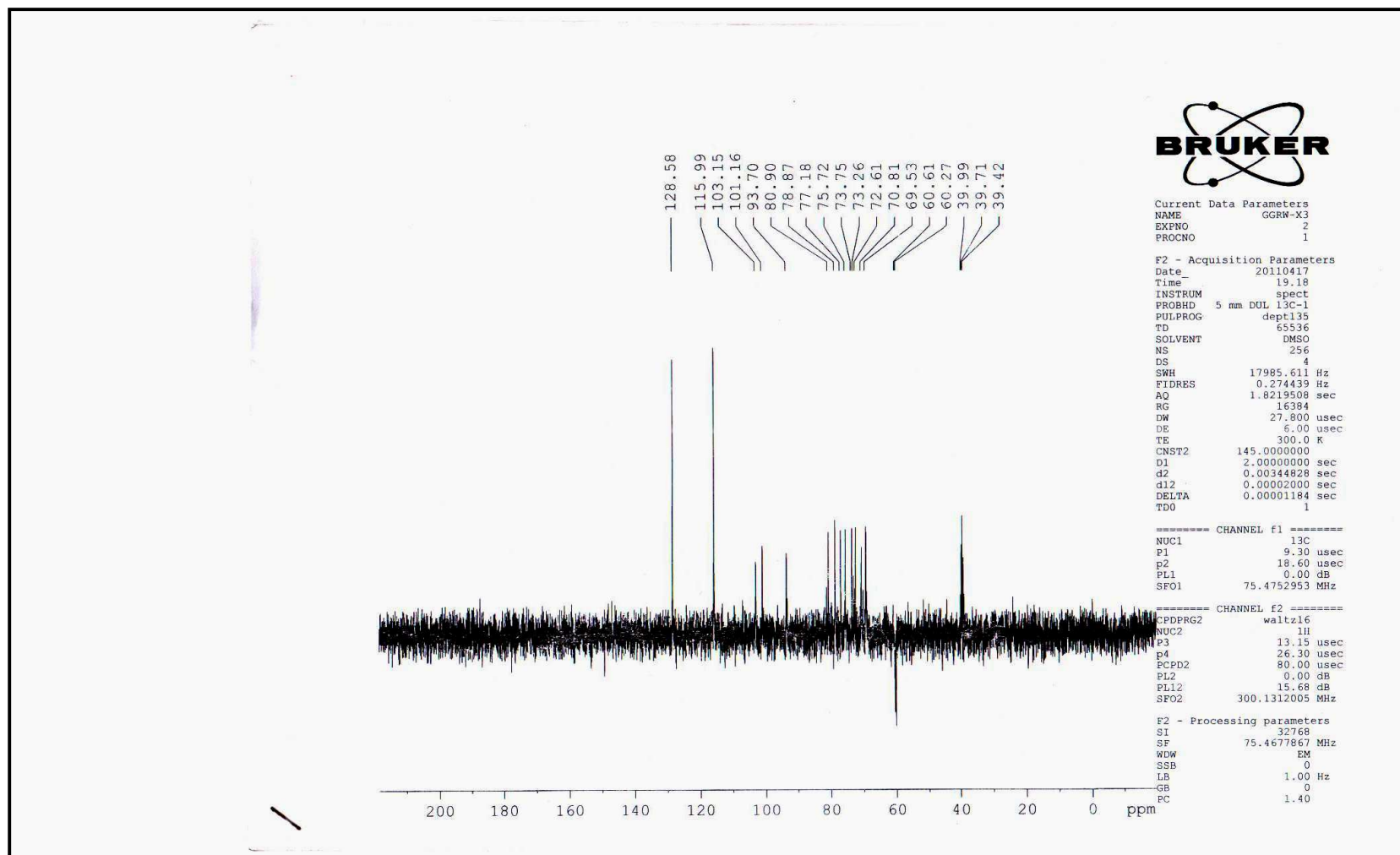
ESI MSMS spectrum of BR-1 (– ve mode)



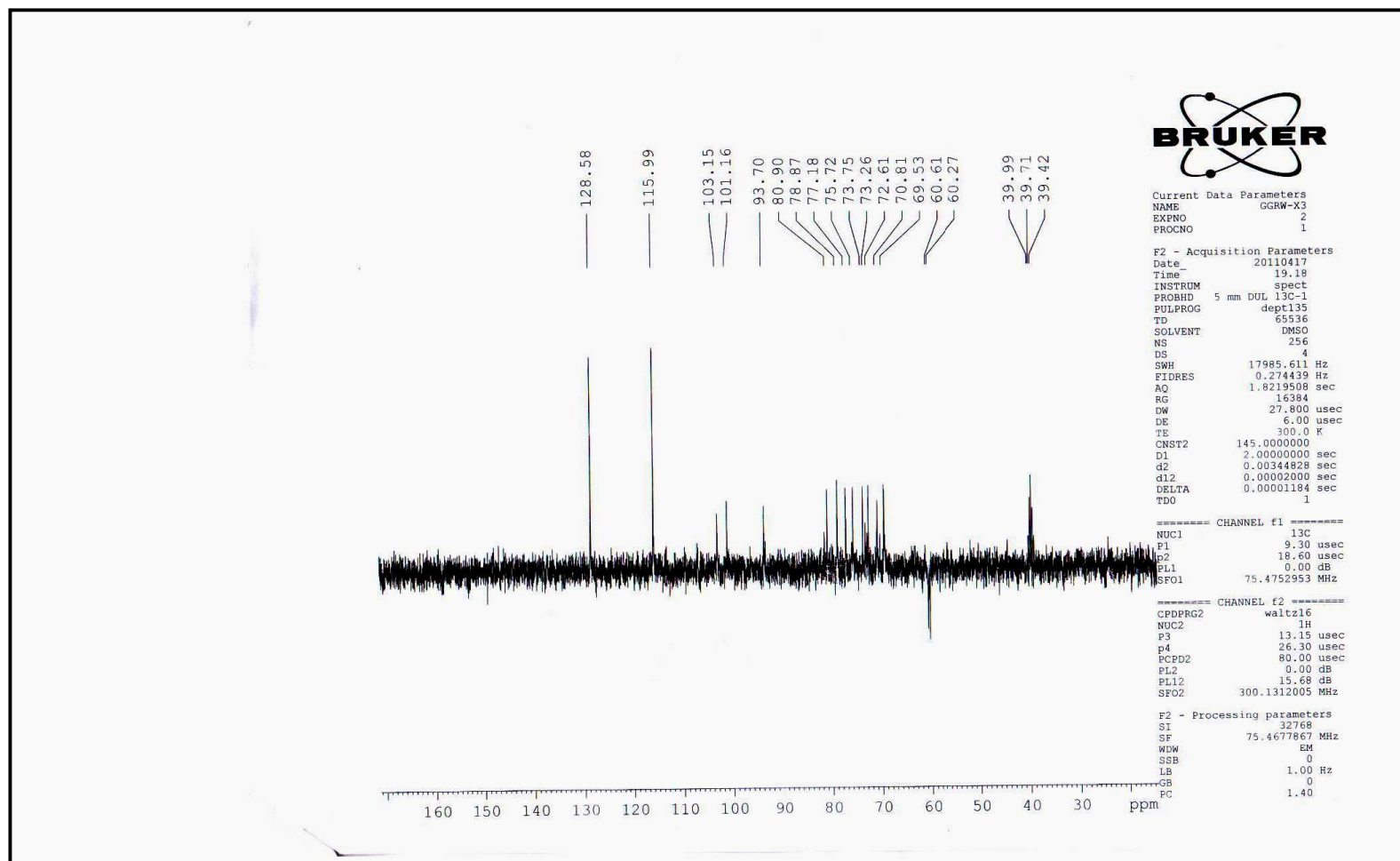
ESI MS spectrum of BR-1 (+ ve mode)



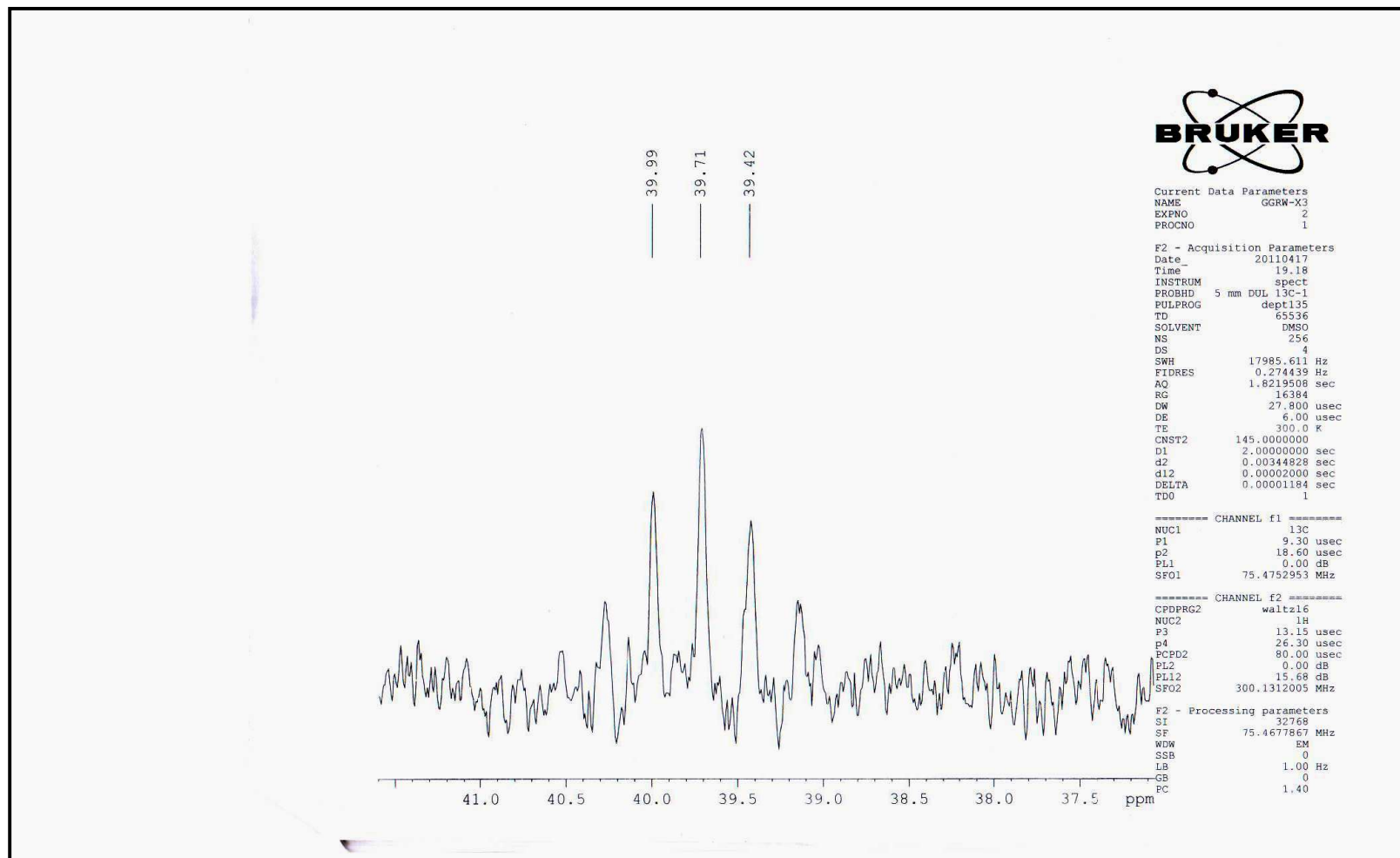
ESI MSMS spectrum of BR-1 (+ ve mode)



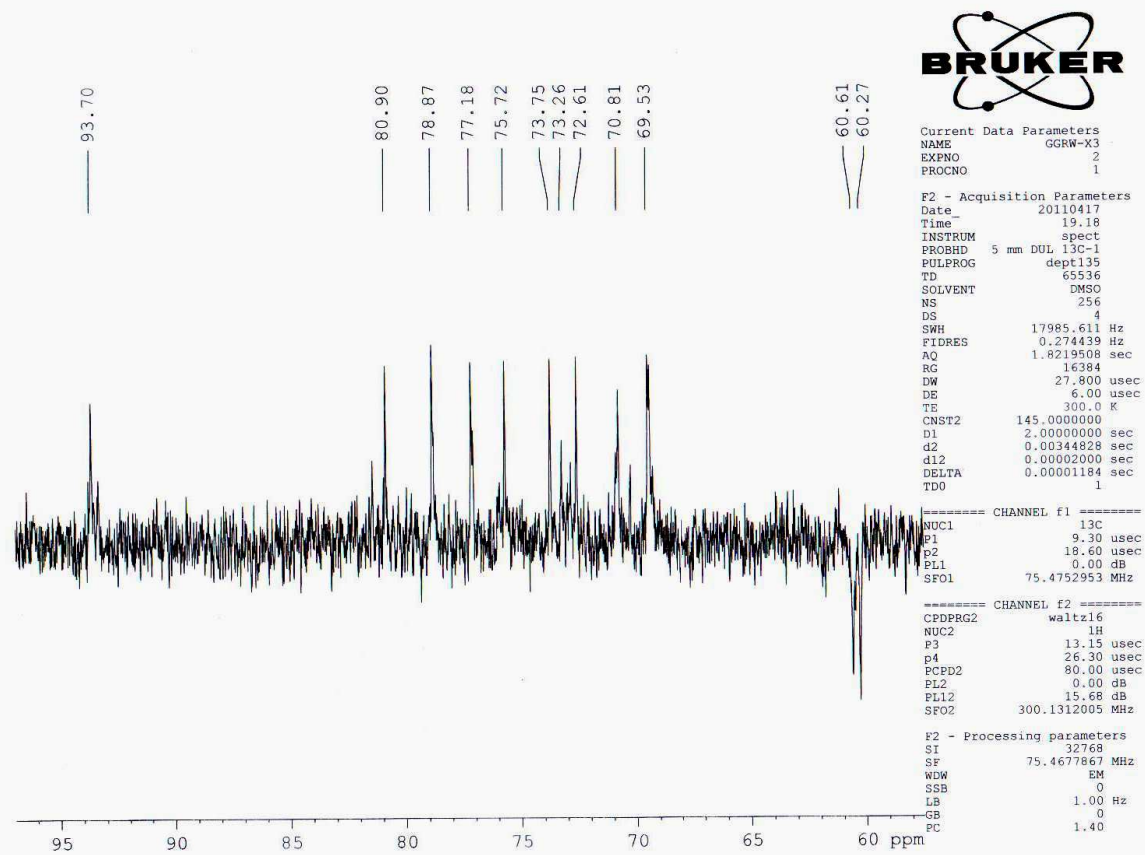
DEPT 135 Spectrum of BR-1



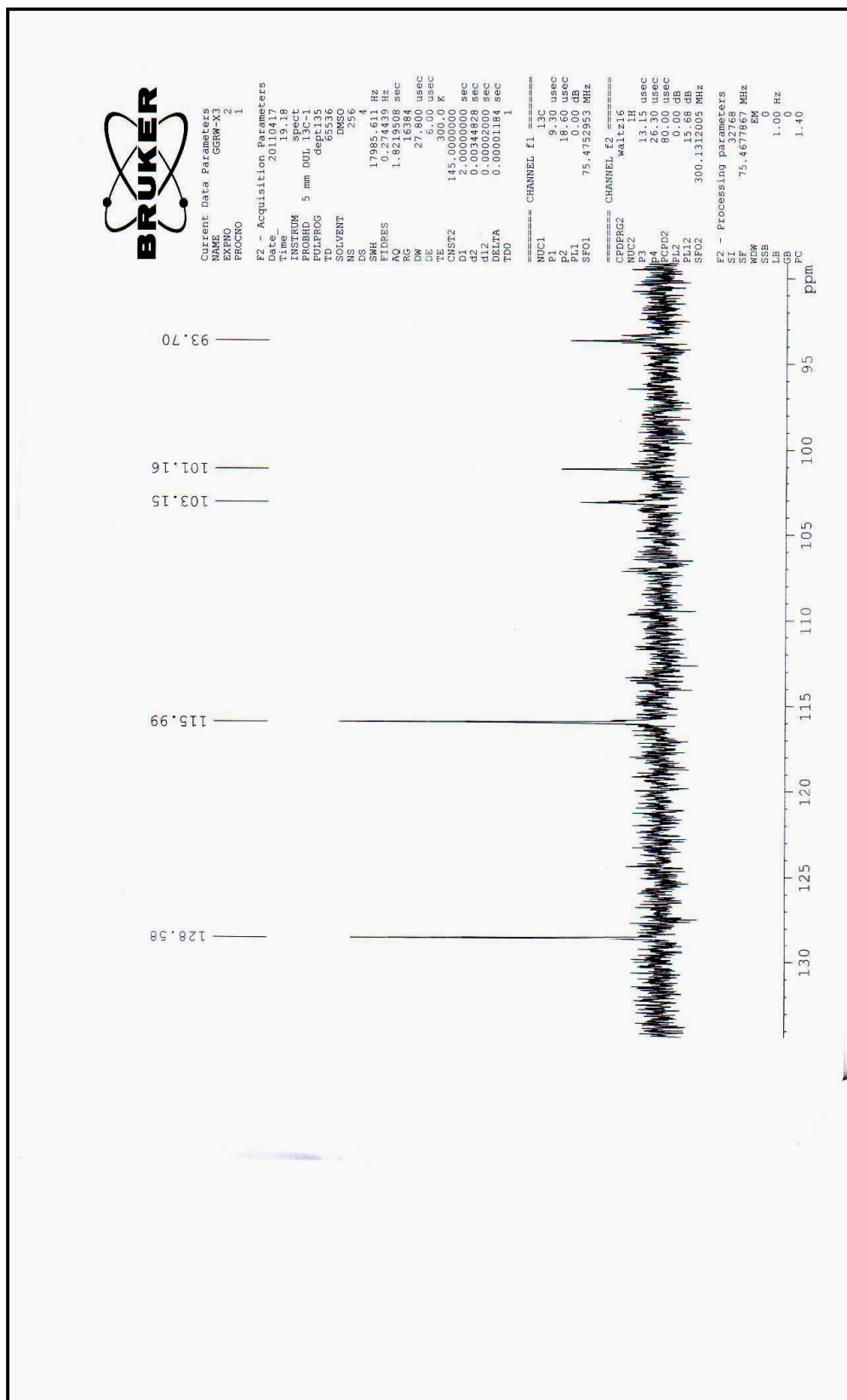
DEPT 135 Spectrum of BR-1



DEPT 135 Spectrum of BR-1

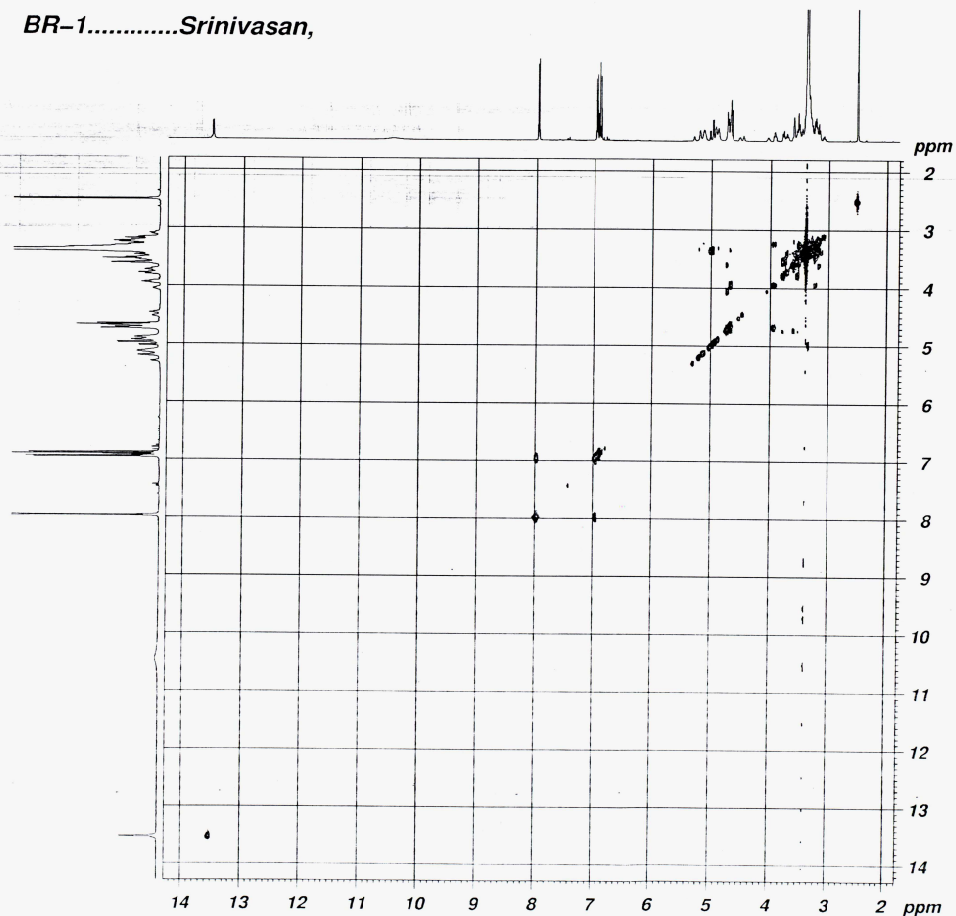


DEPT 135 Spectrum of BR-1



DEPT 135 Spectrum of BR-1

BR-1.....Srinivasan,



Current Data Parameters
NAME May08-2014
EXPNO 11
PROCNO 1

F2 - Acquisition Parameters
Date_ 20140508
Time 8.01
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG cosygpgqf
TD 2048
SOLVENT DMSO
NS 6
DS 8
SWH 6250.000 Hz
FIDRES 3.051758 Hz
AQ 0.1638900 sec
RG 64
DW 80.000 usec
DE 6.50 usec
TE 297.0 K
D0 0.00000300 sec
D1 1.47624195 sec
D13 0.00000400 sec
D16 0.00020000 sec
IN0 0.00016000 sec

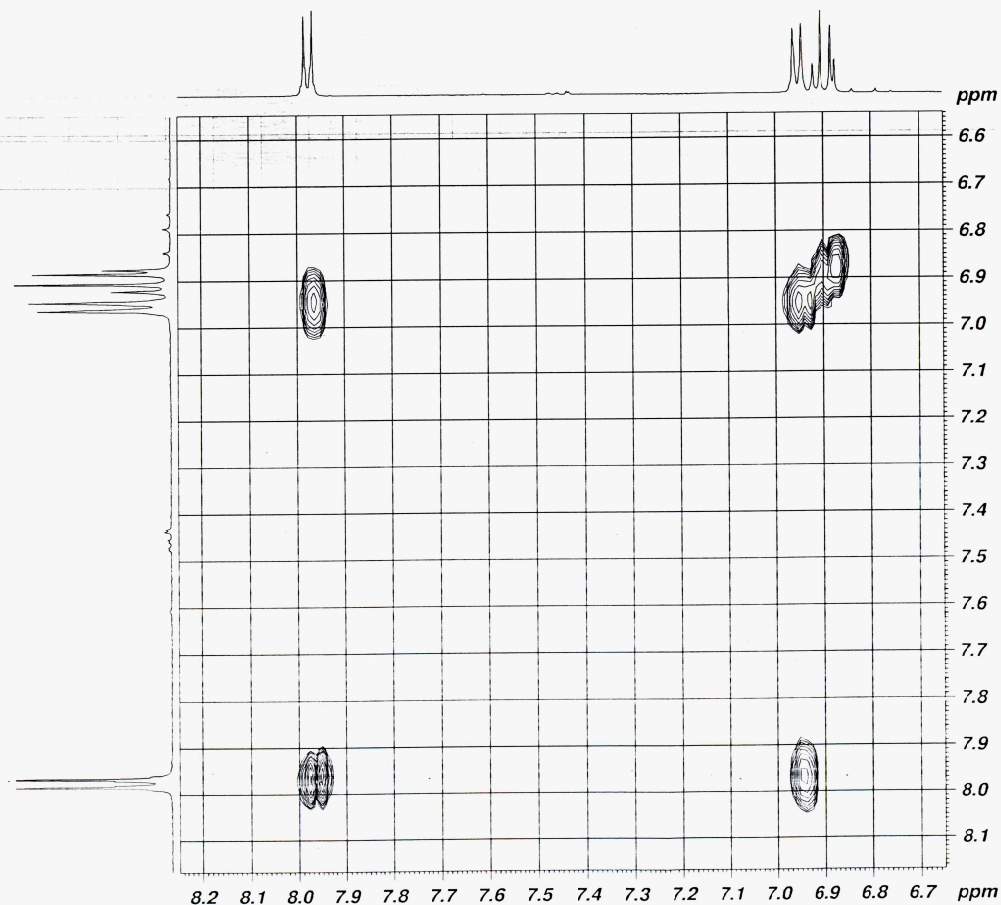
===== CHANNEL f1 =====
NUC1 1H
P0 10.65 usec
P1 10.65 usec
PL1 0.00 dB
PL1W 23.53637505 W
SFO1 500.1340221 MHz

===== GRADIENT CHANNEL
GPNAM1 SINE.100
GPZ1 10.00 %
P16 1000.00 usec

F1 - Acquisition parameters
ND0 1
TD 128
SFO1 500.134 MHz

H-H COSY Spectrum of BR-1

BR-1.....Srinivasan,



Current Data Parameters
NAME May08-2014
EXPNO 11
PROCNO 1

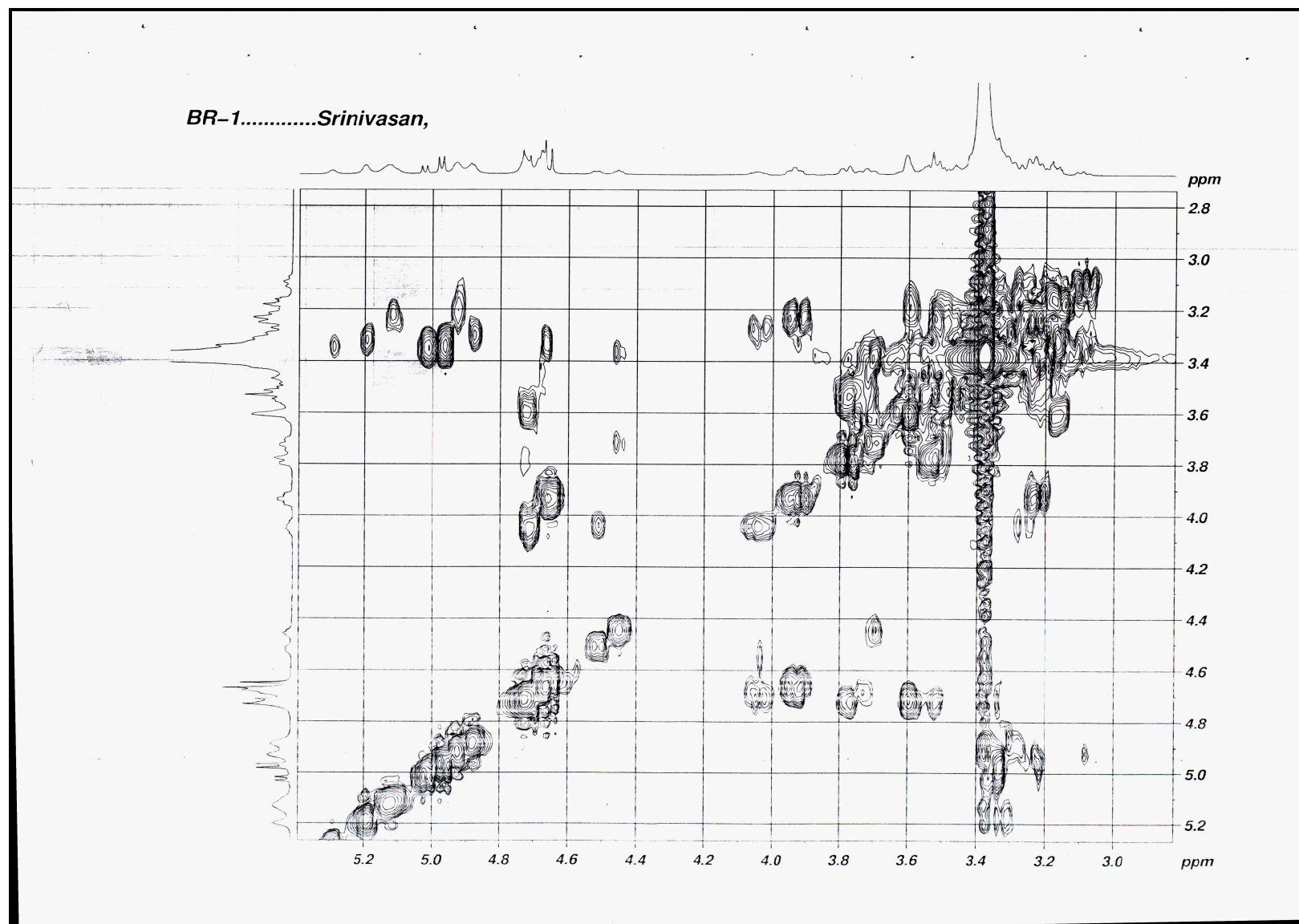
F2 - Acquisition Parameters
Date_ 20140508
Time 8.01
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG cosygpgf
TD 2048
SOLVENT DMSO
NS 6
DS 8
SWH 6250.000 Hz
FIDRES 3.051758 Hz
AQ 0.1638900 sec
RG 64
DW 80.000 usec
DE 6.50 usec
TE 297.0 K
D0 0.00000300 sec
D1 1.47624195 sec
D13 0.00000400 sec
D16 0.00020000 sec
IN0 0.00016000 sec

===== CHANNEL f1 =====
NUC1 1H
P0 10.65 usec
P1 10.65 usec
PL1 0.00 dB
PL1W 23.53637505 W
SFO1 500.1340221 MHz

===== GRADIENT CHANNEL
GPNAM1 SINE.100
GPZ1 10.00 %
P16 1000.00 usec

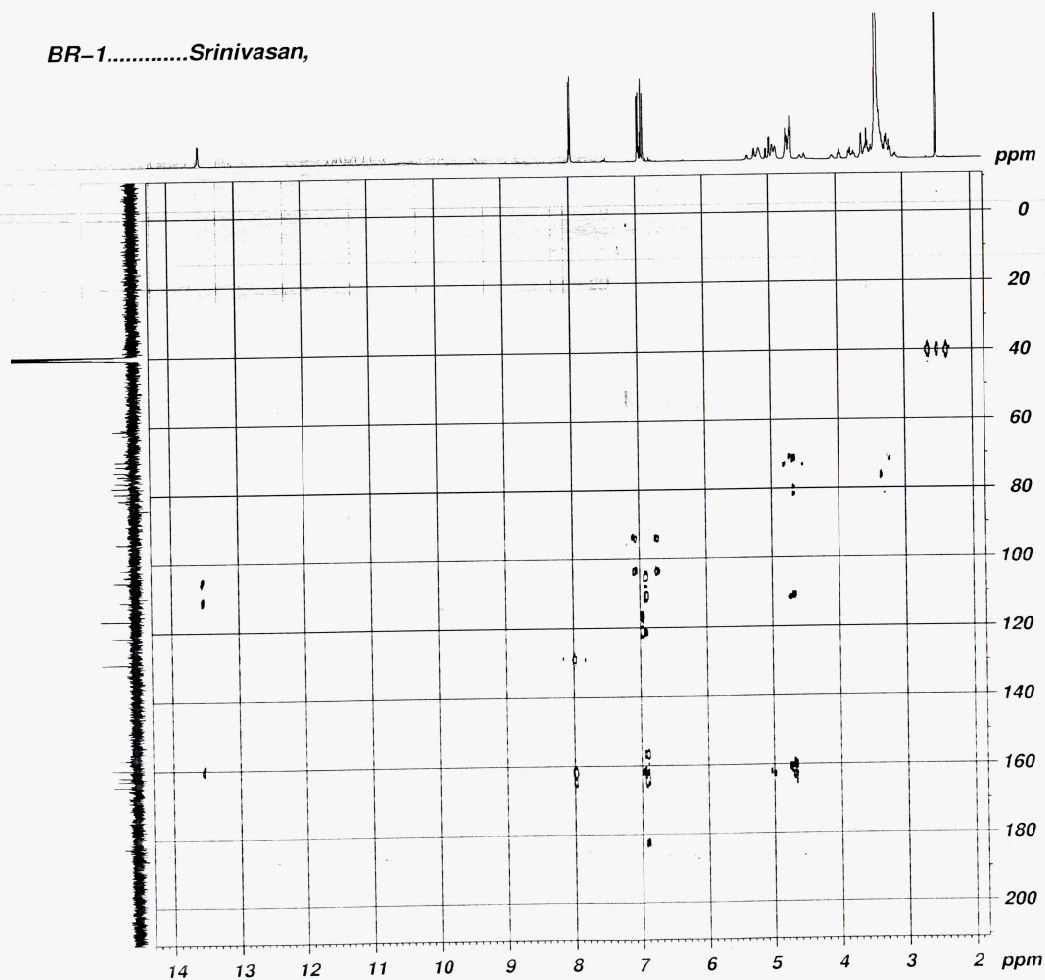
F1 - Acquisition parameters
ND0 1
TD 128
SFO1 500.134 MHz

H-H COSY Spectrum of BR-1



H-H COSY Spectrum of BR-1

BR-1.....Srinivasan,



Current Data Parameters
NAME May08-2014
EXPNO 13
PROCNO 1

F2 - Acquisition Parameters
Date_ 20140508
Time 9.07
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG hmbcgpndqf
TD 4096
SOLVENT DMSO
NS 32
DS 16
SWH 6250.000 Hz
FIDRES 1.525879 Hz
AQ 0.3277300 sec
RG 203
DW 80.000 usec
DE 6.50 usec
TE 296.3 K
CNST13 8.0000000
D0 0.00000300 sec
D1 1.47951996 sec
D6 0.06250000 sec
D16 0.00020000 sec
IN0 0.00001790 sec

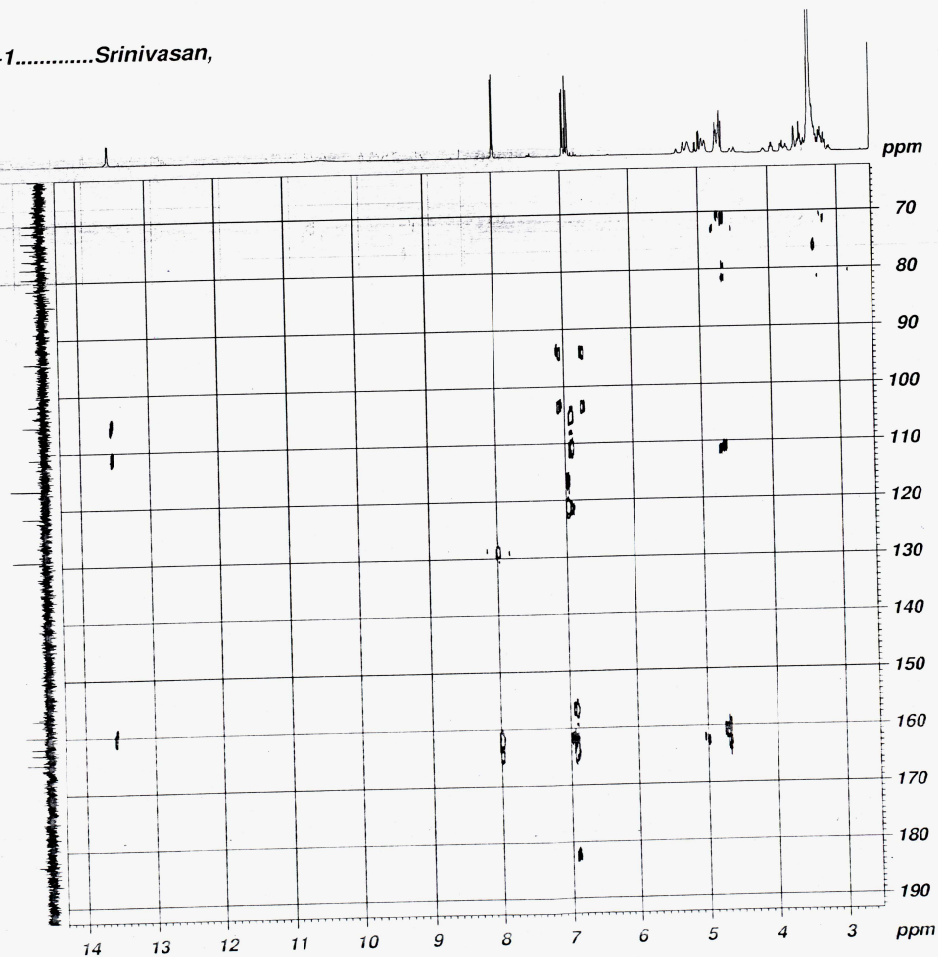
===== CHANNEL f1 =====
NUC1 ^1H
P1 10.65 usec
P2 21.30 usec
PL1 0.00 dB
PL1W 23.53637505 W
SFO1 500.1340221 MHz

===== CHANNEL f2 =====
NUC2 ^{13}C
P3 7.80 usec
PL2 0.00 dB
PL2W 70.83519745 W
SFO2 125.7703443 MHz

===== GRADIENT CHANNEL :

HMBC Spectrum of BR-1

BR-1.....Srinivasan,



Current Data Parameters
NAME May08-2014
EXPNO 13
PROCNO 1

F2 - Acquisition Parameters
Date 20140508
Time 9.07
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG hmbcgpndqf
TD 4096
SOLVENT DMSO
NS 32
DS 16
SWH 6250.000 Hz
FIDRES 1.525879 Hz
AQ 0.3277300 sec
RG 203
DW 80.000 usec
DE 6.50 usec
TE 296.3 K
CNST13 8.0000000
D0 0.00000300 sec
D1 1.47951996 sec
D6 0.06250000 sec
D16 0.00020000 sec
IN0 0.00001790 sec

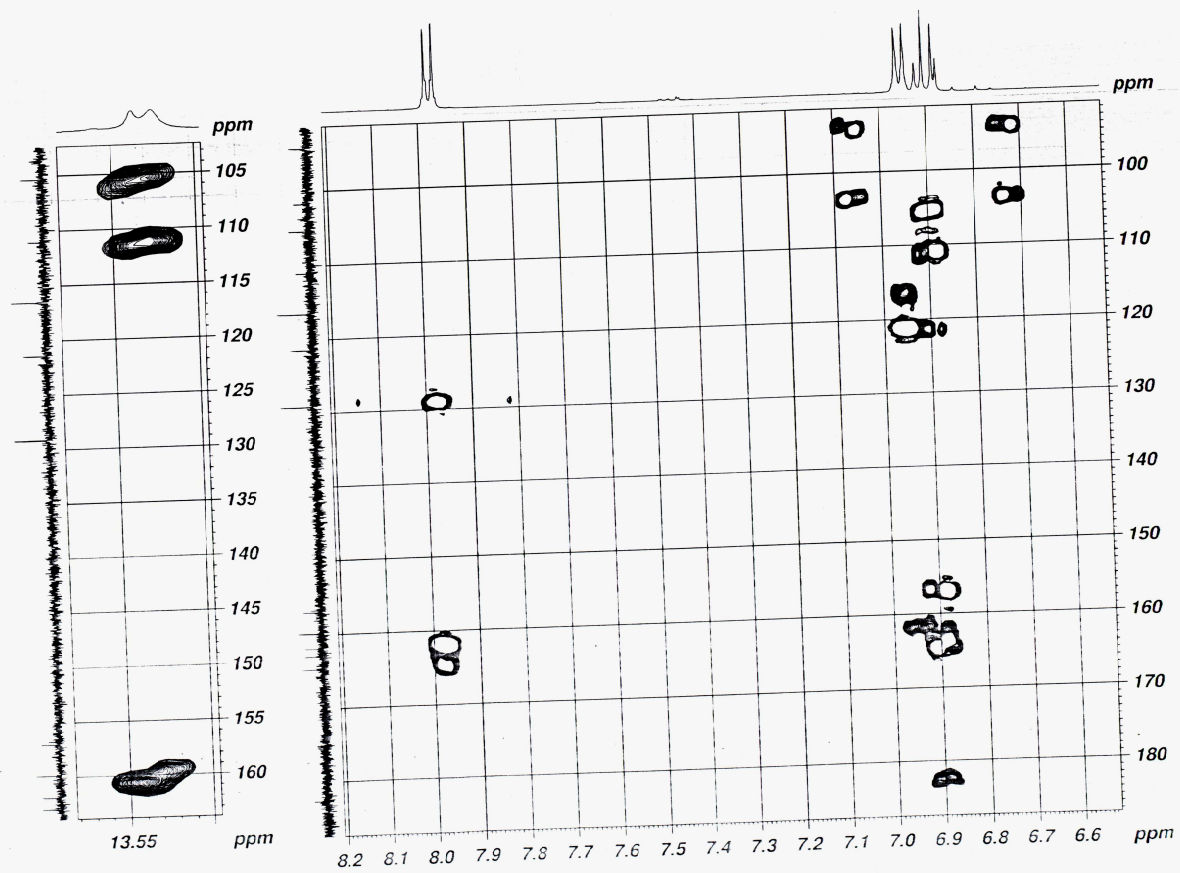
===== CHANNEL f1 =====
NUC1 1H
P1 10.65 usec
P2 21.30 usec
PL1 0.00 dB
PL1W 23.53637505 W
SFO1 500.1340221 MHz

===== CHANNEL f2 =====
NUC2 13C
P3 7.80 usec
PL2 0.00 dB
PL2W 70.83519745 W
SFO2 125.7703443 MHz

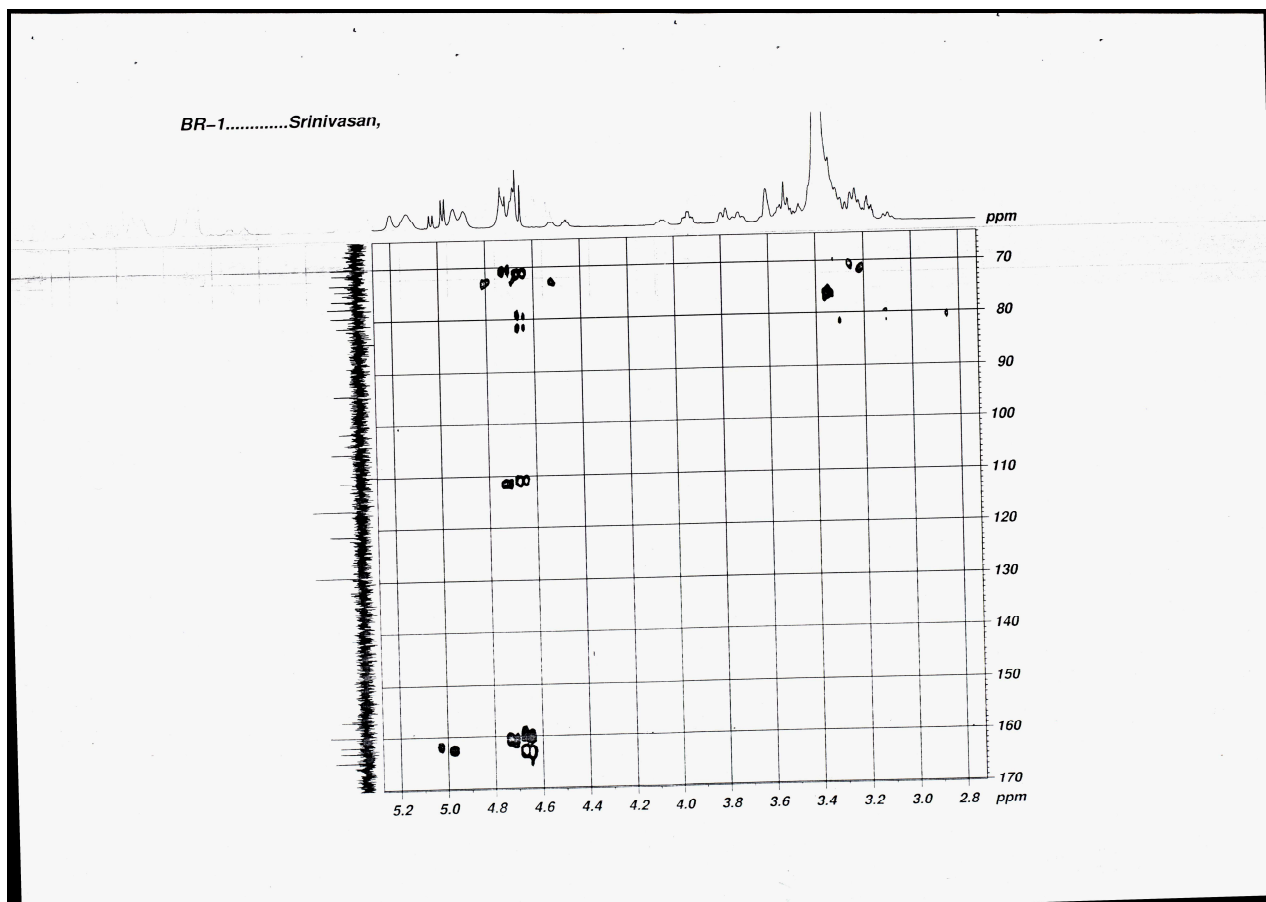
===== GRADIENT CHANNEL :

HMBC Spectrum of BR-1

BR-1.....Srinivasan,

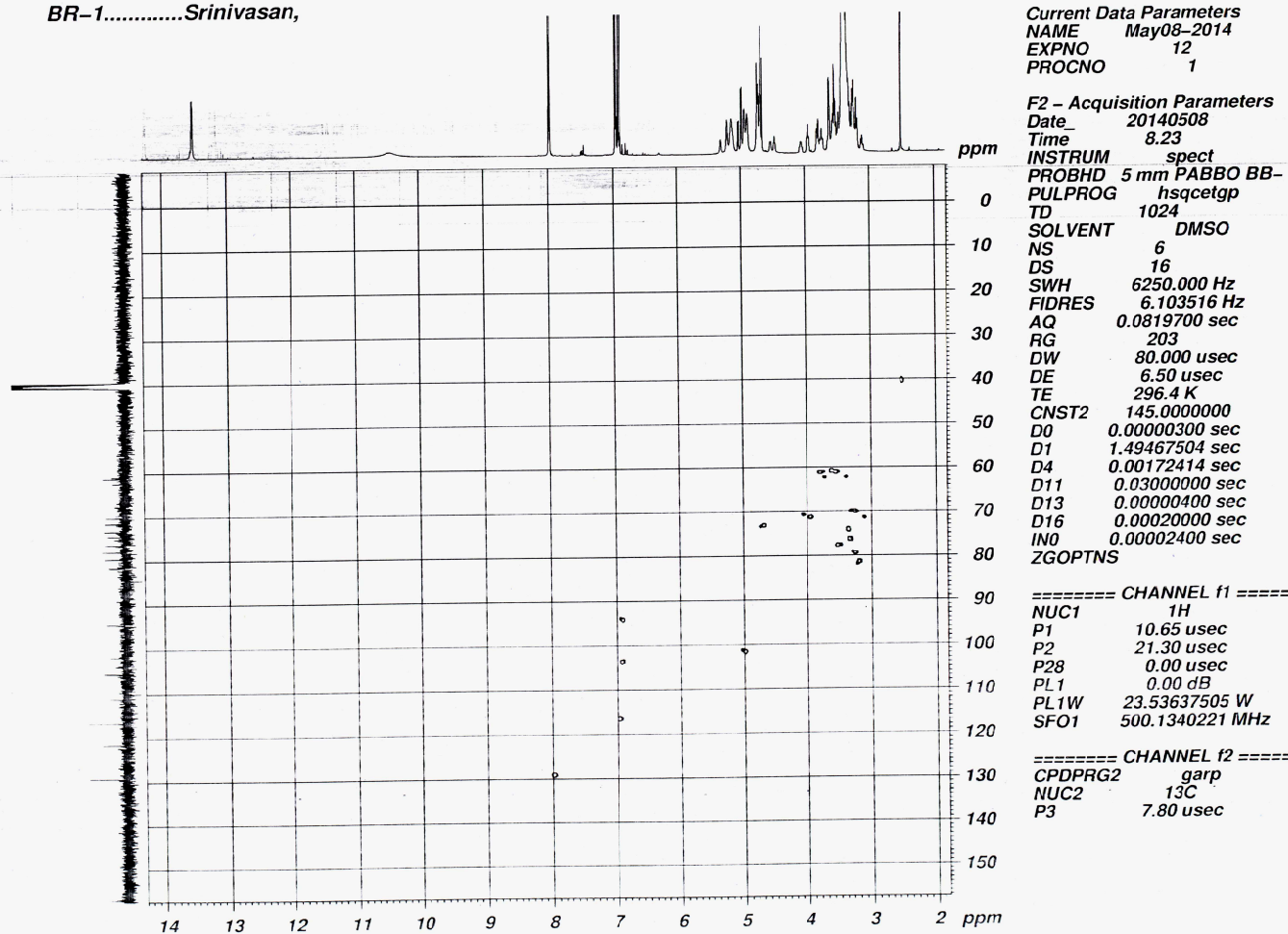


HMBC Spectrum of BR-1

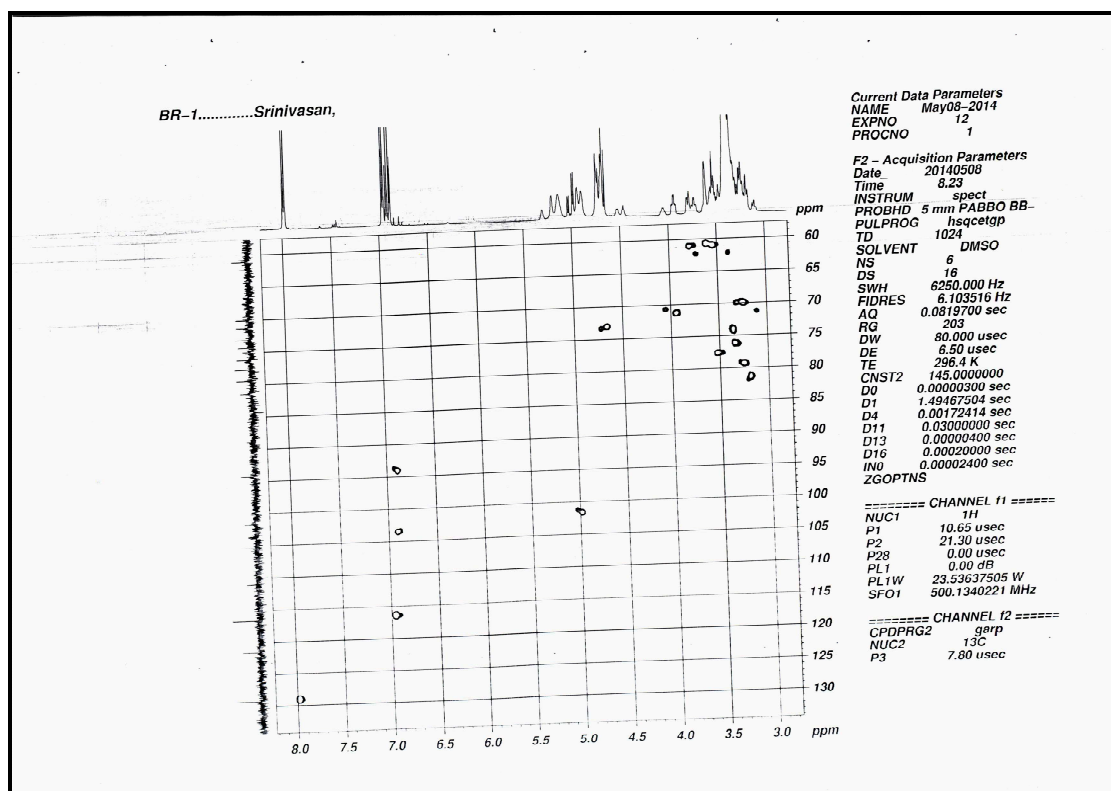


HMBC Spectrum of BR-1

BR-1.....Srinivasan,

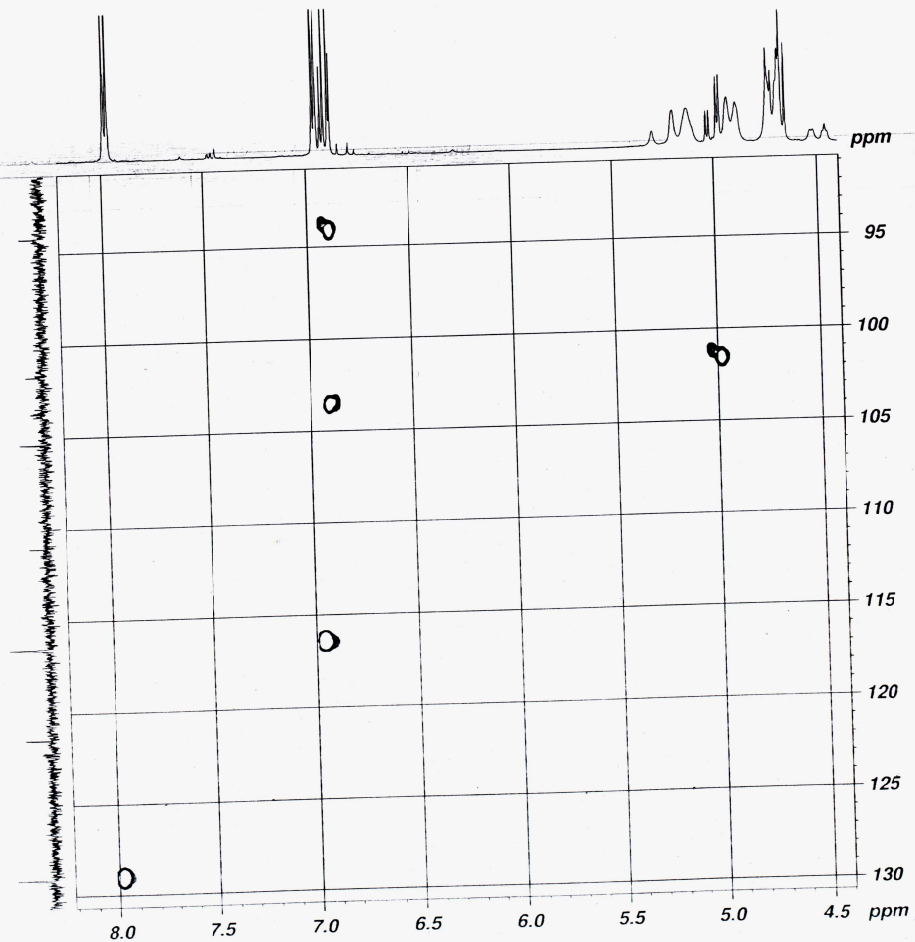


HSQC Spectrum of BR-1



HSQC Spectrum of BR-1

BR-1.....Srinivasan,



Current Data Parameters
NAME May08-2014
EXPNO 12
PROCNO 1

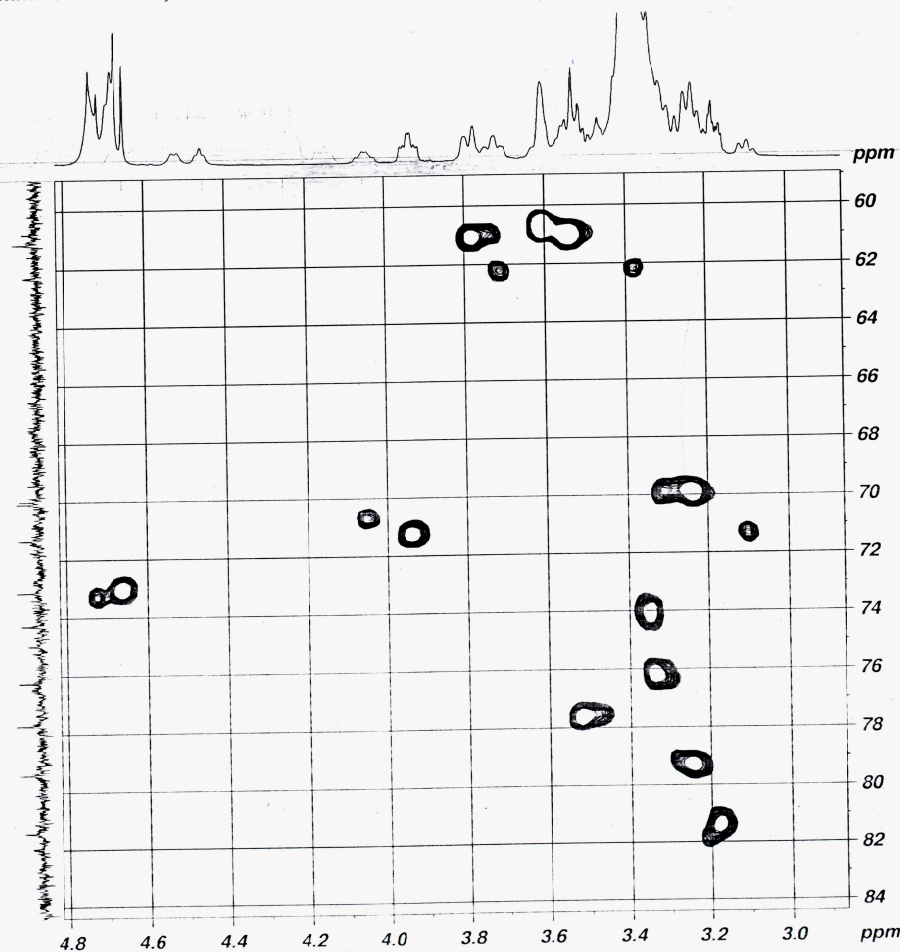
F2 - Acquisition Parameters
Date 20140508
Time 8.23
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG hsqcetgp
TD 1024
SOLVENT DMSO
NS 6
DS 16
SWH 6250.000 Hz
FIDRES 6.103516 Hz
AQ 0.0819700 sec
RG 203
DW 80.000 usec
DE 6.50 usec
TE 296.4 K
CNST2 145.0000000
D0 0.00000300 sec
D1 1.49467504 sec
D4 0.00172414 sec
D11 0.03000000 sec
D13 0.00000400 sec
D16 0.00020000 sec
IN0 0.00002400 sec
ZGPTNS

===== CHANNEL f1 =====
NUC1 1H
P1 10.65 usec
P2 21.30 usec
P28 0.00 usec
PL1 0.00 dB
PL1W 23.53637505 W
SFO1 500.1340221 MHz

===== CHANNEL f2 =====
CPDPRG2 garp
NUC2 13C
P3 7.80 usec

HSQC Spectrum of BR-1

BR-1.....Srinivasan,



Current Data Parameters
 NAME May08-2014
 EXPNO 12
 PROCNO 1

F2 - Acquisition Parameters
 Date 20140508
 Time 8.23
 INSTRUM spect
 PROBHD 5 mm PABBO BB-
 PULPROG hsqcetgp
 TD 1024
 SOLVENT DMSO
 NS 6
 DS 16
 SWH 6250.000 Hz
 FIDRES 6.103516 Hz
 AQ 0.0819700 sec
 RG 203
 DW 80.000 usec
 DE 6.50 usec
 TE 296.4 K
 CNST2 145.0000000
 D0 0.00000300 sec
 D1 1.49467504 sec
 D4 0.00172414 sec
 D11 0.03000000 sec
 D13 0.00000400 sec
 D16 0.00020000 sec
 IN0 0.00002400 sec
 ZGPTNS

===== CHANNEL f1 =====
 NUC1 1H
 P1 10.65 usec
 P2 21.30 usec
 P28 0.00 usec
 PL1 0.00 dB
 PL1W 23.53637505 W
 SFO1 500.1340221 MHz

===== CHANNEL f2 =====
 CPDPRG2 garp
 NUC2 13C
 P3 7.80 usec

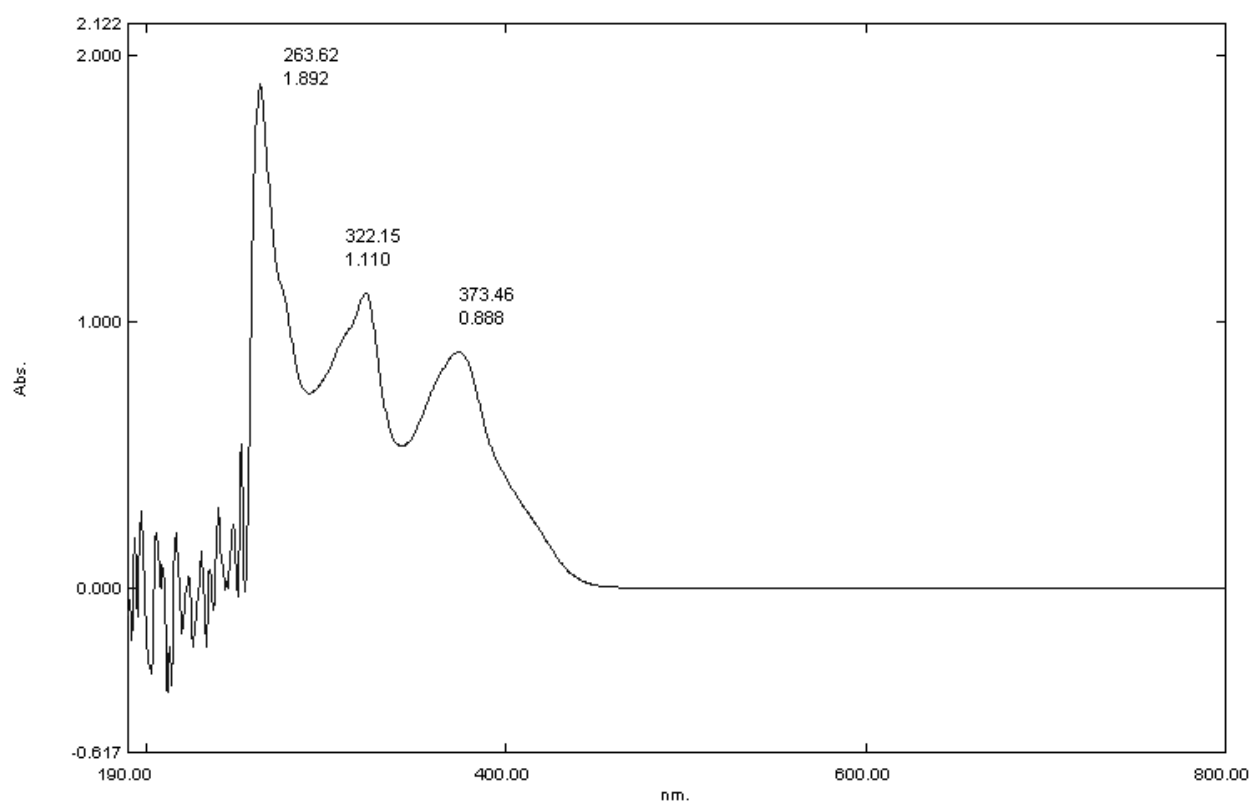
234

HSQC Spectrum of BR-1

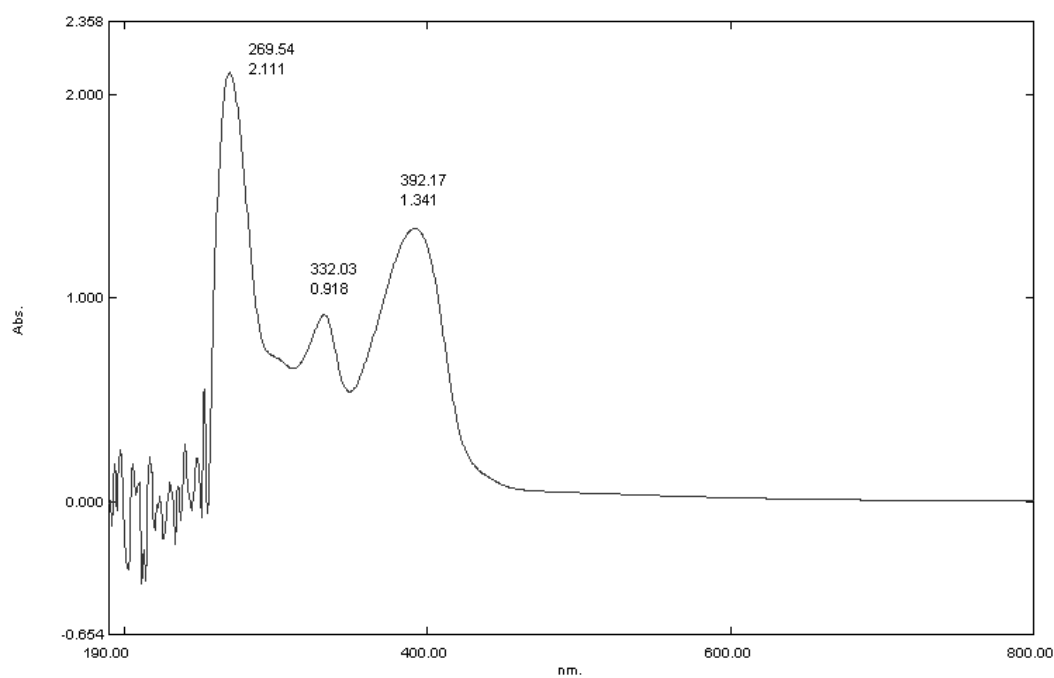
Appendix IV

Spectra of Mangiferin (SF-1) isolated from the roots of

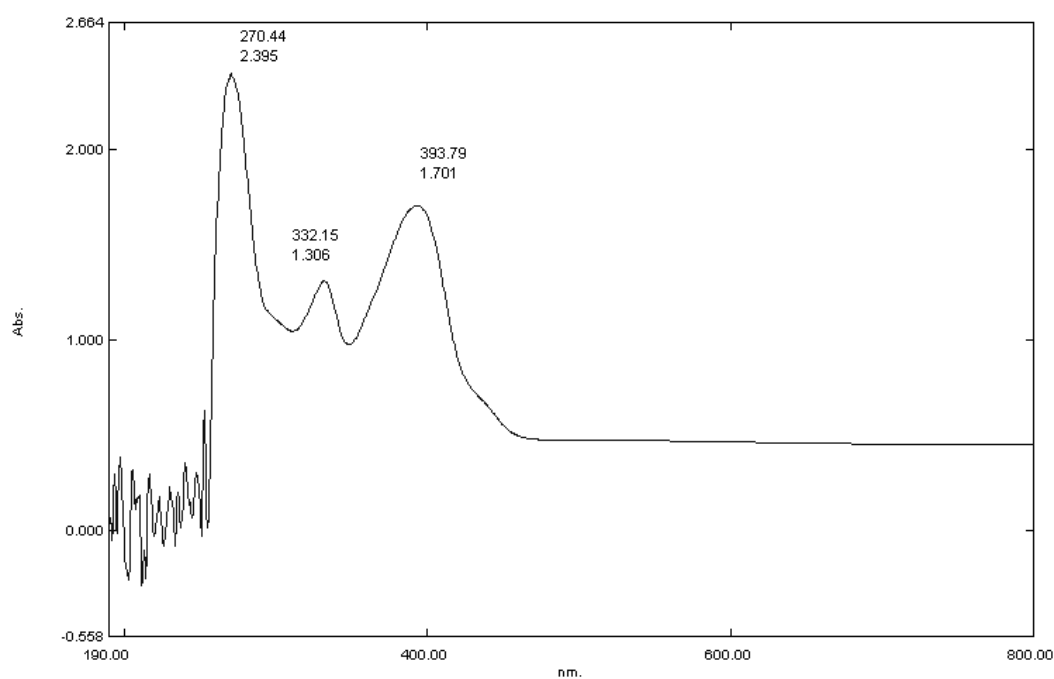
Salacia fruticosa



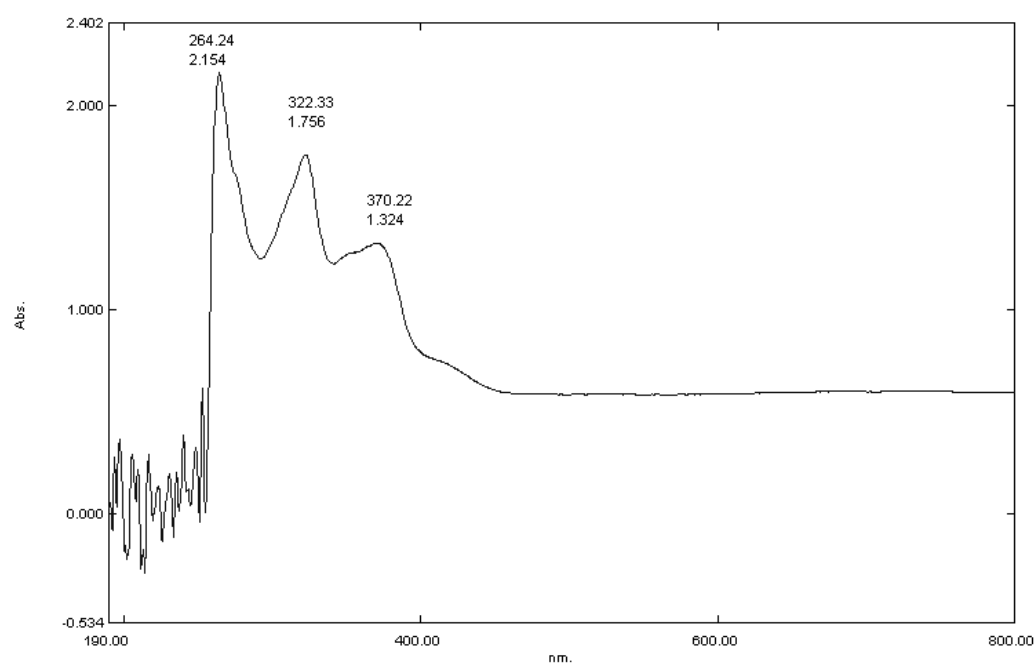
UV- Vis Spectrum of SF-1 (in DMSO)



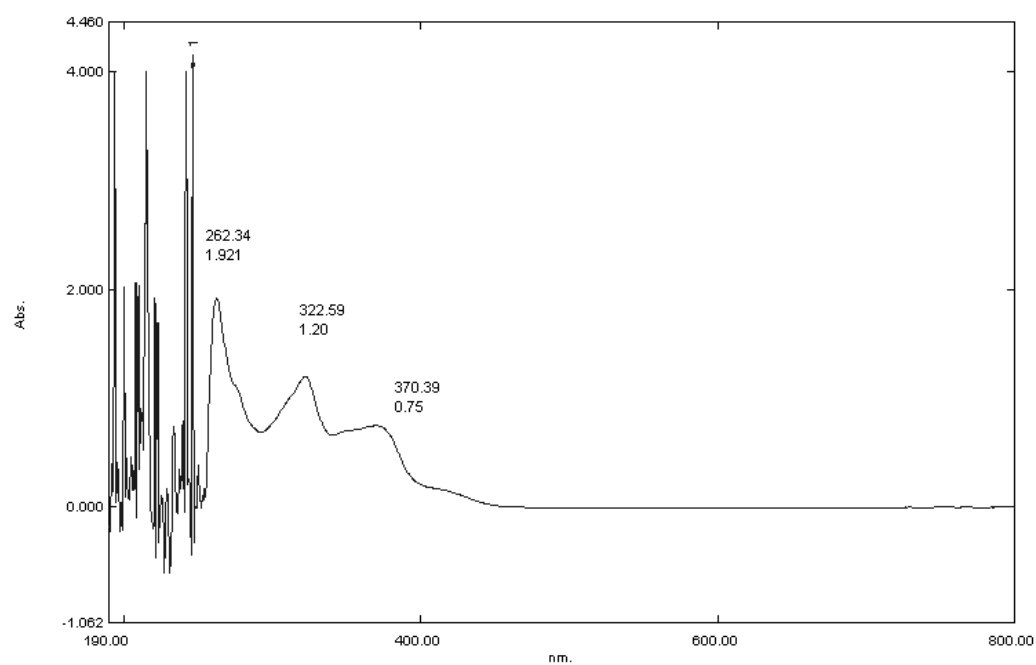
UV- Vis Spectrum of SF-1 + AlCl₃ (initial reading)



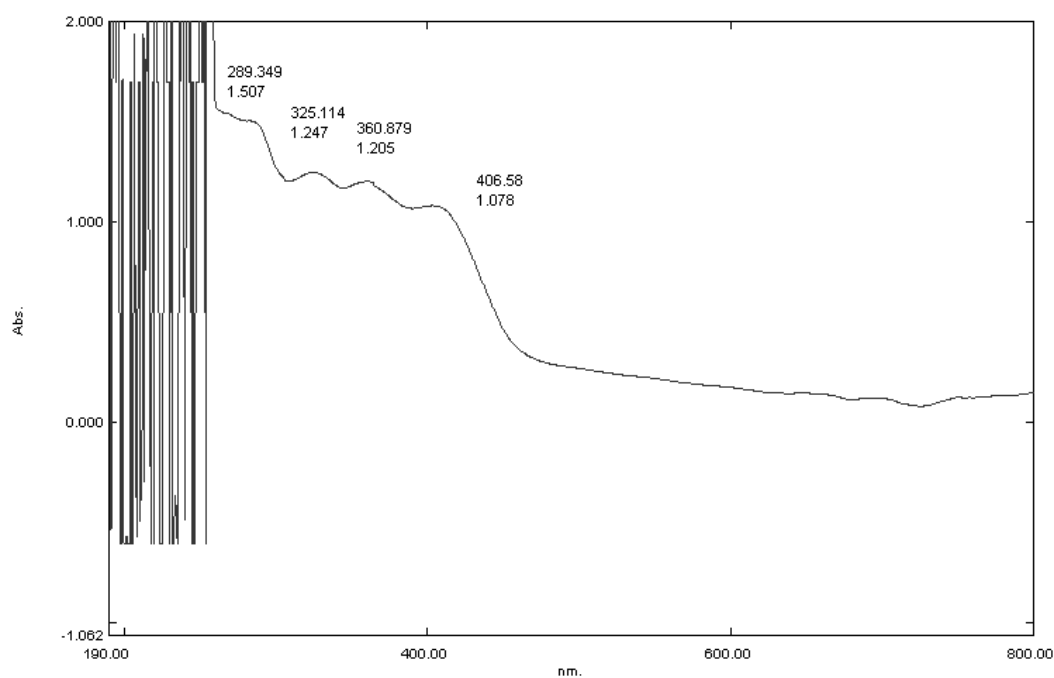
UV- Vis Spectrum of SF-1 + AlCl₃ (after 10 min)



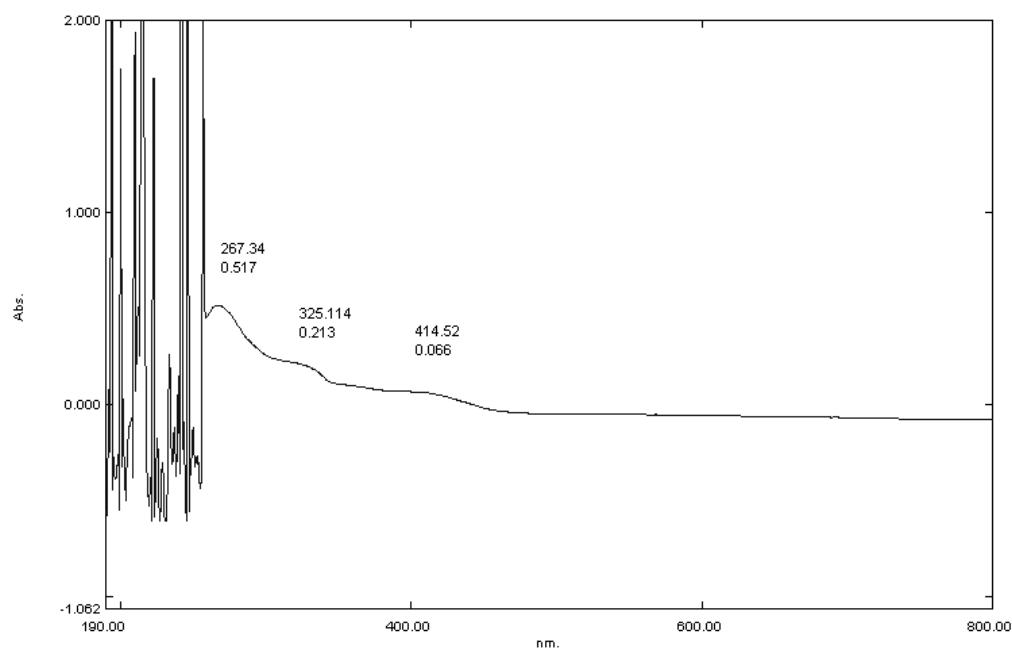
UV- Vis Spectrum of SF-1 + AlCl₃ + 2N HCl (initial reading)



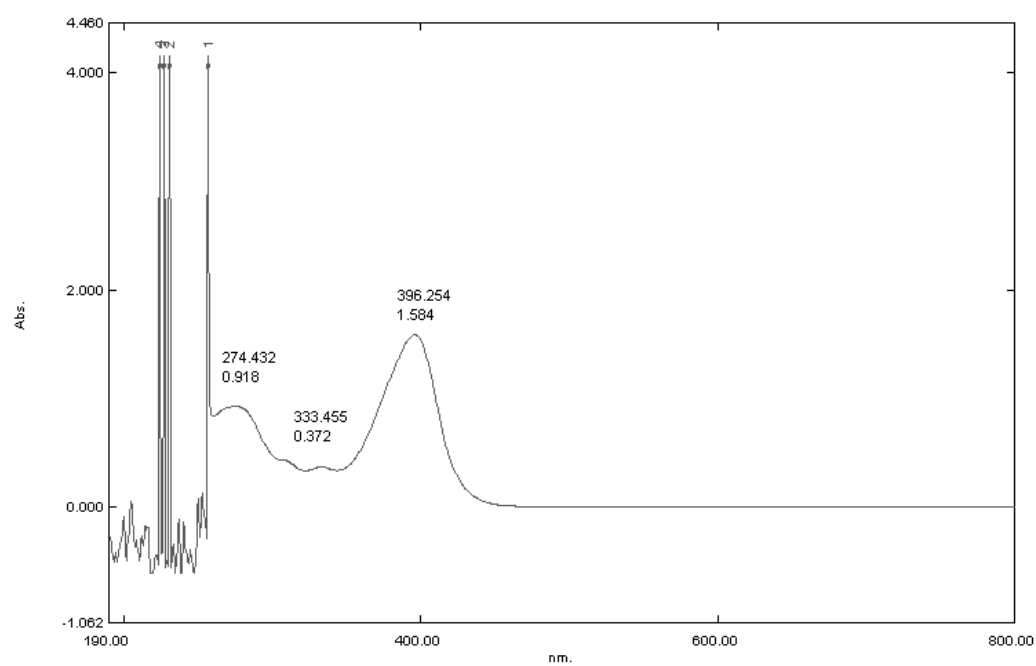
UV- Vis Spectrum of SF-1 + AlCl₃ + 2N HCl (reading after 30 min)



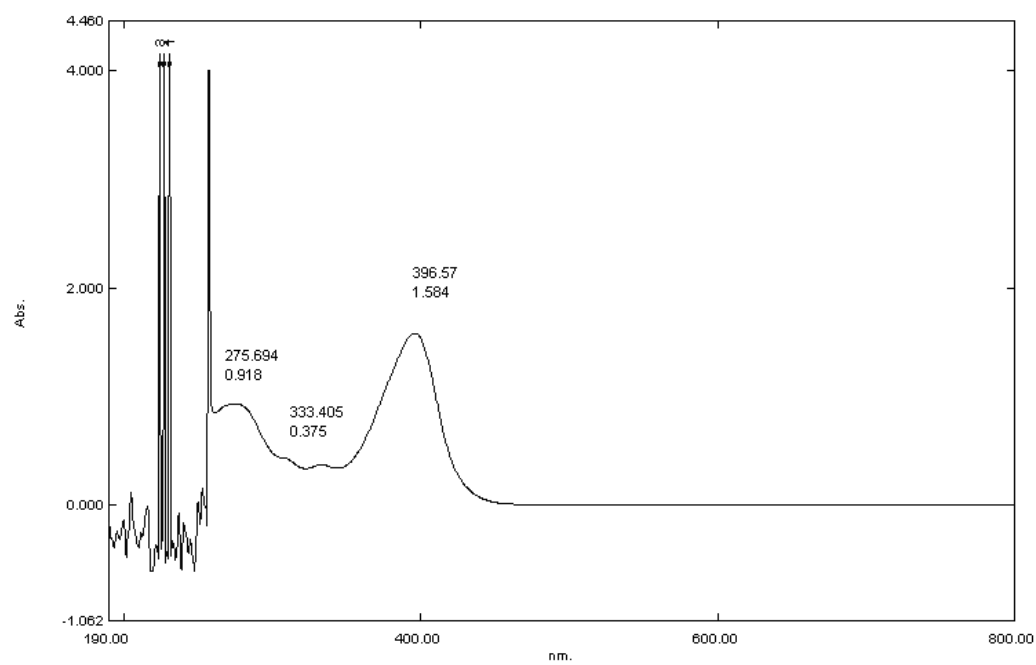
UV- Vis Spectrum of SF-1 + CH₃ONa (initial reading)



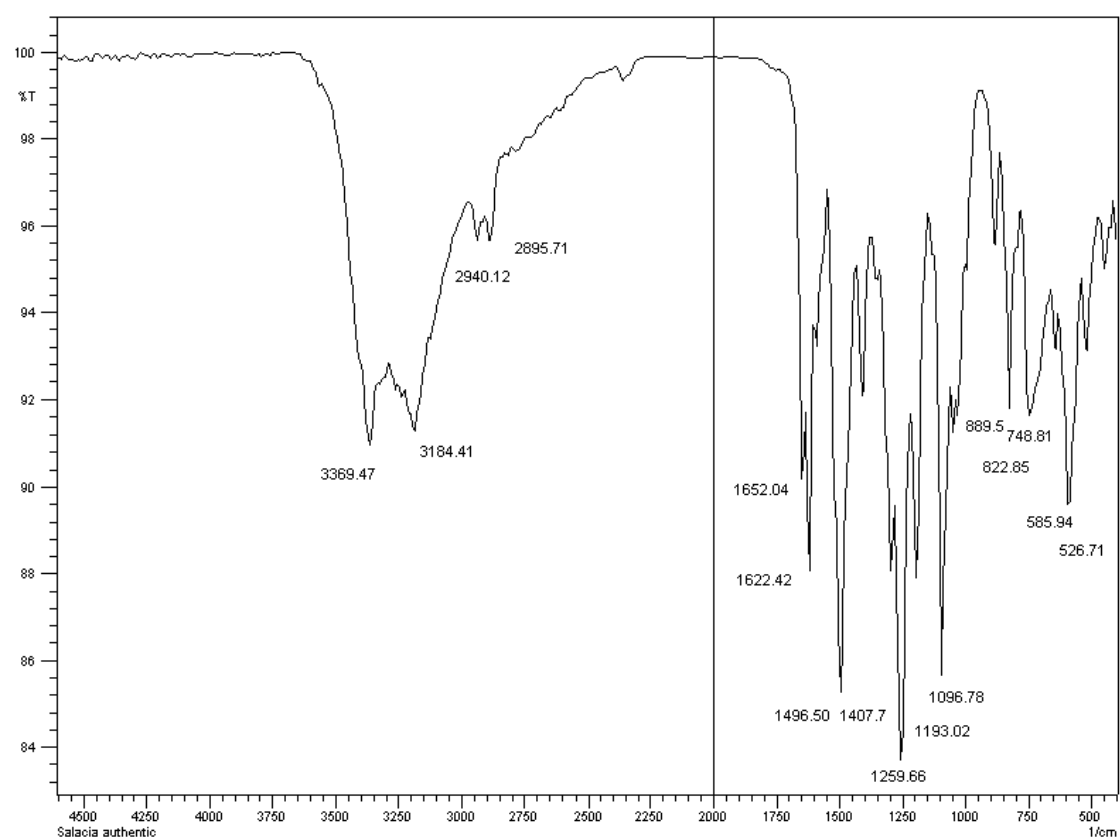
UV- Vis Spectrum of SF-1 + CH₃ONa (reading after 10 min)

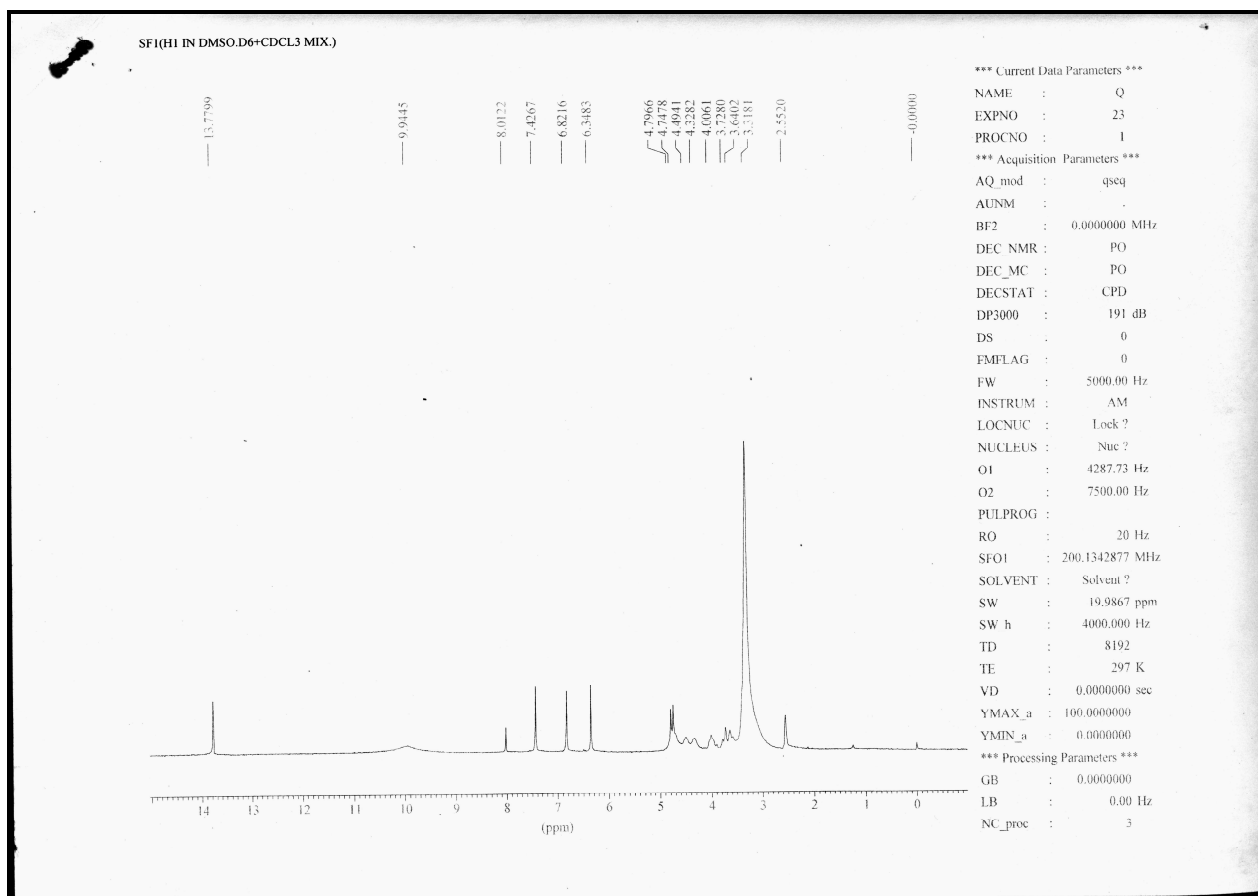


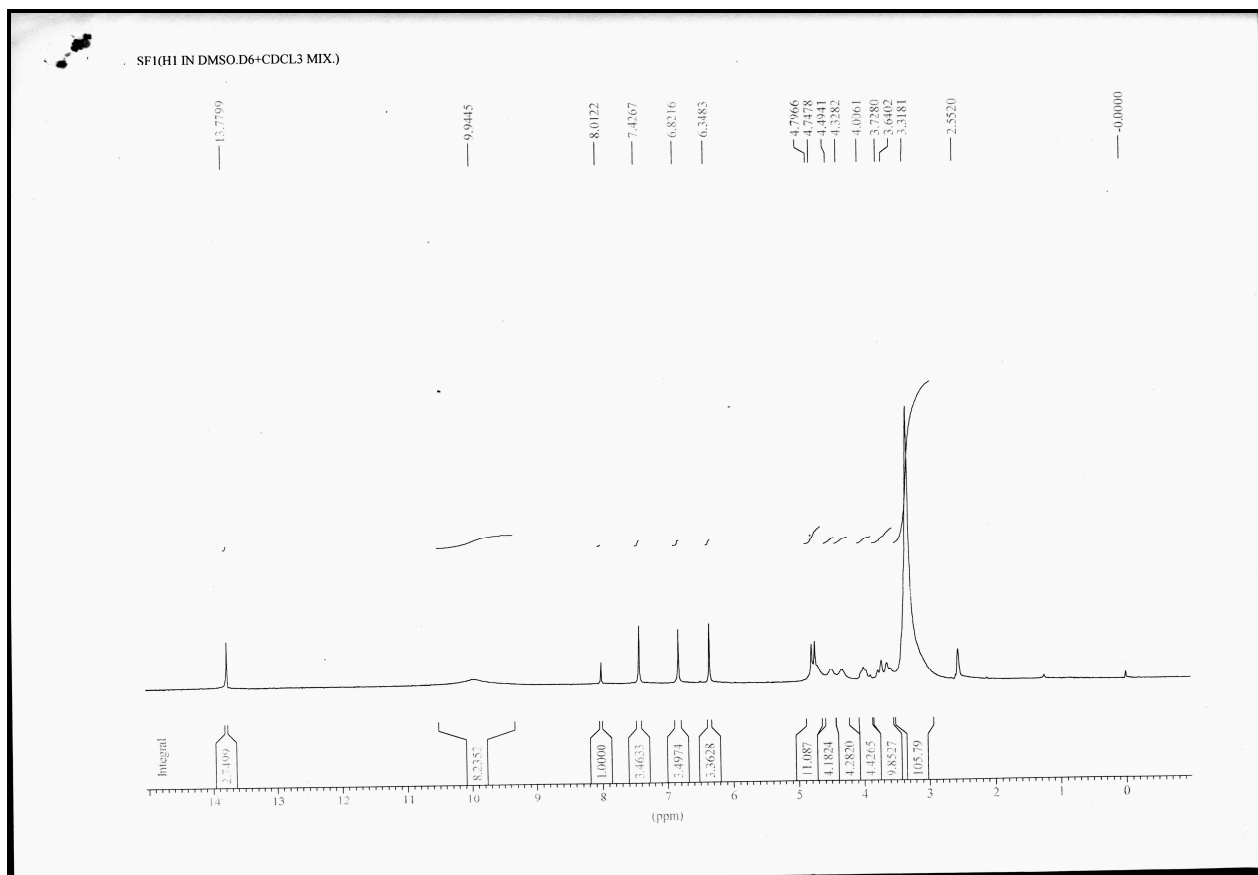
UV- Vis Spectrum of SF-1 + CH₃COONa (initial reading)



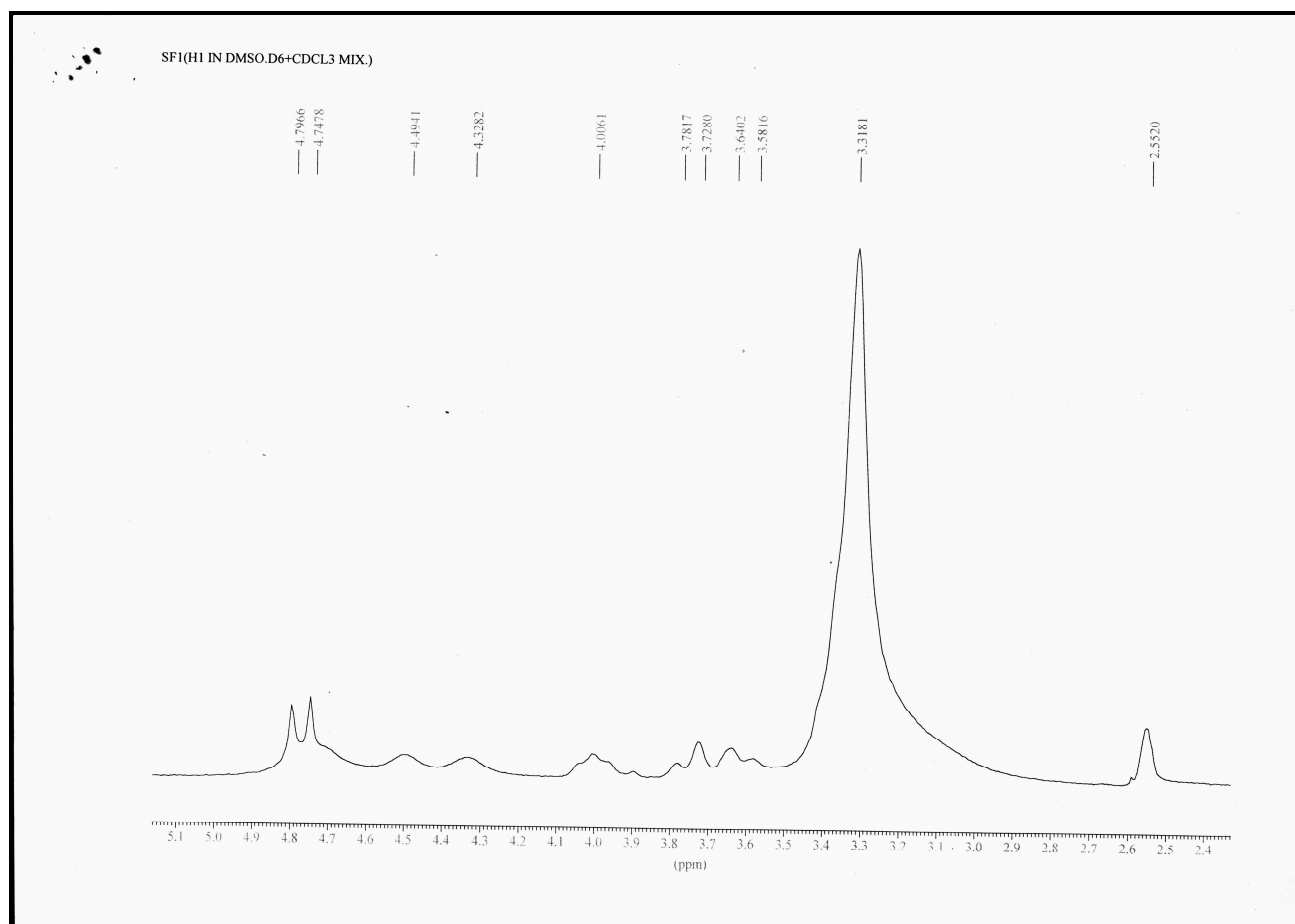
UV- Vis Spectrum of SF-1 + CH₃COONa (reading after 30 min)

**IR spectrum of SF-1**

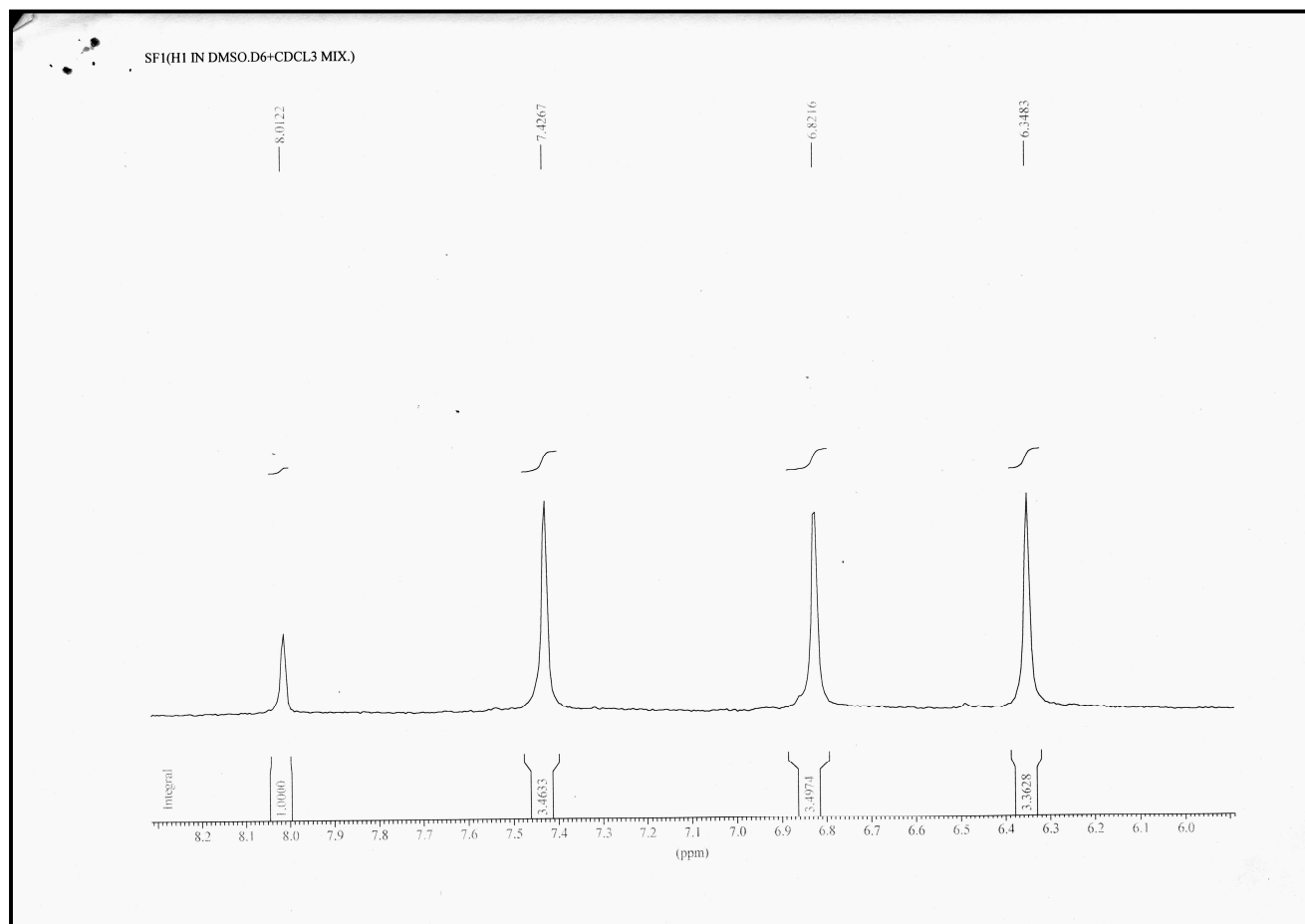
 **^1H NMR Spectrum of SF-1**



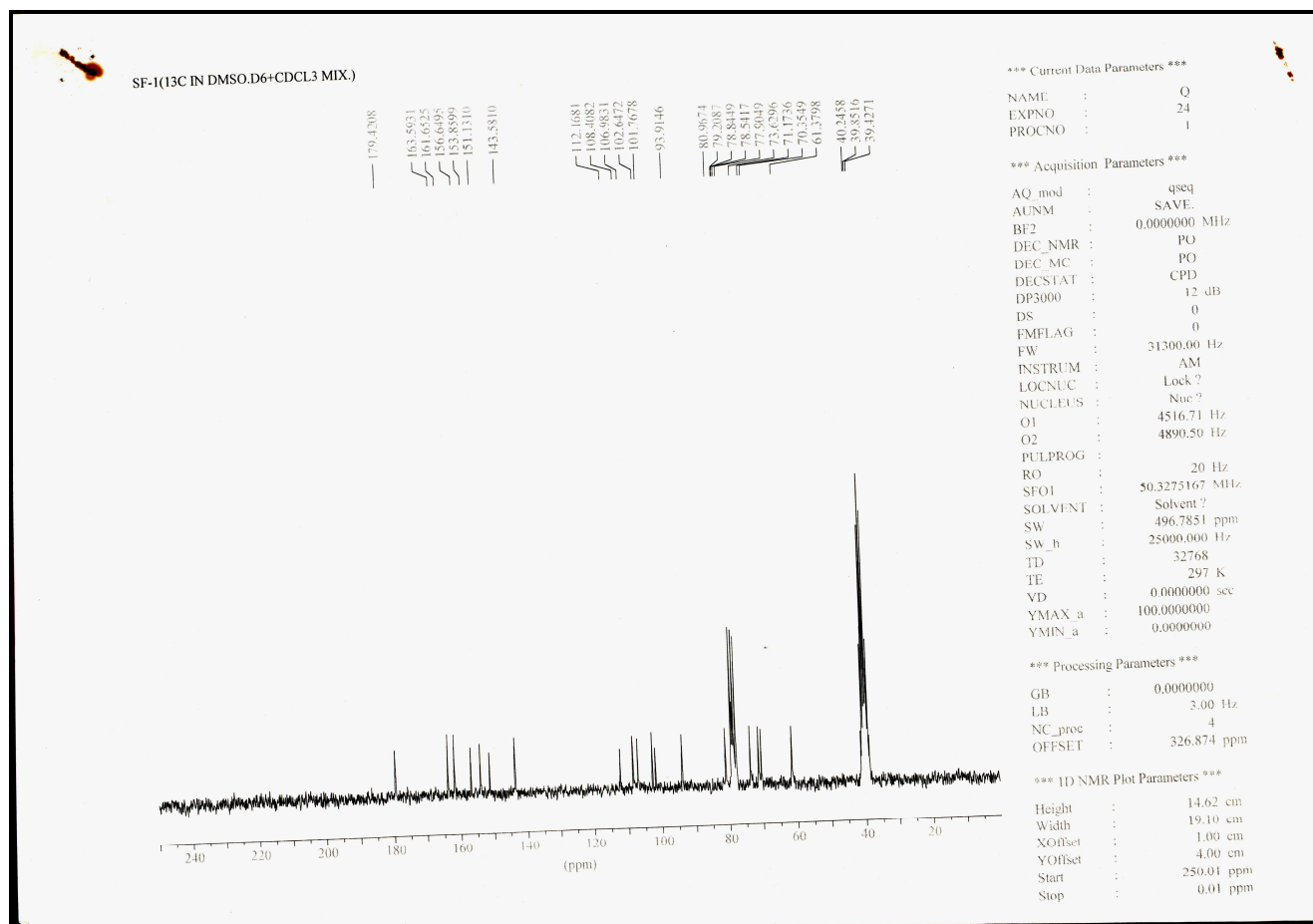
^1H NMR Spectrum of SF-1

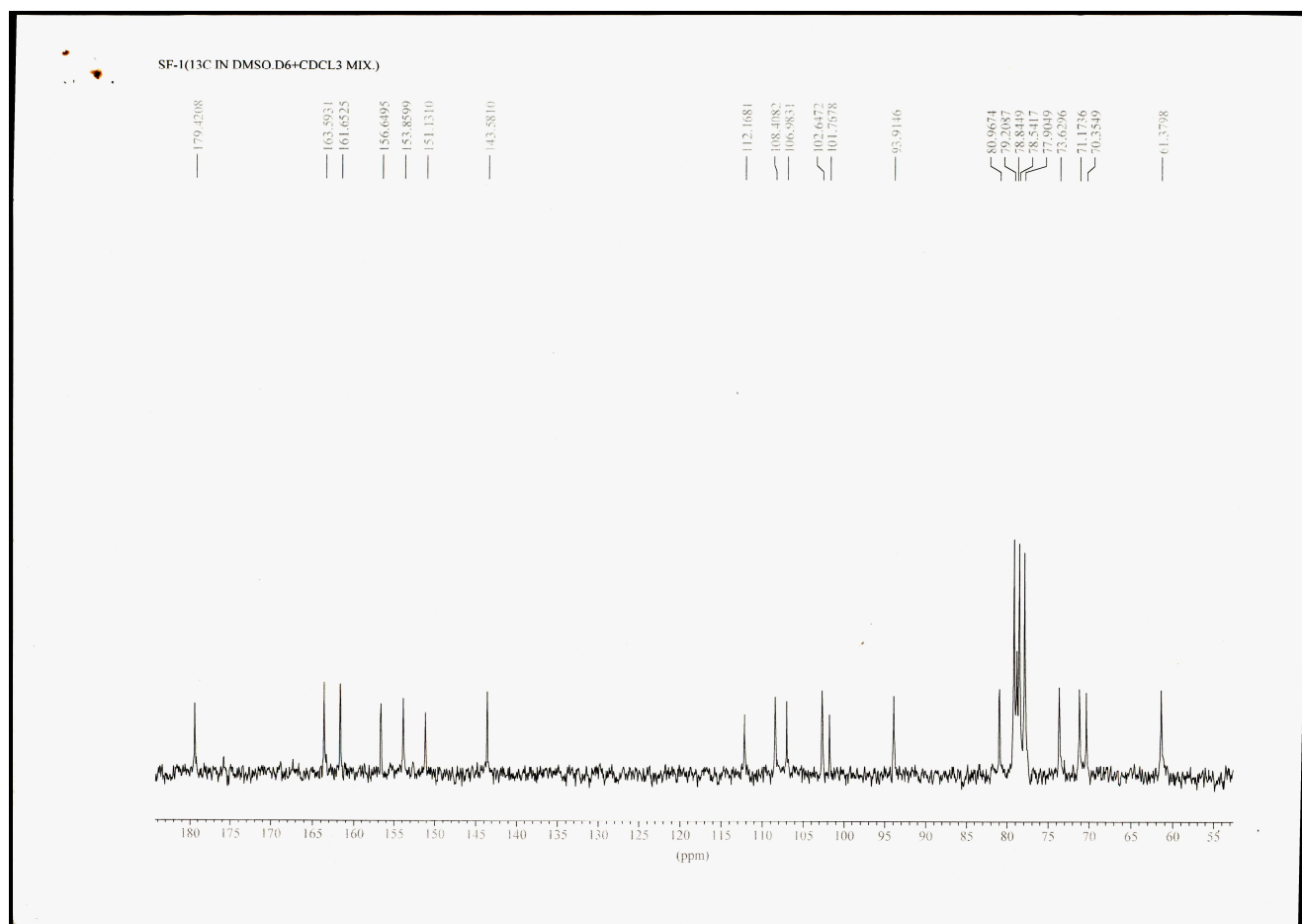


^1H NMR Spectrum of SF-1

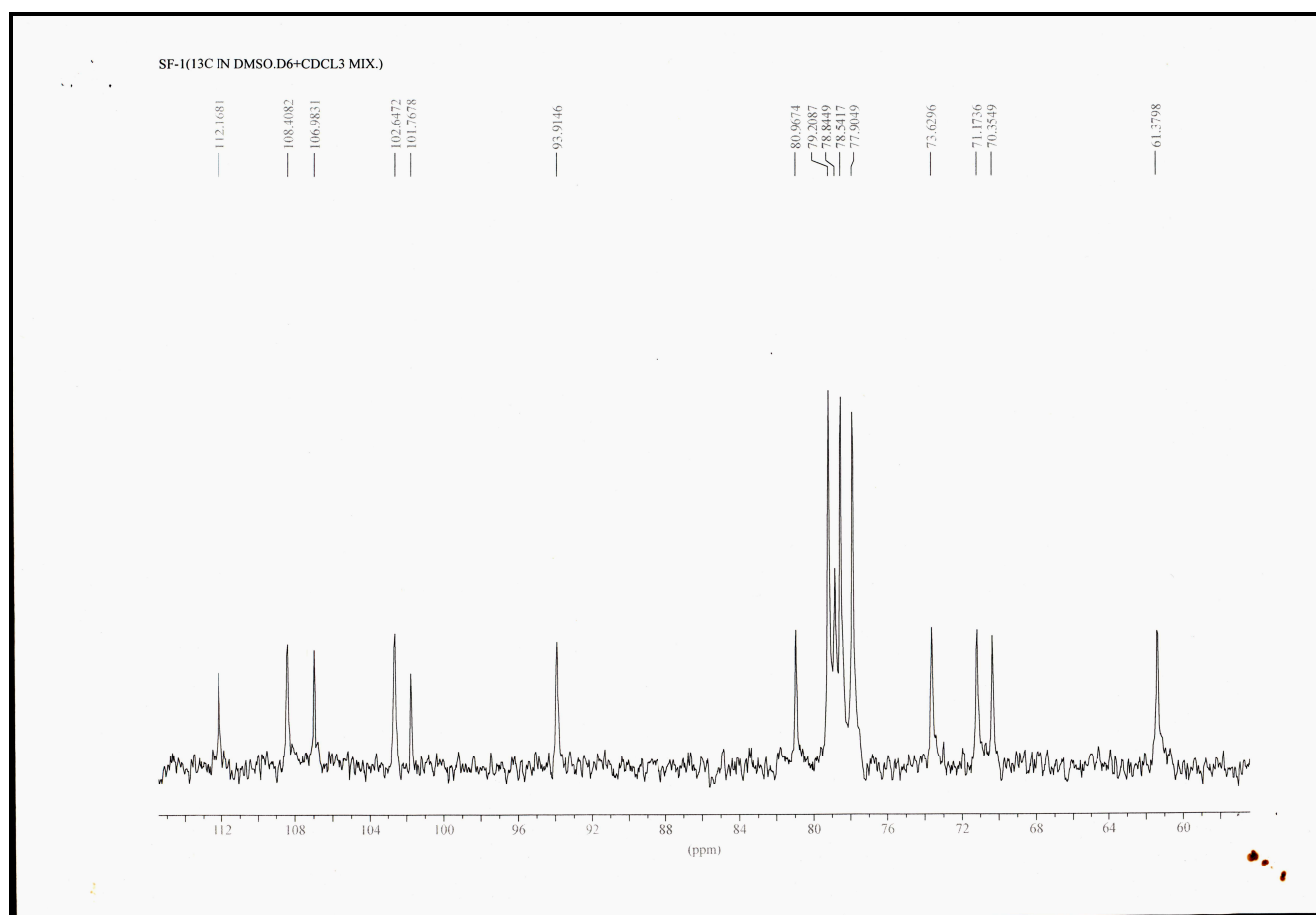


^1H NMR Spectrum of SF-1

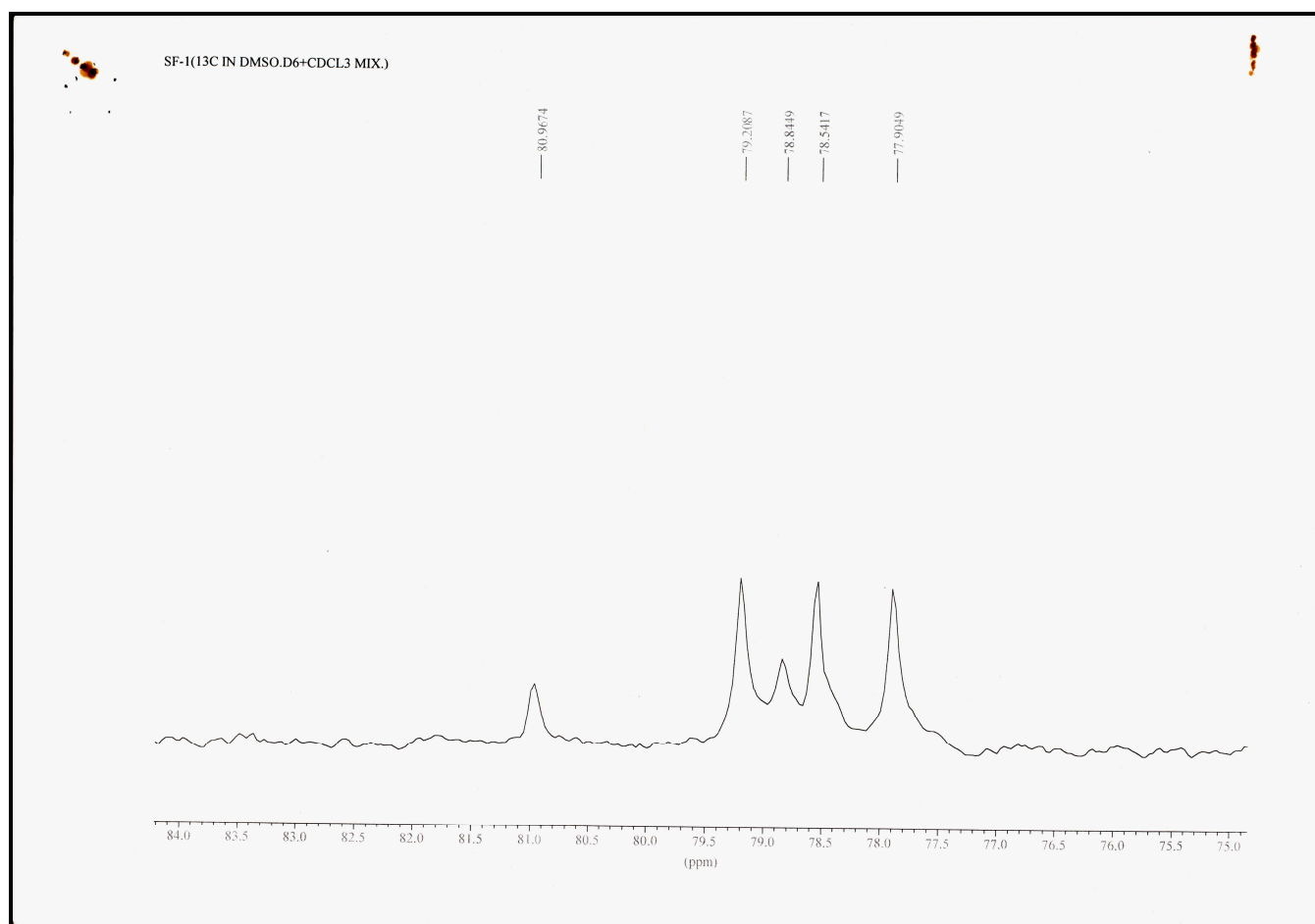
¹³C NMR Spectrum of SF-1



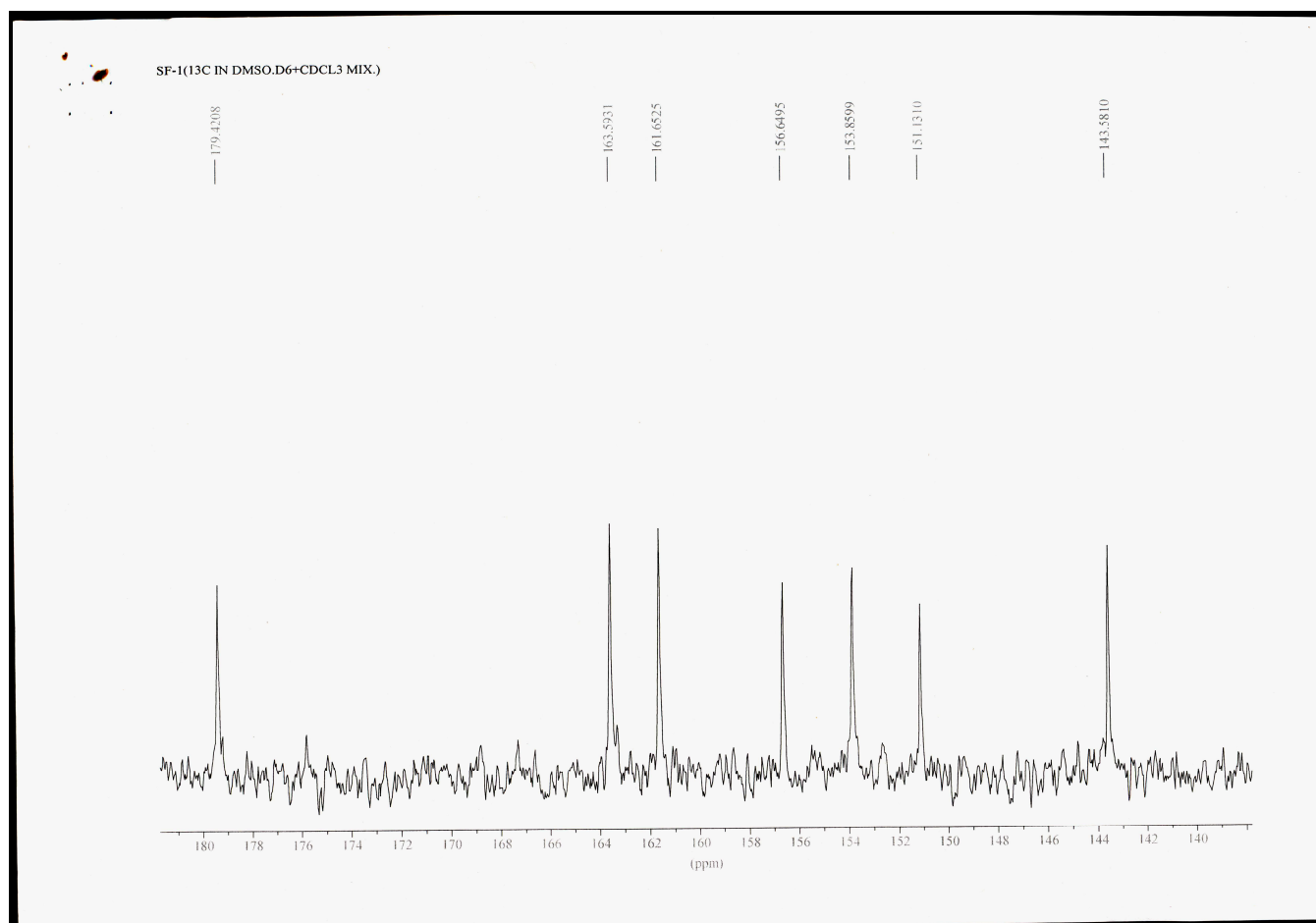
¹³C NMR Spectrum of SF-1



¹³C NMR Spectrum of SF-1



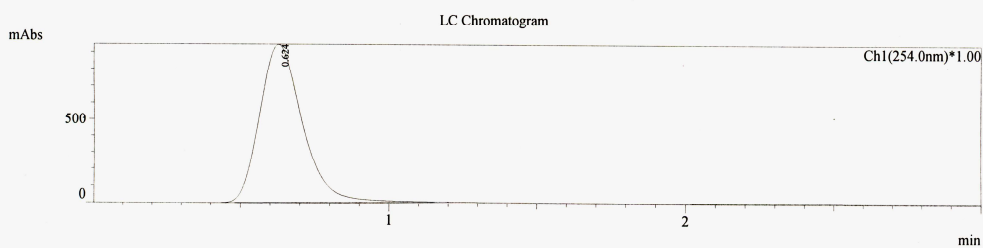
¹³C NMR Spectrum of SF-1



^{13}C NMR Spectrum of SF-1

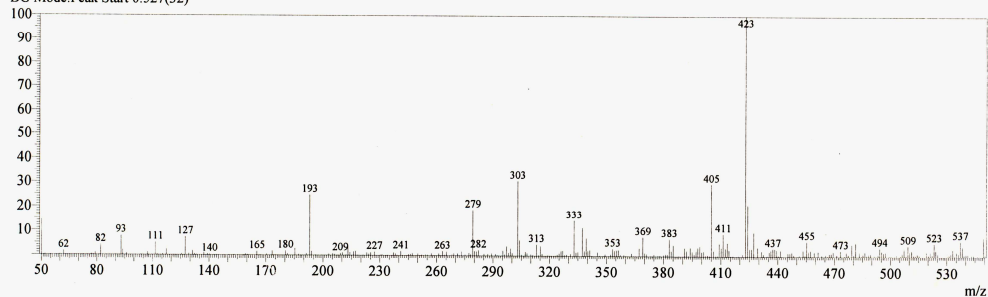
LCMS-2010A DATA REPORT SHIMADZU

Date and Time : 5/29/2007 15:40:45 PM
User : Admin
Vial # : 4
Sample : SF1
Inj. Volume : 5.000
Data Name : C:\LCMSsolution\User\Data\SF1-APCI-POS1.qld
Method Name : C:\LCMSsolution\User\Method\JAY-4-APCI.qlm



MS Spectrum

Line#:1 R.Time:0.668(Scan#:40) Positive
MassPeaks:353 BasePeak:422.95(45632)
RawMode:Single 0.668(40)
BG Mode:Peak Start 0.527(32)

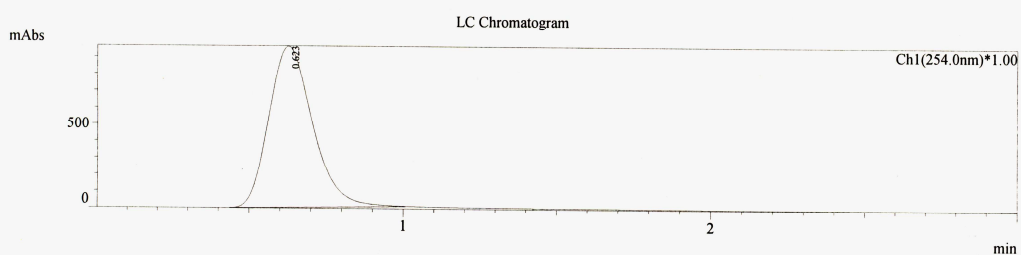


MS Peak Table											
Peak#	R.Time	I.Time	F.Time	Area	Height	A/H	Mark	%Total	Name	Base m/z	Base Int.
1	0.668	0.527	0.843	2645559	297294	8.89		100.00		422.95	45632
				2645559	297294			100.00			

APCI Mass Spectrum (+ve mode) of SF-1

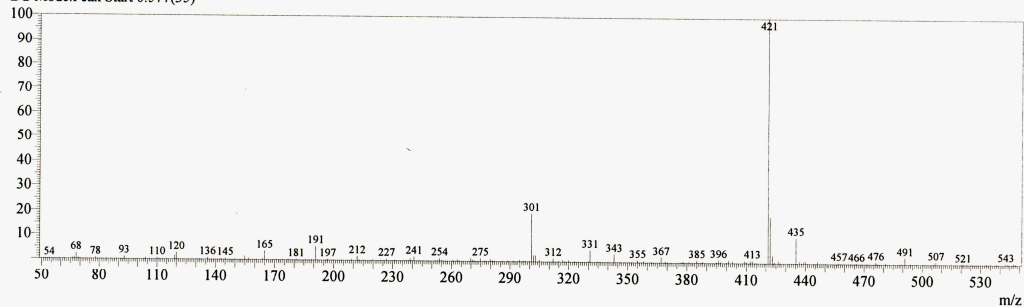
LCMS-2010A DATA REPORT SHIMADZU

Date and Time : 5/29/2007 16:32:22 PM
User : Admin
Vial # : 4
Sample : SF1
Inj. Volume : 5.000
Data Name : C:\LCMSsolution\User\Data\SF1-APCI-NEG1.qld
Method Name : C:\LCMSsolution\User\Method\JAY-4-APCI.qlm



MS Spectrum

Line# 1 R.Time:0.671(Scan# 41) Negative
MassPeaks:271 BasePeak:421.00(47074)
RawMode:Single 0.671(41)
BG Mode:Peak Start 0.577(35)



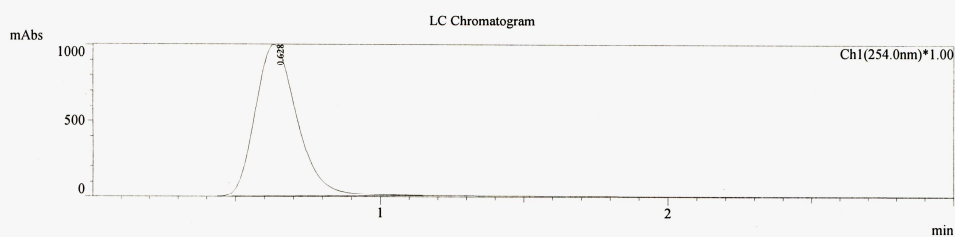
MS Peak Table

Peak#	R.Time	I.Time	F.Time	Area	Height	A/H	Mark	%Total	Name	Base m/z	Base Int.
1	0.671	0.577	0.793	590755	95056	6.21		100.00		421.00	47074
				590755	95056			100.00			

APCI Mass Spectrum (-ve mode) of SF-1

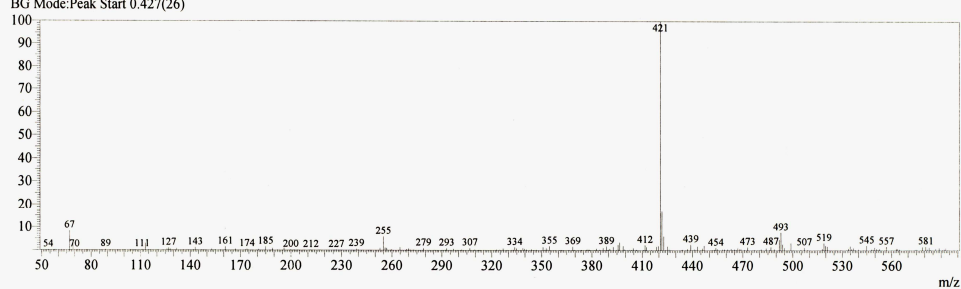
LCMS-2010A DATA REPORT SHIMADZU

Date and Time : 5/29/2007 18:13:29 PM
User : Admin
Vial # : 4
Sample : SF1
Inj. Volume : 5.000
Data Name : C:\LCMSsolution\User\Data\SF1-ESI-NEG1.qld
Method Name : C:\LCMSsolution\User\Method\JAY-4-APCI.qldm



MS Spectrum

Line#:1 R.Time:0.733(Scan#:44) Negative
MassPeaks:288 BasePeak:420.80(59447)
RawMode:Single 0.733(44)
BG Mode:Peak Start 0.427(26)



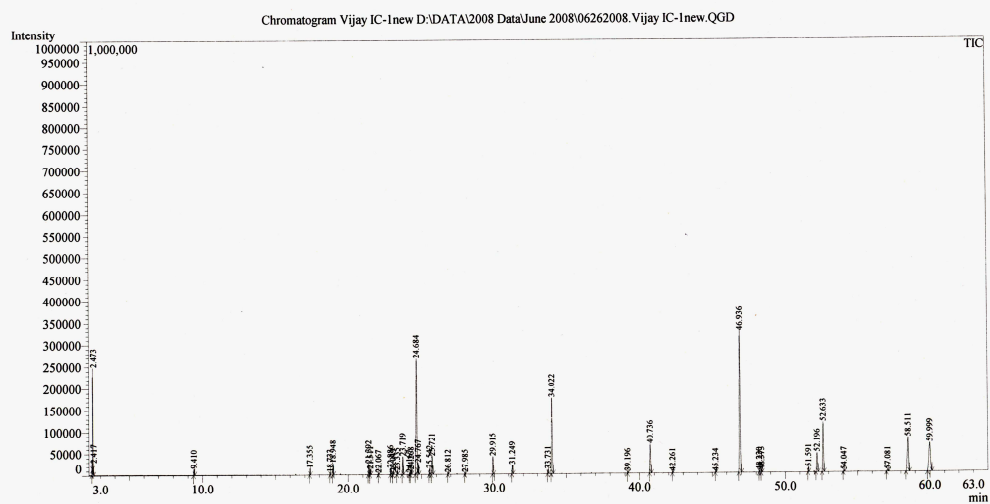
ESI Mass Spectrum (-ve mode) of SF-1

Appendix V

*TIC of the essential oil & cold hexane extract of Ixora
coccinea roots and the MS Spectra of some identified
compounds*

Analyzed by : Leela
Analyzed : 6/26/2008 1:00:41 PM
Sample Type : Unknown
Sample Name : Vijay IC-1new
Sample ID : Vijay IC-1new
Sample Amount : 1.000
Injection Volume : 1.000
Data File : D:\DATA\2008 Data\June 2008\06262008.Vijay IC-1new.QGD
Method File : D:\METHOD\Essoil-IISR-modified (1ml) wax.qgm

Sample Information

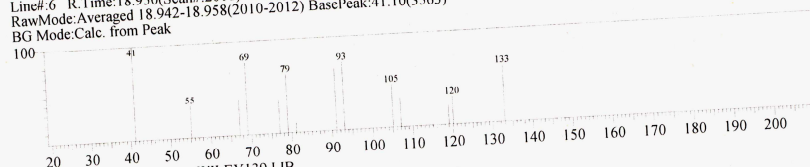


1 / 1

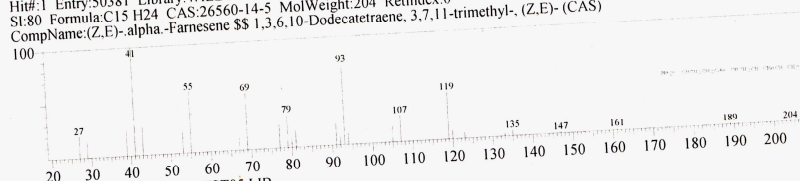
TIC of the essential oil of *Ixora coccinea* roots

6/26/2008 14:24:44

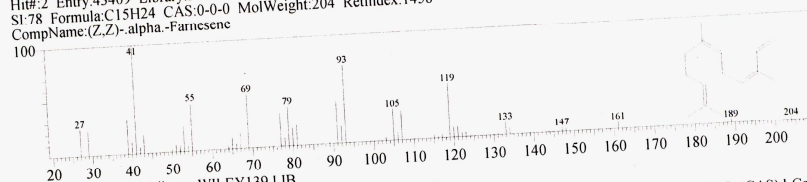
<< Target >>
 Line# 6 R.Time:18.950(Scan#:2011) MassPeaks:14
 RawMode:Averaged 18.942-18.958(2010-2012) BasePeak:41.10(3563)
 BG Mode:Calc. from Peak



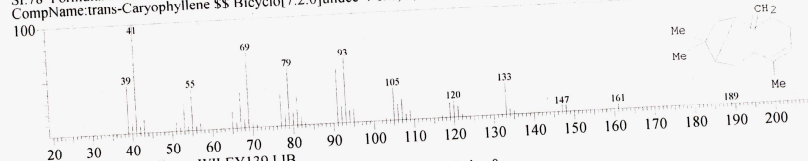
Hit#:1 Entry:50381 Library:WILEY139.LIB
 SI:80 Formula:C15 H24 CAS:26560-14-5 MolWeight:204 RetIndex:0
 CompName:(Z,E)-alpha-Farnesene \$1,3,6,10-Dodecatetraene, 3,7,11-trimethyl-, (Z,E)- (CAS)



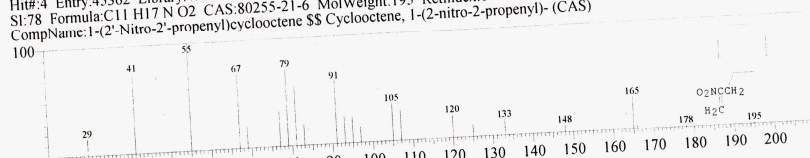
Hit#:2 Entry:43409 Library:NIST05.LIB
 SI:78 Formula:C15H24 CAS:0-0-0 MolWeight:204 RetIndex:1458
 CompName:(Z,Z)-alpha-Farnesene



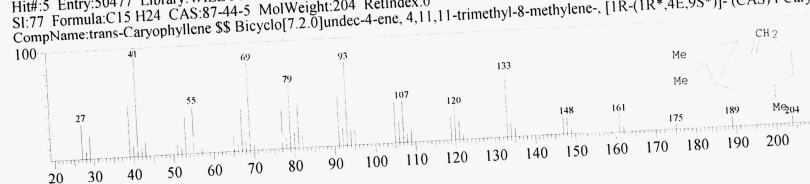
Hit#:3 Entry:50482 Library:WILEY139.LIB
 SI:78 Formula:C15 H24 CAS:87-44-5 MolWeight:204 RetIndex:0
 CompName:trans-Caryophyllene \$Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-, [1R-(1R*,4E,9S*)]- (CAS) l-Cary



Hit#:4 Entry:45362 Library:WILEY139.LIB
 SI:78 Formula:C11 H17 N O2 CAS:80255-21-6 MolWeight:195 RetIndex:0
 CompName:1-(2'-Nitro-2'-propenyl)cyclooctene \$Cyclooctene, 1-(2-nitro-2-propenyl)- (CAS)



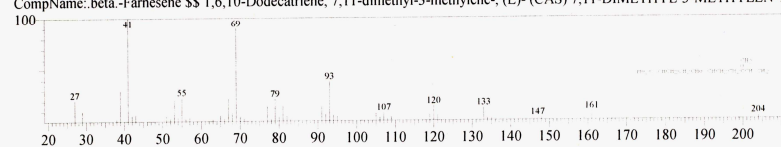
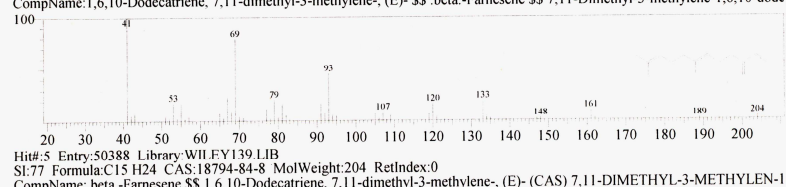
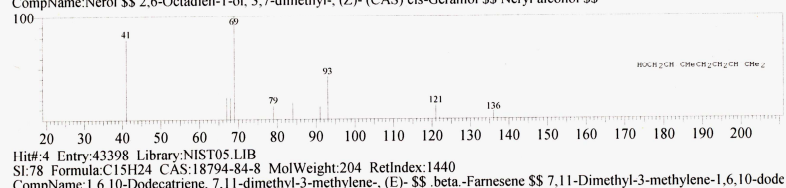
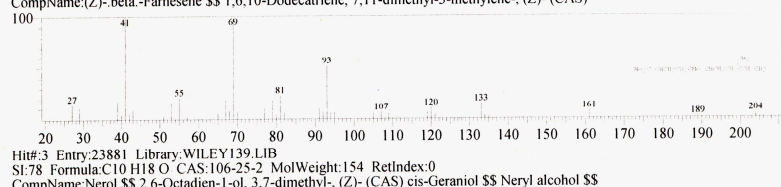
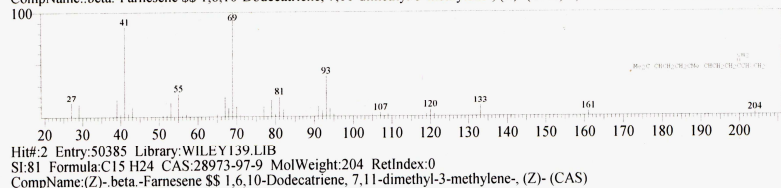
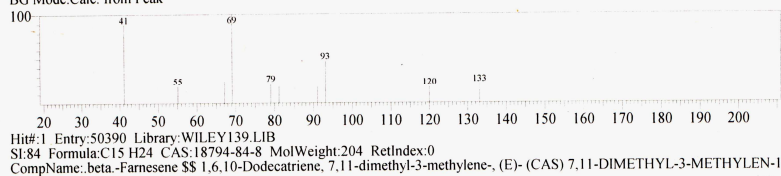
Hit#:5 Entry:50477 Library:WILEY139.LIB
 SI:77 Formula:C15 H24 CAS:87-44-5 MolWeight:204 RetIndex:0
 CompName:trans-Caryophyllene \$Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-, [1R-(1R*,4E,9S*)]- (CAS) l-Cary



6/26/2008 14:24:22

<< Target >>

Line# 7 R Time: 21.392 (Scan#: 2304) MassPeaks: 10
 RawMode: Averaged 21.383-21.400 (2303-2305) BasePeak: 69.10 (6524)
 BG Mode: Calc. from Peak

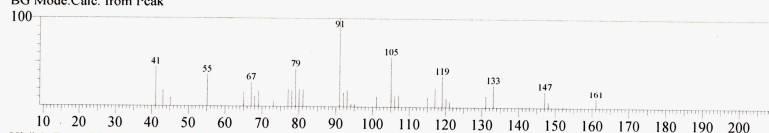


EI Mass Spectrum of β -farnesene along with library search results

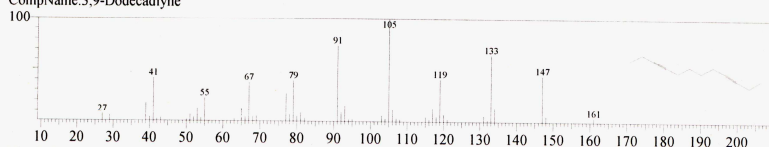
6/26/2008 14:26:29

<< Target >>

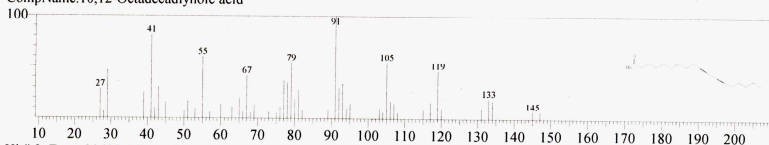
Line#: 38 RTime: 58.508 (Scan#: 6758) MassPeaks: 33
 RawMode: Averaged 58.500-58.517 (6757-6759) BasePeak: 91.05 (11233)
 BG Mode: Calc. from Peak



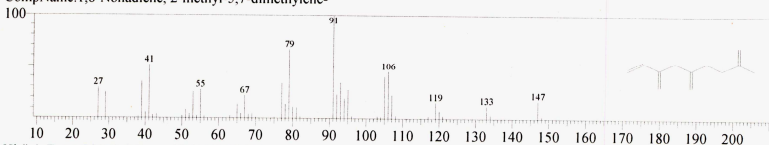
Hit#: 1 Entry: 20620 Library: NIST05.LIB
 SI: 82 Formula: C₁₂H₁₈ CAS: 61827-89-2 MolWeight: 162 RetIndex: 1249
 CompName: 3,9-Dodecadiyne



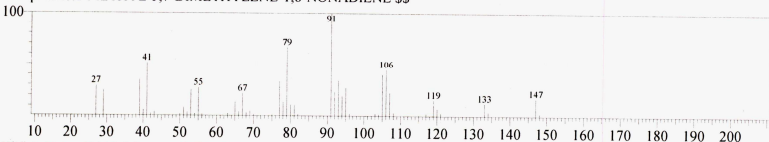
Hit#: 2 Entry: 87068 Library: NIST05.LIB
 SI: 81 Formula: C₁₈H₂₈O₂ CAS: 7333-25-7 MolWeight: 276 RetIndex: 2202
 CompName: 10,12-Octadecadienoic acid



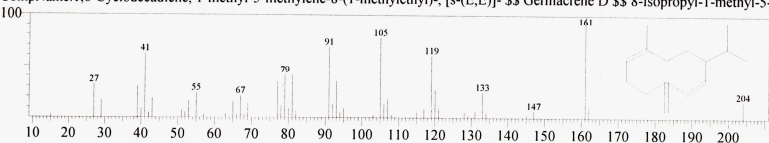
Hit#: 3 Entry: 20600 Library: NIST05.LIB
 SI: 80 Formula: C₁₂H₁₈ CAS: 0-0-0 MolWeight: 162 RetIndex: 1106
 CompName: 1,8-Nonadiene, 2-methyl-5,7-dimethylene-

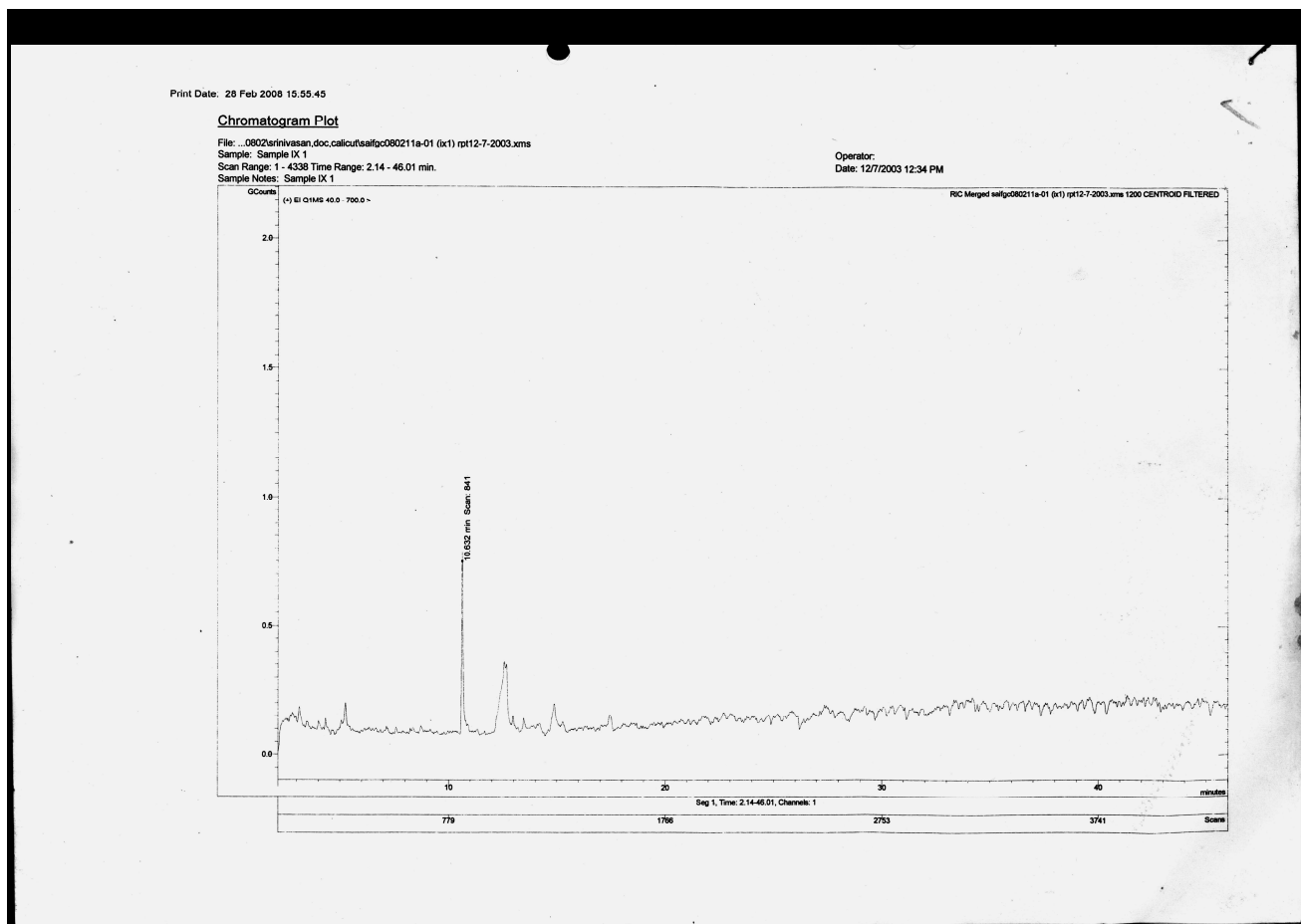


Hit#: 4 Entry: 28078 Library: WILEY139.LIB
 SI: 80 Formula: C₁₂H₁₈ CAS: 0-0-0 MolWeight: 162 RetIndex: 0
 CompName: 2-METHYL-5,7-DIMETHYLENE-1,8-NONADIENE \$\$



Hit#: 5 Entry: 43544 Library: NIST05.LIB
 SI: 80 Formula: C₁₅H₂₄ CAS: 23986-74-5 MolWeight: 204 RetIndex: 1515
 CompName: 1,6-Cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethyl)-, [s-(E,E)]- \$\$ Germacrene D \$\$ 8-Isopropyl-1-methyl-5-

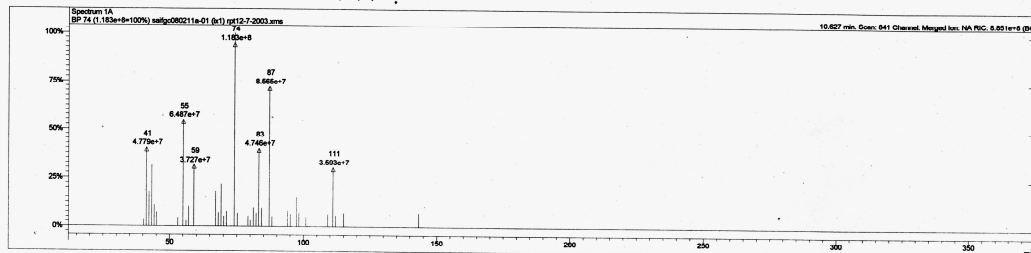




TIC of the cold hexane extract of *Ixora coccinea* roots

Print Date: 29 Feb 2008 09:59:33

Scan 841 from ...vasan.doc\calicut\saifgc080211a-01 (ix1) rpt12-7-2003.xms



Spectrum from ...calicut\saifgc080211a-01 (ix1) rpt12-7-2003.xms
Scan No: 841, Time: 10.627 minutes
No averaging. Background corrected.
Comment: 10.627 min. Scan: 841 Channel: Merged Ion: NA RIC: 8.851e+8
Pair Count: 35 MW: 0 Formula: None
CAS No: None Acquired Range: 40 - 700 m/z

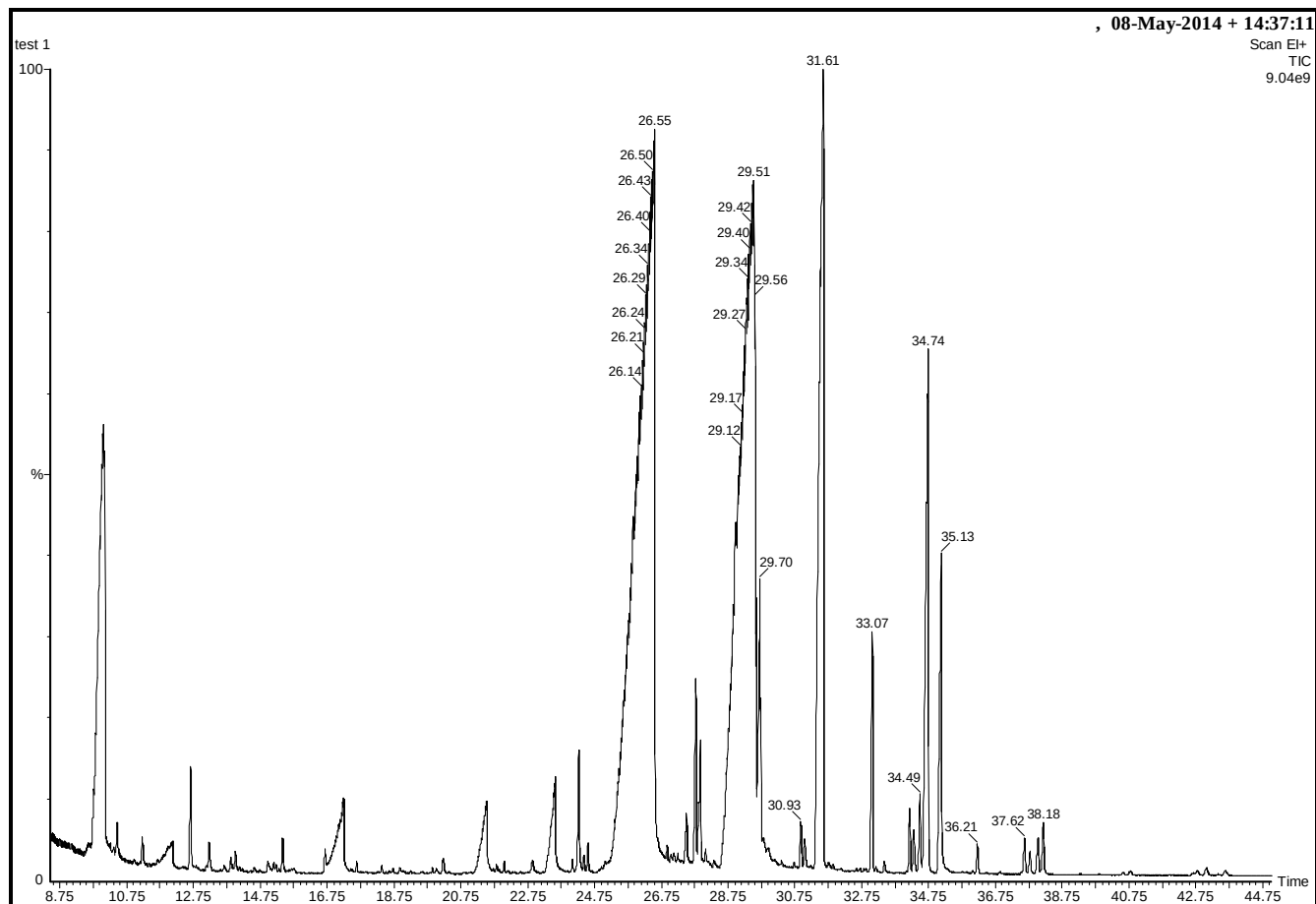
Method Description: EI
Scan 1 Channel Description: 40.0 - 700.0 >
Scan Information: cp = 0.1 PSI
Precursor Mass Range: 40 - 700 m/z

Ion	Int	BP	Ion	Int	BP	Ion	Int	BP	Ion	Int	BP
40	4.089e+6	3	57	1.243e+7	11	79	6.791e+6	6	95	7.842e+6	7
41	4.779e+7	40	59	3.727e+7	31	80	3.878e+6	3	97	1.818e+7	15
42	2.093e+7	18	67	2.147e+7	18	81	1.167e+7	10	98	8.838e+6	7
43	3.707e+7	11	68	8.647e+6	7	82	6.399e+6	7	101	6.055e+6	5
44	1.313e+7	11	69	2.596e+7	22	83	4.745e+7	40	109	7.990e+6	7
45	8.913e+6	8	70	6.473e+6	5	84	1.149e+7	10	111	3.593e+7	30
53	5.520e+6	5	71	9.600e+6	8	87	8.565e+7	72	112	7.175e+6	6
55	6.487e+7	55	74	1.193e+8	100	88	6.419e+6	5	115	8.379e+6	7
56	3.555e+6	3	75	8.298e+6	7	94	1.012e+7	9	143	8.809e+6	7

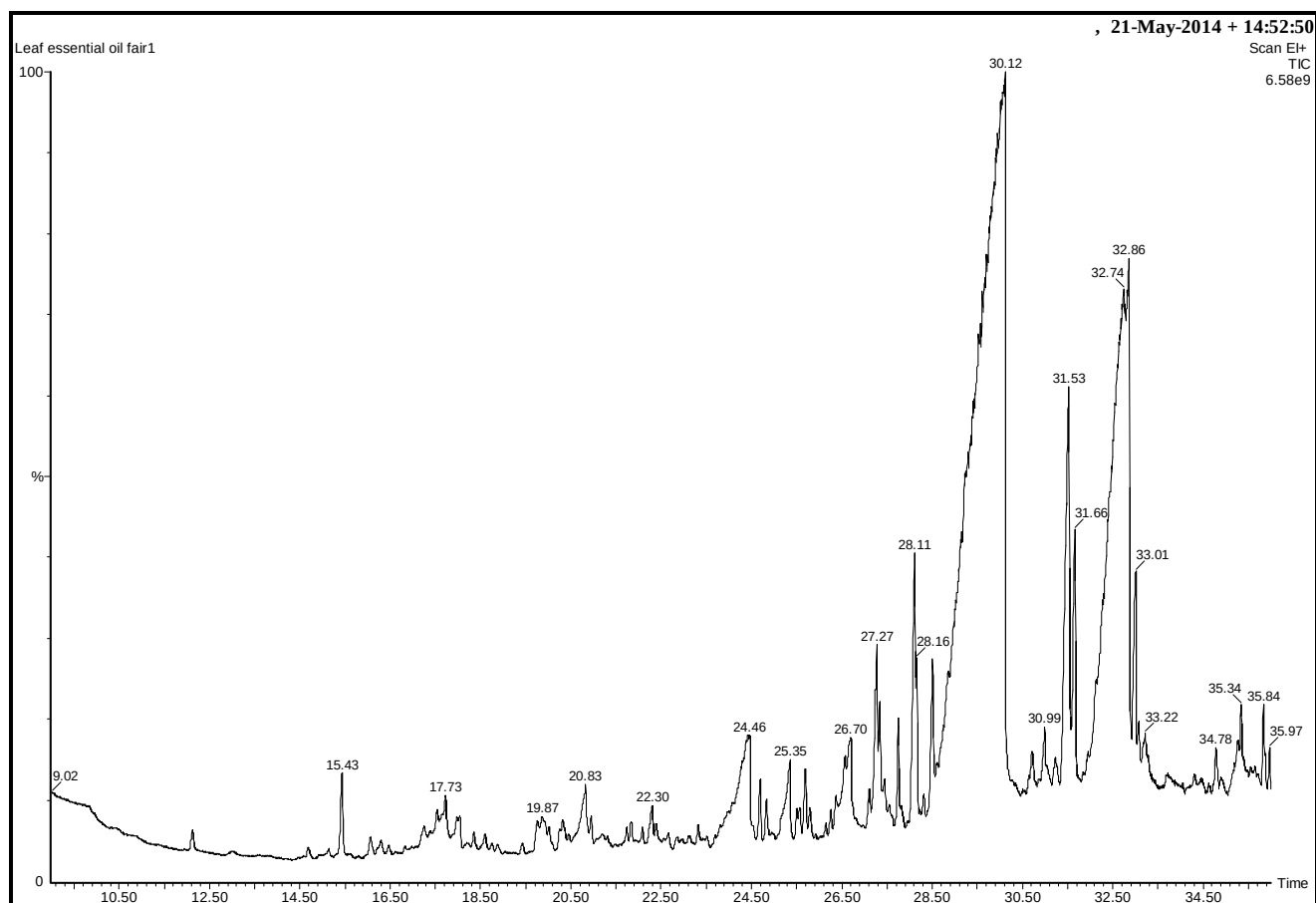
EI Mass Spectrum of nonanoic acid

Appendix VI

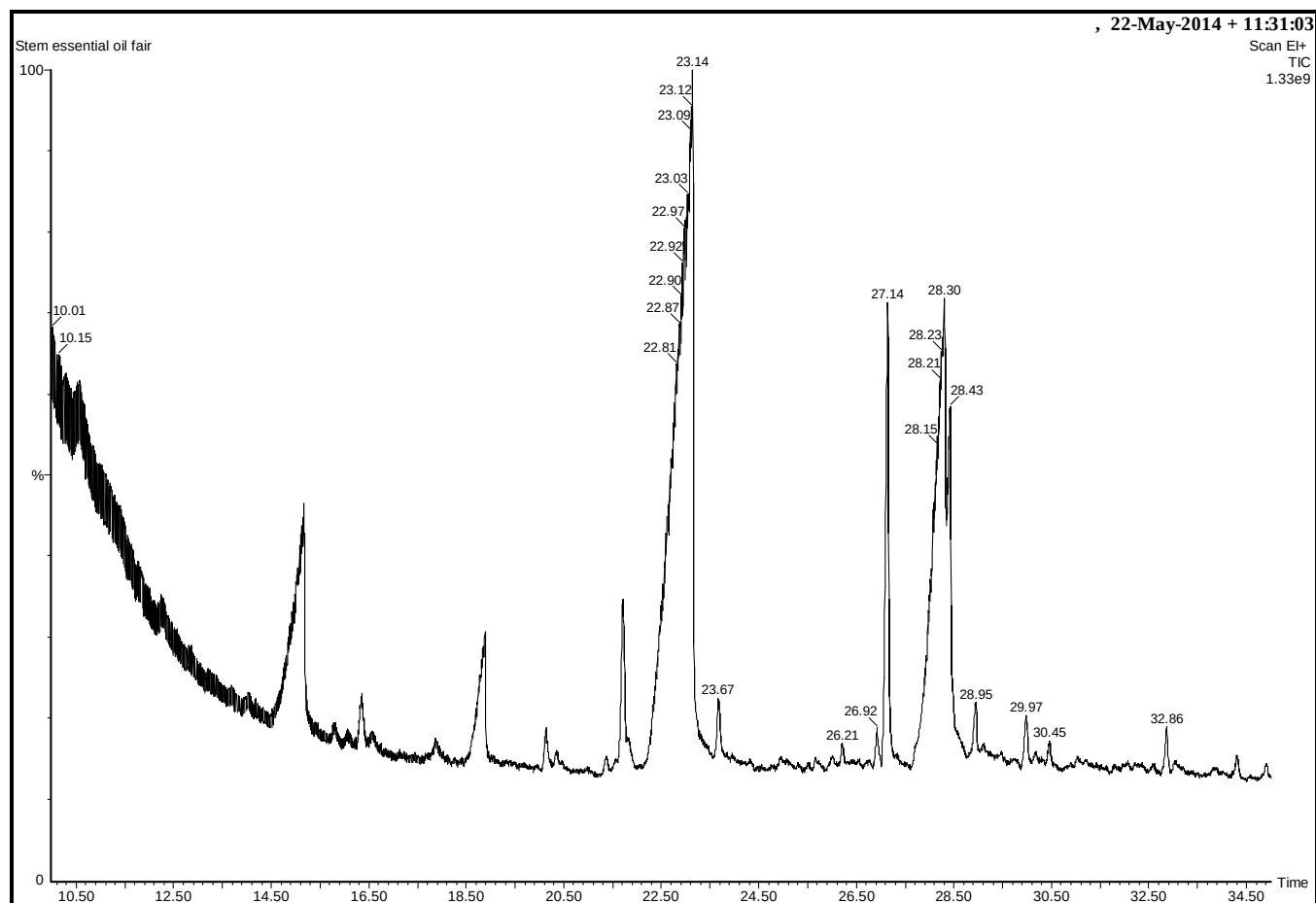
*TIC of the Hydrodistillate of Costus pictus flower,
leaves, stem, rhizome & root and the MS Spectra of
some identified compounds*



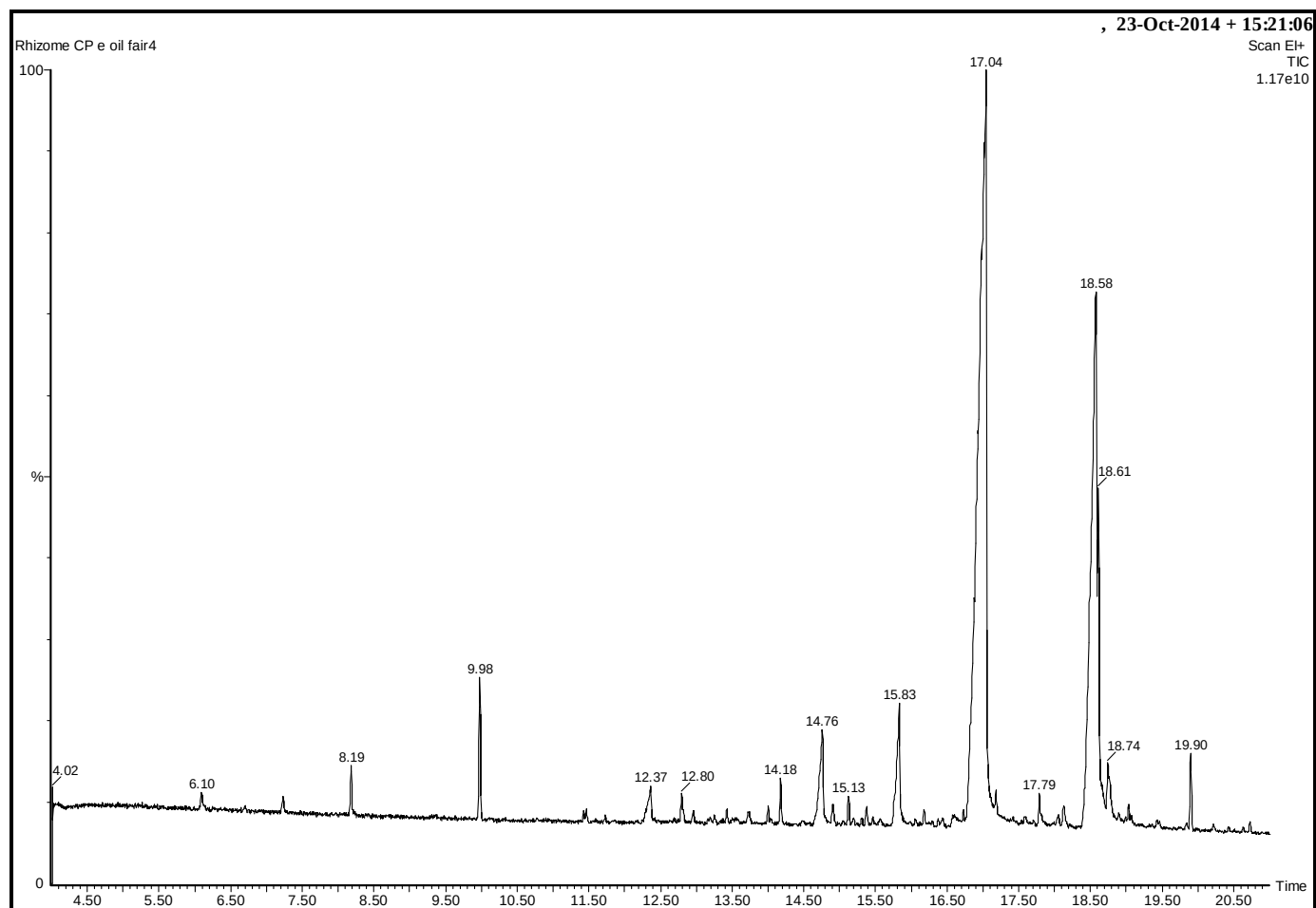
TIC of the hydrodistillate of *C. pictus* flower



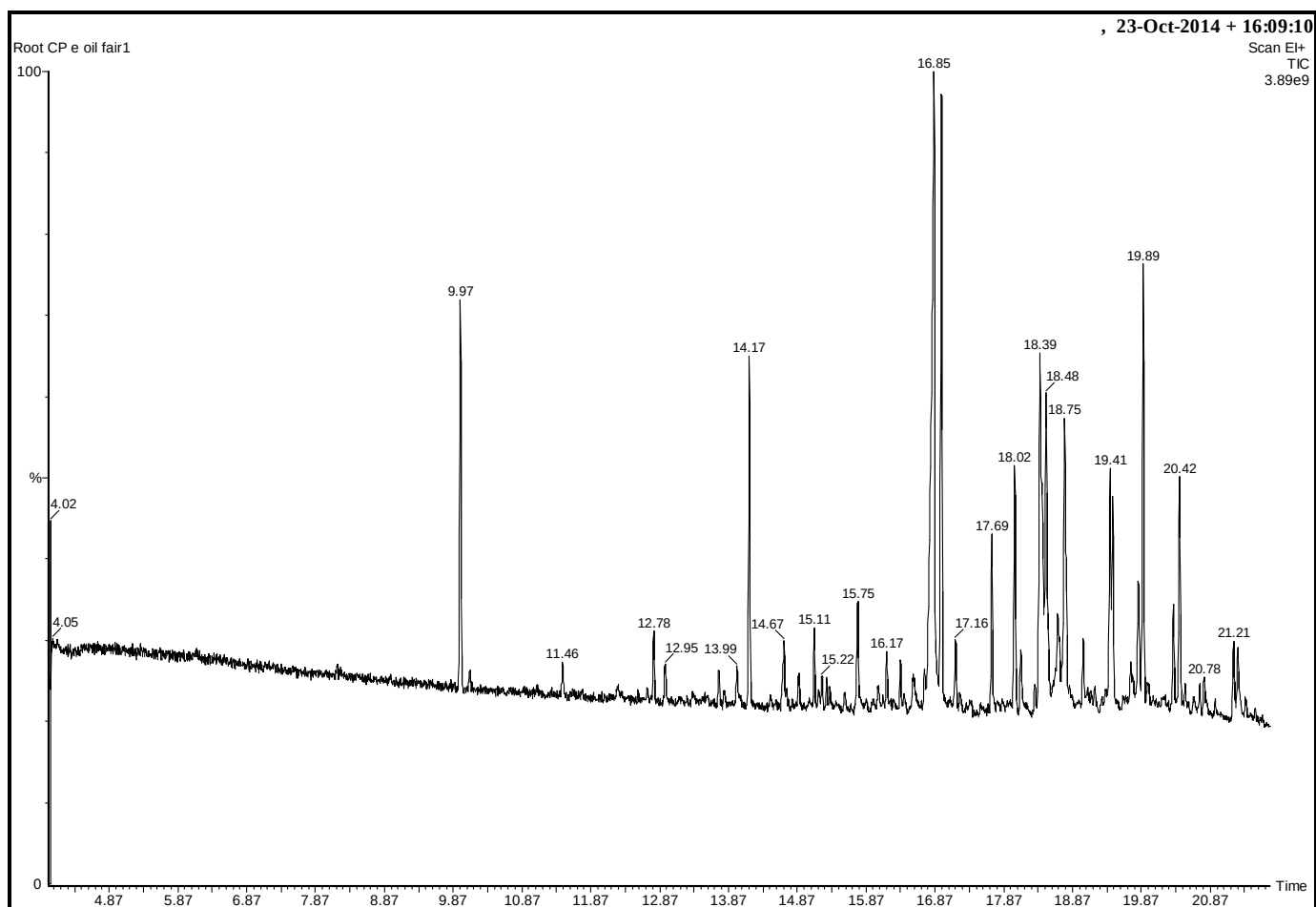
TIC of the hydrodistillate of *C. pictus* leaf



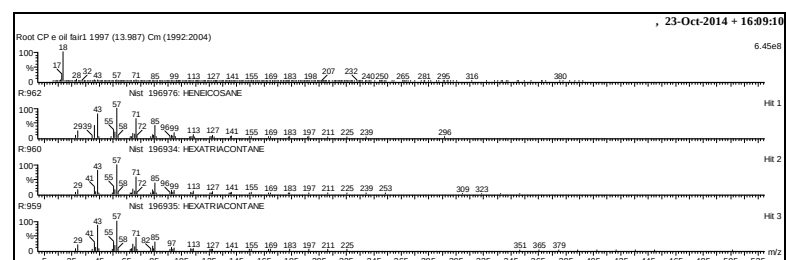
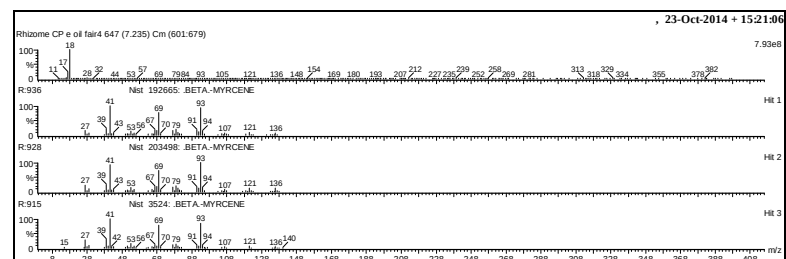
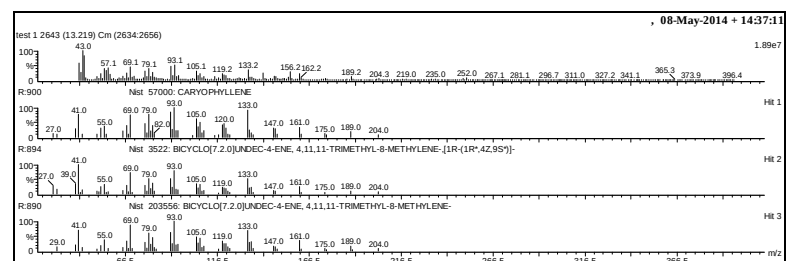
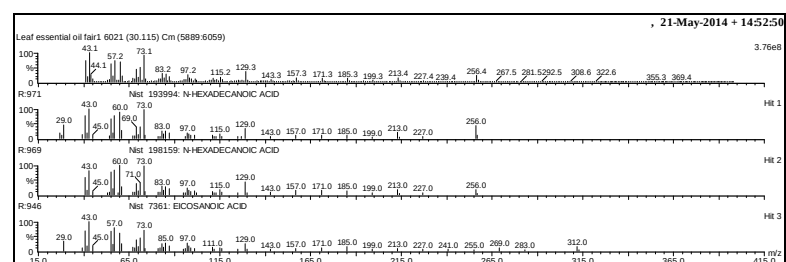
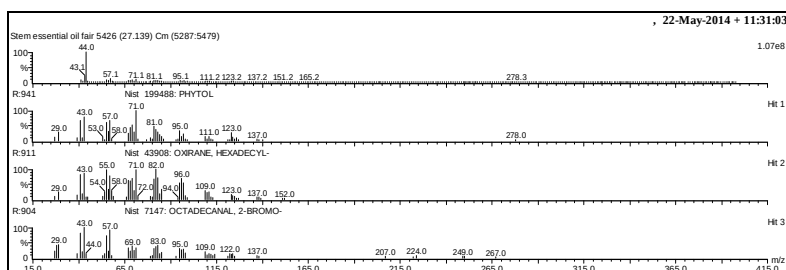
TIC of the hydrodistillate of *C. pictus* stem



TIC of the hydrodistillate of *C. pictus* rhizome



TIC of the hydrodistillate of *C. pictus* root



EI Mass Spectrum of some identified compounds from the flower, leaf, stem, rhizome & root hydrodistillate of *C. pictus* along with library search results

Appendix VII

*Spectra of n-butylgallate (D_2) isolated from the leaves
and TIC of the essential oil from the flowers of *Leea*
*indica**


STIC
**Sophisticated Test &
Instrumentation Centre**
*(An autonomous Institution established
by the Govt. of Kerala in collaboration with
Cochin University of Science & Technology)*

Cochin University P.O.,
Kochi-682 022, Kerala, India
Tel.: +91-484-2575908
Fax: +91-484-2576699
e-mail: stic@vsnl.com
URL: www.sticindia.com

SAIF-Cochin

Analytic functional testing
VarioEL III CHNS
Serial Number 11035060

26/12/2005

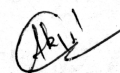
Date of receipt : 09-12-2005

Date of analysis :23-12-2005

Ref. No. SAIF/CH/051209A

Sample No	Sample Name	N%	C%	H%
1	D2	0.00	57.99	7.01

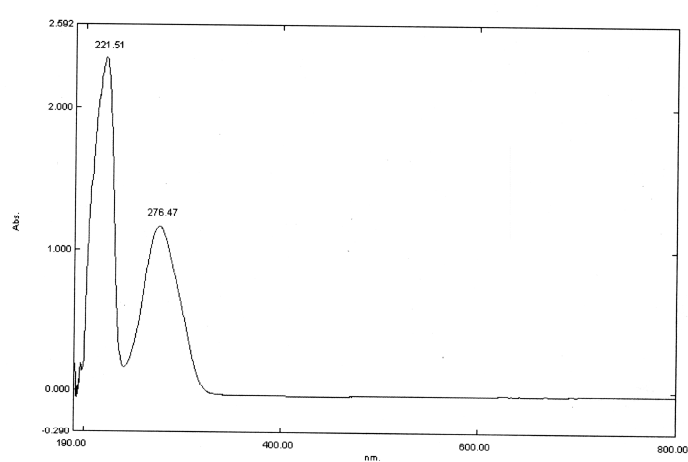

Analyzed by


Scientist in charge

1

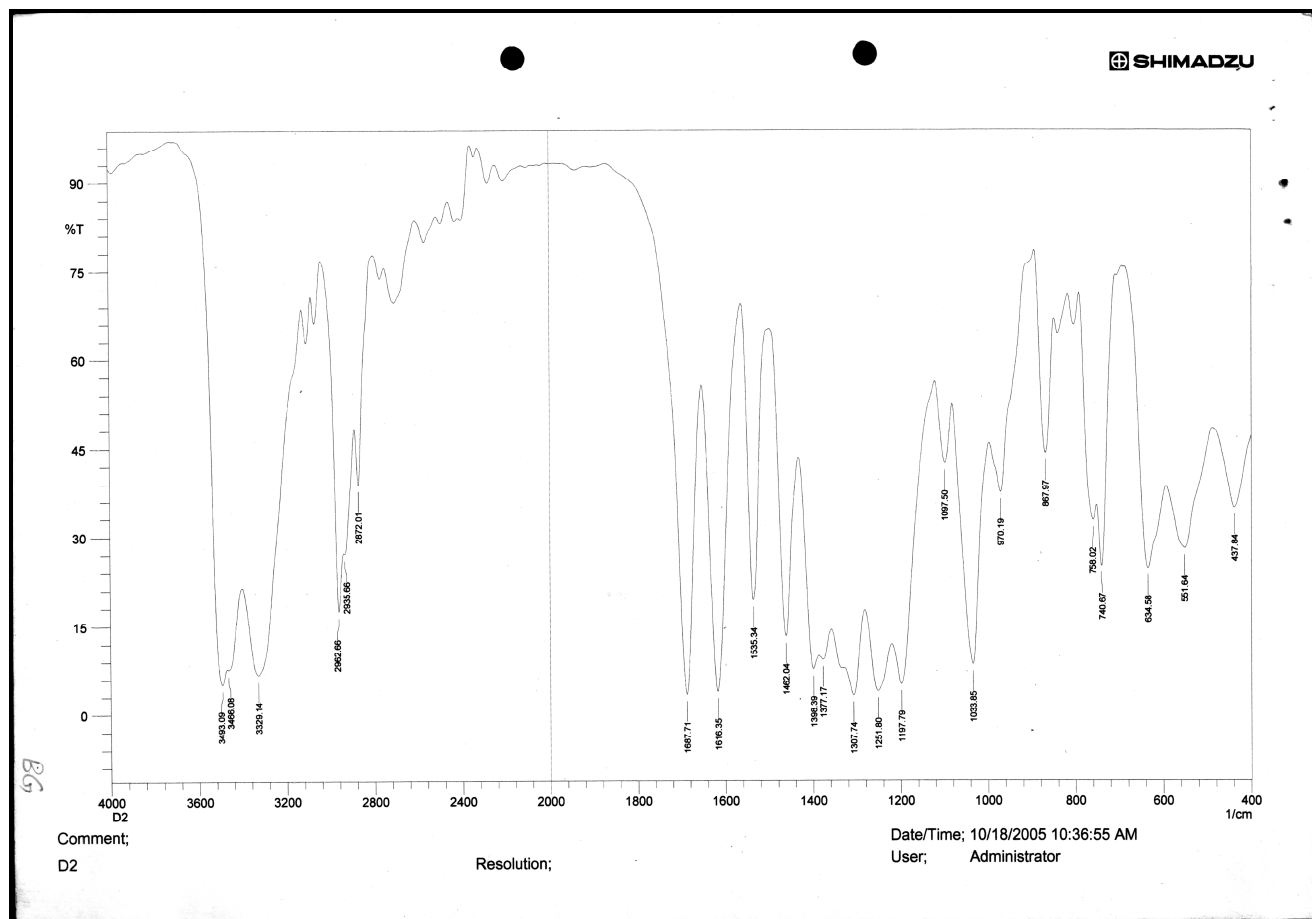
n-butyl gallate (BG)

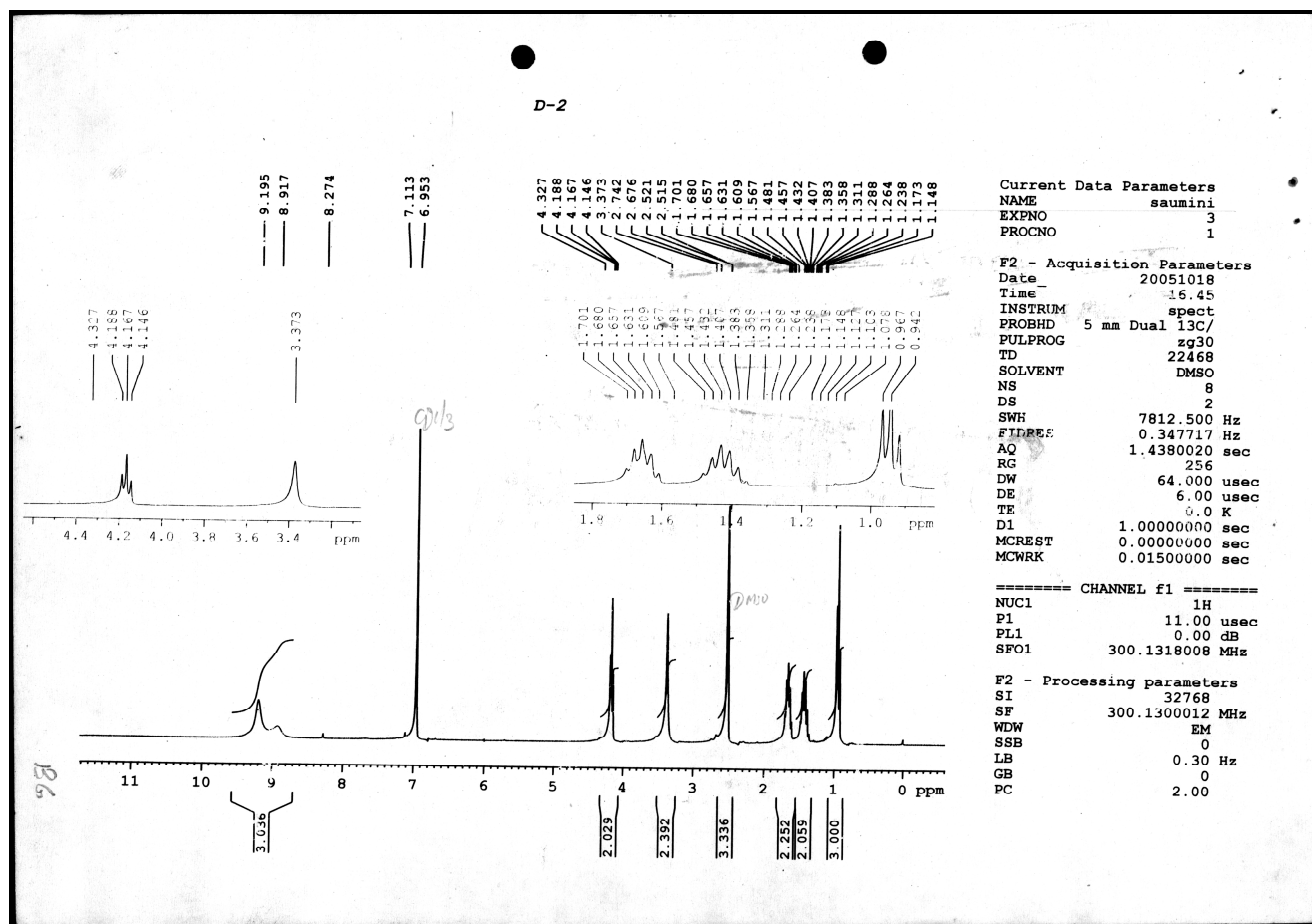
Elemental analysis of D₂

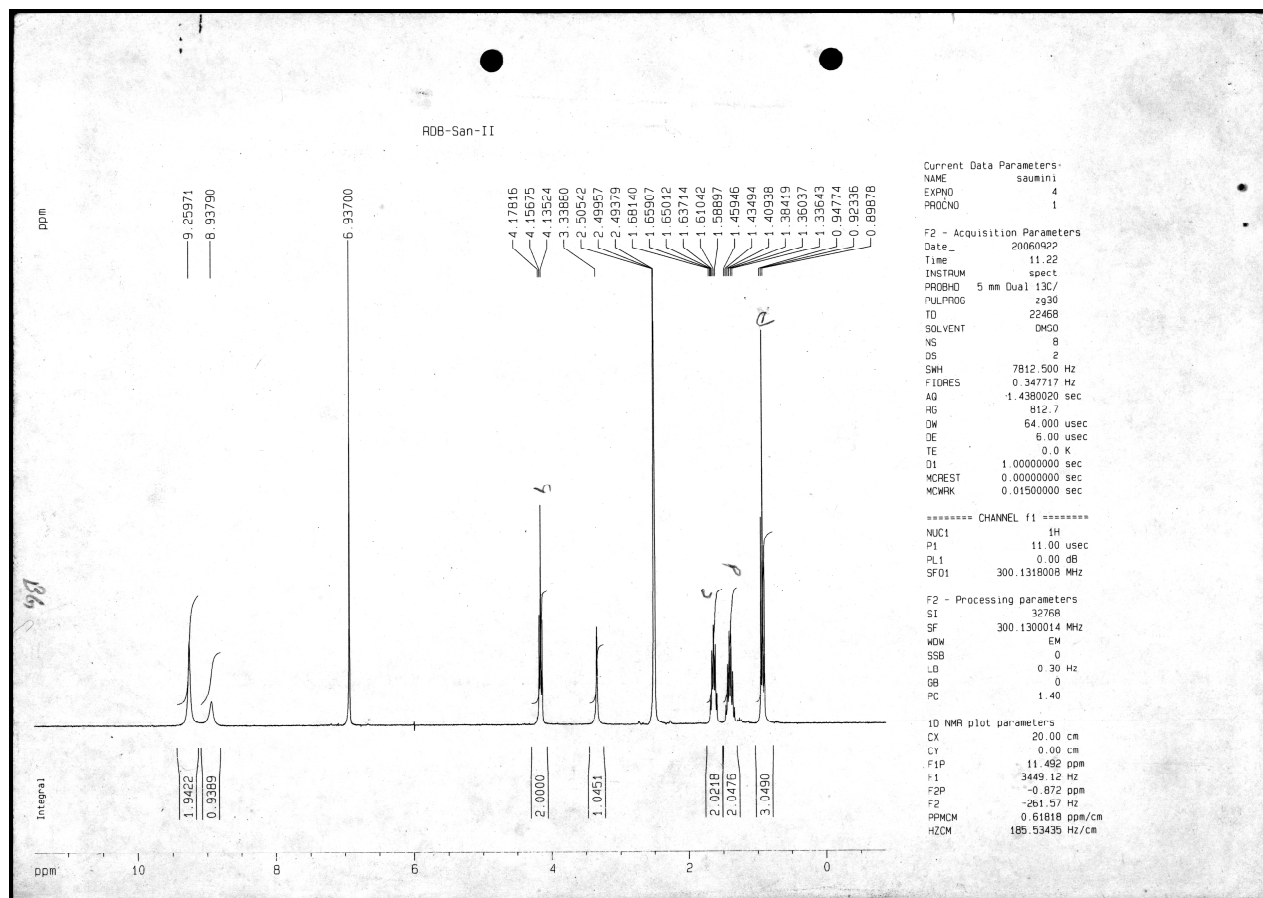
UV Spectrum of D₂

BG

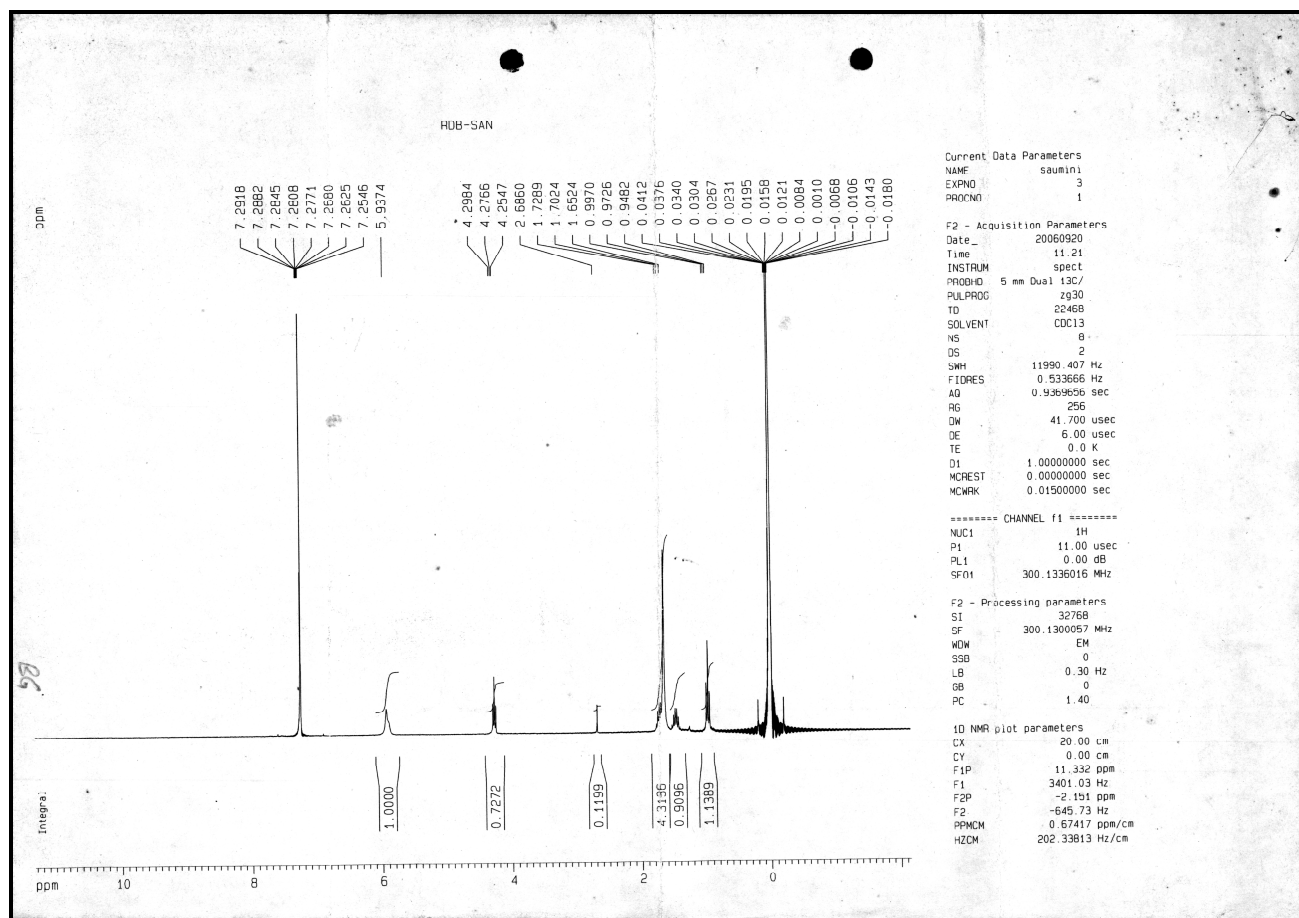
UV-Vis spectrum of D₂

IR spectrum of D₂

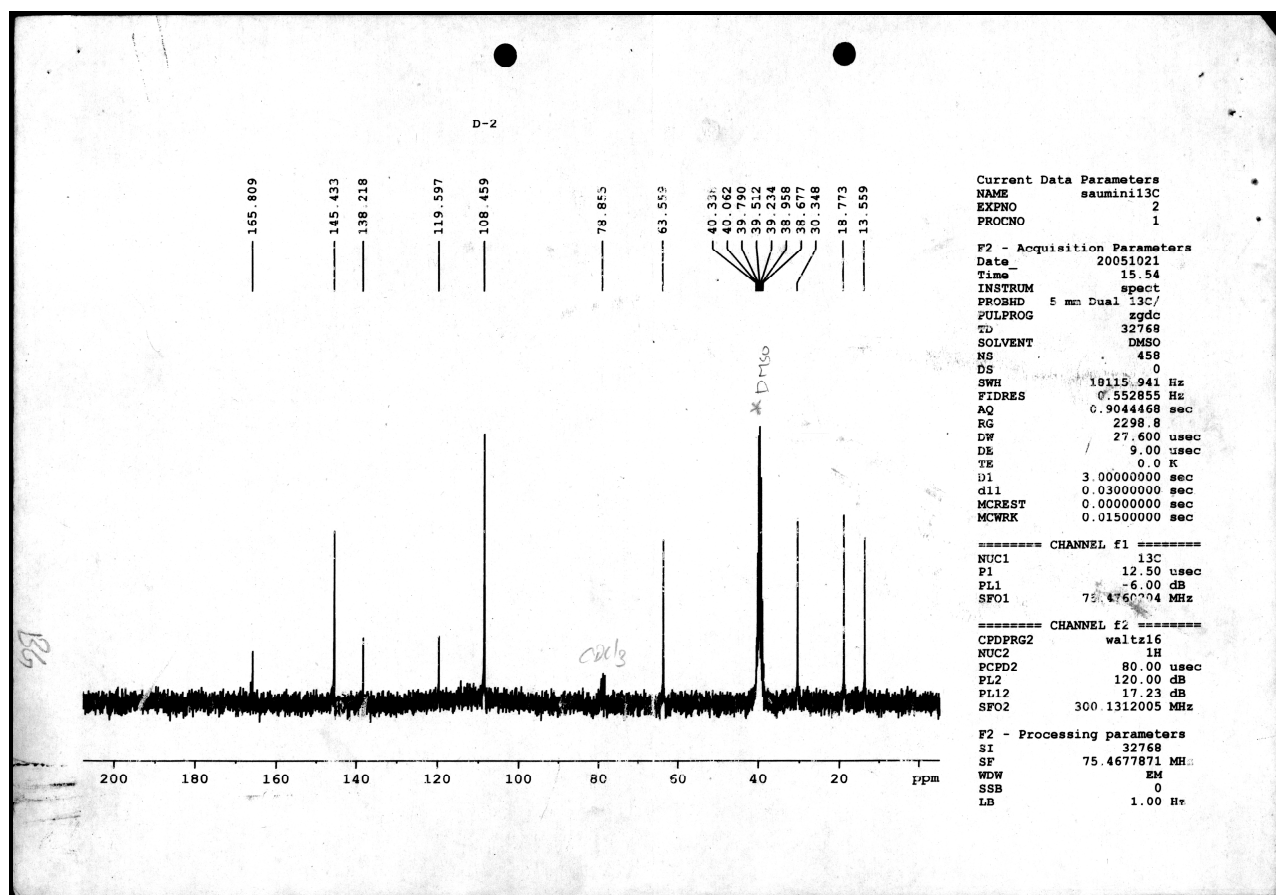
 ^1H NMR spectrum of D_2



^1H NMR spectrum of D_2 (before adding D_2O)

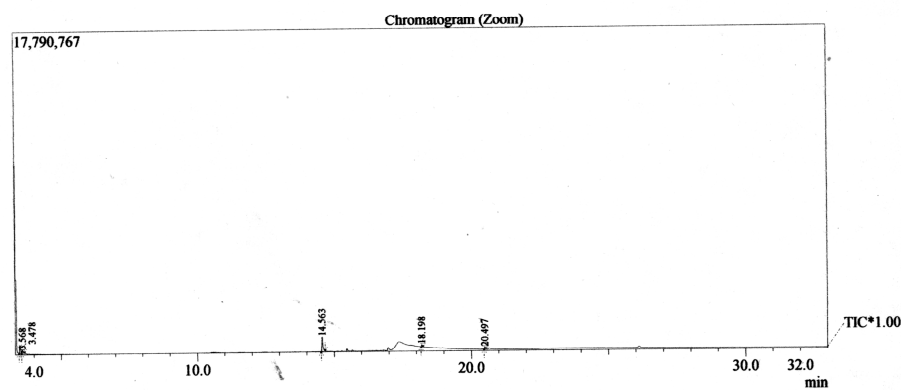
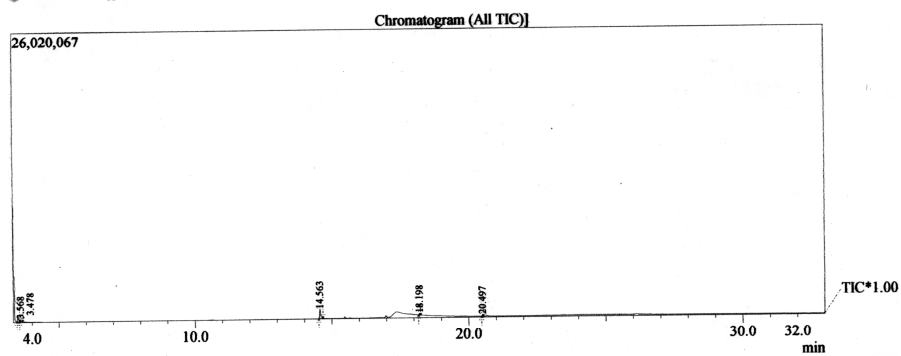


^1H NMR spectrum of D_2 (after the addition of D_2O)

 ^{13}C NMR spectrum of D_2

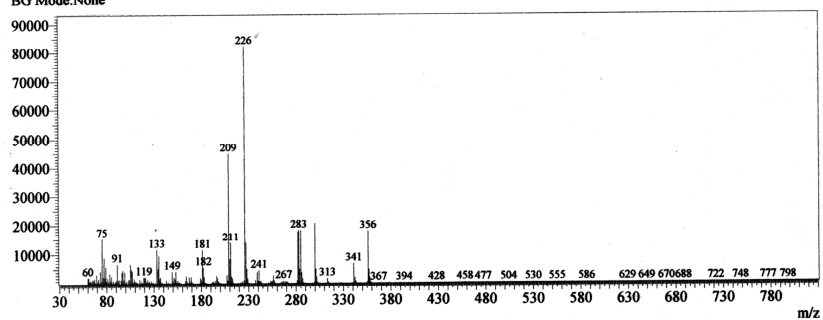
10/18/2005 12:11:10

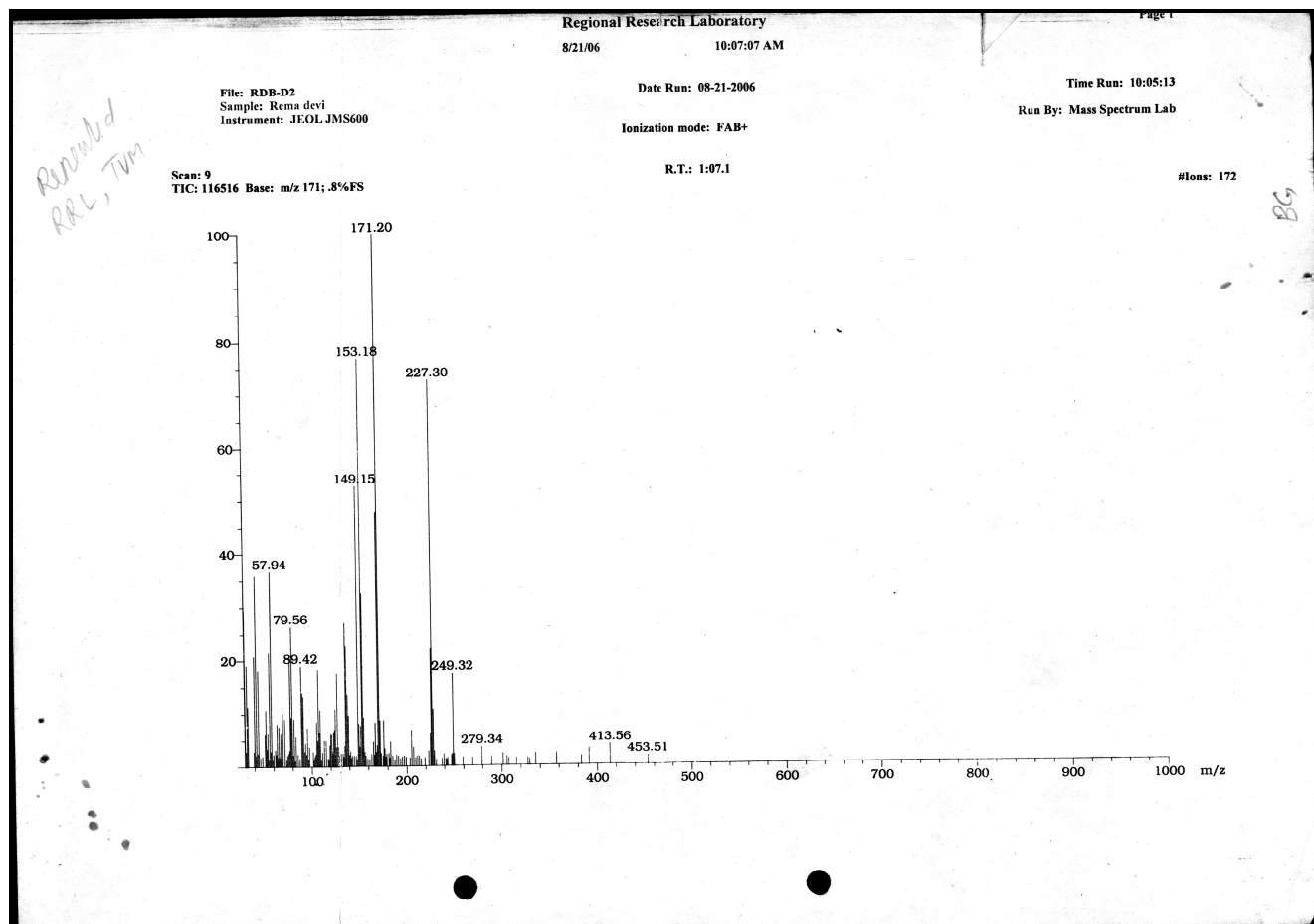
D:\OCT05\VD-2.qgd

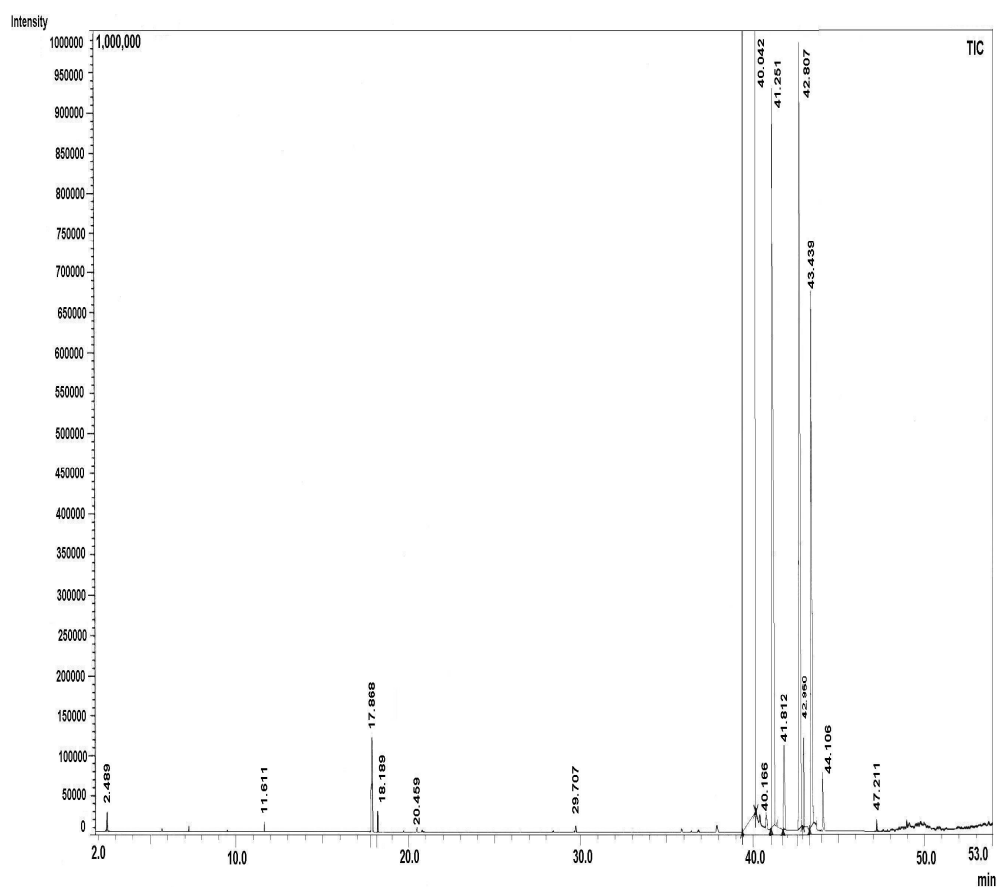


Spectrum

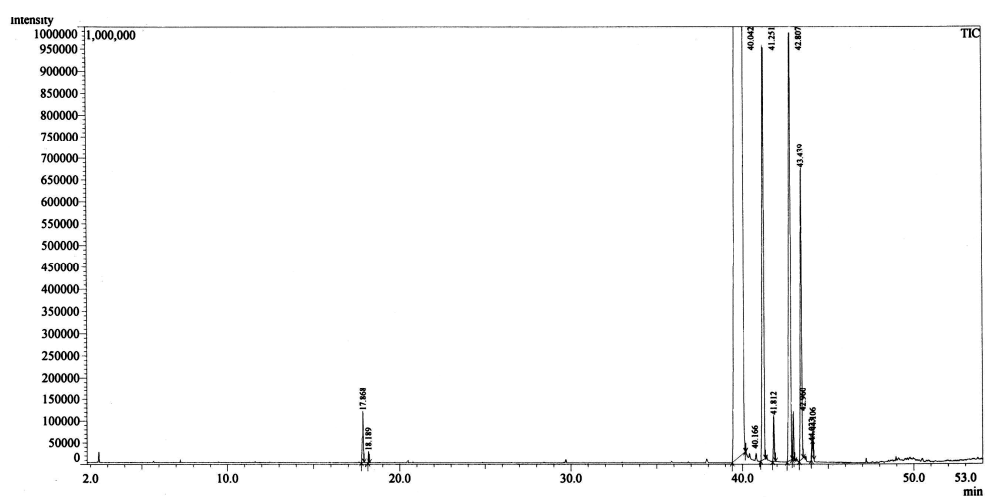
Line#:1 R.Time:17.358(Scan#:1688)
MassPeaks:528 BasePeak:226.00(82184)
RawMode:Single 17.358(1688)
BG Mode:None

EI Mass spectrum of D₂

FAB Mass spectrum of D₂



TIC of the flower essential oil of *Leea indica* from accession 1



TIC of the flower essential oil of *Leea indica* from accession 2
