In the present study, a novel two step CombiFlash chromatographic method was
developed for the fast isolation of glycyrrhizic acid, the major bio-active marker of
G. glabra. Further, glycyrrhizic acid was quantitatively estimated in the root part of
G. glabra and two of its commercial samples such as Yastimadhu churna and Artin
by employing a rapid and validated HPTLC method. The method is rapid, simple and reproducible. The marker compound, glycyrrhizic acid was found to present in 0.88% in the roots of G. glabra and 0.78% in Yastimadhu churna. But surprisingly, it could not be detected in Artin capsules. The developed method can be successfully employed for quality control and quality assurance of G. glabra, where the root part is used.

Dr. Dhananjay Vithalrao Mane is presently working as Regional Director, Yashwantrao Chavan Maharashtra Open University, Nashik since Feb. 2018. He has teaching experience of more than 3 decades. Till today under his guidance 12 research scholars Obtained Ph. D. degree and 10 research scholars are working for their Ph. D. He has written 12 books.

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Dhananjay Mane
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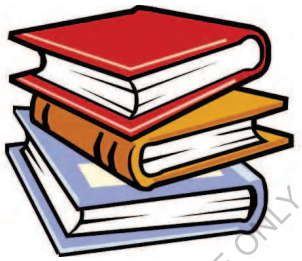
Chemical, Chromatographic and Biological Studies on Some Indian Medicinal Plants

YAGNAMBHATLA APARNA



Dedicated

To

My Beloved Parents

\

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Yagnambhatla Aparna

ABBREVIATIONS

AcOH	:	Acetic acid
Bn	:	Benzyl
bs	:	Broad singlet
calcd	:	Calculated
$CDCl_3$	:	Deuterated chloroform
cm	:	Centimeter
COX	:	Cyclooxygenase
d	:	Doublet
dd	:	Doublet of doublets
DCFDA	:	2',7'-Dichlorofluorescin diacetate
DCM	:	Dichloromethane
DMEM	:	Dulbecco's modified Eagle medium
DMF	:	Dimethylformamide
DMSO-d_6	:	Deuterated dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
EI	:	Electron ionization
ELISA	:	Enzyme-linked immunosorbent assay
ESI	:	Electron spray ionization
EtOAc	:	Ethyl acetate
EtOH	:	Ethanol
FBS	:	Foetal bovine serum

FTIR	:	Fourier transform infrared spectroscopy
g	:	Gram
GSH	:	Glutathione
h	:	Hour (s)
HRMS	:	High resolution mass spectrometry
Hz	:	Hertz
IL	:	Interleukin
J	:	Coupling constant
JNK	:	c-Jun N-terminal kinase
m	:	Multiplet
Me	:	Methyl
MeOH	:	Methanol
mg	:	Milligram
min	:	Minute (s)
mL	:	Milliliter
mmol	:	Millimole
MMP-9	:	Matrix metalloproteinase-9
mp	:	Melting point
MS	:	Mass spectrometry
MTT	:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	:	Nicotinamide adenine dinucleotide phosphate
NF-κB	:	Nuclear Factor Kappa B
nM	:	Nanomolar

NMR	:	Nuclear magnetic resonance
PBS	:	Phorbol myristate acetate
Ph	:	Phenyl
q	:	Quartet
quint	:	Quintet
rt	:	Room temperature
s	:	Singlet
t	:	Triplet
td	:	Triplet of doublets
THF	:	Tetrahydrofuran
TLC	:	Thin layer chromatography
TNF	:	Tumor necrosis factor
μg	:	Microgram
μM	:	Micromolar

GENERAL INFORMATION

- ^{1}H and ^{13}C NMR spectra were recorded on Bruker 300 MHz or Varian 400 MHz or Varian 500 MHz in $CDCl_3$ with TMS as internal standard. Chemical shifts were expressed as δ values in parts per million (ppm) and coupling constants (*J*) in Hertz (Hz).
- HRMS spectra were recorded on Agilent-ESI QTOF or JEOL mass spectrometers.
- IR spectra were recorded on Nicolet 740 or Nicolet Nexus 670 spectrophotometer using KBr pellets and values are given in cm^{-1}.
- Melting points were determined on Buchi or Stuart SMP3 digital melting point apparatus and are uncorrected.
- All reactions were monitored by pre-coated silica gel 60 F_{254} glass TLC plates (Merck) with UV irradiation at 254 nm and exposure to iodine vapours for visualization.
- Column chromatography was carried on Acme (India) silica gel (60-120 or 100-200 mesh).
- Solvents were distilled before use. Solvent evaporation was carried out under reduced pressure on Buchi R-3 Rotavapor below 45° C. Reagents were procured from commercial sources (Sigma-Aldrich, Alfa Aesar) and used without further purification.
- The names of all the compounds given in experimental section were taken from Chem Bio Draw, version 12.0.

CONTENTS

"Nature itself is the best physician."

- Hippocrates

Every human depends on basic physiological needs such as food, shelter, clothing and medicine before moving to other requirements[1]. To stay healthy and fit, human needs resistance to fight against diseases. Decrease in mortality leads to increase in life expectancy. Over population is one of the serious problems in the developing countries and the global population is expected to touch 9.2 billion by the year 2050. In order to address these issues, we need to develop potent, efficacious and safe drugs. When a disease is identified then the treatment is mostly based on the drugs[2] originated from :

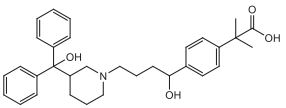
- Synthetic
- Natural
- Natural Products based semi-synthetic

Some of the salient features of these drugs are briefly presented below :

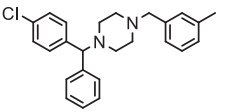
Synthetic Drugs

Synthetic drugs are man created medicine with chemicals. These are also called as designer drugs[3]. Even today for some disease areas the synthetic drugs are only the answers. These include antihistamines, diuretics and hypnotics. Some of the important examples are:

i) Antihistamines: Antihistamines are drugs which treat allergic rhinitis and other allergies[4]. Some of the potent synthetic antihistamines are:

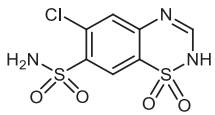


Fexofenadine (**1**)



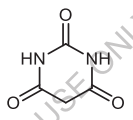
Meclizine (**2**)

ii) Diuretics: A diuretic is a substance that promotes diuresis, that is, the increased production of urine[5]. A well known synthetic dug of his class is:



Chlorothiazide (**3**)

iii) Hypnotics: Commonly known as sleeping pills. These are a class of psychoactive drugs, whose primary function is to induce sleep and to be used in the treatment of insomnia or sleeplessness due to surgery[6]. Barbutirates are commonly used for this purpose. Barbituric acid (4) is the most widely used drug of this class[7].

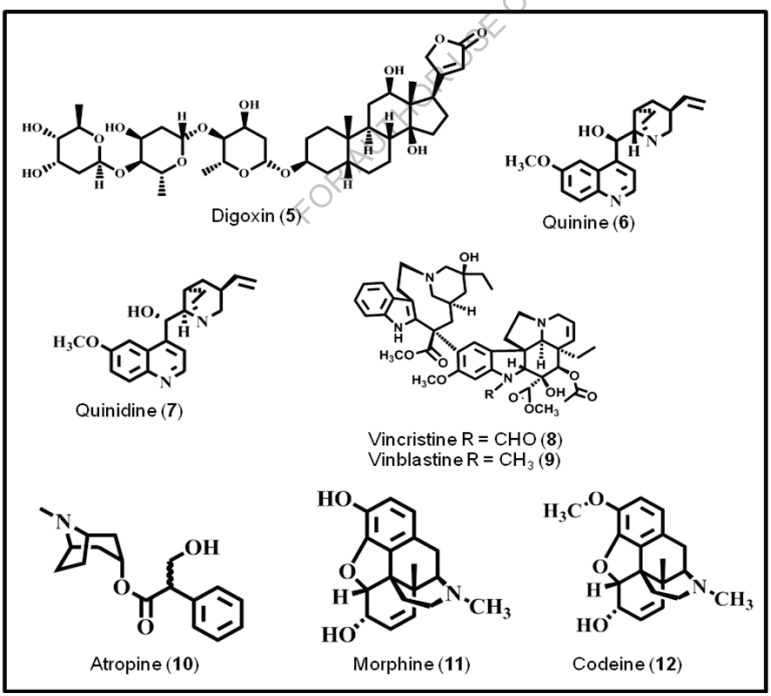


Barbituric acid **(4)**

But overall history reveals that treatments of diseases are not safe. In fact most of the synthetic drugs induce toxicity and carcinogenicity[8]. Hence, we need to explore indigenous medicine or traditional medicine to combat various dreaded and chronic diseases.

Traditional medicine, also known as indigenous or folk medicine, comprises of medical aspects of traditional knowledge that developed over generations within various societies before the era of modern medicine. Traditional medicine refers to any ancient and culturally based health care practice differing from scientific medicine and is largely transmitted orally by communities of different cultures. Traditional system of medicine has been the only option available for health care prior to the induction of modern medicine for prevention, diagnosis, and treatment of social, mental and physical illness[9]. Hippocrates (ca. 470–471 BCE) was the first to reject divine causality in medicine and

to develop a new approach to diseases based on scientific observation of the human body. Hippocrates work was in opposition with the mainstream Aesclepion beliefs, based on religion. Hippocrates is nowadays considered as the 'Father of Medicine'. The Hippocratic Oath being an ethical reference and motto in the medical community worldwide[10]. WHO states that "traditional medicine" implies the knowledge and practices of herbal healing for the prevention, diagnosis, and elimination of physical, mental, or social imbalance[11]. Some of important drugs obtained from plants[12] are digoxin (**5**) from *Digitalis* sp., quinine (**6**) and quinidine (**7**) from *Cinchona* sp. vincristrine (**8**) and vinblastine (**9**) from *Catharanthus roseus*, atropine (**10**) from *Atropa belladonna* and morphine (**11**) and codeine (**12**) from *Papaver somniferum*.



World Health Organization has shown great interest in documenting various remedies of medicinal plants used by tribal communities in different parts of the world. In 1991, WHO proposed the guidelines for the assessment of herbal medicine, which were subsequently approved in the 6th International conference of drug regulatory authorities held at Ottawa. The salient features of WHO guidelines[13] are:

1) Quality assessment: Crude plant materials, extracts and finished products need to be assessed for their quality by generating fingerprints and marker compound based studies.

2) Stability: Shelf life of the preparations need to established for their stability.

3) Safety assessment: Documentation of safety based on experience and Toxicological studies is also a key parameter.

4) Assessment of efficacy: This is a very important parameter and can be established by documentary evidence of traditional use and *in vivo* bio-evaluation in animals and humans.

Natural products offer 100 times higher hit rate when compared to synthetic drugs. New guidelines issued by the Food and Drug Administration for the approval of herbal mixtures with the evidence of safety and efficacy, even though the active constituents are not identified[14]. For many disease areas no single isolated compound found responsible for the therapeutic potential of the source plant used. However, it has been realised that certain combination of compounds of the plant source work in synergistic fashion to produce the total therapeutic activity of the medicinal plant[15]. This concept works very well for many traditional systems of the world. Generally, traditional systems of medicine function through two streams[16] such as:

- Classical Health Traditions (CHT*)* -- highly organized, classified and codified.
- Oral Health Traditions - very rich and diverse, but not organized or codified.

Some of the important traditional medicinal systems of the world are

1. Traditional Chinese Medicine (TCM)

2. Indian System of Medicine (ISM)

3. Kampo (Traditional Japanese Medicine)

Details of these three systems are briefly discussed below:

1. Traditional Chinese Medicine (TCM)

Western medicine was introduced in the sixteenth century, but it did not undergo any development until the nineteenth century. Before that TCM was the dominant form of medical care in the country. Chinese medicine principles rely on the theory that the human body is an open organism operating in continuous biological and mental exchange process with the outside environment. When exchanges are in a balanced state, then the health of the human organism is optimal. It has an extremely valuable, rich, lengthy, and extensive treatment history[7].CHM was firstly described by *Shen-Nong*, who is said to have lived from 2737 BCE to 2697 BCE, nearly 5,000 years ago[17,18]. Of the hundreds of herbs tested, more than 70 herbs found with medicinal value and are suitable as remedies[19]. As a result of extensive efforts, numerous herbs ("herbal" medicine) have been used routinely for health care in ancient china[20]. In terms of the literature on CHM, the theoretical aspects and practical experiences of several thousand years of usage are documented in more than 8,000 books. The total number of ancient literature on both CHM and TCM reached more than 13,000 books. The documentation of knowledge in CHM is unique in the world [21,22].

1.1. Importance of Chinese Herbal medicine in World Pharmacy

For the past 2,000 years, more than 40 types of foreign herbs were imported into China and eventually adopted by TCM. They include kelp from Korea, turmeric and styrax from Southeast Asia, and others such as boreal, clove, frankincense, myrrh, benzoin,

senna and saffron[23]. China has more than 31,000 higher plants, 256 endemic genera, and 15,000–18,000 endemic species (50–60% of the total species on earth). Many of which are living fossils, such as dawn redwood (Metase quoiaglyp to stroboides Hu and Cheng), ginkgo (Ginkgo biloba L.)[24]. The total number of species and protected plant species in china are shown in Figure 1.01

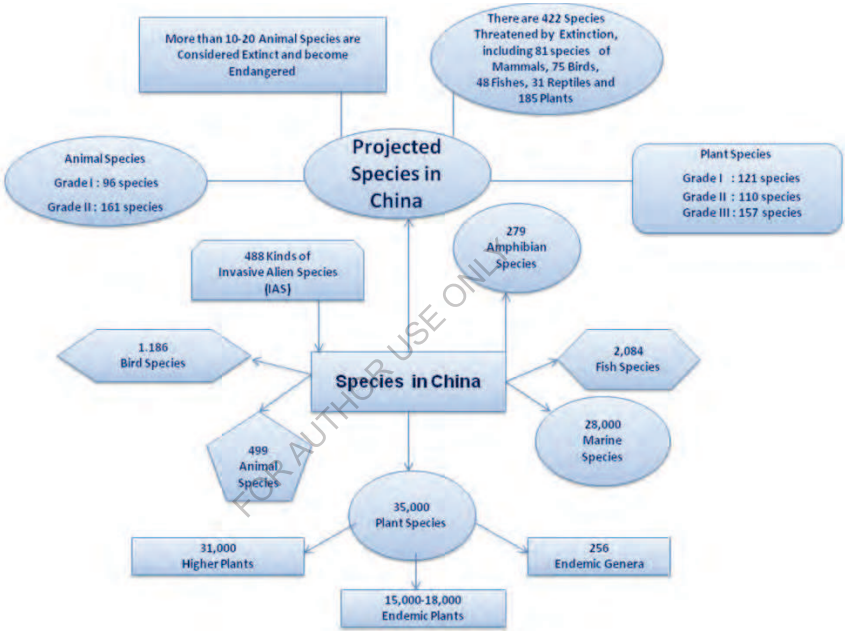


Figure 1.01. Species and protected species in China

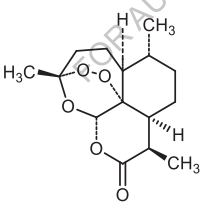
The traditional herbal medicine along with other ethnic herbal medicines of China, forms as a gold mine for potential modern medicine and healthcare products. Recently "nanomized" and "aerosol" herbs have emerged as new dosage forms of CHM[25,26]. Over the past few years, herbal exports have steadily increased[27] from US $1.09 billion in 2006 to US $1.46 billion in 2009. Now China is putting great human affords and financial resources on research and development in the area of Chinese herbal

medicine (CHM). These tremendous efforts are un-matched by other countries in the world.

1.2 Some Drugs or Compounds Isolated from Plants of Chinese Herbal Medicine:

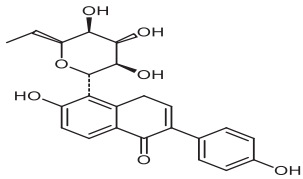
a) *Artemisia annua L.*[28,29]

Artemisinin (**13**), also called as qinghaosu, is an antimalarial drug derived from the sweet wormwood plant, *Artemisia annua*. Artemisinin is a sesquiterpene lactone (a compound made up of three isoprene units bound to cyclic organic esters) distilled from the dried leaves or flower clusters of *A. annua*. Artemisinin is effective against all the malaria-causing protozoal organisms in the genus *Plasmodium*. The drug is particularly useful in the treatment of infections involving chloroquine-resistant parasites and infections involving multidrug-resistant *P. falciparum*, which is the deadliest of the malaria protozoans.



Artemisinin **(13)**

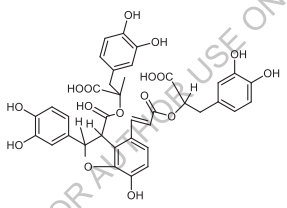
b) *Pueraria lobata (Willd.) Ohwi* --- Its Marker compound is Puerarin[30]. It is used in diabetes. It is a isoflavonoid. Puerarin (**14**) is the most abundant constituent of RP. Administration of RP extract improved glucose tolerance and decreased fasting plasma glucose levels in ob/ob mice and that puerarin supplementation reduced body weight gain and lipid levels in liver and serum of high-fat-diet (HFD) fed-induced obese mice.



Puerarin (**14**)

c) *Salvia miltiorrhiza* Bunge[31,32]

Also known as red sage or Chinese sage, It is a perennial plant in the genus *Salvia*, highly valued for its roots in traditional Chinese medicine. Its Marker compound is Salvianolic acid (**15**), which is used to treat cardiovascular and cerebrovascular diseases.

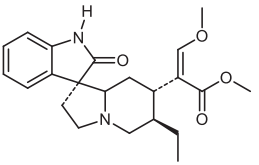


Salvianolic acid (**15**)

d) *Uncaria rhynchophylla* (Miq.) Jacks[33]

Its Marker compound was isolated and identified as rhynchophylline (**16**). It is used as a antihypertensive agent. Six alkaloids, namely, corynoxine, corynoxine B, corynoxeine, isorhynchophylline, isocorynoxeine, and rhynchophylline were isolated from the extract of *Uncaria rhynchophylla*. Among them, rhynchophylline and isorhynchophylline significantly decreased A*β*-induced cell death, intracellular calcium overloading and tau protein hyperphosphorylation in PC12 cells. These results suggest that rhynchophylline and isorhynchophylline are the major active ingredients responsible for the protective action of *Uncaria rhynchophylla* against A*β*-induced neuronal toxicity, and their neuro-

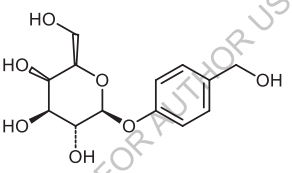
protective effect may be mediated, at least in part, by inhibiting intracellular calcium overloading and tau protein hyper phosphorylation.



Rhynchophylline (**16**)

e) *Gastrodia dlata Bl* [34].

Its marker compound was identified as gastrodin (**17**). It is a phenolic compound and the main constituent of the traditional Chinese herb *Tianma (Gastrodia elata Blume*). It has been reported to be effective in relieving trigeminal neuralgia, migraine and vascular headache in clinical trials in China.



Gastrodin (**17**)

The main advantage of TM's therapeutics is the "synergism"; that is, often multiple components in TMs play a synergistic role which is greater than that of the individual drug. The "1 disease, 1 target, 1 drug" mode cannot treat some complex diseases effectively, such as cardiovascular disease and diabetes. In future, multidisciplinary collaborative research, closely cooperated with new ideas, such as network pharmacology and big data, will be possible to explain the synergism and other mechanisms of natural products and TMs from which more and better new drugs and treatments will be discovered and inspired.

2. Indian System of Medicine (ISM)

Indian traditional medicine is rich knowledge with thousands of plants[35-38]. India has been known to be the richest repository of medicinal plants. About 8000 herbal remedies have been codified in Ayurveda. They are still used in the classical formulations of Ayurvedic system of medicine[39]. India is known for its traditional medicinal systems like Ayurveda, Siddha, and Unani. Some of these systems found mentions even in the ancient Vedas and other scriptures. The Ayurvedic concept appeared and developed between 2500 and 500 BC in India.

2.1 Ayurveda

In India, the history of using plant resources for treating diseases can be dates back to 6,000 to 4,000 BCE. *Bhava Prakasha Nighantu*, written by Bhava-Mishra, is the most important text on herbs/plants and is considered as the authority by modern Ayurvedic practitioners[40-42]. In ancient times, Ayurvedic texts were much respected in the neighbouring countries and they were also translated[43] into Greek (300 BCE), Tibetan and Chinese (300 CE), Persian and Arabic (700 CE). India possesses almost 8% of the estimated biodiversity of the world with about 1,26,000 species belonging to 400 families of flowering plants of the world. Out of which at least 315 of these can be found in India[44]. In India, approximately 25,000 plant-based formulations are used in traditional and folk medicines[45]. The Indian Herbal Medicines (IHMs) are derived either from the whole plant or from different organs like leaves, stem, bark, root, flower, seed etc. Some IHMs also include animals and minerals. Few are prepared from excretory plant products such as gum, resins, and latex. Currently, about 45,000 species (nearly 20% of the global species) are found in the Indian subcontinent, out of which 3,500species of plants are of medicinal value. Figure 1.02 Shows the plants and their related species of India used in Indian herbal medicine.

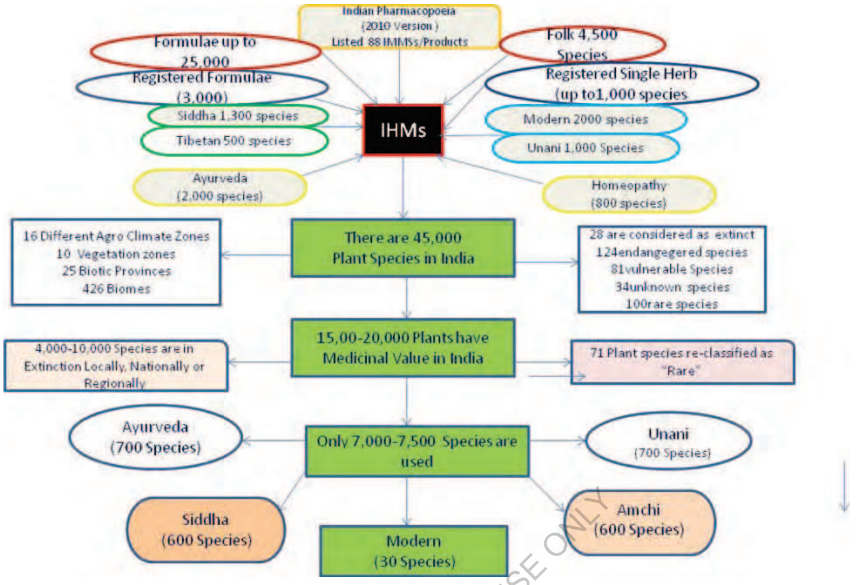


Figure 1.02. Indian Herbal Medicine

On the Earth botanical reviews quantify the presence of approximately 2,50,000 to 3,50,000 plant species. Out of which, only 35,000 species are reported to be used for the treatment of various ailments in different parts of the World[46]. It is estimated that ~15% of the medicinal plants only subjected to phytochemical analysis and ~6% for biological screening. Rest of the plants are untouched. Herbal remedies are the most popular form of traditional medicine and are highly lucrative in the global market place according to an estimate of W.H.O. The demand for medicinal plants is likely to increase more than US $5 trillion in 2050. In India, the medicinal plant related trade is estimated to be approximately US $1 billion per year [47]. Over all international herbal trade market is revolving around China and India. The annual herbal drugs exports of China is estimated over 1,20,000 tonnes followed by India with approximately 32,000 tones. In contrast, Europe is the primary importer of remedial plants and around

4,00,000 tonnes are imported each year by different European countries to meet the local demand of herbal formulations[48-50]. Some common medicinal plants and their primary use in traditional medicine having Nutraceutical potential[51] are presented in Table 1.01.

Table 1.01. Some common medicinal plants and their primary use in traditional medicine having Nutraceutical potential

Plant name	Common name	Uses
Asparagus acemosus Willd	Shatavari	A potent Ayurvedic rejuvenative. It supplies many female hormones and mostly recommended for those women who have hysterectomies. It also helps to maintain urinary tract and strengthens the immune system and also purifies the blood.
Commiphora mukul Engl. .	Guggul	A major ingredient in joint and immune care and regarded as a remedy in Ayurvedic medicine. It increases white blood cell count to possess strong immuno-modulating properties. It also protects against the common cold as well as used in various other conditions like lower cholesterol and triglycerides, while maintaining the HDL to LDL ratio.
Garcinia cambogia Dr	Garcinia	Fruits contain biologically active compound viz. (−) hydroxycitric acid, which is known to inhibit the synthesis of lipids and fatty acids. HCA inhibits the enzyme ATP-citrate lyase that leads to reduce production of acetyl CoA, which is a key substance in fat and carbohydrate metabolism. Therefore, formation of LDL and triglycerides is very low. It also suppresses appetite by promoting synthesis of glycogen. That way the brain gets signals of fullness and satisfaction sooner. Garcinia contains significant amounts of vitamin C and used as a heart tonic.

Glycyrrhiza glabra L.	Yashtimadhu, Licorice	It is a versatile medicine in India and China for gastrointestinal health. It is a mild laxative, soothes and tones the mucous membranes, and relieves muscle spasms. It is an antioxidant, cancer protecting, botanical boosting and certain immune functions such as interferon production. Its mode of action is as an antimutagen, preventing damage to genetic material that can eventually result in cancer.
Gymnema sylvestre R. Br.	Gurmarar	Its Sanskrit name means literally "sugar destroyer". It has aglycolytic action and reduces the strength of a glucose solution. It has been used in Ayurveda to regulate sugar metabolism for several centuries. It increases insulin production and regeneration of pancreas cells. Another property is abolishing the taste of sugar. Gurmarar has been effective to suppress and neutralize the craving for sweets.
Terminalia chebula Retz.	Haritaki	Haritaki is a safe and effective purgative, expectorant and tonic. It is an important ingredient of the classical Ayurvedic formulation "Triphala" which has a combination of three fruits. Tiphalpha is an important Ayurvedic medicine, which promotes health through successive steps of purification and detoxification. It is known to have strong antimutagenic activity, due to its very rich content of vitamin C.

3. Kampo (Traditional Japanese Medicine)

Kampo is the well known traditional system of Japan. Between the fifth and sixth centuries, TCM was introduced to Japan from China. Since then TCM has been significantly altered and adapted by Japanese practitioners to meet their particular circumstances and gradually evolved into Kampo[52]. A recent study has found that some

physicians in Japan use Kampo medicines in their daily practice sometimes as the preferred medication[53-55]. Together with radiotherapy or chemotherapy, some Japanese physicians frequently utilize Kampo medicines in treating cancer patients. This indicates how modern Western medicine can be well integrated with TM[56,57]. As the use of Kampo continues to rise in conjunction with Western medicine, there is growing realization of the urgent need to study the interactions between these two types of medicines[58]. This indicates how modern, Western Medicine can be well integrated with TM. Kampo medicine and Kampo-derived compounds may provide exquisite possibilities for such tailor-made treatment strategies as shown in Figure 1.03.

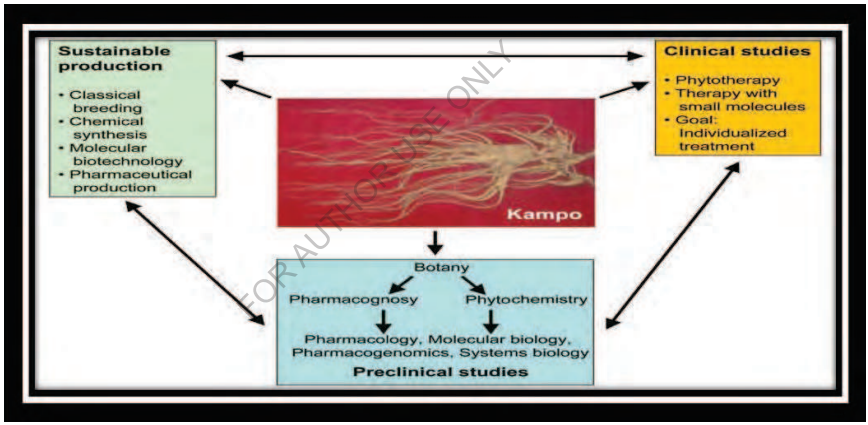


Figure 1.03. Kampo Treatment Strategies

India has diverse population coming from different origins, different cultures and different living habits. Hence, it is not possible to get complete literature about traditional knowledge. Insufficiency of awareness about the basic principles and histories of the systems from different ethnic origins, there is some missing of exchange of information from systems around the globe. To date, there have been several reviews detailing Ayurveda. However, very few reviews detail the modalities of

the basic principles and history of Ayurveda. The above mentioned three medicinal systems of the world provide excellent prior art and clues for modern drug development. A number of potent therapeutic agents have been developed based on traditional systems of the world. Some of the salient features of modern drug development are given below:

4. Drug Discovery and Development

Any drug development process must proceed through several stages in order to produce a product that is safe, efficacious, and meet all the regulatory requirements[59] as presented in Figure 1.04.

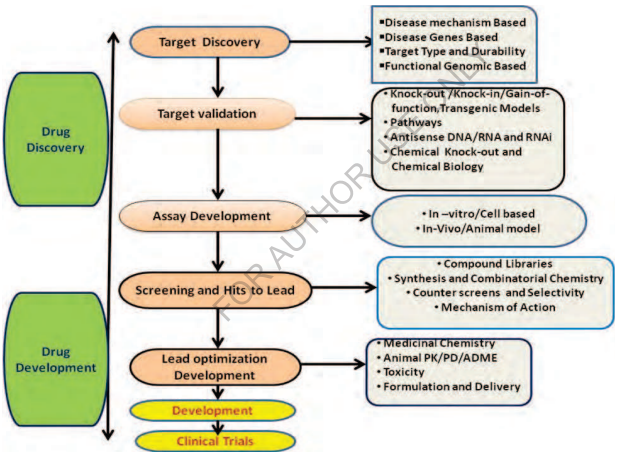


Figure 1.04. Drug Discovery Process and Development

One of the classical example of modern drug discovery is that of a 21 year old pharmacy apprentice named Friedrich Sertürner, who isolated the first pharmacologically active compound, morphine from opium produced by cut seed pods of the poppy plant, *Papaver somniferum* some 200 years ago[60]. Newman estimates that about 60 % of the drugs that are now available-including household names such a artemisinin, camptothecin, lovastatin, maytansine, paclitaxel, penicillin, reserpine and silibinin were either directly or

indirectly derived from natural products[61]. Subsequently, a large number of well known natural compounds were identified, analysed and synthesized. Recently, Butler et al.[62] reported that a total of 25 natural product (NP) based drugs were approved for marketing worldwide., Out of which, 5 are classified as NP, 10 as semi-synthetic NPs, and 10 as NP derived drugs. At present, around 10 compounds (purely plant derived) are either in clinical phase III trials or in registration phase. Some of the potent plant derived drugs are listed below in Table 1.02.

Table 1.02. Natural product derived drugs launched since 2008

Year	Generic name (trade name)/ Company name	Lead compound	Type	Disease area
2008	Methylnaltrexone 8 (Relistor®)/Wyeth and Progenics, Canada	Morphine	NPD	Opioid induced Constipation
2009	Vinflunine 15 (Javlor®)/ Pierre Fabre M´edicament	Vinorelbine (vinblastine)	SSNP	Cancer
2009	Nalfurafne (Remitch®)/ Toray International, Japan	Morphine	SSNP	Pruritus
2010	Cabazitaxel (Jevtana®)/ Sanofi Aventis	Paclitaxel	SSNP	Cancer
2010	Zucapsaicin (Zuacta®)/ Winston Pharmaceuticals, Canada	Capsaicin	NPD	Pain
2012	Ingenol mebutate (Picato®)/ Leo Pharma	Ingenol mebutate	NP	Actinic Keratosis
2012	Dapagliflozin (Forxiga®)/ AstraZeneca and Bristol Myers Squibb	Phlorizin	NPD	Type 2 Diabetes
2012	Arterolane /piperaquine (Synriam™)/Ranbaxy, INDIA	Artemisinin	NPD	Antiparasitic
2013	Canagliflozin (Invokana®)/ Mitsubishi Tanabe Pharma/	Phlorizin	NPD	Type 2 Diabetes

NP-Natural Product, SSNP-Semi synthetic natural products, NPD-natural product derived.

Some of the major categories of the drug molecules are listed in Table 1.03

Table 1.03. Major Categories of drug molecules

S. No	Code	Brief definition/year
1	B	Biological macromolecule, 1997
2	N	Unaltered natural product, 1997
3	NB	Botanical drug (defined mixture), 2012
4	ND	Natural product derivative, 1997
5	S	Synthetic drug, 997Synthetic drug (NP pharmacophore), 11997
6	S*	Synthetic drug (NP pharmacophore), 1997
7	V	Vaccine, 2003
8	/NM	Mimic of natural product, 2003

New approved drugs from 1981−2014 are (n) = 1562. These drugs are from all sources. The drugs derived from each category are given Figure 1.05.

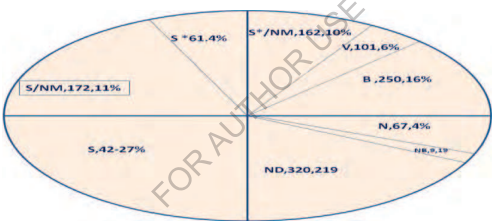


Figure 1.05. N, NB, ND, and S* categories of approved drugs during 1981−2014

The four leading chronic diseases in India, as measured by their prevalence, are in descending order: cardiovascular diseases (CVDs), diabetes mellitus, chronic obstructive pulmonary disease (COPD) and cancer. The projected cumulative loss of national income for India due to non-communicable disease mortality for 2006–2015 is expected to be USD237 billion. By 2030, this productivity loss is expected to double to 17.9 million years lost[63]. India is currently experiencing an epidemic of Type 2 diabetes mellitus (T2DM) and has the largest number of diabetic patients. It is often referred to as the diabetes capital of the world. India currently has the highest prevalence of oral

cancer cases in the world as a result of the popularity of chewing tobacco in its rural regions[64]. The scientific community engaged in drug discovery and development worldwide working feverishly in developing safe and efficacious natural product based drugs. Although, India has rich traditional knowledge and huge wealth of medicinal plants, very little work has been done on the chemistry and biology of Indian medicinal plants. Systematic and detailed chemical and pharmacological evaluation certainly yields novel and therapeutically useful molecules.

It is evident from the above review that there exist tremendous scope and opportunities in the plants used in Indian systems of medicine to develop novel and potent bioactive molecules, leads and drugs. With this objective in mind a detailed programme has been taken up to exploit the following plants used in Indian traditional systems for novel, taxonomically useful and therapeutically useful molecules and marker compounds:

i) *Cochlospermum gossypium*

ii) *Chloroxylon swietenia*

iii) *Boerhavia diffusa*

iv) *Glycyrrhiza glabra*

The results obtained in each plant are presented in the subsequent chapters 2-5.

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The genus *Cochlospermum* belongs to Cochlospermaceae family (some classifications place this genus in the family Bixaceae), which consists of about 15 species. Out of which, 3 occur as far north as northern Mexico and southwestern United States. *Cochlospermum vitifolium,* popularly known *as* buttercup tree, found widespread in central America and the West Indies. It has bright-yellow cup-shaped flowers with about 10 cm (4 inches) across. In some areas rope is made out of its bark. Some of the *Cochlospermum* species are known to yield dyes. The seeds of *C. angolense,* an African species, yield a red dye, whereas the roots of *C. planchoni,* also an African species, yield an yellow dye. Notably, the Indian and Burman *Cochlospermum* species, *C. gossypium* (syn: *C. religiosum*) produces a gum that can substitute the gum tragacanth[1]. *C. gossypium (L)* species is native to India. This species is now in the threatened or endangered list. The salient features of some of the most important species of this genus are given below :

1. *Cochlospermum planchonii* Hook.f

Cochlospermum planchonii (syn : *C. niloticum* Oliv; *C. niloticum* var. glabrum A. Chev., ver : False cotton (English); Faux cotonnier (French); Soasga (Mooré); N'Dribala (Dioula); Family: - *Cochlospermaceae*), a West African species[2], can go up to 0.5-1.5m in height (Figure 2.01) and growing from Guinea region to Cameroon[3].

Figure 2.01. *Cochlospermum planchonii* Hook. f.

Traditional Uses

It has various medicinal applications in different parts of Africa. In Ivory Coast, the root is used to treat schistosomiasis, jaundice, fever and back pains. Whereas in Senegal, it is used to treat jaundice, intestinal worms, bilharziasis and hepatitis[4].

Chemistry

Qualitative evaluation were carried out using chemical test on the samples following standard procedure for identification of the constituents as described by Harbone (1973)[5]; Trease and Evans[6] and Sofowora[7]. The quantification of the detected phytochemicals (Table 2.01) was carried out as described for saponins[8], flavonoids, tannins, steroids, triterpenes, phenolics and phlobatannins[9], alkaloids[10], anthraquinones[11].

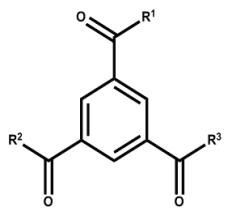
Table 2.01: Phytochemical classes of *C. planchonii* root

S. No.	Phytochemicals	Quantity (%)
1.	Alkaloids	2.920
2.	Tannins	0.147
3.	Phenolics	3.156
4.	Cardiac glycosides	ND
5.	Saponins	7.575
6.	Flavonoids	0.067
7.	Steroids	0.885
8.	Flobatannins	0.028
9.	Triterpenes	0.094
10.	Anthraquinones	0.186
11.	Cardenolides / Dienolides	ND

ND : Not Detected

Achenbach et al reported[12] four cochlospermines (A, B, C, D) from the root part of *C. planchonii* (Table 2.02).

Table 2.02: Phytochemical Constituents of *Cochlospermum planchonii* root.

	Compound	R^1	R^2	R^3
	Cochlospermine A	$H_3C(CH_2)_{10}$	$H_3C(CH_2)_{10}$	$H_3C(CH_2)_{10}$
	Cochlospermine B	$H_3C(CH_2)_{12}$	$H_3C(CH_2)_{10}$	$H_3C(CH_2)_{10}$
	Cochlospermine C	$H_3C(CH_2)_{12}$	$H_3C(CH_2)_{12}$	$H_3C(CH_2)_{10}$
	Cochlospermine D	$H_3C(CH_2)_{12}$	$H_3C(CH_2)_{12}$	$H_3C(CH_2)_{12}$

Pharmacology

The various polar extracts of different parts of *C. planchonii* reported to exhibit interesting biological activities as detailed below :

i) *Antimalarial activity:* The root dichloromethane extract inhibited the growth of the *Plasmodium falciparum* K1 chloroquine-resistant strain (IC50 4.4 µg/ml) (Vonthron-Sénécheau et al., 2003)[13]. Leaf oil yielding the best antimalarial effect (IC50 = 22–35 µg/ml)[14].

ii) Antibacterial activity: The leaf and stem methanol extracts, inhibited the growth of *S. aureus*, *S. pyrogenes*, *S. typhi* and *P. mirabilis* at a concentration of 10 mg ml^{-1}. The MBC showed that at 20 mg ml^{-1}, *S. pyrogenes* and *P. mirabilis* were exterminated, while it was observed for *S. aureus, S. typhi, P. aeruginosa* and Trychophyton sp. at 40 mg ml^{-1} of the plant extract[15].

iii) *Anti-diabetic activity*: The roots aqueous extract[16] have been demonstrated to reduce the blood glucose (86.7%) of alloxanized rats at a dose of 25.5 mg/kg b.w..

iv) *Anti-inflammatory and analgesic activities*: The aqueous methanol root extract at 250 and 500 mg/kg bw (p.o.) significantly ($p < 0.05$) decreased the number of abdominal writhings with percentage inhibition of 65.14% and 74.05%, respectively (Anaga & Oparah, 2009)[17].

v) *Safety data*: The IC_{50} values of single dose toxicity on K562 cell line (human erythroblastic cell line) and L6 cells (rat skeletal muscle myoblasts) were reported to be 1600 µg/mL and 67.3 µg/mL respectively.[18]

vi) *Adverse Effects*: Nausea, dizziness.

2. *Cochlospermum tinctorium* (A. Rich) (*Cochlospermaceae*)[19]

Figure 2.02. *Cochlospermum tinctorium*

The common vernacular names of *Cochlospermum tinctorium* (Figure 2.02) in Nigeria include *rawaya, kyamba* (Hausa), *obazi, abanzi* (Igbo) and *sewutu* (Yoruba).[20] It is a bushy plant attaining about 50 cm in height. It has widespread occurrence in the savannah and shrub land throughout the drier areas of West Africa region.

Traditional Uses

The rhizomes of the plant are used traditionally to treat fever, hepatitis, abdominal pain, and helminthes, and bilharzias infestations.[21] The decoctions of the whole roots are used as remedy for gonorrhea, jaundice and gastrointestinal disease.[22] Some previous studies on the plant reported anti-ulcer, radical scavenging,

immunomodulating,[23] anti-malarial,[24] hepatoprotective,[25,26] antibacterial,[27,28] and anti-convulsant[29] activities.

Chemistry

Aqueous methanol leaf extract (AMLE), aqueous methanol root extract (AMRE), and aqueous methanol root bark extract (AMRBE), respectively. Fresh aqueous solutions of the extracts were prepared for each study. The extracts were screened for the presence of alkaloids, saponins, tannins, flavonoids etc. using standard protocol.[30] Preliminary phytochemical studies shows that all the extracts were found to contain saponins, flavonoids, tannins, steroids, cardiac glycosides, and alkaloids. Phytochemical constituents present in the aqueous methanol extracts of *C.tinctorium* (Table 2.03).

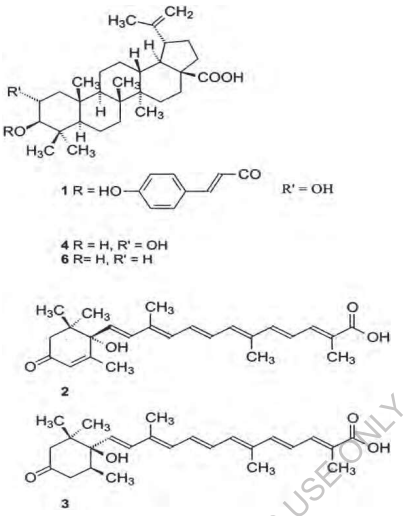
Table 2.03: Phytochemical Constituents of *Cochlospermum tinctorium*

S. No.	Phytochemical Constituents	AMRE	AMRBE	AMLE
1.	Flavonoids	+	+	+
2.	Tannins	+	+	+
3.	Steroids / terpenoids	+	+	+
4.	Cardiac glycosides	+	+	+
5.	Alkaloids	+	+	+
6.	Saponins	+	+	+

+ Indicates : Presence of Phytochemical Constituents

Fractionation of an ethanol extract of roots of *C. tinctorium* afforded five compounds: 3-O-E-p-coumaroylalphitolic acid (1), cochloxanthin (2), dihydrocochloxanthin (3), alphitolic acid (4) and 1-hydroxytetradecan-3-one (5). 1-hydroxyhexadecan-3-one, which was found in the steam distillate from roots of *C. tinctorium* and characterized by the mass spectrum. Its three related analogues such as 1-hydroxyundecan-3-

one, 1-hydroxytridecan-3-one, and 1-hydroxyheptadecan-3-one were detected in the essential oil from leaves of *C. tinctorium* and *C. planchonii* by GC-MS.[31]



Pharmacology

Aqueous leaf, root and root bark extracts of *C. tinctorium* possess significantly analgesic and anti-inflammatory activities. The median lethal dose of the extracts found to be less than 300 mg/kg suggested that they are relatively toxic, intraperitoneally[32]. However, they may be relatively safe at the analgesic and anti-inflammatory doses used for this study.

Decoction prepared from the comminuted roots of *C. tinctorium* is used as a drug against malaria in Burkina Faso. The oil obtained by hydrodistillation of the leaves of *C. tinctorium* showed significant antiplasmodial activity, Whereas the aqueous decoction of the roots showed no antiplasmodial activity *in vitro*, but showed a pronounced activity (1-2 *i*g/mL).[33] Partitioning between water and dichloromethane of the ethanol extract revealed that the major activity was found in the organic layer.

Antiplasmodial activity and activity against Phytohaemagglutinin A activated Human Lymphocytes of Compound **1**, **4**, and **5***a* are presented in Table 2.04.

Table 2.04 : Antiplasmodial Activity of *C. tinctorium* **compounds 1,4 & 5**

Compound	IC_{50} (μM)		
	3d7	Dd2	PHA
1	2.3 ±1.1	3.8 ± 1.9	43 ± 12
4	35		
5	68 ± 19		
Chloroquine	(22± 9) $\times 10^{-3}$	(172 ± 25) $\times 10^{-3}$	

The *P. falciparum* strain 3d7 is chloroquine sensitive, whereas Dd2 is chloroquine resistant. PHA represents phytohaemagglutinin A provoked lymphocyte proliferation. Compound **1** showed an interesting antiplasmodial activity, whereas the activity towards human lymphocytes was only moderate, indicating some selectivity. The activity of the coumaroyl derivative (**1**) was significantly higher than that of alphitolic acid (**4**) and the related betulinic acid (**6**).[34]

3*. Cochlospermum regium (Mart. & Schr.)*

Cochlospermum regium (Mart. & Schr.) Pilger (Figure 2.03) is a shrub found

Figure 2.03. *Cochlospermum regium*

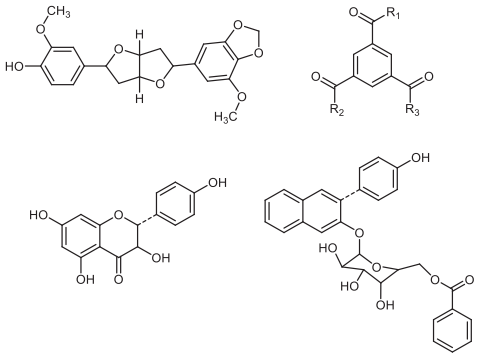
common in the Brazilian cerrado, where it is considered as animal fodder, an ornamental plant and as a medicinal plant.

Traditional Uses

There are reports of *C. regium* (popularly known as “algodãozinho”) use in folk medicine in the states of Goias, Mato Groso, Mato Grosso do Sul, Distrito Federal, and São Paulo. Saint-Hilaire described the use of this plant by the population of Paracatu City, mainly for relieving internal pains and the healing of already formed abscesses. Other reports of the use of this species have been cited in regional books.[35] Roots of *C. regium*, which have been used for the treatment of various diseases related to inflammation (arthritis, rheumatism, and acne) and genitourinary infection. Two other species of *Cochlospermum* are considered native to Brazil, *C. vitifolium* and *C. onirocense*. *C. vitifolium* is cited as being native to the north and northeast regions of Brazil, whereas *C. onirocense* is located predominantly in the northern region of the country.[36]

Chemistry

By using silica-gel column with a chloroform: methanol gradient as a solvent (92:5 to 25:75 v/v) ethyl acetate fraction (2 g) compounds was isolated. Dihydrokaempferol 3-*O*-b-glucopyranoside (**5**)[37] was isolated from these materials. The isolation of acetophenone 1-hydroxytetradecanone-3, an ester of *p*-hydroxycinnamic acid, flavonoids, naringenin, and dihydrokaempferol from the roots of *C. regium*.[38] Phytochemical investigation produced seven phenol derivatives: ellagic acid, gallic acid, dihydrokaempferol, dihydrokaempferol- 3-*O*-β-glucopyranoside, dihydrokaempferol-3-*O*-b-(6''-galloyl)-glucopyranoside, pinoresinol, and excelsin. Two triacylbenzenes, known as cochlospermines A and B.



Pharmacology

Some of the isolated compounds of *C. regium* have been reported to exhibit antinociceptive activity.[39] *C. regium* justifies its use in medical infections and inflammation.[40] The presence of gallic acid provides sufficient support to justify the popular use of this species in treating infections. Antimicrobial activity of the extract, fractions and compounds isolated from the root of *C. regium* against the microorganisms *S. aureus* and *P. aeruginosa*. Table 2.05.

Table 2.05: Antinociceptive Activity of *C. regium* Extracts and Fractions

S. No.	Samples	Inhibition Zone (mm)	
		S. aureus	*P. aeruginosa*
1.	Hydroethanolic extract	16.4 ± 1.3	9.0 ± 1.0
2.	Hexane fraction	9.8 ± 0.4	8.3 ± 0.5
3.	$CHCl_3$ fraction	10.0 ± 0.4	8.0 ± 1.2
4.	EtOAc fraction	15.0 ± 0.7	9.5 ± 0.5
5.	BuOH fraction	13.8 ± 1.9	7.7 ± 0.8
6.	Marc	12.8 ± 1.3	7.0 ± 0.0
7.	1	--	--
8.	3	14.0 ± 0.7	00
9.	Gentamicin	22.0 ± 0.0	22.0 ± 2.8
10.	DMSO	00	00

4. *Cochlospermum gossypium*

Cochlospermum gossypium (syn: *Cochlospermum religiosum*, *Bombax gossypium, Maximilianea gossypium; ver :* Buttercup tree, Cotton tree, Silk Cotton Tree, Yellow Cotton Tree) (Figure 2.04) grows widely in India and Burma. In India it is popularly known[41] as Galgal (Hindi); Pita Karpasa (Sanskrit); Kontopalas (Urdu); **Kondagogu (Telugu)**; Tanaku, Koangillam (Tamil); Appa kudakka (Malayalam); Buruga (Kanada). It produces a gum that can substitute gum tragacanth. It is a small deciduous tree, up to 18 feet height, with branching head, barks smooth and ash coloured. Leaves cordate at base, palmately 3-5 lobed, lobes entire acute, glabrous above, white tomentose beneath, petiole up to 17 cm long, pubescent when young. Flowers 10-12 cm in diameter, sub-terminal, sub-corymbose panicle, flowers appearing before the leaves, bright yellow or golden yellow flowers, pedicels stout, up to 10-12 cm long, grooved and twisted, pubescent. Calyx 5, unequal, oblong, concave, silky outside. Corolla 5, obovate, deeply emarginated, bright golden yellow. Stamens linear, falcate, opening by pores. Capsule 8 cm long, obovoid, striate outside. Seeds reniform, many, 6 mm long, covered with an abundance of silky wool. Flowering and fruiting: January –September[42].

Figure 2.04. *Cochlospermum gossypium*

Traditional Uses

Its seed cake is used for cattle feed and manure; gum in cosmetics and book binding (Katira gum); gel as agent in tissue culture media[43]. Ethno medicinal uses of *C. gossypium* plant recorded for preparing herbal medicine to treatment and increases immunity system and memory power of the village children, who are below 12 years old[44-49]. The *C. gossypium* is also used as stimulant, sedative and is useful in cough, gonorrhoea, syphilis and trachoma[50]. It is also employed in the cigar paste, calico printing, leather dressing, and ice cream industry[51]. The young leaves were used as a cooling, wash for the hairs. The gum is useful in diarrhoea, dysentery and pharyngitis. The medicine men were claiming that, this treatment showed the effective and successful results in improving immunity and the memory power especially in children[52].

Chemistry

The physico-chemical characterisation of the gum of *C. gossypium* has been reported. The FT-IR analysis of the gum kondagogu revealed the presence of hydroxyl, acetyl and carbonyl as major functional groups. The gum kondagogu contains α-D-glucose, β-D-glucose, mannose, fructose and β-D-glactopyranose, in addition to other monosaccharides reported in gum karaya[53]. However, systematic phytochemical analysis of *C. gossypium* not yet been reported.

Pharmacology

The gum of *C. gossypium* has been reported as a good emulsifying agent even at low concentrations with many potential applications in the food and pharmaceutical industries[54]. However, the detailed pharmacological activities of various extracts of different plant parts of *C. gossypium* have not yet been reported.

Present Work

From the above review it is evident that virtually no chemical and pharmacological work has been done on *Cochlospermum gossypium,* although it is the most commercially and traditionally important Indian plat species. Hence, there exists excellent scope to screen *C. gossypium* for newer and biologically active secondary metabolites. With this background in view, systematic and extensive chemical, analytical and pharmacological studies have now been carried out on *C. gossypium* bark under the following four sections:

i) Section A : Isolation of a novel flavonol and major secondary metabolites from the bark of *Cochlospermum gossypium*

ii) Section B : Effect of solvent and extraction method on the accumulation of naringenin, the major metabolite of the bark of *C. gossypium* : HPLC based quantitative studies

iii) Section C : Chemical modification of naringenin, the major metabolite of *C. gossypium* bark : Synthesis of some diverse ether analogues

iv) Section D : α-Glucosidase inhibitory screening of extracts & isolated metabolites of the bark of *C. gossypium* and naringenin analogues: Identification of potent antidiabetic leads

The results obtained in each section are presented in the following pages.

From the above review of literature, it is evident that *Cochlospermum gossypium* (syn: *C. religiosum)* is the only *Cochlospermum* species grown in India. This species is now become endangered. Only the gum part of this plant is commercially exploited so far and detailed physico-chemical studies have been carried out on this part. However, its other plant parts like leaf, bark, wood and roots have not yet been chemically and biologically screened. Especially, the bark of *C. gossypium* from which the gum exudates is a highly potent source as it is extensively used in Unani medicine to treat gonorrhea syphilis, eye troubles, to soften skin and coughs[55]. With this objective in mind, the detailed chemical screening of the stem bark of *C. gossypium* has now been taken up. It is expected that the stem bark of the *C. gossypium* elaborate new chemical structures with interesting biological activities. The details of chemical examination of *C. gossypium* bark are presented below.

Source Collection

The bark material (5 kg) of *C. gossypium* was collected from Jaipur forest area, Mancherial District, Telangana (Figure 2A.01) in the month of November 2015. The authenticity of the plant material was confirmed by taxonomists of Botany Department, Osmania University, Hyderabad and Trans Disciplinary University (TDU) Bangalore. The voucher Specimen was deposited in the Natural Products Chemistry division, CSIR-IICT, Hyderabad.

Figure 2A.01 Area of collection of *C. gossypium*

Processing

The plant material was shade dried for one week and the dried plant material was subjected to cutting and powdering using a pulveriser (Figure 2A.02).

Figure 2A.02 Processing of *C. gossypium* bark

Extraction

The powdered *C. gossypium* bark material (3 kg) was extracted successively with different polar solvents such as n-hexane, ethyl acetate ad methanol under hot conditions by using soxhlet extractor (Figure 2A.03; Chart 2A.01).

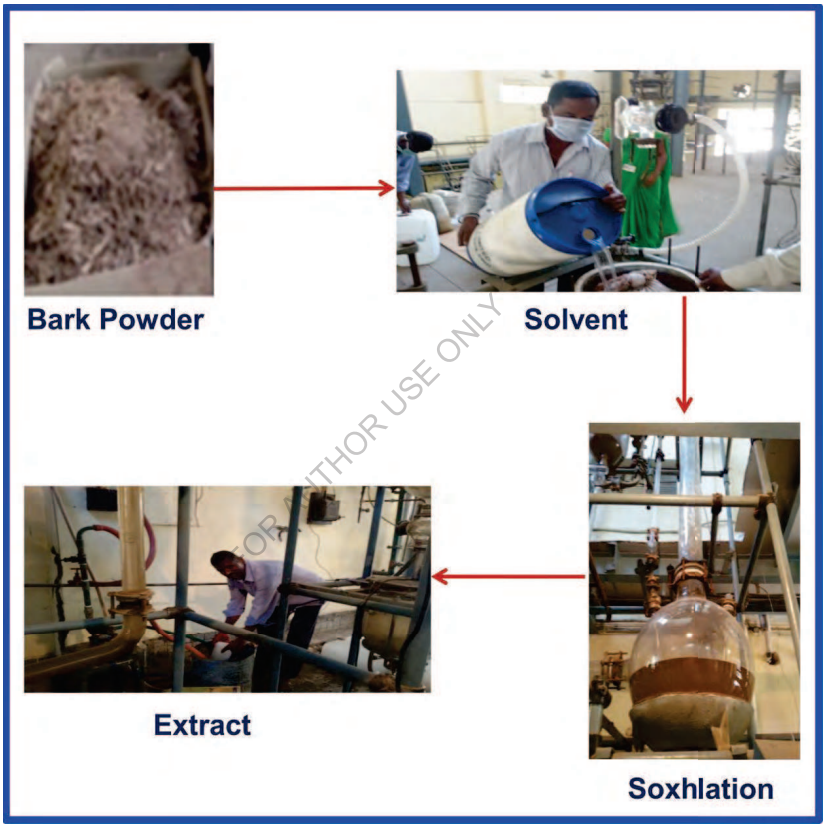


Figure 2A.03 Various steps involved in the extraction of *C. gossypium* bark

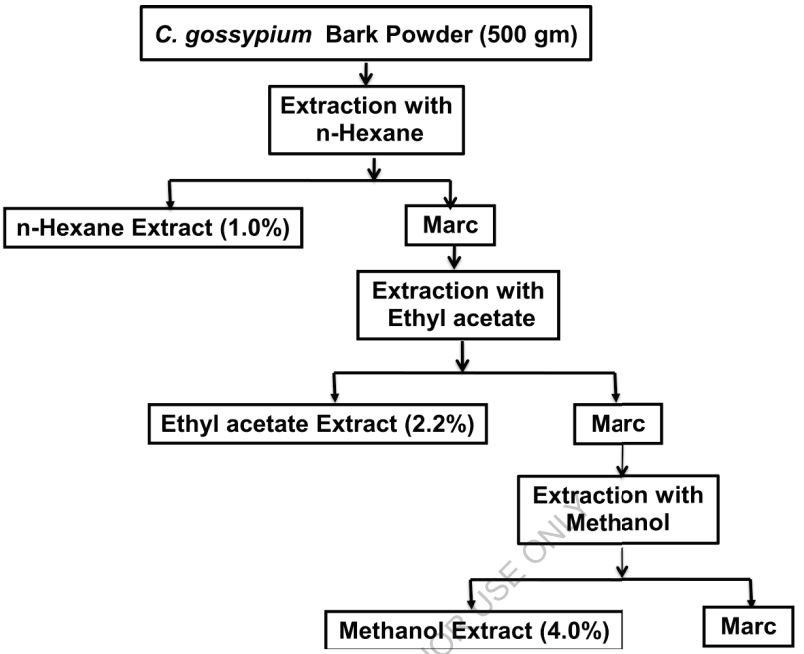


Chart 2A.01 Protocols employed for extraction of *C. gossypium* bark

The three soluble portions on evaporation under reduced pressure yielded the respective extracts as shown in the Chart 2A.01. Among the three extracts, the methanol extract was obtained in high yield. In order to identify major classes of secondary metabolites in these three extracts, they were subjected to preliminary phytochemical screening.

Preliminary phytochemical screening of *C. gossypium* extracts:

Formation of intense colours by a particular class of compounds with some reagents is specific and vital in natural products chemistry. The n-hexane, ethyl acetate and methanol extracts of *C. gossypium* were treated with four commonly used reagents to identify phenols ($FeCl_3$ test), terpenoids/steroids (LB test), alkaloids (Mayer's test),

glycosides (Molisch test) and falvonoids (Shinoda's test) [56 57, and 58]. The results are presented in Table 2A.01

Table 2A.01 Phytochemical Constituents of *C. gossypium* Extracts

S. No.	Extract	Test					Inference
		$FeCl_3$	LB	Shinoda	Molisch	Mayer's	
1.	n-Hexane	+	+	+	-	-	Phenols/Tannins, Terpenoids/Steroids, Flavonoids
2.	Ethyl acetate	+	+	+	+	-	Phenols/Tannins, Terpenoids/Steroids, Flavonoids, Sugars/Glycosides
3.	Methanol	+	+	+	+	-	Phenols/Tannins, Terpenoids/Steroids, Flavonoids, Sugars/Glycosides

From the above results it is evident that the n-hexane extract contains phenols or tannins, terpenoids or steroids and flavonoids. Whereas, the ethyl acetate and methanol extracts showed the presence of sugars or glycosides in addition to the classes of compounds present in n-hexane extract. Three polar extracts have been subjected to detailed TLC studies. The n-hexane and ethyl acetate extracts showed well resolved spots on TLC plate, whereas the methanol extract is unresolved and did not show any spots. Hence, the n-hexane and ethyl acetate extracts were independently subjected to column chromatographic separations.

Chromatographic Separation of n-Hexane Extract

The brown coloured n-hexane extract, showed five major spots on TLC plate (n-hexane-ethyl acetate, 80:20) along with some minor spots. The extract (100 gm) was chromatographed over a column of silica gel and eluted with solvent gradient from 100% n-hexane to 100% ethyl acetate. Several fractions of 100ml capacity were collected and the fractions with similar TLC nature were combined and evaporated to yield five major groups.

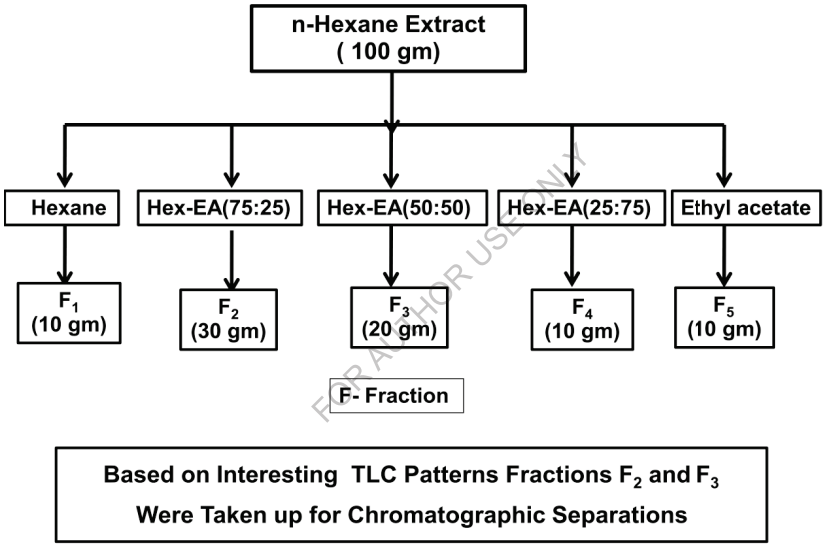


Chart 2A.02 Fractionation of n-Hexane extract of *C. gossypium* bark

As fractions F2 and F3 showed interesting TLC patterns, they have been subjected to independent column chromatographic purifications to isolate single and pure compounds.

Chromatographic purification of F_2 Fraction of n-Hexane Extract

The fraction F_2 (5 gm) was chromatographed over a column of Si gel and eluted with solvents of increasing polarity from n-hexane to ethyl acetate yielded a residue (3.8 gm), which on repetitive chromatographic purification followed by recrystallisation afforded the single and pure compound CGBH 1 (**1**) in 0.1% (Chart 2A.03).

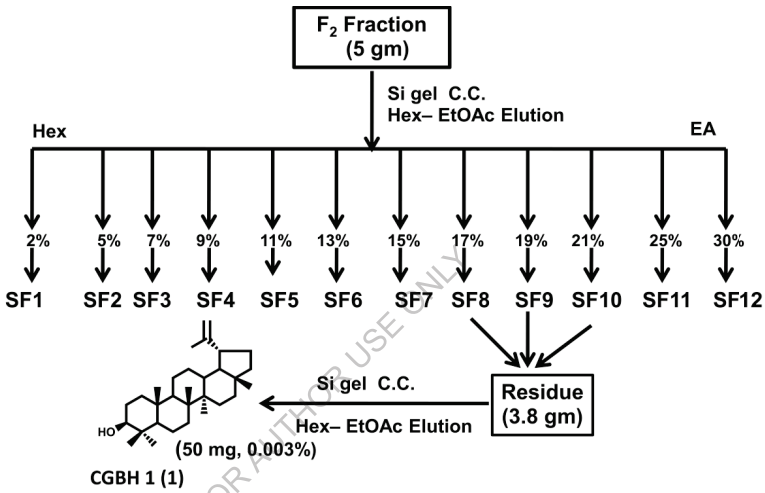


Chart 2A.03 Chromatographic Purification of F_2 Fraction of n-Hexane Extract

Identification of CGBH 1 (1) : 3β-hydroxylup-20(29)-ene (Lupeol)

It was obtained as colorless flakes from methanol, 50 mg (0.003%), m.p. 206-210^0 C, $[\alpha]_D$: - 26.0^0 (c 0.8, $CHCl_3$). It showed homogeneity on TLC plate (R_f : 0.7, n-hexane-ethyl acetate, 85 : 15). It gave positive Libermann - Burchard test for triterpenes. The IR spectrum (Fig. 2A.05a) showed a broad band at 3380 (hydroxyl), 1640 (olefinic), 1456, 1382, 1185, 1106, 1038 and 881 cm^{-1}. The 400 MHz ^{1}H NMR spectrum (Fig. 2A.04a) of the compound showed seven sharp singlets between δ 1.70-0.79 corresponding to seven methyls. The multiplet appeared at δ 3.20 and a pair of

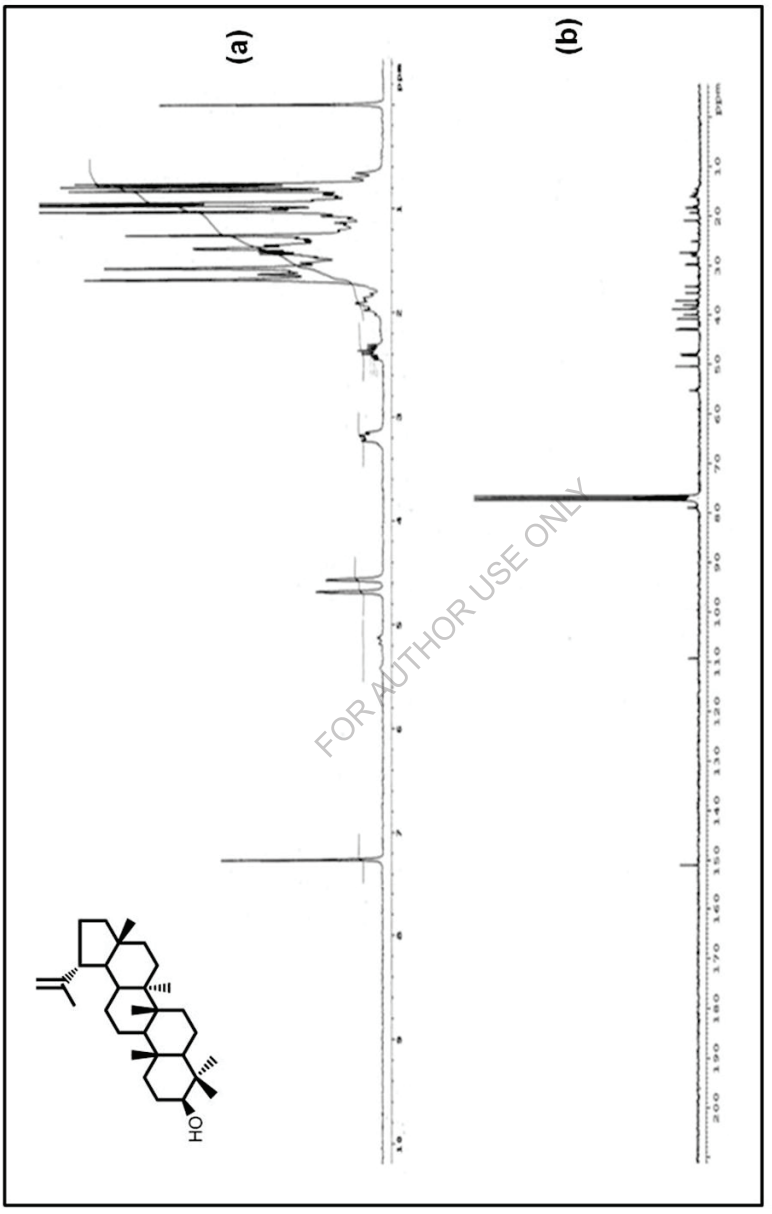


Figure 2A.04 : NMR Spectra of Compound 1 in $CDCl_3$ (a) ^1H (400 MHz); (b) ^{13}C (75 MHz)

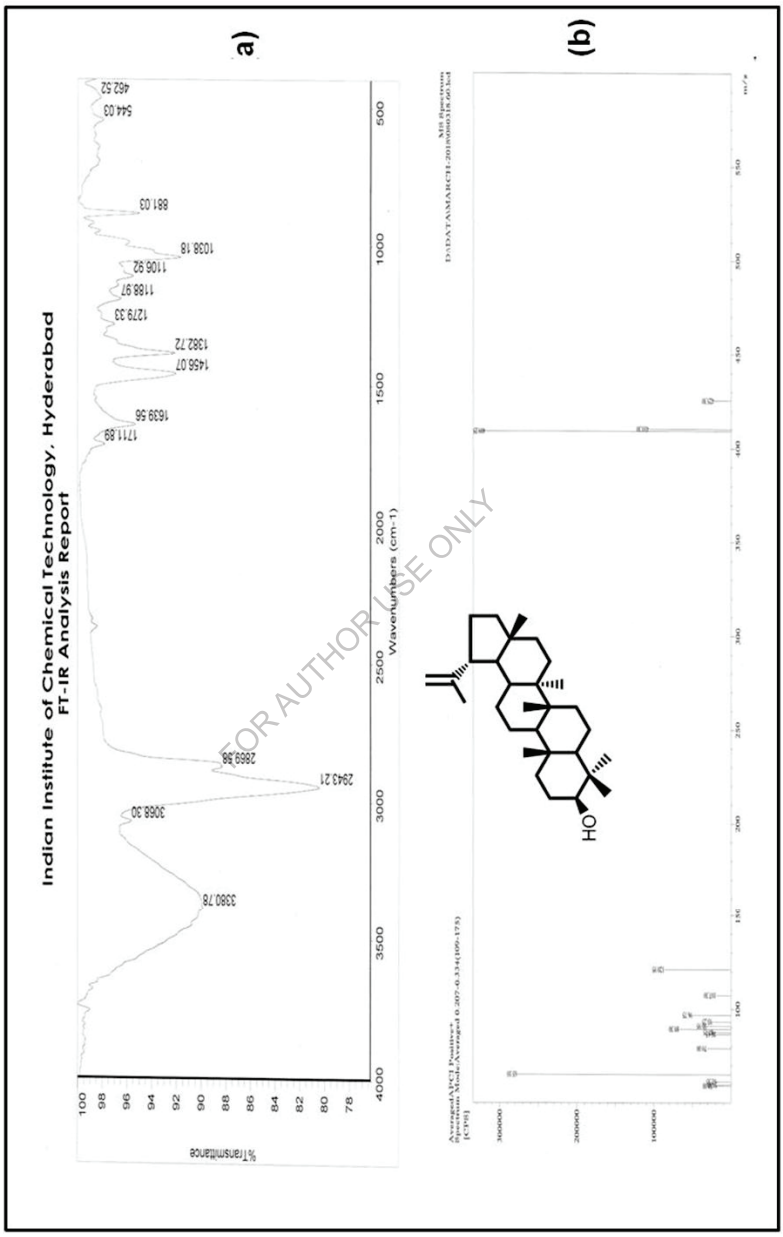
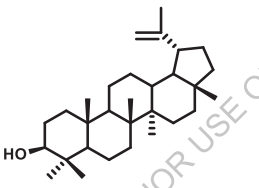


Figure 2A.05 : Spectra of Compound 1 (a) IR; (b) APCI Mass

doublets at δ 4.70 & 4.58 correspond to the 3α-H and C-29 methylene protons respectively of the lupane system[59]. Its ^{13}C NMR spectrum (Fig. 2A.5b), while confirming the lupane skeleton to the compound exhibited the characteristic C_{20} and C_{29} carbon signals at δ 150.8 and 109.6. It also showed the hydroxyl attached C_3 carbon at δ 79.0. The APCI mass spectrum (Fig. 2A.05b) of the compound showed the pseudo molecular ion (M^+-H) at m/z 425.30 corresponds to the molecular formula $C_{30}H_{49}O$. From the above spectral analysis and comparison of its physical and spectroscopic data with the reported values,, **CGBH 1 (1)** was identified as 3β-hydroxylup-20(29)-ene (lupeol)[60].



3β-Hydroxylup-20(29)-ene (1)

Chromatographic purification of F_3 Fraction of n-Hexane Extract

The fraction F_3 (5 gm) was chromatographed over a column of Si gel and eluted with solvents of increasing polarity from n-hexane to ethyl acetate yielded a residue (2.7 gm), which on repetitive chromatographic purification followed by recrystallisation afforded the single and pure compound CGBH 2 (**2**) in 0.2% (Chart 2A.04).

Identification of CGBH 2 (2) : 3β,28-Dihydroxylup-20(29)-ene (Betulin)

This compound was obtained as colourless needles, 100 mg (0.004%) from chloroform-methanol, m.p. 256-257^0 C, R_f : 0.4 (n-hexane-ethyl acetate, 80 : 20). it gave positive LB test for triterpenes. Its APCI mass spectrum (Fig. 2A.07b) showed the pseudo molecular ion (M^++H) at m/z 443.60. Its IR spectrum (Fig. 2A.07a)

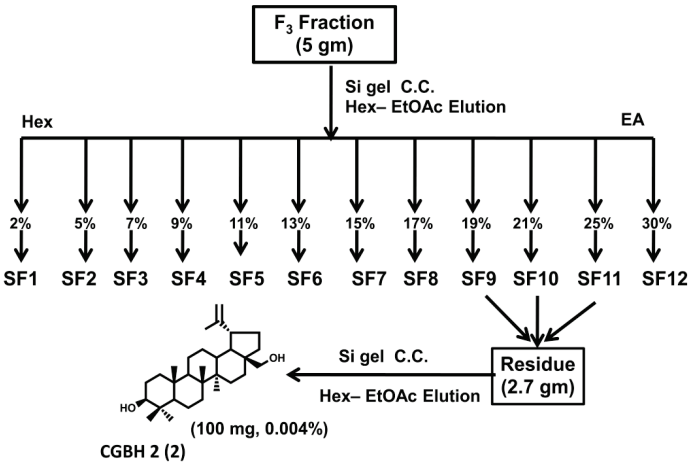
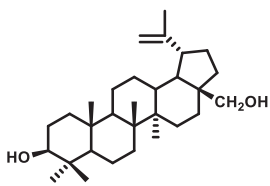


Chart 2A.04 Chromatographic Purification of F_3 Fraction of n-Hexane Extract

revealed the presence of hydroxyl (3386 cm^{-1}) and olefinic (1643 cm^{-1}) functionalities in the compound. The ^1H NMR spectrum (Fig. 2A.06a) found similarities with compounds CGBH 1 in the methyl, secondary hydroxyl and olefinic range, but differs significantly. A pair of doublets corresponding to hydroxymethyl protons appeared at δ 3.8 and 3.3 indicating that the compound contains an additional hydroxyl group. This observation is confirmed by its ^{13}C NMR spectrum (Fig. 2A.06b), which showed two oxygen attached carbon signals at δ 78.97 and 60.53. Based on the above physical and spectral data and comparison with reported values, this compound was identified as 3β,28-dihydroxylup-20(29)-ene (betulin) [61].



3β,28-Dihydroxylup-20(29)-ene (Betulin)

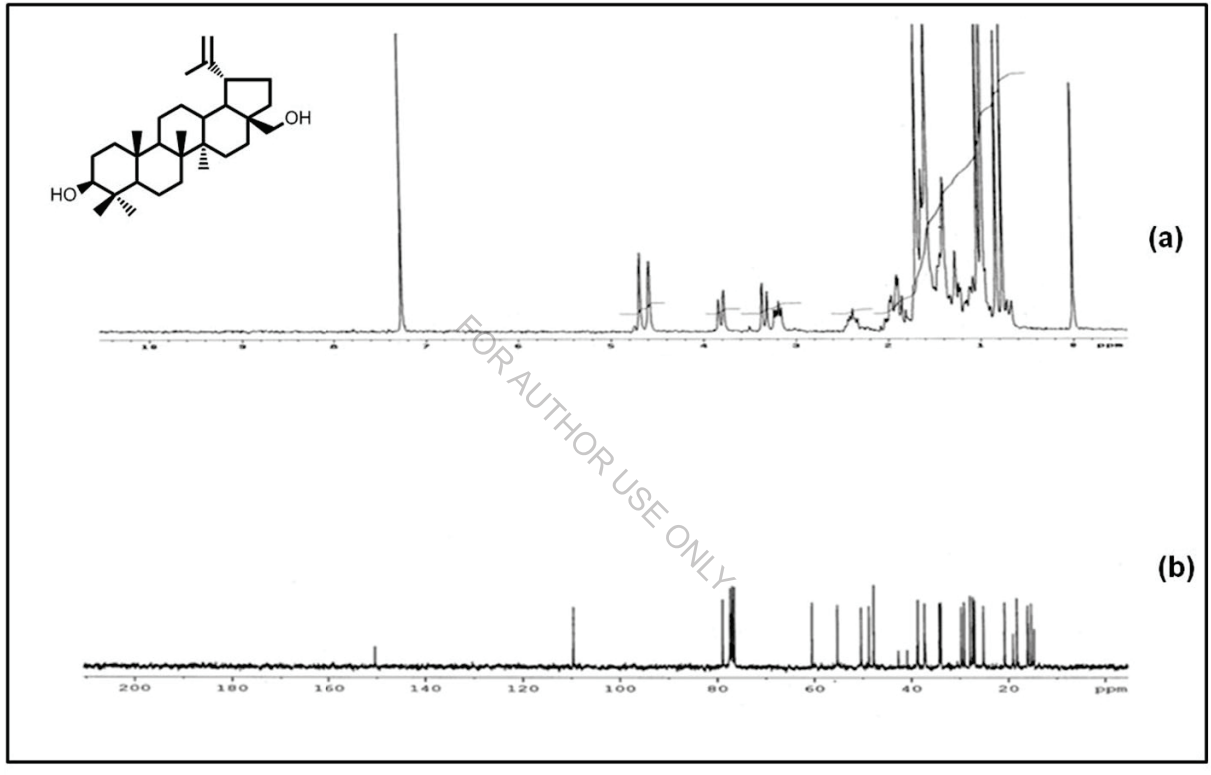


Figure 2A.06 : NMR Spectra of Compound 2 in $CDCl_3$ (a) ^1H (400 MHz); (b) ^{13}C (75 MHz)

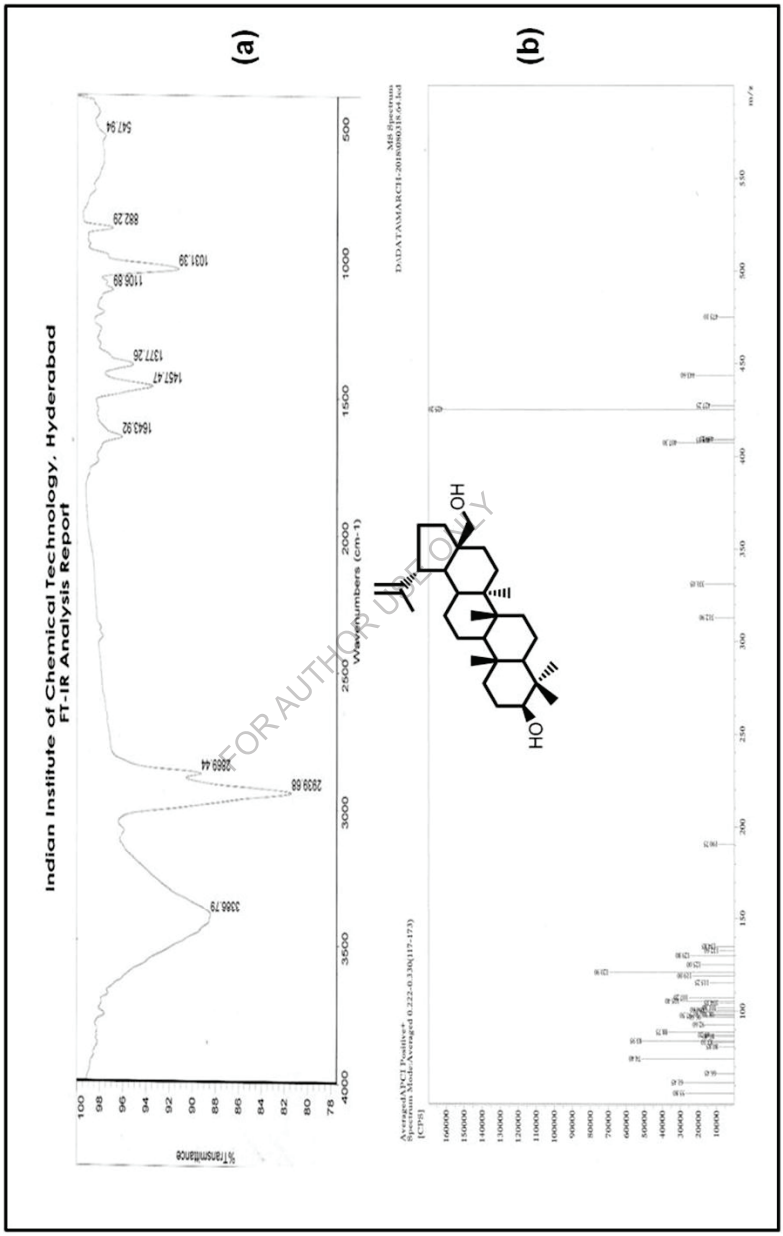


Figure 2A.07: Spectra of Compound 2 (a) IR; (b) APCI Mass

Chromatographic Separation of Ethyl acetate Extract

The reddish brown coloured ethyl acetate extract, showed some prominent spots along with few minor spots on TLC plate (n-hexane-ethyl acetate, 70:30). The extract (27.74 gm) was chromatographed over a column of silica gel and eluted with solvent gradient from 100% n-hexane to 100% ethyl acetate. Several fractions of 100ml capacity were collected and the fractions with similar TLC nature were combined and evaporated to yield seven major groups (Chart 2A.05).

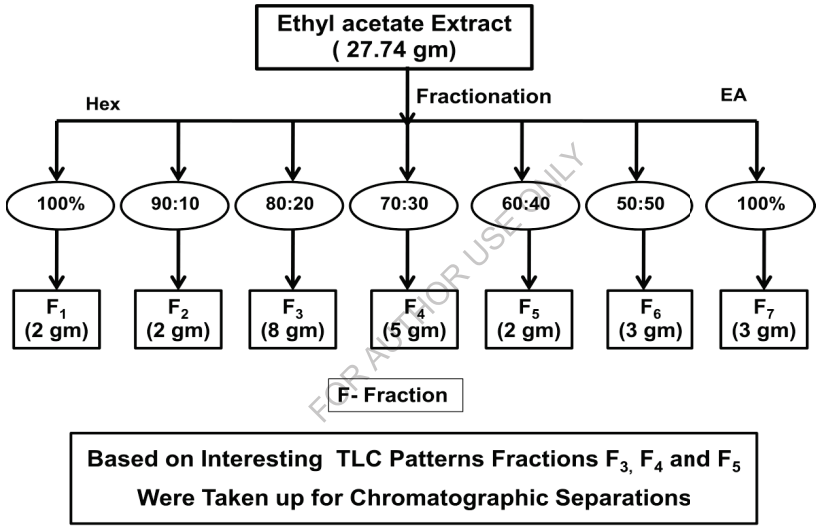


Chart 2A.05 Fractionation of Ethyl acetate extract of *C. gossypium* bark

Based on interesting TLC patterns fractions F3, F4 and F5 were taken up for further chromatographic purification to isolate single and pure compounds.

Chromatographic purification of F_3 Fraction of Ethyl acetate Extract

The fraction F_3 (6 gm) was chromatographed over a column of Si gel and eluted with solvents of increasing polarity from n-hexane to ethyl acetate yielded a residue

(4.0 gm), which on repetitive chromatographic purification followed by recrystallisation afforded the single and pure compound CGBE 1 (**3**) in 0.003% (Chart 2A.06).

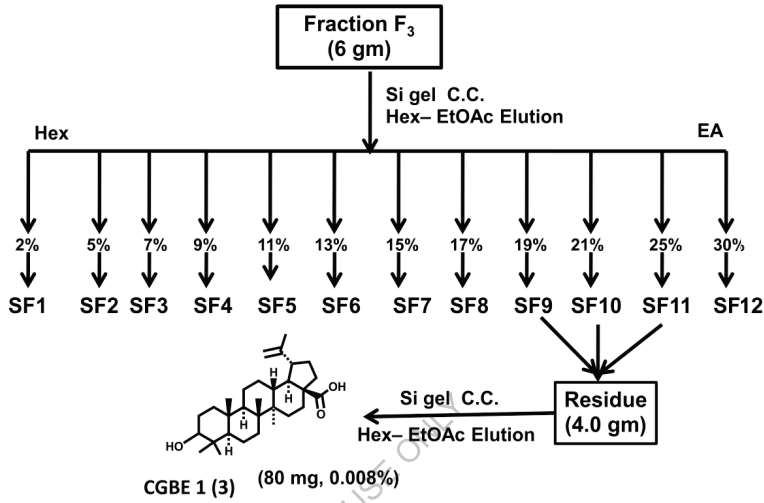


Chart 2A.06 Chromatographic Purification of Fraction F_3 of Ethyl acetate Extract

Identification of CGBE 1(3) : 3β-Hydroxylup-20(29)-en-28-oic acid (Betulinic acid)

It was obtained as colourless powder from chloroform, 80 mg (0.008%), R_f : 0.6 (n-hexane: ethyl acetate, 70: 30), m.p. 316-318^0 C. It gave positive LB test for triterpenes. Its IR spectrum (Fig. 2A.09a) showed strong absorption bands for hydroxyl (3451 cm^{-1}), carboxylic acid (1688 cm^{-1}) and double bond (1642 cm^{-1}) functionalities. Its 300 MHz ^{1}H NMR (Fig.2A.08a) showed singlets at δ 4.75 and 4.60 corresponding to C_{20}-C_{29} end methylene and multiplet at 3.20 of C_3 hydroxyl attached α proton. The ^{13}C NMR spectrum (Fig.2A.08b) suggests that it is a lupane class of triterpenic acid by exhibiting carbon signals at δ 179.5 (-COOH), 150.5 & 109.6 (end methylene carbons) and 78.9 (hydroxyl attached carbon). The APCI mass spectrum (Fig. 2A.09b) showed the pseudo molecular ion (M^+-H) at m/z 455.30. The above

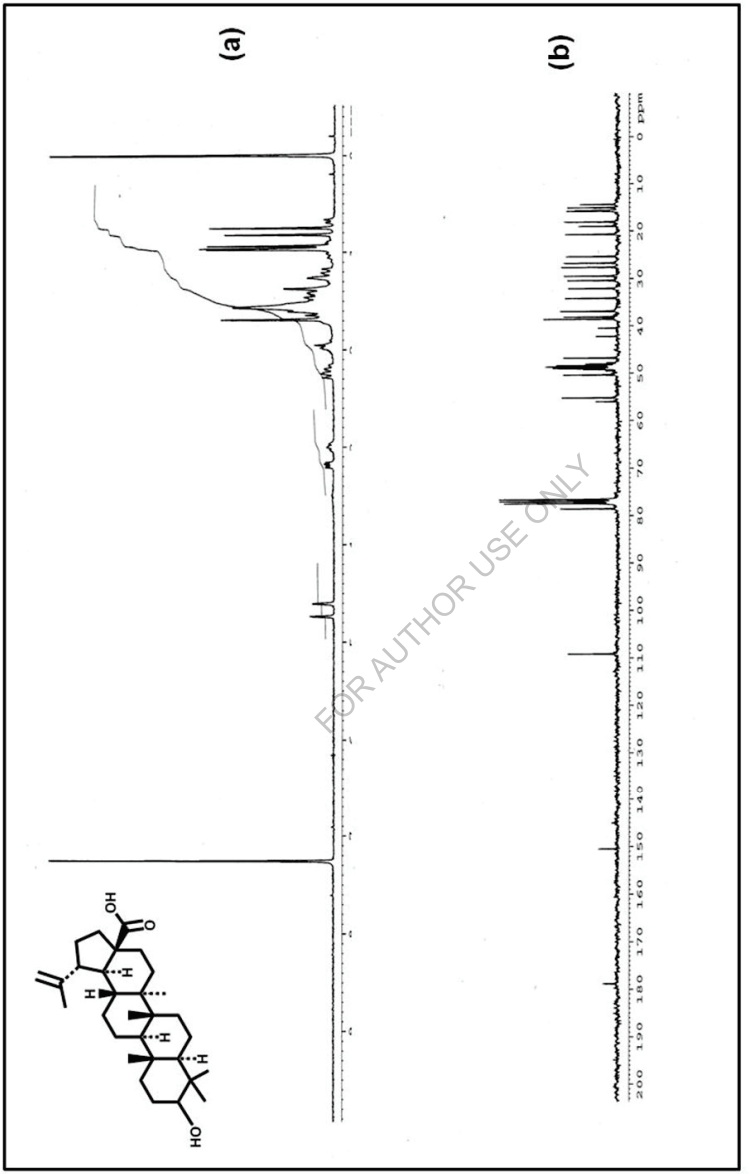


Figure 2A.08 : NMR Spectra of Compound **3** (a) ^{1}H (300 MHz) $CDCl_3$; (b) ^{13}C (75 Hz) DMSO+$CDCl_3$

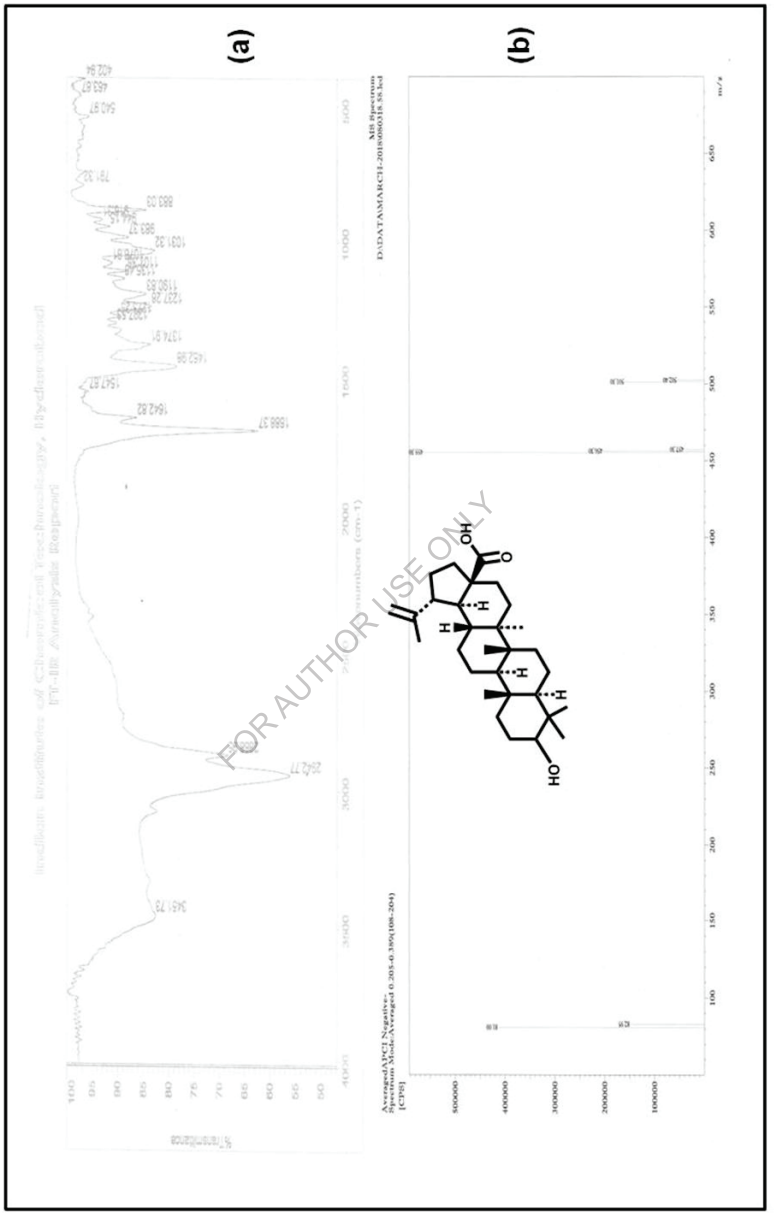
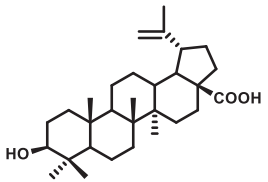


Figure 2A.09: Spectra of Compound 3 (a) IR; (b) APCI Mass

spectral data identifies **CGBE 1(3)** as 3β-hydroxylup-20(29)-en-28-oic acid (betulinic acid). This is further confirmed by comparison of its spectra with the reported values[45].



3β-Hydroxylup-20(29)-en-28-oic acid (3)

Chromatographic purification of F_4 Fraction of Ethyl acetate Extract

The fraction F_4 (4 gm) was chromatographed over a column of Si gel and eluted with solvents of increasing polarity from n-hexane to ethyl acetate yielded a residue (2.0 gm), which on repetitive chromatographic purification followed by recrystallisation afforded two single and pure compounds CGBE 2 (**4**) and CGBE 3 (**5**) in 0.06% and 0.02% respectively (Chart 2A.07).

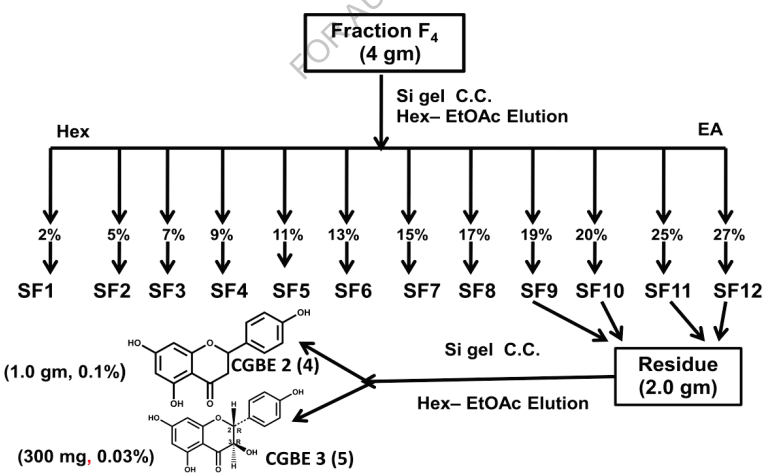


Chart 2A.07 Chromatographic Purification of Fraction F_4 of Ethyl acetate Extract

Identification of CGBE 2 (4) : 2,3-dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one (Naringenin)

It was obtained as colourless flakes, 1.0 gm (0.1%), R_f : 0.3 (n-hexane: ethyl acetate, 70: 30), m.p. 251^0 C. It gave positive tests with ferric chloride (phenols) and Shinoda's reagent (flavonoids). Its IR spectrum (Figure 2A.11) showed strong absorption bands at 3119 and 1633 cm^{-1} corresponding to hydroxyl and carbonyl functionalities. Its EI mass spectrum (Figure 2A.13a) showed the molecular peak at 271 [M^+-H] and EIHRMS spectrum (Figure 2A.12) showed the molecular ion at m/z 273.07713 corresponding to the molecular formula C_{15} H_{13} O_5.. The 400 MHz ^{1}H NMR Spectrum (Figure 2A.10a) revealed that it is a flavanone by exhibiting characteristic C_2 and C_3 protons at δ 5.44 (1H, dd) and 2.67 & 3.25 (each 1H, dds) in addition to two phenolic hydroxyl peaks at δ 12.14 (chelated) & 10.82, a pair of doublets at δ 7.35 & 6.80 corresponding to a para-disubtituted system and a doublet δ 5.90 corresponding to a meta coupled aromatic protons. The ^{13}C NMR (100 MHz) spectrum (Figure 2A.12b) of the compound while confirming the flavanone skeleton, exhibited the carbonyl carbon signal at δ 195.85 and four oxygenated aromatic carbons at δ 166.21,163.06, 162.0 & 157.0 and one oxygenated carbon at δ 77.98, The DEPT 135 spectrum (Figure 2A.13) of the compound reveals that there are seven quaternary, one methylene and seven methane carbons present in the compound. Based on the above physical and spectroscopic data and comparison with reported values, the compound was identified as 2,3-dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one (naringenin). The present isolation

Figure 2A.10 : NMR Spectra of Compound 4 in DMSO (a) ^{1}H (400 MHz); (b) ^{13}C (100 MHz)

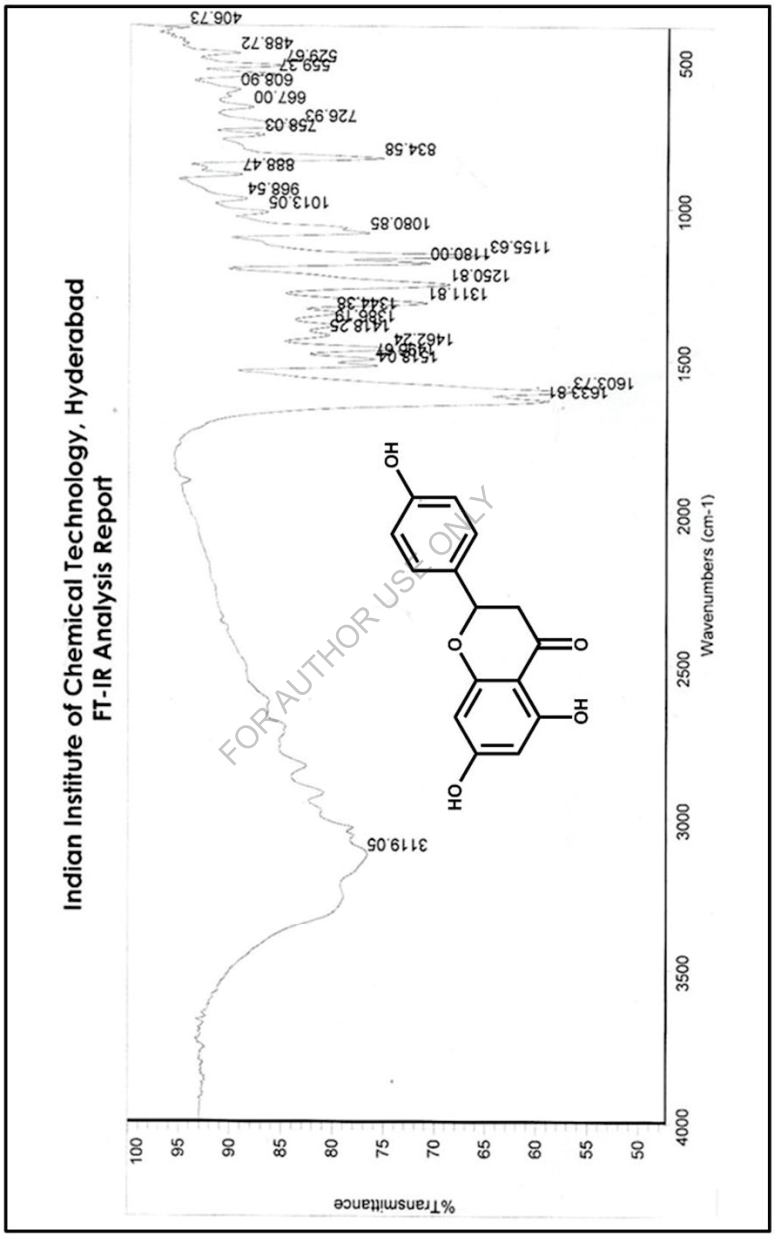


Figure 2A.11 : IR Spectrum of Compound 4

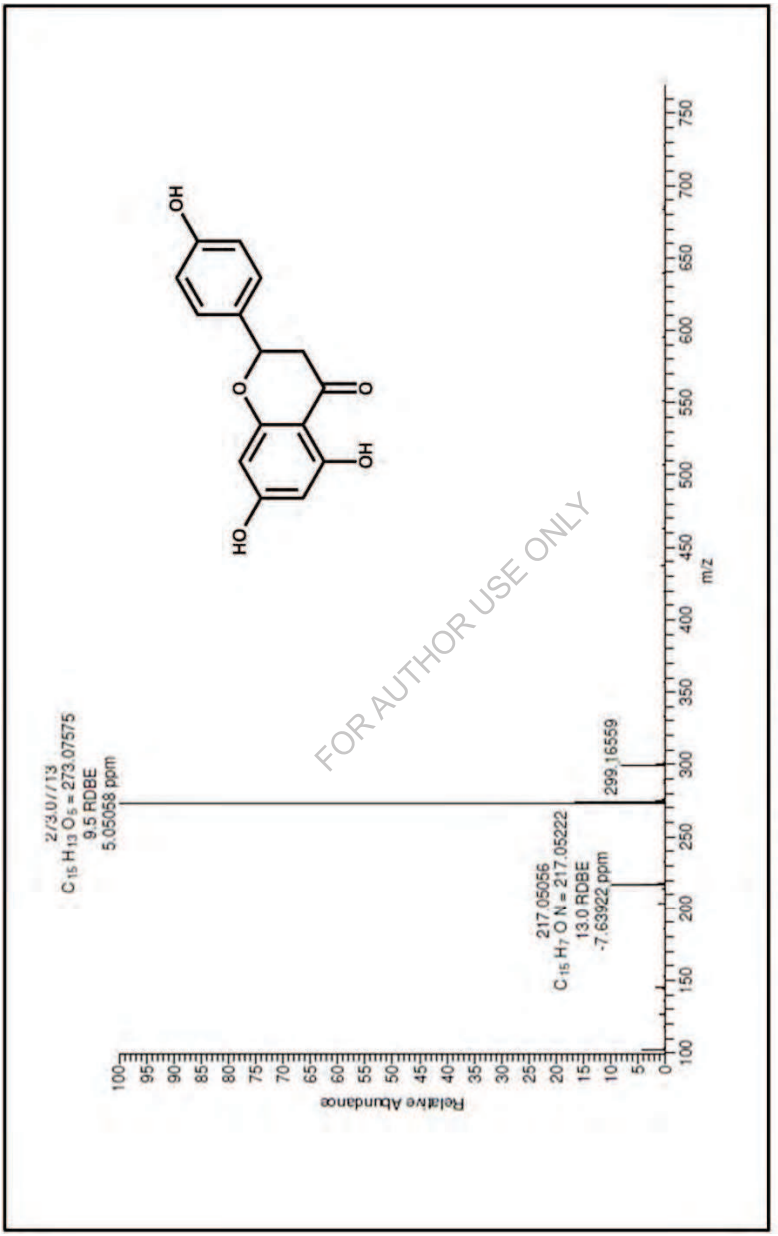


Figure 2A.12 : HRMS Spectrum of Compound 4

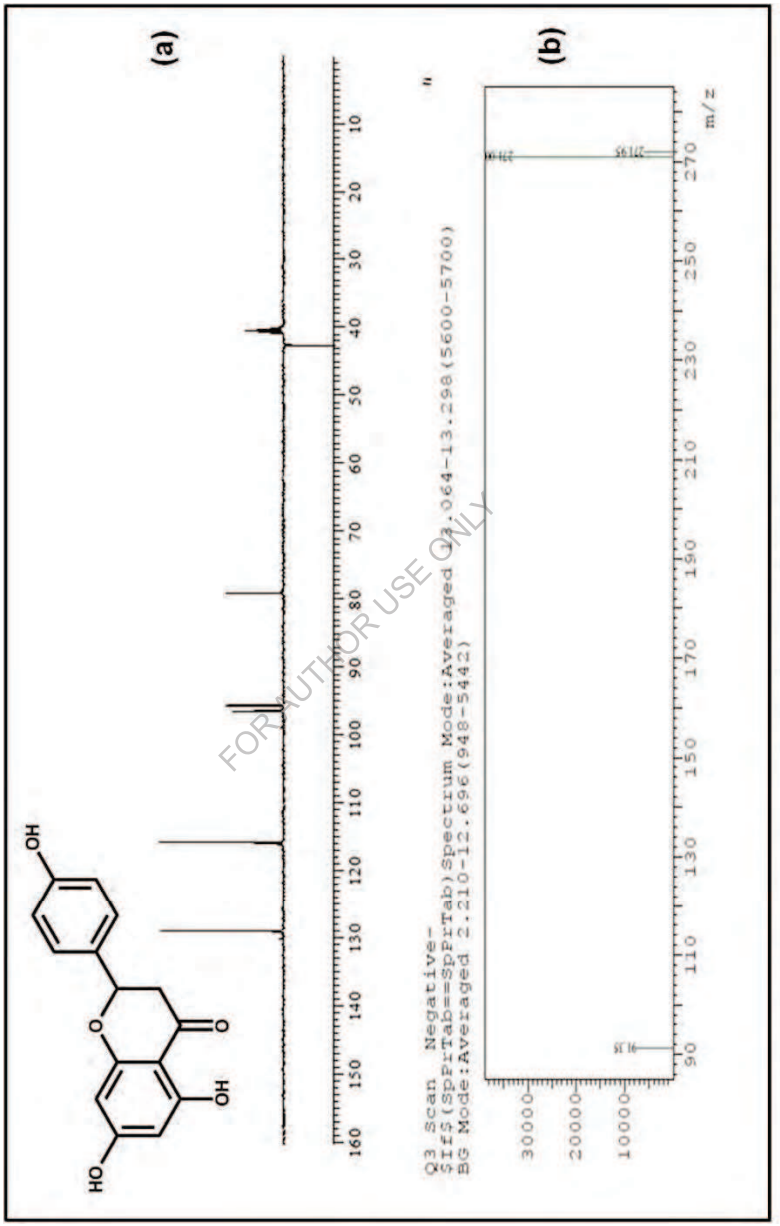
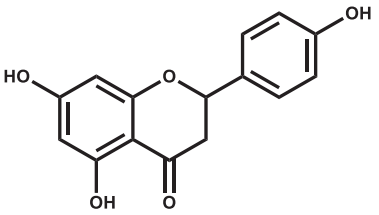


Figure 2A.13 : Spectra of Compound 4 (a) DEPT 135; (b) Mass

of naringenin assumes significance as it is now isolated from a non-citrus plant and more significantly it is isolated in large quantity.



2,3-dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one (Naringenin, 4)

Identification of CGBE3 (5) : (2R,3R)-2,3-dihydro-3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one (Dihydrokaempferol)

It was obtained as colourless crystals, 300 mg (0.03%), m.p. 202-205^0 C, R_f : 0.55 (n-hexane: ethyl acetate, 60: 40) $[\alpha]_D$ -11.43^0 (methanol). it gave positive tests with ferric chloride (phenols) and Shinoda's reagent (flavonoids).. Its EIHRMS spectrum (Figure 2A.16b) showed the pseudo molecular ion $[M^+ + H]$ at m/z 289.07184 corresponding to the molecular formula $C_{15}H_{13}O_6$. The IR spectrum (Figure 2A.15) of the compound showed the characteristic carbonyl and hydroxyl absorption bands at 1642 and 3541 cm^{-1} respectively. The 400 MHz ^{1}H NMR Spectrum (Figure 2A.14a; Table 2A.02) revealed that it is a 3-hydroxy-flavanone by exhibiting characteristic C_2 and C_3 protons at δ 5.16 (d, J=11.2 Hz) and 4.49 (d, J=11.2 Hz) in addition to three phenolic hydroxyl peaks at δ 11.70 (chelated), 10.36 & 9.19, a pair of doublets at δ 7.33 (d, J=8.8 Hz) & 6.87 (d, J=8.8 Hz) corresponding to a para-disubtituted system and another pair of doublets at δ 5.98 (d, J=1.8 Hz) & 5.95 (d, J=1.8 Hz) corresponding to meta coupled aromatic protons. The ^{13}C NMR

Figure 2A.14 : NMR Spectra of Compound 5 in DMSO+$CDCl_3$ (a) ^1H (500 MHz); (b) ^{13}C (75 MHz)

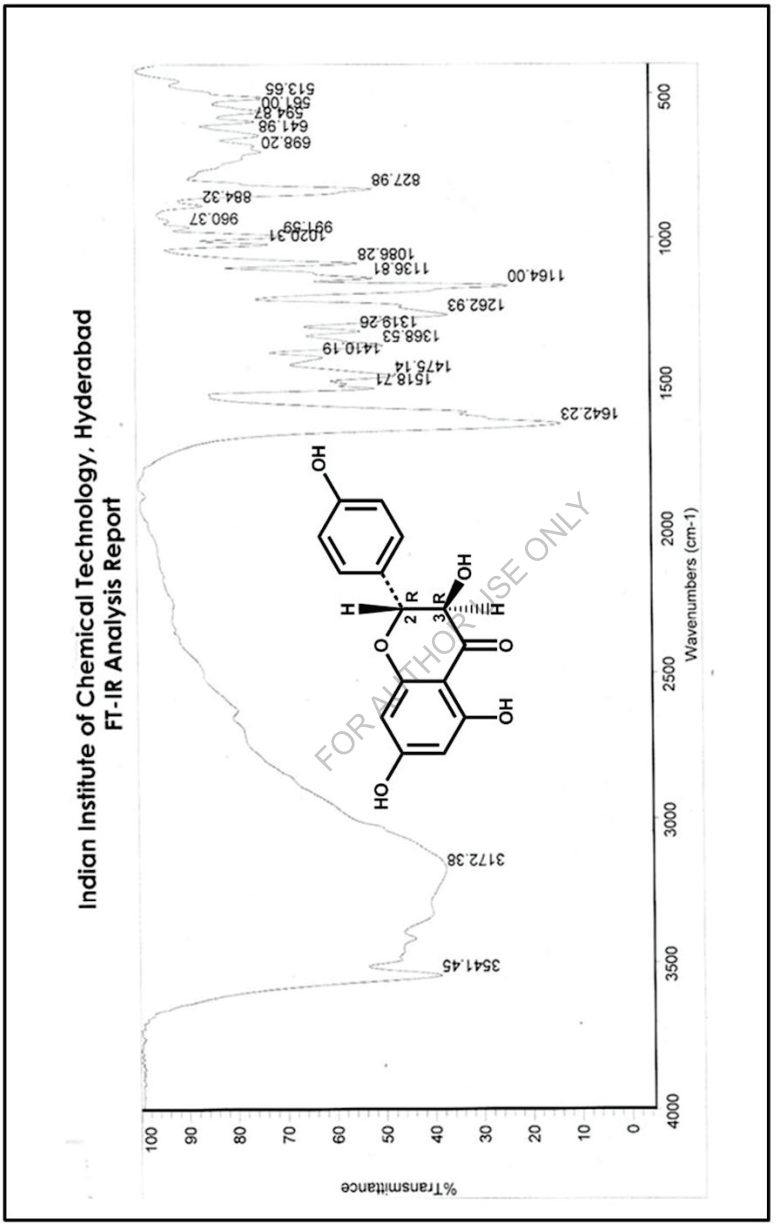


Figure 2A.15 : IR Spectrum of Compound 5

spectrum (Figure 2A.14b; Table 2A.02) while confirming the above observations, exhibiting the characteristic carbon signals of C_2 and C_3 at δ 82.9 and 71.9 respectively. It also showed the carbonyl carbon signal δ 196.9 and three hydroxyl attached aryl carbons at δ 167.05 (C_7), 163.4 (C_5) and 157.6 ($C_{4'}$). The DEPT spectrum (Figure 2A.16a) of the compound reveals that there are seven quaternary and eight methine carbons present in the compound. Based on the above physical and spectral analysis, the compound was identified as 3,4',5,7-tetrahydroxy-flavanone.

Table 2A.02. NMR Chemical Shift Values of Compound 5

Carbon No.	Chemical Shift Values (δ)	
	^{1}H NMR	^{13}C NMR
1	-	-
2	5.16 (d, J=11.2 Hz)	82.9
3	4.49 (d, J=11.2 Hz)	71.9
4	-	196.9
5 OH	11.70 (s)	163.4
10	-	100.2
6	5.98 (d, J=1.8 Hz)	96.4
7 (-OH)	10.36 (s)	167.05
8	5.95 (d, J=1.8 Hz)	95.3
9	-	162.6
1'	-	127.2
2'	7.33 (d, J=8.8 Hz)	128.7
3'	6.87 (d, J=8.8 Hz)	115.14
4' (-OH)	9.19 (s)	157.6
5'	6.87 (d, J=8.8 Hz)	115.14
6'	7.33 (d, J=8.8 Hz)	128.7

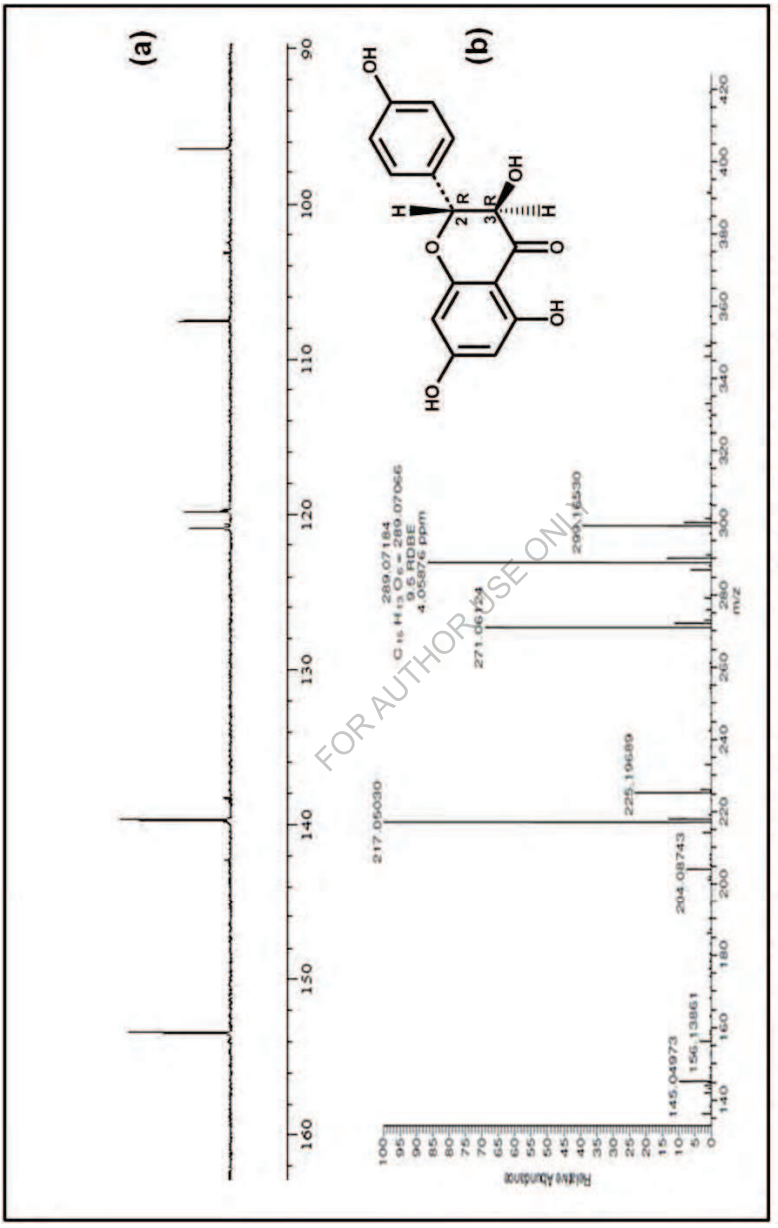


Figure 2A.16 : Spectra of Compound 5 (a) DEPT 135; (b) HRMS

The identity of compound **5** was further confirmed by its NOESY (Figure 2A.17), HSQC (Figure 2A.18) and HMBC (Figure 2a.19) 2D NMR correlation spectra. The NOESY spectrum showed the interactions between H-2 with H-3, H-6 with H-8 and H-2' with H-3'. The HMBC spectrum clearly showed the H-2 and H-3 long range couplings with C_4 carbonyl carbon. In addition it showed the interactions of H-3 and H-3' with $C_{1'}$; H-6 with C_{10}; H-8 with C_6 (Figure 2A.20).

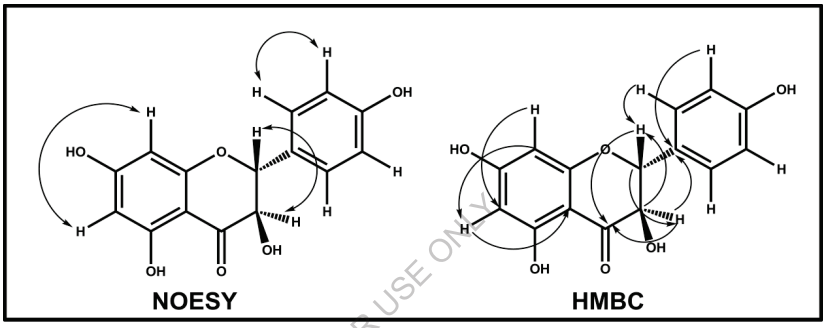


Figure 2A.20: NOESY and HMBC Correlations of Compound 5

The absolute configuration of C_2 and C_3 of compound **5** was established by single crystal X-ray crystallography (Figure 2A.21), which clearly shows that the compound with molecules of methanol trapped in the crystal lattices. These are typical "host - guest" type inclusion crystals[62]. Close observation of the crystal structure clearly shows that H-2 as β (▬) and H-3 as α (''''''). Based the above observations, the absolute configuration of compound 5 to be 2R,3R. Hence, compound **5** was characterised as (2R,3R)-2,3-dihydro-3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one. Literature search reveals that this compound was earlier isolated from few plant species as dihydrokaempferol[63] or aromadendrin[64,65,66].

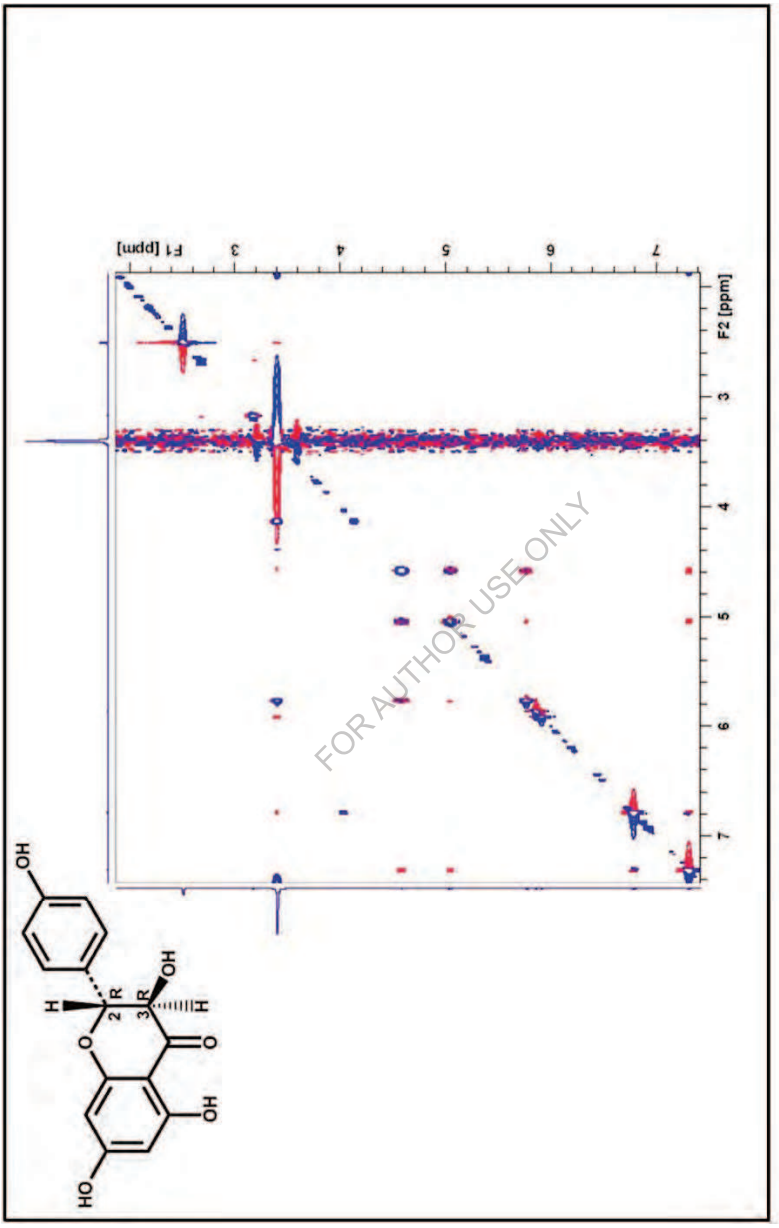


Figure 2A.17 :NOESY Spectrum of Compound 5

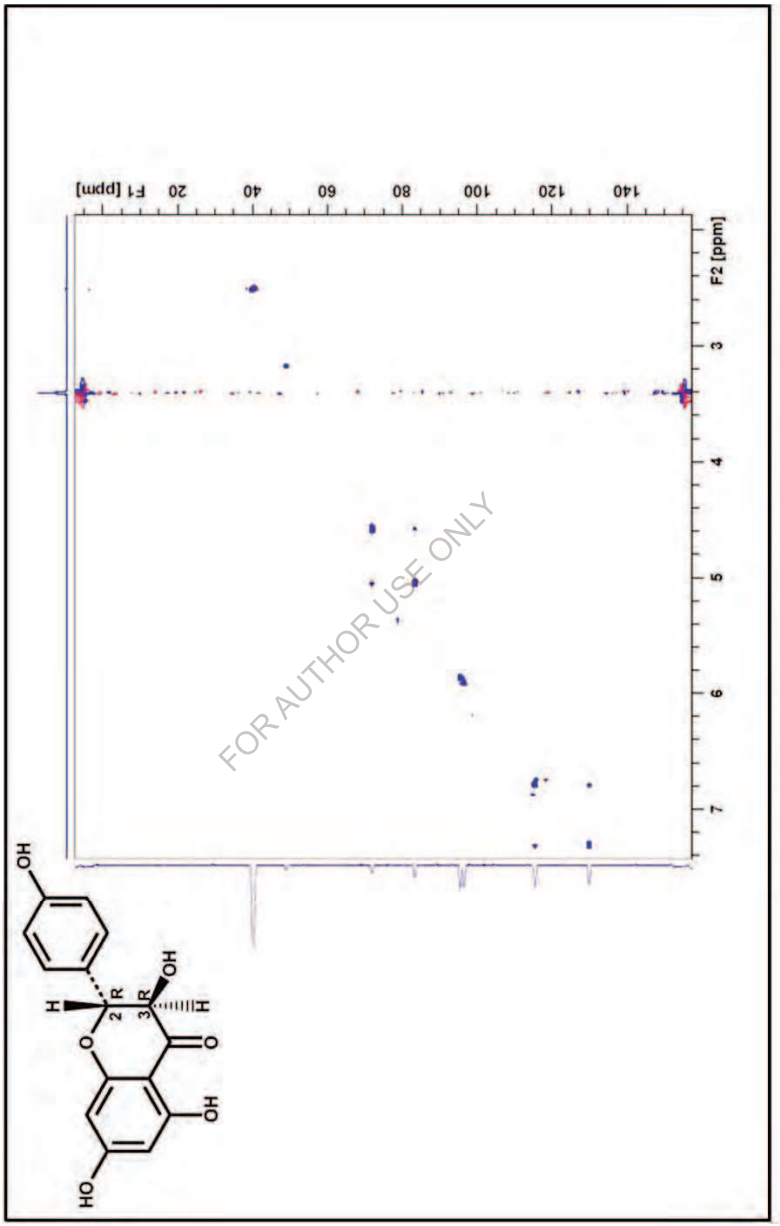


Figure 2A.18 : HSQC Spectrum of Compound 5

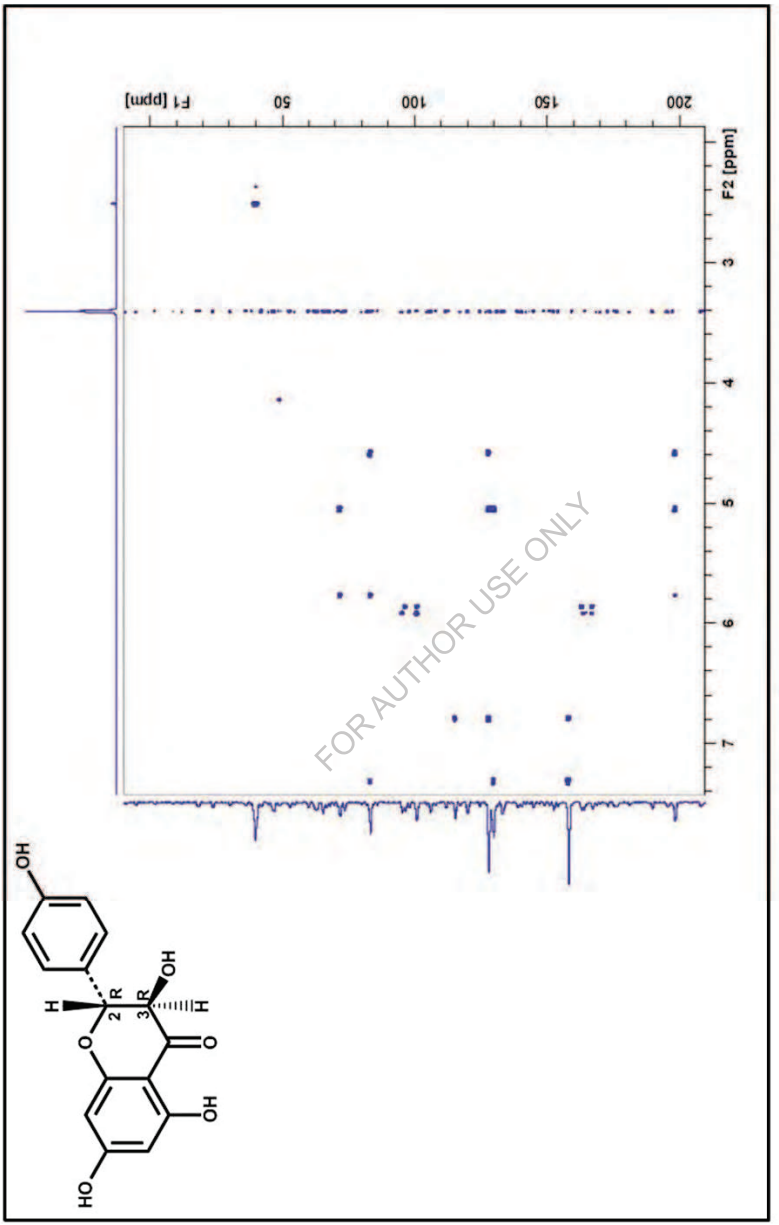


Figure 2A.19 : HMBC Spectrum of Compound 5

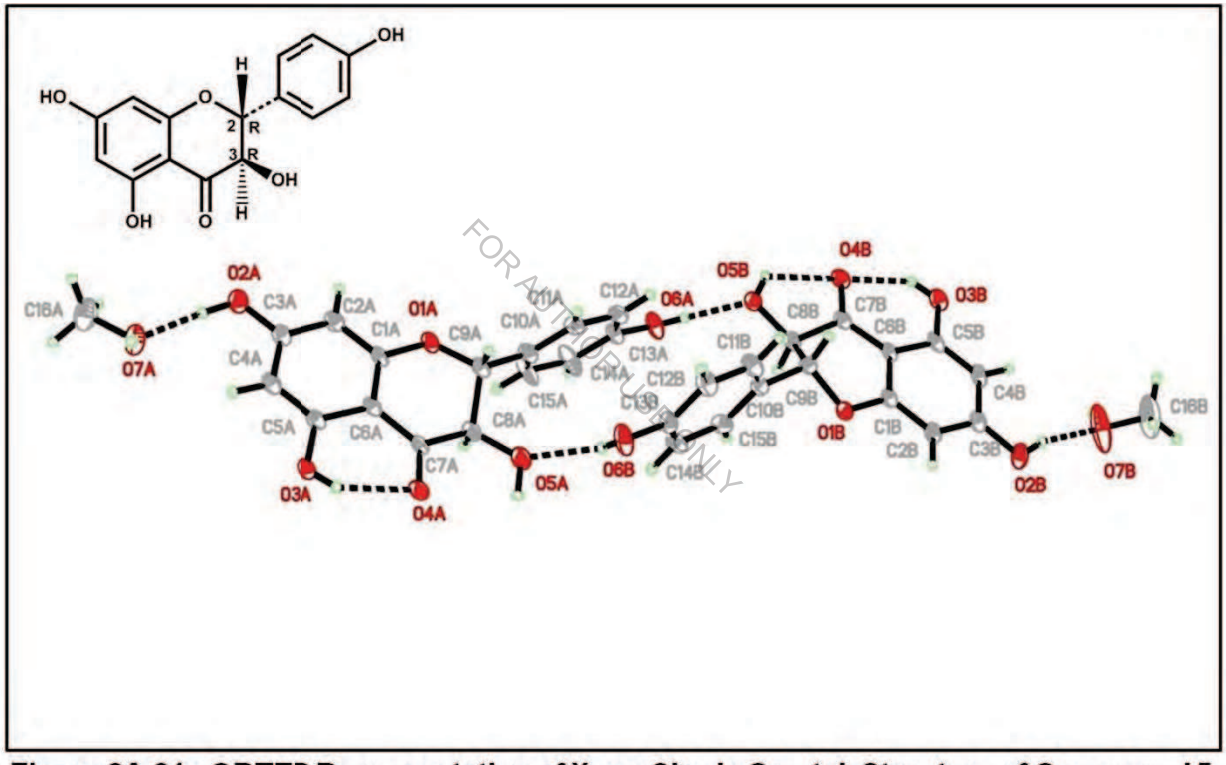
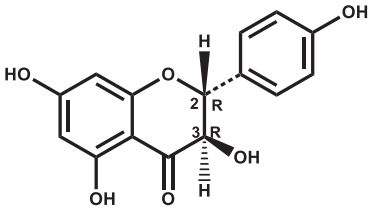


Figure 2A.21 : ORTEP Representation of X-ray Single Crystal Structure of Compound 5

(Numbering Shown on the Crystal Structure is as per the ORTEP Drawing)*



(2R,3R)-2,3-dihydro-3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one

(Dihydrokaempferol,5)

The present isolation of dihydrokaempferol assumes taxonomic significance as it is now isolated for the first time from a *Cochlospermum* species.

Chromatographic purification of F_5 Fraction of Ethyl acetate Extract

The fraction F_5 (1.5 gm) was chromatographed over a column of Si gel and eluted with solvents of increasing polarity from n-hexane to ethyl acetate yielded a residue (750 mg), which on repetitive chromatographic purification followed by recrystallisation afforded two single and pure compounds CGBE 4 (**6**) and CGBE 5 (**7**) in 0.04% and 0.004% respectively (Chart 2A.08).

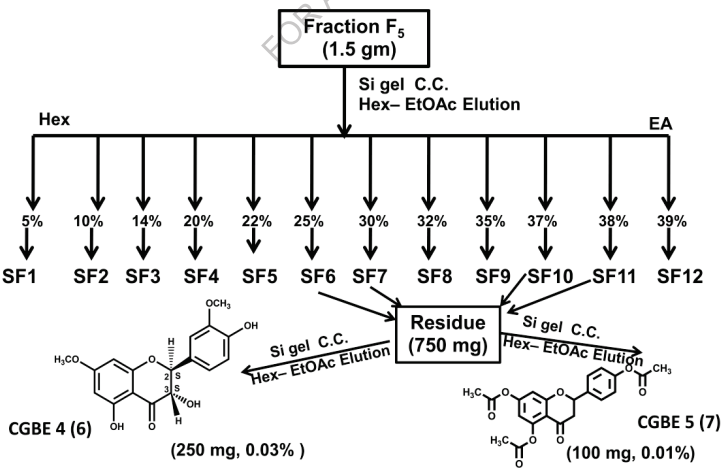


Chart 2A.08 Chromatographic Purification of Fraction F_5 of Ethyl acetate Extract

Identification of CGBE 4 (6) : New (2S,3S)-2,3-dihydro-3,5-dihydroxy-7-methoxy-2-(3-methoxy-4-hydroxyphenyl)-4H-1-benzopyran-4-one

It was obtained as colourless crystals from chloroform, 250 mg (0.03%), R_f : 0.5 (n-hexane: ethyl acetate, 60: 40), m.p. 195-196^0 C. $[\alpha]_D$: -1.42^0 (methanol). It gave positive tests with ferric chloride (phenols) and Shinoda's reagent (flavonoids). Its EIHRMS spectrum (Figure 2A.26) showed the pseudo molecular ion $[M^+ + H]$ at m/z 333.09688 corresponding to the molecular formula $C_{17}H_{17}O_7$. The IR spectrum (Figure 2A.24) of the compound showed the characteristic carbonyl and hydroxyl absorption bands at 1639 and 3429 cm^{-1} respectively. The 400 MHz ^{1}H NMR Spectrum (Figure 2A.22; Table 2A.03) revealed that it is a 3-hydroxy-flavanone by exhibiting characteristic C_2 and C_3 protons at δ 5.05 (d, J=11.2 Hz) and 4.55 (d, J=11.2 Hz) in addition to two phenolic hydroxyl peaks at δ 11.73 (chelated) & 8.60, a pair of doublets at δ 5.48 (d, J=1.8 Hz) & 6.05 (d, J=1.8 Hz) corresponding to two meta coupled aromatic protons and two sharp signals at δ 3.90 and 3.80 corresponding two methoxyls. It also showed the presence of a 1,3,4-trisubstituted aromatic system by exhibiting characteristic signals at δ 7.15 (d, J=1.8 Hz), 6.91 (d, J=8.8 Hz) and 7.03 (d, J=8.8 Hz). The ^{13}C NMR spectrum (Figure 2A.14b; Table 2A.23) while confirming the above observations exhibiting the characteristic carbon signals of C_2 and C_3 at δ 83.1 and 71.9 respectively. It also showed the carbonyl carbon signal δ 197.31, five oxygen attached aryl carbons at δ 167.52 (C_7), 163.70 (C_5), 162.90 (C_9), 147.09 ($C_{3'}$) & 146.65 ($C_{4'}$) and two methoxy carbon signals at δ 52.02 (C_7-OCH_3) & 51.98 ($C_{3'}$-OCH_3). The DEPT spectrum (Figure 2A.25) of the compound reveals that there are eight quaternary, seven methine and two methyl carbons present in the compound. Based on the above

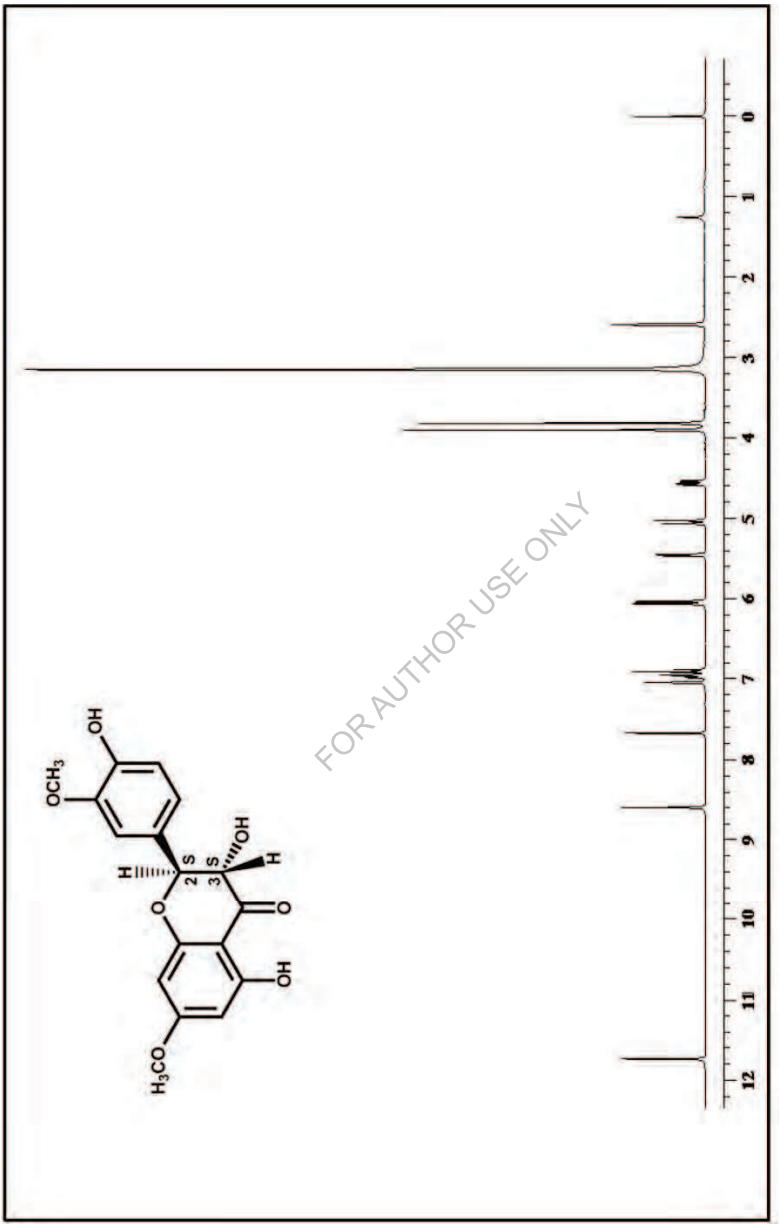


Figure 2A.22 : ^{1}H (300 MHz) NMR Spectrum of Compound 6 in DMSO

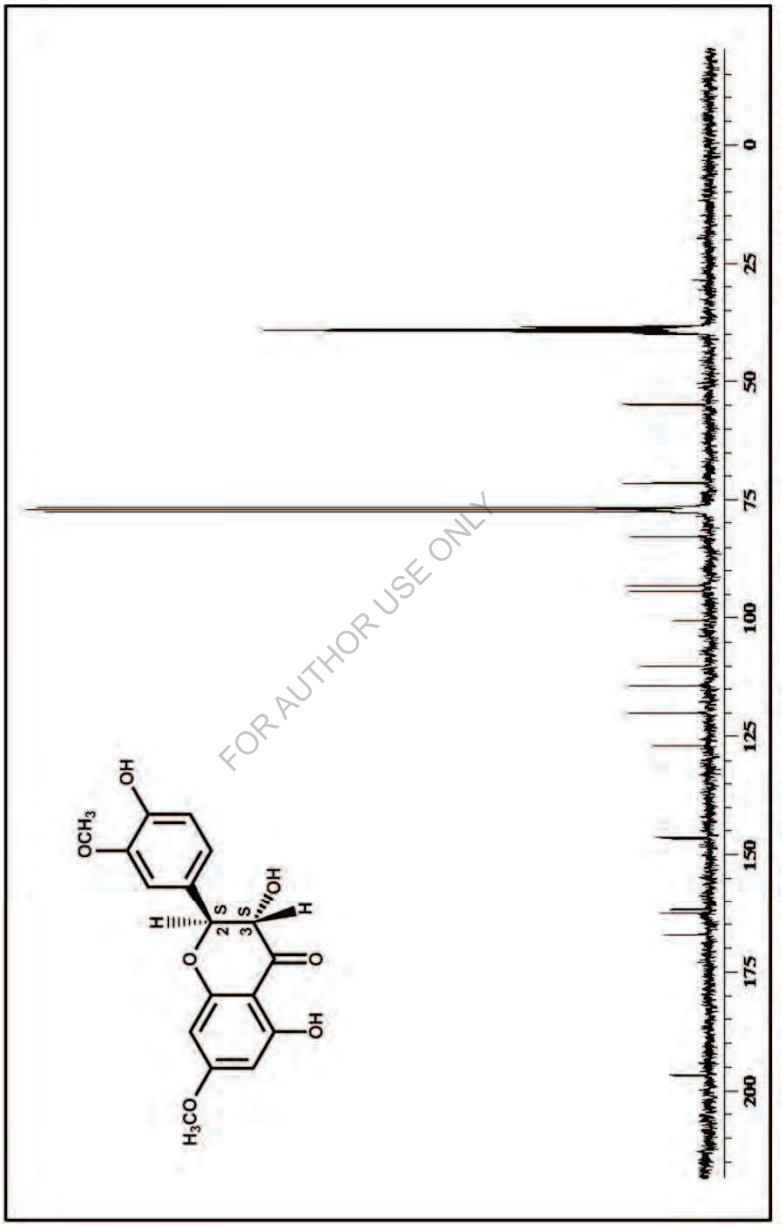


Figure 2A.23 : ^{13}C (75 MHz) NMR Spectrum of Compound 6 in DMSO+$CDCl_3$

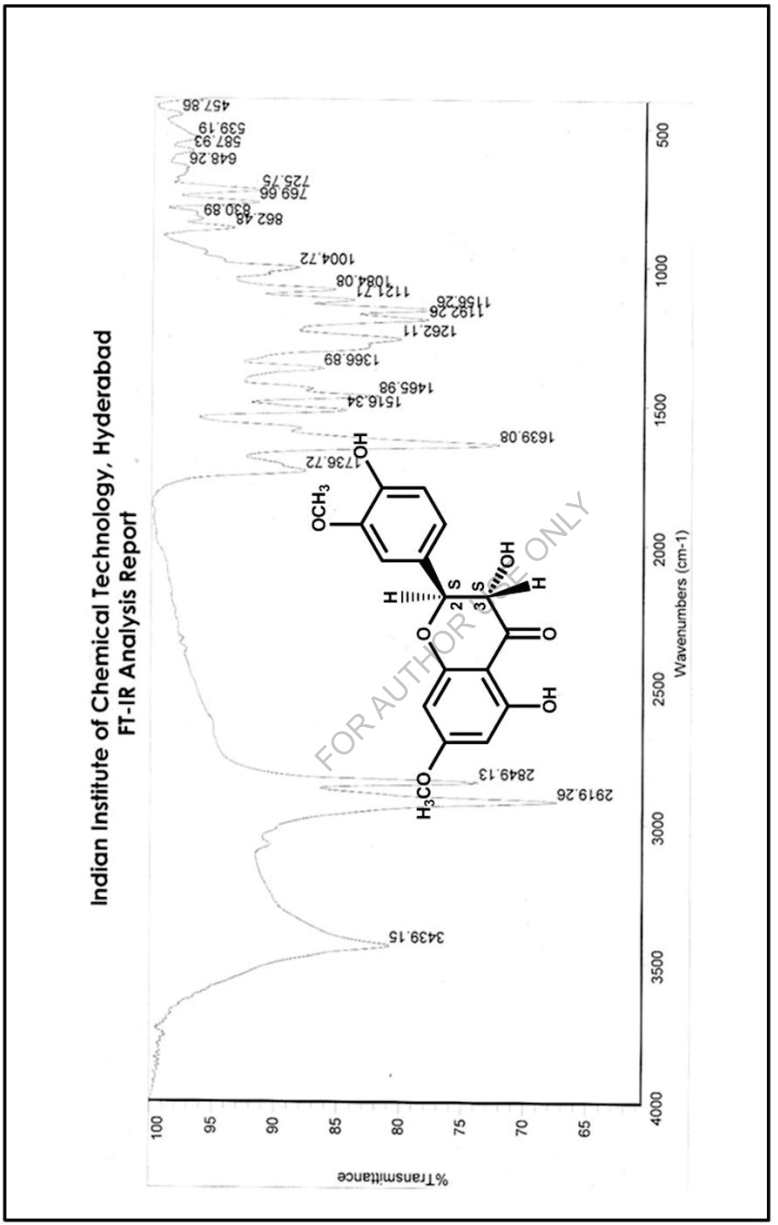


Figure 2A.24 : IR Spectrum of Compound 6

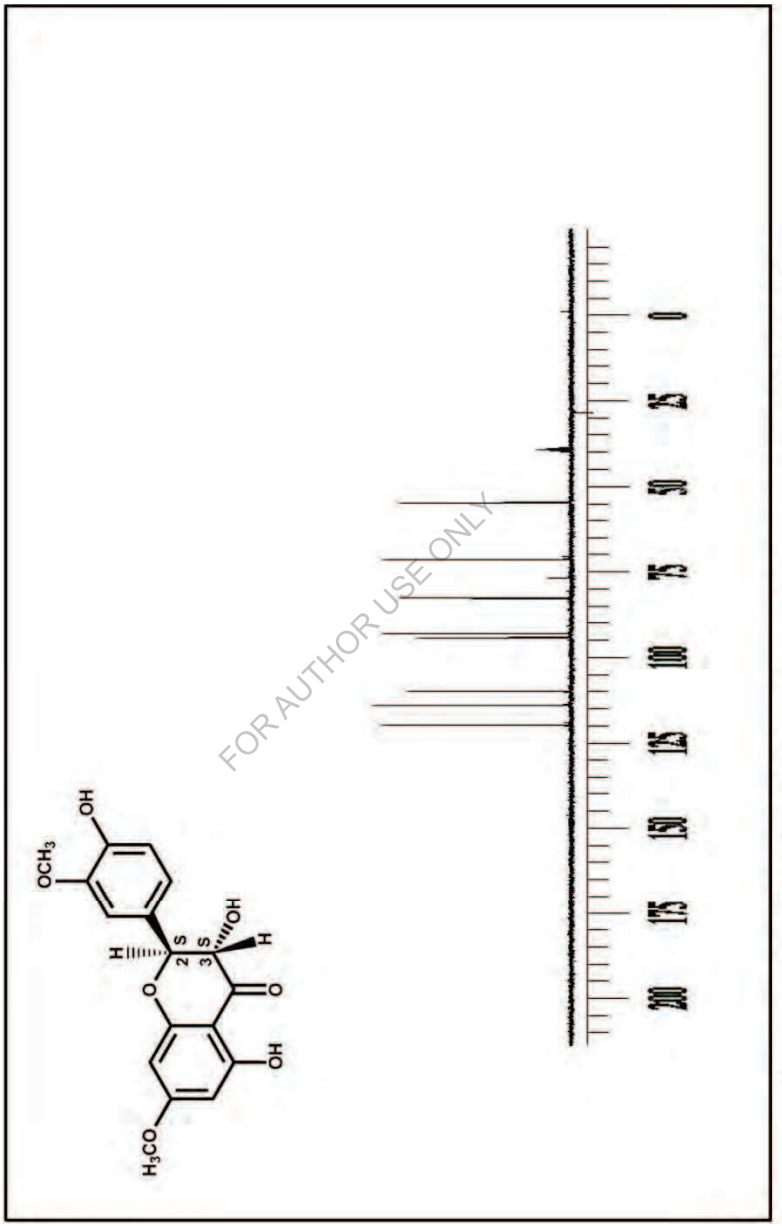


Figure 2A.25 : DEPT 135 Spectrum of Compound 6 in DMSO

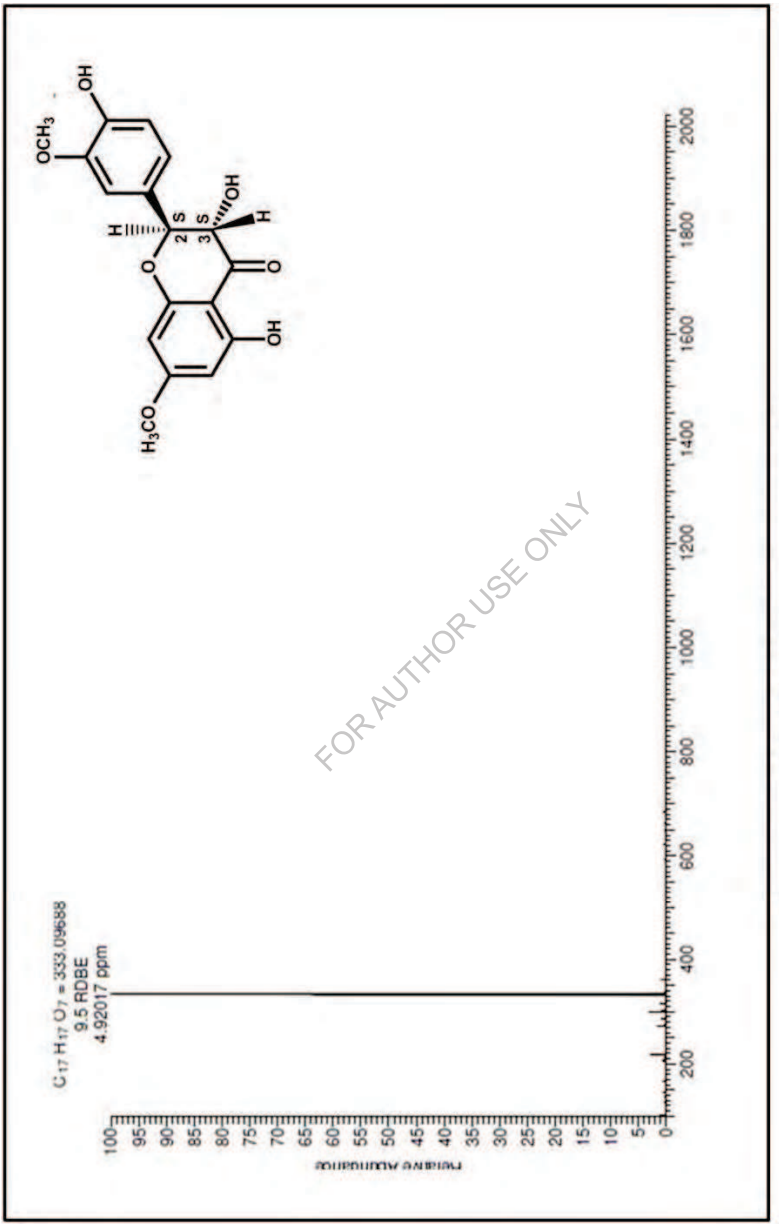


Figure 2A.26 : HRMS of Compound 6

physical and spectral analysis, the compound was identified as 3,4',5-trihydroxy-3',7-dimethoxy-flavanone.

The identity of compound **6** was further confirmed by its NOESY, HSQC and HMBC 2D NMR correlation spectra. The NOESY spectrum (Figure 2A.27) showed the interactions between H-2 with H-3, H-7OCH_3 with H-8 and H-3' OCH_3 with H-2'. The HSQC spectrum (Figure 2A.28) showed connectivites between H2-C_2, H_3-C_3 and -OCH_3-C_7 and –OCH_3-C_3'. The HMBC spectrum (Figure 2a.29) clearly showed the H-2 and H-3 long range couplings with C_4 carbonyl carbon. In addition it showed the interactions of H-2' and H-6' with C_2 ; H-6 with C_{10}; H-8 with C_{10}; H-2' and H-6' with C_2; 3'-OCH_3 with C1' and C4' and 7-OCH_3 with C_6.

Table 2A.03. NMR Chemical Shift Values of Compound 6

Carbon No.	Chemical Shift Values (δ)	
	^{1}H NMR	^{13}C NMR
1	-	-
2	5.05 (d, J=11.2 Hz)	83.10
3	4.55 (d, J=11.2 Hz)	71.90
3(-OH)	7.68 (s)	--
4	-	197.31
5 (-OH)	11.73 (s)	163.70
10	-	101.03
6	5.48 (d, J=1.8 Hz)	94.72
7 (-OCH_3)	3.90 (s)	167.52 52.02 (-OCH_3)
8	6.05 (d, J=1.8 Hz)	93.56
9	-	162.90
1'	-	127.30
2'	7.15 (d, J=1.8 Hz)	110.60
3' (-OCH_3)	3.80 (s)	147.09

		51.98 (-OCH_3)
4' (-OH)	8.60 (s)	146.65
5'	6.91 (d, J=8.8 Hz)	114.80
6'	7.03 (d, J=8.8 Hz)	120.57

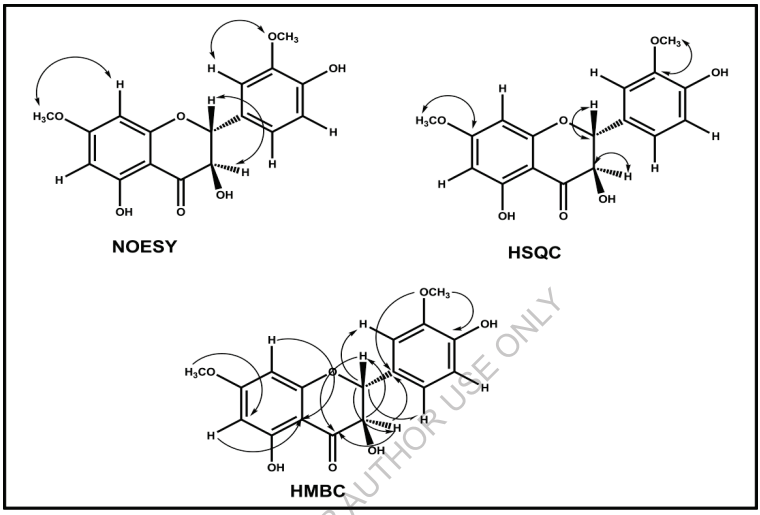


Figure 2A.30: NOESY, HSQC and HMBC Correlations of Compound 6

The absolute configuration of C_2 and C_3 of compound **6** was established by single crystal X-ray crystallography (Figure 2A.31), which clearly shows H-2 as α (''''''') and H-3 as β (▬). Based on the above observations, the absolute configuration of compound **6** is to be 2S,3S. Hence, compound **6** was characterised as (2S,3S)-2,3-dihydro-3,5-dihydroxy-7-methoxy-2-(3-methoxy-4-hydroxyphenyl)-4H-1-benzopyran-4-one. Literature search reveals that this is a new compound and reported for the first time from a *Cochlospermum* species [67].

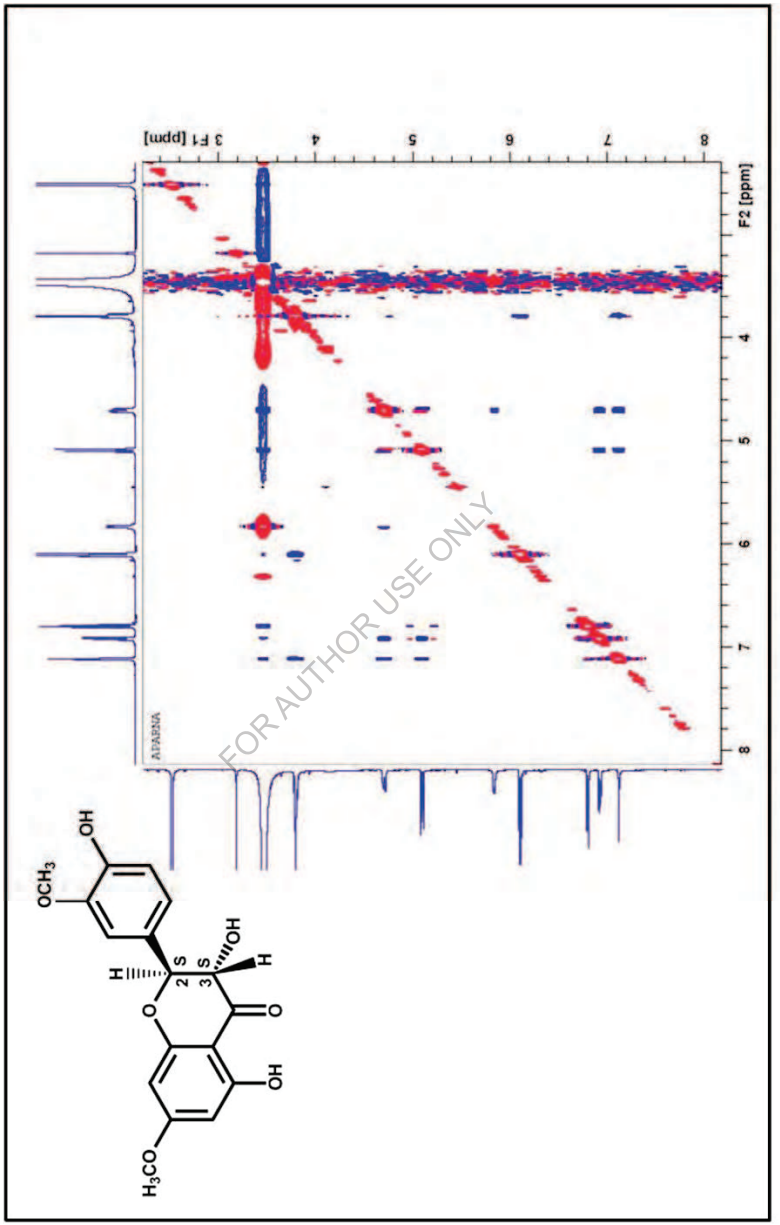


Figure 2A.27 :NOESY Spectrum of Compound 6

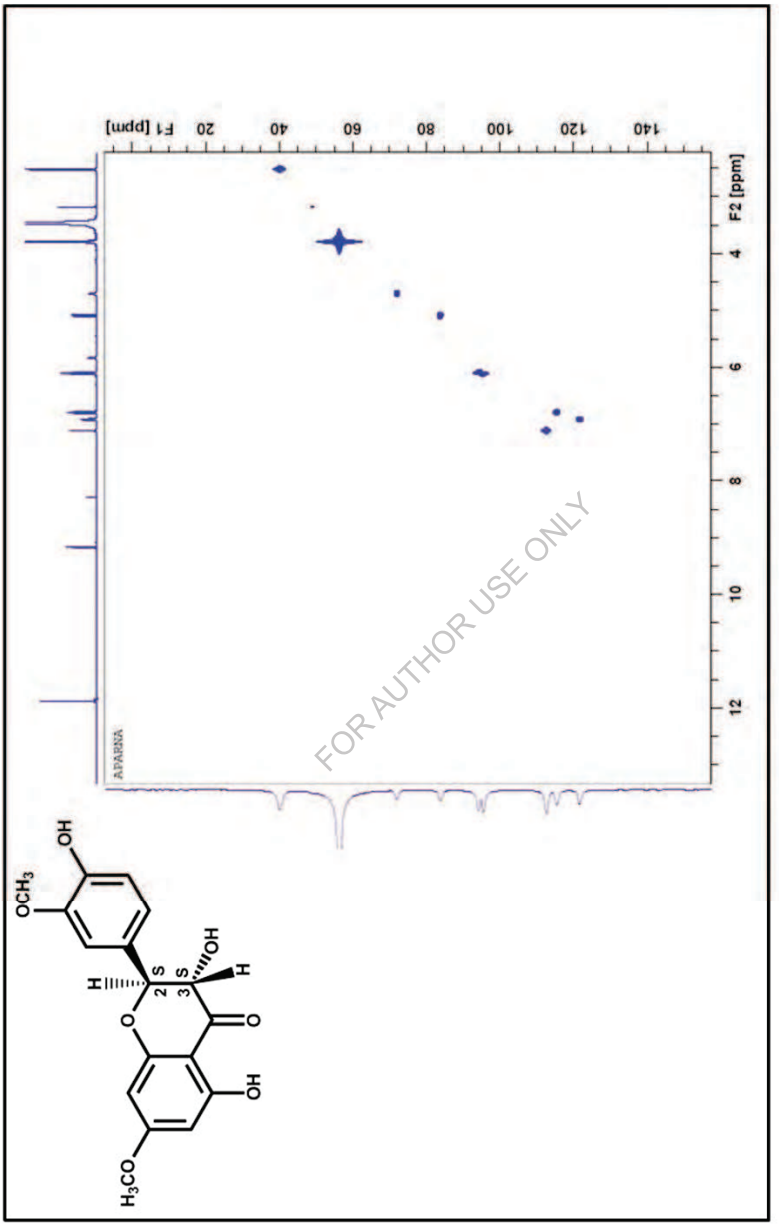


Figure 2A.28 : HSQC Spectrum of Compound 6

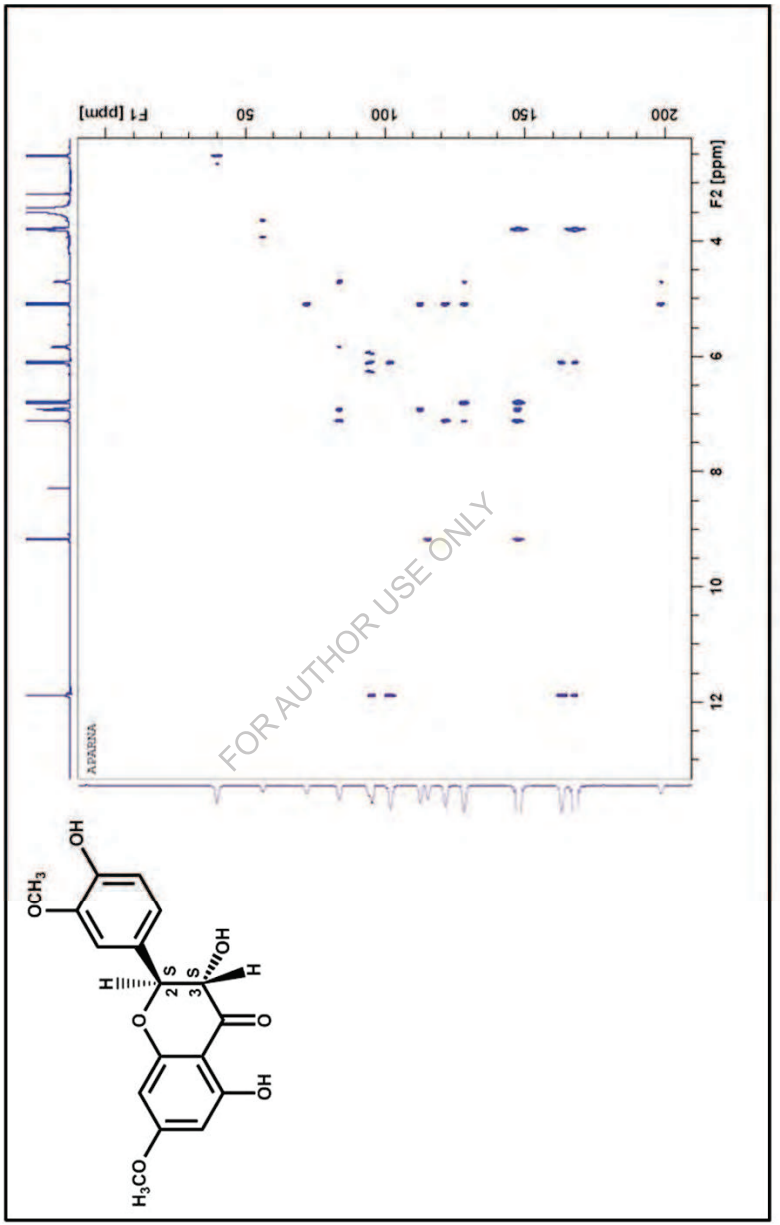


Figure 2A.29 : HMBC Spectrum of Compound 6

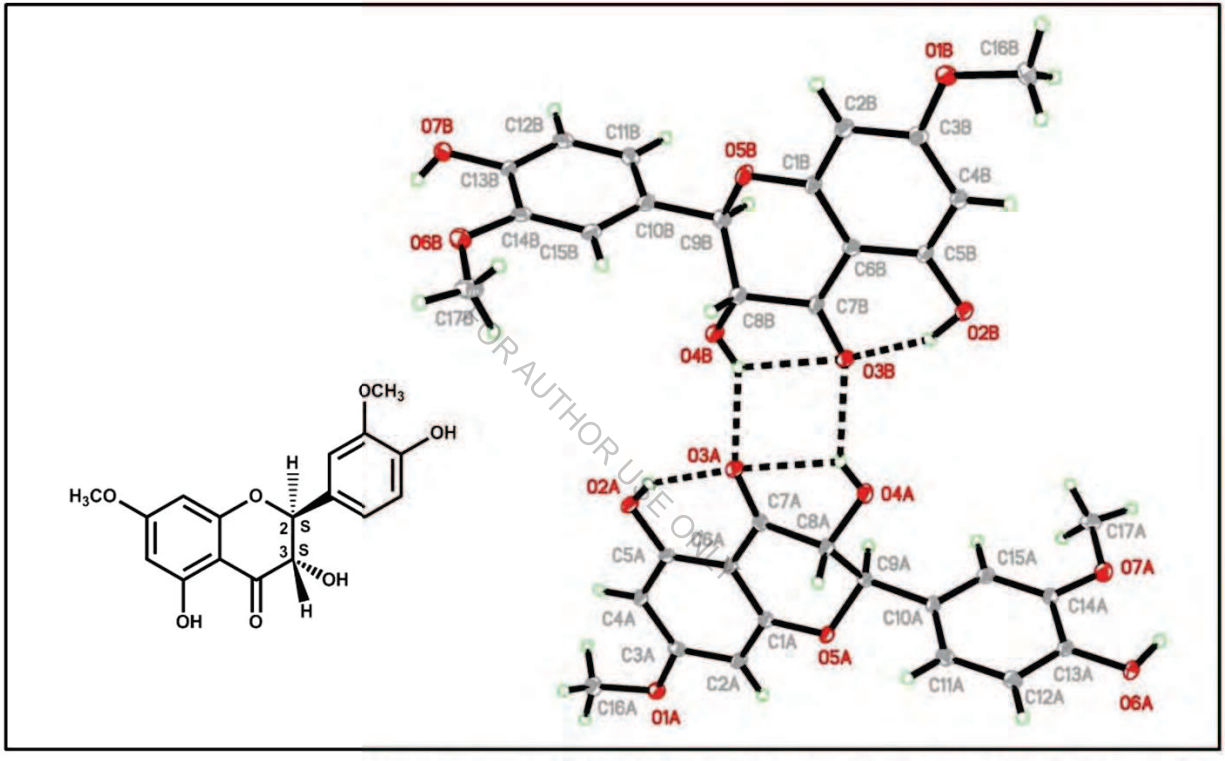


Figure 2A.31: ORTEP Representation of X-ray Single Crystal Structure of Compound 6

(** Numbering Shown on the Crystal Structure is as per the ORTEP Drawing*)

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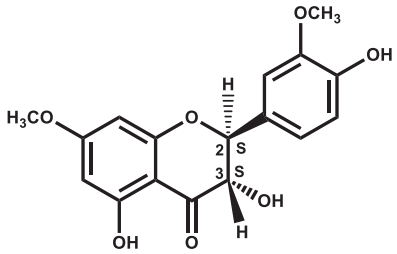


(2S,3S)-2,3-dihydro-3,5-dihydroxy-7-methoxy-2-(3-methoxy-4-hydroxyphenyl)-4H-1-benzopyran-4-one (6)

Identification of CGBE 5 (7) : **2,3-dihydro-5,7-diacetoxy-2-(4-acetoxyphenyl)-4H-1-benzopyran-4-one *(Naringenin triacetate)***

It was obtained as colourless flakes from chloroform, 100 mg (0.01%), R_f : 0.45 (n-hexane: ethyl acetate, 60: 40), m.p. 220^0 C. It gave positive test with Shinoda's reagent (flavonoids). But it did not give any colouration with $FeCl_3$, suggesting that there are no free phenolic hydroxyls present in the molecules. Its IHRMS spectrum (Figure 2A.34b) showed the sodiated molecular ion at m/z 421.08939 corresponding to the molecular formula $C_{21}H_{18}O_8Na$. Its IR spectrum (Figure 2A.33) showed strong absorption bands at 1768 and 1690 cm^{-1} corresponding to ester and carbonyl functionalities. The 400 MHz ^1H NMR Spectrum (Figure 2A.32a) revealed that it is a flavanone by exhibiting characteristic C_2 and C_3 protons at δ 5.70 (1H, dd, J=12.4 & 2.9 Hz) and 3.30 (1H, m) in addition to a pair of doublets at δ 7.79 & 7.20 corresponding to a para-disubtituted system and another pair of doublets δ 6.89 and 6.71 corresponding to two meta coupled aromatic protons. Significantly, it showed a strong nine proton singlet at δ 2.28 corresponding to three acetoxyls. The ^{13}C NMR (100 MHz) spectrum (Figure 2A.34b) of the

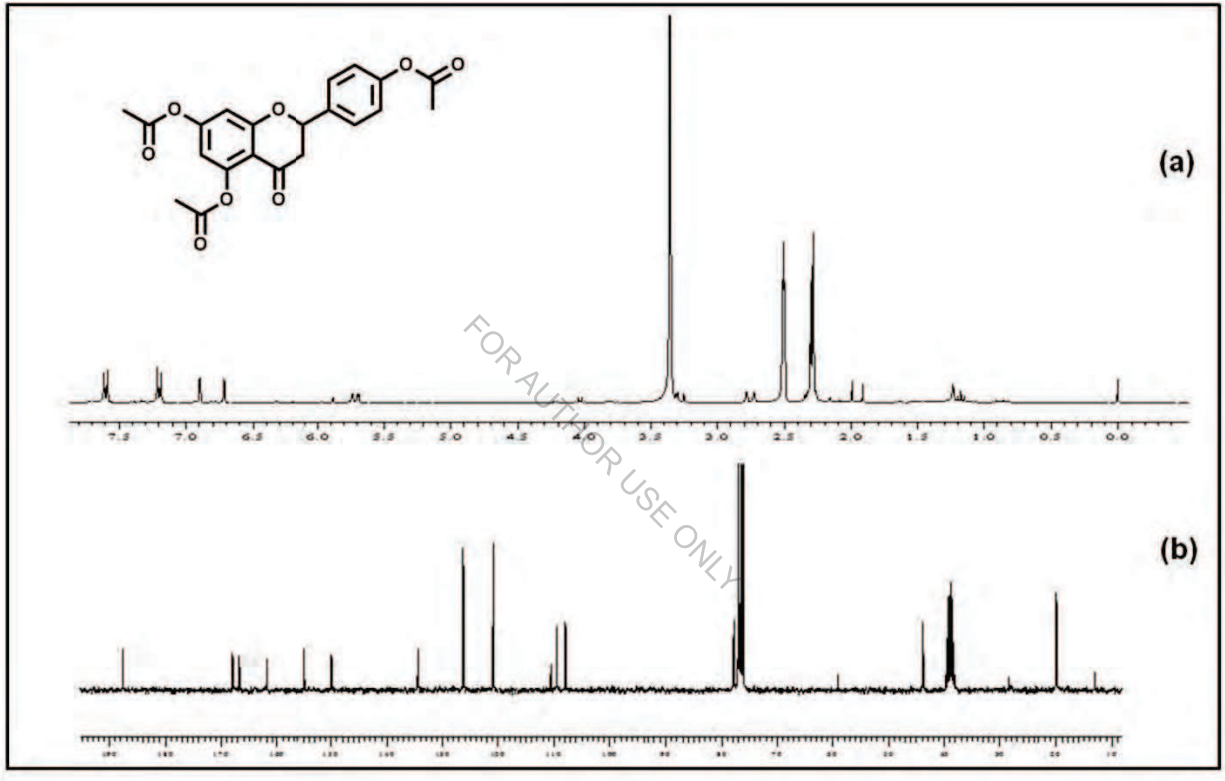


Figure 2A.32 : NMR Spectra of Compound 7 (a) ^{1}H (300 MHz) DMSO ; (b) ^{13}C (75 MHz) in DMSO+$CDCl_3$

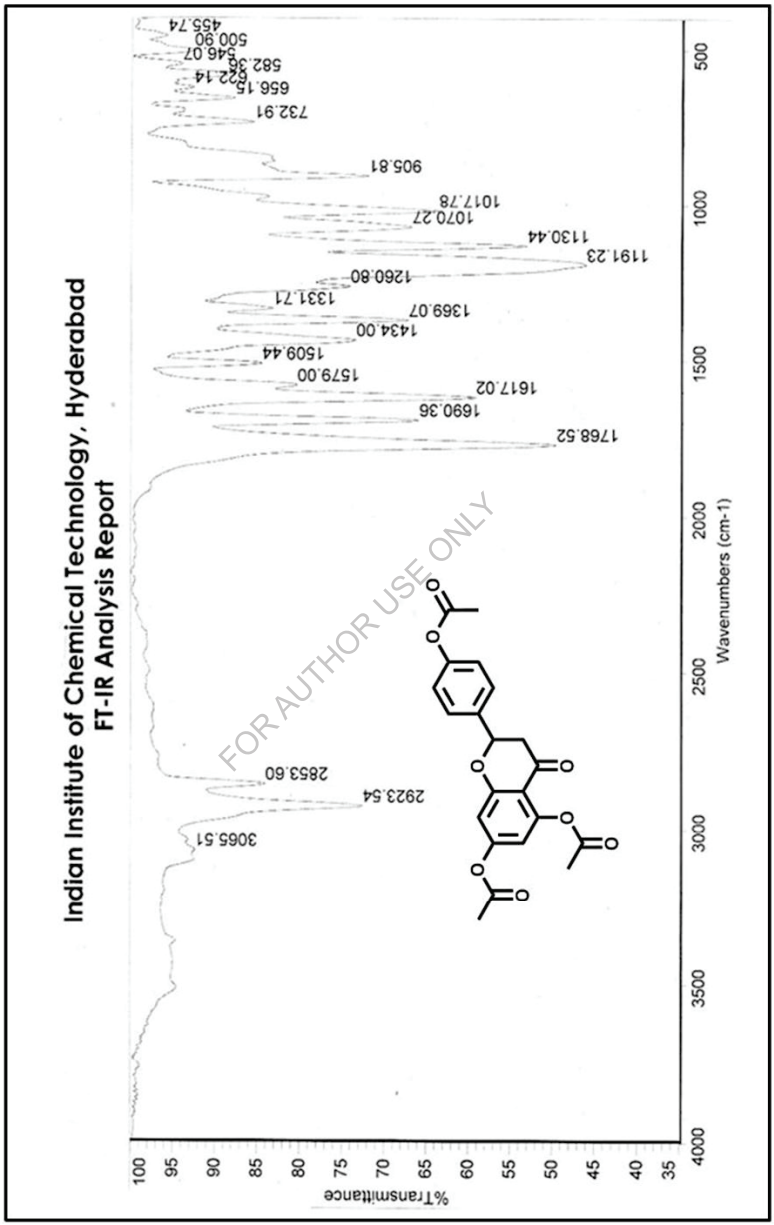


Figure 2A.33 : IR Spectrum of Compound 7

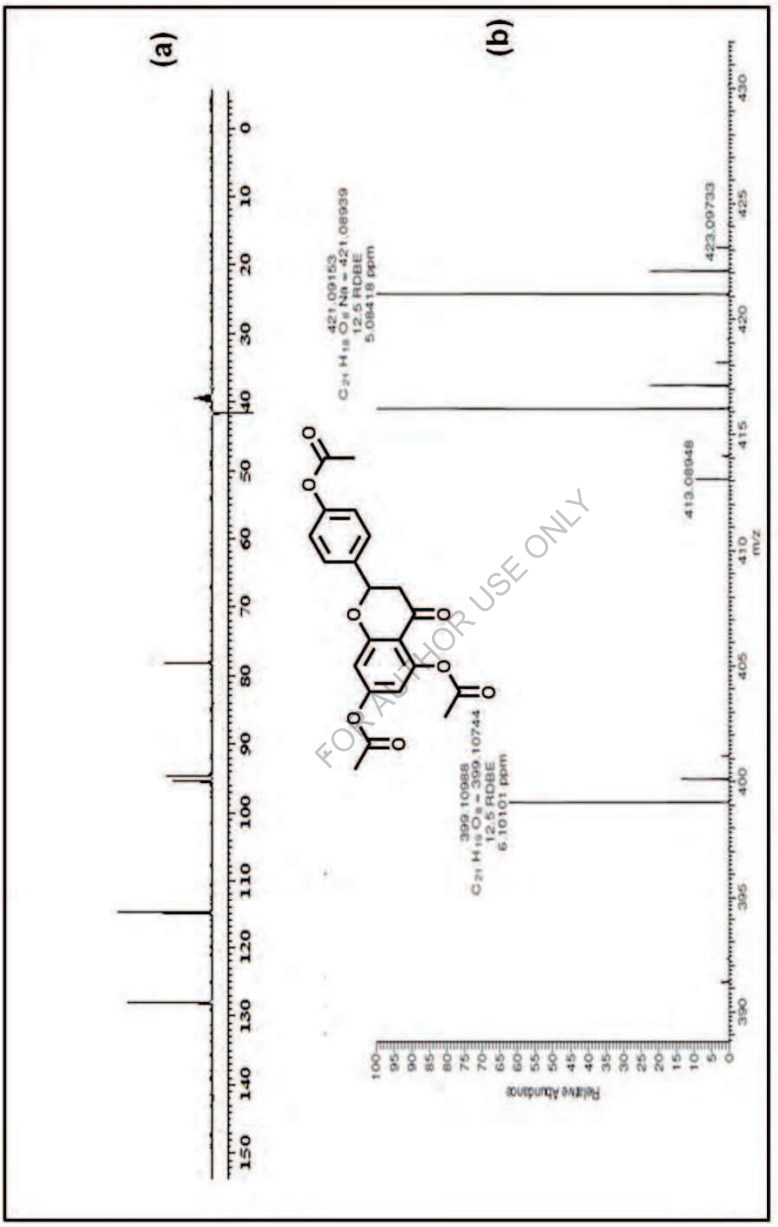
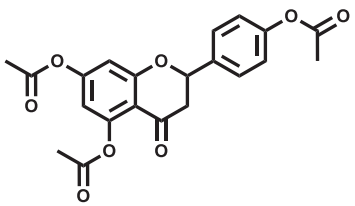


Figure 2A.34 : Spectra of Compound 7 (a) DEPT 135; (b) HRMS

compound while confirming the flavanone skeleton, exhibited the carbonyl carbon signal at δ 188.0, three acetoxyl carbons at d 168.10, 167.80 & 166.80 and the two flavonone ring junction carbons at δ 162.4 (C_9) and 110.99 (C_{10}), It also showed the characteristic C_2 and C_3 carbons at δ 78.13 and 42.16 respectively. The DEPT 135 spectrum (Figure 2A.34b) of the compound reveals that there are ten quaternary, three methyl, one methylene and seven methine carbons present in the compound. Based on the above physical and spectroscopic data, the compound was identified as 2,3-dihydro-5,7-diacetoxy-2-(4-acetoxyphenyl)-4H-1-benzopyran-4-one(naringenin triacetate). Literature search reveals that this compound is not yet reported either naturally or synthetically. Hence, it is a new compound. However, the possibility of its formation as an artefact cannot be ruled out as it is isolated from the ethyl acetate extract of *C. gossypium*. It is noteworthy to mention here that when naringenin is tried to acetylate with acetic anhydride in presence of pyridine, it underwent diacetylation and gave only 4',7-diacetoxyl analogue. This suggests that the triacetoxy analogue is difficult to get chemically under conventional acetylation conditions. Hence, the present isolation of naringenin triacetate assumes significance.



2,3-dihydro-5,7-diacetoxy-2-(4-acetoxyphenyl)-4H-1-benzopyran-4-one (Naringenin triacetate, 7)

Conclusion

In view of virtually no chemistry reported on *C. gossypium*, one of the potent Indian traditional plant, its bark is now subjected to exhaustive extraction and extensive chromatographic studies. A new flavanone, (2S,3S)-2,3-dihydro-3,5-dihydroxy-7-methoxy-2-(3-methoxy-4hydroxyphenyl)-4H-1-benzopyran-4-one was isolated along with six other compounds such as lupeol, betulin, betulinic acid, naringenin, dihydrokaempferol and naringenin triacetate. Structures of the isolated compounds were established by advanced chromatographic analysis (1D & 2D NMR, IR, HR Mass, X-ray crystallography). The isolation of new flavanone and naringenin triacetate from *Cochlospermum gossypium* assumes very high taxonomic significance.

EXPERIMENTAL

Plant Material collection

The bark material (5 kg) of *C. gossypium* was collected from Jaipur forest, Mancherial District, Telangana (Fig. 2A.01) in the month of November 2015. The authenticity of the plant material was confirmed by taxonomists of Botany Department, Osmania University, Hyderabad and Trans Disciplinary University (TDU), Bangalore. The voucher Specimen was deposited in the Natural Products Chemistry division, CSIR-IICT, Hyderabad.

Extraction

The shade dried and powdered bark of the *C. gossypium* (0.5 kg) was placed in a soxhlet extractor and successively extracted with n-hexane, ethyl acetate and methanol solvents under hot condition for 24 hr. Concentration of the three soluble under vacuum gave the respective extracts in 5.13 gm (1.0%), 11.2 gm (2.2%) and 21.8079 gm (4.0%) (Chart 2A.01). The initial TLC studies showed well resolved and different patterns to the n-hexane and ethyl acetate extracts, hence they were subjected to individual column chromatographic separation to isolate single and pure compounds .The methanol extract could not be taken up further, as it showed unresolved TLC pattern.

Chromatographic Separation of n-Hexane Extract

The yellow coloured n-hexane extract, showed prominent spots on TLC plate (n-hexane: ethyl acetate, 8:2) along with some minor overlapping spots. In order to minimise the complexicity the n-hexane extract (100 gm) was fractionated on a column of silica gel (1000 gms, 100-200 mesh) and eluted with solvent gradient from 100% n-hexane to 100% ethyl acetate. Several fractions of 1000 ml capacity were collected and the fractions with similar TLC nature (visualization of spots was

carried out under UV light or Iodine vapours ,or by spraying 5% methanolic H_2SO_4 followed by heating at 110^0 C) were combined and evaporated to yield two major fractions F_2 and F_3 (Chart 2A.02). These fractions on repetitive silica gel column chromatographic separations furnished two single and pure compounds **CGBH1** and **CGBH2** (Chart.2A.03 and Chart.2A.04).

CGBH1 (1): Lup-20(29)-en-3-ol (Lupeol)

It was obtained as colourless crystals from F_2 fraction of n-hexane extract eluted with n-hexane- ethyl acetate (80:20) solvent system, 50 mg (0.003%), m. p. 206-210^0 C. It showed homogeneity on TLC plate (R_f : 0.7, n-hexane-ethyl acetate, 85 : 15).

IR (KBr, cm^{-1})	3380,3068,2943,2869,1711,1639,1456,1382,127, 1038,881,544 & 462.
APCI Mass (m/z)	425.30 [M^+-H] corresponds to the molecular formula $C_{30}H_{49}O$
Mass (m/z)	426 [M^+], 411, 393, 383 [M-side chain-2H], 370 [M-side chain -CH3]
^{1}H NMR ($CDCl_3$, 400 MHz)	δ 4.70, 4.55(2H, s, H-29a, 29b), 3.2(1H, m, H-3), 0.77, 0.79,0.85, 0.94, 0.97,1.05,1.65 (each 3H, s).
^{13}C NMR ($CDCl_3$, 100 MHz)	δ 150.0(C-20), 109.0(C-29), 79.0(C-3), 55.5(C-5), 50.5(C-9), 48.3(C-18), 48.0(C-19), 43.0(C-17), 42.9(C-14), 40.9(C-8), 40.0(C-22), 38.9(C-4), 38.7(C-1), 38.1(C-13), 37.2(C-10), 35.5(C-16), 34.2(C-7), 29.9(C-21), 28.0(C-23), 27.4(C-2), 27.1(C-15), 25.2(C-12), 21.0(C-11), 19.5(C-30), 18.5(C-6), 18.0(C-28), 16.1(C-25), 16.0(C-26),

15.5(C-24), 14.8(C-27).

CGBH2 (2): Lup-20(29)-ene-3β, 28-diol (Betulin)

It was obtained as colourless powder from F_3 fraction of n-hexane extract eluted with n-hexane-ethyl acetate (80:20) solvent system, 100 mg (0.004%), m. p. 256-257° C. It showed homogeneity on TLC plate (R_f: 0.4, n-hexane – ethyl acetate, 80: 20).

IR (KBr, cm^{-1})	3386, 2938, 2869,1643, 1457, 1377, 1106, 1031, 882.
APCI Mass (m/z)	443.60 [M^++H]
^{1}H NMR ($CDCl_3$, 400 MHz)	δ 4.70 (1H, d, H- 29b), 4.58 (1H,d, H-29a), 3.79 (1H, d, J = 10.8, H-28b), 3.33 (1H, d, J = 10.8, H-28a), 3.18 (1H, dd, J =5.3, H-3α), 1.67 (3H, s, H- 30), 0.99 (3H, s, H-27), 0.97 (3H, s, H-26), 0.96 (3H, s, H-23),0.80 (3H, s, H-25), 0.75 (3H, s, H-24).
^{13}C NMR ($CDCl_3$, 75 MHz)	δ 150.6 (C- 20),109.8 (C-29), 79.2 (C-3), 60.6 (C-28), 55.4 (C-5), 50.5 (C-9), 48.8 (C-19), 47.9 (C- 17),47.9 (C-18), 42.8 (C-14), 41.0 (C-8), 38.9 (C-1), 38.8 (C-4), 37.4 (C-10), 37.2 (C- 13),34.3 (C-7), 34.1 (C-22), 29.8 (C-21), 29.2 (C-16), 28.1 (C-23), 27.5 (C-2), 27.1 (C- 15),25.3 (C-12), 20.9 (C-11), 19.2 (C-30), 18.4 (C-6), 16.2 (C-25), 16.1 (C-26), 15.4 (C-24),14.8 (C-27).

Chromatographic Separation of Ethyl acetate Extract

The dark brown coloured ethyl acetate extract of *C. gossypium* bark on TLC showed spots orange coloured spots, but not resolved properly, as they are super imposable in n-hexane-ethyl acetate (70:30) solvent system. In order to minimise the complexicity, a portion of the extract (27.74 gm) was adsorbed on silica gel (100 gm) and chromatographed over a column of silica gel (500 g, 100-200 mesh) and eluted with solvent gradient from 100% n-hexane to 100% ethyl acetate (Chart 2a.05). Several fractions of 250 ml capacity were collected and the fractions with similar TLC nature (visualization of spots were carried out under UV light or Iodine vapours, or by spraying 5% methanolic H_2SO_4 followed by heating at 110^0 C) were combined and evaporated to yield seven major fractions (F1-F_7), of these fractions F_3, F_4 and F_5 showed well resolved TLC patterns. Hence, they were subjected to independent and repetitive again sub fractionated on repetitive silica gel column chromatographic separations (Chart 2A.06 to Chart 2A.08) to yield five single and pure compounds **CGBE1 (3)-CGBE5 (7)**.

CGBE1 (3): (3β)-3-Hydroxy-lup-20(29)-en-28-oic acid (Betulinic acid)

It was obtained as pale brown color granules from the F_3 fraction of ethyl acetate extract eluted with n-hexane- ethyl acetate (70:30) solvent system, 80 mg (0.008%), m.p. 316- 318° C. It showed homogeneity on TLC plate (R_f: 0.60 n-hexane – ethyl acetate, 70: 30).

IR (KBr, cm^{-1})	3451, 1688 and 1642.
APCI Mass (m/z)	455.30 [M^+-H]
^{1}H NMR ($CDCl_3$, 300 MHz)	δ 4.59 (s, 2H, H-12), 4.56 (s, 2H, H-11), 3.38 (s, 2H, H-7), 3.17 (t, 2H, J = 7 Hz, H-2), 2.13

	(m, 2H, H-14), 2.09 (m, 3H, H-1 and H-9), 1.51 (m, 4H, H-18, H-19 and H-15), 1.45 (m, 2H, H-20), 1.38 (m, 2H, H-16), 1.37 (m, 2H, H-21), 0.65, 0.77, 0.98, 1.14 and 1.34 (5s, 15H, all tertiary –CH3).
^{13}CNMR($CDCl_3$+DMSO)75 MHz	δ C: 39.2 (C-1); 27.9 (C-2); 77.8 (C-3); 39.2 (C-4); 55.6 (C-5); 18.4 (C-6); 34.5 (C-7); 40.8 (C-8); 49.4 (C-9); 37.2 (C-10); 20.9 (C-11); 25.8 (C-12); 38.3 (C-13); 42.5 (C-14); 29.9 (C-15); 32.5 (C-16); 56.3 (C-17); 47.4 (C-18); 49.4 (C-19); 151.0 (C-20); 30.9 (C-21); 37.2 (C-22); 28.3 (C-23); 16.1 (C-24); 16.0 (C-25); 19.1 (C-26); 14.6 (C-27); 178.5 (C-28); 109.6 (C-29); 19.1 (C-30).

CGBE 2 (4): *2,3-dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one* (Naringenin)

It was obtained as colourless flakes from the F_4 fraction of ethyl acetate extract eluted with n-hexane-ethyl acetate (70:30) solvent system, 1.0 gm (0.10%), m.p. 251° C. It showed homogeneity on TLC plate (R_f: 0.30 n-hexane – ethyl acetate, 70: 30).

IR (KBr, cm^{-1})	3119, 1633 and 1603.
EI Mass (m/z)	273 [M^+]
HR Mass	273.07575 corresponding to the molecular formula $C_{15}H_{13}O_5$

^{1}H NMR (DMSO,400 MHz)	δ 12.14 (s,1H, OH), 10.82 (s,1H, OH), 7.32 (2H, dd, J=8.0 Hz, H-2' & H-6')), 6.79 (2H, dd, J=8.0 Hz, H-3' & H-5'), 5.88 (1H, br.s, H-6 & H-8), 5.44 (1H, dd, J=12.4, 2.9 Hz, H-2), 3.26 (1H, dd, J=13.2 Hz, H-3b) and 2.68 (1H, dd, J=13.2 Hz, H-3a).
^{13}CNMR (DMSO+CDCL$_3$100 MHz)	δ 195.85 (C_4), 166.21 ($C_{7'}$), 163.06 ($C_{5'}$), 162.0 (C_9), 157 ($C_{4'}$), 128.33 ($C_{1'}$), 127.83 (C2'-$C_{6'}$), 114.68 ($C_{3'}$-$C_{5'}$) 101.14 (C_{10}), 95.76 (C_6), 94.13 (C_8), 77.98 (C_2), 41.33 (C_3)

CGBE3 (5): ***(2R,3R)-2,3-dihydro-3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one*** **(Dihydrokaempherol)**

It was obtained as colourless crystals from the F_4 fraction of ethyl acetate extract eluted with n-hexane- ethyl acetate (70:30) solvent system, 300 mg (0.03%), m.p. 202-205° C. $[\alpha]_D$ -11.43^0 (methanol). It showed homogeneity on TLC plate (R_f: 0.55 n-hexane – ethyl acetate, 60: 40).

IR (KBr, cm^{-1})	3541, 1642 and 1518.
EI HRMS Mass (m/z)	289.07066 [M^++H] corresponding to the molecular formula $C_{15}H_{13}O_6$
^{1}H NMR (DMSO+CDCL$_3$) 500 MHz)	δ: 11.70, 10.36, 9.19 (each 1H, s, 3x-OH), 7.33 (2H, d), 6.87 (2H, d), 5.98 (1H, d), 5.95 (1H, d), 5.16 (1H, d), 4.49 (1H, dd).
^{13}CNMR(DMSO+CDCL$_3$)75 MHz	δ:196.6 (C_4), 167.05 (C_7), 163.4 (C_5), 162.6 (C_9), 157.6 ($C_{4'}$), 128.7 ($C_{2'}$, $C_{6'}$), 127.2

($C_{1'}$), 115.14 ($C_{3'}$, $_{5'}$), 100.2 (C_{10}), 96.4 (C_6), 95.3 (C_8), 82.9 (C_2), 71.9 (C_3).

CGBE4 (6) : *(2S,3S)-2,3-dihydro-3,5-dihydroxy-7-methoxy-2-(3-methoxy-4-hydroxyphenyl)-4H-1-benzopyran-4-one* (New compound)

It was obtained as colourless crystals from the F5 fraction of ethyl acetate extract eluted with n- hexane- ethyl acetate (70:30), 250 mg (0.03%), m.p.195-196° C. $[\alpha]_D$: -1.42^0, (methanol). It showed homogeneity on TLC plate (R_f: 0.5, n-hexane – ethyl acetate, 60: 40).

IR (KBr, cm^{-1})	3439, 1639 and 1516.
HRESI Mass (m/z)	333.09688 $[M^+ + H]$ corresponds to the molecular formula $C_{17}H_{17}O_7$
^{1}H NMR (DMSO) 300 MHz)	δ:11.73 (1H, s, 5-OH), 8.60 (1H, s, 4'-OH), 7.68 (1H, s, 3-OH), 7.15 (1H, d, J=1.8 Hz, 2'-H), 7.03 (1H, d, J=8.8 Hz, 6'-H), 6.91 (1H,d, J=8.8 Hz, 5'-H), 6.05 (1H,d, J=1.8 Hz, 8-H), 5.48 (1H,dd, J=1.8 Hz, 6-H), 5.05 (1H,d, J=11.2 Hz, 2-H), 4.55 (1H,d, J=11.2 Hz, H-3), 3.90 (3H,s, 7-OCH_3), 3.80 (3H,s, 3'-OCH3).
^{13}CNMR(DMSO+$CDCL_3$)75 MHz	δ:197.31 (C_4), 167.52 (C_7), 163.70 (C_5), 162.90 (C_9), 147.09 ($C_{3'}$), 146.65 (C4'), 127.30 ($C_{1'}$), 120.57 ($C_{6'}$), 114.80 ($C_{5'}$), 110.60 ($C_{2'}$), 101.03 (C_{10}), 94.72 (C_6), 93.56 (C_8), 83.10 (C_2), 71.90 (C_3), 52.02

(C_7, -OCH_3), 51.98 ($C_{3'}$, -OCH_3).

CGBE5 (7) : 2,3-dihydro-5,7-diacetoxy-2-(4-acetoxyphenyl)-4H-1-benzopyran-4-one (Naringenin triacetate)

It was obtained as colourless flakes from the F_5 fraction of ethyl acetate extract eluted with n-hexane- ethyl acetate (62:38) solvent system, 100 mg (0.010%), m.p. 220^0 C. It showed homogeneity on TLC plate (R_f: 0.45 n-hexane-ethyl acetate, 60: 40).

IR (KBr, cm^{-1})	1768, 1690, 1617 and 1579.
EIHRMS Mass (m/z)	421.08939 [M^++Na] corresponds to the molecular formula $C_{21}H_{18}O_8Na$
^{1}H NMR (DMSO, 300 MHz)	**δ:** 7.79 (2H, d, J=8.8 Hz, H-2' & H-6'), 7.20 (2H, d, J=8.8 Hz, H-3' & H-5'), 6.89 (H, d, J=1.8 Hz, H-6), 6.71 (H, d, J=1.8 Hz, H-8), 5.70 (H, dd, 12.4, 2.9 Hz, H-2), 3.30 (1H, m, H-3), 2.28 (9H, s, 3x-OCOCH$_3$).
^{13}CNMR(DMSO+$CDCl_3$, 75 MHz)	**δ:**188.0 (C_4), 168.10 (-OCOCH$_3$), 167.80 (-OCOCH$_3$), 166.80 (-OCOCH$_3$), 162.44 (C_9), 155.17 (C_7), 150.59 (C_5), 149.50 ($C_{4'}$), 134.83 ($C_{1'}$), 127.6 ($C_{2'}$,$C_{6'}$), 120.5 ($C_{3'}$,$C_{5'}$), 110.99 (C_{10}), 109.69 (C_6), 108.16 (C_8), 78.13 (C_2), 42.16 (C_3), 20.02 (-OCOCH$_3$), 20.01 (-OCOCH$_3$) and 19.90 (-OCOCH$_3$).

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Naringenin, chemically known as (2R,3R)-2,3-dihydro-3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, is one of the most important naturally occurring flavonoid, predominantly found in some edible fruits, like citrus species, tomatoes and figs belonging to smyrna-type Ficus caricar [1,2,3]. It is particularly abundant in citrus fruits, such as lemon, orange, clementine and grapefruit[4]. As per the flavonoid classification, naringenin is a flavanone and exists naturally in both free and glycosidic forms. Naturally it derives mostly from naringin or narirutin (its 7-O-glycosides) precursors by hydrolysis[5].

Naringenin is a bitter and colorless compound insoluble in water and soluble in organic solvents. It is readily soluble in the binary system of ethanol and water[6]. Naringenin[7] has the molecular weight of 272.26 corresponding to the molecular formula $C_{15}H_{12}O_5$.

Biological activity

Naringenin is widely used as nutritional supplement and medicinal agent. It is widely accepted as safe additive in many countries. Naringenin is reported to exhibit broad spectrum of biological activities[8-11](Chart 2B.01). Several *in vitro* and *in vivo* studies on naringenin demonstrated various pharmacological effects, including antidiabetic activity by reducing hepatic and pancreatic inflammation; anti-metastatic effect by enhancing trans glutaminase activity and polyamine depletion; cytotoxic activity by

Chart 2B.01 Pharmacological Activities of Naringenin

inhibiting lung large cell carcinoma (COR-L23), amelanotic melanoma (C32), prostate adenocarcinoma cells (LNCaP) and human cancer cell proliferation; neuroprotective effects against colchicine-induced cognitive dysfunction; renoprotective effects against cadmium (Cd)-induced renal dysfunction; anti-ulcerative effect by inhibiting histidine decarboxylase; anti-inflammatory activity by suppressing Toll-like receptor 4/NF-κB signaling; anti-photocarcinogenic activity by stimulating melanogenesis and anti-bacterial activity by inhibiting *Salmonella typhi* in comparison with chloramphenicol[12]. Naringenin has broad cytoprotective properties and slow down the progression of the multifactorial Alzheimer's disease. The oral administration of naringenin (50 mg/kg body weight) attenuated the oxy-tetracycline induced nephrotoxicity by significantly decreasing levels of serum urea and creatinine with the significant normalization of creatinine clearance[13]. Intense activity is going on worldwide on clinical and safety evaluation of naringenin before putting into the commercial level for human healthcare.

In view of excellent therapeutic potential of naringenin, now the focus is on its large scale production. Due to low yield, time-consuming extraction and the high cost of purification steps, an alternative process of naringenin production by identifying high yielding natural plant source and efficient extraction protocol is highly warranted. Some of the key aspects such as search for new plant sources, efficient extraction protocols and rapid quantitative method development will greatly help in producing naringenin on large scale .

Search for new plant sources :

Naringenin is mainly found in fruits (grapes, lemons and oranges) and vegetables[14]. Due to the poor production efficiency from plants and chemical synthesis, research groups have directed their attention to the heterologous production of flavonoids in microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae* using metabolic engineering and synthetic biology[15,16,17], But these also have limitations such as reproducibility and handling huge aqueous volumes. Hence, search for new plant sources especially non citrus plants is the most viable alternative to identify high naringenin yielding plant source.

Efficient extraction protocols:

Naringenin was mostly extracted by liquid-liquid extraction[18], solvent extraction[19], supercritical extraction[20], solid phase extraction (SPE)[21] and by Magnetic Stirring-Assisted Dispersive Liquid–Liquid Microextraction[22] methods. These methods have both advantages and limitations. Microwave assisted extraction and ultrasound mediated extraction[23] are the recent additions for extraction of botanicals. Hence, these methods can be tried for high naringenin extraction.

Rapid quantitative method development :

UV Spectroscopic method [24,25] HPLC[26,27,28], HPLC-MS-MS[29] and HPLC-SPE[30] methods are being used for estimation of naringenin. HPLC based quantification found rapid and highly reproducible for naringenin estimation in various plant extracts.

From the above review, it is evident that naringenin is a 'Hot Molecule' due to its multiple therapeutic benefits. In order to carry out detailed clinical and safety studies and also to make more potent analogues of naringenin, its high yielding plant source need to be identified and develop efficient extraction protocol.

Present work

Keeping the above background in mind and also isolation of naringenin as the major metabolite (0.1%) from the bark of *C. gossypium* during our search for natural anti diabetic agents, detailed extraction and HPLC based quantitative studies have now been carried out on the bark of *C. gossypium* collected from two different areas and identified potent plant sample and high yielding extraction method. The details of work done are given below.

2. Materials and methods

2.1 Plant Material,

The bark material of *Cochlospermum gossypium* was collected from two different localities, one from Jaipur forest (79.5786^0 E, 18.8486^0 N), Telangana state and the other from Trichur (76.214729^0 E, 10.530345^0 N), Kerala state. The identity of the collected samples was established by taxonomists from Osmania University, Hyderabad and Trans Disciplinary University (TDU), Bangalore. The voucher specimens were deposited in the Centre for Natural Products & Traditional Knowledge, CSIR-IICT, Hyderabad.

2.2 Chemicals, Solvents,

For extraction and isolation of the bio-active naringenin, commercial grade solvents such as *n*-hexane, ethyl acetate, chloroform, acetone and methanol were used after drying and distillation following standard purification procedures. Silica gel G and silica gel (100–200 mesh) of ACME (Mumbai, India) grade were used for thin-layer chromatography (TLC) and column chromatography respectively.

2.3. Extraction

C. gossypium bark was extracted by the following two methods:

i) Soxhlet extraction with a range of polar solvents under hot conditions

ii) Ultrasound mediated extraction with a range of polar solvents

2.3.1. Soxhlet Extraction

The powdered *C. gossypium* bark material was extracted using soxhlet extractor under hot conditions with a range of polar solvents in two different ways: i) directly with individual solvents such as methanol, methanol-chloroform (1:1) and acetone and ii) successively with n-hexane, ethyl acetate and methanol (Table 2B.01)

2.3.2. Ultrasound mediated Extraction

The powdered bark material was also extracted using an ultrasound extraction system with same polar solvents under two different conditions i.e. individually as well as successively (Table 2B.01)

In total 24 different extracts of *C. gossypium* bark are generated using the above two methods.

S. No.	Bark Collection Area	Bark Powder for Extraction (gm)	Extraction Method	Solvent	Direct / Successive	Extract Code	Extract yield	
							gm	%
1	Trichur (Kerala)	20	Soxhlet	Methanol	Direct	E1	1.300	6.500
2	“	20	"	Methanol+ chloroform(1:1)	Direct	E2	0.600	3.000
3	“	20	"	Acetone	Direct	E3	0.600	3.000
4	“	20	"	Hexane		E4	0.350	1.750
5	“	20	"	Ethyl acetate	Successive	E5	0.340	1.700
6	“	20	"	Methanol		E6	1.000	5.000
7	Jaipur (Telangana)	20	"	Methanol	Direct	E7	1.700	8.500
8	“	20	"	Methanol+ chloroform(1:1)	Direct	E8	0.900	4.500
9	“	20	"	Acetone	Direct	E9	0.770	3.850
10	“	20		Hexane		E10	0.500	2.500
11	“	20	"	Ethyl acetate	Successive	E11	0.580	2.900
12	“	20	"	Methanol		E12	1.300	6.500
13	Trichur (Kerala)	3	**Ultrasound**	Methanol	Direct	E13	0.400	2.000
14	“	3	"	Methanol+ chloroform(1:1)	Direct	E14	0.200	1.000
15	“	3	"	Acetone	Direct	E15	0.003	0.015
16	“	3	"	Hexane		E16	0.030	0.150
17	“	3	"	Ethyl acetate	Successive	E17	0.019	0.095
18	“	3	"	Methanol		E18	0.150	0.750
19	Jaipur (Telangana)	3	"	Methanol	Direct	E19	0.220	1.100
20	“	3	"	Methanol+ chloroform(1:1)	Direct	E20	0.120	0.600
21	“	3	"	Acetone	Direct	E21	0.069	0.345
22	“	3	"	Hexane		E22	0.040	0.200
23	“	3	"	Ethyl acetate	Successive	E23	0.030	1.500
24	“	3	"	Methanol		E24	0.170	0.850

Table 2B.01 Extraction of *C. gossypium* bark with different polar solvents under Soxhlet and Ultrasound conditions

2.4. Isolation of naringenin

Naringenin was isolated from the ethyl acetate extract of *C. gossypium* bark as per the protocol given in Chapter 2A. It was obtained in 99.9% purity as shown by LCMS.

2.5. Characteristics of the sample

24 different extracts of *C. gossypium* bark were prepared with extractive yields ranging from 3 mg to 1.7 gm (0.095 to 8.5%) (Table 2B.01). Almost all the extracts showed the presence of naringenin when tested on TLC [R_f : 0.3 (n-hexane: ethyl acetate, 70: 30),.

2.6. Quantification of Naringenin by HPLC

For the determination of naringenin in 24 different polar extracts of the bark of *C. gossypium*, a validated HPLC method has been used as described below.

2.6.1. Instrumentation and Chromatographic conditions.

The chromatographic analysis was performed on a Shimadzu nexera X2 UPLC system (Shimadzu Corporation, Nakagyo, Kyoto, Japan) equipped with a quaternary pump, an online degasser (DGU-20A 5R), a thermo stated auto sampler (SIL-30AC), a thermostatically controlled column compartment (CTO-20AC), and a diode array detector (PDA) (SPD-M20A), which were communicated through bus module (CBM-20A).

The mobile phase consisting of water with 0.1% formic acid in water (solvent A) and acetonitrile with 0.1% formic acid (solvent B) was pumped at a flow rate of 1.0 mL/min. The gradient elution program was as follows: 0 min, 10%B; 5.00 min, 30% B; 9.00min, 85%B; 12.50min, 10%B; 17.00 min 10% B; 18.00 min 5% B. Equilibration time was 1 min and the injection volume was 20 µL. Column oven maintained at 50 °C and the Auto sampler temperature maintained at 10 °C using an

Waters SunFire C_{18} column (250 mm X 4.6 mm i.d., 5 µm particle size) (Waters, Milford, MA, USA).

2.6.2. Preparation of standard solution

Reference standard solution of marker compound, naringenin was prepared by dissolving 5 mg in 5 mL of acetonitrile. Calibration standards ranging from 0.5-600 ppm were subsequently prepared by diluting with water: acetonitrile (50:50).

2.6.3. Sample preparation

Twenty four extracts of *C. gossypium* bark (**E1-E24**) were prepared for the quantification of naringenin as per the procedure given section 2.3. (Table 2B.01) and the samples were reconstituted using mobile phase (water: acetonitrile: 50:50, v/v), then filtered using membrane filters of 0.45 µ m pore size.

2.7. Method of validation

The method was validated according to the ICH guide lines using standard naringenin [linearity, limit of detection (LOD) and limit of quantification (LOQ)] and *C. gossypium* bark extract samples (accuracy and precision)[31,32].

2.7.1. Linearity and calibration curve

Linearity of naringenin was determined with seven concentration levels over the range of 0.5-600 ppm. The calibration curve was established by plotting the peak area (Y-axis) against concentration (X-axis) of the calibration solutions with linear regression analysis. Calibration curves showed that there was a linear correlation between peak area and concentration of standard solution. Linear regression analysis was followed and the correlation of coefficient (r^2) was used as a measure of linearity.

2.7.2. LOD and LOQ

The limits of detection and quantification for each compound were determined by the signal- to-noise (S/N) ratio for naringenin. LOD was calculated as the amount of the injected sample gave a signal to noise ratio of 3, and LOQ was determined when the S/N ratio was 10.

2.7.3. Precision

For each analyte method precision was tested at three concentration levels (QC_L, QC_M and QC_H) in three replicates for each kind of 24 *C. gossypium* bark extract samples in order to calculate the percentage of RSD of the determination.

2.7.4. Accuracy

C. gossypium bark extract samples spiked with standard solution at three concentration levels (QC_L, QC_M and QC_H) were used for the determination of the accuracy of the method, samples were spiked in triplicates. Runs were accomplished in triplicates.

2.7.4. Selectivity

Selectivity of the method was evaluated comparing standard calibration curves in the methanol with standard calibration curves using the *C. gossypium* bark extract samples containing the lowest concentration of the standard compound.

3. Results and Discussion

In order to identify the high naringenin yielding *C. gossypium* bark sample and extraction solvent, detailed HPLC based studies have now been carried out. In total 24 different polar extracts (E1-E24) from C. gossypium bark samples collected from two different localities such as Jaipur (Telangana) and Trichur (Kerala). The UHPLC system was chosen for method development and optimization to achieve higher efficiency for separation and quantification of the flavanone, naringenin. The

chromatographic separation on the stationary phase is based on various retention mechanisms due to hydrophilic and hydrophobic interactions. The mobile phase consisting of water with 0.1% formic acid in water (solvent A) and acetonitrile with 0.1% formic acid (solvent B) was pumped at a flow rate of 1.0 mL/min. The gradient elution program was as follows: 0 min, 10%B; 5.00 min, 30% B; 9.00min, 85%B; 12.50min, 10%B; 17.00 min 10% B; 18.00 min 5% B. Equilibration time was 1 min and the injection volume was 20 µL. Column oven maintained at 50 °C and the Auto sampler temperature maintained at 10 °C using an Waters SunFire C_{18} column (250 mm X 4.6 mm i.d., 5 µm particle size) (Waters, Milford, MA, USA).

To decrease the system back pressure, a column temperature of 50°C was used. The gradient elution program needed to achieve the desired analyte separation.

3.1. UPLC method validation

The UPLC method was validated using analyte mixtures in order to evaluate Linearity, LOD , LOQ and extracts samples to evaluate accuracy and precession.

3.1.1. System suitability

The system suitability was assessed by seven replicate analysis of the analyte at concentration 0.1 ppm to 600 ppm .As represented in Table 2B.02(Figure 2B.01) .All concentrations, SD, % RSD .

Table 2B.02 System Suitability

S No	Conc.	Area 01	Area 02	Area 03	Avg. Area	SD	%RSD
1	0.1	2182	2287	2490	2319.666667	156.577	6.74998
2	0.5	9401	9553	9588	9514	99.4133	1.04492
3	1	19220	19588	19588	19465.33333	212.465	1.0915
4	5	97090	95321	97341	96584	1100.97	1.13991
5	10	195987	197495	196132	196538	831.951	0.4233
6	100	1956298	1953313	1953901	1954504	1581.22	0.0809
7	600	17131041	17568907	1760371 4	17434554	263425	1.51094

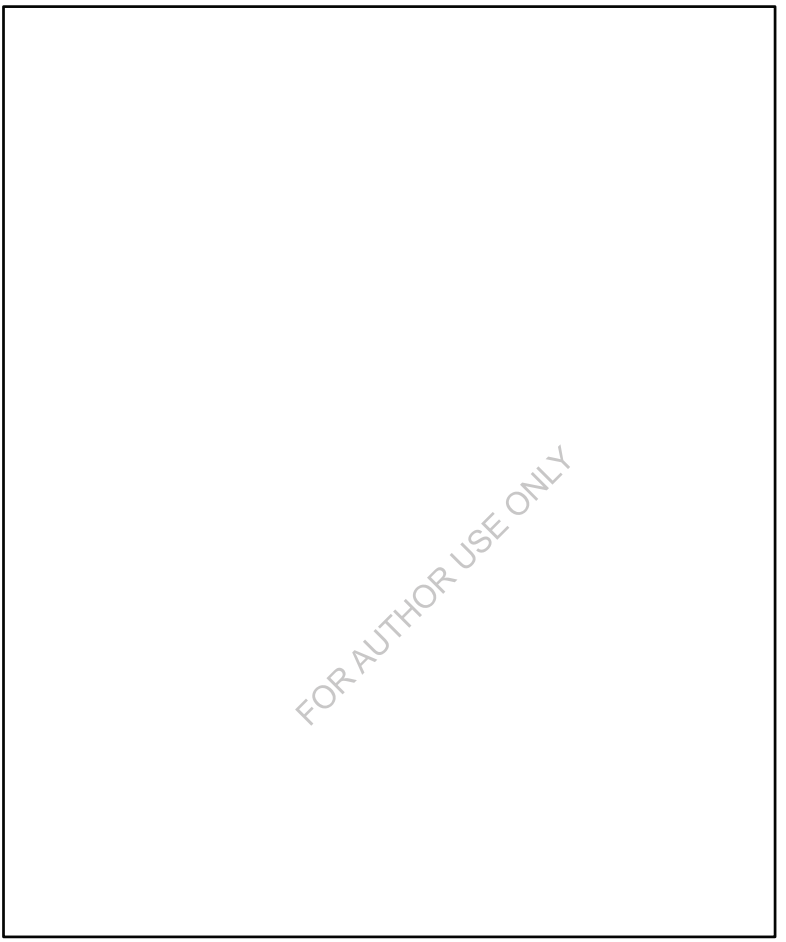

Figure 2B.01 LC Chromatograms of naringenin at various concentrations

3.1.2 Linearity

The linearity of the standard compound under investigation was estimated using 24 extracts at different concentration levels (QC_L, QC_M, $QCH_)$.The slope obtained from

the extracts is different from that of the standard solution. Standard addition methodology was used during the quantification step in order to obtain reliable results. The calibration curves were drawn in the range of 0.5 – 600 ppm to the peak area of standard .the calibration curves were linear over the range of 0.5 – 600 ppm

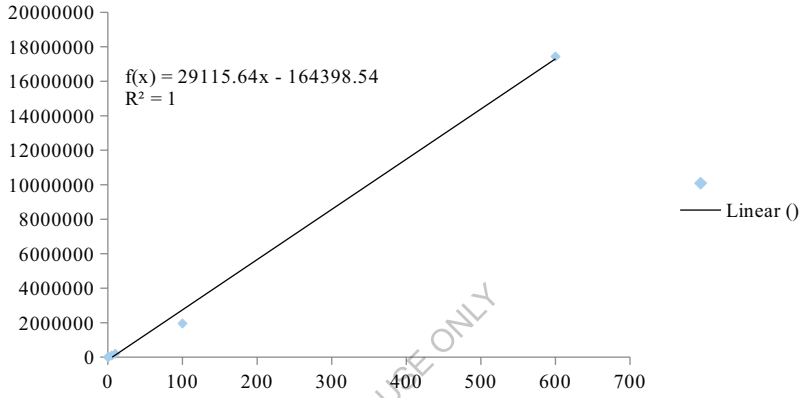

Figure 2B.02 Linearity curve

3.1.3. LOD – LOQ

The LOD of the method was defined according to the international guidelines ICH Q2 (R1) 2005 at the concentration of the lowest standard on the calibration curve for which (a) the analyte peak is identifiable and discrete ,(b) the analyte response is at least ten times the response of the blank sample. (c) The analyte response is reproducible with a precision less than 20% and trueness better of 80-120% .The LOD and LOQ were estimated on the basis of the results for three replicates of real samples spiked at different concentration levels, considering a signal-to-noise ratio of 3 and 10, respectively .The LOQ values for each analyte were assessed to be 0.15 ppm. On the basis of the signal-to-noise ratio of the chromatogram, the LOD of the method was also set at 0.15 ppm .

Figure 2B. 03 LC chromatogram of naringenin at 0.05 ppm

3.1.4. Accuracy and precision

Data for intra and inter day precession and accuracy, obtained from the analysis of three batches of LOD-LOQ samples at three different concentration levels of all analytes and the QC samples at in duplicate on the same day and for standard for three days.

3.1.5. Quantification

Quantification of naringenin in 24 *C. gossypium* bark extracts (**E1-E24**) was carried out using UPLC. The chromatographic conditions with respect to mobile phase, gradient elution and column were optimized based on peak shape, response and peak resolution of naringenin. The gradient elution of the mobile phase is used for the best resolution in the crude extracts (Figure 2B.04-Figure 2B.08). Based on peak area method the amount of naringenin present in each *C. gossypium* bark extract samples is given in Table 2B.03.

Figure 2B.04 LC Chromatograms of *C. gossypium* bark extract samples (E1-E5)

Figure 2B.05 LC Chromatograms of *C. gossypium* bark extract samples (E6-E10)

Figure 2B.06 LC Chromatograms of *C. gossypium* bark extract samples (E11-E15)

E20

Figure 2B.07 LC Chromatograms of *C. gossypium* bark extract samples (E16-E20)

E24

Figure 2B.08 LC Chromatograms of *C. gossypium* bark extract samples (E21-E24)

Table 2B.03 Amount of naringenin present in *C. gossypium* bark extracts (E1-E24)

S No	Sample Code	Area 01	Area 02	Area 03	Avg Area	SD	%RSD	Amount Present (ppm)
1	UVM-CG-MN-01	379692	377102	376009	377601	1891.526632	0.50093263	13.5334524
2	UVM-CG-MN-02	449901	449714	448704	449439.667	643.9303792	0.14327404	16.0007785
3	UVM-CG-MN-03	277829	277043	276456	277109.333	688.8993637	0.24860201	10.0822821
4	UVM-CG-MN-04	403630	407303	404052	404995	2009.892783	0.49627595	14.47430966
5	UVM-CG-MN-05	822429	825477	817032	821646	4276.601805	0.52049201	28.78434538
6	UVM-CG-MN-06	1323005	1324692	1323996	1323897.67	847.787906	0.06403727	46.03436827
7	UVM-CG-MN-07	12576	12648	12421	12548.3333	116.0014368	0.924437	0.995580895
8	UVM-CG-MN-08	1510159	1517851	1518695	1515568.33	4703.588984	0.3103515	52.6173696
9	UVM-CG-MN-09	124246	123526	123662	123811.333	382.5249447	0.30895704	4.816950588
10	UVM-CG-MN-10	6580	8014	7736	7443.33333	760.4796732	10.2169235	0.820247745
11	UVM-CG-MN-11	1184413	1219691	1144532	1182878.67	37602.98465	3.17893844	41.19111754
12	UVM-CG-MN-12	124756	128923	128575	127418	2311.916737	1.81443496	4.940822915
13	UVM-CG-MN-13	8040	8422	8619	8360.33333	294.384669	3.52120732	0.851742455
14	UVM-CG-MN-14	357219	357315	356532	357022	427.058544	0.11961687	12.82665888
15	UVM-CG-MN-15	191244	214901	214641	206928.667	13583.94186	6.56455293	7.671646746
16	UVM-CG-MN-16	ND	ND	ND	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
17	UVM-CG-MN-17	1021992	1037792	1037466	1032416.67	9029.497513	0.87459819	36.02334341
18	UVM-CG-MN-18	324187	325533	326388	325369.333	1109.590165	0.34102481	11.73953611
19	UVM-CG-MN-19	318969	357746	357550	344755	22331.5461	6.47751188	12.40534414
20	UVM-CG-MN-20	307691	307544	307474	307569.667	110.7534801	0.03600923	11.12819984
21	UVM-CG-MN-21	36518	37017	36217	36584	404.0631139	1.10448041	1.821064931
22	UVM-CG-MN-22	376703	381432	360968	373034.333	10713.92367	2.87210123	13.37660851
23	UVM-CG-MN-23	205348	202120	203496	203654.667	1619.838675	0.795385	7.559159982
24	UVM-CG-MN-24	512767	513943	512694	513134.667	700.9881121	0.136609	18.18840729

	Sample Conc 400 ppm
m	29116
c	16439

* UVM-CG-MN-01 to UVM-CG-MN-24 represent sample codes of the extracts E1-E24

Based on the above data, the amount of naringenin present in the plant samples is calculated and presented in Table 2B.04. Close analysis of the data reveals that naringenin is present in almost all samples except E16 (the n-hexane extract of C.

gossypium bark sample collected from Trichur (Kerala) and extracted using soxhlet. Naringenin is found to present in the range 0.0002 – 0.59%. In general, naringenin found to present in higher levels in the extracts (E1-E12) generated by soxhlet extraction than the ultrasound mediated extracts (E13-E24). This clearly reveals that for high naringenin extraction hot conditions are necessary. The highest naringenin content (0.59%) was found in methanol + chloroform (1:1) extract (E8) generated by direct soxhlet extraction of C. gossypium bark collected from Jaipur (Telangana). This is followed by 0.57% in the successive methanol extract (E6) of Trichur (Kerala) C. gossypium bark sample and 0.29% in the successive ethyl acetate extract (E11) using soxhlet extraction of C. gossypium bark sample collected from Jaipur (Telangana).

S. No.	Collection Area	Extract	Direct / Successive	Extract Code	Naringenin Content (%)
1	Trichur (Kerala)	Methanol	Direct (SX)	E1	0.21
2	"	Methanol+ chloroform(1:1)	Direct (SX)	E2	0.21
3	"	Acetone	Direct (SX)	E3	0.075
4	"	Hexane		E4	0.06
5	"	Ethyl acetate	Successive (SX)	E5	0.12
6	"	Methanol		**E6**	**0.57**
7	Jaipur (Telangana)	Methanol	Direct (SX)	E7	0.021
8	"	Methanol+ chloroform(1:1)	Direct (SX)	**E8**	**0.59**
9	"	Acetone	Direct (SX)	E9	0.04
10	"	Hexane		E10	0.0005
11	"	Ethyl acetate	Successive (SX)	**E11**	**0.29**
12	"	Methanol		E12	0.08
13	Trichur (Kerala)	Methanol	Direct (US)	E13	0.004
14	"	Methanol+ chloroform(1:1)	Direct (US)	E14	0.03
15	"	Acetone	Direct (US)	E15	0.0002
16	"	Hexane		E16	Not detected
17	"	Ethyl acetate	Successive (US)	E17	0.008
18	"	Methanol		E18	0.02
19	Jaipur (Telangana)	Methanol	Direct (US)	E19	0.003
20	"	Methanol+ chloroform(1:1)	Direct (US)	E20	0.016
21	"	Acetone	Direct (US)	E21	0.0015
22	"	Hexane		E22	0.006
23	"	Ethyl acetate	Successive (US)	E23	0.02
24	"	Methanol		E24	0.03

SX – Soxhlet; US - Ultrasound

Table 2B. 04 Naringenin content in various C. gossypium bark extracts

Among the ultrasound extracts, highest naringenin content (0..03%) was found in the E14 and E24 extracts followed by 0.02% in E18 and E23 extracts. It is observed that n-hexane extracts in general afforded naringenin low yields suggesting non polar solvents like n-hexane are not at all suitable for naringenin extraction.

Conclusion

As naringenin is gaining importance as 'Hot Molecule' in view of high therapeutic potential, detailed HPLC based quantitative studies were now carried out on *C. gossypium* bark, which contains significant levels of naringenin. In total 24 different polar extracts were generated from two bark samples collected from Jaipur (Telangana) and Trichur (Kerala) under soxhlet and ultrasound extraction conditions. The highest naringenin content (0.59%) was found in the methanol + chloroform (1:1) extract (E8) generated by direct soxhlet extraction of C. gossypium bark collected from Jaipur (Telangana).

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Flavonoids form an important group of natural products having low-molecular-weight phenolic substitution patterns and found widely in fruits, vegetables and certain beverages. Their occurrence is not restricted to flowers, but also found in all parts of classified plants. They have miscellaneous favourable biochemical and antioxidant effects associated with various diseases such as cancer, Alzheimer's disease (AD), atherosclerosis, etc. They are also known to be potent inhibitors for several enzymes, such as xanthine oxidase (XO), cyclooxygenase (COX), lipoxygenase and phosphoinositide 3-kinase. According to substitution patterns, degree of oxidation, annularity of ring C and connection position of ring B, flavonoids can be classified into different sub-classes such as flavones, flavanones, flavonols, chalcones etc. providing higher degree of chemical and bio diversity[1] .

Incidentally, in the present study naringenin was isolated in abundance (1%) by repetitive chromatographic purifications of the ethyl acetate extract of the bark part of *Cochlospermum gossypium* (Chapter 2A). Further, extensive extraction, isolation and HPLC based quantitative studies were carried out on naringenin in various polar extracts of *C. gossypium* bark samples collected from two different areas to identify high naringenin yielding *C. gossypium* bark sample, suitable solvent system and method of extraction (Chapter 2B). As naringenin is gaining importance as 'Hot Molecule' due to its chemical and biological diversity, intense activity is going on worldwide on the chemical modification of naringenin to synthesise New Chemical Entities (NCEs). With this background in view, work has now been taken up on chemical transformation of naringenin at 4',5 and 7 hydroxyls to synthesise some new saturated and unsaturated O-alkyl ethers and to evaluate the synthesized ether analogues for their α-glucosidase inhibitory potential *in vitro* and *in vivo* to identify potent antidiabetic agents.

Naringenin (**1**) belongs to flavanone sub class of flavonoid group. Chemically it is known as (2S)-2,3-dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one (Figure 2C.01). Naringenin consists of a 1,4-benzopyrone scaffold, the A- and the C- ring fused system with two hydroxyl groups at positions 5 and 7. The C-ring contains a carbonyl group at position 4. The aryl ring B with a hydroxyl group at para position (4') is attached at position C2.

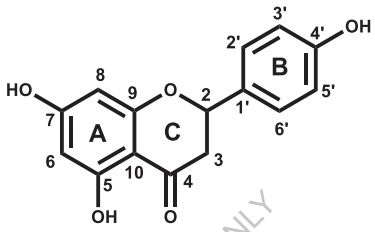

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Figure 2C.01 Chemical Structure of Naringenin

The calculated log P value of naringenin is 2.52, which means it is a slightly lipophilic compound. Naringenin has a molecular weight of 272.26 g/mol. In order to fulfill further requirements, as nitrogen atoms, heterocycles and increased hydrophobicity, the following two different strategies of chemical transformations on naringenin were pursued (Figure 2C.02):

1) Transformation of the hydroxyl groups by alkylation or acylation to synthesise ether and ester analogues;
2) Addition of nitrogen nucleophiles at C-4 carbonyl to synthesise hydrazones and hydrazides

Structure Activity Relationship (SAR) studies on naringenin suggests that the manner of hydroxylation of the molecule is essential. A hydroxyl group at position 5 is required, whereas the hydroxyl at position 7 is detrimental for the activity, but

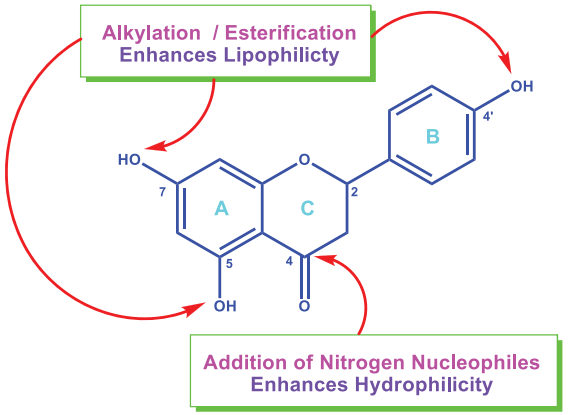
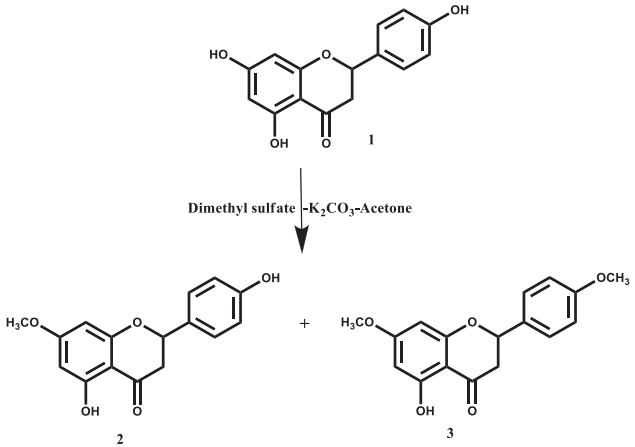


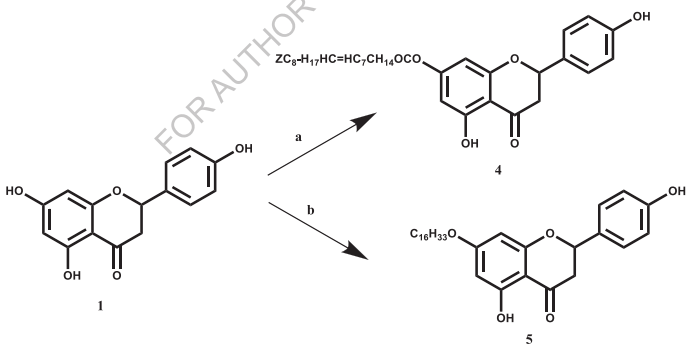
Figure 2C.02 Possible Chemical Modifications of Naringenin

alkylation of this hydroxyl group is thought to be slightly beneficial[2]. As the three hydroxyl groups at 4', 5 and 7 are responsible for the biological activity of naringenin, fine tuning of these three hydroxyls by alkylation or acylation enhances the lipophilicity of the molecule and is expected to exhibit enhanced and newer therapeutic activities. In this connection detailed literature search has been done and reviewed briefly on the work done so far on the alkylation and acylation reactions carried out on naringenin and their effect on overall biological profile of naringenin.

In order to increase the lipophilicity, naringenin was methylated at the hydroxyl groups in position 7 and 4' to yield 7-methoxy naringenin (sakuranetin) and 4',7-dimethoxy naringenin (narigenin-4',7-dimethyl ether)[3,4]. The reaction can take place in presence of alkaline carbonates either in aqueous solution or organic solvent. In this particular case, naringenin was dissolved in acetone and potassium carbonate was added in suspension[5].

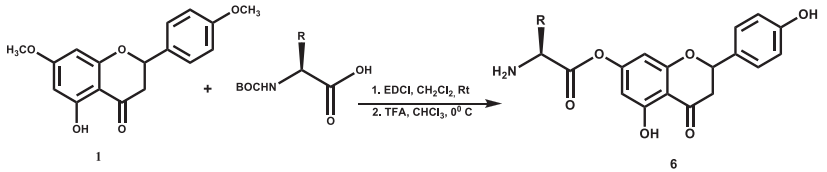


Two classes of naringenin derivatives were evaluated for anti-atherogenic activity. Naringenin 7-O-oleic ester (**4**) and naringenin 7-O-cetyl ether (**5**) inhibited the formation of aortic atherosclerotic lesions in high cholesterol-fed rabbits[6].

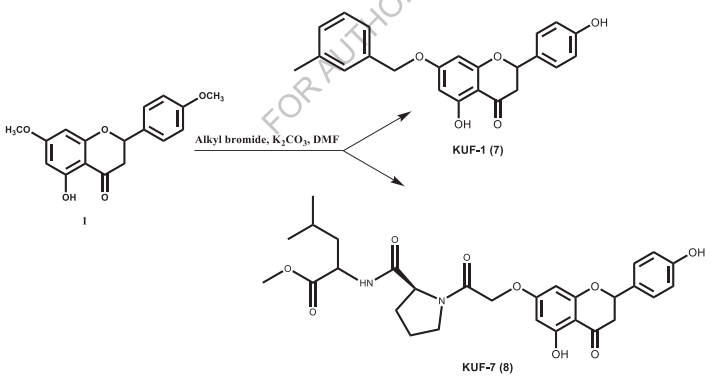


Scheme 1. (a) Z-$C_8H_{17}CH$=$CHC_7H_{14}COCl$, Et_3N, CH_2Cl_2; (b) $C_{16}H_{33}Br$, Na_2CO_3, DMF, 80^0 C.

In order to maintain the lipophilic and hydrophilic balance, the 7-hydroxyl group of naringenin was substituted with amino acids and to synthesise some 7-O-amino acid esters of naringenin. The synthesised compounds were tested for their Cyclin Dependent Kinase 2 capabilities[7].

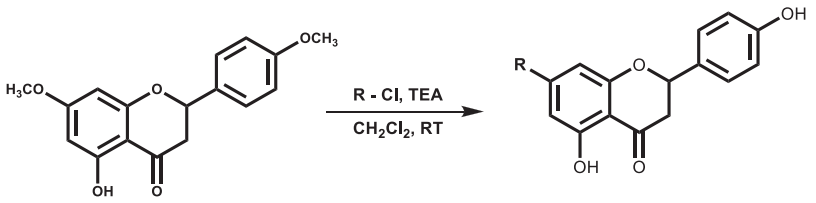


In order to determine the importance of the OH group or substitution of the 5 or carbon-7 of ring A of naringenin, several modified naringenin derivatives, including 7-O-benzyl naringenin (KUF-1) and 7-O-(MeO-L-Leu-D-Pro-carbonylmethyl) naringenin (KUF-7) were synthesised by Eung-Ryoung et al. KUF-1 and KUF-7 differentially regulate the apoptosis of A549 cells via intracellular ROS production coupled with the concomitant activation of the caspase cascade signaling pathway, thereby implying that hydroxylation or substitution at Carbon-7 is critical for the apoptosis-inducing activity of naringenin[8].



Several naringenin esters modified at position 7 with bulky substituents were designed and synthesized, and their inhibitory effects on HCT116 human colon cancer cells were tested using a clonogenic assay. The IC50 value of five naringenin

derivatives ranged between 1.20 μM and 20.01 μM which are much higher than naringenin used as a control [9].



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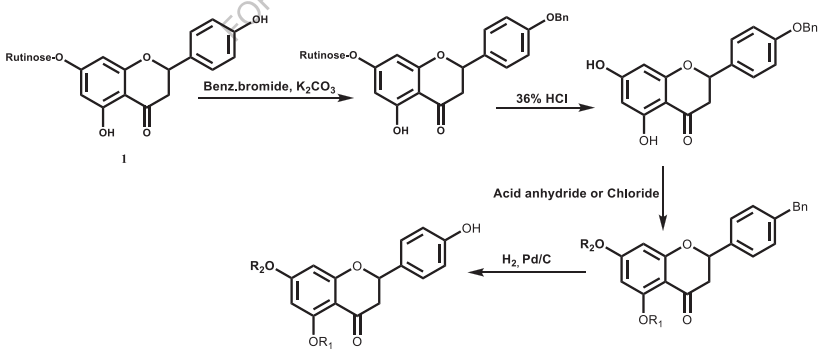
9. R = thiophenecarboxylate
10. R = methylbenzoate
11. R = isobutyrate
12. R = allyloxy
13. R = phenylcarbonate

Three acylated derivatives of naringenin such as naringenin-7-O-acetate (14), naringenin-5,7-O-di-propionate (15) and naringenin-7-O-valerate (16), were synthesized from naringin by employing a four step protocol consisting of benzylation-hydrolysis-acylation-hydrogenation.



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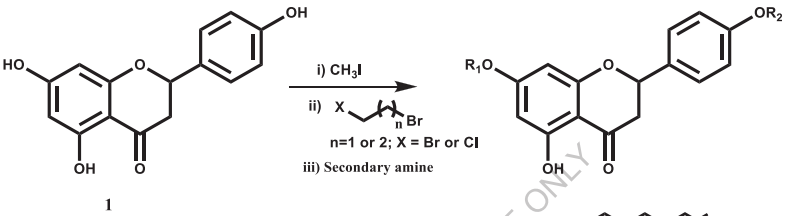
14. $R_1 = H$, $R_2 = COCH_3$
15. $R_1 = R_2 = COCH_2CH_3$
16. $R_1 = H$, $R_2 = COCH_2CH_2CH_2CH_3$

Interestingly the water solubility of synthesised compounds increased without affecting the anti-platelet aggregation activity of naringenin[10].

A series of naringenin derivatives with a tertiary amino side chain were prepared and evaluated their antiproliferative potential on four human cancer cell lines such as MCF-7, HCT116, Hela, and A549. Compounds **17-19** exhibited enhanced growth inhibition against Hela cervical cancer cell line[11].



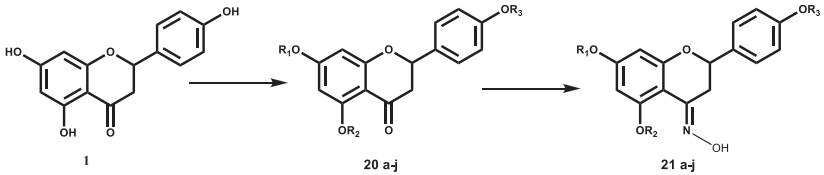
1

17. R_1 = ; R_2 = H

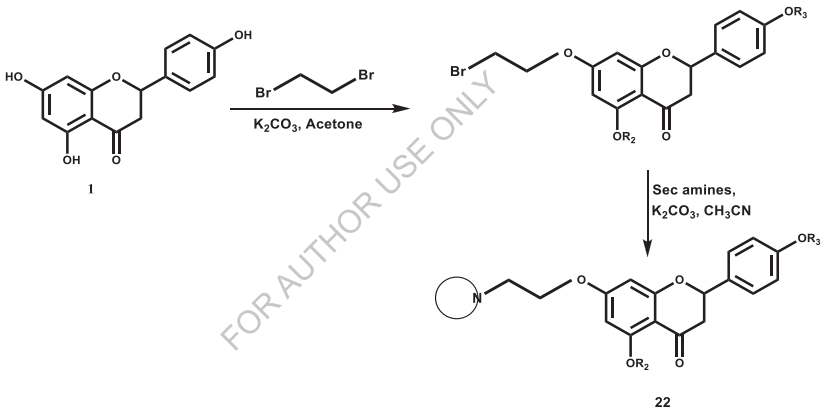
18. R_1 = CH_3 ; R_2 =

19. R_1 = CH_3 ; R_2 =

Ten O-alkyl derivatives (20a–j) of naringenin were synthesised from naringenin using the corresponding alkyl iodides and anhydrous potassium carbonate. The resulting products were used to synthesise ten oximes (21a–j). All compounds were tested for antimicrobial activity and found active against *E. coli* ATCC10536, *S. aureus* DSM799, *C.albicans* DSM1386, *A. alternata* CBS1526, *F. linii* KB-F1, and *A. niger* DSM1957[12].



Some new (±)-naringenin cyclic aminoethyl derivatives were synthesised and analyzed for cytotoxic and anti-proliferative activities using MTT assay. Some of the synthesised naringenin derivatives showed potential anticancer activity against human cervix and breast cancers[13].

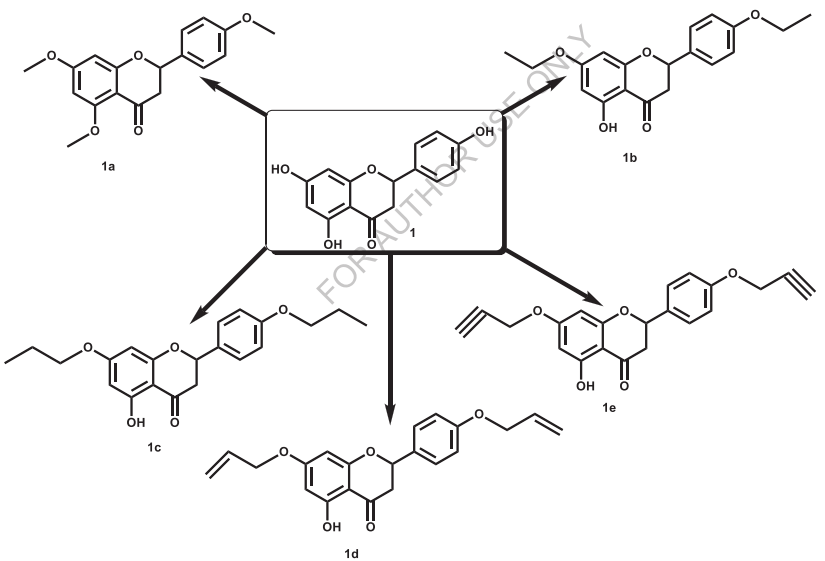


Among the alkylation and acylation reactions, the latter has a disadvantage as the product esters are susceptible to hydrolysis under strong acidic and basic conditions. In contrast the alkylated products are stable and induce sufficient lipophilicity, which is the desired parameter for enhanced therapeutic profiles of the lead molecules.

Present Work

From the above literature review it is evident that the three hydroxyls at 4',5 and 7 positions of naringenin influence its therapeutic potential. Further, alkylation at these positions increases the lipophilicity and enhance or induce newer biological activities of naringenin. With this objective, naringenin is now subjected to alkylation reactions with both saturated and unsaturated alkyl halides to synthesise a range of diverse alkyl ethers for detailed *in vitro* and *in vivo* α-glucosidase inhibitory activity.

The protocols employed to synthesise naringenin alkyl ethers are presented in Scheme 2C.01.



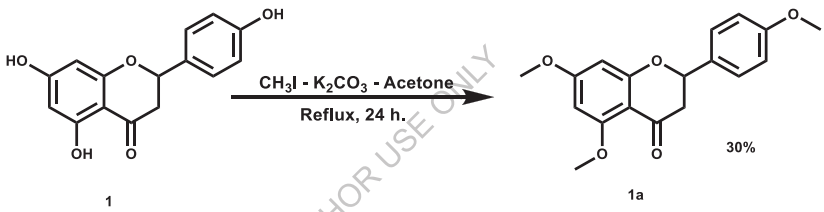
Scheme 2C.01 Protocols for synthesis of naringenin alkyl ethers

In total five alkyl ether analogues (**1a-1e**) of naringenin were synthesized in good to very good yields. Among the synthesized analogues, three are saturated alkyl (methyl, ethyl and propyl) ethers (**1a-1c**), whereas the other two are unsaturated

alkyl (allyl and proporgyl) ethers (**1d-1e**). The details of their synthesis are given below :

Synthesis of 4', 5, 7-trimethoxy-naringenin (1a)

Naringenin (1 eq.) was treated with methyl iodide (3 eq.) and K_2CO_3 (3 eq.) in acetone under reflux conditions for 24 hr. After usual work followed by chromatographic purification and crystallization afforded the product as colourless powder (30.07%), m. p. 126–129^0 C. It gave negative ferric chloride test suggesting the absence of phenolic hydroxyls in the compound. The IR spectrum (Figure 2C.04) of the compound showed strong absorption peak at 1669 cm^{-1} corresponding to



Scheme: 2C.02 Synthesis of 4',5,7-trimethoxy-naringenin

carbonyl functionality. These observation are confirmed by its ^{1}H NMR Spectrum [Figure 2C.03 (i)], which reveals the absence of peaks corresponding to phenolic hydroxyl protons. Most diagnostically, it showed the three methoxyl group protons as three sharp singlets at δ 3.90, 3.83 and 3.82 in addition to the naringenin 2-CH and 3-CH_2 peaks at δ 5.359 (dd) and a pair of doublets at δ 3.04 & 2.78. The ^{13}C NMR spectrum [Figure 2c.03 (ii)], while confirming these observations showed the characteristic peaks at δ 189.1 (carbonyl) and 56.1, 55.59 & 55.39 (C_7, C_5 & $C_{4'}$-3xmethoxyls). Its molecular formula was deduced from its HRESI mass spectra (Figure 2C.05) as $C_{18}H_{18}O_5$, which showed the pseudo molecular ion $[M+H]^+$ at m/z

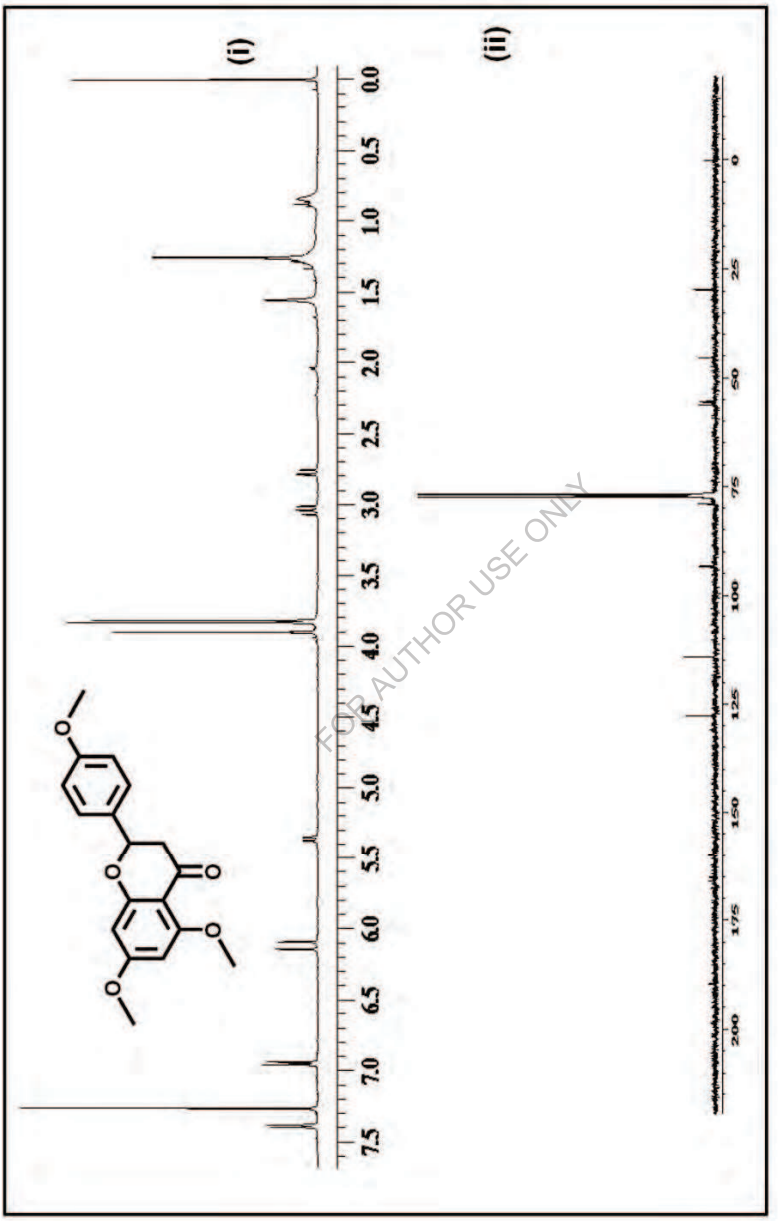


Figure 2C.03 : NMR Spectra of Compound 1a in $CDCl_3$ (i) ^1H (500 MHz); (ii) ^{13}C (100 MHz)

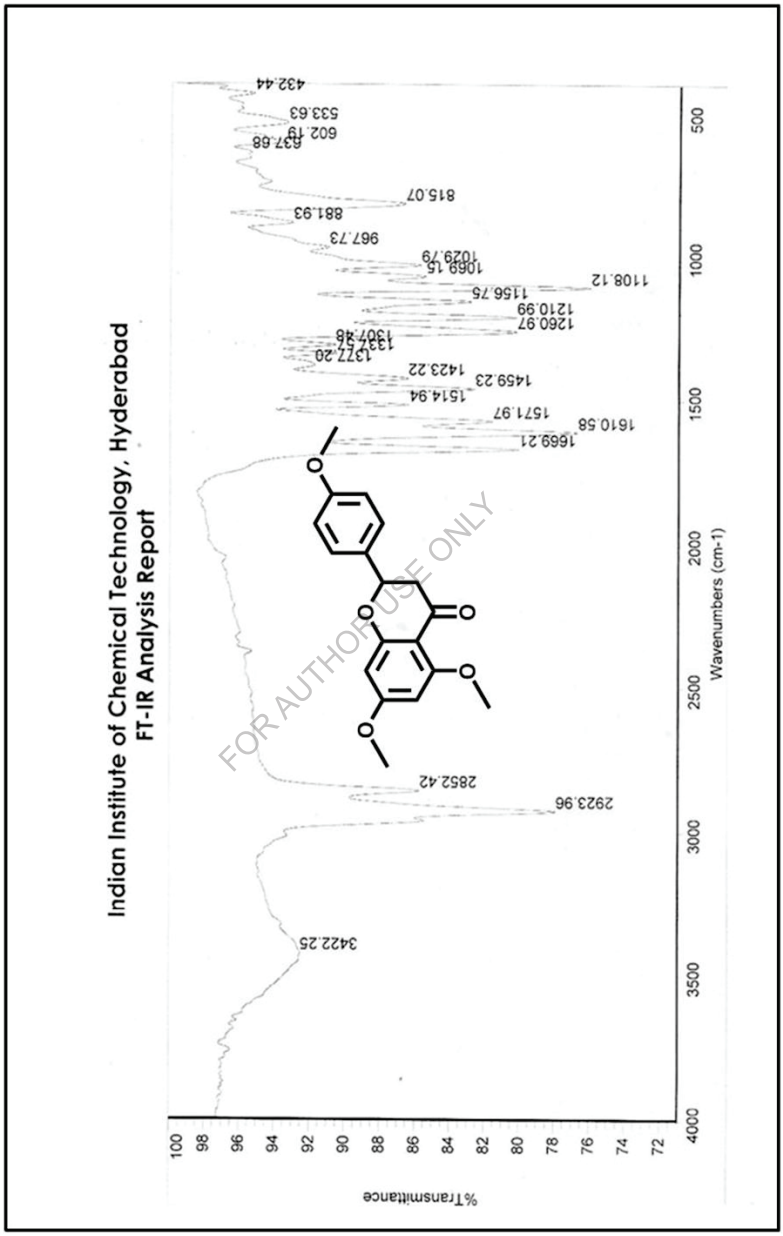


Figure 2C.04 : IR Spectrum of Compound 1a

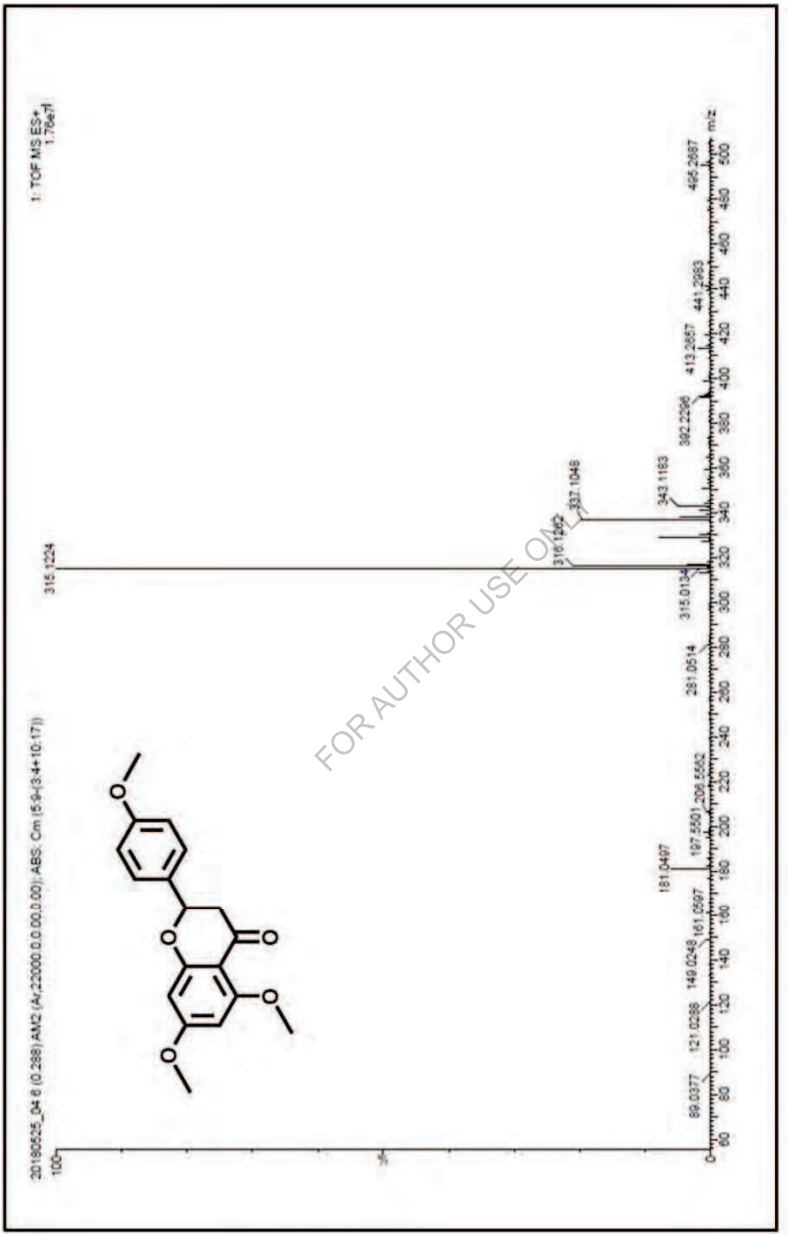
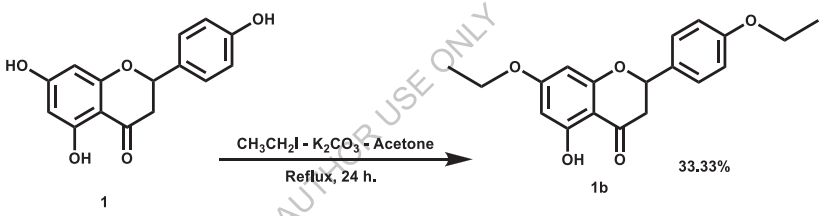


Figure 2C.05 : HRMS Spectrum of Compound 1a

315.1224. Based on the above spectral data and comparison with literature values, this compound was identified as 4',5,7-trimethoxy-naringenin (**1a**).

Synthesis of 5, 7-diethoxy-naringenin (1b)

Naringenin (1 eq.) was treated with ethyl iodide (3 eq.) and K_2CO_3 (3 eq.) in acetone under reflux conditions for 24 hr. After usual work followed by chromatographic purification and crystallization afforded the product as colourless powder (33.35%), m. p. 97–102^0 C. It gave positive ferric chloride test suggesting the presence of phenolic hydroxyl group in the compound. The IR spectrum (Figure 2C.07) of the compound showed strong absorption peak at 1638 cm^{-1} corresponding to



Scheme: 2C.03 Synthesis of 5,7-diethoxy-naringenin (1b)

hydrogen bonded carbonyl functionality. These observation are confirmed by its ^{1}H NMR Spectrum [Figure 2C.06 (i)], which showed a peak at δ 12.0 corresponding to a chelated hydroxyl proton. Most diagnostically, it showed a triplet at δ 1.41 (6H) and a quartet at δ 4.05 (2H) corresponding to two ethyl groups in addition to the naringenin 2-CH and 3-CH_2 peaks at δ 5.36 (dd) and a pair of doublets at δ 3.09 & 2.79. The ^{13}C NMR spectrum [Figure 2c.06 (ii)] while confirming these observations showed the characteristic peaks at d 196.0 (carbonyl) and three oxygenated carbon signal at δ 78.9, 64.0, 63.5 (C_2, $C_{1'}$ & $C_{1''}$). Its molecular formula was deduced from its HRESI

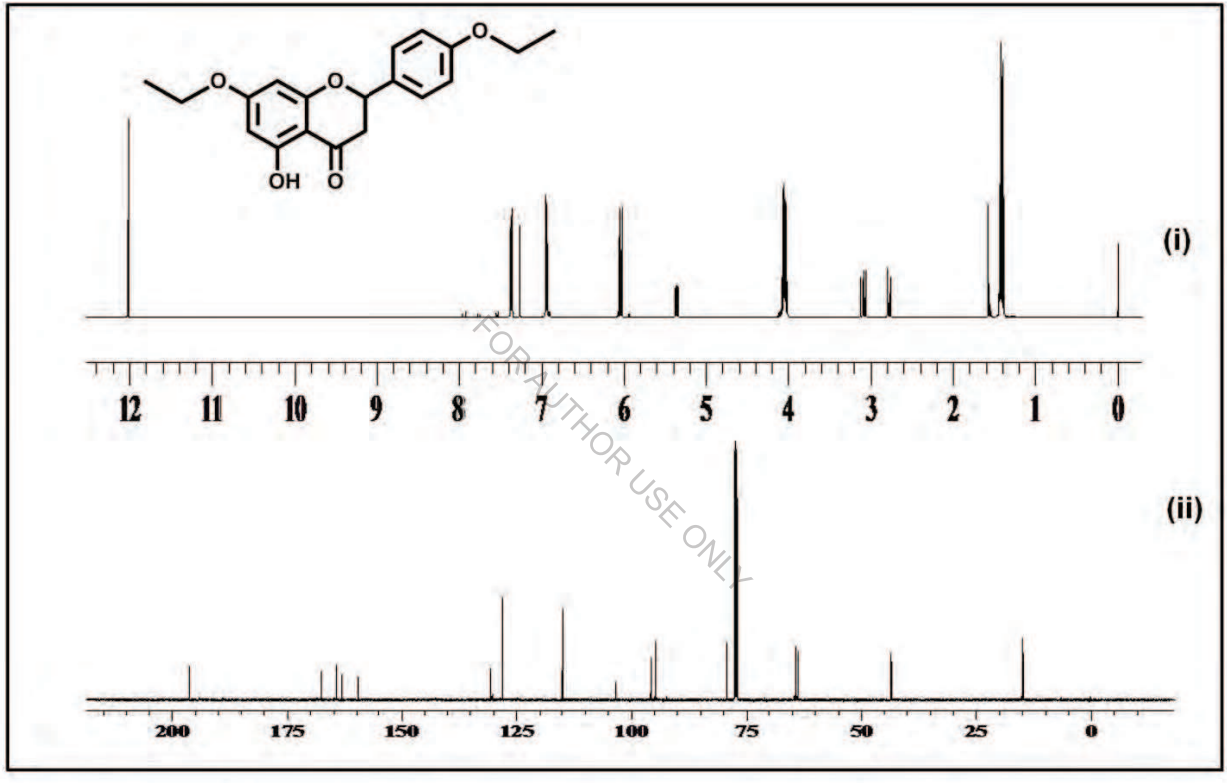


Figure 2C.06 : NMR Spectra of Compound 1b in $CDCl_3$ (i) ^1H (500 MHz); (ii) ^{13}C (125 MHz)

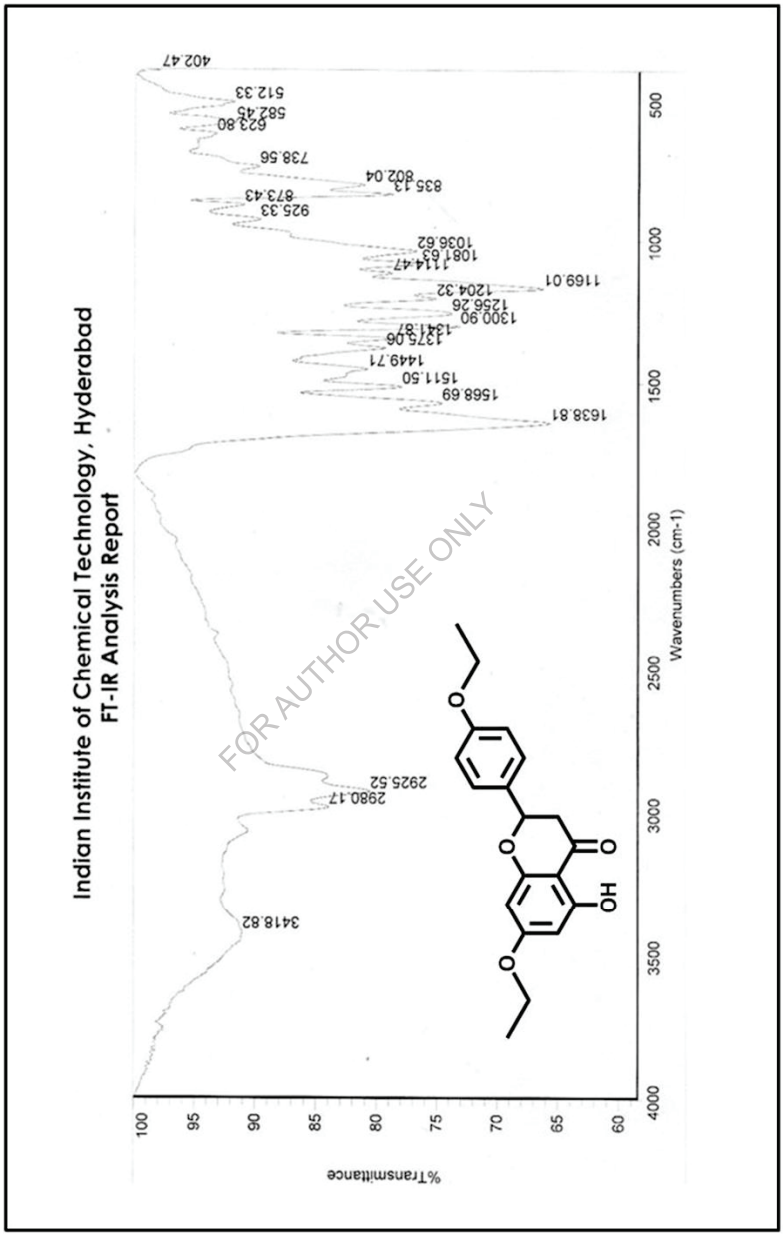


Figure 2C.07 : IR Spectrum of Compound 1b

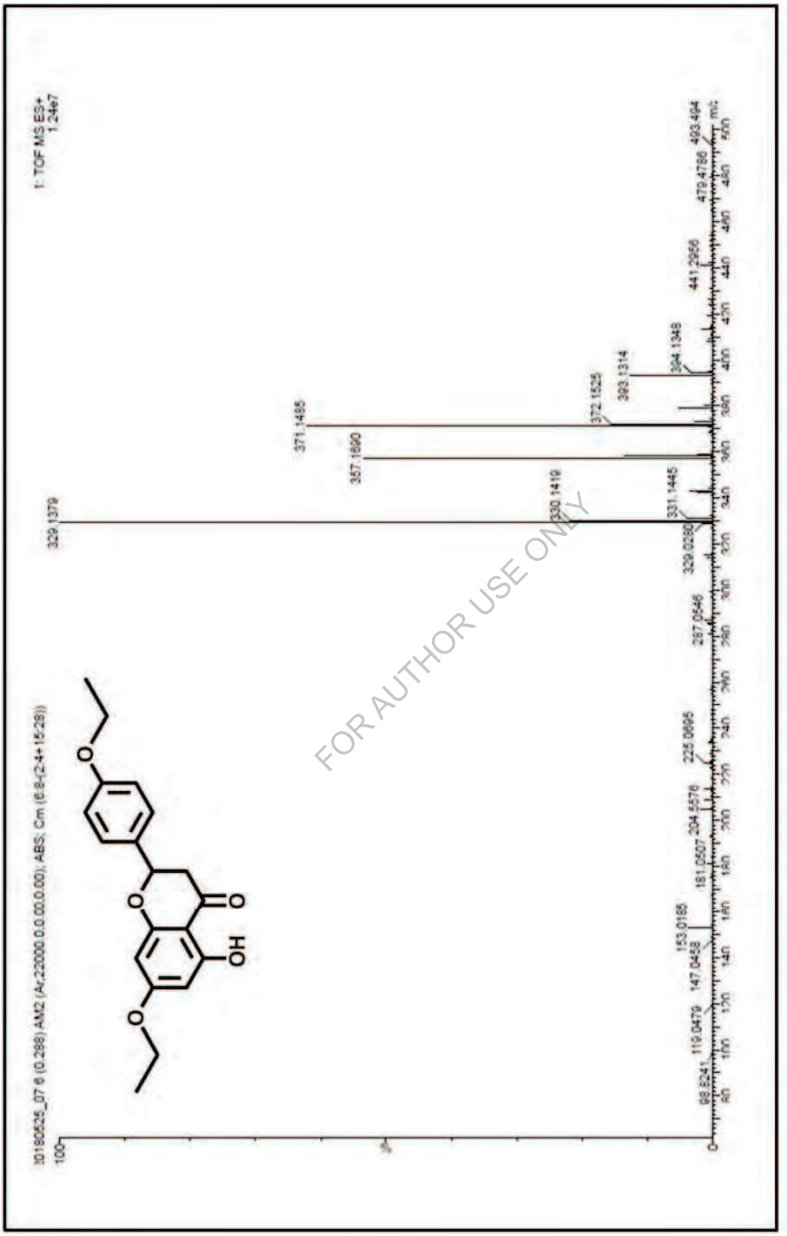
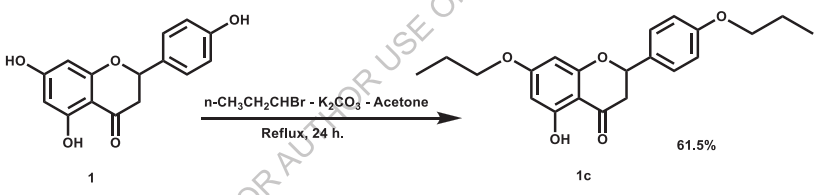


Figure 2C.08 : HRMS Spectrum of Compound 1b

mass spectra (Figure 2C.08) as $C_{19}H_{20}O_5$, which showed the pseudo molecular ion $[M+H]^+$ at m/z 329.1379. Based on the above spectral data and comparison with literature values, this compound was identified as 5,7-diethoxy-naringenin (**1b**).

Synthesis of 5, 7-dipropyloxy-naringenin (1c)

Naringenin (1 eq.) was treated with propyl bromide (3 eq.) and K_2CO_3 (3 eq.) in acetone under reflux conditions for 24 hr. After usual work followed by chromatographic purification and crystallization afforded the product as colourless powder (61.5%)., m. p. 122^0 C. It gave positive ferric chloride test suggesting the presence of phenolic hydroxyl group in the compound. The IR spectrum (Figure 2C.10) of the compound showed strong absorption peak at 1631 cm^{-1}



Scheme: 2C.04 Synthesis of 5,7-dipropyloxy-naringenin (1c)

corresponding to hydrogen bonded carbonyl functionality. These observation are confirmed by its ^{1}H NMR Spectrum [Figure 2C.09 (i)], which showed a peak at δ 12.02 corresponding to a chelated hydroxyl proton. Most diagnostically, it showed a triplet-multiplet-triplet system at δ 3.93(4H), 1.81(4H) and 1.01(6H) corresponding to two propyl groups in addition to the naringenin 2-CH and 3-CH2 peaks at δ 5.35 (dd) and a pair of doublets at δ 3.08 & 2.80. The ^{13}C NMR spectrum [Figure 2C.09 (ii)] while confirming these observations showed the characteristic peaks at d 195.5 (carbonyl) and two oxygenated carbon signal at δ 78.2 (C2), 69.6 (C1' & C1"). Its

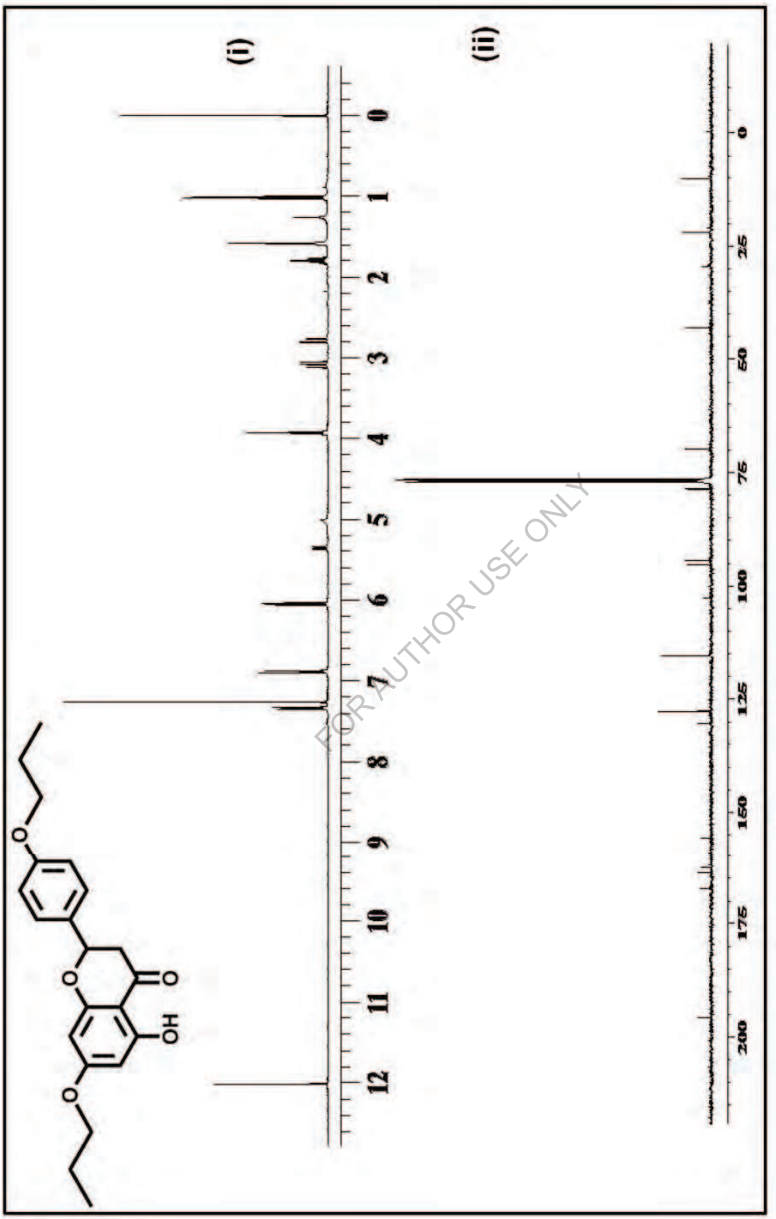


Figure 2C.09 : NMR Spectra of Compound 1c in $CDCl_3$ (i) ^1H (500 MHz); (ii) ^{13}C (100 MHz)

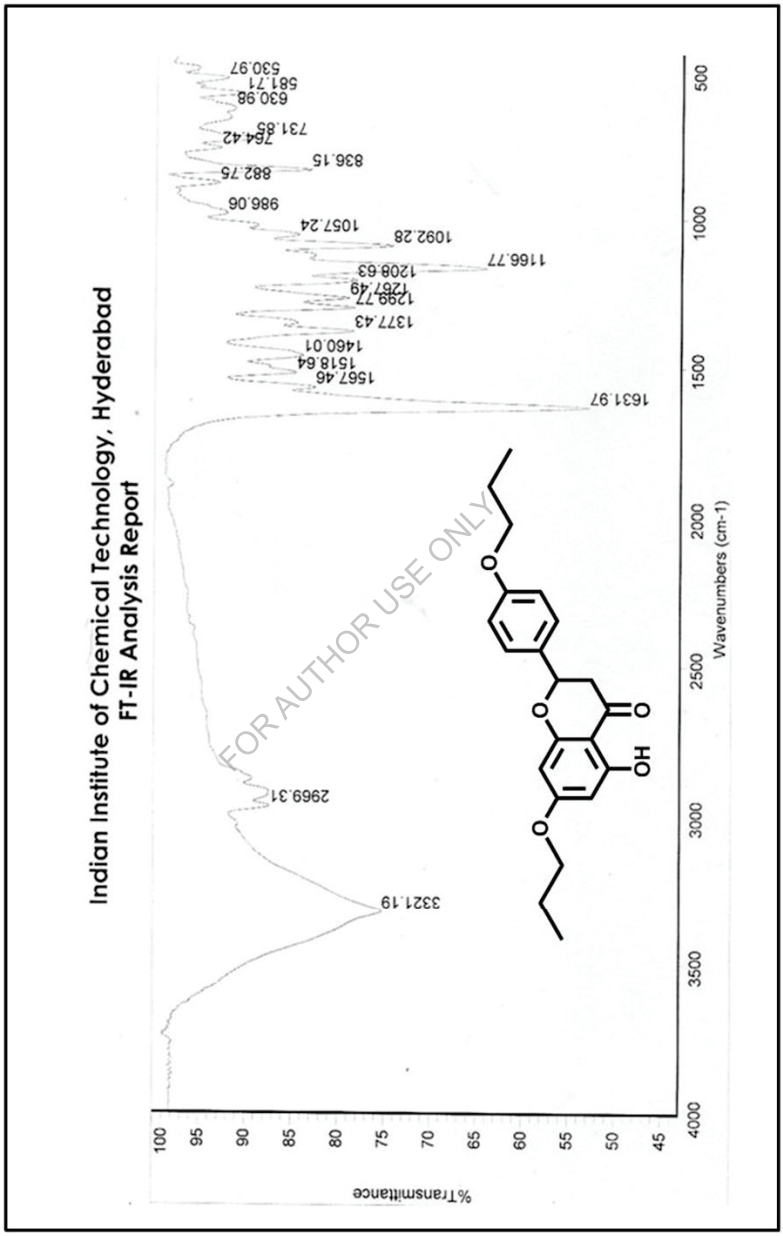


Figure 2C.10 : IR Spectrum of Compound 1c

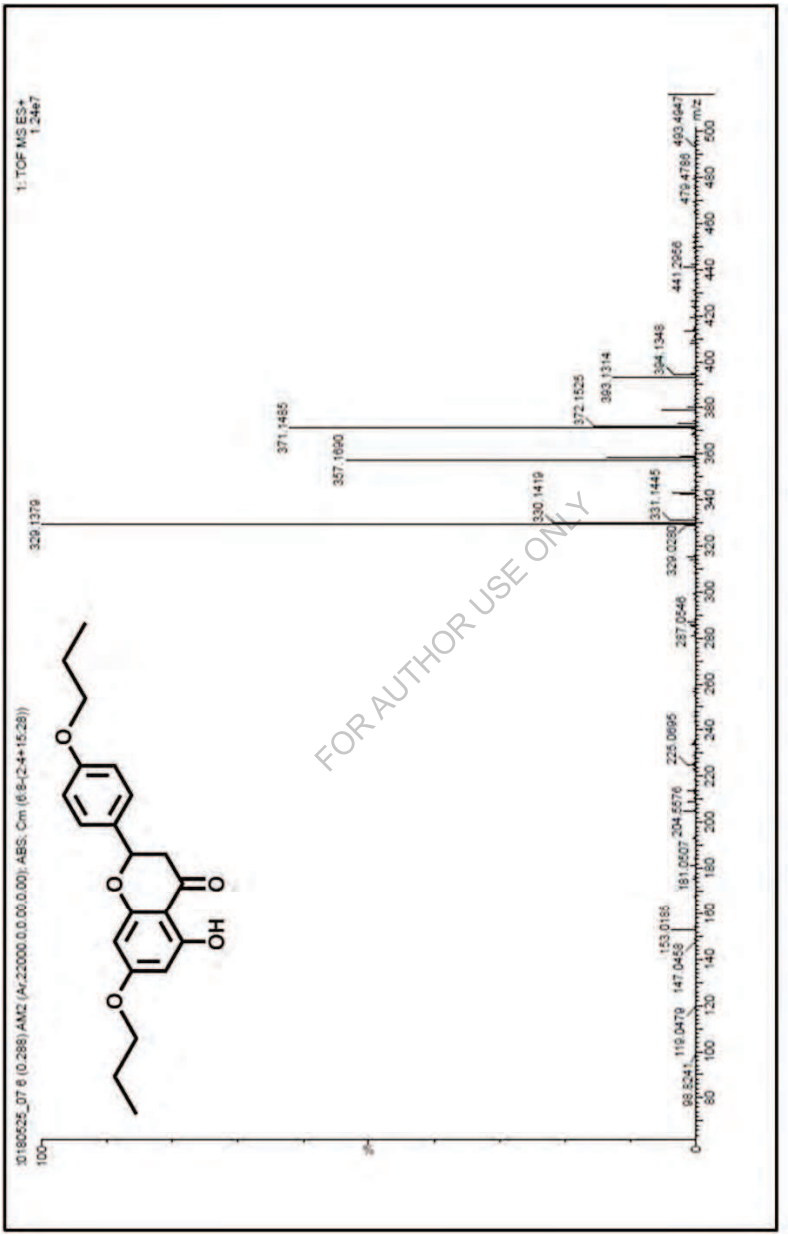
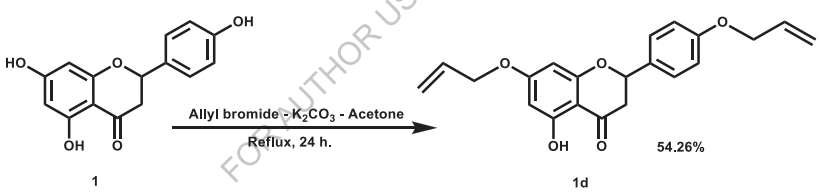


Figure 2C.11 : HRMS Spectrum of Compound 1c

molecular formula was deduced from its HRESI mass spectra (Figure 2C.11) as $C_{21}H_{24}O_5$, which showed the pseudo molecular ion [M+H]+ at m/z 357.1690. Based on the above spectral data and comparison with literature values, this compound was identified as 5,7-dipropyloxy-naringenin (**1c**).

Synthesis of 5, 7-diallyloxy-naringenin (1d)

Naringenin (1 eq.) was treated with allyl bromide (3 eq.) and K_2CO_3 (3 eq.) in acetone under reflux conditions for 24 hr. After usual work followed by chromatographic purification and crystallization afforded the product as colourless powder (54.20%), m. p. 98^0 C. It gave positive ferric chloride test suggesting the presence of phenolic hydroxyl group in the compound. The IR spectrum (Figure 2C.13) of the compound showed strong absorption peak at 1636 cm^{-1}



Scheme: 2C.05 Synthesis of 5,7-diallyloxy-naringenin (1d)

corresponding to hydrogen bonded carbonyl functionality. These observation are confirmed by its ^{1}H NMR Spectrum [Figure 2C.12 (i)], which showed a peak at δ 12.0 corresponding to a chelated hydroxyl proton. Most diagnostically, it showed a set of multiplets at δ 6.05 (2H), 5.42 (4H) and 4.55 (4H) corresponding to two allyloxy groups in addition to the naringenin 2-CH and 3-CH2 peaks at δ 5.32 (dd) and a pair of doublets at δ 3.08 & 2.80. The ^{13}C NMR spectrum [Figure 2C.12 (ii)] while confirming these observations showed the characteristic peaks at d 195.7 (carbonyl)

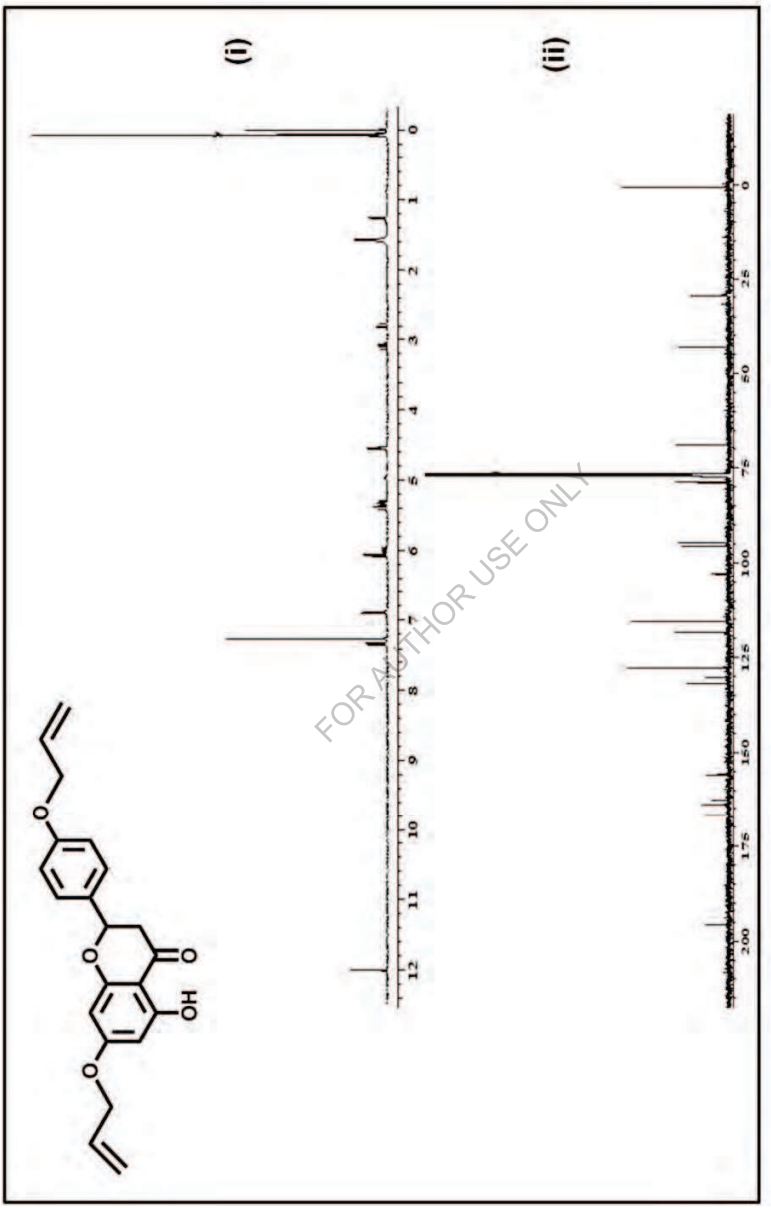


Figure 2C.12 : NMR Spectra of Compound 1d in $CDCl_3$ (i) ^1H (400 MHz); (ii) ^{13}C (125 MHz)

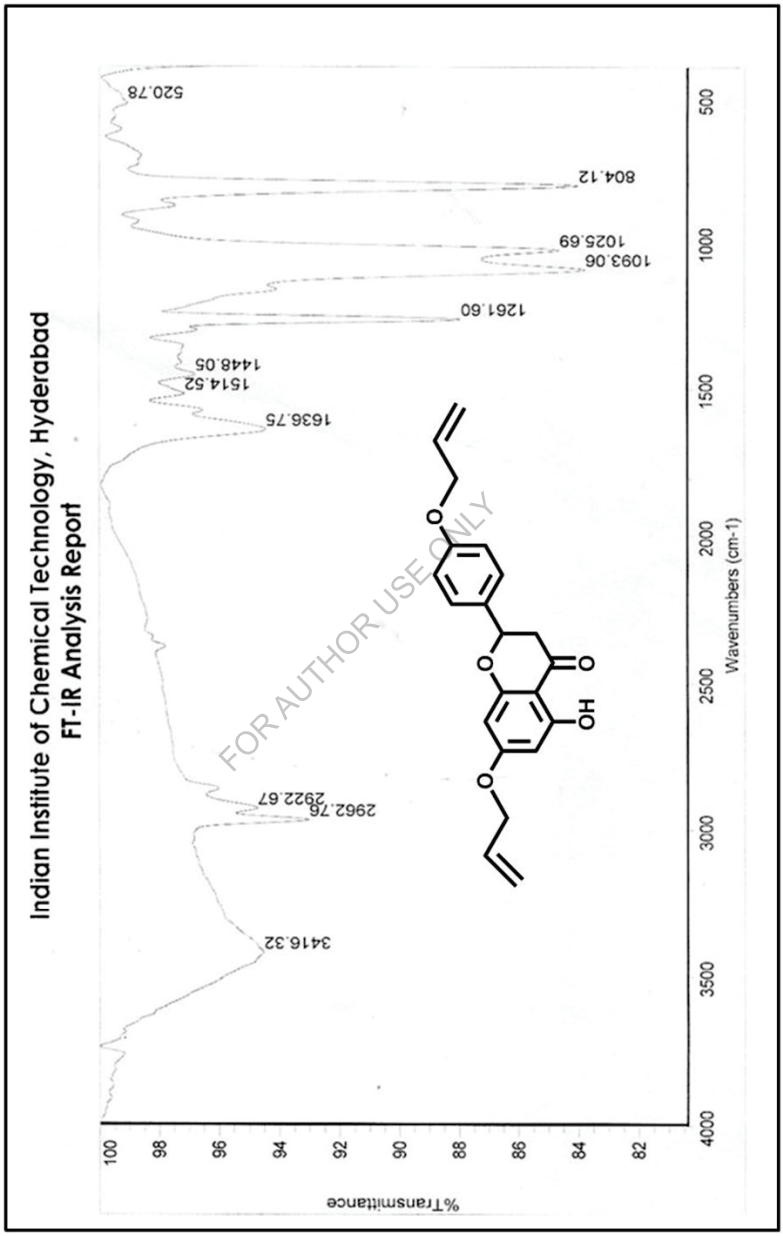


Figure 2C.13 : IR Spectrum of Compound 1d

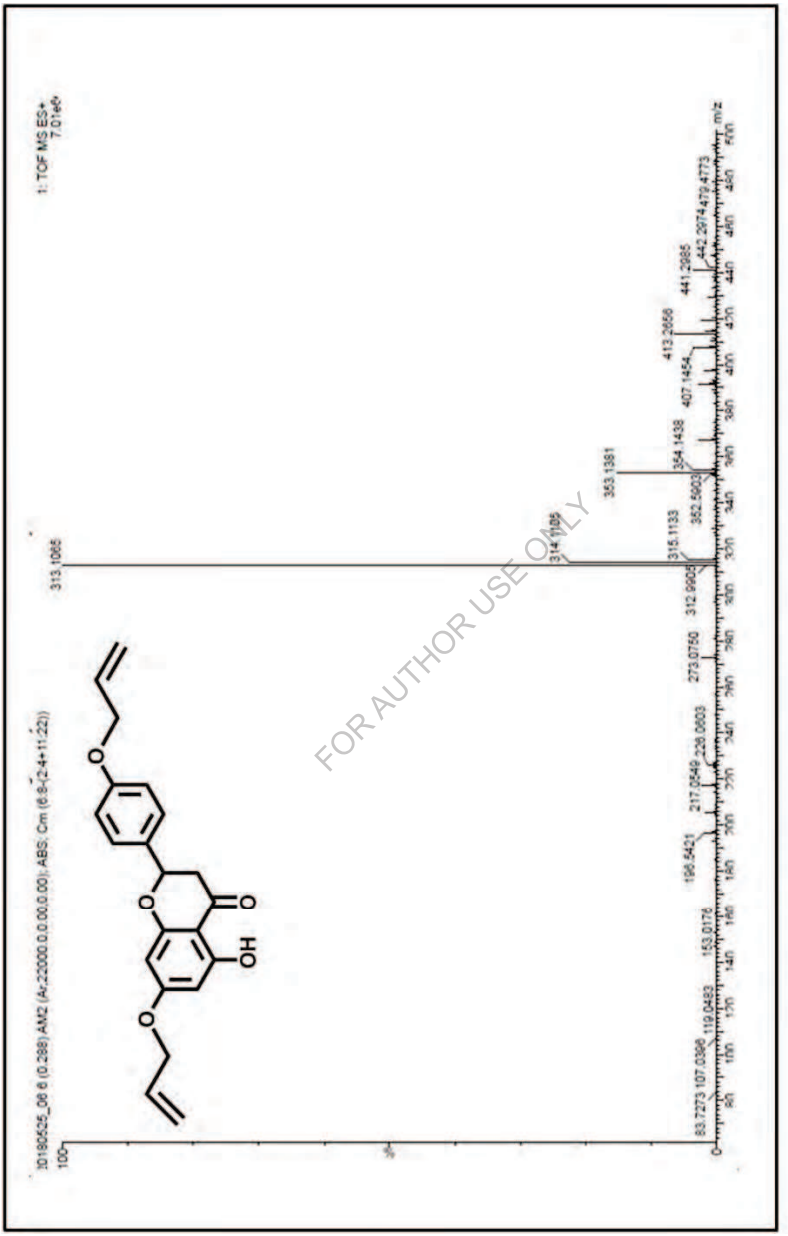
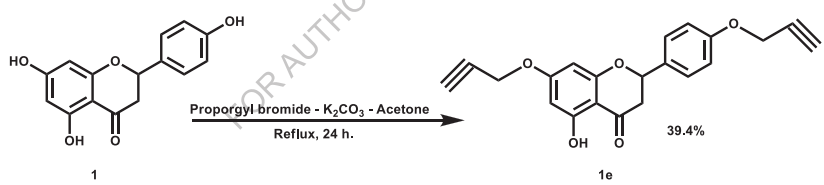


Figure 2C.14 : HRMS Spectrum of Compound 1d

and three oxygenated carbon signal at δ 78.6 (C2), (68.8 C1' & C1"). Its molecular formula was deduced from its HRESI mass spectra (Figure 2C.14) as $C_{21}H_{20}O_5$, which showed the pseudo molecular ion [M+H]+ at m/z 353.1381. Based on the above spectral data and comparison with literature values, this compound was identified as 5,7-diallyloxy-naringenin (**1d**)

Synthesis of 5,7-dipropargyloxy-naringenin (1e)

Naringenin (1 eq.) was treated with ethyl iodide (3 eq.) and K_2CO_3 (3 eq.) in acetone under reflux conditions for 24 hr. After usual work followed by chromatographic purification and crystallization afforded the product as colourless powder (39.30%), m. p. 273^0 C (decomp.). It gave positive ferric chloride test suggesting the presence of phenolic hydroxyl group in the compound. The IR spectrum (Figure 2C.16) of the compound showed strong absorption peaks at 2121 and 1638 cm^{-1}



Scheme: 2C.06 Synthesis of 5,7-dipropargyloxy-naringenin (1e)

corresponding to acetylenic and hydrogen bonded carbonyl functionalities respectively. These observation are confirmed by its ^{1}H NMR Spectrum [Figure 2C.15 (i)], which showed a peak at δ 12.0 corresponding to a chelated hydroxyl proton. Most diagnostically, it showed a singlet at δ 2.57 and a multiplet at δ 4.69 corresponding to acetylenic group in addition to the naringenin 2-CH and 3-CH2 peaks at δ 5.37 (dd) and a pair of doublets at δ 3.10 & 2.79. The ^{13}C NMR spectrum

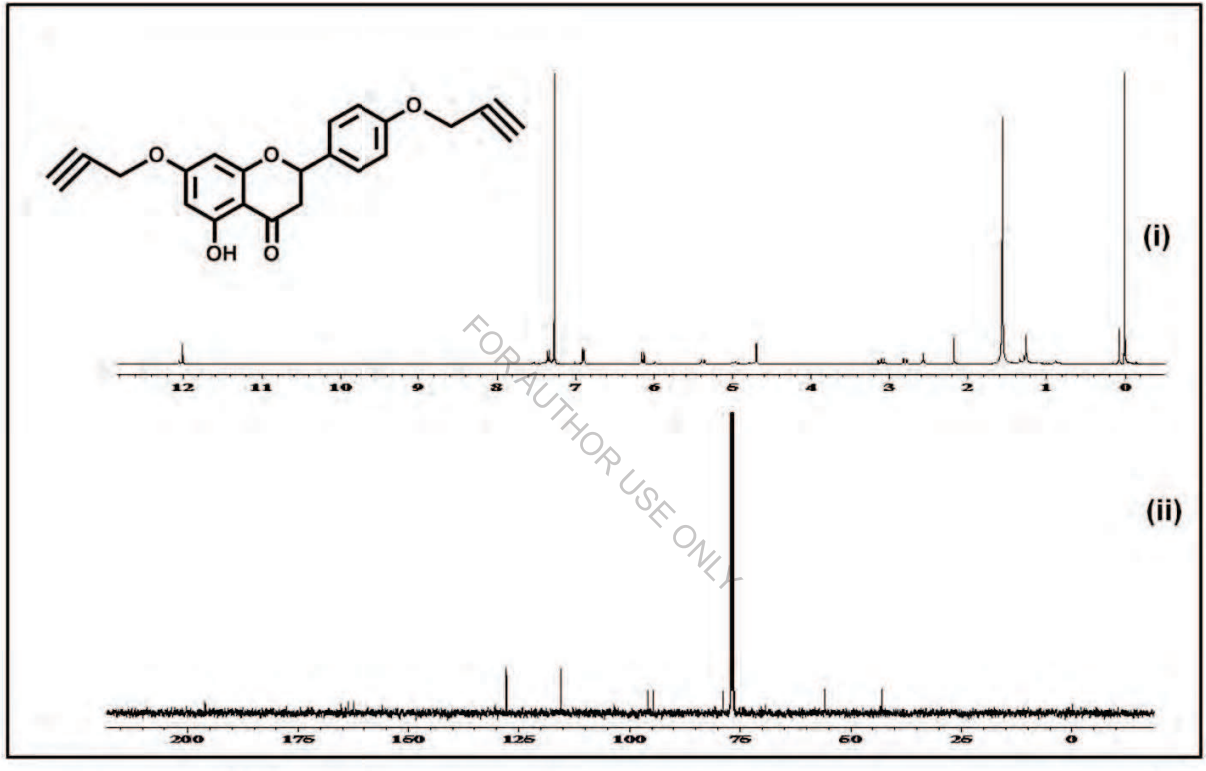


Figure 2C.15 : NMR Spectra of Compound 1e in $CDCl_3$ (i) ^1H (400 MHz); (ii) ^{13}C (125 MHz)

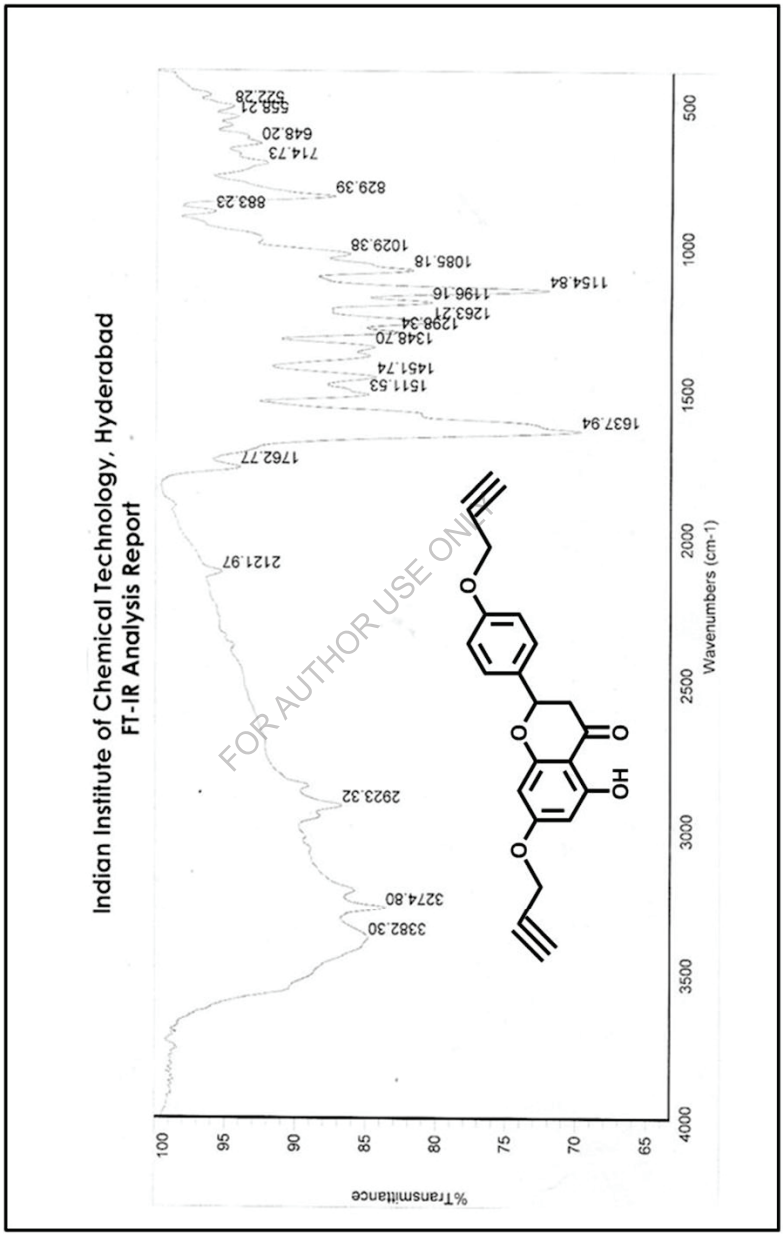


Figure 2C.16 : IR Spectrum of Compound 1e

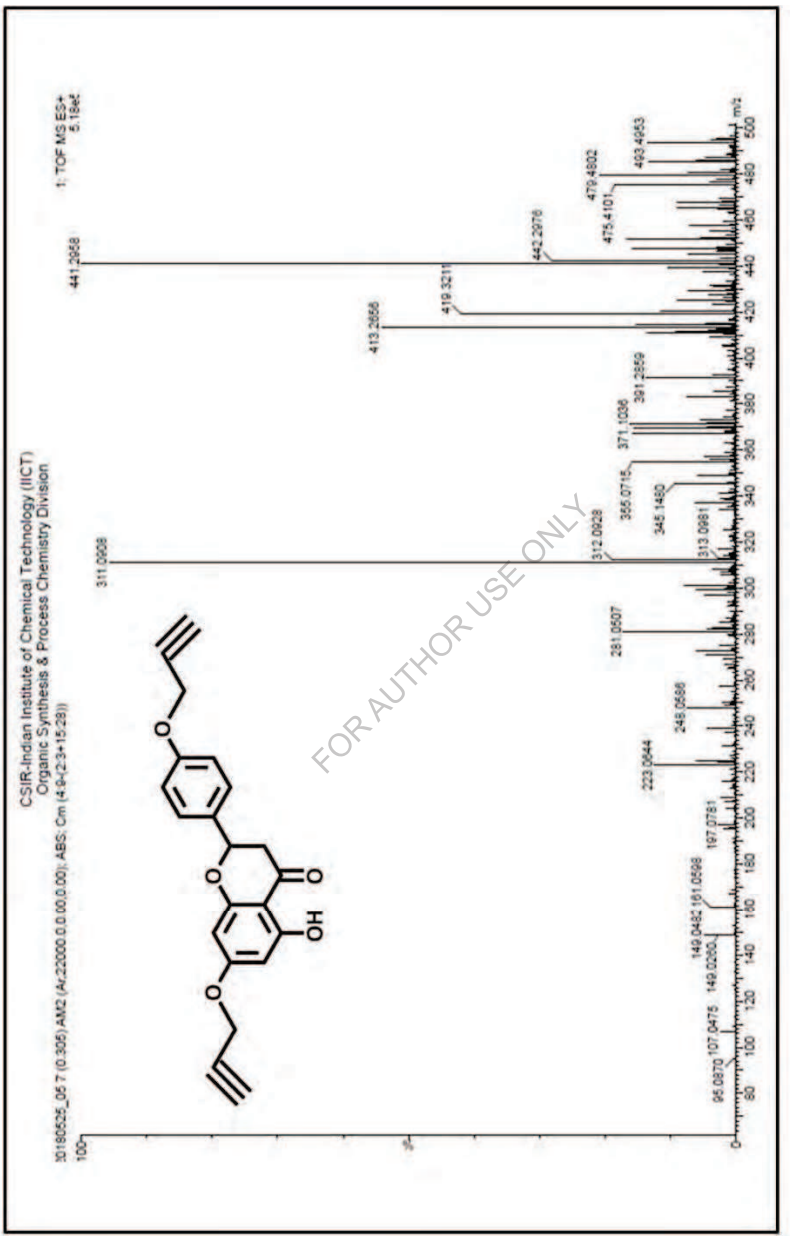


Figure 2C.17 : HRMS Spectrum of Compound 1e

[Figure 2C.15 (ii)] while confirming these observations showed the characteristic peaks at d 196.1 (carbonyl) and the propargylyoxy carbon signals at δ 78.6, 76.2 and 56.9. Its molecular formula was deduced from its HRESI mass spectra (Figure 2C.17) as $C_{21}H_{16}O_5$, which showed the pseudo molecular ion [M+Na]+ at m/z 371.1036. Based on the above spectral data and comparison with literature values, this compound was identified as 5,7-dipropargyloxy-naringenin (**1e**).

Conclusion

As naringenin is gaining importance as 'Hot Molecule' in view of its high natural abundance, interesting skeletal features and wide range of therapeutic applications, its chemical modification is now taken up to synthesise some diverse ether analogues. In total five alkyl ethers were synthesized in good to excellent yields. Out of the synthesized ether analogues, three are saturated (methyl, ethyl, propyl) and two are unsaturated (allyl, propargyl) alkyl ethers. All the synthesized ether analogues were characterized by their physical and spectroscopic data.

Experimental

General procedures for the synthesis of naringenin **(1**) *saturated* **(1a-1c)** *and unsaturated* **(1d & 1e)** *ether analogues*

Naringenin (0.1 g, 1 eq.) was treated with appropriate alkyl halide (0.130~0.171 g, 3 eq.) in the presence of K_2CO_3 (0.16 g, 3 eq.) in acetone (5.0 ml) for 24 h. After completion of the reaction as indicated by TLC, the reaction mixture was extracted with ethyl acetate (3 x 5 ml). The combined organic layer was washed with water (3 x 5 ml), dried over anhydrous sodium sulphate, filtered and concentrated under reduced pressure to give crude product, which was chromatographed over silica gel column to yield pure compounds (**1a-1e**). The structures of the pure compounds were established by their physical and spectroscopic (IR, 1H & 13C NMR and Mass).

4', 5, 7-trimethoxy-naringenin (1a)

Naringenin (0.1 g, 1 eq.) was treated with methyl iodide (0.156, 3 eq.) and K_2CO_3 (0.152g, 3 eq.) in acetone (5 ml) under reflux conditions for 24 hr. After usual work followed by chromatographic purification and crystallization afforded the product as colourless powder (35 mg, 30.07%)., m.p. 126–129^0 C. It gave negative ferric chloride test suggesting the absence of phenolic hydroxyls in the compound.

IR (KBr, cm^{-1}) : 3422, 1669, 1610 and 1571.

HRESI Mass (m/z) : 315.1224 $[M+H]^+$; M.F. : $C_{18}H_{18}O_5$

^{1}H NMR ($CDCl_3$, 500 MHz) : *δ*:7.39 (2H, d, J=8.69 Hz), 6.94 (2H, d, J=8.69 Hz), 6.14 (1H, d, J=2.3, Hz), 6.09 (1H, d, J=2.29 Hz), 5.35 (1H, dd, J=2.89,13.27 Hz), 3.90 (3H, s), 3.83 (3H, s), 3.82 (3H, s), 3.04 (1H, dd,

J=3.35,16.47 Hz), 2.78 (1H, dd, J=2.89,16.5 Hz).

^{13}C NMR ($CDCl_3$, 100 MHz) : *δ*: 189.1 (C4), 166.0 (C7), 162.0 (C5), 159.0 (C4'), 131.0 (C-1′), 127.6 (C-2′,6'), 114.3 (C3′,5'), 107.0 (C10), 93.5 (C8), 93.1 (C6), 78.9 (C2), 56.1 (-O<u>C</u>H$_3$), 55.59 (-O<u>C</u>H$_3$), 55.39 (-OCH3), 45.0 (C3)

5, 7-diethoxy-naringenin (1b)

Naringenin (0,1 g, 1 eq.) was treated with ethyl iodide (0.171 g, 3 eq.) and K_2CO_3 (0,152 g, 3 eq.) in acetone (5 ml) under reflux conditions for 24 hr. After usual work followed by chromatographic purification and crystallization afforded the product as colourless powder (40 mg, 33.35%)., m.p. 97–102^0 C. It gave positive ferric chloride test suggesting the presence of phenolic hydroxyl group in the compound.

IR (KBr, cm^{-1}) : 3418, 1638, 1568, and 1375.

HRESI Mass (m/z) : 329.1379 $(M+H)^+$; M.F. : $C_{19}H_{20}O_5$

^{1}H NMR ($CDCl_3$, 500 MHz) : *δ*: 12.0 (1H, s), 7.37 (2H, d, J=8.6 Hz), 6.95 (2H, d, J=8.69 Hz), 6.05 (2H, dd, J=2.28,11.139 Hz), 5.36 (1H, dd, J=3.05,13.122 Hz), 4.05 (4H, m), 3.09 (1H, dd, J=4.12, 30.21), 2.79 (1H, dd, J=3.05,17.24) 1.41 (6H, m).

^{13}C NMR ($CDCl_3$, 125 MHz) : *δ*: 196.0 (C4), 167.3 (C7), 162.8 (C5), 159.3 (C9), 130.2 (C-1′), 127.6 (C-2′, 6'), 114.7 (C-3′, 5'), 103.0 (C10), 95.4 (C6), 94.5 (C8), 78.9 (C2), 64.0 (C-1″), 63.5 (C-1″), 43.1 (C3), 14.7 (C-2″),

14.5 (C-2″).

5, 7-dipropyloxy-naringenin (1c)

Naringenin (0.1 g, 1 eq.) was treated with propyl bromide (0.135 g, 3 eq.) and K_2CO_3 (0.152 g, 3 eq.) in acetone 95 ml) under reflux conditions for 24 hr. After usual work followed by chromatographic purification and crystallization afforded the product as colourless powder (80 mg, 61.5%)., m.p. 122^0 C. It gave positive ferric chloride test suggesting the presence of phenolic hydroxyl group in the compound.

IR (KBr, cm^{-1}) : 3321,1631,1567,1460 and 1377.

EI Mass (m/z) : 357.1690 $(M+H)^+$; M.F. $C_{21}H_{24}O_5$

^{1}H NMR ($CDCl_3$, 500 MHz) : *δ*:5.35 (d, 2-H), 3.08 & 2.80 (each dd, 3-H), 12.02 (5-OH), 6.03 (d, 6-H), 3.93 (t, 1''-H), 1.81 (m, 2''-H), 1.01 (t, 3''-H), 6.06 (d, 8-H), 3.93 (t, 1'''-H), 1.81 (m,2'''-H), 1.01 (t, 3'''-H), 6.89 (H-2', H-6'), 7.34 (H-3', H-5').

^{13}C NMR ($CDCl_3$, 125 MHz) : *δ*: 195.5 (C4), 167.2 (C7), 163.7 (C5), 162.5 (C9), 155.7 (C4′), 130.3 (C1'), 127.6 (C2′,C6'), 115.3 (C3′, C5'), 102.6 (C10), 95.2 (C6), 94.2 (C8), 78.2 (C2), 69.6 (C1′ ′, C1'''), 21.9 (C2″, C2'''), 10.0 (C3″, C3''').

5, 7-diallyloxy-naringenin (1d)

Naringenin (0,1 g, 1 eq.) was treated with allyl bromide (0.133 g, 3 eq.) and K_2CO_3 (0.152 g, 3 eq.) in acetone (5 ml) under reflux conditions for 24 hr. After usual work followed by chromatographic purification and crystallization afforded the product as

colourless powder (70 mg, 54.20%), m. p. 98^0 C. It gave positive ferric chloride test suggesting the presence of phenolic hydroxyl group in the compound.

IR (KBr, cm^{-1}) : 3416, 1636, 1514, 1448 and 1261.

HRESI Mass (m/z) : 353. 1381 $(M+H)^+$; M.F. $C_{21}H_{20}O_5$

^{1}H NMR ($CDCl_3$, 300 MHz) : δ 2.80 (1H, dd, J=3.05, 17.11 Hz), 3.08 (1H, dd, J=12.16,17.11 Hz), 4.53 (1H, t, J=1.34Hz), 4.55 (1H, t, J=1.46 Hz), 5.32 (1H, dd, J=3.05 Hz), 5.42 (2H, d, J=1.22 Hz), 5.99 (1H, m), 6.05 (1H, d, J=2.32 Hz), 6.08 (2H, d, J=2.32 Hz), 6.90 (2H, d, J=8.55 Hz), 7.33 (2H, d, J=8.43 Hz),12 (1H, s).

^{13}C NMR ($CDCl_3$, 125 MHz) : δ:195.7 (C4), 166.6 (C7), 163.7 (C5), 162.1 (C9), 155.8 (C4′), 131.7 (C2", C2"'), 130.2 (C1′), 127.6 (C2′, C6'), 118.1 (C3′, C5'), 115.4 (C3", C3"'), 95.5 (C6), 94.5 (C8), 78.6 (C2), 68.8 (C1", C1"'), 42.9 (C3).

5,7-dipropargyloxy-naringenin (1e)

Naringenin (0.1, 1 eq.) was treated with propargyl bromide (0.130 g, 3 eq.) and K_2CO_3 (0.152 g, 3 eq.) in acetone (5 ml) under reflux conditions for 24 hr. After usual work followed by chromatographic purification and crystallization afforded the product as colourless powder (50 mg, 39.30%)., m. p. 273^0 C (decomp.). It gave positive ferric chloride test suggesting the presence of phenolic hydroxyl group in the compound.

IR (KBr, cm^{-1})	:	3382, 1762, 1637 and 2121.
HRESI Mass (m/z)	:	371.1036 $(M+Na)^+$; M.F. $C_{21}H_{16}O_5$
^1H NMR ($CDCl_3$, 500 MHz)	:	δ 5.37 (d, 2-H), 2.79 & 3.10 (each dd 3-H), 12.0 (5-OH.), 6.12 (d, 6-H), 4.69 (s, C1", C1"'), 2.57 (s, C3", C3"'), 6.15 (d, 8-H), 6.90 (d, 2'-H, 6'-H), 7.36 (d, 3'-H, 5'-H) .
^{13}C NMR ($CDCl_3$, 125 MHz)	:	δ:196.1 (C4), 165.6 (C7), 163.9 (C5), 162.8 (C9), 101.0 (C10), 130.3 (C1′), 127.9 (C2′, C6'), 115.6 (C3′, C5'), 95.9 (C6), 94.8 (C8), 80.5 (C2), 78.6 (C2″, C2"'), 76.2 (C3″, C3"'), 56.9 (C1″, C"'), 43.2 (C3).

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In the present investigation, *Cochlospermum gossypium* bark was subjected to extensive extraction, chromatographic separation, isolation and quantification studies for the first time (Chapters 2A, 2B & 2C). These studies resulted in the identification of high secondary metabolite yielding *C. gossypium* bark sample collected from Jaypore forest area of Telangana state and isolated in total seven secondary metabolites. Out of these, three are lupane class of pentacyclic triterpenes such as lupeol, betulin and betulinic acid and four flavonoids such as naringenin, naringenin triacetate, dihydro kaempferol and a new flavonol. Further, optimized the solvent and method of extraction for the major metabolite naringenin from *C. gossypium* bark.

Literature search reveals that some Cochlospermum species exhibit significant antidiabetic activity. *Cochlospermum vitifolium* (Willd.) Sprengel is a Mexican medicinal plant that is used in the folk medicine for the treatment of hypertension, diabetes, hepatitis and related diseases. Its bark hexane extract was reported to show a significant decrease of blood glucose levels ($p < 0.05$) on normoglycemic rats at a dose of 120 mg/kg[1]. *Cochlospermum planchonii*, known as 'Gbehutu' or 'Feru' is a slow growing shrub in Nigeria, Guinea and Sudan. Especially, its rhizomes and leaves are used to treat diabetes, jaundice, malaria and diarrhea. Its roots aqueous extract showed significant reduction in blood glucose levels in diabetic rats at a concentration of 100 mg/kg of body weight[2]. It was also reported that the aqueous extract *C. planchonii* leaves significantly reduced the blood glucose level in a dose-dependent manner, with the highest dose producing a 74.52% reduction after 21 days of administration, which is comparable with that of the control and metformin-treated groups. Similarly, STZ induced diabetic mediated alterations in the serum lipids were significantly ($P<0.01$) restored by the extract[3]. In another study, the saponin rich extract of *C. planchonii* root was reported to exhibit moderate

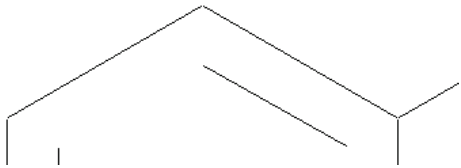
antidiabetic effects possibly by the inhibition of the carbohydrate enzymes, α-amylase and α-glucosidase. This study corroborates the earlier reported traditional use of the plant in tropical Africa[4].

The chemotaxonomy of the *Cochlospermum* genus is the elaboration of flavonoids and triterpenes as the major metabolites and carotenoids, organic acids, carbohydrates and tannins as the minor constituents[5,6]. This is found true, as in the present investigation pentacyclic triterpenes and flavonoids were isolated from the bark of *C. gossypium*. Interestingly, pentacyclic triterpenes and flavonoids were reported to exhibit significant antidiabetic activity *in vitro* and *in vivo*. Especially, the pentacyclic triterpenes (**A-D**) inhibit enzymes involved in glucose metabolism, prevent the development of insulin resistance and normalize plasma glucose and insulin levels.

These triterpene metabolites produce hypoglycemic effect and also found to manifest

hypolipidemic and anti-obesity activities. Triterpenes are also promising candidates in the prevention of diabetic complications. They have strong antioxidant activity and inhibit the formation of advanced glycation end products, implicated in the pathogenesis of diabetic nephropathy, embryopathy, neuropathy or impaired wound healing[7].

Similarly, flavonoids (**E-J**) exhibit antidiabetic properties, which are usually large as a result of their modulatory effects on blood sugar transporter by enhancing insulin secretion, reducing apoptosis and promoting proliferation of pancreatic β-cells, reducing insulin resistance, inflammation and oxidative stress in muscle and promoting translocation of GLUT4 via PI3K/AKT and AMPK[8].



In this context it is to mention here that flavonoids represent a large class of at least 6000 phenolic compounds found in fruits, vegetables, nuts, grain seeds, cocoa,

chocolate, tea, soy, red wine, herbs and beverage products. The hypothesized relation between diabetes and inflammation[9,10]. and the potential for flavonoids to protect the body against free radicals and other pro-oxidative compounds[11,12] , it is biologically plausible that consumption of flavonoids or flavonoid-rich foods may reduce the risk of diabetes[13,14]. Recent investigations have demonstrated that flavonoids are very promising antidiabetic agents[15].. Non-insulin-dependent diabetic mellitus (NIDDM) is characterized by impaired insulin activity and deficient insulin secretion, which cause decreased peripheral glucose uptake and thereby lead to postprandial hyperglycemia. Hyperglycemia increases the risk of developing long-term micro vascular and neuropathic complications and should therefore be controlled. NIDDM patients are treated with orally delivered compounds that have α-glucosidase inhibitory activity. These drugs inhibit disaccharide hydrolases that convert disaccharides into monosaccharides. Their administration thus impedes the digestion and absorption of glucose and thereby attenuates the increase in postprandial plasma glucose levels. These include foodstuffs capable of inhibiting α-glucosidase in animals and humans[16]. Many patients with type 2 diabetes mellitus are treated with α-glucosidase inhibitors, which inhibit the activity of disaccharidases in the brush border membrane of the small intestine, often in combination with other drugs such as insulin and sulfonylureas. Compared with other oral glucose lowering drugs, α-glucosidase inhibitors reduce glucose fluctuations by inhibiting postprandial hyperglycemia and are associated with less frequent hypoglycemia. Acarbose, a pseudotetrasaccharide, is a competitive inhibitor of sucrase, glucoamylase, and isomaltase. Voglibose, an N-substituted valiolamine derivative, has been reported to have stronger α-glucosidase inhibitory activity against maltase and sucrase[17.] Currently, the National Institutes of Health Clinical Center is investigating the use of

quercetin on glucose absorption in obesity, and obesity with type 2 diabetes patients on oral glucose tolerance test[18]. Diabetes mellitus is a chronic disease reaching epidemic levels in both developed and developing countries. According to WHO by 2030, there will be 366 million diabetic patients worldwide and 80 million diabetics in India alone, significant morbidity, mortality and cost are associated with this disease due to progressive nature resulting in many micro and macrovascular complications. It requires continuous medical care and self-management by the patient to prevent both acute and chronic complications related to uncontrolled glycemic status[19]. Plant-based traditional medical systems continue to provide the primary health care to more than three quarters of the world's population. Indigenous herbal drugs widely used to treat diabetes and metabolic disorders. Plants continue to play an important role in the treatment of various diseases due to multiple beneficiary compositions having lesser or no side effect and plant-based medicines can be used as an alternative approaches to treat diabetes. India with rich traditional knowledge and wealth of medicinal plants, is one of the 'Hot Spots' for plant based antidiabetic drug development. There exists tremendous scope and opportunities in developing safe and efficacious antidiabetic drugs based on Indian plant species or their products.

Present Work

In view of the above background and virtually no work has been reported so far on the antidiabetic potential of *Cochlospermum gossypium*, work has now been taken up for detailed and systematic antidiabetic screenings *in vitro* and *in vivo*. The main objective of the present study is to evaluate the antihyperglycemic effect of extracts, fractions and isolated single pure compounds of *C. gossypium* bark. Antihyperglycemic activity was studied *in vitro* through the inhibitory tests on α-glucosidase of Saccharomyces cerevisiae and *In vivo* in normal and diabetic mice

using various tests including: the decrease of post prandial hyperglycemia, oral glucose tolerance test (OGTT), hypoglycemic effect and inhibition of glucose intestinal transporters. The details of work done are given below:

✓-Glucosidase Inhibitory Screening of *Cochlospermum gossypium*: Identification of Potent Antidiabetic Leads

Plant Material

Out of the two *C. gossypium* bark samples collected from Jaipur (Telangana) and Trichur (Kerala), the Jaipur sample found to accumulate high content of the major metabolite. Hence, chemical and biological studies have been carried out on this sample. The details of the collection and processing of the plant material are given in Chapter 2B.

Extraction

The *C. gossypium* bark was extracted successively with solvents of increasing polarity such as n-hexane, ethyl acetate and methanol using a soxhlet extractor under hot conditions as per the protocols with given in Chapter 2A (Chart 2A.01). The three extracts of *C. gossypium* bark are coded as CGBHE (n-hexane extract), CGBEE (ethyl acetate extract) and CGBME (methanol extract). The yields of the three extracts are : CGBHE (1.0%), CGBEE (2.2%) and CGBME (4.0%).

In vitro ✓ ɣ glucosidase inhibitory potential of C. gossypium bark extracts

The non-polar (CGBHE), medium polar (CGBEE) and polar (CGBME) extracts of *C. gossypium* bark are subjected to *in vitro* ✓-glucosidase inhibitory assay as per the following protocol to identify active extracts.

α-Glucosidase Assay [20]

Principle

The ✔-glucosidase inhibitory activity of the compound depends upon the ability of the compound to block the action of α-glucosidase enzyme on the substrate p-nitophenyl α-D-glucoside (PNP-Gluc).

Procedure

The extracts were dissolved in DMSO to obtain a stock solution of 1mg/ml. Assay was performed by adding sample to 0.1 U/ml enzyme (✔-glucosidase isolated from the yeast, *Saccharomyces cerevisiae*) and incubating along with 2.5 mM of substrate at 37ºC for 45 min and the reaction was stopped by the addition of 100 mM of sodium carbonate. The absorbance of the final product was measured at 405 nm. Screening was done for all the compounds starting at a concentration of 1mg/ml and making further serial dilutions. Acarbose was used as a reference standard. The per cent growth inhibition and IC_{50} values of the tested samples were tabulated in Table 2D.01.

Table 2D.01 : ✔-Glucosidase inhibitory potential of various extracts of *C. gossypium* bark

S. No.	Extract	Sample Code	a-Glucosidase Inhibitory Activity	
			Growth Inhibition (%)	IC_{50}
1.	n-Hexane	CGBHE	97.75	0.092 ± 0.002
2.	Ethyl acetate	CHBEE	87.47	0.168 ± 0.005
3.	Methanol	CGBME	38.73	ND
4.	Acarbose	Standard	73.66	0.673 ±0.079

ND : Not Determined

From the above data it is evident that the n-hexane (**CGBHE**) and ethyl acetate (**CGBEE**) extracts are highly active, whereas the methanol extract (**CGBME**) weakly active. Interestingly, **CGBHE** (IC_{50}: 0.092 ± 0.002) and **CGBEE** (IC_{50}: 0.168 ± 0.005) are several folds more active than the standard acarbose (IC_{50}: 0.673 ±0.079). Further, **CGBHE** is 1.83 times more active than **CGBEE.**

The two active extracts (**CGBHE** and **CGBEE)** were subjected to independent

fractionation studies to identify the active fractions.

Fractionation of n-hexane extract of *C. gossypium* bark

As the n-hexane extract showed several closely related spots on TLC, it was initially separated into five fractions (**F1-F5**) to minimise the complexicity (Chart 2D. 01) on a chromatographic column using n-hexane and ethyl acetate solvent mixtures.

Not Active

Chart 2D.01 : Fractionation of n-hexane extract of C. gossypium bark

The five fractions (**F1 - F5**) of n-hexane extract were subjected to ✔-glucosidase inhibitory activity. While three fractions **F2, F3** and **F4** showed potent activity, the two fractions **F1** and **F5** did not showed any significant activity. Among the three active fractions, two fractions **F2** (Inhibition (%) : 96, IC_{50} : 0.092 ± 0.002) and **F3** (Inhibition (%) : 87.82, IC_{50} :0.168 ± 0.005) are highly potent. Hence, these fractions were taken up independently for further fractionation to identify the active sub fractions, which were subsequently subjected to chromatographic purification to isolate active molecules. The details are given below:

Further fractionation of F2 fraction of n-hexane extract: Isolation of active molecule CGBHC1 (1)

The F2 fraction of the n-hexane extract, which showed highest ✔-glucosidase inhibitory activity (Inhibition (%) : 96; IC_{50} : 0.092 ± 0.002) was subjected to further fractionation (Chart 2D.02) using n-hexane and ethyl acetate solvents and made into 12 sub fractions (**SF1-SF12**). Out of these, two sub fractions **SF10** and **SF11** showed maximum activity. Hence, these two were combined and subjected to chromatographic purification to isolate one single and pure compound (**CGBHEC1**).

Column Chromatography

Chart 2D.02 : Further fractionation of F2 fraction of n-hexane extract of *C. gossypium* bark and isolation of active molecules

Its identity was established as lupeol by its physical and spectral data and by comparing its data with the one reported in Chapter 2A. Interestingly, this compound showed exceptionally potent ✔-glucosidase inhibitory activity (Growth Inhibition (%) : 90.13; IC_{50} : 0.017 ± 0.009).

Further fractionation of F3 fraction of n-hexane extract : Isolation of active molecule CGBHEC2 (2)

The F3 fraction of the n-hexane extract, which showed very high ✔-glucosidase inhibitory activity (Inhibition (%) : 87.82, IC_{50} :0.168 ± 0.005) was subjected to further fractionation (Chart 2D.03) using n-hexane and ethyl acetate solvents and made into12 sub fractions (**SF1-SF12**). Out of these, two sub fractions **SF5** and **SF8** showed maximum activity. Hence, these two were combined and subjected to chromatographic purification to isolate one single and pure compound (**CGBHEC2**).

Column Chromatography

Chart 2D.03 : Further fractionation of F3 fraction of n-hexane extract of *C. gossypium* bark and isolation of active molecules

Its identity was established as betulin by its physical and spectral data and by comparing its data with the one reported in Chapter 2A. Interestingly, this compound showed exceptionally potent ✔-glucosidase inhibitory activity (Growth Inhibition (%) : 96; IC_{50} : 0.010±0.018).

Fractionation and chromatographic purification of ethyl acetate extract of *C. gossypium* bark : Isolation of active molecules CGBEEC 1 (3) - CGBEEC 5 (7)

As the ethyl acetate extract of *C. gossypium* bark (CGBEE) showed potent ✔-glucosidase inhibitory activity (Inhibition (%) : 87.47; IC50 : 0.168 ± 0.005) and

well resolved spots on TLC, it was subjected to Si gel column chromatographic purification using n-hexane and ethyl acetate solvents as eluents (Chart 2D.04).

Column Chromatography

Chart 2D.04 : Fractionation and identification of active molecules from ethyl acetate extract of *C. gossypium* bark

In total five single and pure compounds (**CGBEEC1-CGBEEC5**) were isolated, out of which one is lupane class pentacyclic triterpenic acid (**CGBEEC1**) and four flavonoids (**CGBEEC2-CGBEEC5**). Their identity was established as betulinic acid (**CGBEEC1**), naringenin (**CGBEEC2**), dihydrokaempferol (**CGBEEC3**), a new dimethoxyflavonol (**CGBEEC4**) and naringenin triacetate (**CGBEEC5**) by their physical and spectral data and by comparing its data with the ones reported in Chapter 2A.

The five isolated compounds were screened for their ✔-glucosidase inhibitory potential. Interestingly betulinic acid (**CGBEEC1**) showed highest inhibitory activity (Inhibition (%):97.12. IC_{50} : 0.036±0.002) followed by dihydrokaempferol (**CGBEEC3,** Inhibition (%) : 86.93, IC_{50} : 0.466±0.04) and naringenin (**CGBEEC2,** Inhibition (%) : 82.25, IC50 : 0.609±0.08). While naringenin triacetate (**CGBEEC5)** showed good activity (Inhibitory (%) : 69.28, IC50 : 0.724 ± 0.020), the new dimethoxyflavonol (**CGBEEC4**) did not show any activity.

In total seven compounds (**CGBHEC1** and **CGBHEC2, CGBEEC1-CGBEEC5**) were isolated from the bio-assay (✔-glucosidase inhibition) guided screening of the two active extracts (**CGBHE** and **CGBEE**) of C. gossypium bark. Among the isolated compounds, three are lupane class of pentacyclic triterpenes and four are flavonoids. The ✔-glucosidase inhibitory activity of the isolated compounds is presented in Table 2D.02. Critical analysis of the data reveals that the pentacyclic triterpenes

(**CGBHEC1, CGBHEC2** and **CGBEEC1**) are more active than the flavonoids (**CGBEEC2-CGBEEC5**). The ✔-glucosidase inhibitory potential of the three triterpenes is in the range : 90.13-97.12% (growth inhibition) and 0.010-0.036 (IC_{50}), whereas the inhibitory potential of flavonoids is in the range: 69.28-86.93% (growth inhibition) and 0.466-0.724 (IC_{50}). Among the triterpenes, lupeol (**CGBHEC1**) and betulin (**CGBHEC2**) showed almost same inhibitory potential. Whereas in case of flavonoids, dihydrokaempferol (**CGBEEC**3) showed maximum activity followed by naringenin (**CGBEEC2**) and naringenin triacetate (**CGBEEC5**). Surprisingly, the new dimethoxyflavonol (**CGBEEC4**) did not show any inhibitory activity.

Table 2D.02: ✔-Glucosidase inhibitory potential of compounds isolated from *C. gossypium*

S. No.	Compound		✔ – Glucosidase Inhibitory activity	
	Code	Identification	Inhibition (%)	IC_{50}
1.	CGBHEC1 (**1**)	Lupeol	90.13	0.017 ± 0.009
2.	CGBHEC2 (**2**)	Betulin	96.00	0.010±0.018
3.	CGBEEC1 (**3**)	Betulinic acid	97.12	0.036±0.002
4.	CGBEEC 2 (**4**)	Naringenin	82.20	0.609±0.08
5.	CGBEEC 3 (**5**)	Dihydrokaempferol	86.93	0.466±0.04
6.	CGBEEC 4 (**6**)	New(2S,3S)-2,3-dihydro-3,5-dihydroxy-7-ethoxy-2-(3-ethoxy-4-hydroxyphenyl)-4H-1-benzopyran-4-one	-21.60	ND
7.	CGBEEC 5 (**7**)	Naringenin triacetate	69.28	0.724 ± 0.020
8.	Standard	Acarbose	73.66	0.673 ±0.079

ND : Not Determined

✔-Glucosidase inhibitory activity of naringenin analogues : Identification of potent leads

As mentioned above, the bio-assay guided (✔ -glucosidase inhibitory activity) of the n-hexane and ethyl acetate extracts of *C. gossypium* bark resulted in the isolation of seven compounds (**CGBHEC1-CGBHEC2, CGBEEC1-CGBEEC5**). In fact these compounds were now found to be the major metabolites of *C.gossypium* bark (Chapter 2A). The accumulation of these compounds in C. gossypium bark is given in Table 2D.

Table 2D.03: Accumulation (%) ọfcompounds (1-7) in *C. gossypium* bark

S. No.	Compound		Accumulation (%)
	Code	Identification	
1.	CGBHEC1 (**1**)	Lupeol	0.003
2.	CGBHEC2 (**2**)	Betulin	0.004
3.	CGBEEC1 (**3**)	Betulinic acid	0.008
4.	CGBEEC 2 (**4**)	Naringenin	0.1
5.	CGBEEC 3 (**5**)	Dihydrokaempferol	0.03
6.	CGBEEC 4 (**6**)	New(2S,3S)-2,3-dihydro-3,5-dihydroxy-7-ethoxy-2-(3-ethoxy-4-hydroxyphenyl)-4H-1-benzopyran-4-one	0.03
7.	CGBEEC 5 (**7**)	Naringenin triacetate	0.01

From the above Table, it is evident that the four flavonoid metabolites (**CGBEEC2-CGBEEC5**) accumulated more than triterpene metabolites are (**CGBHEC1, CGBHEC2** and **CGBEEC1**). Further, naringenin (**CGBEEC3**) was found to be the major metabolite (0.1%) of *C. gossypium* bark. In view of high accumulation and significant ✔ -glucosidase inhibitory activity, naringenin can be taken up as the scaffold for chemical modification to synthesise a range of new chemical entities (NCEs) for detailed biological evaluation to identify potent a-glucosidase inhibitory

leads. Further, structure activity relationship of naringenin suggests that alkylation of hydroxyls at 4', 5 and 7 positions enhance the biological activity (Chapter 2C).

With this background, naringenin (4) was subjected to synthesise a total of five saturated and un)saturated ether analogues (**4a-4e**) in good to very good yields (Chart 2D.05). Out of the five synthesized naringenin analogues, three are saturated ethers (**4a, 4b** and **4c)** and two are unsaturated ethers **(4d** and **4e).**

4e

Chart 2D.05 : Fractionation and identification of active molecules from ethyl acetate extract of *C. gossypium* bark

The synthesized analogues (**4a-4e**) along with the parent naringenin (**4**) were subjected to *in vitro* ✔-glucosidase inhibitory activity by employing the above mentioned procedure. The activity was expressed in terms of per cent growth inhibition and IC50 values and the data is presented in Table 2D.04.

Table 2D.04: ✔ - Glucosidase Inhibitory Potential of naringenin alkyl ethers

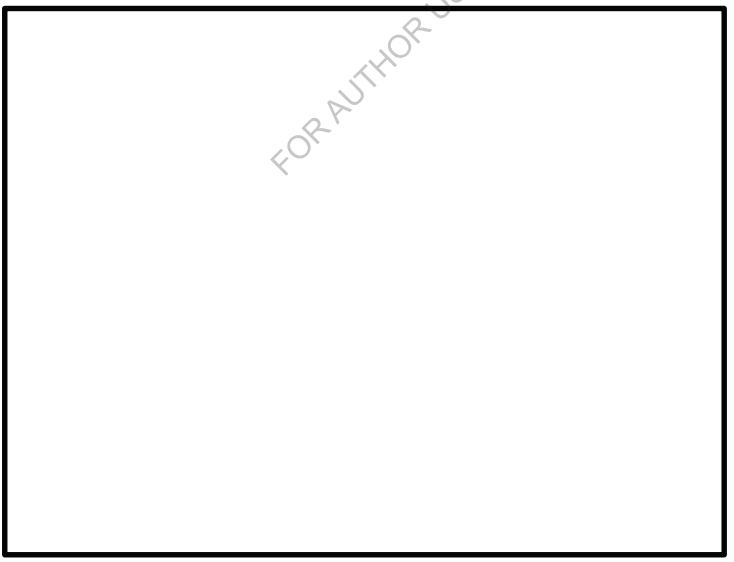
S. No.	Compound	✔ – Glucosidase Inhibitory activity	
		Growth Inhibition (%)	IC_{50}
1.	**4**	82.2	0.609±0.08
2.	**4a**	-11	ND
3.	**4b**	-18	ND
4.	**4c**	64.58	2.215±0.05
5.	**4d**	-33	ND
6.	**4e**	85.44	0.491±0.02
7.	**Acarbose**	73.66	0.673 ±0.079

ND : Not Determined

From the data presented in the above table, it is evident that the chemical modification of naringenin did altered the ✔-glucosidase potential of naringenin. It

affects both positively and negatively. Especially, the 3-carbon alkyl (**4c**) and alkenyl (**4e**) ether analogues have significant effect on the overall inhibitory activity of naringenin (**4**). Most significantly, the 3-carbon alkenyl (propargyl) ether analogue (**4e**) showed enhanced and highly potent ✔-glucosidase inhibitory activity (Inhibition (%): 85.44, IC_{50} : 0.491±0.02), which is 1.25 times more than that of naringenin (Inhibition (%) : 82.25, IC_{50} : 0.609±0.08). Although, the 3-carbon alkyl (propyl) ether analogue (**4c**) showed significant growth inhibition, its ✔-glucose inhibitory activity is less than that of the parent naringenin. Surprisingly, the methyl (**4a**), ethyl (**4b**) and allyl (**4d**) analogues did not show any inhibitory activity.

From the above *in vitro* ✔-glucosidase inhibitory screening of *C. gossypium* bark extracts, pure isolated compounds and naringenin analogues the following three compounds were identified as potent molecules :



In order to see their potentiality further, they have been subjected to detailed *in vivo* ✔-glucosidase enzyme inhibitory screening by using sucrose loaded model in wistar Rats[21]. The details of *in vivo* screening are given below:.

***In vivo* α- Glucosidase Assay:**

Principle

The ✔-glucosidase inhibitory activity of the compound depends upon the ability of the compound to block the action of α-glucosidase enzyme on the substrate p-nitro phenyl α-D-glucoside (PNP-Gluc).

Procedure

The compounds were dissolved in DMSO to obtain a stock solution of 10 mM/ml. Assay was performed by adding sample to 0.1 U/ml enzyme and incubating along with 2.5 mM of substrate at 37ºC for 45 min and the reaction was stopped by the addition of 100 mM of sodium carbonate. The absorbance of the final product was measured at 405 nm. Screening was done for all the compounds starting at a concentration of 10mM/ml and making further serial dilutions. Acarbose (1mg/ml) was used as a reference standard. The results are expressed as IC_{50} values.

Chemicals and kits

Acarbose (≥95%), *α*-glucosidase enzyme (*Saccharomyces cerevisiae*), p-nitophenyl-*α*-D-glucoside (PNP-Gluc), dimethyl sulfoxide (DMSO) and sucrose were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade unless otherwise specified.

Test Compounds:

1. Lupeol (**1**)

2. Naringenin (**4**)

3. Naringeni-4',7-bis propargyl ether (**4e**)

Test animals

Male Albino Wistar rats weighing between 180 and 200g were used for the present study. Animals were quarantined and acclimatized to laboratory conditions for 7 days before study initiation. They were kept in polypropylene cages under standard laboratory conditions (12:12 light/dark cycle) at 24±2 °C and were provided with commercial rodent diet (Safe diets, France) and water throughout the study period. The experiment was conducted after obtaining approval from Institutional Animal Ethical Committee of Indian Institute of Chemical Technology.

Assessment of anti-hyperglycemic activity in normal rats (OSTT)

Wistar rats weighing between 180-200g were used for the present study. Rats were fasted overnight and animals showing fasting blood glucose levels between 60 to 90 mg/dl were selected and divided into four groups consisting 6 animals each. Rats from experimental group were orally administered with a suspension of either test compounds (100 mg/kg body weight) or Acarbose (20 mg/kg body weight) in 1.0% gum acacia. Animals in the control group were given an equal volume of 1.0% gum acacia. All the experimental animals were loaded orally with sucrose (3.0 g/kg) 15 min following the administration of the test compound or vehicle. Blood samples were collected from all the groups of rats at intervals of 30, 60, 90, and 120 minutes, post administration, to estimate the blood glucose levels. Food, but not water, was with held from the cages during the course of experimentation. The percentage increase in the glucose levels was calculated for all the test groups of animals comparing with zero hour glucose concentration.

Results & Discussion

***In vivo* antihyperglycemic activity of compounds 1, 4 and 4a**

Postprandial hyperglycemia is the key problem in diabetes mellitus. Ingestion of carbohydrate rich diet causes elevation in blood glucose level by the rapid absorption of carbohydrates in the intestine aided by the action of glycoside hydrolyses, which break complex carbohydrates into simple sugars. Glycosidase inhibitors recently gained significance on the basis of their interesting role in delineating enzyme mechanisms and the control of postprandial hyperglycemic excursion in diabetes mellitus[22].

The *in vivo* anti-hyperglycemic activity of the compounds **1**, **4** and **4e** were evaluated using sucrose loaded model in Wistar rats. The results are presented Table 2D.05 and Figure 2D.01.

Table 2.05 Antihyperglycemic activity of compounds 1, 4 and 4e

Sample	Glucose Concentration (mg/dl)				
	0 min	**30 Min**	**60 Min**	**90 Min**	**120 Min**
SCR	83.2	124	122	123	114
ACR	83.6	96.4	105.6	105.6	105.4
1	79.6	117.8	117.2	120.8	108.2
4	78.6	125.4	125.4	126.4	118.2
4a	89.4	130.8	117	117.2	106.6

SCR : Sucrose Control Group ; ACR : Acarbose

The oral administration of test compound **4e**, at the dose of 100 mg/kg significantly inhibited the upsurge in the blood glucose levels at 30min, 60min & 90 min ($p < 0.05$) and at 120 min($p < 0.01$) compared with the sucrose control group at all the time intervals of the study. Rats administered with standard drug acarbose also showed a significant ($p < 0.001$) at 30min, ($p < 0.01$) at 60min &90 min and ($p < 0.05$) at

120min reduction in the blood glucose levels compared with the sucrose alone group.

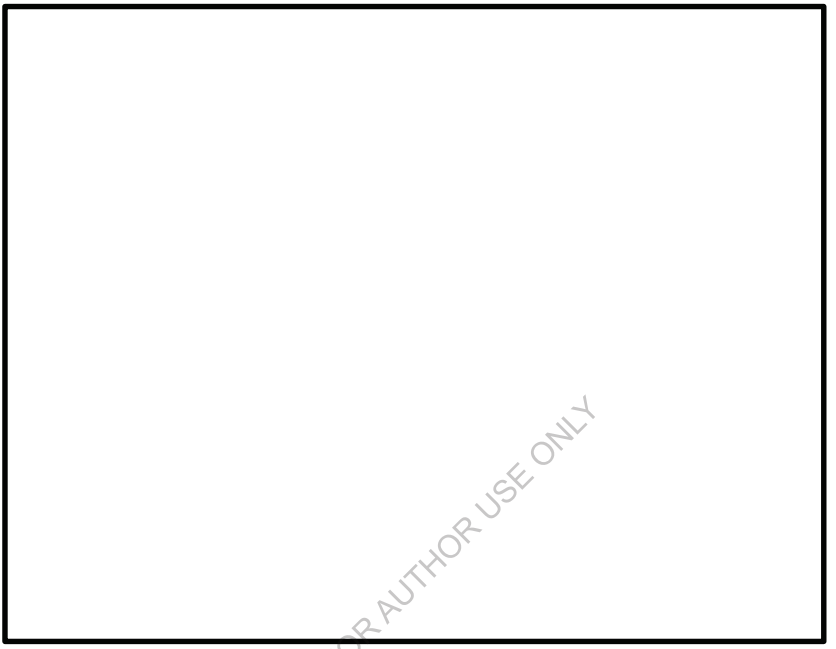


Figure 2D.01 Anti hyperglycemic effect of **1**, **4** and **4e** by sucrose loaded model in wistar Rats. Glucose lowering effect of compound **4e** when administered by oral route at the dose of 100 mg/kg in sucrose loaded model in Wistar rats. Values are expressed as Mean ± S.E.M, n = 6, S.C = Sucrose Control.

From the above results it is concluded that the hypoglycemic potential of compounds are in the orders of **4e** > **1** > **4** with regards to their α-glucosidase inhibitory potential. Pretreatment with compound **1** (100mg/kg p.o.) displayed moderate activity, while compound **4** (100mg/kg p.o.) did not show any activity.

Exceptionally Potent Molecules

From the above in vitro and in vivo a-glucosidase inhibitory assays, the highly potent molecules are found to be : naringenin-4',7-bis propargyl ether (**4e**) and lupeol (**1**). Interestingly, **4e** is a semi synthetic analogue of the flavanone, naringenin. Whereas,

Lupeol (**1**)

compound **1** is a natural lupane class of pentacyclic triterpene. These two compounds have interesting functional groups, which can be fine tuned further to develop therapeutically useful antidiabetic agents.

Conclusion

In conclusion, the non-polar, medium polar and polar extracts of *Cochlospermum gossypium* bark were subjected to detailed and systematic *in vitro* ✔-glucosidase inhibitory screening and identified the n-hexane and ethyl acetate extracts as the active extracts. These extracts were fractionated and sub-fractionated further to identify the highly active fractions, which on column chromatogaraphic purification yielded in total seven compounds. Out of the seven isolated compounds, three are lupane class of pentacyclic triterpenes and four are flavonoids. These compounds were tested for their ✔-glucosidase inhibitory potential. Interestingly, the three triterpenes (lupeol, betulin and betulinic acid) and two flavonoids (naringenin and dihydrokaempferol) showed potent activity. Based on abundance and very good activity, the major metabolite, naringenin (**4**) was chemically modified and synthesized five saturated and unsaturated ether analogues (**4a-4e**). The synthesized analogues along with the parent compound were tested for their *in vitro* ✔-glucosidase inhibitory activity. Most significantly, the unsaturated 3-carbon alkenyl (propargyl) analogue (**4e**) showed significantly enhanced activity than the parent naringenin. In order to evaluate their potentiality further, lupeol (**1**), naringenin (**4**) and

naringenin-4',7-bis propargyl ether (**4e**) were subjected to in vivo anti hyperglycemic activity using sucrose loaded model in wistar Rats. These studies clearly shows that naringenin-4',7-bis propargyl ether (**4e**) is the most potent one followed by lupeol (**1**). In view of excellent *in vitro* and *in vivo* activities and interesting functional groups, these two compounds can be considered as lead compounds for further fine tuning to develop therapeutically useful antidiabetic agents.

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Plants produce two types of metabolites such as primary and secondary metabolites. **Primary metabolites** are produced continuously during the growth phase and are involved in primary metabolic processes such as respiration and photosynthesis. Primary metabolites, which are identical in most organisms, include sugars, amino acids, tricarboxylic acids, the universal building blocks, and energy sources. Other than the above compounds, proteins, nucleic acids, and polysaccharides are also considered as primary metabolites. The importance of primary metabolism is in the cell's growth, reproduction, and development[1].

Plant secondary metabolites (**SMs**) are derivatives of primary metabolites produced by plants because of diverse physiological changes. The SMs significantly improve plant growth and survival under different environmental stresses[2]. Unique carbon skeleton structures are basic properties of plant secondary metabolites. Secondary metabolites are used as especially chemical such as drugs, flavours, fragrances, insecticides, and dyes by human because of a great economic value[3,4]. Plants have several metabolic pathways leading to tens of thousands of secondary products capable of effectively responding to stress situations imposed by biotic and abiotic factors[5]. They protect plants against stresses, both biotic and abiotic. The synthesis of secondary metabolites is generally increasing when plants suffer with biotic and abiotic stresses[6,7]. There are three major groups of SMs in plants based on their biosynthetic[8] pathway. These groups include nitrogen-containing compounds (cyanogenic glycosides, alkaloids, and glucosinolates), phenolic compounds (flavonoids and phenylpropanoids) and terpenes (isoprenoids)[9]. Secondary metabolites are involved in protective functions in response to both biotic and abiotic stress conditions. Plants challenged with different types of abiotic and biotic stresses results into the reduction of different

morphological characters such as height, leaf number, leaf area, number of branches, root volume, etc[10].

Biotic Stresses: Biotic stresses in plants are caused by diverse living organisms that include nematodes, bacteria, virus, and fungi[11]. Plants under pathogen attack show enhanced biosynthesis of SMs. By comparing transcriptomic data from microarray experiments after 22 different forms of biotic damage on eight different plant species, Bilgin et al reported that transcript levels of photosynthesis light reaction, carbon reduction cycle and pigment synthesis genes decreased regardless of the type of biotic attack[12]. Secondary metabolites like phenolics show changes in their concentrations against fungal infection by *Colletotrichum lupini* in lupin (*Lupinus angustifolius*)[13]. The concentration of SMs is significantly decreased during stress recovery[14].

Abiotic Stresses: Plant have potential to adopt some strategies to neutralize the effects of various abiotic stresses such as[15]

- Temperature ,
- UV- irradiation
- Light exposure,
- Altitude,
- Solar radiation,
- Shifting latitudes,
- Enriched carbon dioxide atmosphere,
- Exposure to red light (600~700 nm),
- Drought
- High concentrations of Cu and Mn nutrition,

Each abiotic factor influence responses of secondary metabolism (Table 3.01)[16].

Table 3.01 Abiotic factors influencing responses of secondary metabolism

S. No.	Abiotic factor	Plant	Effect
1	Lower temperature	*Artemisia spp.*	Higher levels of artemisin
2	Lower temperature	*Nicotiana tabacum*	Higher levels of anthocyanins
3	Lower temperature; UV-B irradiation	*Malus sp.*	Higher levels of anthocyanins
4	Longer light exposure	*Panax quinquefolius*	Higher levels of ginsenosides
5	Altitude	*Leontodon autumnalis*	Shifting flavonoids contents
6	Higher altitudes and lower temperature	*Arnica montana*	Phenolics derivatives
7	Continuous solar radiation	*Vaccinium myrtillus*	Flavonoid biosynthesis pathway activation
8	Shifting latitudes	*Several (review paper)*	Intragenus shifts of flavonoid contents
9	Enriched carbon dioxide atmosphere	*Quercus spp.; Galactia elliottii (meta-analysis)*	Higher tannin levels
10	Exposure to red light (600~700 nm)	*Ocimun basilicum*	Rosmarinic acid accumulation
11	Drought stress	*Quercus ilex*	Lower monoterpene emissions
12	High conditions of Cu and Mn nutrition	*Eugenia uniflora*	Lower both tanins and flavonoids contents

13	Temperature and light variations	*Populus canescens*	Shifts on transcript levels of terpene biosynthesis-related gene
14	Exposure to UV	*Catharanthus roseus (cell culture)*	Enhanced production of catharanthine

From the above table it is clearly seen the effects of different abiotic stresses on the elaboration of secondary metabolites in plants. However, altitudinal variation of secondary metabolite profiles of higher plants is not well investigated so far. Only a few studies deal with this subject and most of them are based on plants growing in their natural habitats. The crucial factors governing the altitudinal variation in the field has not been thoroughly investigated so far[17]. In general, plant species diversity declines with altitude[18]. Altitudinal variation in plant secondary metabolites is like latitudinal variation. There are two prevailing but partially contradicting theories.

- Theory 1: according to this theory, amounts (and diversity) of bioactive natural products decreases from the equator to the poles[19]. In analogy, decreasing numbers and diversity of herbivores from low to high altitude sites are to be expected and in consequence a decrease in anti-herbivory compounds from low land to high altitude growing sites is postulated[20].
- Theory 2: according to this theory bioactive compounds alleviating the impact e.g. low temperatures or enhanced UV-B radiation will increase in high latitudes and high altitudes[21]. While UV-B radiation can damage DNA directly, the harmful effect of low temperature is indirect.

Example of first theory is the high altitude populations of *Lupinus argenteus* Pursh contain significantly lower amounts of toxic quinolizidine alkaloids in their leaves[22]. The second theory was supported by studies on the ferns *Pteridium arachoideum* (Kaulf.)

Maxon and *Pteridium caudatum* (L.) Maxon, which contained higher amounts of photo-7 protective and radical scavenging phenolics in higher altitude sites than in lower growing stands[23]. Despite niche fragmentation, proximity and number of life zones in mountain ranges, the available surface area for plant growth decreases with altitude due to the conical shape of most mountains. The overall effect is that plant species richness decreases with elevation. The overall balance was loss of phytodiversity relative to larger areas found in lower elevations. The higher the altitude and the lower the latitude, the greater the gradient[24]. High altitude plants are subject to various powerful abiotic stresses as we have seen, acting simultaneously or with considerable variation depending on season or even time of the day. As altitude and annual average temperature rather than rainfall or other variables explained most of the differentiation, it was concluded that elevation entails strong selective environmental pressures for ecotype establishment in *P. dodecandria*, which changes rapidly as altitude increases. If it is true that species richness generally declines with altitude, total alkaloid accumulation generally decreases with altitude. Molecular damage (UV energy reaching the ground, even at low altitude, may be quite damaging[25]. Many chromophoric compounds in plants will absorb UV-B photons chiefly by two processes: (1) photoactivation followed by intersystem crossing towards excitation of oxygen molecules to highly reactive singlet oxygen, its anion radical and other free radicals. Acquired photo-energy can also be relaxed as heat; and (2) absorption to promote chemical reactions. The first mechanism comprises a widespread form of defense collectively known as phototoxicity (Chart 3.01), which is aimed at plant predators[26].

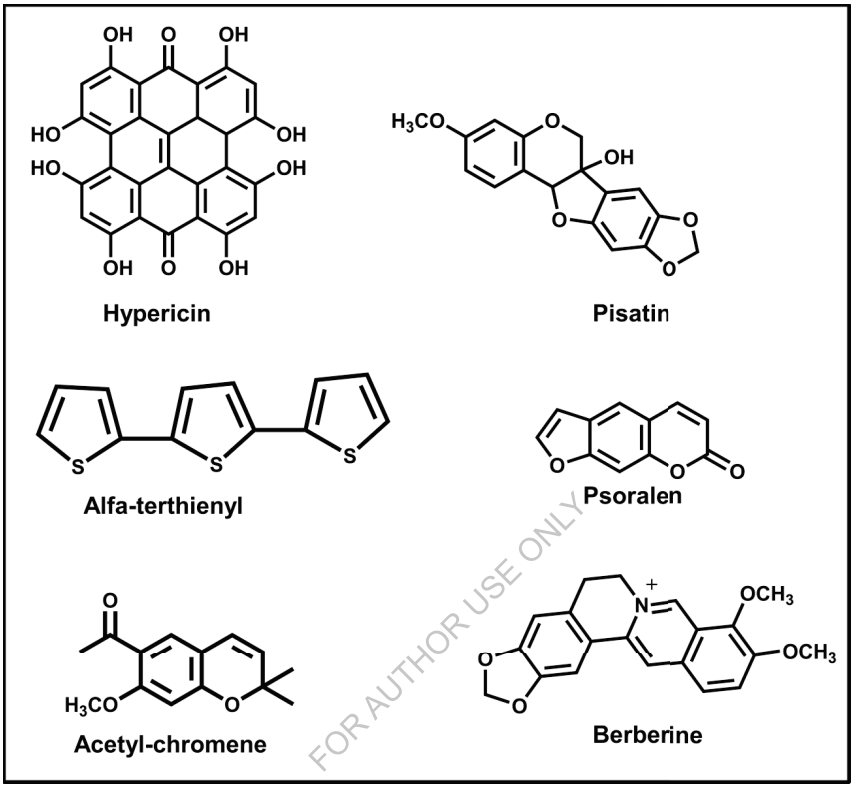


Chart 3.01. Examples of photosensitizers activated by UV-B solar radiation found in plants

Enhanced UV-B radiation affect the secondary metabolism of plants growing in high altitudes. Enhanced biosynthesis of UV-B absorbing and antioxidant phenolic compounds was interpreted as a protective response against damage from excessive UV-B radiation in plants due to the UV-shielding properties of these compounds.

Altitudinal trends for various classes of secondary metabolites are summarized in Chart 3.02. It is feasible that the proportion of induced and inherited variation differs not only between particular species but also across species between compound classes.

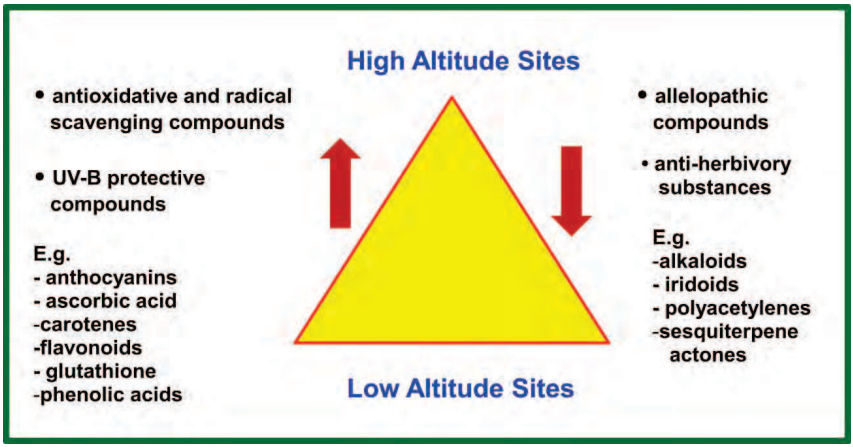


Chart 3.02 Altitudinal trends predicted for various classes of plant secondary metabolites with differing ecological functions

A classical example for the effect of altitude on the secondary metabolite accumulation is that of Zidorn[27], who has done extensive work on the extraction and HPLC based analysis of the flowering heads collected at different altitudes ranging from 180 to 1,060 m (*Crepis capillaris*), from 190 to 1,290 m (*Hieracium pilosella*) and from 20 to 1,290 m (*Hypochaeris radicata*) respectively. Results showed a positive correlation between the altitude of the growing site and the contents of flavonoids and phenolic acids for all investigated taxa.

It is clearly seen from the above that high altitude plants have developed highly specialized traits to withstand high levels of UV-B solar radiation, which may be necessary to elaborate diverse secondary metabolites. India is one of the 17 mega bio-diverse countries of the world with 15 agro climatic zones, 10 bio-geographic zones, 45000 different plant species, 17000 ~ 18000 flowering plants, 6000 ~ 7000 medicinal plants, 2400 plant species in codified ISM, 960 plants are in current trade[28]. As far as medicinal plant diversity in India is concerned, about 25~30% species grown in high

altitudes. Till date very few high altitude plant species of India could be exploited chemically and biologically. Still a large number of these plants remain to be explored. As these plants grow at high altitude and at low temperatures, they are expected to elaborate diverse biologically active natural products.

Recently, *Chloroxylon swietenia* DC., a highly potent Indian folklore medicinal plant has attracted the attention of chemists and biologists due to its widespread accumulation in different geographical regions of India. It is of interest now to see the effect of various growth parameters, especially altitude on its phytochemical constituents and biological activities. In this connection, detailed literature search has been made on botanical, traditional, chemical and pharmacological properties of *C. swietenia* and presented the brief review below.

***Chloroxylon swietenia* : A Highly Potent Indian Folklore Medicinal Plant**

Chloroxylon swietenia DC. (Fam : Rutaceae), popularly known as Yellow wood, East Indian satin wood, Ceylon satin wood, is native to India and Sri Lanka[29]. In India it is commonly known as Bhirra (Hindi), Bhillotaka (Sanskrit), Billudu (Telugu), Vaaimaram (Tamil)[30]. In India, it is found wild in dry deciduous forests up to an altitude of 1100 m, extending in the north to the Satpuras and Chota Nagpur[31].

C. swietenia DC. (Figure 3.01) medium sized deciduous tree with height up to 9 -15 m and 1.0-1.2 m girth. The leaves are 15 - 23 cm long and abruptly pinnate. The leaflets (10 - 20 pairs) are sub-opposite or alternate, oblong, obtuse, glabrous and glaucous. Flowers are white or cream in colour and present in terminal or axillary panicles. Flowers appear during March-April, fruits produce seeds[32].

Figure 3.01 *Chloroxylon swietenia* DC. plant

The International Union for Conservation of Nature (IUCN) in its updated version of 2014 included *Chloroxylon swietenia* as vulnerable species due to over exploitation as timber.

Traditional uses

C. swietenia is considered as a potent medicinal plant having several medicinal uses in the folklore remedies[33]. The Malasar tribes of Tamil Nadu, South India apply the leaf paste on wounds, cuts, burns and skin diseases for quick recovery[34]. While dried leaves are useful for pains, the crushed leaves are useful for the treatment of wounds, snakebites, and rheumatism. Leaves and roots are made into paste then taken internally or applied externally to relieve from headache. The wood is used as decorative timber, agricultural equipment, railway sleepers and in heavy construction and boat building due to its heavy, hard and durable nature. In addition, it is used as fuel wood[35,36]. The stem bark is credited for its effectiveness in the treatment of common cold and cough[37], ophthalmic infection & cataract, wounds[38,39] and as an stringent[40]. The dried stem barks alone or in combination with sesame oil (*Sesamum indicum*) in the form of a paste is applied externally to treat itches[41,42].

Chemistry

The various phytochemical constituents reported[43-62] from different plant parts of *C. swietenia* are listed in Table 3.03.

Table 3.03 Phytochemical constituents reported from different plant parts of *C. swietenia*

S.No.	Plant part	Chemical Constituents
1	Leaves	Geraniol, geranyl acetate, limonene, linalool, α– terpinene, α– terpineol, α– pinene, α– phellandrene
2	Leaves	Myrcene, alloocimene, β- ocimene, β- pinenes
3	Stems and leaves	Copaene, β- caryphyellene oxide
4	Stems and leaves	α- Humulune, Germacerene-D
5	Leaves	Xylotenin, Xanthoxyletin, 7-Demethyl-suberosin, Luvangetin
6	Heart wood and bark	Aesculetin Dimethyl ether, Nodakenetin
7	Heart wood and bark	Swietenol, Alloxanthoxyletin
8	Heart wood and bark	Tert-Butylketoles, Swietenone
9	Heart wood and bark	Rutamarin
10	Leaves	Isopimpinellin, Bergaptan, Heliettin
11	Bark	Swietenocoumarin A, B, C, D, E and Swietenocoumarin F
12	Bark	Chalpein, Suberosin
13	Leaves	Coumarindiol
14	Bark	Hinokinin, Savinin, Collinusin, Syringaresinol
15	Heart wood	2,4-dihydroxy-5-prenyl-cinnamic acid
16	Leaves	Isoquercetrin
17	Bark	Skimmianine, γ- Fagarine
18	Bark	Switenidin A, Switenidin B

The chemical structures of some of the isolated metabolites of *C.swietenia* are presented below in Chart 3.03.

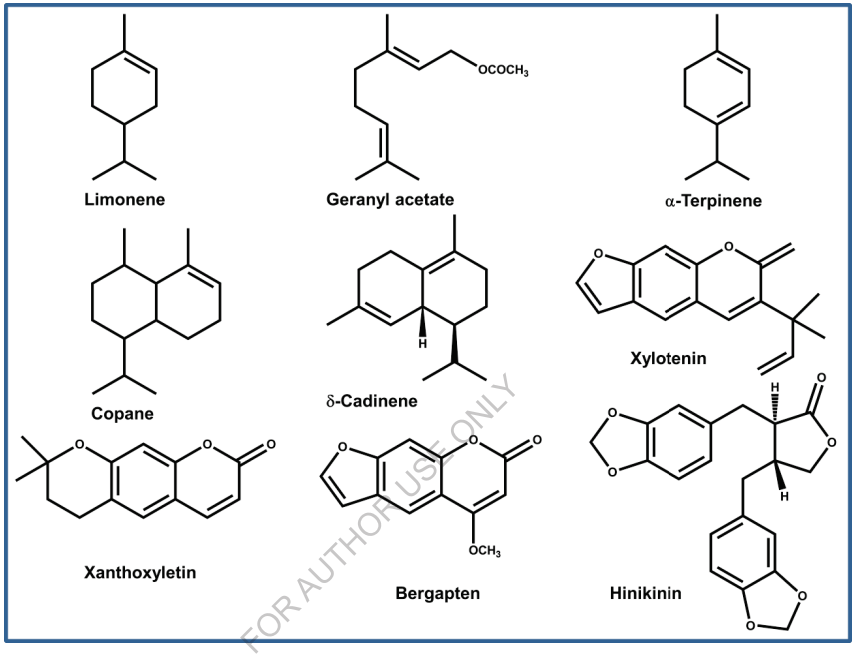


Chart 3.03 Chemical structures of some of the isolated metabolites of *C. swietenia*

Pharmacology

The chloroform extract of the leaves exhibited significant anti-inflammatory response at various doses of 50, 100 and 200 mg/kg, when administered orally[63]. The essential oil produced mosquitocidal activity for the three-vector. Among the major sesquiterpenes of the oil tested at different doses, germacrene D exhibited better mosquitocidal activity and proved to be the potent one followed by pregeijerene and geijerene. Further, the oil and the isolated compounds were reported to be particularly active against *Anopheles gambiae*[64]. The ethanolic extract of the whole plant was reported to possess

hepatoprotective activity at a dose of 25 mg/kg administered by oral gavage[65]. The secondary metabolites of *C. swietenia* crude extract and its fractions, such as hexane and n-butanol fractions showed good tyrosinase inhibition activity[66]. However, the variation in phytochemistry and biological activities of *Chloroxylon swietenia* leaf growing in different altitudes have not yet been reported.

Present work

From the above review it is clearly seen that altitude affect the accumulation of phytoconstituents and their biological activities considerably. Further, *Chloroxylon Swietenia* a potent Indian folklore medicinal plant attracted the attention of researchers recently due to its excellent biological and commercial applications. With this background in view, detailed and systematic chemical and biological studies were now carried out on *Chloroxylon swietenia* leaves collected from two different altitudes. The leaves of *C. swietenia* were collected from Jaipur forest area, Telangana state (**CSL-J**) and from Gadchiroli, Maharashtra state (**CSL-G**). These two leaf samples were independently subjected to chemical and antiproliferative studies and the results are presented in three sections as detailed below.

Section A: Isolation of major secondary metabolites from the leaves of *Chloroxylon swietenia* collected from Jaipur forest area, Telangana state (CSL-J)

Source Collection

The leaf material (2 kg) of *C. swietenia* (**CSL-J**) was collected from Jaipur forest, Mancherial District, Telangana state (Figure 3A.01) in the month of November 2017.

Figure 3A.01 Area of collection of *C. Swietenia* leaves from Jaipur

The authenticity of the plant material was confirmed by taxonomists of Botany Department, Osmania University, Hyderabad and Trans Disciplinary University (TDU) Bangalore. The voucher Specimen was deposited in the Natural Products Chemistry division, CSIR-IICT, Hyderabad. Geographical characteristics of the area of collection are given below. (Table 3A.01).

Table 3A.01 Geographical characters of the area of collection of *C. swietenia* leaves

Location	Latitude	Longitude	Altitude (Above sea level)
Jaipur Forest (Telangana)	18˚ 50’ 47.7” N	79˚ 35’ 12.1” E	159 meters.

Processing

The plant material was shade dried for one week and the dried plant material was subjected to cutting and powdering using a pulveriser as shown in Figure 2A.02.

Extraction

The powdered *C. Swietenia* leaf material (0.25 kg) was extracted successively with different polar solvents such as n-hexane, ethyl acetate ad methanol under hot conditions by using Soxhlet extractor (Chart 3A.01).

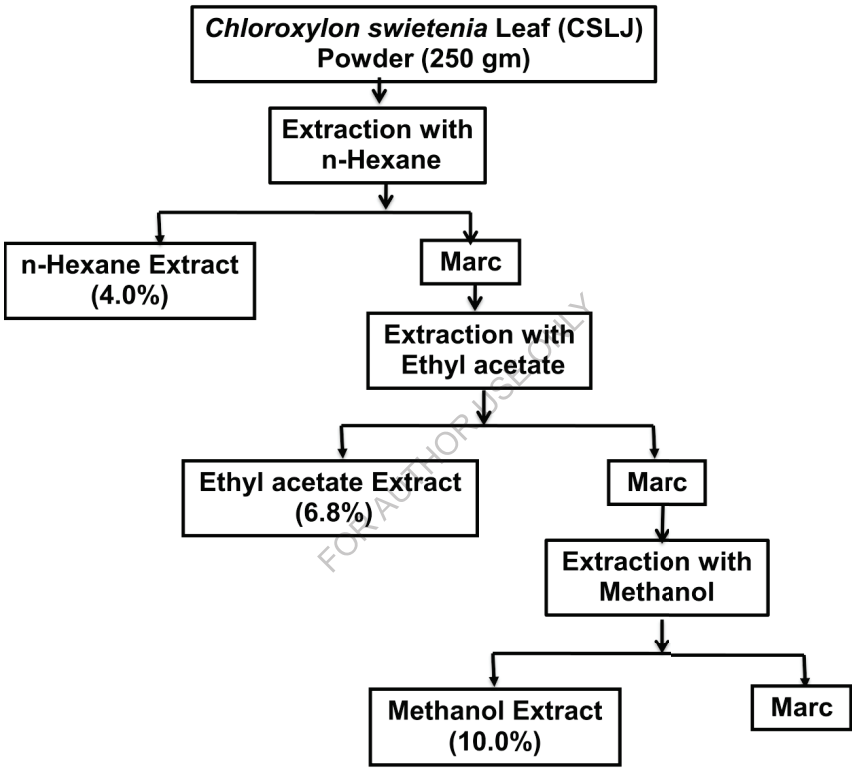


Chart 3A.01 Protocols employed for extraction of *C. Swietenia leaf*

The three soluble portions on evaporation under reduced pressure yielded the respective extracts as shown in the above chart. Among the three extracts, the methanol extract was obtained in high yield (10%). In order to identify major classes of secondary metabolites in these three extracts, they were subjected to preliminary phytochemical screening.

Preliminary phytochemical screening of *C. swietenia extracts*:

Formation of intense colours by a particular class of compounds with some reagents is specific and vital in natural products chemistry. The n-hexane, ethyl acetate and methanol extracts of *C. swietenia* were treated with four commonly used reagents to identify phenols ($FeCl_3$ test), terpenoids/steroids (LB test), alkaloids (Mayer's test), glycosides (Molisch test) and falvonoids (Shinoda's test). The results are presented in Table 3A.02.

Table 3A.02 Phytochemical screening of various polar extracts of *C. swietenia* leaves (CSLJ)

S.No.	Extract	Test					Inference
		$FeCl_3$	LB	Shinoda	Molisch	Mayer's	
1.	n-Hexane	+	+	+	-	-	Phenols/Tannins, Terpenoids/Steroids, Flavonoids
2.	Ethyl acetate	+	+	+	+	-	Phenols/Tannins, Terpenoids/Steroids, Flavonoids, Sugars/Glycosides
3.	Methanol	+	+	+	+	-	Phenols/Tannins, Terpenoids/Steroids, Flavonoids Sugars/Glycosides

From the above results it is evident that the n-hexane extract contains phenols or tannins and terpenoids/steroids. Whereas, the ethyl acetate and methanol extracts showed the presence of sugars or glycosides in addition to the classes of compounds present in n-hexane extract. The three polar extracts have been subjected to detailed TLC studies. The ethyl acetate extracts showed well resolved spots on TLC plate, compared to n-

hexane and methanol extracts, which showed unresolved patterns. Hence, the ethyl acetate extract was subjected to extensive column chromatographic separation and purifications.

Chromatographic purification of ethyl acetate extract of *C. swietenia* leaves (CSLJ)

The green coloured ethyl acetate extract was subjected to column chromatography (Chart 3B.02) over Silica gel (100 – 200 mesh) and eluted with non-polar (n-hexane) to medium polar (ethyl acetate) solvents and made into seven major fractions (F_1-F_7).

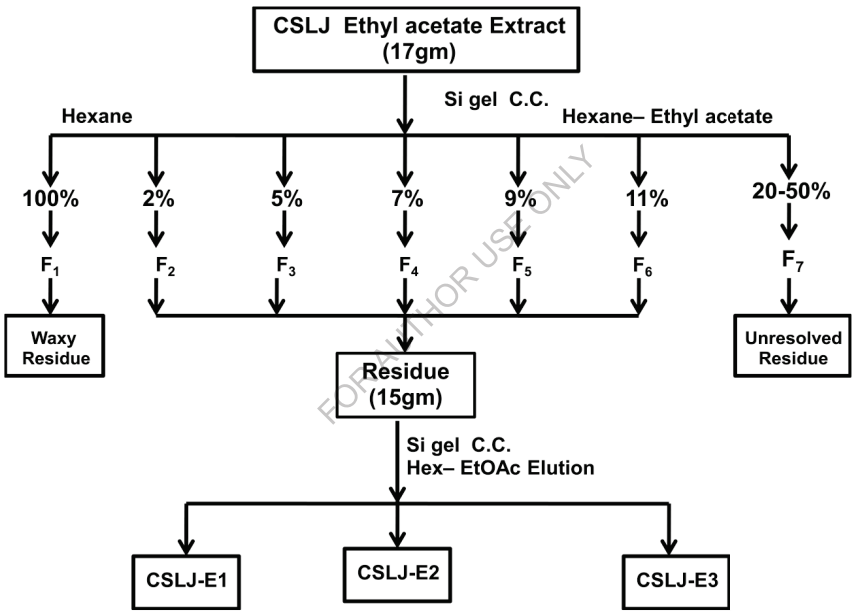


Chart: 3A.02. Chromatographic separation of ethyl acetate extract

Based on TLC nature, fractions F2-F6 were combined and the resultant residue (15 gm) was subjected to further column chromatographic purification over Si gel using n-hexane-ethyl acetate gradient elution to yield three single and pure compounds **CSLJ-E1, CSLJ-E2** and **CSLJ-E3** (Chart 3A.03).

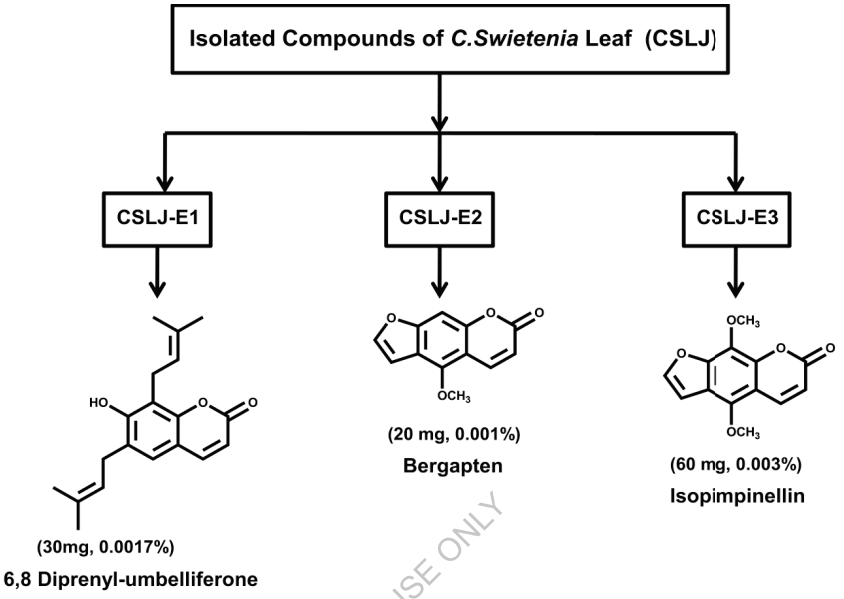


Chart: 3A .03 Compounds isolated from *C. swietenia* leaves (CSLJ)

Structures of these three compounds were established by their physical and spectroscopic (IR, 1H & 13C NMR and HRMS) analysis.

Identification of CSLJ-E1: 6,8 –Diprenyl-umbelliferone

It was obtained as colour less flakes from chloroform, 30 mg (0.0017%), m.p 230^0 C. It showed homogeneity on TLC plate (R_f : 0.5, n-hexane-ethyl acetate, 80:20). It gave positive ferric chloride test for phenols. Its IR spectrum showed absorption peaks at 3300 and 1730 cm^{-1} corresponding to hydroxyl and lactone functionalities. The 400 MHz ^1H NMR spectrum (Figure 3A.02a) of the compound showed four sharp singlets at δ 1.87, 1.79, 1.76 and1.58 corresponding to four double bonded methyls. Further, it showed a

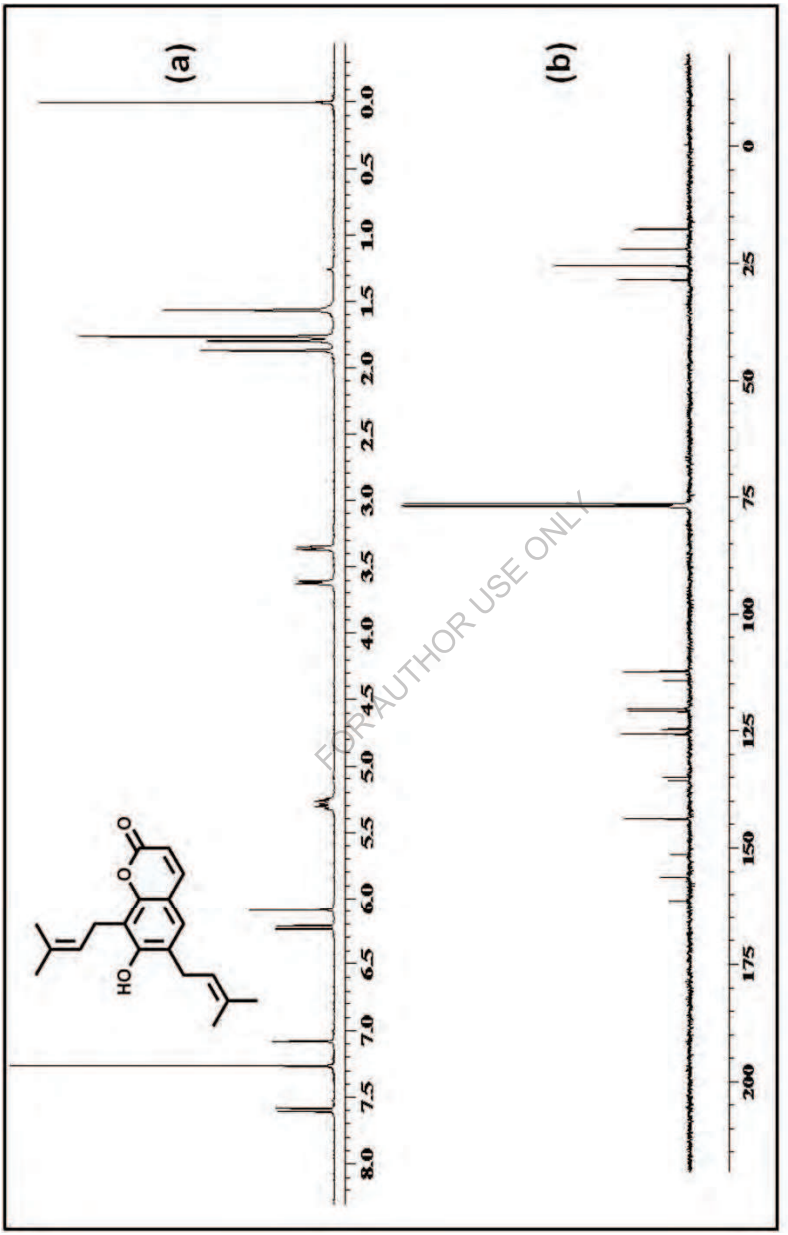
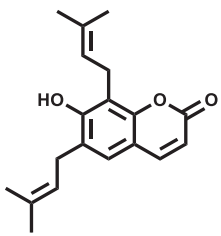


Figure 3A.02 : NMR Spectra of CSLJ-E1 in $CDCl_3$ (a) ^1H (400 MHz); (b) ^{13}C (100 MHz)

pair of doublets at δ 3.36 (2H) and 3.62(2H) corresponding to two methylenes, a multiplet at δ 5.27 (2H) corresponding to two methine protons, a pair of doublets at δ 6.22(1H) and 7.59 (1H) corresponding to two olefinic protons of an α ,ß unsaturated system and singlets at δ 7.08 and δ 6.09(1H) corresponding to an aromatic and phenolic hydroxyl protons respectively. The above ^{1}H NMR data suggests that the compound is 6,8-diprenyl- umbelliferone[67]. The ^{13}C NMR spectrum (Figure 3A.02b) of the compound, while confirming the above structure to the compound exhibited the characteristic carbon signals of α ,ß unsaturated lactone corbons at δ 161.5 (C-2), 144.1 (C-3) and 114.2 (C-4). Further it showed two oxygenated carbons at δ 156.7 (C-7) and 151.6(8a). The DEPT 135 spectrum (Figure 3A.03) revealed the presence of 8 quaternary, 4 methyl, 2 methlene and 5 methine carbons. The HRMS (Figure 3A.04) of the compound showed the pseudo molecular ion $[M+H]^+$ at m/z 299.1646 indicating the molecular formula of the compound as $C_{19}H_{22}O_3$. Based on the above physical and spectral data and comparition with reported value, this compound was identified as 6, 8 –diprenyl-umbelliferone.



6, 8 –Diprenyl-umbelliferone

Identification of CSLJ-E2 : Bergaptan (4-methoxy-7H-furo[3,2-g]chromen-7-one)

It was obtained as colour less powder, 20 mg (0.001%) from chloroform, m.p. 188-190^{0}C. It showed homogeneity on TLC plate (R_f : 0.29, n-hexane-ethyl acetate, 80:20). It

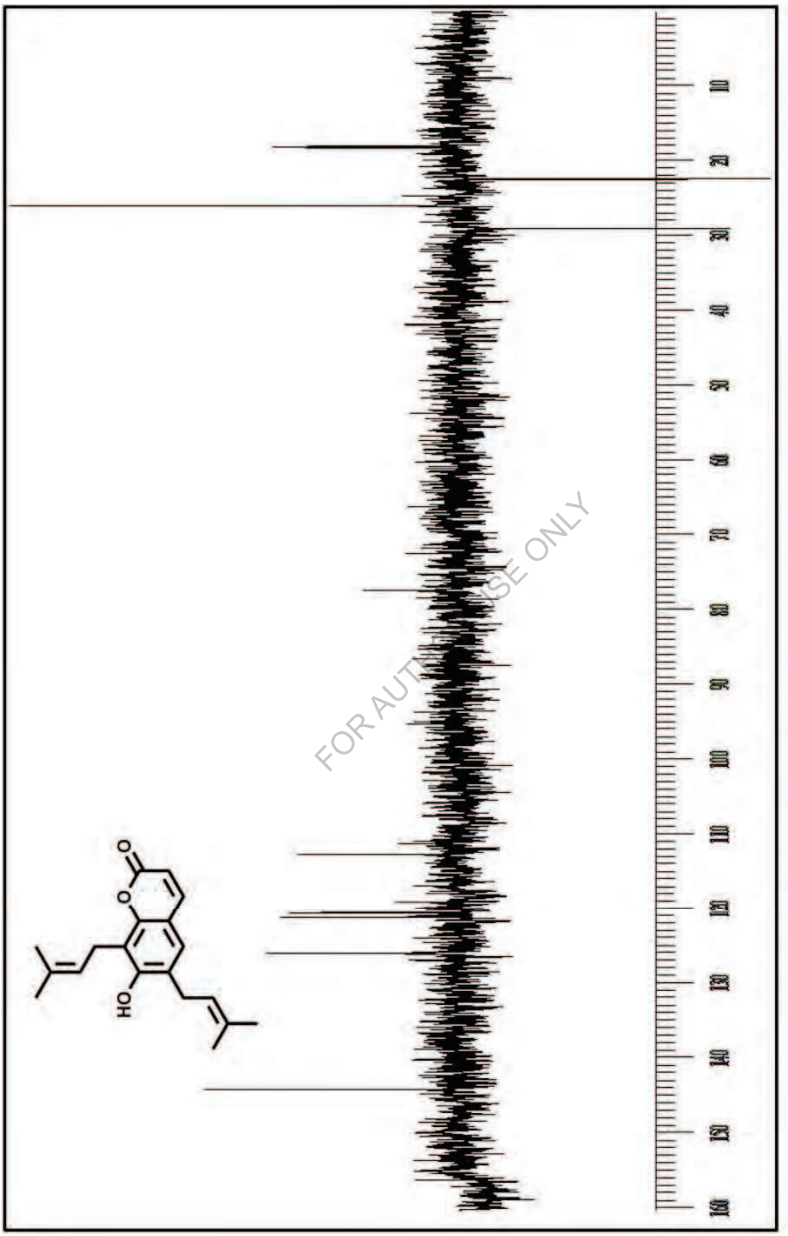


Figure 3A.03: DEPT 135 Spectrum of CSLJ-E1

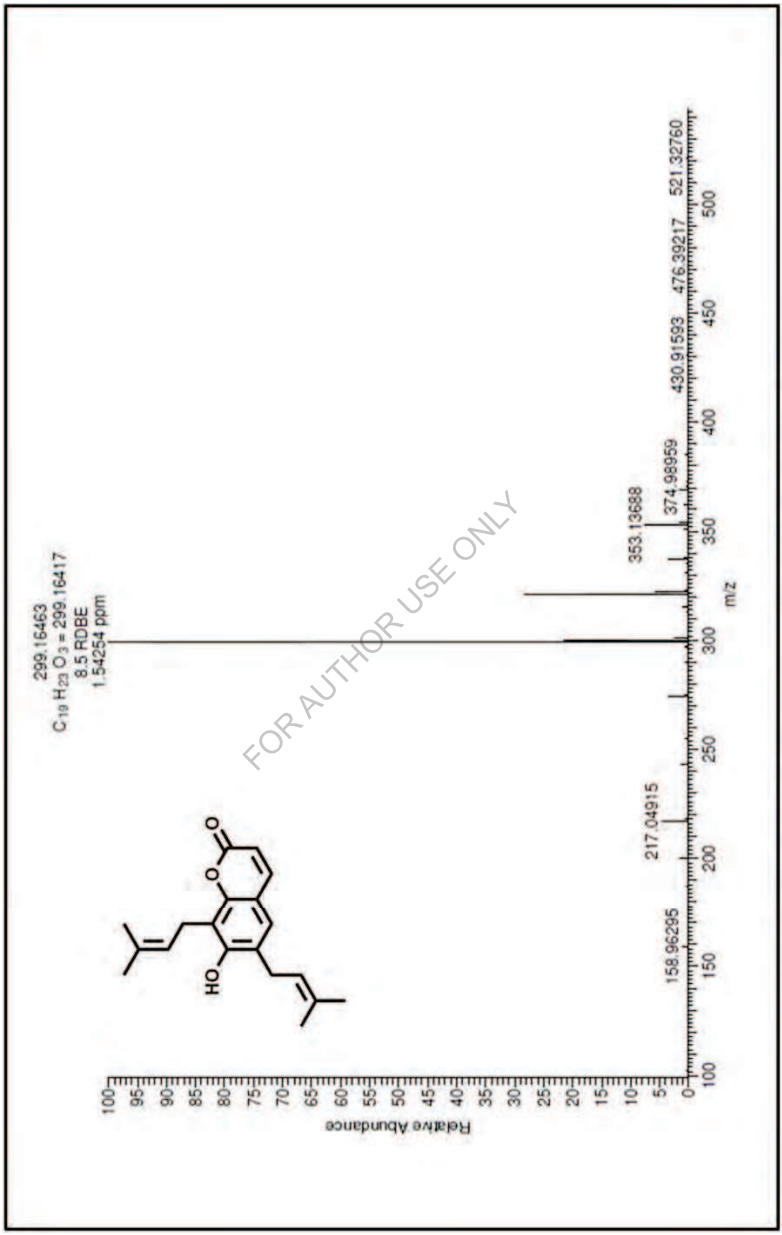
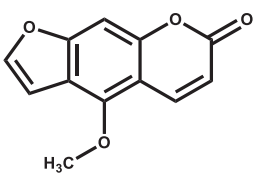


Figure 3A.04: HRMS Spectrum of CSLJ-E1

gave positive ferric chloride test for phenols. Its IR spectrum (Figure 3A.05) showed strong absorption band at 1729 cm^{-1} corresponding to a lactone moeity. The 300 MHz ^1H NMR spectrum (Figure 3A.06a) showed a three proton singlet at δ 4.27 corresponding to methoxyl group and a singlet at δ 7.15 corresponding to an aromatic proton. Further, it showed a pair of doublets at δ 8.17 & 6.29 corresponding to C3 and C4 protons of a coumarin moeity. It also showed another pair of doublets at δ 7.60 & 7.03 corresponding to C2 and C3 protons of a furan ring. The above data suggests that the compound is 5-methoxy-furanocoumarin. The ^{13}C NMR spectrum (Figure 3A.06b), while conforming these observations exhibiting the charecteric α,β-unsaturated carbonyl system of coumarin moeity at δ 168.37 (C-2), 138.9 (C-4), 112.5, (C-3). It also showed the olefinic carbons of furan moiety at δ 144.3 & 104.9 and C-5 methoxy carbon at δ 60.33. The HRMS spectrum (Figure 3A.07) of the compound showed the pseudo molecular ion at m/z 217.04915 suggesting the molecular formula of the compound as $C_{12}H_8O_4$. Based on the above physical and spectral analysis and comparition with reported values[68,69] the compound was identified as Bergaptan (4-methoxy-7H-furo[3,2-g]chromen-7-one) .



Bergaptan

Identification of CSLJ-E3: **Isopimpinellin (4,9-dimethoxy-7H-furo[3,2-g]chromen-7-one)**

It was obtained as colourless powder, 60 mg (0.003%), m.p.147-149^0 C. It showed homogeneity on TLC plate (R_f: 0.20, n-hexane-ethyl acetate, 80:20). It did not give any

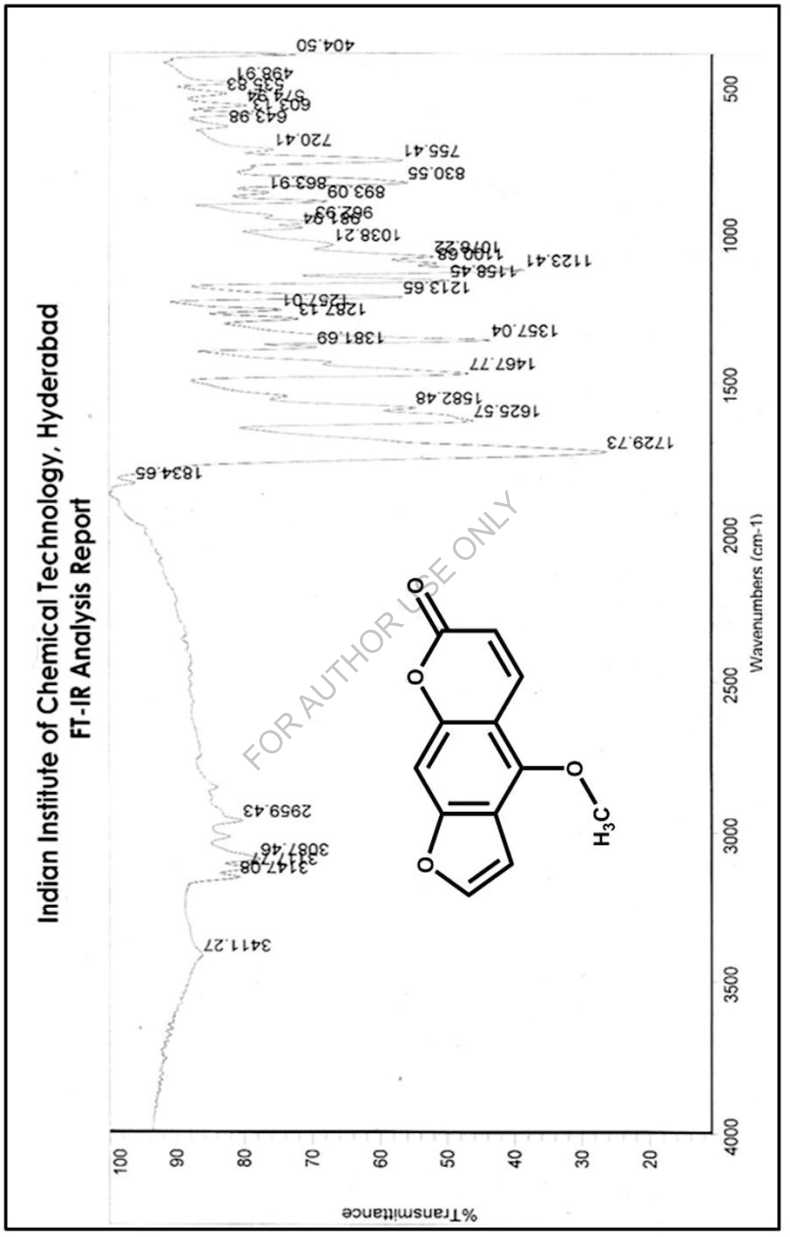


Figure 3A.05: IR Spectrum of CSLJ-E2

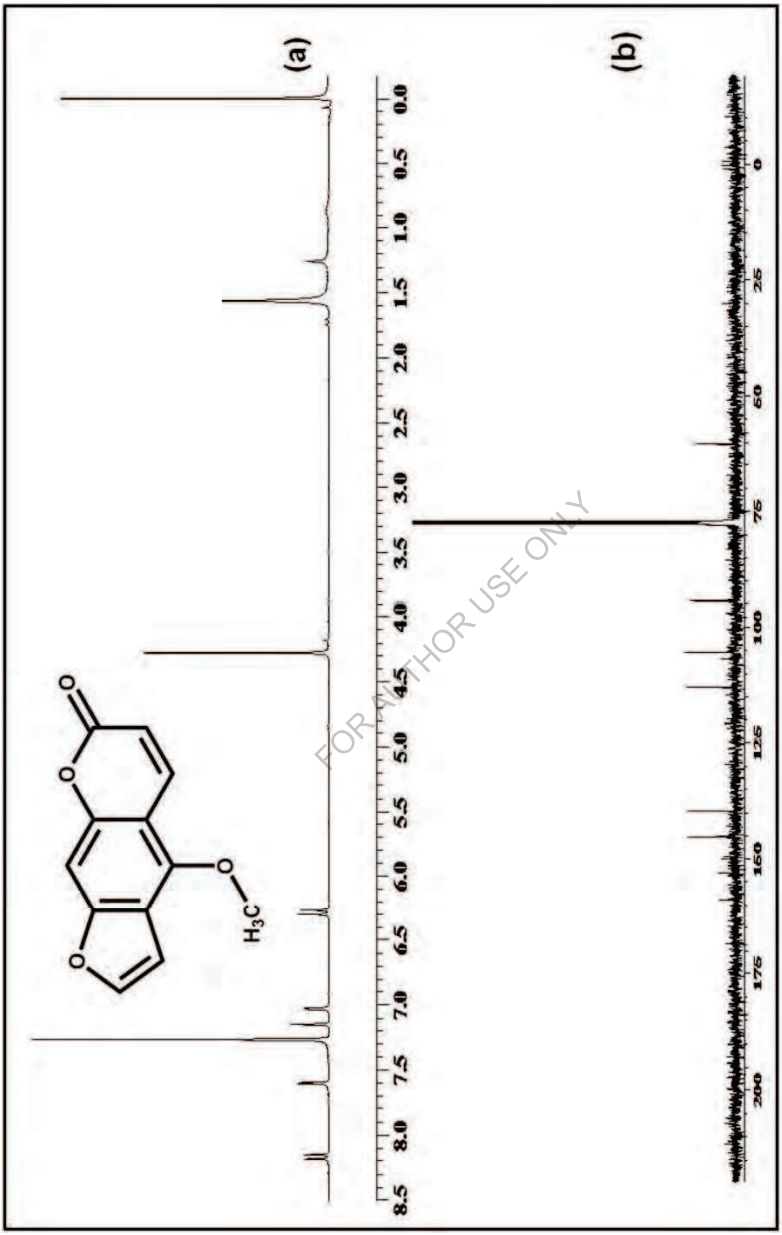


Figure 3A.06: NMR Spectra of CSLJ-E2 in $CDCl_3$ (a) ^1H (300 MHz); (b) ^{13}C (100 MHz)

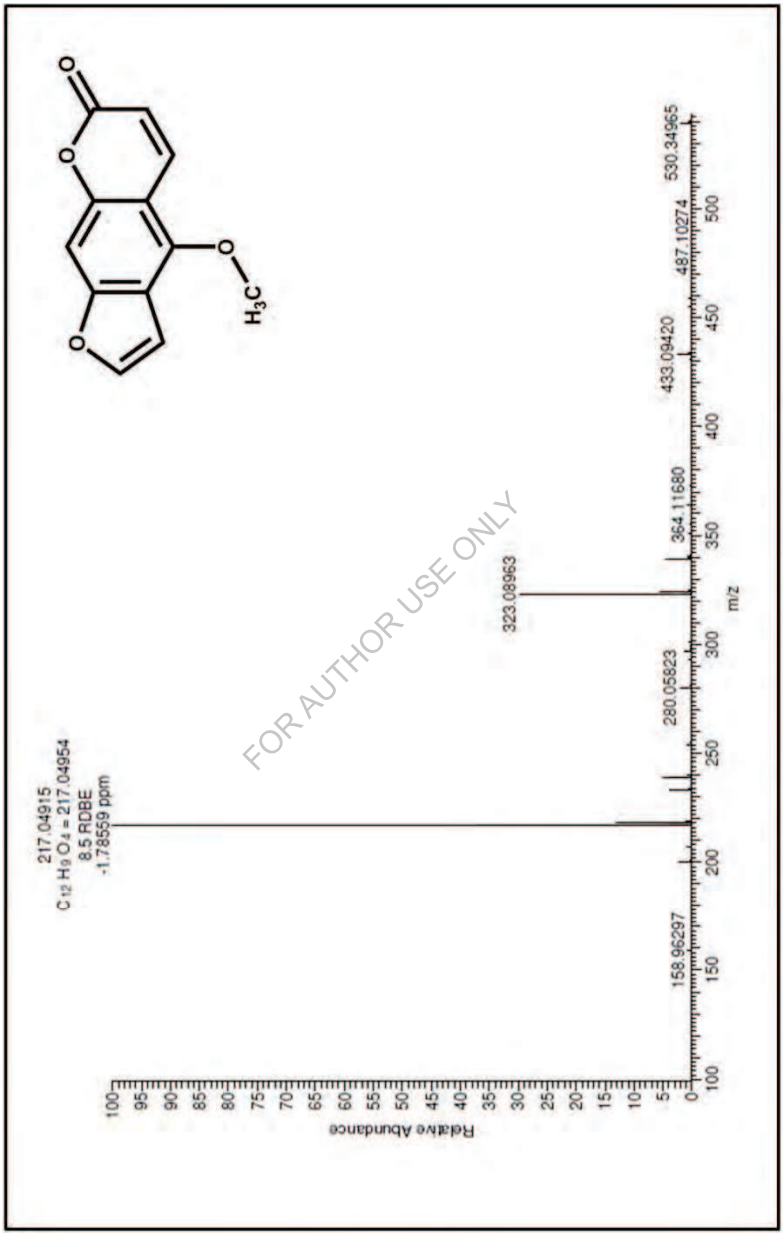
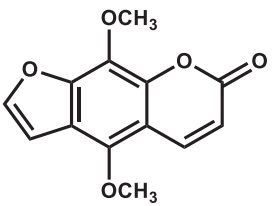


Figure 3A.07 : HRMS Spectrum of CSLJ-E2

colouration with $FeCl_3$, suggesting that there are no free phenolic hydroxyls present in the molecule. Its IR spectrum (Figure 3A.08), showed strong absorption band at 1718 cm^{-1} corresponding to a lactone moiety. Its HRMS (Figure 3A.10) showed the pseudomolecular ion $[M+H]^+$ at m/z 247.06097 suggesting the molecular formula of the compound as $C_{13}H_{10}O_5$ .The 300 MHz ^1H NMR spectrum (Figure 3A.09a) revealed that it is a methoxy substituted furano coumarin by exhibiting the characteristic methoxy protons at δ 4.17, C-3 and C-4 protons of coumarin ring at δ 8.14 (1H,d, J = 9.78 Hz) & 6.29, (1H,d, J = 9.7 Hz) and C8-C9 protons of furan ring at δ 7.63 (1H,d, , J = 2.32 Hz)& 7.0 (1H,d, J = 2.23 Hz),.The ^{13}C NMR spectrum (Figure 3A.09b) of the compound, while conforming the methoxy substituted furano-coumarin skeleton to the compound, exhibited the characteristic carbon signals of C-2 carbonyl at δ 160.3; four oxygenated carbon signals at δ 149.7 (C-5), 144.7 (C-7), 143.9 (C-8a), 144.3 (C-2'); the oelefinic carbons of coumarin moiety at δ 112.7 & 139.3 and furan moiety at δ 105.08 & 145.3; methoxy carbons at δ 61.6 & 60.7. Based on the above physical and spectroscopic data and comparison with reported value[70,71,72] the compound was identified as isopimpinellin (4,9-dimethoxy-7H-furo[3,2-g]chromen-7-one).



Isopimpinellin

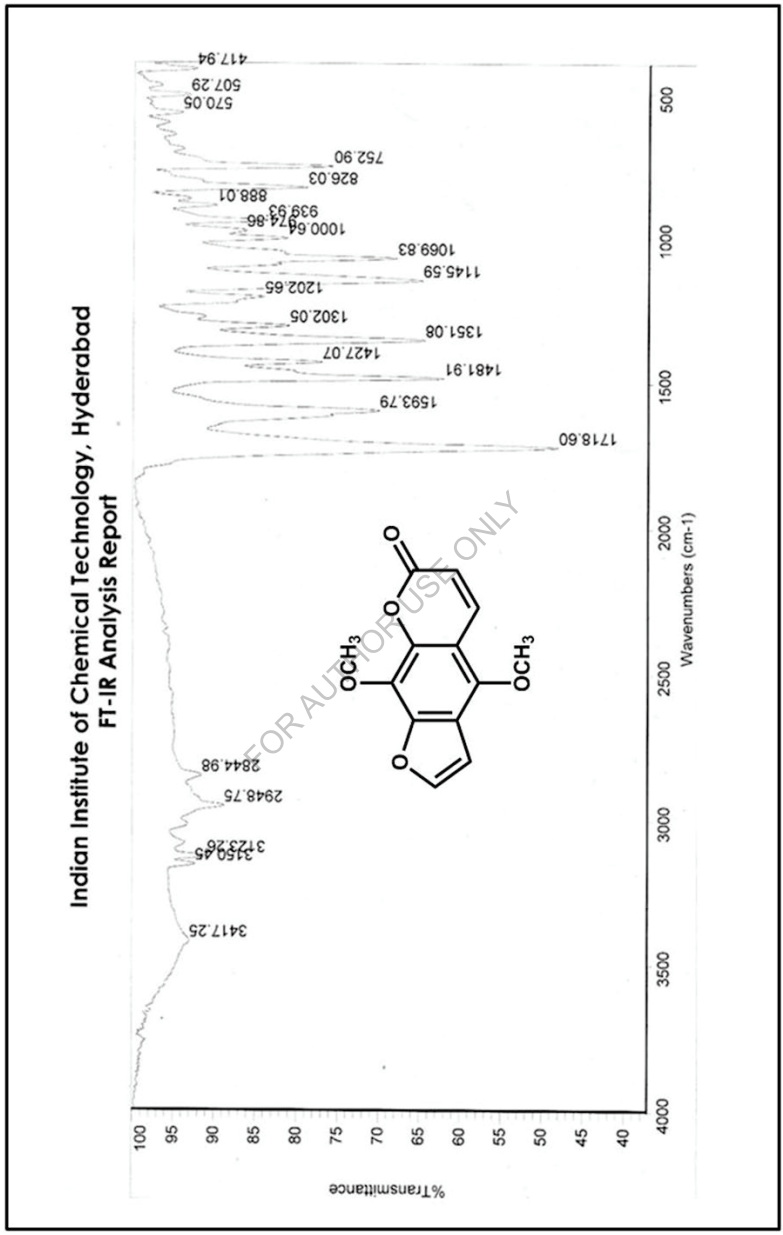


Figure 3A.08: IR Spectrum of CSLJ-E3

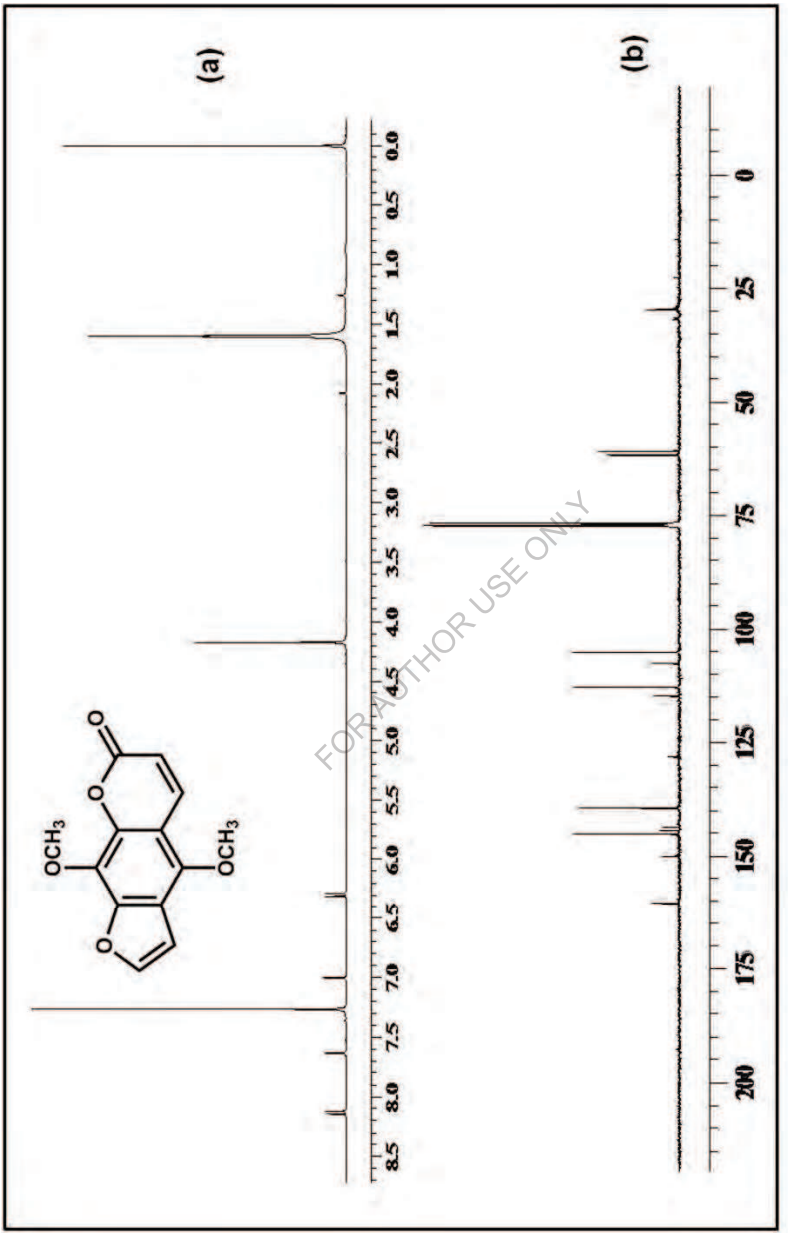


Figure 3A.09 : NMR Spectra of CSLJ-E3 in $CDCl_3$ (a) ^1H (300 MHz); (b) ^{13}C (100 MHz)

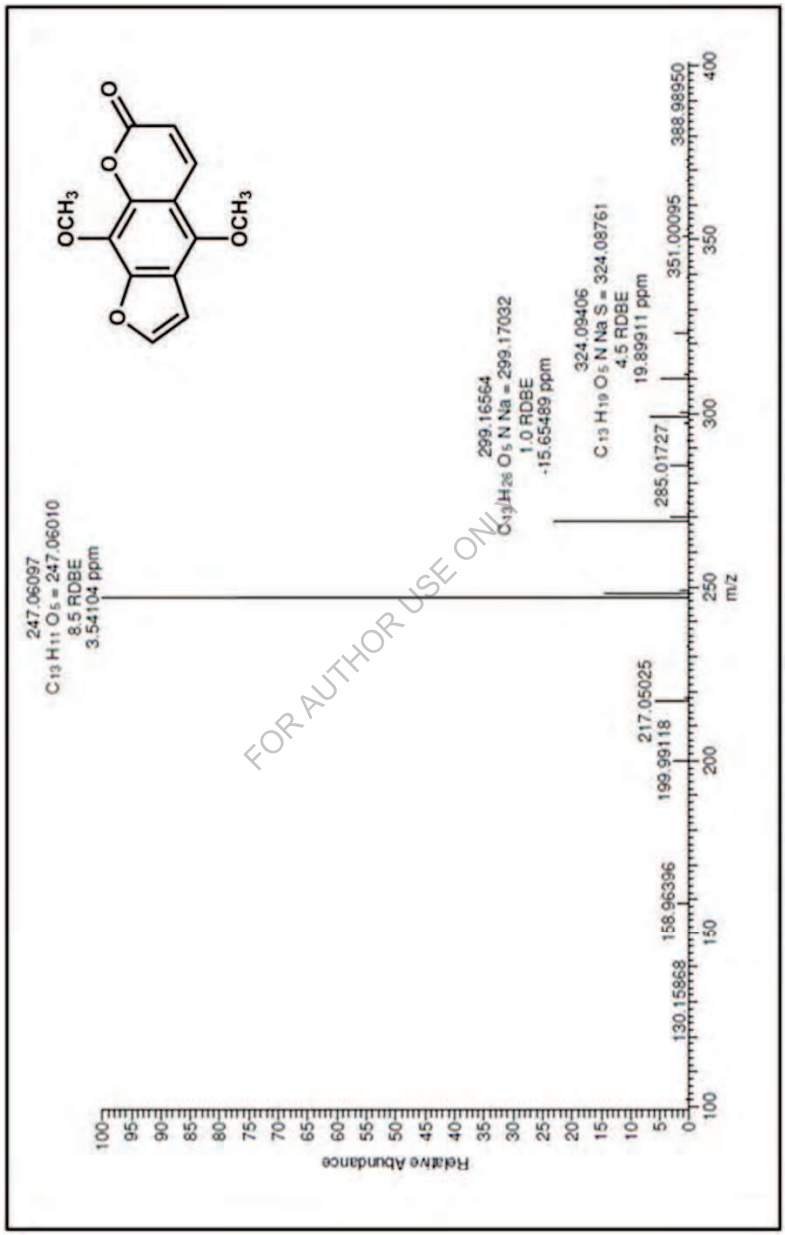


Figure 3A.10 : HRMS Spectrum of CSLJ-E3

EXPERIMENTAL

Plant Material collection

The leaf material (3 kg) of *C. swietenia* was collected from Jaipur forest, Mancherial District, Telangana State (Figure 3A.01) in the month of November 2017. The authenticity of the plant material was confirmed by taxonomists of Botany Department, Osmania University, Hyderabad and Trans Disciplinary University (TDU), Bangalore. The voucher Specimen was deposited in the Natural Products Chemistry division, CSIR-IICT, Hyderabad.

Extraction

The shade dried and powdered leaf of the *C. swietenia* (0.25 kg) was packed in a soxhlet extractor and extracted successively with n-hexane, ethyl acetate and methanol solvents under hot condition for 24 hr. Concentration of the three solubles under vacuum gave the respective extracts in 11 gm (4%), 17 gm (6.8%) and 25 gm (10%) respectively (Chart 3A.01). The initial TLC studies showed well resolved and interesting patterns to ethyl acetate extract, whereas the n-hexane and methanol extracts showed feeble and unresolved patterns. Hence, the ethyl acetate extract was only taken up for column chromatographic separation to isolate single and pure compounds.

Chromatographic Separation of Ethyl acetate Extract

The dark green coloured ethyl acetate extract (17 gm) of *C. swietenia* leaf on TLC showed some blue coloured spots, but not resolved properly, as they are super imposable in n-hexane-ethyl acetate (90:10) solvent system, In order to minimise the complexicity, the extract (17 gm) was adsorbed on silica gel and fractionated over a column of silica gel (500 g, 100-200 mesh) and eluted with solvent gradient from 100% n-hexane to 50% ethyl acetate (Chart 3A.02). Several fractions of 250 ml capacity were collected and the fractions with similar TLC nature (visualization of spots were carried out

under UV light or Iodine vapours, or by spraying 5% methanolic H_2SO_4 followed by heating at 110^0 C) were combined and evaporated to yield seven major fractions (F_1-F_7). Out of these fractions, F_2–F_7 showed well resolved TLC patterns. Hence, they were combined and the resultant residue (15 gm) was subjected to further column chromatographic separation using n-hexane-ethyl acetate gradient elution followed by recrystallization with n-hexane and chloroform to yield three single and pure compounds (**CSLJ-E1 to CSLJ-E3**).

CSLJ-E1: 6,8 –Diprenyl-umbelliferone

It was obtained as colour less flacks from the residue eluted with 5% n-hexane- ethyl acetate (95:5), 30mg (0.0017%). m.p. 230^0 C. It showed homogeneity on TLC plate (R_f: 0.5, n-hexane-ethyl acetate, 8:2).

IR (KBr, cm^{-1})	:	1705 (C=O), 1690 and 1620.
EI Mass (m/z)	:	298 [M^+]
HR Mass		299.1646 $[M+H]^+$ corresponding to the molecular formula $C_{19}H_{23}O_3$.
^{1}H NMR ($CDCL_3$,400 MHz)	:	δ 7.60 (1H, d, J=9.5 Hz), 7.08 (1H, s), 6.08 (1H, s), 6.22 (1H, d, J=9.4 Hz), 3.62 (2H, d, J=7.2 Hz), 5.29 (2H, m), 3.36 (2H, d, J=7.2 Hz), 1.87 (3H, s), 1.79 (3H, s), 1.76 (6H, s).
^{13}CNMR (DMSO+$CDCL_3$100 MHz)	:	δ 161.5 (C-2), 156.7 (C-7), 151.6 (8a),144.1 (C-4), 135.7 (C-3'), 135.0 (C-3), 125.7 (C-6), 124.3 (C-5), 120.7 (C-2'), 120.1 (C-2"), 114.2 (C-3), 112.2 (C-4a), 112.0 (C-8), 28.6 (C-1'), 25.4

(C-5"), 17.7 (C-4'), 112.0 (C-8), 28.6 (C-1'), 25.4 (C-5"), 17.7 (C-4').

CSLJ-E2 : Bergaptan (4-methoxy-7H-furo[3,2-g]chromen-7-one)

It was obtained as colourless powder from the residue eluted with 7% n-hexane- ethyl acetate (93:7), 20mg (0.001%). m.p 190^0 C. It showed homogeneity on TLC plate (R_f : 0.29, n-hexane-ethyl acetate, 8:2).

IR (KBr, cm^{-1}) : 1729 (C=O), 1625, 1582.

EI Mass (m/z) : 217 $[M+H]^+$

HR Mass 217.04915 corresponding to the molecular formula $C_{12}H_9O_4$.

^{1}H NMR ($CDCL_3$,400 MHz) : δ 8.17(1H, d, J=9.9Hz), 7.60 (1H, d, J=2.3 Hz,), 7.15 (1H, s), 7.03 (1H, d, J=2.3 Hz,), 6.29 (1H, d , J=9.9Hz), 4.27 (3H, s, -OCH3).

^{13}CNMR (DMSO+$CDCL_3$100 MHz) : δ 161.5 (C-2), 157.8 (C-7), 152.4 (C-8a), 144.3 (C-10), 138.9 (C-4), 112.5 (C-3), 112.2 (C-5), 106 (C-4a), 104.9 (C-8), 104.7 (C-9), 93.5 (C-8), 59.66 (C-5 -OCH3).

CSLJ-E3: **Isopimpinellin (4,9-dimethoxy-7H-furo[3,2-g]chromen-7-one)**

It was obtained as colourless powder, from the residue eluted with n-hexane- ethyl acetate (91:9), 60 mg (0.003%). m.p 147-149^0 C. It showed homogeneity on TLC plate. (R_f : 0.20, n-hexane:ethyl acetate, 8:2).

IR (KBr, cm^{-1}) : 1718.6 (C=O), 1593 (aromatic).

EI Mass (m/z) : 246 $[M^+]$,

HR Mass 247.0610 $[M+H]^+$ corresponding to the

molecular formula $C_{13}H_{11}O_5$.

^{1}H NMR ($CDCL_3$,400 MHz)	:	δ 8.14 (1H, d, J=9.78 Hz), 7.63 (1H, d, J=2.32 Hz), 7.0 (1H, d, J=2.23 Hz), 6.29 (1H, d, J=9.7 Hz), 4.17 (6H, d, J=0.85 Hz,).
^{13}CNMR (DMSO+$CDCL_3$100 MHz)	:	δ 160.5 (C-2), 149.93 (C-5), 145.3 (C-7), 144.2 (C-2 '), 143.62 (C-8a), 139.3, 4 (C-4), 128.1 (C-8), 114.7 (C-6), 112.7 (C-3), 107.5 (C-4a), 105.08 (C-3), 61.6 (C-8-OCH3), 60.7 (C-5-OCH3).

Section B: Isolation of major secondary metabolites from the leaves of *Chloroxylon swietenia* collected from Gadchiroli Forest, Maharashtra (CSL-G)

Source Collection

The leaf material (2 kg) of *Chloroxylon swietenia* was collected from Gadchiroli forest area, Maharashtra state (Figure 3B.01) in the month of November 2017.

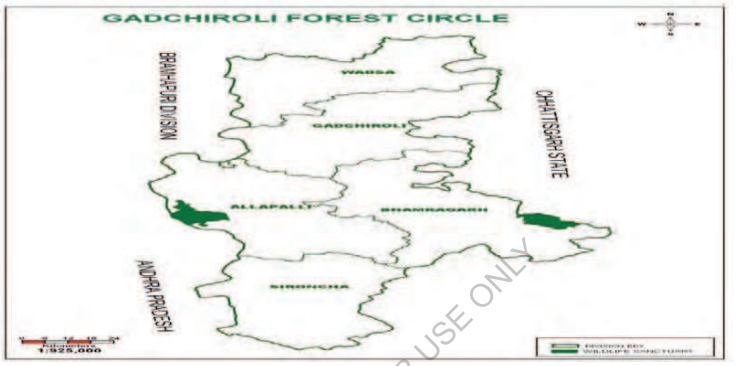


Figure 3B.01 Area of collection of *C. Swietenia* leaves from Gadchiroli

The authenticity of the plant material was confirmed by taxonomists of Botany Department, Osmania University, Hyderabad and Trans Disciplinary University (TDU) Bangalore. The voucher Specimen was deposited in the Natural Products Chemistry division, CSIR-IICT, Hyderabad. Geographical characteristics of the area of collection are given below. (Table 3B.01).

Table 3B.01 Geographical characters of the area of collection of *C. swietenia* leaves

Location	Latitude	Longitude	Altitude (Above sea level)
Gadchiroli Forest (Maharashtra)	20° 10' 56.66"N	80° 00' 12.06" E	217 meters

Processing

The plant material was shade dried for one week and the dried plant material was subjected to cutting and powdering using a pulveriser as shown in Figure 2A.02..

Extraction

The powdered *C. Swietenia* leaf material (0.5 kg) was extracted successively with different polar solvents such as n-hexane, ethyl acetate ad methanol under hot conditions using soxhlet extractor (Chart 3B.01).

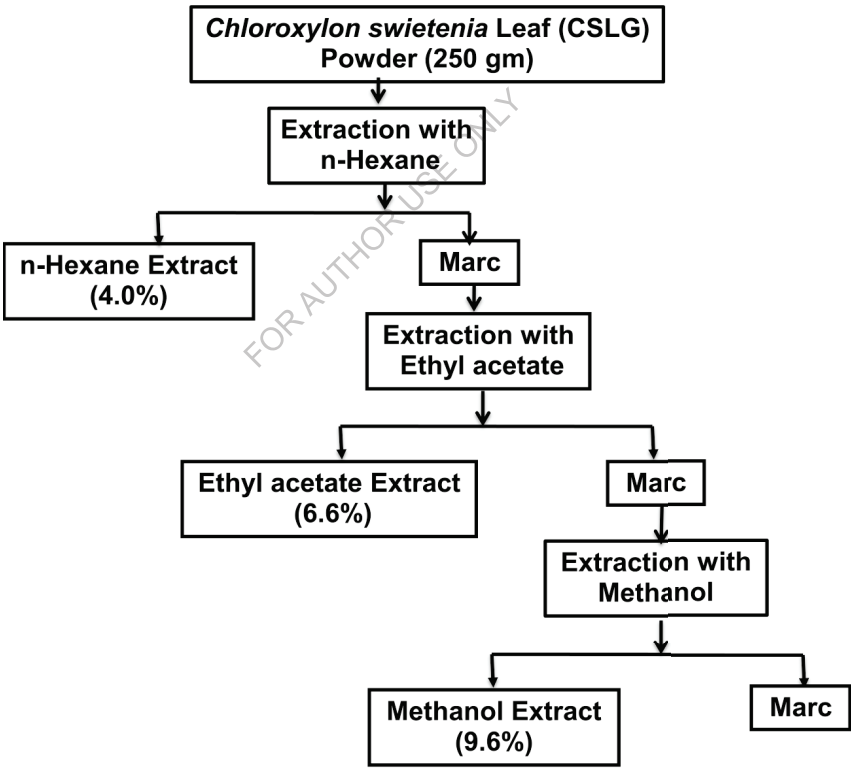


Chart 3B.01 Protocols employed for extraction of *C. Swietenia leaf*

The three soluble portions on evaporation under reduced pressure yielded the respective extracts as shown in the above Chart. Among the three extracts, the methanol extract was obtained in high yield (9.6%). In order to identify major classes of secondary metabolites in these three extracts, they were subjected to preliminary phytochemical screening.

Preliminary phytochemical screening of *C. Swietenia extracts*

Formation of intense colours by a particular class of compounds with some reagents is specific and vital in natural products chemistry. The n-hexane, ethyl acetate and methanol extracts of *C. swietenia* were treated with five commonly used reagents to identify phenols ($FeCl_3$ test), terpenoids/steroids (LB test), alkaloids (Mayer's test), glycosides (Molisch test) and flavonoids (Shinoda's test). The results are presented in Table 3B.02.

Table 3B.02 Phytochemical screening of various polar extracts of *C. swietenia* leaves (CSLG)

S.No.	Extract	Test					Inference
		$FeCl_3$	LB	Shinoda	Molisch	Mayer's	
1.	n-Hexane	+	+	+	-	-	Phenols/Tannins, Terpenoids/Steroids, Flavonoids
2.	Ethyl acetate	+	+	+	+	-	Phenols/Tannins, Terpenoids/Steroids, Flavonoids, Sugars/Glycosides
3.	Methanol	+	+	+	+	-	Phenols/Tannins, Terpenoids/Steroids, Flavonoids Sugars/Glycosides

From the above results it is evident that the n-hexane extract contains phenols or tannins and terpenoids/steroids. Whereas, the ethyl acetate and methanol extracts showed the presence of sugars or glycosides in addition to the classes of compounds present in n-hexane extract. The three polar extracts were subjected to detailed TLC studies. The ethyl acetate extracts showed well resolved spots on TLC plate, compared to n-hexane and methanol extracts, which showed unresolved patterns. Hence, the ethyl acetate extract was subjected to extensive column chromatographic separation and purifications.

Chromatographic purification of ethyl acetate extract of *C. swietenia* leaves (CSLG)

The green coloured ethyl acetate extract was subjected to column chromatography (Chart 3B.02) over Silica gel (100 – 200 mesh) and eluted with non-polar (n-hexane) to medium polar (ethyl acetate) solvents and made into seven major fractions (F_1-F_7).

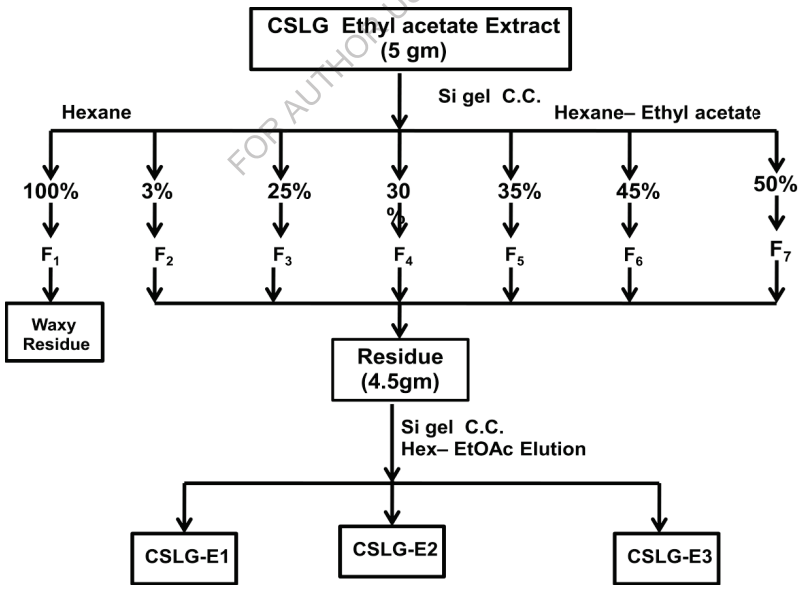


Chart: 3B.02. Chromatographic separation of ethyl acetate extract

Based on TLC nature fractions F_2-F_7 were combined and the resultant residue (4.5 gm) was subjected to further column chromatographic purification over Si gel using n-hexane-ethyl acetate gradient elution to yield three single and pure compounds **CSLG-E1, CSLG-E2** and **CSLG-E3** (Chart 3B.03).

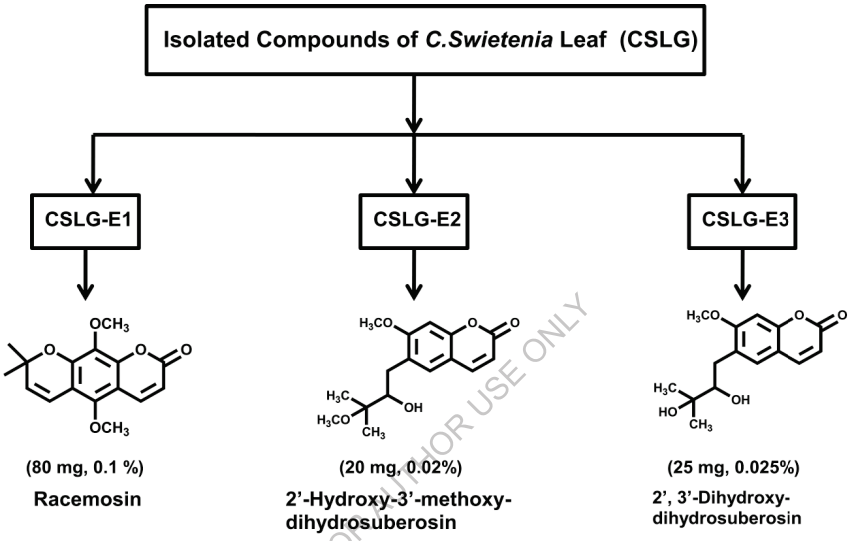


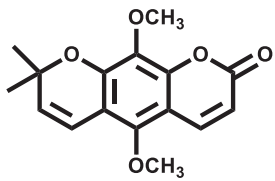
Chart: 3B .03 Compounds isolated from *C. swietenia* leaves (CSLG)

Structures of these three compounds were established by their physical and spectroscopic (IR, ^1H & ^{13}C NMR and Mass) analysis.

Identification of CSLG-E1: Racemosin (5,10-dimethoxy-8,8-dimethyl-8,9a-dihydro-pyrano[3,2-g]chromen-2(5aH)-one)

It was obtained as colourless granules from chloroform, 80 mg (0.1 %), 125-130^0 C, It showed homogeneity on TLC plate (R_f : 0.5, n-hexane:ethyl acetate, 80:20). it did not give any colouration with $FeCl_3$, suggesting thet there are no free phenolic hydroxyls present in the molecule. Its UV spectrum showed the absorption maxima at λ_{max} 266 and 348 nm, which are characteristic of a pyranocoumarin moiety and found to be closely

related to xanthyletin[73]. The IR spectrum of the compound showed a strong absorption band at 1727 cm^{-1} suggesting the presence of coumarin ring. The 300 MHz ^1H NMR spectrum (Figure 3B.02) of the compound showed two geminal methyls as a six proton singlet at δ 1.25, two methoxyl groups as a six proton singlet at δ 4.15, a pair of doublet (J=9.9 Hz) at δ 6.30 and δ 8.15 corresponding to H-3 and H-4 protons of a coumarin moiety, a pair of broad doublets at at δ 7.00 and δ 7.63 corresonding to a chromene olefinic system. These observations suggest that it is a dimethoxy furnocoumarin. The ^{13}C NMR spectrum (Figure 3B.03) of the compound while confirming these observations showed the characteristic carbon signals at δ 160.5 (coumarin carbonyl); 144.5 & 113.0 (C3 & C4 of coumarin moiety); 150.0, 145.0 &144.8 (phenolic oxygen connected carbons), 139.5 & 115.0 (olefinic carbons of chromene ring); 61.5 & 60.5 (methoxy carbons) and 29.0 (gem dimehtlys). The mass spectrum of the compound showed the sodiated molecular ion [M+Na] at m/z 311 suggesting the molecular formula of the compound as $C_{16}H_{16}O_5$. Based on the above physical and spectroscopic data and by comparison with reported values, this compound was identified as 5,10-dimethoxy-xanthyletin. It is trivially known as racemosin (5,10-dimethoxy-8,8-dimethyl-8,9a-dihydro-pyrano[3,2-g]chromen-2(5aH)-one).



Racemosin

The present isolation of this compound from *Chloroxylon swietenia* assumes taxonomic significance, as this compound was earlier reported[74] from entirely different genus plant species, *Atalantia racemosa* Wt. and Am.

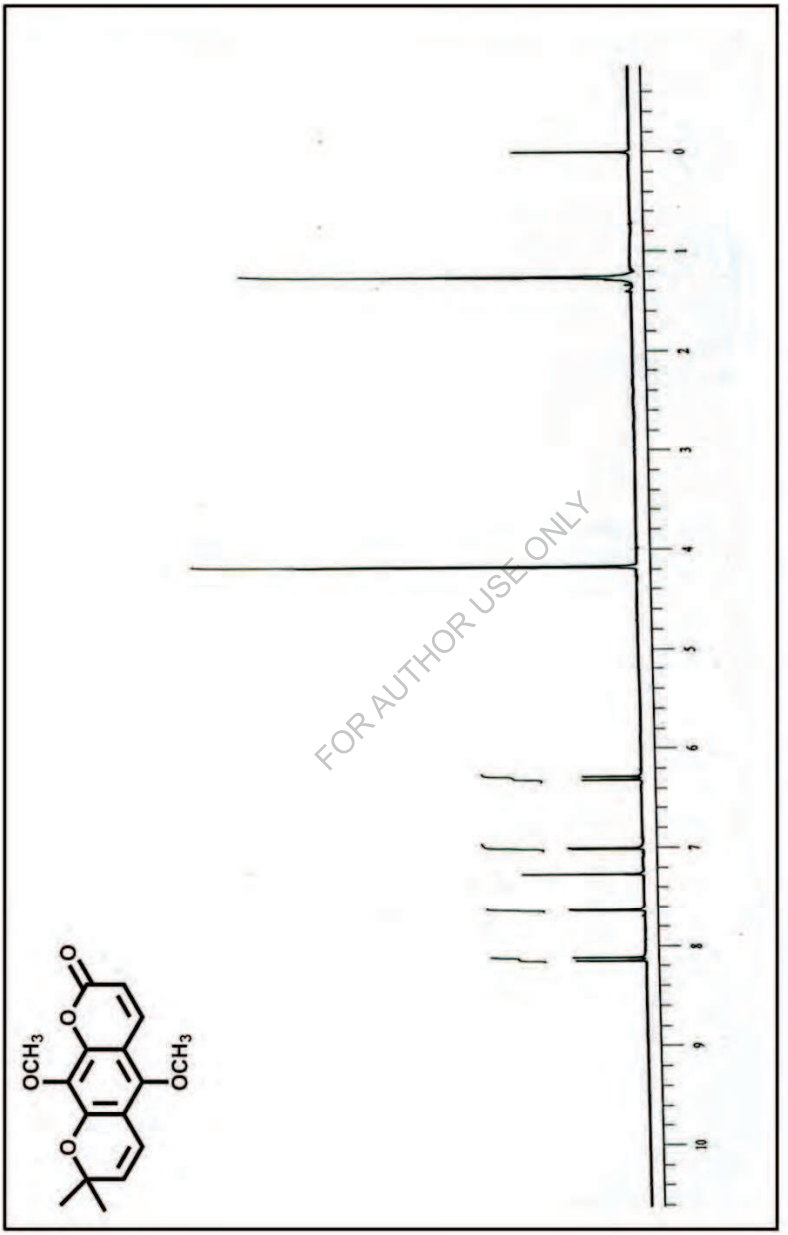


Figure 3B.02 : ^{1}H (400) MHz NMR Spectra of CSLG-E1 in $CDCl_3$

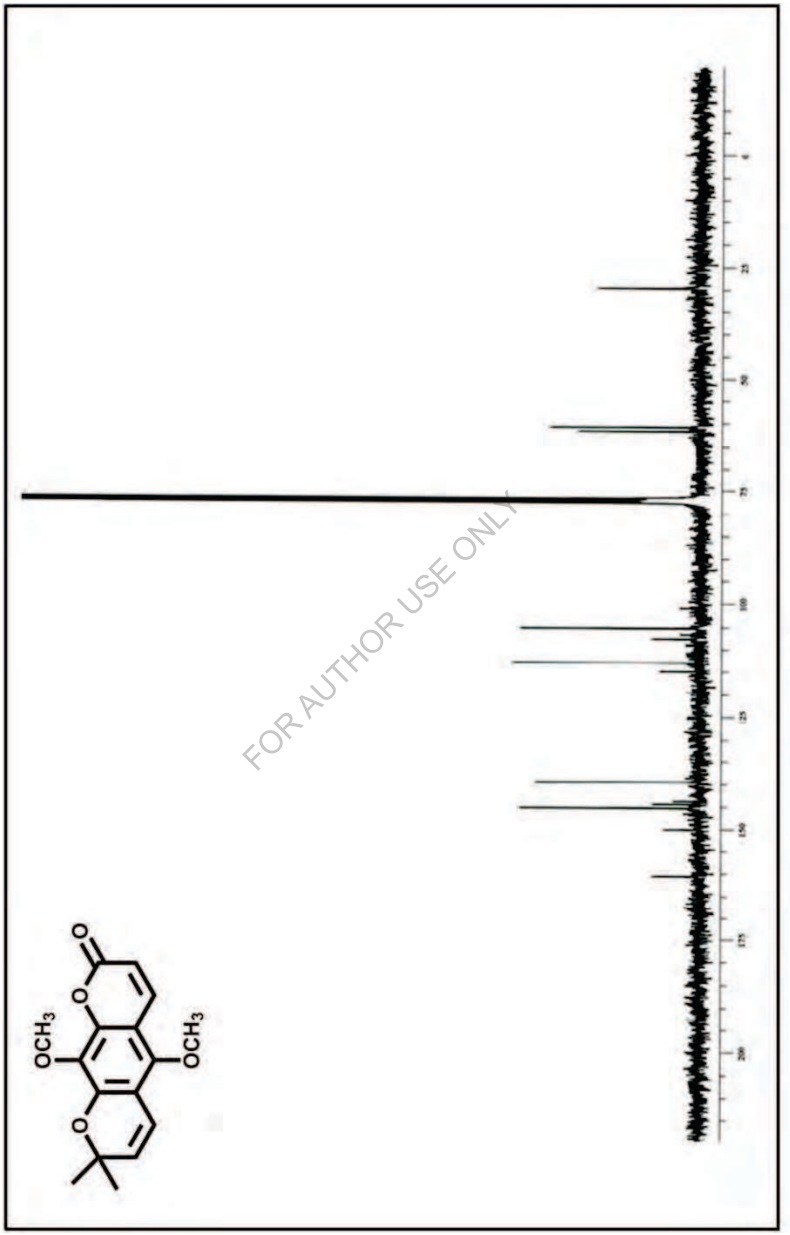


Figure 3B.03 : ^{13}C (100 MHz) NMR Spectra of CSLG-E1 in $CDCl_3$

Identification of CSLG-E2: 2'-Hydroxy-3'-methoxy-dihydrosuberosin [6-(2-Hydroxy-3-methoxy-3-methylbutyl)-7-methoxy-2H-chromen-2-one]

It was obtained as a semisolid, 20 mg (0.02%). It showed homogeneity on TLC plate (R_f 0.22, n-hexane:ethyl acetate , 70:30). It is determined to be a coumarin derivative by the presence of characteristic absorption signal at λ_{max} 328 nm in its UV spectrum. The IR spectrum of the compound showed strong absorption band at 1730 cm^{-1} corresponding to the carbonyl of a lactone ring. The 300 MHz ^1H NMR spectrum (Figure 3B.04) of the compound showed two sharp singlet at δ 1.24 & 1.21 .integrating each for three protons corresponding to two geminal methyls; two three proton singlets at δ 3.28 and 3.90 corresponding to one aliphatic and one aromatic methoxyl groups; a pair of doublet at δ 6.25 (1H,d,H-3,J=9.9Hz) and 7.64 (1H,d,H-3,J=9.9Hz) corresponding to H-3 and H-4 of a coumarin ring; two singlets at δ 7.38 & 6.80 corresponding to para substituted aromatic protons. Further, the ^1H NMR spectrum also showed a benzylic methylene as a pair of multiplts at δ 3.00 and 2.50 in addition to an oxygenated methine proton at δ 3.78 as a multiplet. These observations suggests that the compound is a suberosin analogue substituted at 2' and 3' positions of the prenyl unit. The ^{13}C NMR spectrum (Figure 3B.05), while supporting these obsevations, showed the characteristic oxygenated carbon signals at δ 99.0 and 75.6 suggesting that the prenyl unit is substituted with two oxygenated functions. The ^{13}C NMR spectrum also showed carbon signals at 162.0 (coumarin carbonyl); 161.0 & 154.5 (phenolic oxygen attached aromaric carbons); 144.0 & 113.0 (coumarin C-4 and C-3 olefinic carbons); 56.0 & 49.5 (methoxy carbons) and 32.0 (benzylic methylene). ESI mass spectrum of the compound showed the pseudo molecular ion [M+H] at m/z 293 suggesting the molecular formula $C_{16}H_{20}O_5$ to the compound. Based on the above physical and spectroscopic data and comparison of its data with the reported values, the compound was identified as 2'-hydroxy-3'-methoxy-

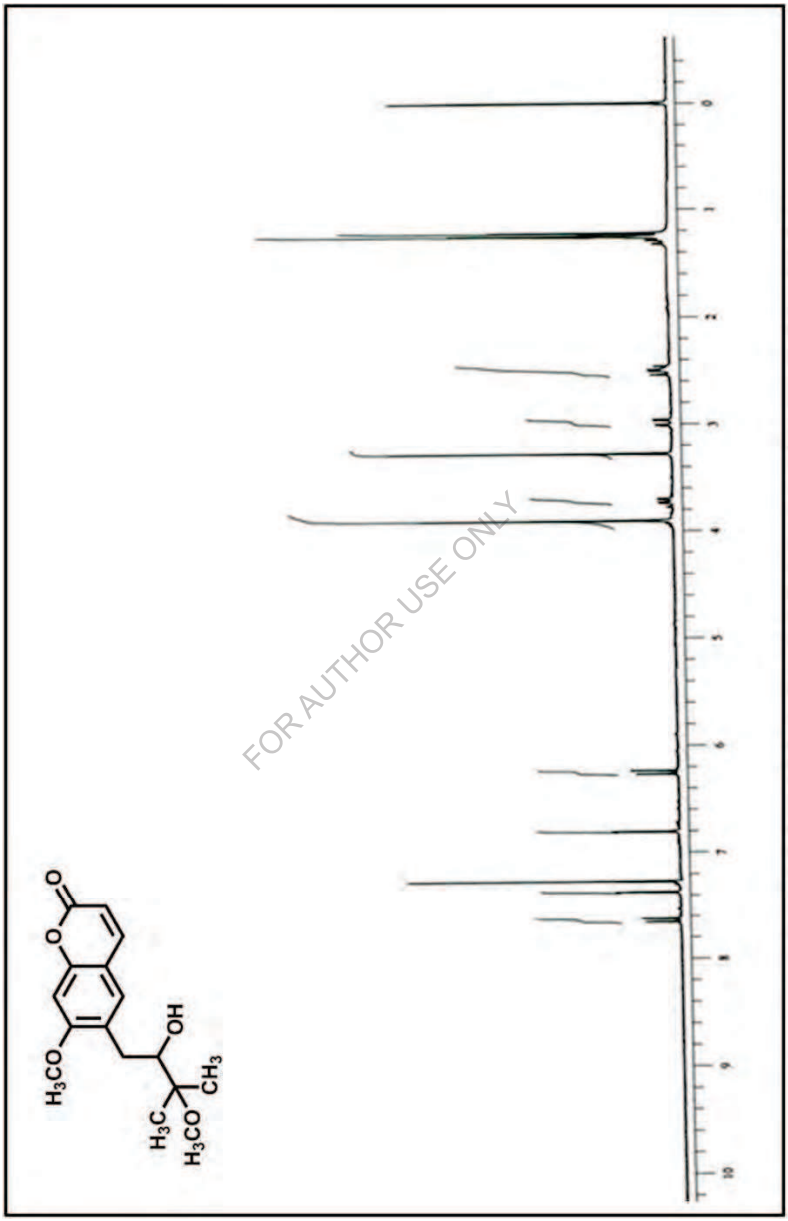


Figure 3B.04 : ^{1}H (400 MHz) NMR Spectra of CSLG-E2 in $CDCl_3$

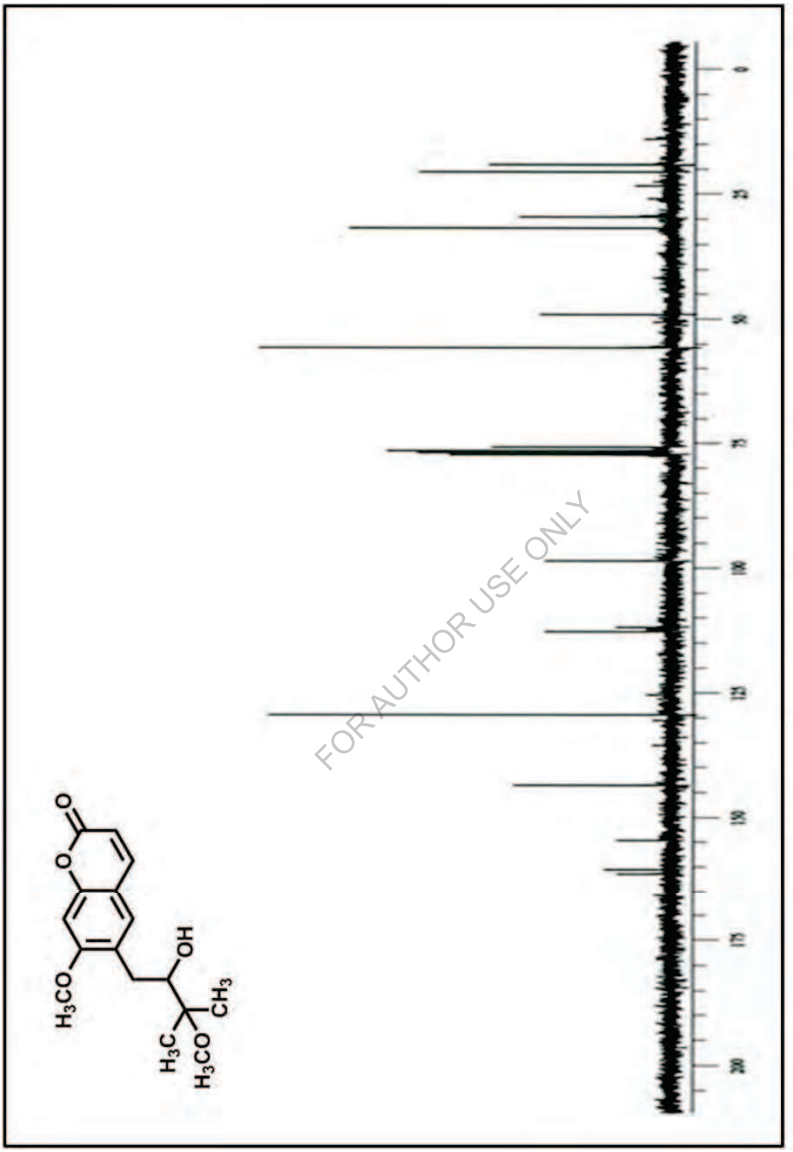
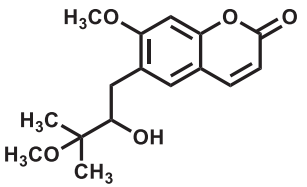


Figure 3B.05 : ^{13}C (100 MHz) NMR Spectra of CSLG-E2 in $CDCl_3$

dihydrosuberosin (6- (2-Hydroxy-3-methoxy-3-methylbutyl) - 7-methoxy-2H-chromen-2-one).



2'-Hydroxy-3'-methoxy-dihydrosuberosin

The present isolation of a suberosin analogue from *C. swietenia* assumes taxonomic significance as this class of compounds were reported earlier from *Harbouria trachypleura* (A. Gray) J. Coulter & Rose, which belongs to the Apiaceae (Umbelliferae), family[75].

Identification of CSLG-E3 : 2', 3'-Dihydroxy-dihydrosuberosin [6-(2,3-Dihydroxy-3-methylbutyl)-7-methoxy-2H-chromen-2-one]

It was obtained as a semisolid, 25 mg (0.025%). It showed homogeneity on TLC plate (R_f 0.19, n-hexane-ethyl acetate, 70:30). It is determined to be a coumarin derivative by the presence of characteristic absorption signal at λ_{max} 328 nm in its UV spectrum. The IR spectrum of the compound showed strong absorption band at 1725 cm^{-1} corresponding to the carbonyl of a lactone ring. The 300 MHz ^1H NMR spectrum (Figure 3B.06) of the compound showed close similarities with that of CSLG-E2. But the major difference found is the disappearance of the peak at δ 3.28 suggesting that the methoxyl group attached at C-3' of prenyl unit is absent in this compound. This observation is well supported by its ^{13}C NMR spectrum (Figure 3B.07), which also showed only one methoxyl carbon at δ 52.0. Another major difference found in this compound is the chemical shift values of C-2' and C-3', which appeared at δ 62.0 and 76.5 as against

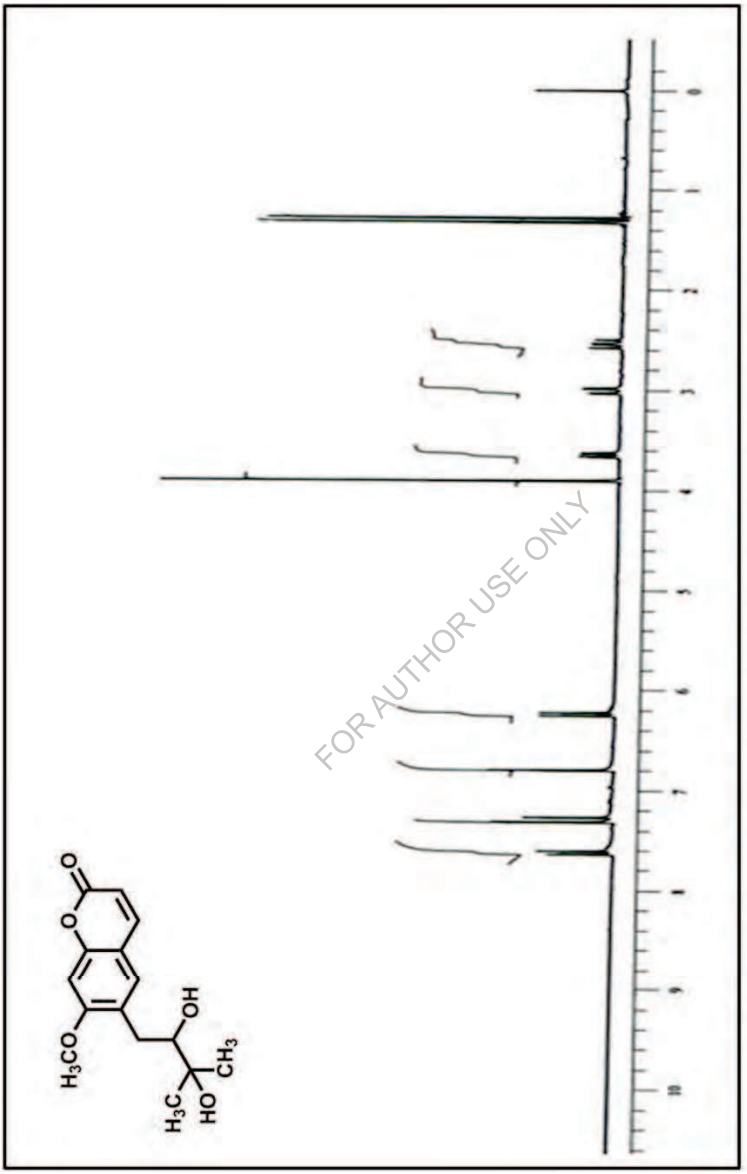


Figure 3B.06 : ^1H (400 MHz) NMR Spectra of CSLG-E3 in $CDCl_3$

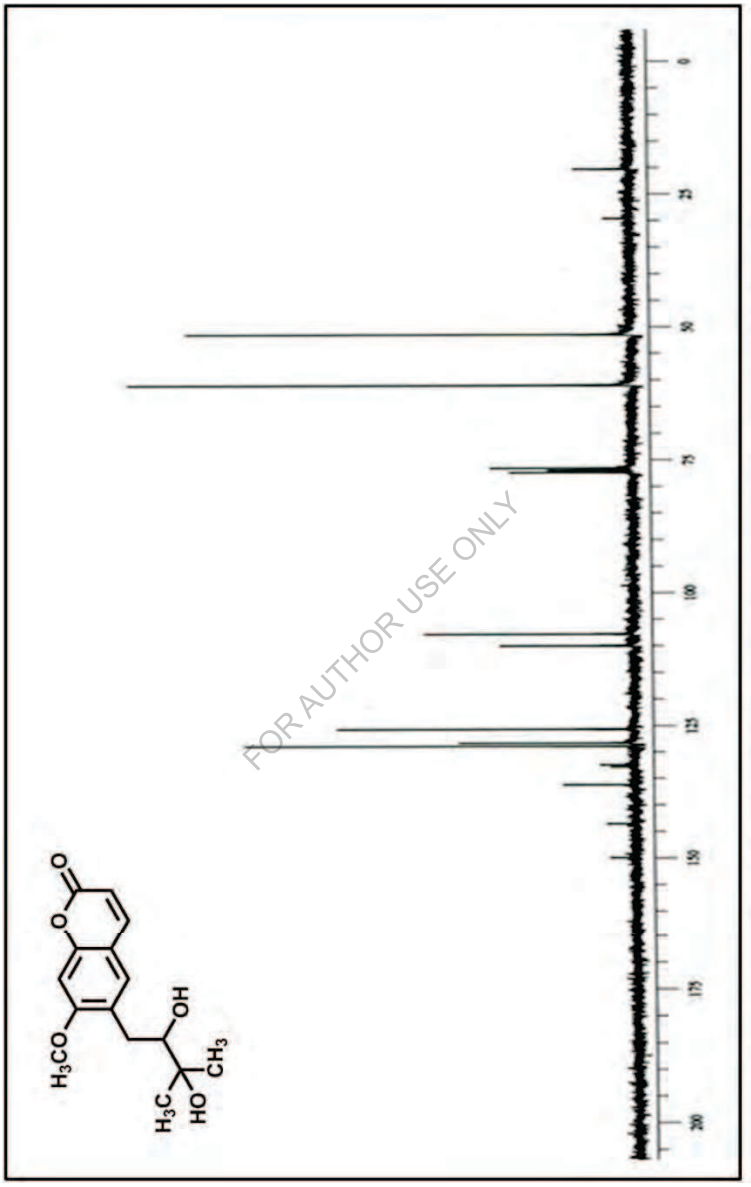


Figure 3B.07 : ^{13}C (100 MHz) NMR Spectra of CSLG-E3 in $CDCl_3$

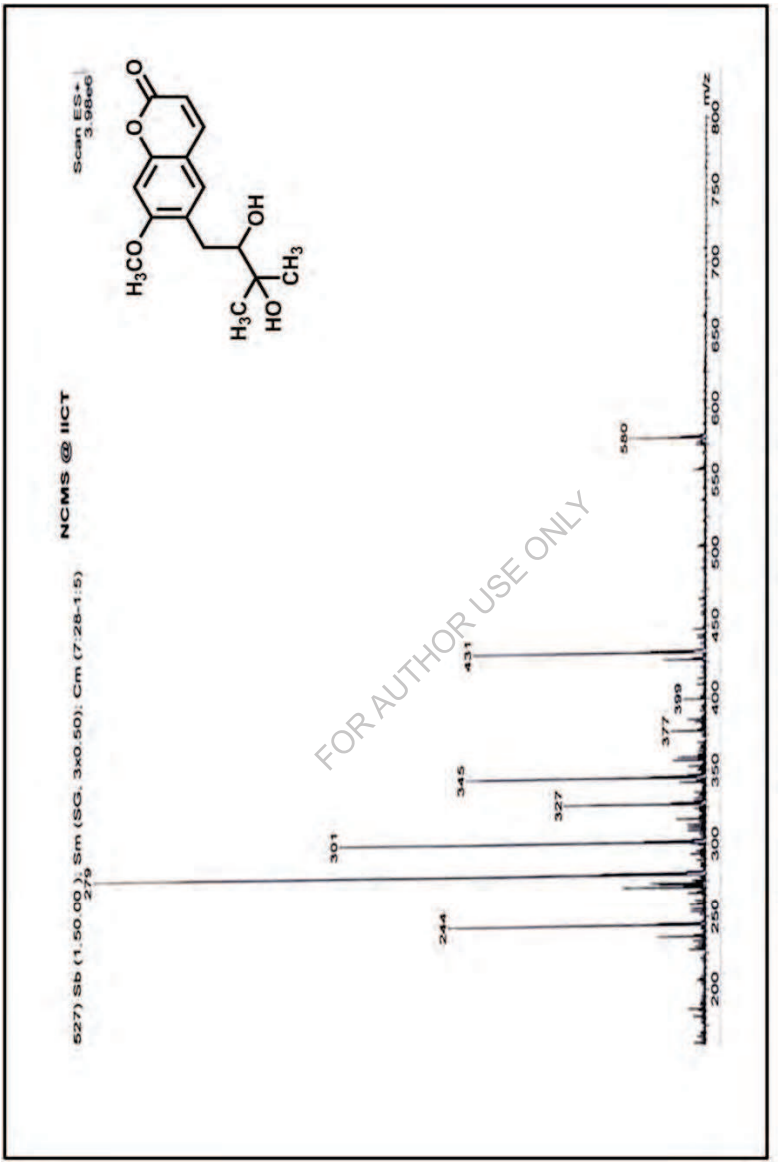
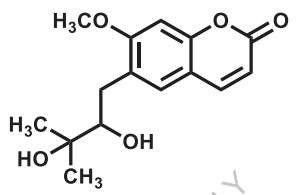


Figure 3B.08 : Mass Spectrum of CSLG-E3

δ 99.0 and 75.6 of CSLG-E2 (Figure 3B.05). Rest of the proton and carbon chemical shift values are almost matching. The ESI mass spectrum (Figure 3B.08) of the compound showed the pseudo molecular ions [M+H] and [M+Na] at m/z 279 and 301 suggesting the molecular formula of the compound as $C_{15}H_{18}O_5$. Based on the above physical and spectral data and comparition with reported value, this compound was identified as 2', 3'-gihydroxy-dihydrosuberosin.



2', 3'-gihydroxy-dihydrosuberosin.

The present isolation of this compound from *Chloroxylon swietenia* assumes taxonomic significance as this compound was earlier isolated from an entirely different species, *Coleonema album* (*Biosma album*) of Rutaceae family[76].

EXPERIMENTAL

The leaf material (2 kg) of *Chloroxylon swietenia* (CSLG) was collected from Gadchiroli forest, Maharashtra state, (Fig. 3B.01) in the month of November 2017. The authenticity of the plant material was confirmed by taxonomists of Botany Department, Osmania University, Hyderabad and Trans Disciplinary University (TDU) Bangalore. The voucher specimen was deposited in the Natural Products Chemistry Division, CSIR-IICT, Hyderabad.

Extraction

The shade dried and powdered leaf of the *C. Swietenia* (0.5 kg) was placed in a soxhlet extractor and successively extracted with n-hexane, ethyl acetate and methanol solvents under hot condition for 24 hr. Concentration of the three solubles under vacuum gave the respective extracts in 20 gm (4%), 33 gm (6.6%) and 48 gm (9.6%) (Chart 3B.01).The initial TLC studies showed well resolved and different patterns to the ethyl acetate extract as compared to the n-hexane extract. Hence, ethyl acetate extract was subjected to column chromatographic separation to isolate single and pure compounds. The methanol extract could not be taken up further, as it showed unresolved TLC patterns.

Chromatographic Separation of Ethyl acetate Extract

The dark green coloured ethyl acetate extract of *C. Swietenia* leaf on TLC showed blue coloured spots, but not resolved properly, as they are super imposable in n-hexane-ethyl acetate (90:10) solvent system, In order to minimise the complexicity, the extract (5 gm) was adsorbed on silica gel and chromatographed over a column of silica gel (500 g, 100-200 mesh) and eluted with solvent gradient from 100% n-hexane to 50% ethyl acetate (Chart 3B.02). Several fractions of 250 ml capacity were collected and the fractions with similar TLC nature (visualization of spots were carried out under UV light or Iodine vapours, or by spraying 5% methanolic H_2SO_4 followed by heating at 110^0 C) were

combined and evaporated to yield seven major fractions (F1-F7)**.** Based on TLC nature fractions F2-F7 were combined and the resultant residue (4.5 gm) was subjected to further column chromatographic purification over Si gel using n-hexane- ethyl acetate gradient elution to yield three single and pure compounds CSLG-E1, CSLG-E2 and CSLG-E3 (Chart 3B.03).

CSLG-E1: Racemosin (5,10-dimethoxy-8,8-dimethyl-8,9a-dihydro-pyrano[3,2-g]chromen-2(5aH)-one)

It was obtained as colourless granules from chloroform, 80 mg (0.1 %), 125-130^0 C, It showed homogeneity on TLC plate (R_f : 0.5, n-hexane-ethyl acetate, 80:20).

IR (KBr, cm^{-1})	:	1727
UV (MeOH, λ_{max}, nm)		266, 348
EI Mass (m/z)	:	311 [M+Na] suggesting the molecular formula of the compound as $C_{16}H_{16}O_5$
^{1}H NMR ($CDCL_3$, 400 MHz)	:	δ 8.15 (1H, d, H-4, J=9.9Hz), 7.63 (1H, s, H-5), 7.00 (1H, s, H-10), 6.30 (1H, d, H-3, J=9.9Hz), 4.15 (6H, s, 2 x-OCH_3), 1.25 (6H, s, 2x-CH_3).
^{13}CNMR (DMSO+$CDCl_3$,100 MHz)	:	δ 160.5 (C = O) , 150.0 (C-9a) , 145.0 (C-5), 144.8 (C-10a), 144.5 (C-3), 139.5 (C-10), 115.0 (C-6), 113.0 (C-4), 105.0 (C-4a), 61.5 (Ar-OCH_3), 60.5 (Ar-OCH_3), 29.0 (2x-methyls)

Identification of CSLG-E2: 2'-Hydroxy-3'-methoxy-dihydrosuberosin [6-(2-Hydroxy-3-methoxy-3-methylbutyl)-7-methoxy-2H-chromen-2-one]

It was obtained as a semisolid, 20 mg (0.02%). It showed homogeneity on TLC plate (R_f 0.22, n-hexane:ethyl acetate , 70:30).

IR (KBr, cm^{-1})	:	1730
UV (MeOH, λ_{max}, nm)	:	328
EI Mass (m/z)	:	293. [M+H] suggesting the molecular formula of the compound as $C_{16}H_{20}O_5$.
^{1}H NMR (300MHz,$CDCl_3$)	:	δ 7.64 (1H, d, H-4, J=9.9Hz), 7.38 (1H, s, H-5), 6.80 (1H, s, H-8), 6.25 (1H, d, H-3, J=9.9Hz), 3.90 (3H, s, Ar-OCH_3), 3.78 (1H, m, -CHOH), 3.28 (3H, s, OCH_3), 3.00 & 2.50 (multiplets, each 1H, benzylic methylene), 1.24 (3H, s, CH_3), 1.21 (3H, s, CH_3) .
^{13}CNMR (DMSO+$CDCL_3$100 MHz)	:	δ 162.0 (C=O), 161.0 (C-7) , 154.5 (C-8a), 144.0 (C-4), 130.0 (C-5), 113.0 (C-3), 112.6 (C-6), 112.0 (C-8), 99.0 (C-3'), 75.6 (C-2'), 56.0 (C7-OCH_3), 49.5 (C3'-OCH_3), 32.0 (C-1'), 21.0 (CH_3), 19.5 (CH_3).

CSLG-E3 : 2', 3'-Dihydroxy-dihydrosuberosin [6-(2,3-Dihydroxy-3-methylbutyl)-7-methoxy-2H-chromen-2-one]

It was obtained as a semisolid, 25 mg (0.025 %). It showed homogeneity on TLC plate (R_f 0.19, n-hexane-ethyl acetate, 70:30).

IR (KBr, cm^{-1})	:	1725
UV (MeOH, λ_{max}, nm)	:	328
EI Mass (m/z)	:	279 [M+H], 301 [M+Na] suggesting the

HR Mass		molecular formula $C_{15}H_{18}O_5$.
^{1}H NMR (300MHz,$CDCl_3$)	:	δ 7.62 (1H, d, H-4, J=9.9Hz), 7.29 (1H, s, H-5), 6.80 (1H, s, H-8), 6.23 (1H, d, H-3, J=9.9Hz), 3.90 (3H, s, 7-OCH_3), 3.75 (1H, m, H-2'), 3.01 & 2.54 (each 1H, multiplets, H-1'), 1.2-1.4 (6H, 2x-CH_3).
^{13}CNMR (DMSO+$CDCL_3$100 MHz)	:	δ 161.0 (C= O), 155.0 (C-7), 150.0 (C-8a), 143.5 (C-4), 125.5 (C-5), 110.5 (C-6, C-5a), 108.0 (C-3, C-8), 76.5 (C-3'), 62.0 (C-2'), 52.0 (C7-OCH_3), 34.0 (C-1') , 29.0 and 20.0 (C3'-CH3).

Section C : Antiproliferative activity of isolated compounds (CSLJ-E1 - CSLJ-E3 and CSLG-E1 - CSLG-E3) *Chloroxylon swietenia*

In the present investigation detailed chemical screening of the leaf samples of *Chloroxylon swietenia* collected from two different altitudes yielded two different classes of fused coumarin metabolites. The leaves of *C. swietenia* collected from Jaipur forest area, Telangana state (altitude:159 meters) yielded exclusively the furano-coumarin metabolites (**CSLJ-E2** and **CSLJ-E3)** or its precursor (**CSLJ-E1**). Whereas the leaves collected from Gadchiroli forest area, Maharashtra state (altitude : 217 meters) yielded exclusively the pyarano-coumarin metabolite (**CSLG-E1)** or its precursors (**CSLG-E2** and **CSLG-E3**). As altitude has significant effect on the elaboration of diverse coumarin metabolites in *C. swietenia*, it is expected that it will also affect the biological activity of the metabolites isolated from its leaf samples collected from two different altitudes. Literature search reveals that significant anticancer activity was reported to both furano-coumarins[77] and pyrano-coumarins[78]. With this background, the isolated compounds of *C. swietenia* such as furano-coumarins (**CSLJ-E1–CSLJ-E3**) and pyrano-coumarins (**CSLG-E1–CSLG-E3**) were subjected to antiproliferative screening against some human cancer cell lines to identify the potent and lead compounds. The details of the antiproliferative screening of both the series are given below.

Anti-proliferative activity furano-coumarin compounds (CSLJ-E1-CSLJ-E3)

The chemical screening of the leaves of *C. swietenia* (CSLJ) collected from Jaipur, Telangana state afforded three compounds such as 6,8–diprenyl-umbelliferone (**CSLJ-E1**), bergaptan (**CSLJ-E2**) and isopimpinellin (**CSLJ-E3**). These three compounds were subjected to antiproliferative screening using MTT assay[79] on six cancer cell lines viz. HT-29 (colon cancer), MDA-MB-231 (breast Cancer), MG-63

(osteosarcoma), SNU-449 (hepatocellular carcinoma), SKMEL-28 (skin melanoma) and 4T1 (mouse breast carcinoma). The details of the assay are presented below.

MTT assay

To the tumor cells after respective drug treatments, 10 µl MTT (100 mg MTT/20 ml DMEM) was added and incubated for 6 h at room temperature with gentle shaking. To stop the reaction 200 µl DMSO was added and the absorbance was recorded at 590 nm in an ELISA reader. The formazan values obtained are converted to percent values, data interpreted such that decreased percentage indicate decreased survival potential. The antiproliferative potential of the tested compounds is presented as IC50 values and are presented in Table 3C.01.

Table 3C.01 Anti-proliferative potential of furano-coumarin (CSLJ-E1-CSLJ-E3)

Compound	IC_{50} Concentration (µM)					
	SKMEL-28	SNU-449	MG-63	HT-29	MDA-MB-231	4T1
CSLJ-E1	**85.8 ± 1.9**	**70.37 ± 0.68**	NA	NA	**64.24 ± 1.03**	**49.9 ± 1.78**
CSLJ-E2	NA	NA	NA	NA	NA	NA
CSLJ-E3 .	NA	NA	NA	NA	NA	NA

NA : NOT ACTIVE

Close analysis of the above data reveals that compound **CSLJ-E1** (6,8–diprenyl-umbelliferone) showed moderate activity against four cancer cell lines such as SKMEL-28 (skin melanoma), SNU-449 (hepatocellular carcinoma), MDA-MB-231 (breast Cancer) and 4T1 (mouse breast carcinoma). Surprisingly, the other two compounds, **CSLJ-E2** and **CSLJ-E3** did not show any activity. Interestingly, **CSLJ-E1** (6,8–diprenyl-umbelliferone) showed highest activity against 4T1 (mouse breast carcinoma) with an IC_{50} of 49.9 ± 1.78 followed by MDA-MB-231 (breast Cancer), SNU-449 (hepatocellular carcinoma) and SKMEL-28 (skin melanoma) with IC_{50} values 64.24 ± 1.03, 70.37 ± 0.68

and 85.8 ± 1.9 respectively. The screening data also reveals that the precursor ortho-hydroxydiprenyl compound (**CSLJ-E1**) exhibited moderate activity, whereas the cyclised fused fuarano-coumarins did not show any activity.

Anti-proliferative activity pyrano-coumarin compounds (CSLG-E1-CSLG-E3)

The chemical screening of the leaves of *C. swietenia* (**CSLJ**) collected from Gadchiroli Forest, Maharashtra state afforded three compounds such as racemosin (**CSLG-E1**), 2'-Hydroxy-3'-methoxy-dihydrosuberosin (**CSLG-E2**) and 2', 3'-Dihydroxy-dihydrosuberosin (**CSLG-E3**). These three compounds were subjected to antiproliferative screening using MTT assay[79] on two human cell lines viz. A-549 (lung cancer) and IMR-32 (neuroblastoma cell line). The details of the assay are presented below.

Cell culture maintenance and treatments

Human lung cancer (A-549) and neuroblastoma (IMR-32) tumor cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (50 mg/ml) in a humidified incubator chamber (37^0 C) supplied with 5% CO2. Exponentially growing tumor cells (1x104/well) in complete medium in 96-well plates were treated with 2.0, 5.0, and 10.0 µM concentrations of the three compounds **CSLG-E1**, **CSLG-E2**, and **CSLG-E3** (Figure 3C.01). The treatments were continued for 20 h at culture conditions, and then the cells were harvested for MTT assay. Doxorubicin is used as a positive control in this assay.

Table 3C.02 Anti-proliferative potential of pyrano-coumarin (CSLG-E1-CSLG-E3)

Cell line	CSLG-E1	CSLG-E2	CSLG-E3	Doxorubicin
A-549	**3.6**	**4.2**	**2.7**	**2.2**
IMR-32	**3.8**	**3.6**	**1.6**	**1.9**

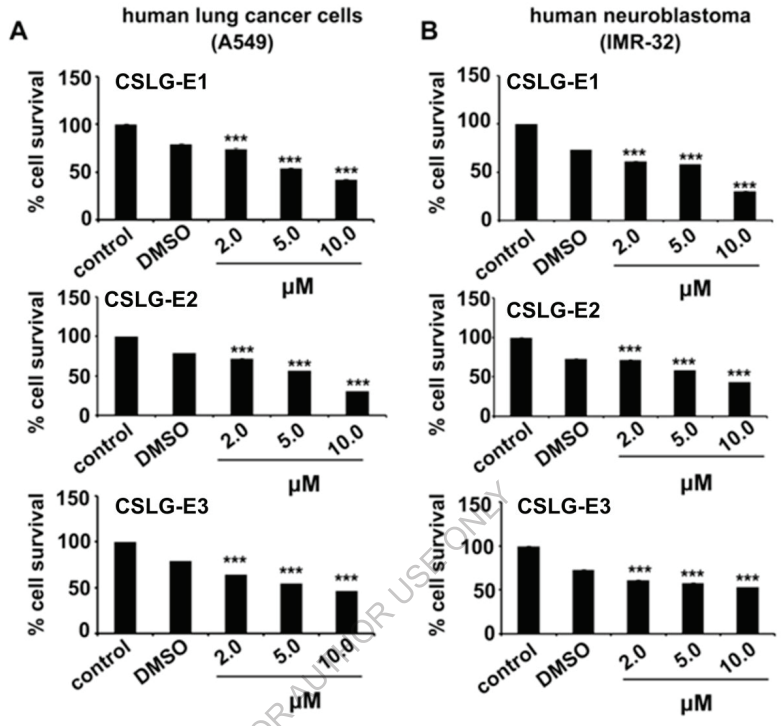


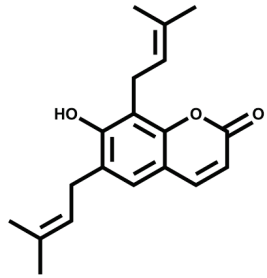
Figure 3C.01. Effect of coumarin compounds against A549 and IMR-32 cell survival. (A) A549 and (B) IMR-32 tumor cells were treated with 2.0, 10.0, and 20.0 μM of compounds **CSLG-E1, CSLG-E2, and CSLG-E3** and the percent cell survival calculated by MTT assay. DMSO used as solvent control. X-axis represents the treatment and Y-axis represents percent cell survival after the treatment. Significance values calculated comparing DMSO controls with treatments. $p<0.001$.

Statistical analysis of data

Data in the bar diagrams are reported as mean±SD. The control groups are compared with treated ones and the significance values were calculated by paired student's t-test. Data represented are from minimum three independent sets of experiments in each group. The *p* values $p<0.05$; , $p<0.01$, and, $p<0.001$.

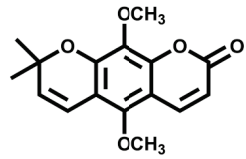
Close analysis of the above data reveals that all the three compound **CSLJ-E1** (racemosin), **CSLG-E2** (2'-Hydroxy-3'-methoxy-dihydrosuberosin) and **CSLG-E3** (2', 3'-Dihydroxy-dihydrosuberosin) highly potent activity against both A-549 (lung) and IMR-32 (neuroblastoma) cancer cell lines. The IC_{50} of these three compounds against the two tested ell lines are very close to that of the standard, doxorubicin. Most significantly, compound **CSLG-E3 (**2', 3'-dihydroxy-dihydrosuberosin) showed highest activity on both the cell lines than the other two compounds, **CSLJ-E1** (racemosin) and **CSLG-E2** (2'-hydroxy-3'-methoxy-dihydrosuberosin). In fact, the compound **CSLG-E3** (2', 3'-dihydroxy-dihydrosuberosin) showed ~1.2 times more activity (IC_{50}: 1.60) than the standard, doxorubicin (IC_{50}:1.90) against IMR-32 (neuroblastoma) cancer cell line. Further, this compound (IC_{50}:2.7) showed almost identical activity as that of standard, doxorubicin (IC_{50}:2.2) against A-549 (lung cancer cell line). In general the suberosin analogues (**CSLG-E2** and **CSLG-E3**) showed higher activity than the fused pyrano-coumarin (**CSLG-E1**).

From the above screening results of the compounds isolated from the leaf samples of *C. swietenia* collected from Jaipur, Telangana state (**CSLJ**) and Gadchiroli, Maharashtra state (CSLG), it can be concluded that the Gadchiroli (CSLG) compounds such as suberosin analogues (**CSLG-E2** and **CSLG-E3**) and racemosin (**CSLG-E1**) are more active than the Jaipur (**CSLJ**) compounds. This suggests that the high altitude (217 meters) Gadchiroli sample elaborate more potent antiproliferative compounds than the relatively low altitude (159 meters) Jaipur sample. The potent molecules from these two series are given below:



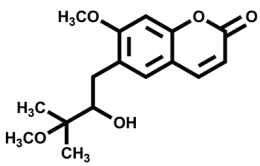
CSLJ-E1

IC_{50} : 49.9 (4T1-mouse breast)



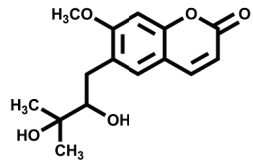
CSLG-E1

IC_{50} : 3.6 (A 549-lung);
3.8 (IMR 32- neuroblastoma)



CSLG-E3

IC_{50} : 4.2 (A 549-lung);
3.6 (IMR 32- neuroblastoma)



CSLG-E3

IC_{50} : 2.7 (A 549-lung);
1.6 (IMR 32- neuroblastoma)

These compounds with interesting functional groups have potentiality to develop further as anticancer therapeutic agents.

Conclusion

The abiotic parameter, altitude is reported to effect the elaboration of secondary metabolite in natural plant species. In order to validate this concept, the detailed chemical and biological studies have now been carried out on the leaves of *Chloroxylon swietenia*, one of the potent Indian folklore medicinal plant. The leaf samples of *Chloroxylon swietenia* were collected from two different altitudes The leaves of *C. swietenia* collected from Jaipur forest area (altitude:159 meters), Telangana state yielded exclusively the furano-coumarin metabolites (**CSLJ-E2** and **CSLJ-E3**) or its precursor (**CSLJ-E1**). Whereas, the leaves collected from Gadchiroli forest area (altitude : 217 meters), Maharashtra state yielded exclusively the pyarano-coumarin metabolite (**CSLG-E1**) or its precursors (**CSLG-E2** and **CSLG-E3**). As significant anticancer activity was reported in literature to both furano-coumarins and pyrano-coumarins, the isolated compounds of *C. swietenia* were now subjected to antiproliferative screening against some human cancer cell lines to identify potent lead compounds. The Gadchiroli (**CSLG**) compounds such as suberosin analogues (**CSLG-E2** and **CSLG-E3**) and racemosin (**CSLG-E1**) are more active than the Jaipur (**CSLJ**) compounds. This suggests that the high altitude (217 meters) Gadchiroli sample elaborate more potent antiproliferative compounds than the relatively low altitude (159 meters) Jaipur sample. The identified potent compounds with interesting functional groups have potentiality to develop further as anticancer therapeutic agents.

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4.1. Introduction

The biologically active agents from natural sources have always been of great interest in developing potent drugs for various diseases[1]. The pivotal role of natural products in modern drug discovery has been realised worldwide and reviewed in literature[2-7].

The main advantages with natural products are :

- rate of introduction of new chemical entities of wide structural diversity
- number of diseases treated or prevented by these substances
- frequency of use in the treatment of disease

The medicinal plants have been the object of research in both systematic and advanced areas of plant sciences[8]. The traditional knowledge of healing methods mainly by the use of wild plants is now fast disappearing because of modernization, globalization and also the tendency to change traditional lifestyles[9]. Herbal medicine is now a multibillion dollar industry. In developing countries, up to 80% of people depend on plant-based medicines. The identity, authenticity, and quality of crude plants are often uncertain and difficult to assess. Standardization is possible for the few herbs for which the dossier of all active ingredients is known, but is technically difficult and would make drugs unaffordable in developing nations[10]. This lack of standardization and quality control is seen as one of the major disadvantages of traditional/complementary and alternative medicine[11]. Chemical fingerprinting patterns based on one or more marker compounds through chromatographic techniques are more commonly used for herbal and traditional medicine standardization, so the quality control and quality assurance of herbal medicine are possible. Keeping this objective in mind, detailed and systematic quantitative studies have now been carried out on *Boerhavia diffusa* Linn. one of the high demand Indian

medicinal herb, with respect to its major marker compound, eupalitin-galactoside using **H**igh **P**erformance **T**hin **L**ayer **C**hromatography (**HPTLC**).

4.2. *Boerhavia diffusa* Linn: A High Demand Indian Medicinal Herb

Figure 4.01: *Boerhavia diffusa* Linn.

Boerhavia diffusa Linn. (Fam. Nyctaginaceae) is a perennial creeping weed (Figure 4.01) found abundantly in India and other parts of the world[12]. It is popularly known as 'Punarnava' in Indian Ayurvedic system of medicine and classified as "Rasayana" herb, which is said to posses properties like anti-aging, re-establishing youth, strengthening life and brain power and disease prevention, which imply that they increase the resistance of the body against immunomodulation[13]. It is also reported in traditional medicine for various ailments such as diuretic, oedema and ascites from early cirrhosis of liver and chronic peritonitis, jaundice, anasarca, strangury and gonorrhoea. Its root is reportedly used as analgesic, laxative and anti-inflammatory. Leaf juice is used in ophthalmia[14]. In Ayurvedic texts more than 35 formulations of different types contain *B. diffusa* as the major ingredient were recorded[15]. It was reported in literature that alcoholic extract of the whole plant is showed potent hepatoprotective activity against experimentally induced carbon tetra chloride hepatotoxicity in rats and mice. The extract also showed strong choleritic

activity[16, 17]. It was also reported that the whole plant alcoholic extract exhibited cardiotonic effect in perfused frog heart preparation and anaesthetised dog heart *in situ*[18]. *B. diffusa* whole plant reported to contain a large number of secondary metabolites such as rotenoids, flavonoids, alkaloids, steroids and triterpenoids. The major metabolites identified in this plant are punarnavine, borhahavone, boeravinones (A-F), diffusarotenoid, quercetrine, kaempferol, eupalitin-3-O-β-D-galactopyranoside, punarnavoside, boerhavine, lirodendrin, boerhavisterol, β-ecdysone, and boeradiffusene[19-21]. Some of the important classes of compounds isolated from *B. diffusa*[22-35] are listed below:

(i) *Rotenoids*

Boeravinone F

(ii) Phenolic glycosides

(iii) Lignans

Punarnavoside

Syringaresinol mono- β- D-glucoside

(iv) *Flavonoids*

Quercetin

Kaempferol

Eupalitin 3-O-galactoside

(v) .*Steroids*

β-sitosterol- β-D-glucoside

In spite of its excellent traditional medicinal uses, significant biological activities attributed to its various extracts and elaborating large number of secondary metabolites, there is no rapid, reproducible and efficacious quantitative method reported so far for *B. diffusa* plant. In this connection detailed literature search has been made on the quantitative evaluation of *B. diffusa* and the highlights presented below.

4.3. Literature Survey

Few analytical methods were reported in literature for the quantitative evaluation of *B. diffusa* based on boeravinones[36] and punarnavoside[37,38]. Boeravinones are bio-active rotenoid constituents isolated from the roots of *B. diffusa*. A UPLC method

was reported for the determination and quantification of eight boeravinones[39-48] in the methanol extract of *B. diffusa* roots using a photodiode array (PDA) detector. However, these methods are only for root part but not for the whole plant material, which is actually the official drug traditionally known as 'punrnava'. Critical analysis of the compounds isolated from *B. diffusa* and their biological activities reveals that eupalitin-3-O-β-D-galactopyranoside found to exhibit immunomodulatory activity[49], similar to that of the plant. Further, eupalitin-3-O-β-D-galactopyranoside found to accumulate in significant levels in the whole plant of *B. diffusa*. Hence, it can be considered as the bio-active marker compound for quantitative evaluation of *B. diffusa* and some of its commercial formulations. In connection with several research programmes on botanical and Ayurvedic drugs, our group found that HPTLC is highly useful analytical tool for their quantitative evaluation[50,51]. With this background, an efficient HPTLC method for quantitative evaluation of *B. diffusa* whole plant based on its major bio-active marker compound, eupalitin-3-O-β-D-galactopyranoside was now developed and the results are presented below.

4.4. Aims and Objectives

1. To develop a rapid, reliable and simple analytical method using HPTLC for establishing a fingerprinting profile, which can be used in quality control and quality assurance.
2. The developed method can serve the purpose of choosing authentic raw plant material for commercial formulations.
3. To establish the method of extraction by various techniques, which can be helpful in obtaining better yield of marker compounds.
4. To apply the developed HPTLC method for identification and quantitative evaluation of *Boerhavia diffusa* and its commercial formulations with respect

to its major bio-active marker, eupalitin-3-O-β-D-galactopyranoside.

5. To validate the developed HPTLC method as per regulatory guidelines

4.5. Experimental

4.5.0. Materials and Methods

4.5.1. Plant Material

The whole plant material of *B. diffusa* was procured through CSIR-IIIM (Formerly RRL), Jammu and its identity was established by taxonomists.

4.5.2. *Chemicals, solvents and commercial formulations*

For extraction and isolation of the marker compound, commercial grade solvents such as n-hexane, ethyl acetate, acetone and methanol were used after drying and distillation following standard purification procedures. Silica gel G and Silica gel (100-200 mesh) of ACME grade were used for TLC and column chromatography respectively. Pre-coated high performance thin layer chromatography (HPTLC) silica gel $60F_{254}$ (E. Merck, Darmstadt, Germany) alumina backed plates were used. Two commercial formulations of *B.diffusa* such as Punarnava mandur and Artrin capsules of M/s Baidyanath and Imis pharmacy respectively were procured from local market.

4.5.3. Extraction *and isolation of marker, eupalitin-3-O-β-D-galactopyranoside*

The powdered plant material (4 kg) was extracted with 50% aqueous ethanol in an aspirator at room temperature for 16 hr. The process was repeated three times. The combined solubles on concentration under reduced pressure yielded a reddish brown extract (450 gm., 11.25%). The extract showed +ve tests for phenols, steroids and glycosides. The extract showed well resolved spots on TLC plate in two different solvent system viz. n-butanol-acetic acid-water (8:1:1) and ethyl acetate–methanol-water (6:2:2). In order to isolate the marker compound, the extract (80 gm) was subjected to detailed column chromatographic separation over silica gel using chloroform-methanol mixtures as eluents. Based on TLC nature of the column

fractions they were regrouped into four major fractions A-D. These fractions on repetitive column chromatographic purifications followed by re-crystallisation furnished a pale yellow amorphous powder (1.8 g, 0.25%), m.p. 179^0 C, R_f : 0.55 (n-butanol-acetic acid-water 8:1:1). It gave +ve tests for phenols and glycosides. IR (KBr, cm^{-1}) (Figure 4.04) : 3446 (hydroxyl), 1658 (carbonyl); ^{1}H NMR (400 MHz, DMSO-d_6) (Figure 4.02) : ● 12.52 (1H, br. s, 5-OH), 10.15 (1H, br. s, 4'-OH), 8.06 (2H, d, J= 8.9 Hz, H-2', H-6'), 6.84 (1H, s, H-8), 6.81 (2H, d, J=8.9 Hz, H-3', H-5'), 5.38 (1H, d, J=7.7 Hz), 5.12 - 4.39 (sugar protons), 3.86 (3H, s, 7-OMe), 3.68 (3H, s, 6-OMe). ^{13}C NMR (100 MHz, DMSO) (Figure 4.03) : 177.7 (C-4), 159.9 (C-7), 158.6 (C-2), 156.6 (C-8a), 151.5 (C-5), 133.1 (C-3), 131.6 (C-6), 130.9 (C-2', C-6'), 120.7 (C-1'), 115.0 (C-3', C-5'), 105.2 (C-1''), 101.4 (C-6a), 91.2 (C-8), 75.7 (C-5'', C-3''), 73.0 (C-2''), 71.1 (C-4'), 67.8 (C-6''), 56.4 (C-7 OCH_3), 60.9 (C-6 OCH_3), ESIMS (+ve) (Figure 4.05) : 515.30 $[M+Na]^+$ corresponding to $C_{23}H_{24}O_{12}Na$. EI MS (Figure 4.06):

Figure 4.02 ^{1}H (400 MHz) NMR Spectrum of Eupalitin-galactoside in DMSO

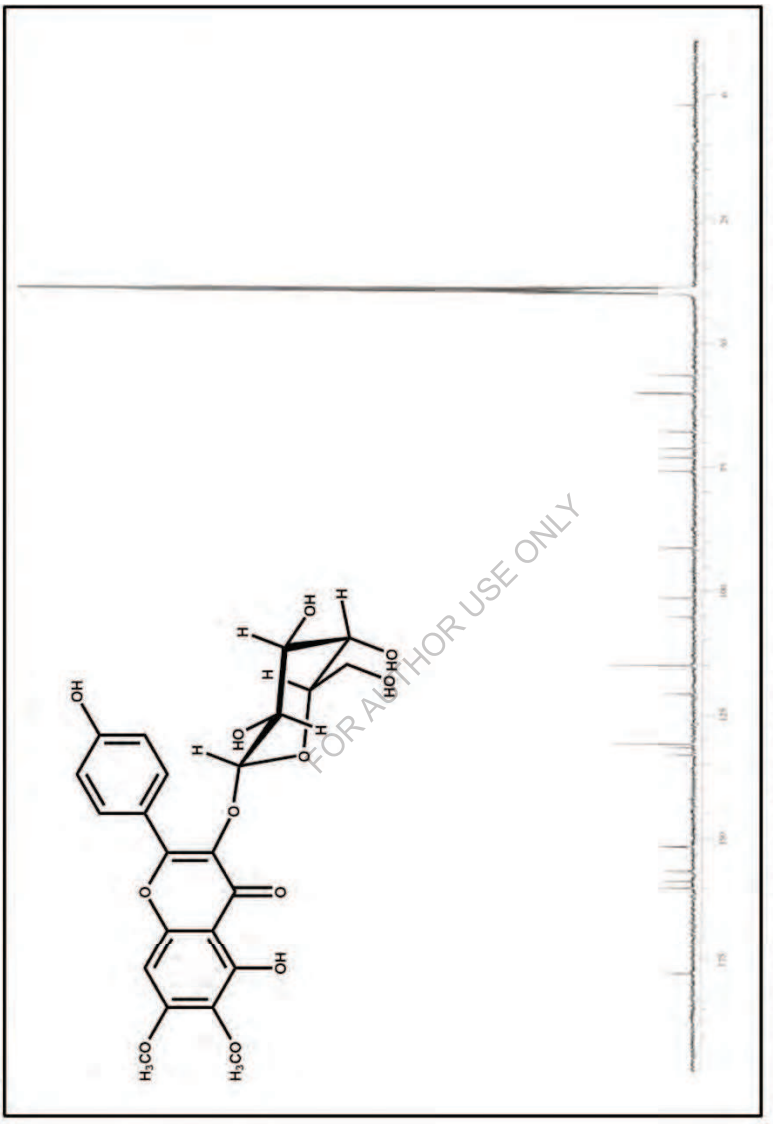

Figure 4.03 ^{13}C (100 MHz) NMR Spectrum of Eupalitin-galactoside in DMSO

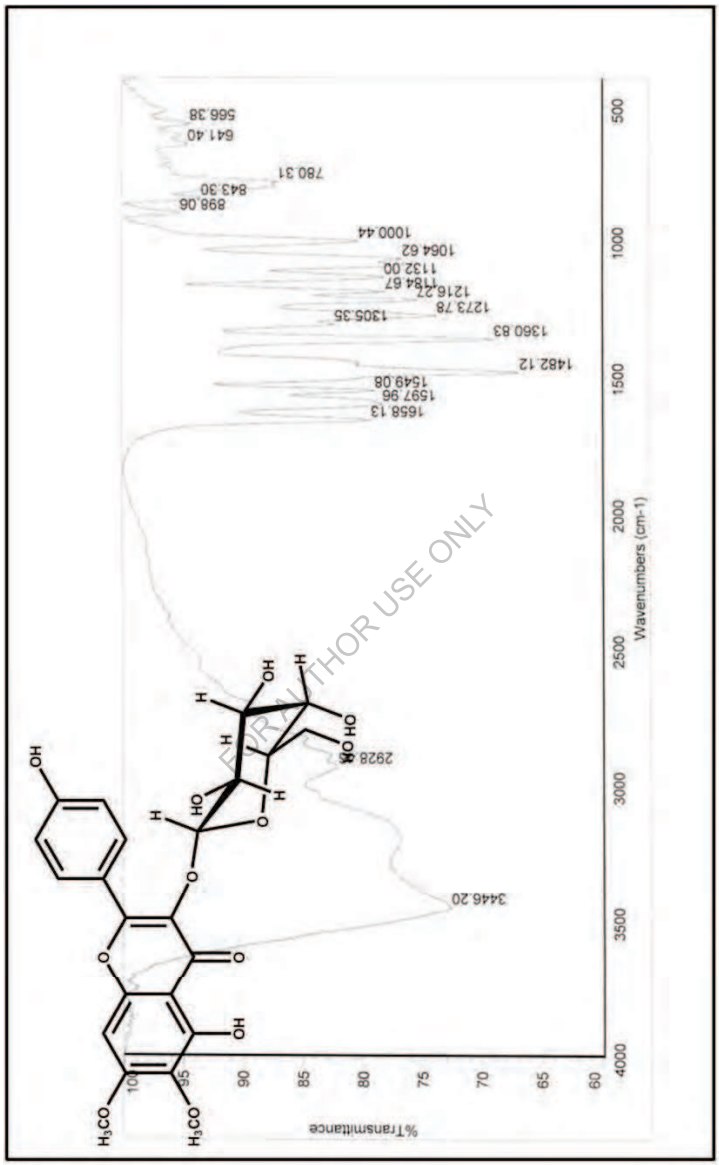

Figure 4.04 IR Spectrum of Eupalitin-galactoside

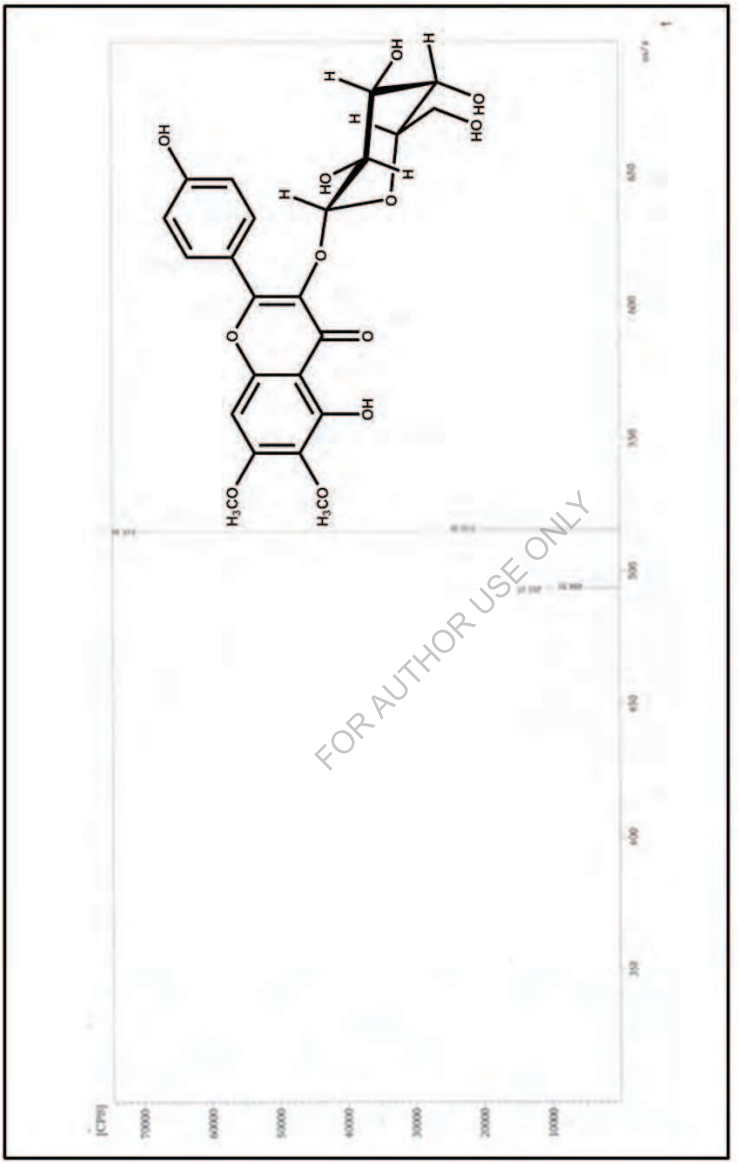

Figure 4.05 ESI (+ve) Mass Spectrum of Eupalitin-galactoside

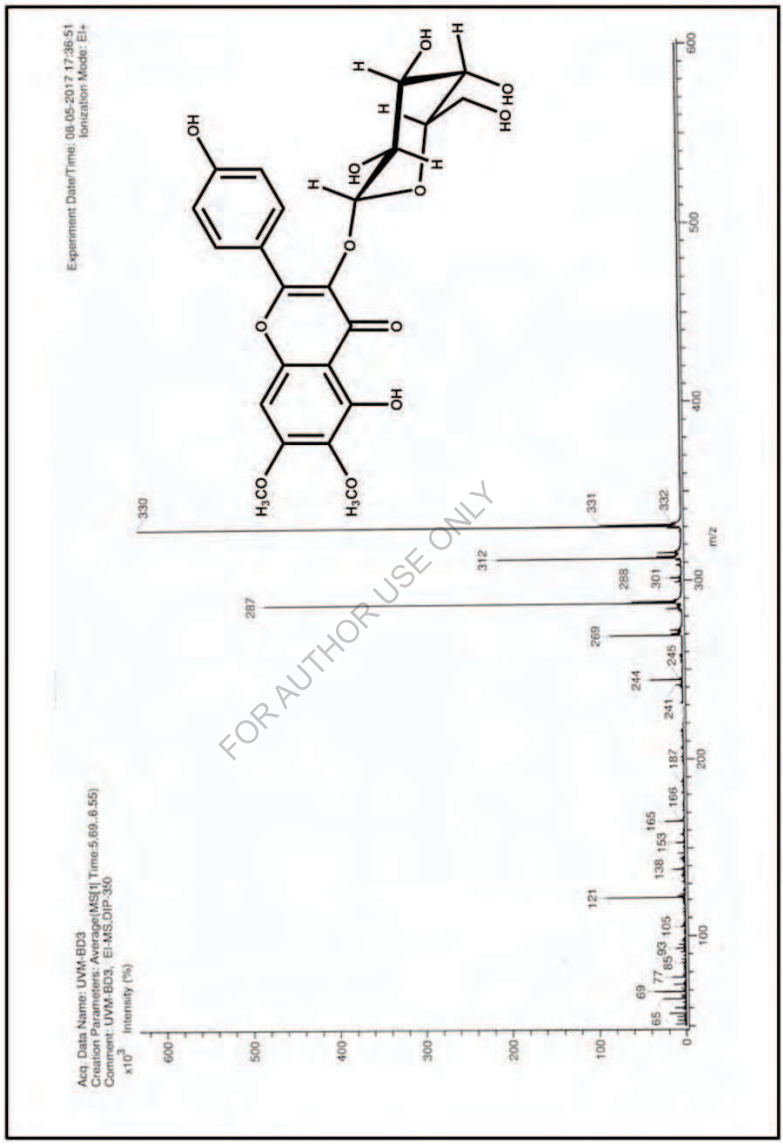

Figure 4.06 EI Mass Spectrum of Eupalitin-galactoside

330 (100%) corresponding to $C_{17}H_{14}O_7$ (eupalitin). Based on the above physical and spectroscopic (Figures 4.02-4.06) data, the marker compound was identified as eupalitin-3-O-β-D-galactopyranoside.

4. 5.4. Extraction and sample preparation

The powdered material (1.00 gm each) was extracted in soxhlet extractor for 7 hours in ethanol. These extracts were concentrated and further dried in a lyophiliser. 100 mg of each extract was dissolved in 10 ml volumetric flasks.

4.6. HPTLC instrumentation and chromatographic condition

4.6.1. HPTLC instrumentation

The chromatographic estimation was performed by spotting standard and extracted samples of *B. diffusa* on pre-coated silica gel aluminium sheet plate 60 F-254 (20 cm×10 cm with 250 mm thickness, E. Merck, Darmstadt, Germany) using a Camag Linomat V sample applicator (Camag, Muttenz, Switzerland) and a 100 ml syringe. The samples, in the form of bands of length 6 mm, were spotted (15 ml)10 mm from the bottom, 35 mm from left margin of the plate and 13 mm apart, at a constant

application rate of 80 nl/s using nitrogen aspirator. Plates were developed using a mobile phase consisting of n-butanol–acetic acid-water (8:1:1, v/v/v). Linear ascending development was carried out in 20 cm×10 cm twin trough glass chamber (Camag Muttenz, Switzerland) equilibrated with mobile phase. The optimized chamber saturation time for mobile phase was 10 min at room temperature. The length of chromatogram run was 5 cm. Approximately, 20ml of the mobile phase (10 ml in trough containing the plate and 10ml in the other trough) was used for each development, which required 15 min. It results in better apparent resolution with more convenient capability of the detecting device to perform integration of peak area. Subsequent to the development, TLC plates were dried in a current of air with the help of an air-dryer for 5 min. The slit dimension settings of length 5.00 mm and width 0.45 mm, and a scanning rate of 20 mm/s was employed. The monochromator bandwidth was set at 20 nm.

Densitometric scanning was performed on Camag TLC scanner III in the absorbance mode at 274 nm and operated by winCATS planar chromatography version 1.1.3.0. The source of radiation utilized was deuterium and tungsten lamp. Concentrations of the compound chromatographed were determined from the intensity of absorbance. Evaluation was via peak areas with linear regression.

4.6.2. Visualization

Visualisation is being done at 254 nm and also after derivatisation with freshly prepared vanillin-H_2SO_4 reagent (Figure 4.07)

4.6.3. Calibration curves of standard

Calibration curves of standard in ethanol containing concentrations of 0.005 mg to 0.25 mg/ml were prepared from the stock solution of 1.0 mg/ml. 10 micro litres from each solution was spotted on the TLC plate to obtain final concentration range of

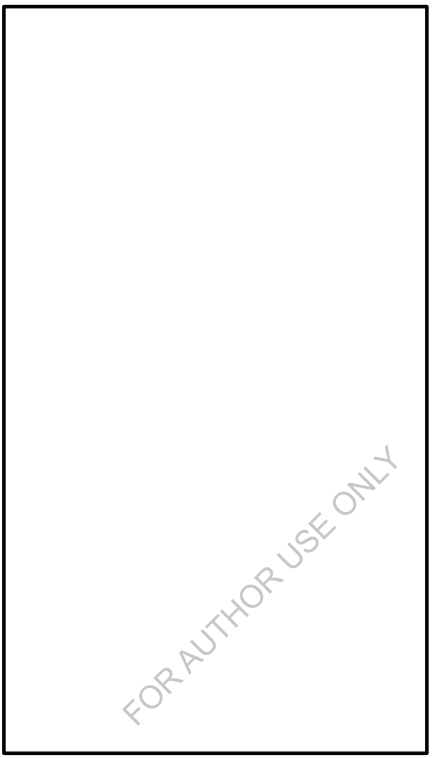

Figure 4.07 TLC chromatograms of B. diffusa and its commercial formulations

(A) UV 254; (B) Vanillin–H_2SO_4 reagent

0.05-2.5 µg/spot.The data of peak area versus sample concentration were treated by linear regression analysis (Fig. 4.08).

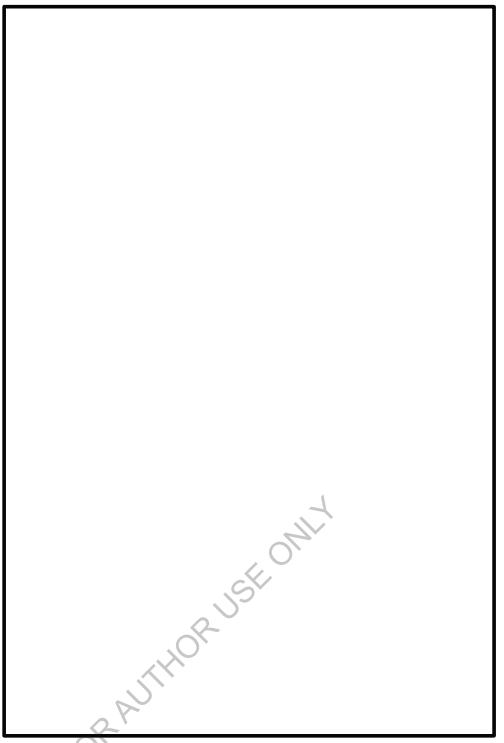

Figure 4.08 Calibration curves of the marker and its commercial samples

4.6.4. Method validation

The HPTLC method developed was validated for following parameters.

4.6.5. LOD & LOQ

The LOD and the LOQ were calculated for the marker compound on the basis of three- and ten-times the noise level, respectively. LOD was determined as 3 times the average noise value of blank ethanol applied (10 µl, 5 times). So the LOD was calculated as 1.38 ng and LOQ was calculated as 4.18 ng.

4.6.6. Specificity

The specificity of the method was ascertained by analyzing standard and test samples. The band for the marker compound in test samples was confirmed by comparing the R_F and spectra of the spot with that of the standard (Figure. 4.09).

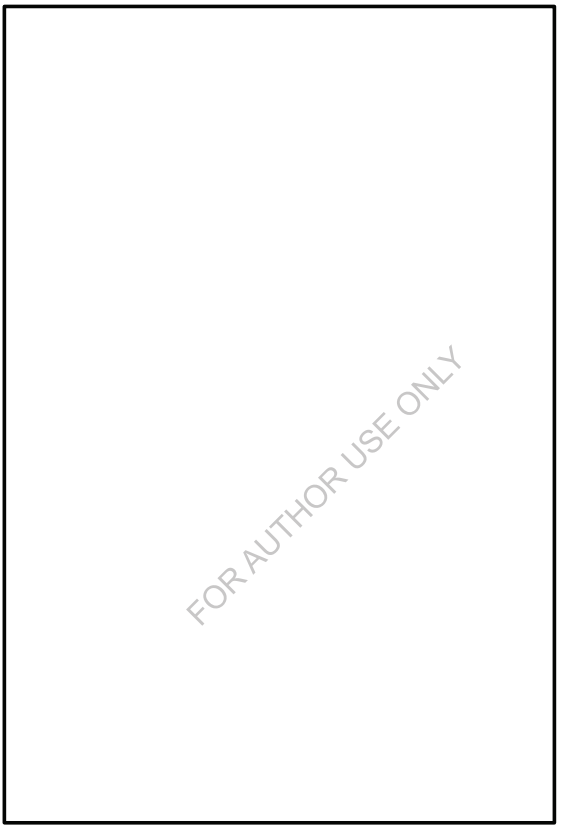

Figure 4.09 HPTLC chromatograms of the marker compound, *B. diffusa* and its commercial formulations

The peak purity of the marker compound was assessed by comparing the spectra at peak start, peak apex and peak end of the band, respectively (Figure 4.10).

Representative overlay spectra of standard and *B. diffusa* extracts are shown in Figure 4.11.

Figure 4.10 UV spectra of the standard and *B. diffusa* samples

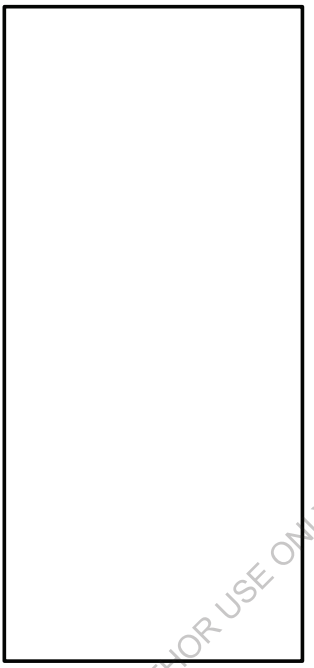

Figure 4.11 3D overlay spectra of the standard and *B. diffusa* samples

4.6.7. Precision

The repeatability of measurement (n=6) of peak area for compounds was expressed in terms of percent coefficient of variation (% RSD). The intra and inter day variation study in the analysis of was carried out at three different concentration levels.

4.6.8. Recovery

Accuracy assessed with the method of standard additions at three concentration levels. Accuracy was assessed by comparing the results with the analysis of the standard reference material over the range of 80–120% of the amount corresponding to the midpoint of the curve.

4.7 Results and discussions

The whole plant material of *B. Diffusa* was exhaustively extracted with 50% aqueous ethanol and the resultant extract on extensive Si gel chromatographic purifications resulted in the isolation of the bio-active marker compound as pale yellow amorphous powder. Preliminary physical and chemical tests identify the compound as a flavonol glycoside. Detailed spectroscopic analysis (IR, ^{1}H & ^{13}C NMR and Mass) confirms its structure as eupalitin-3-O-β-D-galactopyranoside**.** Detailed thin layer chromatographic studies on this compound optimised the solvent system as n-butanol-acetic acid-water (8:1:1), which showed single spot at Rf: 0.55 (Figure 4.06). This compound was taken as marker compound and quantitatively evaluated *B. Diffusa* whole plant along with two of its commercial samples such as punarnava mandur (Baidyanath) and artrin capsules (Imis Pharma) using HPTLC.

4.7.1 Sensitivity

The sensitivity of the method was determined with respect to LOD, LOQ, linearity range and correlation coefficient. Solutions containing 0.05-2.5 µg of standard were spotted on TLC plate. The LOD was calculated as 1.38 ng (3 times the noise level) and LOQ was calculated as 4.18 ng (10 times the noise level). The regression data for the four samples are given in the Table 4.01, which shows good linear relationship in that range studied.

Table 4.01. Results of Linearity

Linearity range	r^2	Slope	Intercept
0.05-2.5 µg	0.99872	8.719	100.702

4.7.2. Recovery study

Recovery of standard was calculated by spiking 1.0 mg to the *formulation,* punarnava mandur (1.00 gm) and extracted in ethanol and analysed three times as

described in Section 2.4. The recovery was calculated by comparing the resultant peak areas with the standard (Table 4.02).

Table 4.02 Recovery and RSD of method

Sample	Amount spotted (ng)	Amount detected (ng) (Mean ± SD, n=3)	RSD (%)	Recovery (%)
PM	500	491.54 ± 5.32	1.08	98.30

4.7.3. Precision and accuracy

Different amounts punarnava mandur (spiked) samples are spotted on TLC plate. These spots were analyzed by using above described HPTLC method (Table 4.03). Precision was expressed as the percent relative standard deviation (% R.S.D.).

Table 4.03 Results of precision and accuracy

Actual amount of standard spotted (ng)	Amount detected (ng ± SD, n=5)	RSD (%)
300	294.57 ± 4.75	1.61
500	587.24 ± 9.39	1.59
1000	972.46 ± 19.57	2.01

4.7.4. Reproducibility

The repeatability was evaluated (Table 4.04) by analyzing the known amounts of punarnava mandur (spiked) samples spotted on TLC plate in replicates (n = 5). The inter day and intra day precision was evaluated by analyzing the same amount of analyte over a period of 3 days (n = 5) and expressed in terms of % R.S.D.

Table 4.04 Results of Reproducibility

Amount spotted (ng)	Amount detected (ng ± SD)	RSD (%)
Inter day (n=3)		
300	281.39 ± 5.17	1.83
500	573.36 ± 11.48	2.00
1000	972.64 ± 22.71	2.33
Intra day (n=5)		
300	287.65 ± 4.92	1.71
500	585.36± 9.47	1.61
1000	977.18± 18.84	1.93

4.8 Sample analysis

The validated HPTLC method was applied for quantitative determination of the marker, eupalitin-3-O-β-D-galactopyranoside in *B. diffusa* whole plant sample and two of its commercial formulations such as punarnava mandur and artrin capsules and the results are presented in Table 4.05. The marker compound was found to accumulate in 0.075% in the *B. diffusa* whole plant sample. Surprisingly, it was found absent in both the commercial samples. In order to rationalise these observations, the composition of the two commercial samples were closely observed. Both the commercial samples were found to contain about ten herbal or herbo-mineral ingredients. Further, it was observed that the in both the commercial formulations it is the root part of *B. diffusa* was used. Hence, the marker compound in these two formulations might be lost during their manufacturing process or due to different plant part used. But marker compound could be detected accurately in the whole plant samples. The developed method can be used for successfully for quality control and quality assurance of *B. Diffusa* formulations, where the whole plant is used.

Table 4.05 Accumulation of the marker in various *B. diffusa* samples

S. No	Samples	Eupalitin-galactoside [%]
1.	Extract	0.075
2.	Punarnava mandur	Not detected
3.	Artin capsule	Not detected

Conclusion

In the present study, eupalitin-3-O-ఐ-D-galactopyranoside the bio-active marker of *B. diffusa* was isolated and quantitatively estimated in the whole plant material and two of its commercial formulations by employing the validated HPTLC method. The method is rapid and reproducible. the marker compound was found to present in 0.075% in the whole plant of *B. diffusa*. The developed method can be successfully employed for quality control and quality assurance of *B. diffusa*, where the whole plant part is used.

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5.1. Introduction

Medicinal herbs form an important component of modern life style and are rich sources of important therapeutic agents for alienating human ailments[1]. Herbal formulations contain a specific combination of herbs to get required dosage forms for the management of a wide range of diseases. The quality assessment of herbal formulations is of importance in order to justify their acceptability in modern system of medicine. Standardization of herbal formulations is essential in order to assess the quality of drugs based on physical, chemical and phytochemical parameters, concentration of active principles, *in vitro* and *in vivo* parameters. Standardization is a system that ensures a predefined amount of quality, quantity and therapeutic effect of ingredients in each dose. Markers are chemically defined constituents independent of whether they have any therapeutic activity or not. These are of interest for quality control and quality assurance of herbal formulations[2].

Marker compound based standardization using chromatographic techniques such as HPLC and HPTLC is commonly applied for herbal formulations. Chemical marker can be an indicator of the quality of an herbal medicine but not related to therapeutic activity, whereas a bio-active marker is the compound which has the therapeutic activity. It is always a promising signal in the search for bio-active markers, which are to be used for the purpose of standardization. If the active bio-active markers are not available, chemical markers can be used for this purpose. In the process of standardization, quantification of markers will be carried out in the herbal ingredients as well as in the herbal formulations. According to European Medicine Agency guidelines (EMEA 2005), quantification of chemical substances with known therapeutic activity or without activity is mandatory. For reliable and reproducible standardisation, marker compounds should be structurally known and present in

adequate amount in both raw plant material and in finished formulations[3]. Chromatography related separation methods and their use as analytical separation tools, serves as means of resolution of mixtures and for the isolation and identification of the marker components, whose presence may be known or suspected. Modern analytical chromatographic and spectroscopic techniques that are used for screening of plant constituents include TLC, HPTLC, and thin layer electrophoresis, HPLC, NMR, GC-MS, and LCMS. Quality control of herbal formulations for human consumption is highly essential. This can be ensured by regularly monitoring the quality of raw material and manufacturing process for obtaining the finished products. Hence, development of fast isolation of marker compounds and rapid, reliable, simple & acceptable validated analytical method for maintaining the quality of the raw material right from obtaining authentic plant parts to the finished dosage form to be carried out[4].

A prominent group of natural products are the terpenes and derivatized terpenoids. The terpenes are biosynthetically constructed from isoprene (2-methylbutadiene) units[5,6]. Triterpenes are composed of six isoprene units derived from mevalonic acid or deoxyxylulose phosphate, and most of the compounds have 30 carbon atoms[7]. According to their basic molecular carbon skeleton classification, triterpenes can be divided into various subgroups, namely, acyclic triterpenes (squalene), monocyclic triterpenes (achilleol A), bicyclic triterpenes (pouoside A), tricyclic triterpenes (lansioside A), tetracyclic triterpenes (lanostane, dammarane, euphane), pentacyclic triterpenes (oleanane, ursane, lupane, hopane) and miscellaneous compounds[8-13]. Pentacyclic triterpenes are the major class of chemical compounds occurring as free acids or esters or glycosides (saponins) in natural plants and resinous natural

materials[14]. Numerous chromatographic methods have been reported to separate, purify, identify or quantify pentacyclic triterpenes in plant materials[15].

Among the various Indian medicinal plants, *Glycyrrhiza glabra* (Figure 5.01) is rich in such pentacyclic triterpenes. *Glycyrrhiza glabra* Linn. is also known as Liquorice, Mulaithi or Yashtimadu. Glycyrrhiza is a word derived from the ancient Greek term 'glykos', meaning sweet, and 'rhiza', meaning root[16].

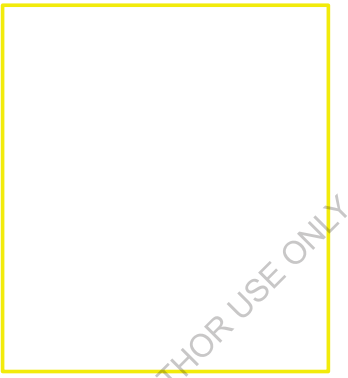

Figure 5.01 *Glycyrrhiza glabra*

Glycyrrhiza glabra, is still in use in India today for the treatment of various diseases as ingredients of official drugs or herbal preparations used in Indian system of medicine and other traditional medicine[17]. Among the various phytoconstituents (Figure 5.02) of *G. Glabra*[18-25], glycyrrhizic acid is the major constituent in terms of both abundance and bio-activity[26].

Glycyrrhizic acid (Figure 5.03), also known as glycyrrhizinic acid, is an oleanane class of pentacyclic triterpene saponin with two glucuronic acid units linked at 3 hydroxyl position. It is chemically known as 18-glycyrrhetinic acid-3-O--D-glucuronopyranosyl-(1→2)--D-glucuronide[27].

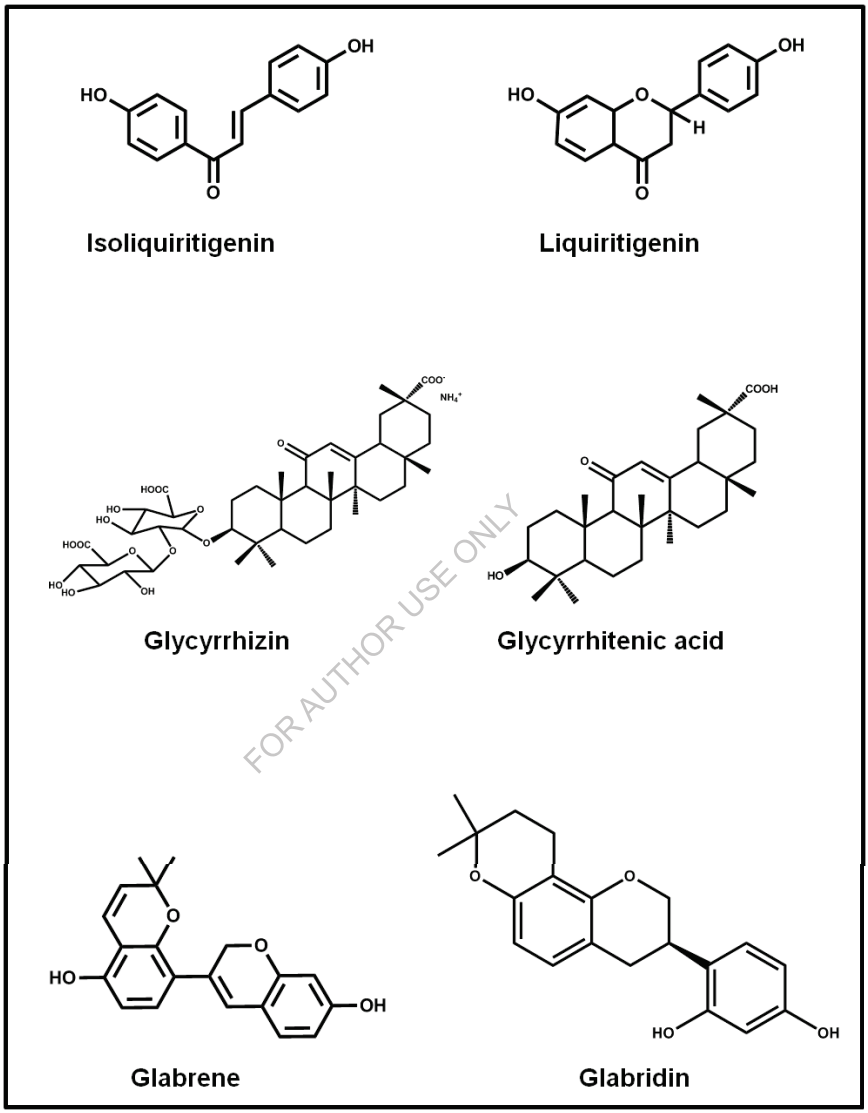

Figure 5.02. Phytoconstituents of *G. glabra*

Figure 5.03 Structure of Glycyrrhizic acid

Glycyrrhizic acid is a natural sweetener with ~ 50 times more than sucrose and most importantly has no calorific value[28]. It has been used clinically for more than 20 years in patients with chronic hepatitis in China and Japan and shows a satisfactory therapeutic effect in many other diseases[29]. It is reported to exhibit a wide range of therapeutic activities like anti-inflammatory, anti-ulcer, anti-allergic, antioxidant, anti-tumour, anti diabetic and hepatoprotective[30]. *In vitro* studies have demonstrated that glycyrrhizic acid inhibits cyclooxygenase activity and prostaglandin formulation as well as indirectly inhibiting platelet aggregation[31,32]. Glycyrrhizic acid has also been shown to inhibit growth and cytopathology of numerous RNA and DNA viruses including hepatitis A & C, herpes zoster, herpes simplex, HIV and CMV[30]. Interestingly, in controlled clinical trials antiulcer potential of glycyrrhizic acid, its aglycone, corresponding extract and root powder of its source *G. glabra* plant material was established[33]. In view of these interesting therapeutic and sweetener properties, glycyrrhizic acid has become as a 'Hot Molecule' and attracted the attention of biologists and chemists all over the world. In order to carryout detailed and systematic research and developmental studies on glycyrrhizic acid, its availability on large scale is highly warranted. Literature search reveals that

glycyrrhizic acid was isolated earlier by a number of research groups from different *Glycyrrhiza* species in milligram to multigram level[34]. The isolation of glycyrrhizic acid involves two major steps such as extraction of the source plant material and separation followed by purification of the desired compound from the crude extract. For the extraction of Glcyrrhiza species, various polar solvents like alcohol, hydroalcohol and water using conventional maceration, hot soxhlet, sonication and microwave methods were employed[35]. Xuejun et al. reported the extraction of licorice with EtOH, EtOH-water, water, ammonia solution and ethanol-water-ammonia using a microwave assisted extractor followed by HPLC analysis[36]. Jiang et al. extracted the licorice roots with 70% aqueous methanol followed by filtration, precipitation with HCl, freeze drying of the resultant precipitate and separation by HSCCC[37]. Boqiang et al. used sonication method for extraction of licorice followed by separation using macro-porous resins[38]. Shen et al. reported the extraction of glycyrrhizic acid from dried licorice slices with aqueous ammonia solution (0.5% vol%) under sonication followed by three liquid phase systems containing four components such as organic solvent, inorganic salt, polymer and the treated licorice extract[39]. Tian et al. have reported that mixture of ethanol-water (70:30) and extraction time of 60 min. at 50^0 C are optimum conditions for extraction of glycyrrhizic acid from licorice[40]. The above listed methods have drawbacks such as use of either specialised extraction equipments or acid - base media. Further, all these methods are mostly to improve the efficiency of extraction of *Glycyrrhiza* species, but not for physical separation or purification of glycyrrhizic acid. Even though, glycyrrhizic acid is a highly wanted bio-active molecule, no systematic quantitative studies have been reported so far to identify its high yielding *G.glabra* sample and also for rapid, reliable and reproducible

analytical method for standardisation. In view of these observations detailed literature search has been made and presented below.

5.2. Literature Survey

Although, glycyrrhizic acid is the major bio-active molecule of *G.glabra* and attracted the attention all over the world, few reports are available on its quantitative evaluation in *G.glabra* formulations using chromatographic methods. However, some LC, HPTLC, capillary electrophoresis methods are available for its calcium or ammonium salt (glycyrrhizine) or its aglycone (glycyrrhitinic acid)[41-47].

As the demand for glycyrrhizic acid is increasing considerably in developing new chemical entities to develop potent therapeutic agents and *G.glabra* for various traditional preparations, there is an urgent need to develop fast isolation and rapid quantitative methods for glycyrrhizic acid from *G. glabra*. Recently, Combiflash chromatographic system found highly useful[48] in isolating ursolic acid from *Diospyros melanoxylon* leaves in 5.5 hrs. with an overall yield of 0.6%. This method has several advantages such as low solvent consumption, short isolation time and high yields of the desired compounds. Hence, this method can be extended to glycyrrhizic acid isolation also. For quantitative evaluation, HPTLC is the most suitable choice of analytical tool as several botanical and traditional preparations have been successfully quantified and standardised by our group[49,50]. With this background, the detailed chemical and chromatographic studies on *G. glabra* have now been carried out and the results are presented in this Chapter.

5.3. Aims and Objectives

1. To develop a fast isolation method for glycyrrhizic acid, the major bio-active marker compound of *Glycyrrhiza glabra.*
2. To develop a rapid, reliable and simple analytical method using HPTLC for establishing a fingerprinting profile, which can be used in quality control and quality assurance.
3. To apply the developed HPTLC method for identification and quantitative evaluation of *Glycyrrhiza glabra* and its commercial formulations with respect to its major bio-active marker, glycyrrhizic acid.
4. To validate the developed HPTLC method as per regulatory guidelines

*5.*4. Experimental

5.4.1. *Reagents and Apparatus*

For extraction and isolation of the marker compound, commercial grade solvents such as n-hexane, ethyl acetate, acetone and methanol were used after drying and distillation following standard purification procedures. Silica gel G was used for Thin Layer Chromatography. Pre-coated high performance thin layer chromatography (HPTLC) silica gel 60F_{254} (E. Merck, Darmstadt, Germany) alumina backed plates were used. The 50% aqueous ethanol extract of *G. glabra* roots was procured from CSIR-IIM (Formerly RRL), Jammu, India. The roots of *G. glabra* were collected through Sri Baidyanath Ayurved Pharmacy, Patna, India. The two commercial formulations of *G. glabra* such as Yastimadhu churna and Artrin capsules of M/s Ganga Pharmaceuticals and Imis pharmacy respectively were procured from local market.

5.4.2. *Isolation of the marker compound, glycyrrhizic acid*

The 50% aqueous ethanol extract (4 g) was absorbed on silica gel (6 g) and subjected fractionation using ISCO CombiFlash Chromatographic System Sg 100c (Chart 5.01). A glass column of 350x25 mm (l x d) with teflon connectors at both ends was packed with silica gel (30 g) of 230-400 mesh size. Then the adsorbed material was poured in to this column slowly and packed uniformly. The column was then eluted initially with chloroform-methanol (1:1) followed by n-butanol at a flow rate of 20 mL/min at 15 psi pressure using UV detector at wavelength of 215 nm. The whole process is controlled by a PC based Peak Track software.

Crystallisation from Methanol

The n-butanol washings were combined and evaporated to get a brown coloured residue (504 mg), which was again subjected to CombiFlash chromatograhy with 100 test tubes using chloroform, chloroform-methanol(1:1), methanol and n-butanol. Concentration of n-butanol fractions (56-83) under reduced pressure afforded the marker compound, glycyrrhizic acid (1).

It was obtained as pale brown amorphous powder (275 mg, 6.9%). m.p. 295^0 C, R_f: 0.6 [n-butanol-acetic acid-water (7:2:1)]. It gave +ve tests for glycosides (Molisch's) and triterpenes (Liebermann–Burchard). IR (KBr, cm^{-1}) (Figure 5.07) : 3447 (hydroxyl), 1731, 1698 and 1654 (-COOH); ^{1}H NMR (400 MHz, DMSO-d_6) (Figure 5.04) : ● 5.50 (s, 1H, 12-H), 5.40 (br.s, 2H, H-1',1"), 4.47 (b.d, 2H, d, J= 7.70 Hz, H-2',2"), 4.31 (b.d, 2H, d, J= 7.50 Hz, H-3',3"), 3.41-3.13 (m, H-3. 4',4".5'.5"), 1.34-0.72 (6xs CH_3-23,24,25,26,27,28,&30). ^{13}C NMR (125 MHz, DMSO) (Figure 5.05) : ● 198.5 (C-11), 177.9 (C-29), 171.3 (C-6'), 171.0 (C-6"), 170.5 (C-13), 126.8 (C-12), 107.5 (C-1'), 102.3 (C-1"), 87.6 (C-2'), 87.5 (C-3), 78.9 (C-4'), 78.3 (C-2"), 75.8 (C-3'), 75.3 (C-4"), 75.2 (C-5'), 74.5 (C-3"), 71.5 (C-5"). 71.3 (C-6'), 60.5 (C-9), 55.9 (C-5), 47.5 (C-18), 44.6 (C-14), 44.3 (C-20), 42.6 (C-8), 42.4 (C-19), 40.2 (C-4), 38.1 (C-22 & C-1), 37.0 (C-10), 33.4 (C-7), 31.9 (C-17), 31.0 (C-21), 30.0 (C-28), 29.3 (C-30), 27.9 (C-23), 27.8 (C-2), 27.3 (C-15 & C-16), 25.0 (C-27), 18.1 (C-26 & C-6),

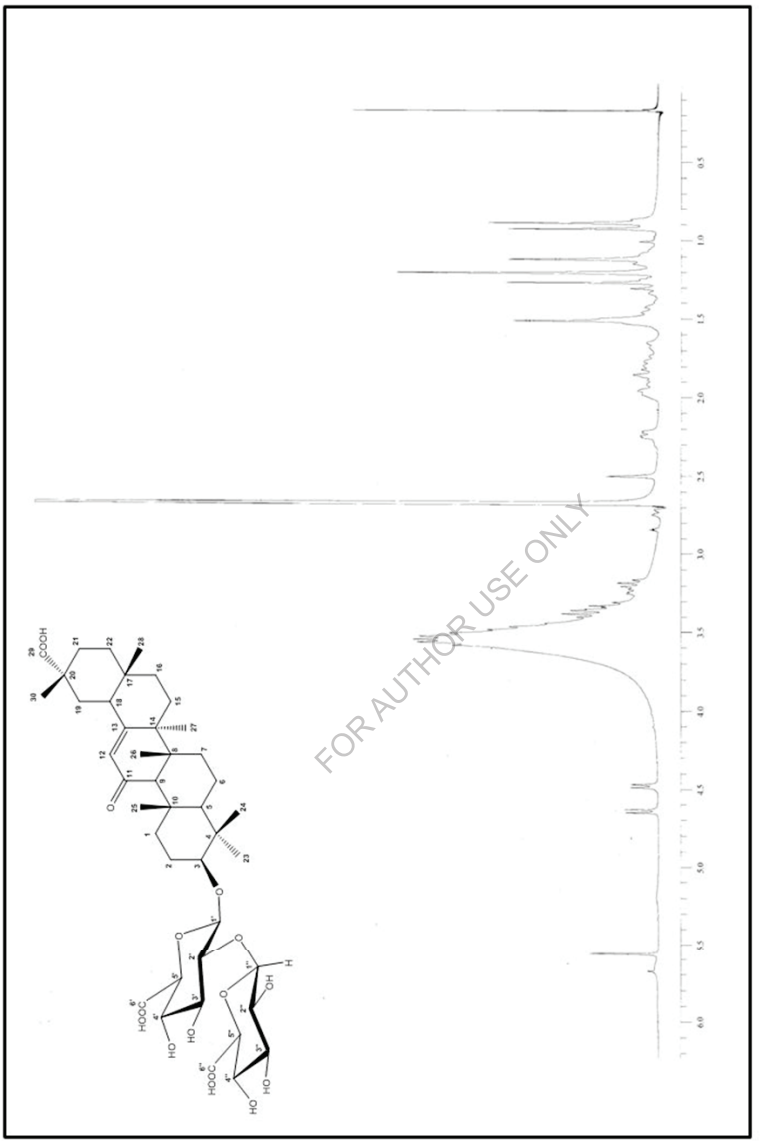

Figure 5.04 : ^{1}H (400 MHz) NMR Spectrum of Glycyrrhizic Acid in DMSO

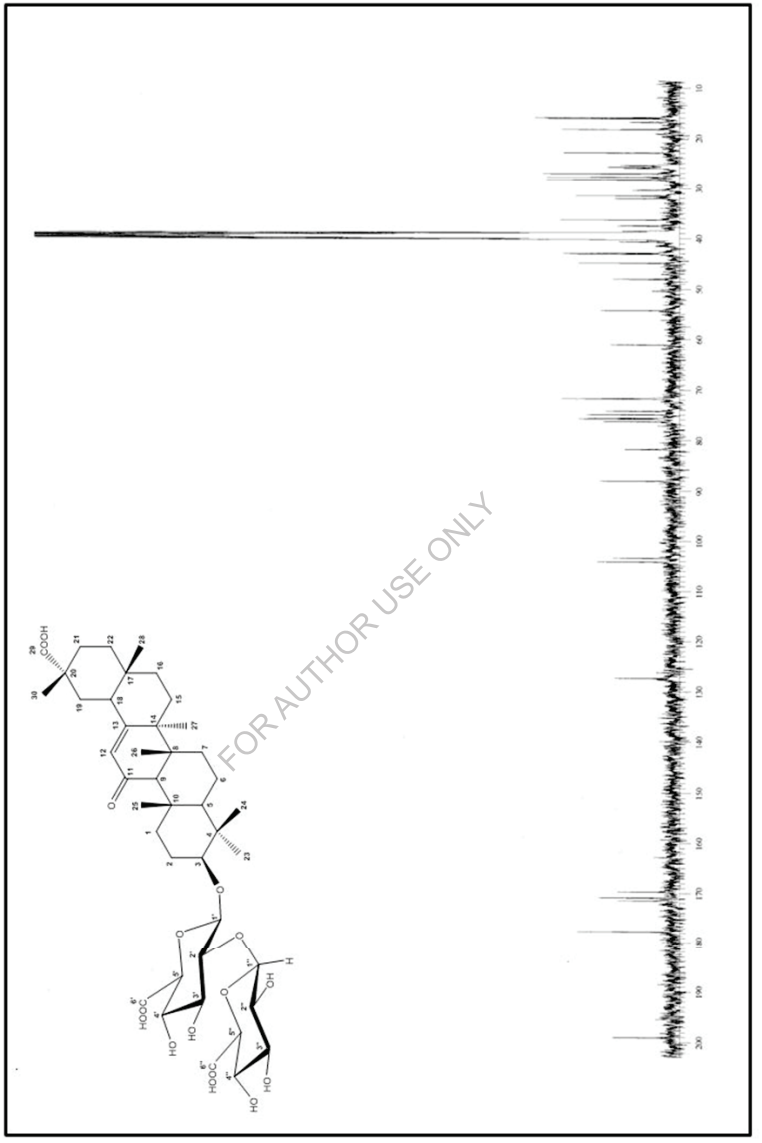

Figure 5.05 : ^{13}C (100 MHz) NMR Spectrum of Glycyrrhizine in DMSO

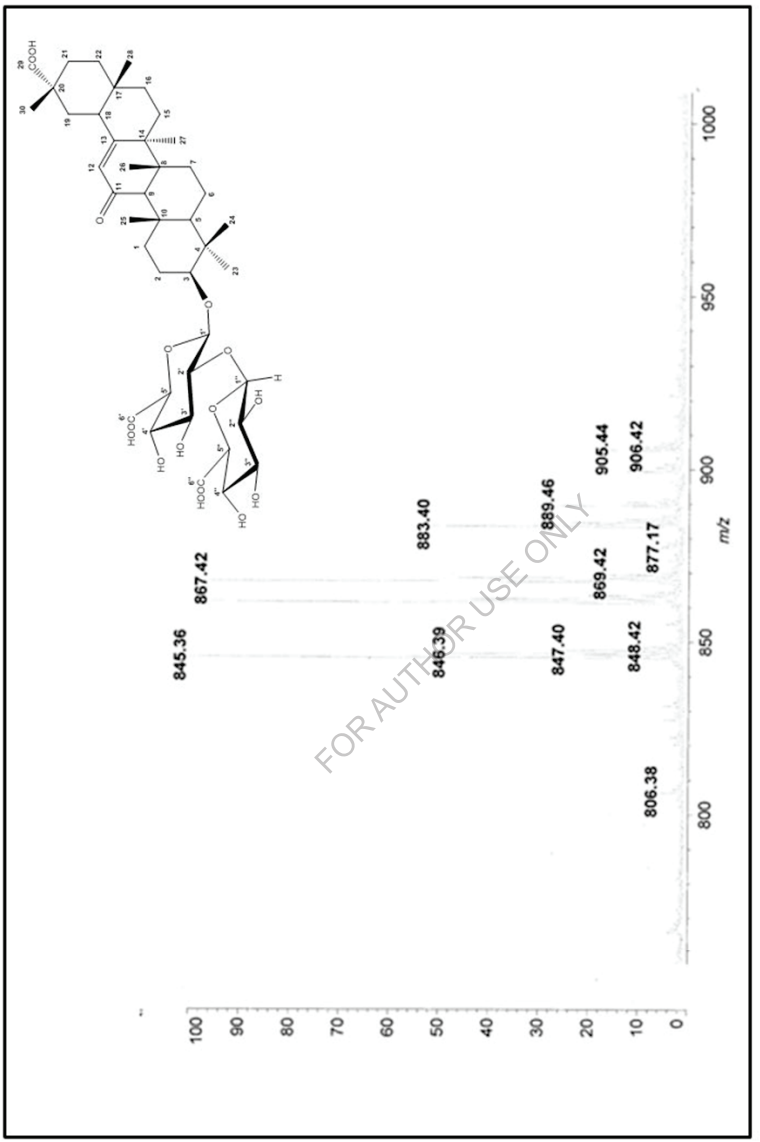

Figure 5.06 : MALDI Spectra of Compound Glycyrrhizine

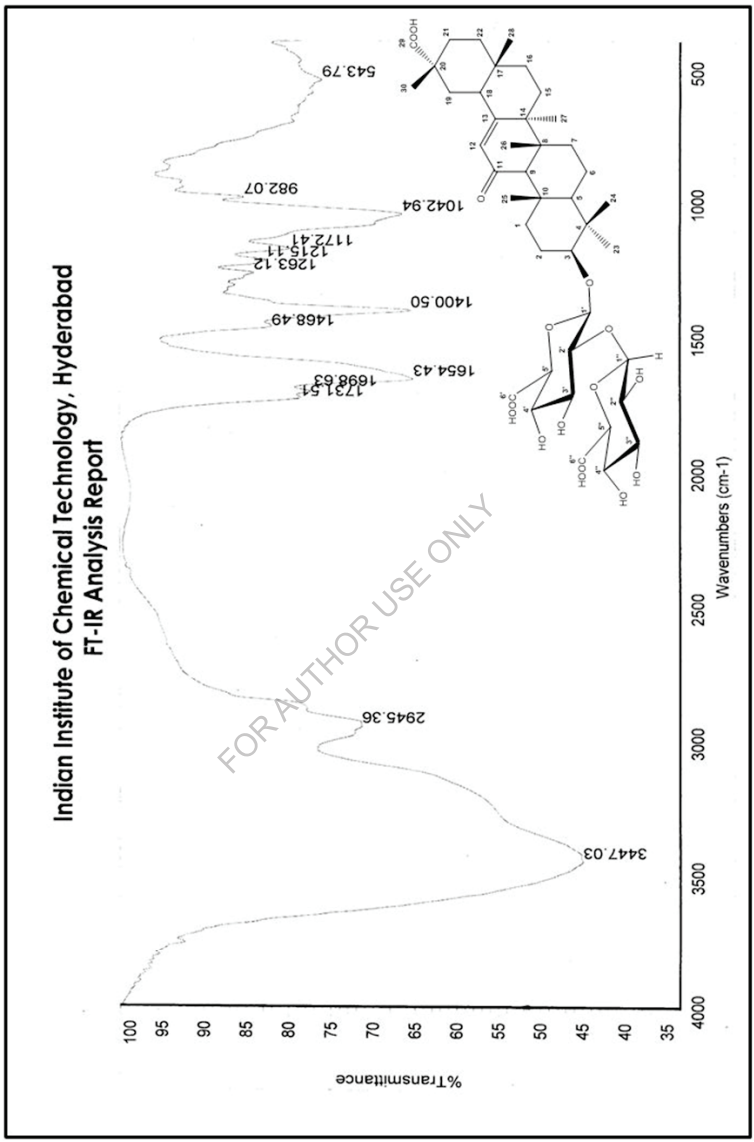

Figure 5.07 : IR Spectra of Compound Glycyrrhizine

15.8 (C-25), 15.5 (C-24). MALDI Mass (Figure 5.06): 845.36 $[M+Na]^+$ corresponding to $C_{42}H_{62}O_{16}Na$. Based on the above spectral data and by comparing its values with the reported values, it was identified as glycyrrhizic acid[18].

5.5. *HPTLC instrumentation and chromatographic condition*

5.5.1. *Extraction and sample preparation*

The powdered materials (1.00 gm each) were extracted in soxhlets for 7 hours in ethanol. These extracts were further dried in freeze drier to get the respective extracts. 100 mg of each extract has been dissolved in 10 ml of volumetric flasks.

5.5.2. *Visualization*

Visualization is being done in 254 nm (Figure 5.08) and also after derivatization with freshly prepared anisaldehyde spray reagent.

5.5.3. *HPTLC instrumentation*

The chromatographic estimation was performed by spotting standards and extracted samples of glycyrrhizin on precoated silica gel aluminium sheet plate 60 F-254 (20 cm×10 cm with 250 µm thickness, E. Merck, Darmstadt, Germany) using a Camag Linomat V sample applicator (Camag, Muttenz, Switzerland) and a 100 µl syringe. The samples, in the form of bands of length 6 mm, were spotted (5 µl each) 10mm from the bottom, 35 mm from left margin of the plate and 13 mm apart, at a constant application rate of 80 nl/s using nitrogen aspirator. Plates were developed using a mobile phase consisting of n-butanol–acetic acid-water (7:2:1, v/v/v). Linear ascending development was carried out in 20 cm×10 cm twin trough glass chamber (Camag Muttenz, Switzerland) equilibrated with mobile phase. The optimized chamber saturation time for mobile phase was 10 min at room temperature. The length of chromatogram run was 5 cm. approximately; 20 ml of the mobile phase (10 ml in trough containing the plate and 10ml in the other trough) was used for each

development, which required 15 min. It results in better apparent resolution with more convenient capability of the detecting device to perform integration of peak area. Subsequent to the development, TLC plates were dried in a current of air with the help of an air-dryer for 5 min. The slit dimension settings of length 5.00 mm and width 0.45 mm, and a scanning rate of 20 mm/s was employed. The monochromator bandwidth was set at 20 nm.

Densitometric scanning was performed on Camag TLC scanner III in the absorbance mode at 254 nm and operated by winCATS planar chromatography version 1.1.3.0. The source of radiation utilized was deuterium and tungsten lamp. Concentrations of the compound chromatographed were determined from the intensity of absorbance. Evaluation was via peak areas with linear regression.

T_1 : Glycyrrhizic acid ; T_2 : Extract ; T_3 : Yastimadhu churna ; T_4 : Artin capsule

Figure 5.08. TLC Chromatogram of *G. glabra* and its commercial formulations

5.5.4. *Calibration curves of standard*

Calibration curves of standard in ethanol containing concentrations of 0.02 mg/ml to 0.25 mg/ml were prepared from the stock solution of 1.0 mg/ml. 10 microlitres from each solution was spotted on the TLC plate to obtain final concentration range of 0.2–2.5 ug/spot. Each concentration was spotted two times on the TLC plate. The data of peak area versus sample concentration were treated by linear regression analysis (Figure 5.09).

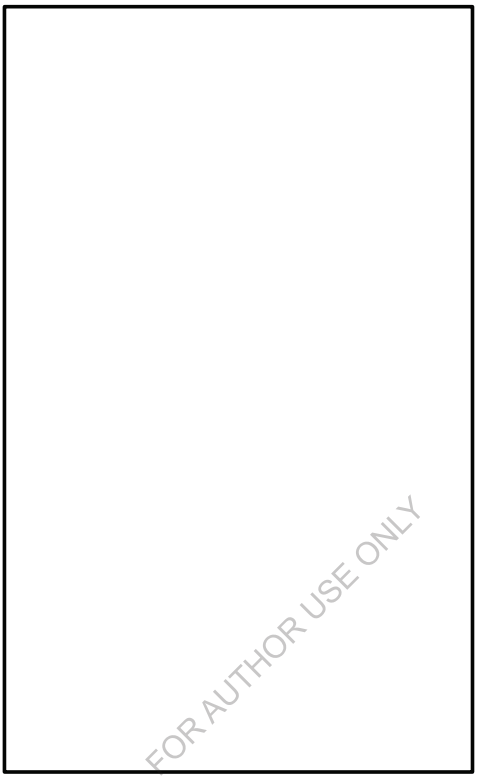

Figure 5.09. Calibration curve of the marker compound and G. glabra samples.

5.5.5. *Method validation*

The HPTLC method developed was validated for following parameters.

5.5.6. *Sensitivity*

The sensitivity of the method was determined with respect to LOD, LOQ, linearity range and correlation coefficient. Solutions containing 0.2–2.5 ug of standard were spotted on TLC plate. The LOD was calculated as 160 ng (3.3 times the noise level) and LOQ was calculated as 487 ng (10 times the noise level). The regression data

for the four samples are given in Figure 5.10, which shows good linear relationship in that range studied.

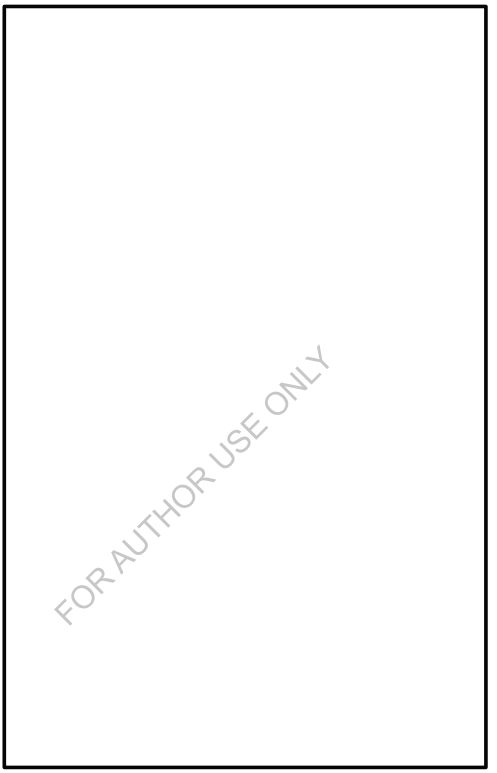

Figure 5.10. HPTLC Chromatograms of the marker compound, *G. glabra* and its commercial formulations of *G. glabra* and its commercial formulations.

The peak purity of the marker compound was assessed by comparing the spectra at peak start, peak apex and peak end of the band, respectively (Figure 5.11). Representative overlay spectra of standard and *B. diffusa* extracts are shown in Figure 5.12.

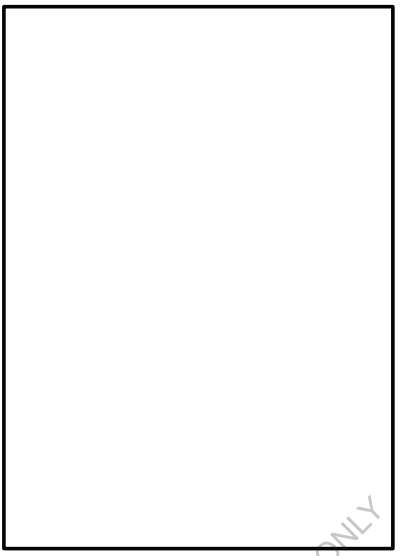

Figure 5.11. UV spectra of the standard and *G. glabra* samples

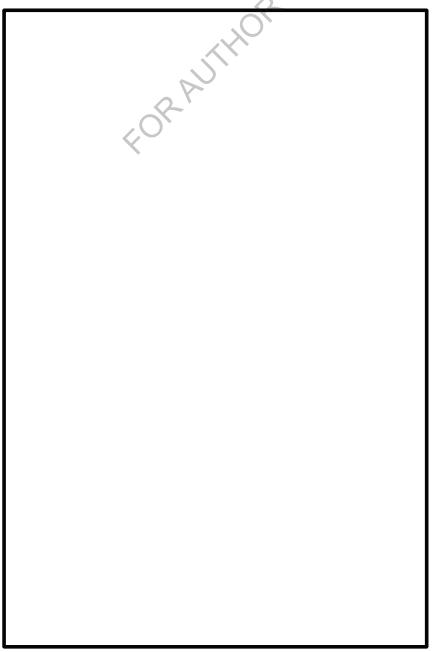

Figure 5.12. 3D Overlay chromatograms of the standard and *G. glabra samples*

5.5.7. *Recovery study*

Recovery of standard was calculated by spiking 1.0 mg to the formulation, Artin capsule (1.00gm) and extracted in ethanol and analysed three times as described in Section 2.2.2. The recovery was calculated by comparing the resultant peak areas with the standard (Table 5.02).

5.5.8. *Precision and accuracy*

Different amounts Artin (spiked) samples are spotted on TLC plate. These spots were analyzed by using above described HPTLC method. Precision was expressed as the percent of relative standard deviation (% R.S.D.) (Table5.03).

5.5.9. *Reproducibility*

The repeatability was evaluated by analyzing the known amounts of samples spotted on TLC plate in replicates (n = 5). The inter day and intraday precision was evaluated by analyzing the same amount of analyte over period of 3 days (n = 5) and expressed in terms of % R.S.D (Table 5.04).

5.6. Results and discussions

The 50% aqueous ethanolic extract of the roots of *G. glabra* was subjected to a novel CombiFlash chromatography over Sigel with the initial elution with chloroform-methanol (1:1) followed by n-butanol, which resulted in the isolation of the bio-active marker compound as colourless amorphous powder. Preliminary physical and chemical tests identify the compound as a triterpene glycoside. Detailed spectroscopic analysis (IR, ^{1}H & ^{13}C NMR and Mass) confirms its structure as glycyrrhizic acid. Detailed thin layer chromatographic studies on this compound optimised the solvent system as n-butanol-acetic acid-water (7:2:1), which showed

single spot at R_f: 0.6 (Fig. 5.05). This compound was taken as marker compound and quantitatively evaluated *G. glabra* roots along with two of its commercial samples such as Yastimadhu churna (Ganga Pharmaceuticals) and Artin capsules (Imis Pharma) using HPTLC.

5.6.1. *Sensitivity*

The sensitivity of the method was determined with respect to LOD, LOQ, linearity range and correlation coefficient. Solutions containing 0.2-2.5 µg of standard were spotted on TLC plate. The LOD was calculated as 160 ng (3 times the noise level) and LOQ was calculated as 487 ng (10 times the noise level). The regression data for the four samples are given in the Table 5.01, which shows good linear relationship in that range studied.

Table 5.01 Results of Linearity

Linearity range	r^2	Slope	Intercept
0.2 – 2.5 µg	0.99867	0.737	101.387

5.6.2. *Recovery study*

Recovery of standard was calculated by spiking 1.0 mg to the *formulation,* artin (1.00 gm) and extracted in ethanol and analysed three times as described in Section 2.3.3. The recovery was calculated by comparing the resultant peak areas with the standard (Table 5.02).

Table 5.02 Recovery and RSD of method

Sample	Amount spotted (ng)	Amount detected (ng) (Mean ± SD, n=3)	RSD (%)	Recovery (%)
Artin	500 (10 µl)	494.19 ± 5.27	1.06	98.83

5.6.3. *Precision and accuracy*

Different amounts Artin (spiked) samples are spotted on TLC plate. These spots were analyzed by using above described HPTLC method (Table 5.03). Precision was expressed as the percent relative standard deviation (% R.S.D.).

Table 5.03 Results of precision and accuracy

Actual amount of standard spotted (µg)	Amount detected (µg ± SD, n=5)	RSD (%)
0.5	0.486 ± 0.013	2.67
1.0	0.979 ± 0.036	3.76
3.0	2.884 ± 0.112	3.88

5.6.4. *Reproducibility*

The repeatability was evaluated by analyzing the known amounts of spiked samples spotted on TLC plate in replicates (n = 5). The inter day and intra day precision was evaluated by analyzing the same amount of analyte over period of 3 days (n = 5) and expressed in terms of % R.S.D. (Table 5.04).

Table 5.0 4 Results of reproducibility

Amount spotted (µg)	Amount detected (µg ± SD)	RSD %
Inter day (n=3)		
0.5	0.483 ± 0.023	4.76
1.0	0.971 ± 0.050	5.14
3.0	2.868 ± 0.164	5.71
Intra day (n=5)		
0.5	0.491 ± 0.012	2.44
1.0	0.983 ± 0.031	3.15
3.0	2.881 ± 0.124	4.30

5.6.5. *Sample analysis*

The validated HPTLC method was applied for quantitative determination of the marker, glycyrrhizic acid in *G. glabra* root sample and two of its commercial formulations such as Yastimadhu churna and Artin capsules and the results are presented in Table 5.05. The marker compound was found to accumulate in 0.88% in the *G. glabra* root sample. The developed HPTLC method found working very well in case of one o the commercial formulations, Yastimadhu churna, where the marker compound was found to present in 0.78%. Surprisingly, the marker compound could not be detected even in traces in Artin capsule, which contain more than ten herbal ingredients including *G. glabra*. The absence of marker in this formulation might be due to its loss during manufacturing process or different plant parts used in this formulation. The developed methods can be used successfully for its fast isolation and quality control and quality assurance of *G. glabra* formulations, where the root part is used. Hence, the marker compound in these two formulations might be lost during their manufacturing process or due to different plant part used. But marker compound could be detected accurately in the whole plant samples. The developed method can be used for successfully for quality control and quality assurance of *G. glabra* formulations where the root part is used.

Table .5.05 Accumulation of the marker in various *G.glabra* samples

S. No.	Samples	Glycyrrhizic acid (%)
1.	Root	0.88
2.	Yastimadhu churna	0.78
3.	Artin capsule	Not detected

Conclusion

In the present study, a novel two step CombiFlash chromatographic method was developed for the fast isolation of glycyrrhizic acid, the major bio-active marker of *G. glabra*. Further, glycyrrhizic acid was quantitatively estimated in the root part of *G. glabra* and two of its commercial samples such as Yastimadhu churna and Artin by employing a rapid and validated HPTLC method. The method is rapid, simple and reproducible. The marker compound, glycyrrhizic acid was found to present in 0.88% in the roots of *G. glabra* and 0.78% in Yastimadhu churna. But surprisingly, it could not be detected in Artin capsules. The developed method can be successfully employed for quality control and quality assurance of *G. glabra*, where the root part is used.

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