PHYTOCHEMICAL AND BIOLOGICAL INVESTIGATION OF MEDICINAL PLANTS

THESIS SUBMITTED TO THE TAMILNADU DR. M.G. R. MEDICAL UNIVERSITY, CHENNAI, FOR THE AWARD OF THE DEGREE OF

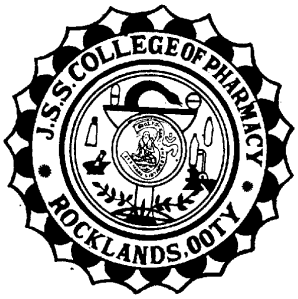
DOCTOR OF PHILOSOPHY

Submitted by

B. GEETHA, M. Pharm.,

Under the Guidance of

Dr. SHRISHAILAPPA BADAMI, M. Pharm., Ph.D.



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DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

J. S. S. COLLEGE OF PHARMACY

OOTACAMUND - 643 001, TAMILNADU, INDIA

J.S.S. MAHAVIDYAPEETHA

J.S.S. COLLEGE OF PHARMACY

Accrediated by : National Board of Accreditation of AICTE - New Delhi

Dr. Shrishailappa Badami
Professor

CERTIFICATE

This is to certify that the thesis entitled **"Phytochemical and biological investigation of medicinal plants"** submitted by **Mrs. B. Geetha**, to The Tamilnadu Dr. M. G. R. Medical University, Chennai, for the award of the Degree of Doctor of Philosophy in Pharmaceutical Sciences, is a record of the independent research work carried out by her at J. S. S. College of Pharmacy, Ootacamund, under my supervision, during 2006-2010. I also certify that this thesis or any part thereof has not formed the basis for the award of any other research degree, of this or any other University, previously.

Dr. Shrishailappa Badami
Research Supervisor

P.B. No. 20, "Rocklands", Ootacamund-643 001, The Nilgiris, Tamil Nadu, S.India
Phone : +91-423-2443393/2443847; Fax : +91 - 423-2442937; E-mail : jaspharm@hotmail.com ; Website : www.jsspharma-ooty.com

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Principal

P.B. No. 20, "Rocklands", Ootacamund-643 001, The Nilgiris, Tamil Nadu, S.India
Phone : +91-423-2443393/2443847; Fax : +91 - 423-2442937; E-mail : jaspharm@hotmail.com ; Website : www.jsspharma-ooty.com

DECLARATION

I hereby declare that the thesis entitled **"Phytochemical and biological investigation of medicinal plants"** submitted by me to The Tamilnadu Dr. M. G. R. Medical University, Chennai, for the award of the Degree of Doctor of Philosophy in Pharmaceutical Sciences, is the result of my original and independent work carried out at J. S. S. College of Pharmacy, Ootacamund, under the supervision of **Dr. Shrishailappa Badami,** Professor, J. S. S. College of Pharmacy, Ootacamund. The thesis or any part thereof has not formed the basis for the award of any degree, diploma, associateship, fellowship, or any other similar title, of this or any other University, previously.

(B. Geetha)

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B. Geetha

CONTENTS

Plants have been always an integral part of the ancient culture of India, China and Egypt as medicine and their importance even dates back to the Neanderthal period . These medicines initially took the form of crude drugs such as tinctures, teas, poultices, powders, and other herbal formulations (Samuelsson, 2004). The applications of plant based formations for various ailments and their methods of preparation were passed through oral history during ancient periods. Eventually information regarding medicinal plants was recorded in herbals which are providing lot of opportunities to explore and isolate unknown plant components.

India represented by rich culture, traditions and natural biodiversity, offers a unique opportunity for drug discovery researchers. This knowledge-based country is well recognized for its heritage of the world's most ancient traditional system of medicine, Ayurveda. Even, Dioscorides who influenced Hippocrates is thought to have taken many of his ideas from India (Gurib-Fakim, 2006). India is a major resource of medicinal plants, two of the 18 hotspots of plant biodiversity in the world namely the Eastern Himalayas and the Western Ghats are found in India. Interestingly, India ranks seventh among the 16 mega diverse countries, where 70% of the world's species occurs collectively. The natural resources found in India are exorbitant accounting for rich flora of endemic plant species consisting of 5725 angiosperms, 10 gymnosperms, 193 pteridophytes, 678 bryophytes, 260 liverworts, 466 lichens, 3500 fungi and 1924 algae (Sanjappa, 2005). Unfortunately, only 65% of the flora of the country has been surveyed so far due to inaccessibility to tough terrains. It is estimated that around 2,50,000 flowering plant species are reported to occur globally. Approximately

half (1,25,000) of these are found in the tropical forests. They continue to provide natural product chemists with invaluable compounds for the development of new drugs. The potential for finding new compounds is enormous as till date only about 1% of tropical species have been studied for their pharmaceutical potential. Hence, natural products are considered to be a treasure for the phytochemists.

In more recent history, the use of plant based medicines has involved the isolation of active compounds. It has its beginning with the isolation of morphine from opium in the early 19th century (Kinghorn, 2001; Samuelsson, 2004). Isolation and characterization of pharmacologically active compounds from medicinal plants continue till today. The techniques for the utilization of plant medicines have been improved to a greater extent in recent years. Various drug discovery techniques have been applied for the standardization of herbal medicines, to elucidate analytical marker compounds. Drug discovery from medicinal plants includes numerous fields of inquiry and various methods of analysis. The process typically begins with a botanist, ethnobotanist, ethnopharmacologist, or plant ecologist. The primary activity involves the identification and collection of plant(s) of interest. Collection is based on the species with known biological activity for which active compound(s) have not been isolated (e.g., traditionally used herbal remedies) or may involve taxa collected randomly for a large screening program giving respect to the intellectual property rights of a given country where plant(s) of interest are collected (Baker et al., 1995). In the next step, Phytochemists (natural product

chemists) prepare extracts from the plant materials, subject these extracts to biological screening in pharmacologically relevant assays, and commence the process of isolation and characterization of the active compound(s) through bioassay-guided fractionation. Finally the isolated compounds subjected to appropriate screening techniques for the determination of physiologically relevant targets by molecular biologists.

There are two types of plant chemicals, primary metabolites such as sugars, proteins, amino acids, chlorophylls etc. The other category of chemicals is called secondary metabolites, which includes alkaloids, terpenoids, saponins and phenolic compounds. These chemicals exert a significant physiological effect on the mammalian system. Secondary metabolites from natural sources have been elaborated within living systems and they are often perceived as showing more "drug-likeness and biological friendliness than totally synthetic molecules" (Koehn and Carter, 2005) making them good candidates for further drug development (Balunas and Kinghorn, 2005; Drahl et al., 2005).

The global market for plant-derived drugs was worth an estimated $18 billion in 2005. It is expected this figure to grow to nearly $19 billion in 2006 and more than $26 billion by 2011, at an average annual growth rate (AAGR) of 6.6% between 2006 and 2011.The U.S. accounts for 50% of the global plant-derived drug market and is expected to grow faster than other markets at an AAGR of 7.5% per year vs. 5.3% .A total of 26 plant-based drugs were approved/launched

during 2000–2006, which also include novel molecule-based drugs like Galanthamine HBr (Reminyl1), Miglustat (Zavesca1) and Nitisinone (Orfadin1).

Despite the recent interest in molecular modeling, combinatorial chemistry, and other synthetic chemistry techniques by pharmaceutical companies and funding organizations, natural products, and particularly medicinal plants, remain an important source of new drugs, new drug leads, and new chemical entities (NCEs) (Newman et al., 2000, 2003; Butler, 2004). Nature has evolved over time to produce a bewildering diversity of secondary metabolites. The large proportion of natural products in drug discovery has stemmed from the diverse structures and the intricate carbon skeletons of natural products. Natural products interrogate a different area of chemical space than synthetic compounds. There are significant differences in the molecular architecture produced by nature when compared to the synthetic molecules of medicinal chemistry (Ganesan, 2004 ;Grabowski and Schneider 2007; Ortholand and Ganesan, 2004)**.** Although both aim to produce biologically active matter, biosynthesis operates under a different set of constraints and guiding principles than the synthetic organic chemist. In nature, a very parsimonious set of building blocks is utilized, whereas we have access to tens of thousands of commercially available chemicals. As a consequence, we achieve numbers by repeating a reliable sequence of reactions over and over again while changing the input. Nature, on the contrary, diversifies by taking its limited building blocks and partitioning them into a multitude of pathways. Further differences occur in the type of synthetic transformation performed. Nature is oxophilic, and has developed

enzymes that exquisitely accomplish site-selective C–H activation (Chen and White, 2007; Hartwig et al., 2005) to introduce oxygen and discriminate between numerous functional groups at different oxidation levels.

Natural products offer large structural diversity (Clardy and Walsh, 2004), and modern techniques for separation, structure elucidation, screening and combinatorial synthesis (Corcoran and Spraul, 2003; Steinbeck, 2004; Ganesan, 2002) have led to revitalization of plant products as sources of new drugs. The importance of plant based drugs, the introduction of herbals in the form of nutraceuticals and dietary supplements are also changing the current trend dominated by synthetic drugs to plant-based drug market (Cardellina, 2002; Raskin, 2002). Hence, in the present study we were interested in natural products research.

There are over 200 species of *Strobilanthes* nearly all in Asia and over 150 occurs in India, especially in Western Ghats and Nilgiris alone claims more than thirty species. Many of the species flower at longer intervals such as between six and twelve years, usually and in some even after 35 years. There exists a strong chemotaxonomical relationship among the genus. The most common and best known species *Strobilanthes Kunthianus* (kurinji), which has the flowering cycle once in twelve years, has not been studied so far chemically and biologically. Hence, we were interested to find out the phytoconstituents present in the plant and subject its extracts for biological investigations.

Free radicals are chemical species, which contains one or more unpaired electrons due to which they are highly unstable and cause damage to other molecules by extracting electrons from them in order to attain stability. Free radicals may be involved in the etiology of ageing, inflammation, arthritis, diabetes, cancer and cardiovascular diseases. Oxygen is the molecule of life for aerobic creatures. Oxygen is not only fundamentally essential for energy metabolism and respiration, but it has been implicated in many diseases and degenerative conditions. Reactive oxygen species (ROS) formed *in vivo*, such as superoxide anion, hydroxyl radical and hydrogen peroxide, are highly reactive and potentially damaging transient chemical species. The ROS play an important role in the pathogenesis of various serious diseases, such as neurodegenerative disorders, cancer, cardiovascular diseases, atherosclerosis, cataracts, and inflammation (Aruoma, 1998; Kris- Etherton et al., 2004). These are continuously produced in the human body, as they are essential for energy supply, detoxification, chemical signaling and immune function.

Mammalian cells possess elaborate defense mechanisms for radical detoxification. Key metabolic steps are the superoxide dismutase (SOD) catalysis of the dismutation of superoxide to hydrogen peroxide and oxygen, and the conversion of H_2O_2 into water and oxygen by catalase (CAT) and glutathione peroxidase (GPX), which destroys toxic peroxides. In addition to antioxidant enzymes, several small-molecule antioxidants play important roles in the antioxidant defense systems. These can be divided into compounds made *in vivo*, and compounds obtained from diet. Glutathione, bilirubin, and melatonin are

examples of the former, and vitamins such as α-tocopherol, β-carotene, and ascorbic acid and micronutrient elements such as zinc and selenium are examples of the latter (Halliwell and Gutteridge, 1998). Antioxidants are vital substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress (Ozsoy et al., 2008). Many synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) are very effective and are used for industrial processing but they possess potential health risk and toxic properties to human health and should be replaced with natural antioxidants.

Natural products now have received special attention as dietary supplements because of their potent antioxidant activity. Most beneficial health effects are attributed to their capacity to transfer electrons to free radicals, chelate metal catalysts, activate enzyme system, reduce α-tocopherol radicals and have capacity to inhibit LDL oxidation (Heim et al., 2002). Ascorbic acid, which is very common in various plants, has ability to act as reducing agent. Vitamin C is effective to some extent in maintaining levels of antioxidant in plasma and liver and reduced glutathione when supplemented to alloxan induced diabetic rats (Garg et al., 1997). As correlation of free radicals and various diseases is obvious, demand for herbal antioxidant is on high. Many of the phytonutrients produced by plant secondary metabolism are excellent antioxidants. Examples are carotenoids as lutein, beta-carotene, lycopene, and zeaxanthin; flavonoids as apigenin, catechin, gentisein, kaempferol, myricetin, quercetin and rutin; phenolic compounds as caffeic acid, ellagic acid, ferulic acid, gallic acid, para-

amino benzoic acid; triterpenoid saponins as ginsenosides glycyrrhizin; diterpenes as ginkgolide A, ginkgolide B, and ginkgolide C, and sesquiterpene bilobade. The leaves of *S. crispus* have been reported to possess potent antioxidant activity (Ismail et al., 2000; Rahmat et al., 2006). However, so far no antioxidant activity has been carried out for *S. kunthianus*. Hence, in the present study we were interested to evaluate the extracts of the plant for their antioxidant potentials.

Inflammation is the body's response to infection, tissue injury or invasion by microorganisms and its main purpose is to remove the cause (e.g., the invading microorganism or the irritating agent) and promote the regeneration of the tissue that was injured. There are many inflammatory mediators that participate in the regulation of inflammatory response. They include vascular amines, metabolites of arachidonic acid (prostaglandin, leukotriene and lipoxin), cytokines (interlukin-1β, interlukin-6β and tumor necrosis factor-α), platelet activating factor, neuropeptides and nitric oxide, all of which are released from cells. Also, generating inflammatory mediators are complementary systems including the kinin system, the clotting system and the fibrinolytic system.

Pain can be simply defined as undesirable physical or emotional experiences. Pain is the most common reason that individuals seek medical attention. It can be divided into two types, acute pain and chronic pain. Acute pain serves as a warning system to remove oneself from particular pain stimuli. Chronic pain can exist for undefined times and undefined reasons and seems to serve no clear purpose.

Drugs which are in use presently for the management of pain and inflammatory conditions are either narcotics e.g. opioids or non-narcotics e.g. salicylates and corticosteroids e.g. hydrocortisone. All of these drugs possess well known side and toxic effects. Moreover, synthetic drugs are very expensive to develop and whose cost of development ranges from 0.5 to 5 million dollars. On the contrary many medicines of plant origin had been used since long time without any adverse effects. It is well established that ROS play a prominent role in the stimulation, propagation and maintenance of both acute and chronic inflammatory processes as well as pain causing tissue damage (Ilavarasan et al., 2006). These adverse effects due to pain and excessive inflammation has been shown to be reduced by the use of suitable antioxidants either by preventing the formation of oxygen free radicals or by scavenging them before they react with sites such as unsaturated lipids in the cell membrane. The plant *S. cusia* commonly known as banlangen reported to possess antipyretic, antiviral, anti-inflammatory and antiinfluenza activities (Ho and Chang, 2002). The root extracts of *S. callosus* were reported for anti-inflammatory activity. However, no work has been carried out to evaluate the anti-inflammatory and analgesic activities of *S. kunthianus*, though the genus has been reported for the treatment of such disorders. Hence, in the present study, the potent antioxidant extracts were tested for *in vivo* anti-inflammatory and analgesic studies.

Liver has a pivotal role in regulation of physiological processes. It is involved in several vital functions such as metabolism, secretion and storage. Furthermore,

detoxification of a variety of drugs and xenobiotics occurs in liver. The bile secreted by the liver has, among other things, an important role in digestion. Liver diseases are among the most serious ailment. They may be classified as acute or chronic hepatitis (inflammatory liver diseases), hepatosis (non inflammatory diseases) and cirrhosis (degenerative disorder resulting in fibrosis of the liver).

Liver diseases are mainly caused by toxic chemicals (certain antibiotics, chemotherapeutics, peroxidised oil, aflatoxin, carbon-tetrachloride, chlorinated hydrocarbons, etc.), excess consumption of alcohol, infections and autoimmune/disorder. Most of the hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation and other oxidative damages in liver (Recknagel, 1983; Wendel et al., 1987; Dianzani et al., 1991; Hiroshi et al., 1987). One among them is carbon tetrachloride (CCl_4) (Kodavanti et al., 1989). Reductive dehalogenation of CCl_4 by the P450 enzyme system to the highly reactive trichloromethyl radical initiates the process of lipid peroxidation which is considered to be the most important mechanism in the pathogenesis of liver damage induced by CCl_4 (Demirdag et al., 2004). Trichloromethyl radical can even react with sulfhydrylgroups of glutathione (GSH) and protein thiols. In addition, CCl_4 also alters the antioxidant profile of the liver including the antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathionetransferase (GST). Enhanced lipid peroxidation produced during the liver microsomal metabolism of ethanol may result in hepatitis and cirrhosis

(Smuckler, 1975). It has been estimated that about 90% of the acute hepatitis is due to viruses. The major viral agents involved are Hepatitis B, A, C, D (delta agents), E and G. Of these, Hepatitis B infection often results in chronic liver diseases and cirrhosis of liver. Primary liver cancer has also been shown to be produced by these viruses. It has been estimated that approximately 14-16 million people are infected with this virus in South east Asia region and about 6% of the total population in the region are carriers of this virus. A vaccine has become available for immunization against Hepatitis B virus. Hepatitis C and Hepatitis E infections are also common in countries of South East Asia region (WHO, 1997).

In spite of the tremendous advances made in allopathic medicine, no effective hepatoprotective medicine is available. Plant drugs are known to play a vital role in the management of liver diseases. There are numerous plants and polyherbal formulations claimed to have hepatoprotective activities. Nearly 150 phytoconsitituents from 101 plants have been aimed to possess liver protecting activity (Doreswamy and Sharma, 1995). At the same time, surprisingly, we do not have readily available satisfactory plant drugs/formulations to treat severe liver disease.

The antioxidant activity or the inhibition of the generation of free radicals is important in providing protection against hepatic damage. A number of plants have been shown to possess the hepatoprotective activity by improving antioxidant properties (Shahjahan et al., 2004). *S. crispus* has been used as

antidiabetic, antilytic, laxative, anti AIDS, antileukemic and hepatitis (Sunarto, 1977; Kusumoto et al., 1992; Ismail et al., 2000; Jaksa et al., 2004; Yogespriya et al., 2005). However, no work has been carried out to evaluate the hepatoprotective effect of *S. kunthianus*, though the gene has been reported for the treatment of hepatic disorders. Hence, in the present study we also aimed to investigate the potent extract for *in vivo* hepatoprotective and antioxidant activities.

Natural products, including plants, animals and minerals have been the basis of treatment of human diseases. History of medicine dates back practically to the existence of human civilization. The current accepted modern medicine or allopathy has gradually developed over the years by scientific and observational efforts of scientists. However, the basis of its development remains rooted in traditional medicine and therapies. The history of medicine includes many ludicrous therapies. Nevertheless, ancient wisdom has been the basis of modern medicine and will remain as one important source of future medicine and therapeutics. Pharmacognostical studies serve as an important tool in plant identification. Detailed microscopic evaluation will be of immense importance in the standardization of plant materials. Isolation of phytoconstituents from the active extracts helps in many ways in plant research. These constituents can serve as marker compounds for their standardization. The determination of the biological activities helps in developing these compounds into drugs or lead molecules for further drug development.

Species of *Strobilanthes* grow wild on the Nilgiri ranges between 6000 to 7000 feet. The genus *Strobilanthes* is known for its various biological activities (Sunarto, 1977; Kusumoto et al., 1992; Ismail et al., 2000; Jaksa et al., 2004; Rahmat et al., 2006; Nair et al., 1988). *S. kunthianus* (Neelakurinji) is a shrub in the grasslands of Western Ghats in India. The Nilgiris, which literally means the blue mountains got its name from the purplish blue flowers of Neelakurinji that blossoms gregariously once in twelve years. Lupeol has been isolated from the

whole plant. Except this study, so far no phytochemical investigations were carried out. Hence, in the present study the isolation of phytoconstituents from *S. kunthianus* was aimed.

In a situation of oxidative stress, reactive oxygen species (ROS) such as superoxide (O_2•-, OOH•), hydroxyl (OH•) and peroxyl (ROO•) radicals are generated. The ROS play an important role in the pathogenesis of various serious diseases, such as neurodegenerative disorders, cancer, cardiovascular diseases, atherosclerosis, cataracts, inflammation etc., Antioxidants are molecules which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. Several synthetic antioxidants such as butylated hydroxyl anisole (BHA), butylated hydroxy toluene (BHT), and tert-butylhydroquinone (TBHQ) may be inappropriate for chronic human consumption, because of their toxic properties for human health and environment (Ito et al., 1986; Stich, 1991). The use of traditional medicine is widespread and plants still represent a large source of natural antioxidants that might serve as leads for the development of novel drugs. Hence, in the present study *in vitro* antioxidant activity of the extracts were carried out. Several anti-inflammatory and analgesics have recently been shown to have an antioxidant and/or radical scavenging mechanism as part of their activity.

Liver is the most important organ concerned with the biochemical activities in the human body. It has great capacity to detoxicate toxic substances and synthesize useful principles. Therefore, damage to the liver inflicted by

hepatotoxic agents is of grave consequences. Hepatitis is one of the most prevalent diseases in the world and drug related hepatotoxicity is the leading cause of acute liver failure among patients referred for liver transplantation. Despite the fact that hepatic problems are responsible for a significant number of liver transplantations and deaths recorded worldwide, available pharmacotherapeutic options for liver diseases are very limited and there is a great demand for the development of new effective drugs. Therefore, herbal products and traditional medicines with better effectiveness and safe profiles are needed as a substitute for chemical therapeutics.

The plant *S. cusia* commonly known as banlangen reported to possess antipyretic, antiviral, anti-inflammatory and antiinfluenza activities (Ho and Chang, 2002). *S. Crispus* has been used as antidiabetic, antilytic, laxative, anti AIDS, antileukemic and hepatitis (Sunarto, 1977; Kusumoto et al., 1992; Ismail et al., 2000; Jaksa et al., 2004; Yogespiriya et al., 2005). The root extracts of *S. callosus* were reported for anti-inflammatory activity. Hence, in the present study, potent extracts of *S. kunthianus* was screened for *in vivo* anti-inflammatory, analgesic, hepatoprotective and antioxidant activities.

OBJECTIVES OF THE WORK

- To select a plant based on their chemotaxonomy and ethnomedical uses.
- Collection and extraction.
- Evaluation of macroscopy, microscopical characters and physicochemical constants of the selected plant.

- To isolate phytoconstituents from the extracts and their characterization by spectral methods.
- To screen the extracts for cytotoxic activity by using *in vitro* methods.
- To screen the extracts for antioxidant activity by using various *in vitro* methods.
- To screen the potent extracts for their *in vivo* antioxidant, analgesic, anti inflammatory and hepatoprotective activities.

PLAN OF THE WORK

Phase-I: Pharmacognostical studies

- Collection, processing and authentication of *S. Kunthianus*
- Macroscopical study
- Microscopical study
- Physicochemical constants

Phase-II: Phytochemical studies of the extracts

- Preparation of extracts
- Qualitative phytochemical analysis
- Estimation of total phenol content

Phase-III: Isolation and characterization of phytoconstituents

- Column chromatography
- Spectral study: UV, ^{1}H NMR, ^{13}C NMR, EIMS and ESIMS.

Phase-IV: *In vitro* antioxidant activity

- DPPH radical scavenging method
- Nitric oxide radical inhibition assay
- Scavenging of ABTS radical cation assay
- Hydrogen peroxide scavenging method
- Hydroxyl radical scavenging by p-NDA and deoxyribose methods
- Total antioxidant capacity by phosphomolybdenum method.

Phase-V: *In vitro* cytotoxicity studies

- Microculture tetrazolium (MTT) assay
- Sulphorhodamine B (SRB) assay.

Phase-VI: Pharmacological screening of the active extracts of *S. kunthianus*

- Acute toxicity studies.
- Acute anti-inflammatory studies of crude methanol extracts by using carrageenan, formalin and histamine induced rat paw edema methods.
- Chronic anti-inflammatory studies of potent extract by using cotton pellet method.
- Analgesic activity of crude methanol extracts by hot plate and tail immersion methods.
- *In vivo* hepatoprotective and antioxidant activities of potent extract.

Strobilanthes Kunthianus

Botanical name	:	*Strobilanthes Kunthianus* Nees T Anders
Family	:	Acanthaceae
Synonyms	:	*Phlebophyllum Kunthianus* *Strobilanthes Kunthiana* *Strobilanthes Nilgirianthsis* *Phlebophyllum Kunthianus*
Vernacular Names	:	Tamil – Neelakurinji, kurinji
Distribution	:	It is found in Western Ghats, The Nilgiris and the Palnis, and the hills of Kerala above 1800 m on grassy downs.

Description : Bushy shrub, 1- 2 m high, in stray clumps or gregarious; stems numerous, erect, quadrangular; nodes prominent. Leaves elliptic-ovate, ca 5 x 2.5 cm, acute at base, crenate-serrate at margin, acute at apex, coriaceous, scabrid above, white-villous between veins beneath; secondary veins 8 or 9 pairs, prominent; petioles ca 5 mm long. Inflorescences spikes, uninterrupted, sometimes branched, ca 8 cm long, supported by leafy bracts, white-villous; bracts elliptic-ovate, ca 1.2 cm long, acute at apex, white villous, floccose at margin and middle. Calyx ca 1.2 cm long, floccose-villous; segments linear-lanceolate, connate almost half way from base. Corolla tubular ventricose portion gradually expanding from base, hairy inside; lobes 5, orbicular, entire. Stamens 2, included, monodelphouus; filaments ca 7 mm long, pilose; stamina

sheath extending just above cylinder base. Ovary glabrous but hairy at tip; style ca 1.5 cm long, included, hairy. Capsules oblong, ca 1.2 cm long, 4-seedded; seeds orbitular; ca 1.5 mm, complanate, densely hairy and hairs spreading when wet except on basal circular areole (Venu, 2006).

Parts used	:	Root, stem, leaves and flowers
Ethnomedical information	:	No ethnomedical information available.
Chemical constituents and biological properties	:	Lupeol has been isolated from the whole plant. No biological activities are reported.

Other *Strobilanthes* species

S. crispus, S. callosus, S. ixocephala, S. auriculatus, S. discolor, S. cusia, S. cuspidatus, S. foliosus, S. consanguineus, S. gossypinus, S. pulneyensis, S. perrottetianus, S. papillous, S. neilgherrensis, S. wightianus, S. urceolaris, S. sessilis, S. asper, S. zenkerianus, S. mincranthus, S. luridus, S. homotropus, S. violaceus and *S. amabilis* (Fyson, 1977; Anonymous, 1989).

Reported chemical constituents from other *Strobilanthes* species

Lupeol was isolated from *S. callosus* and *S. ixiocephala* (Agarwal and Rangari, 2003). Trypanthrin, indigo and indirubin were found in *S. cusia* (Liau et al., 2007). Caffeic acid, p-hydroxy benzoic acid, p-voumeric acid, vanilic acid, gentinic acid, ferulic acid, syryngic acid, β-sitosterol and stigmasterol were isolated from *S. crispus* (Rahmat et al., 2006; Soediro et al., 1983; 1987).

Reported biological activities of other *Strobilanthes* species

The plant *S. cusia* commonly known as banlangen was reported to possess antipyretic, antiviral, anti-inflammatory and antiinfluenza activities (Ho and Chang, 2002). *S. crispus* has been used as antidiabetic, antilytic, laxative, anti AIDS, antileukemic and hepatitis (Sunarto, 1977; Kusumoto et al., 1992; Ismail et al., 2000; Jaksa et al., 2004; Yogespiriya et al., 2005). The root extract of *S. callosus* has been reported for anti-inflammatory activity.

Fig. 1. Different parts of *S. kunthianus*

4.1. INSTRUMENTS

Autoanalyser:

Merck Microlab 200, manufactured by M/s Vital Scientific N. V., Darmslandt, The Netherlands.

Centrifuge:

Remi centrifuge and R-8c Laboratory centrifuge, Remi Motors Ltd., Mumbai, India

Elisa Reader:

Bio-Rad Laboratories Inc, California, USA, model 550.

Fourier Transform Infrared:

1600 Series FT-IR, Perkin Elmer (India) Pvt. Ltd., India.

Homogenizer:

Elvenjan homogenizer, Remi Motors Ltd., Mumbai.

Melting Point Apparatus*:*

Lab India, Mumbai.

NMR:

DDR X – 500 MHz – Bruker Daltonics, Karsruhe, Germany.

Rotary Evaporator:

Rotavapor R-205, Buchi Laboratory Equipments, Flawil, Switzerland.

Rotary Microtome:

Leica RM 2135, Leica Microsystem GmbH, Nussloch, Germany.

Spectrophotometer:

Shimadzu 160-A UV-VIS, Koyoto, Japan.

4.2. CHEMICALS

2,2′-Diphenyl-1-picryl hydrazyl (DPPH), 2,2′-Azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS), 3-(4, 5–Dimethyl thiazol–2–yl)–5–diphenyl tetrazolium bromide (MTT), Sulphorhodamine B (SRB) were obtained from Sigma Aldrich Co, St Louis, USA. p–Nitroso dimethyl aniline (p–NDA) and Rutin were obtained from Acros Organics, New Jersy, USA. Naphthyl ethylene diamine dihydrochloride (NEDD) was obtained from Roch–Light Ltd., Suffolk, UK. Ascorbic acid and Butylated hydroxy anisole (BHA) were obtained from S.D. Fine Chem, Ltd., Biosar, India. Sulphanilic acid, 2-Deoxy-D-ribose, Minimal essential medium (MEM) and minimal essential medium without phenol red (MEM-PR) were procured from Hi-media Laboratories Ltd., Mumbai. Gallic acid, Thiobarbituric acid, Trichloroacetic acid, Ethylenediaminetetra aceticacid (EDTA) and Adrenaline were procured from Loba Chemie Indo Austrand Co., Mumbai. Aspartate aminotransferase (ASAT), Alanine aminotransaminase (ALAT) and Alkaline phosphatase (ALP) diagnostic kits were obtained from Span Diagnostic Ltd., Surat, India. Aluminium backed high performance thin layer chromatography (HPTLC) plates coated with 0.2 mm layers of silica gel 60 F_{254} plates and other diagnostic kits were obtained from E–Merck Ltd., Mumbai. Silymarin was obtained from Ranbaxy India Ltd, New Delhi. Ibuprofen was procured from Overseas Pharma, Bangalore. Commercial animal feed was purchased from Sai Durga Feeds and Food, Bangalore. All chemicals used were of analytical grade.

4.3. COLLECTION AND EXTRACTION

4.3.1. Collection and authentication

The whole plant of *S. kunthianus* was collected from Thalaikuntha region, near Ootacamund, Nilgiris district, Tamilnadu, India. The plant was identified and authenticated at Botanical Survey of India, Coimbatore.

4.4. PHARMACOGNOSTICAL EVALUATION

4.4.1. Anatomical studies

The different organs viz., root, stem, leaf and flowers of *S. kunthianus* were cut and removed from fresh healthy plants and fixed in FAA (formalin-5 ml + acetic acid-5 ml + 70% ethanol-90 ml). After 24 h of fixing, the specimens were dehydrated with graded series of tertiary-butyl alcohol as per the schedule (Sass, 1940). Infiltration of the specimens were carried out by gradual addition of paraffin wax (m.p. 58-60 ^{o}C) until TBA solution attained super saturation. The specimens were cast into paraffin blocks.

4.4.2. Sectioning

The paraffin embedded specimens were sectioned with the help of Rotary microtome. The thickness of the sections was 10-12 µm. Dewaxing of the sections was done by customary procedure (Johansen, 1940). The sections were stained with toluidine blue (O'Brien et al, 1964). Since toluidine blue is a polychromatic stain, the staining results were remarkably good and some cytochemical reactions were also obtained. The dye rendered pink color to the

cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc., wherever necessary sections were also stained with safranin, fast green and IKI (for starch).

For studying the stomatal morphology, venation pattern and trichome distribution, paradermal sections (section taken parallel to the surface of leaf) as well as clearing of leaf with 5% sodium hydroxide or epidermal peeling by partial maceration employing Jeffrey's maceration fluid were prepared (Sass, 1940). Glycerin mounted temporary preparations were made for macerated/cleared materials. Powdered materials of different parts were cleared with sodium hydroxide and mounted in glycerin medium after staining. Different cell components were studied and measured.

4.4.3. Photomicrographs

Microscopic descriptions of tissues are supplemented with micrographs wherever necessary. Photographs of different magnifications were taken with Nickon labphoto 2 microscopic units. For normal observations bright field was used. For the study of crystals, starch grains lignified cells, and polarized light was employed. Since these structures have birefringent property, under polarized light they appear bright against dark back ground. Magnifications of the figures are indicated by the scale-bars. Descriptive terms of the anatomical features are as given in the standard anatomy textbooks (Easu, 1964 and1979).

4.5. EXTRACTION

4.5.1. Pre-extraction

The whole plant was washed thoroughly with water and separated into different parts viz stem, leaves, flowers and roots. These were shade dried and powdered separately using a mechanical blender before extraction.

4.5.2. Successive extraction

The powdered root and stem of *S. kunthianus* (500 g) were extracted successively with 2.5 l each of petroleum ether, chloroform, ethyl acetate and methanol in a Soxhlet apparatus separately for 18-20 h. The extracts were concentrated in a rotary evaporator under reduced pressure at 35-40 ^{o}C and stored at 4 ^{o}C in a refrigerator till further use.

4.5.3. Cold maceration

The powdered leaves and flowers (250 g) were extracted with 1.5 l of methanol by cold maceration separately by agitation for 7 days and filtered. The mass was squeezed out and again subjected for remaceration for 7 days and filtered off. The combined filtrate was concentrated as above.

4.5.4. Crude extraction

The powdered root, stem, leaves and flowers of *S. kunthianus* (500 g) were extracted separately with 2.5 l of methanol in a Soxhlet apparatus for 18-20 h. The extracts were concentrated as above.

4.6. PHYTOCHEMICAL SCREENING

A systematic and complete study of crude drugs should include a complete investigation of both primary and secondary metabolites derived from plant metabolism. The different qualitative chemical tests are to be performed for establishing profiles of given extracts for their nature of chemical composition. All the extracts obtained as above were tested for the following qualitative chemical tests for the identification of various phytoconstituents (Harborne, 1984 ; Kokate, 1997).

Tests for carbohydrates:

Molisch's test, Fehling's test, Benedict's test and Barfoed's test.

Tests for alkaloids:

Mayer's test, Dragendorff's test, Wagner's test and Hager's test.

Tests for steroids and sterols:

Libermann Burchard's test and Salkowski test.

Tests for glycosides:

Legal's test, Baljet's test, Borntrager's test and Keller-Kiliani test.

Tests for saponins:

Foam test and hemolysis test.

Test for flavonoids:

Shinoda test.

Tests for tannins:

Lead acetate test and gelatin test.

Test for triterpenoids:

Tin and thionyl chloride test.

Tests for proteins and amino acids:

Biuret test, Ninhydrin test and Xanthoproteic test.

4.7. ESTIMATION OF TOTAL PHENOL CONTENT

Total phenol content of the extracts was determined by using Folin-Ciocalteau method (Spanos and Wrolstad, 1990). This test is based on the oxidation of phenolic groups with phosphomolybdic and phosphotungstic acids. After oxidation, the green blue complex formed was measured at 750 nm.

4.7.1. Chemicals and Reagents

Commercially available Folin-Ciocalteu reagent was diluted (1:10) with distilled water and used. 20.25 g of sodium carbonate was dissolved in 100 ml of distilled water and used (0.7 M).

4.7.2. Preparation of test and standard solutions

The plant extracts (50 mg each) were dissolved separately in 50 ml of methanol. These solutions were serially diluted with methanol to obtain lower dilutions. Gallic acid monohydrate (50 mg) was dissolved in 50 ml of distilled water. It was serially diluted with water to obtain lower dilutions.

4.7.3. Procedure

In a test tube, 200 µl of the extract (1 mg/ml to 0.1 mg/ml) was mixed with 1 ml of Folin-Ciocalteu reagent and 800 µl of sodium carbonate. After shaking, it was kept for 2 h for reaction. The absorbance was measured at 750 nm. Using gallic acid monohydrate, standard curve was prepared and linearity was obtained in the range of 10-50 µg/ml. Using the standard curve, the total phenol content of the extract was determined and expressed as gallic acid equivalent in mg/g of the extract.

4.8. ISOLATION AND CHARACTERIZATION OF PHYTOCONSTITUENTS

Isolation is a part of natural product chemistry, through which it is possible to separate components. The biologically active ones can be incorporated as ingredients in the modern system of medicine. The column chromatographic technique (adsorption chromatography) is widely used for the separation, isolation and purification of the natural products. The principle involved in this is the adsorption towards the adsorbent packed in the column. By changing the polarity of the mobile phase, the separation can be achieved by column chromatography. Characterization of the isolated compounds can be carried out by different analytical techniques like UV, IR, NMR and Mass spectroscopy (MS).

4.8.1. Column chromatography of petroleum ether root extract

The petroleum ether root extract (6 g) was chromatographed over silica gel 60-120 mesh of column length 60 cm and diameter 3 cm. Elution was carried out with solvents and solvent mixtures of increasing polarities. The fractions were collected in 25 ml portions and monitored on TLC and the fractions showing similar spots were combined (Table 1). The fractions 143-172 eluted with chloroform:ethyl acetate (80:20) gave a white precipitate which showed two major spots on TLC. Hence, it was subjected for recolumn chromatography to isolate the two compounds. The remaining fractions were not worked out because of lower yields.

4.8.1.1. Recolumn chromatography of fractions 143-172

The white precipitate (3.2 g) obtained from fractions 143-172 was further chromatographed over silica gel 100-200 mesh of column length 50 cm and diameter 3 cm. Elution was carried out with solvents and solvent mixtures of increasing polarities. The fractions were collected in 10 ml portions and monitored on TLC (Table 2). The fractions 25–48 eluted with petroleum ether: acetone (90:10) gave a white residue and showed single spot with tailing. Repeated recrystallisation in methanol yielded colorless needle shaped crystals which showed a single spot on TLC with petroleum ether: acetone (85:15). TLC studies were carried out in different solvent systems to prove the homogeneity (Table 10). It was designated as compound 1 (GS1, yield 1.4 g, 23.33%). The next fraction 49-56 which gave colourless white residue was washed several times with petroleum ether and on recrystallisation in chloroform yielded a colorless compound and its homogeneity was confirmed by TLC studies, designated as compound 2 (GS2, yield 0.31 g, 5.17%). Compounds 1 and 2 were subjected to physical and spectral studies for confirming their purity and characterization. The remaining fractions were not worked out because of lower yields.

Table 1. Column chromatography of petroleum ether root extract

Fraction No.	Eluent	Residue on evaporation
1-12	n-Hexane	No residue
13-20	n-Hexane:Petroleum ether (80:20)	No residue
21-25	n-Hexane:Petroleum ether (60:40)	No residue
26-30	n-Hexane:Petroleum ether (20:80)	No residue
31-45	Petroleum ether	No residue
46-86	Petroleum ether:Chloroform (80:20)	Light yellow residue
87-95	Petroleum ether:Chloroform (70:30)	Light yellow residue
96-100	Petroleum ether:Chloroform (60:40)	Light yellow residue
101-110	Petroleum ether:Chloroform (50:50)	Yellow residue
111-115	Petroleum ether:Chloroform (40:60)	Yellow residue
116-120	Petroleum ether:Chloroform (30:70)	Yellow residue
121-125	Petroleum ether:Chloroform (20:80)	Yellow residue
126-136	Petroleum ether:Chloroform (10:90)	Yellow residue
137-142	Chloroform	Yellow residue
143-172	**Chloroform:Ethyl acetate (80:20)**	**White residue**
173-185	Chloroform:Ethyl acetate (60:40)	White residue
186-190	Chloroform:Ethyl acetate (40:60)	White residue
191-195	Chloroform:Ethyl acetate (20:80)	Yellow residue
196-206	Ethyl acetate	Light brown residue
207-215	Ethyl acetate:Methanol (80:20)	Light brown residue
216-224	Ethyl acetate:Methanol (60:40)	Light brown residue
225-230	Ethyl acetate:Methanol (40:60)	Light brown residue
231-238	Ethyl acetate:Methanol (20:80)	Brown residue
239-277	Methanol	Brown residue
278-290	Acetone	Brown residue

Table 2. Recolumn chromatography of fractions 143-172

Fraction No.	Eluent	Residue on evaporation
1-5	Petroleum ether	No residue
6-12	Petroleum ether: Acetone (98:2)	No residue
13-18	Petroleum ether: Acetone (96:4)	No residue
19-24	Petroleum ether: Acetone (92:8)	No residue
25-48	**Petroleum ether: Acetone (90:10)**	**White residue**
49-56	**Petroleum ether: Acetone (88:12)**	**White residue**
57-60	Petroleum ether: Acetone (86:14)	White residue
61-65	Petroleum ether: Acetone (84:16)	White residue
66-71	Petroleum ether: Acetone (82:18)	White residue
72-75	Petroleum ether: Acetone (80:20)	Brown residue
76-80	Petroleum ether: Acetone (60:40)	Brown residue
81-85	Petroleum ether: Acetone (20:80)	Brown residue
86-90	Acetone	No residue

4.8.2. Column chromatography of petroleum ether stem extract

The petroleum ether stem extract (5.6 g) was chromatographed over silica gel 100-200 mesh of column length 100 cm and diameter 1.2 cm. Elution was carried out with solvents and solvent mixtures of increasing polarities. The fractions were collected in 50 ml portions and monitored on TLC and the fractions showing similar spots were combined (Table 3). The fractions 46-64, on elution with chloroform: ethyl acetate (60:40) yielded a yellow residue (2.1 g)

which showed on TLC single spot with tailing. On repeated recrystallisation with methanol it gave a colorless needle shaped crystalline compound and its homogeneity was proved by TLC studies and designated as compound 3 (PES 1, yield 0.9 g, 16.07%). This compound was subjected to physical and spectral studies for confirming the purity and characterization. The remaining fractions were not worked out because of lesser yields.

Table 3. Column chromatography of petroleum ether stem extract

Fraction No.	Eluent	Residue on evaporation
1-4	n-Hexane	No residue
5-10	n-Hexane:Petroleum ether (50:50)	No residue
11-20	Petroleum ether	No residue
21-26	Petroleum ether:Chloroform (80:20)	Colourless residue
27-31	Petroleum ether:Chloroform (60:40)	Colourless residue
32-38	Petroleum ether:Chloroform (20:80)	Light yellow residue
39-42	Chloroform	Light yellow residue
43-45	Chloroform:Ethyl acetate (80:20)	Yellow residue
46-64	**Chloroform:Ethyl acetate (60:40)**	**Yellow residue**
65-71	Chloroform:Ethyl acetate (40:60)	Light brown residue
72-75	Chloroform:Ethyl acetate (20:80)	Light brown residue
76-79	Ethyl acetate	Light brown residue
80-81	Ethyl acetate:Methanol (80:20)	Brown residue
82-88	Ethyl acetate:Methanol (60:40)	Brown residue
89-92	Ethyl acetate:Methanol (20:80)	Brown residue
93-104	Methanol	Brown residue

4.8.3. Column chromatography of chloroform stem extract

The chloroform stem extract (2.5 g) was chromatographed over silica gel 100-200 mesh of column length 100 cm and diameter 1.2 cm. Elution was carried out with solvents and solvent mixtures of increasing polarities. The fractions were collected in 25 ml portions and monitored on TLC and the fractions showing similar spots were combined (Table 4). The fractions 25-55 eluted with petroleum ether: chloroform (80:20) gave a light green residue. On repeated washing with acetone, it yielded a colourless residue. The colourless residue was dissolved in minimum quantity of chloroform and filtered off. The filtrate on evaporation yielded a white semisolid compound, which on repeated washing with acetone and recrystallisation with chloroform yielded a colorless residue and its homogeneity was confirmed with various solvent systems by TLC and designated as compound 4 (SC 1, yield 0.016 g, 0.64%). This compound was subjected to physical and spectral studies for confirming the purity and characterization. The remaining fractions were not worked out because of lesser yields.

Table 4. Column chromatography of chloroform stem extract

Fraction No.	Eluent	Residue on evaporation
1-24	Petroleum ether	Colourless sticky residue
25-55	**Petroleum ether:Chloroform (80:20)**	**Light green residue**
56-66	Petroleum ether:Chloroform (60:40)	Yellow residue
67-87	Petroleum ether:Chloroform (40:20)	Dark yellow residue
88-90	Petroleum ether:Chloroform (20:80)	No residue
91-95	Chloroform	Green residue
96-101	Chloroform:Ethyl acetate (80:20)	Green residue
102-110	Chloroform:Ethyl acetate (60:40)	Green residue
111-119	Chloroform:Ethyl acetate (40:60)	Light green residue
120-126	Chloroform:Ethyl acetate (20:80)	No residue
127-147	Ethyl acetate	Dark green residue
148-151	Ethyl acetate:Methanol (80:20)	Light yellow residue
152-158	Ethyl acetate:Methanol (60:40)	Yellow sticky residue
159-165	Ethyl acetate:Methanol (40:60)	No residue
166-180	Ethyl acetate:Methanol (20:80)	Light yellow residue
181-185	Methanol	yellow residue
186-195	Acetone	No residue

4.8.4. Column chromatography of macerated methanol flower extract

The macerated methanol flower extract (6 g) was chromatographed over silica gel 60-120 mesh of column length 50 cm and diameter 3 cm. Elution was carried out with solvents and solvent mixtures of increasing polarities. The fractions were collected in 50 ml portions and monitored on TLC and the fractions showing similar spots were combined (Table 5). The fractions 59-76 eluted with chloroform: ethyl acetate (60:40) gave a yellow residue and showed one major spot along with other minor impurities on TLC. Repeated recrystallisation with chloroform: methanol (90:10) gave a colorless compound. The colorless compound showed a single spot in petroleuem ether: ethyl acetate (88:12) and was further proved for its homogeneity with different solvent systems by TLC and designated as compound 5 (GSMES3, yield 0.015 g, 0.25%).

The fractions 89-95 eluted with ethyl acetate gave a brown residue. On washing with acetone, followed by methanol washing it gave a white crystalline compound. The white crystalline compound which showed a single spot with TLC by using different solvent systems was designated as compound 6 (G1, yield 0.012 g, 0.20%). These compounds were subjected to physical and spectral studies for confirming their purity and characterization. The remaining fractions were not worked out because of lesser yields.

Table 5. Column chromatography of macerated methanol flower extract

Fraction No.	Eluent	Residue on evaporation
1-5	Petroleum ether	No residue
6-10	Petroleum ether:Chloroform (80:20)	No residue
11-16	Petroleum ether:Chloroform (60:40)	No residue
17-20	Petroleum ether:Chloroform (20:80)	Light yellow residue
21-42	Chloroform	Light yellow residue
43-58	Chloroform:Ethyl acetate (80:20)	Yellow residue
59-76	**Chloroform:Ethyl acetate (60:40)**	**Yellow residue**
77-88	Chloroform:Ethyl acetate (20:80)	Colourless residue
89-95	**Ethyl acetate**	**Light brown residue**
96-115	Ethyl acetate:Methanol (80:20)	Reddish brown residue
116-121	Ethyl acetate:Methanol (60:40)	Reddish brown residue
122-128	Ethyl acetate:Methanol (20:80)	Reddish brown residue
129-149	Methanol	Dark brown residue
150-161	Acetone	No residue

4.8.5. Column chromatography of crude methanol flower extract

The crude methanol flower extract (50 g) was chromatographed over silica gel 60-120 mesh of column length 60 cm and diameter 10 cm. Elution was carried out with solvents and solvent mixtures of increasing polarities. The fractions were collected in 100 ml portions and monitored on TLC and the fractions showing similar spots were combined (Table 6).

The fractions 11-30 eluted with petroleum ether: chloroform (50:50) gave a colorless residue which on purification with acetone gave a fluffy white precipitate and its homogeneity was checked by TLC in n-hexane: ethyl acetate (65:35) and was further confirmed with different solvent systems by TLC. It was designated as compound 7 (CMF1, yield 0.012 g, 0.02%).

The fractions 31-50 eluted in chloroform were mixed together, evaporated to a minimal volume and then added acetone. White amorphous sticky residue was obtained. Repeated washing with acetone and recrystallisation with chloroform yielded a colorless precipitate, showed a single spot on TLC in n-hexane: ethyl acetate (50:20) and it was designated as compound 8 (CMF 2, yield 0.01 g, 0.02%).

The fractions 148-189 eluted with ethyl acetate: methanol (60:40) gave a sticky brown residue. This was washed several times with methanol and repeatedly recrystallized with water. A reddish brown compound was obtained. Its homogeneity was checked with chloroform: methanol: water (8:2:0.1) and designated as compound 9 (CMF 5, yield 0.025 g, 0.05%). These compounds were subjected to physical and spectral studies for confirming their purity and characterization. The remaining fractions were not worked out because of lesser yields and mixtures of compounds.

Table 6. Column chromatography of crude methanol flower extract

Fraction No.	Eluent	Residue on evaporation
1-10	Petroleum ether	No residue
11-30	**Petroleum ether:Chloroform (50:50)**	**Colourless residue**
31-50	**Chloroform**	**Colourless residue**
51-60	Chloroform:Ethyl acetate (90:10)	Light yellow residue
61-70	Chloroform:Ethyl acetate (80:20)	Yellow residue
71-80	Chloroform:Ethyl acetate (70:30)	Yellow residue
81-100	Chloroform:Ethyl acetate (60:40)	Yellow residue
101-115	Chloroform:Ethyl acetate (20:80)	Yellow residue
116-122	Ethyl acetate	Yellow residue
123-128	Ethyl acetate:Methanol (90:10)	Brown residue
129-135	Ethyl acetate:Methanol (80:20)	Brown residue
136-147	Ethyl acetate:Methanol (70:30)	Brown residue
148-189	**Ethyl acetate:Methanol (60:40)**	**Brown residue**
190-199	Ethyl acetate:Methanol (50:50)	Dark brown residue
200-220	Ethyl acetate:Methanol (40:60)	Dark brown residue
221-230	Ethyl acetate:Methanol (20:80)	Dark brown residue
231-250	Methanol	Dark brown residue
251-310	Acetone	Dark brown residue

4.8.6. Isolation by solvent-solvent extraction

The macerated methanol leaves extract (7 g) was fractionated with petroleum ether (500 ml). The petroleum ether layer was separated and evaporated. A colourless semisolid residue was obtained which on repeated washing with acetone and recrystallization with chloroform gave a colourless precipitate. Its homogeneity was checked with various solvent systems by TLC and designated as compound 10 (ML 1, yield 0.01 g, 0.14%). This compound was subjected to physical and spectral studies for confirming its purity and characterization.

4.9. *IN VITRO* ANTIOXIDANT ACTIVITY

The *in vitro* methods for antioxidant activity are based on inhibition of free radicals. Samples were added to a free radical-generating system and inhibition of the free radical action was measured. This inhibition is related to antioxidant activity of the sample. Methods vary greatly as to the generated radical, the reproducibility of the generation process and the end point that is used for the determination.

Even though *in vitro* methods provide a useful indication of antioxidant activities, data obtained from *in vitro* methods are difficult to apply to biological systems and do not necessarily predict a similar *in vivo* antioxidant activity. All the methods developed have strengths and limitations and hence a single measurement of antioxidant capacity usually is not sufficient. A number of different methods may be necessary to adequately assess *in vitro* antioxidant activity of a specific compound or antioxidant capacity of a biological fluid.

In the present study, all the extracts were therefore, tested for their in vitro antioxidant activity using several standard methods. The absorbance was measured spectrophotometrically against the corresponding blank solution. The percentage inhibition was calculated by using the formula,

$$\text{Percentage inhibition} = \frac{\text{OD control - OD sample}}{\text{OD control}} X 100$$

IC_{50}, values, the concentration of the samples required to scavenge 50% of free radicals, were calculated.

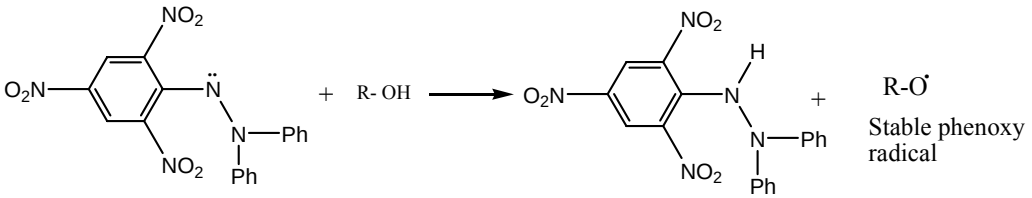
4.9.1. DPPH assay

The DPPH free radical is reduced to a corresponding hydrazine when it reacts with hydrogen donors. The DPPH radical is purple in colour and upon reaction with an hydrogen donor changes to yellow in colour. It is a discoloration assay, which is evaluated by the addition of the antioxidant to a DPPH solution in ethanol or methanol and the decrease in absorbance is measured at 490 nm.

4.9.1.1. Reagents

4.9.1.1.1. DPPH solution (100 μM)

Accurately 22 mg of DPPH was weighed and dissolved in 100 ml of methanol. From this stock solution, 18 ml was diluted to 100 ml with methanol to obtain 100 μM DPPH solution.



Purple DPPH
(2,2 diphenyl-1-picryl hydrazyl)

Yellow

4.9.1.1.2. Preparation of extract solutions

Accurately 21 mg of each of the extracts were weighed and dissolved in 1 ml of freshly distilled DMSO separately to obtain solutions of 21 mg/ml concentration. These solutions were serially diluted separately to obtain lower concentrations.

4.9.1.1.3. Preparation of standard solutions

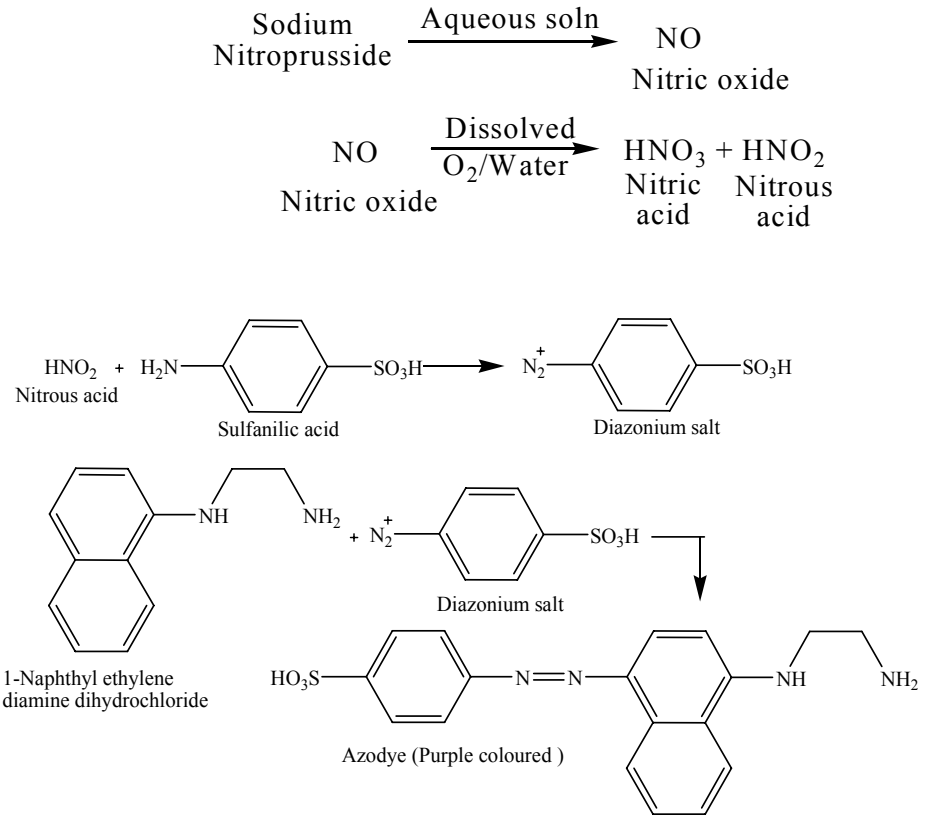
Accurately 10 mg each of ascorbic acid and rutin were weighed and dissolved in 0.95 ml of freshly distilled DMSO separately to obtain 10.5 mg/ml concentration. These solutions were serially diluted with DMSO to get lower concentrations.

4.9.1.2. Procedure

The assay was carried out in a 96 well microtitre plate. To 200 µl of DPPH solution, 10 µl of each of the extract or standard solution was added separately in wells of the microtitre plate. The plates were incubated at 37^{o}C for 30 min and the absorbance of each solution was measured at 490 nm (Hwang et al., 2001), using ELISA reader.

4.9.2. Scavenging of nitric oxide radical

Sodium nitroprusside in aqueous solution at physiological pH, spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be estimated by the use of modified Griess Ilosvay reaction (Garrat, 1964). In the present investigation, Griess Ilosvay reagent was modified by using naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of 1-napthylamine (5%). Nitrite ions react with Griess reagent, which forms a purple azo dye. In presence of test components, likely to be scavengers, the amount of nitrite ions will decrease. The degree of decrease in the formation of purple azo dye will reflect the extent of scavenging. The absorbance of the chromophore formed is measured at 540 nm.



4.9.2.1. Reagents

4.9.2.1.1. Sodium nitroprusside solution (10 mM)

Accurately 0.30 g of sodium nitroprusside was weighed and dissolved in distilled water and the volume was made up to 100 ml in a volumetric flask.

4.9.2.1.2. Naphthyl ethylene diamine dihydrochloride (NEDD, 0.1%)

Accurately 0.1 g of NEDD was weighed and dissolved in 60 ml of 50% glacial acetic acid by heating and the volume was made up to 100 ml with distilled water in a volumetric flask.

4.9.2.1.3. Sulphanilic acid reagent (0.33% w/v)

Accurately 0.33 g of sulphanilic acid was weighed and dissolved in 20% glacial acetic acid by heating and the volume was made up to 100 ml in a volumetric flask.

4.9.2.1.4. Preparation of extract and standard solutions

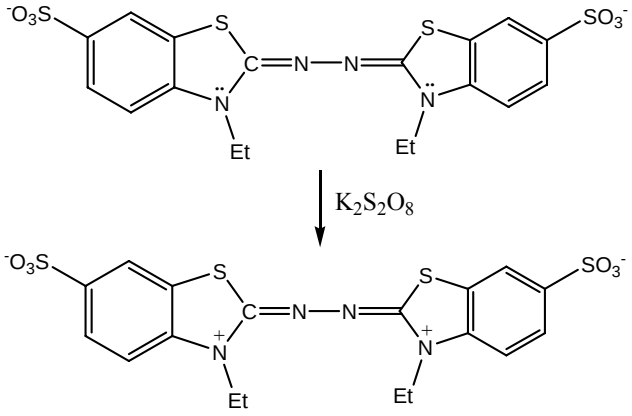
These solutions were prepared as described under DPPH assay.

4.9.2.2. Procedure

The reaction mixture (6 ml) containing sodium nitroprusside (10 mM, 4 ml), phosphate buffer saline (PBS, pH 7.4, 1 ml) and extract or standard (1ml) in DMSO at various concentrations was incubated at 25^{o}C for 150 min. After incubation, 0.5 ml of the reaction mixture containing nitrite ion was removed, 1 ml of sulphanilic acid reagent was added to this, mixed well and allowed to stand for 5 min for completion of diazotisation. Then, 1 ml of NEDD was added, mixed and allowed to stand for 30 min in diffused light. A pink coloured chromophore was formed. The absorbance was measured at 540 nm (Marcocci et al., 1994).

4.9.3. Scavenging of ABTS radical cation assay

ABTS assay is relatively a recent one, which involves a more drastic radical, chemically produced and is often used for screening complex antioxidant mixtures such as plant extracts, beverages and biological fluids. The solubility in both the organic and aqueous media and the stability in a wide pH range raised the interest on the use of $ABTS^{\bullet+}$ for the estimation of the antioxidant activity (Nenadis et al., 2004).



ABTS radical cation

4.9.3.1. Preparation of extract and standard solutions

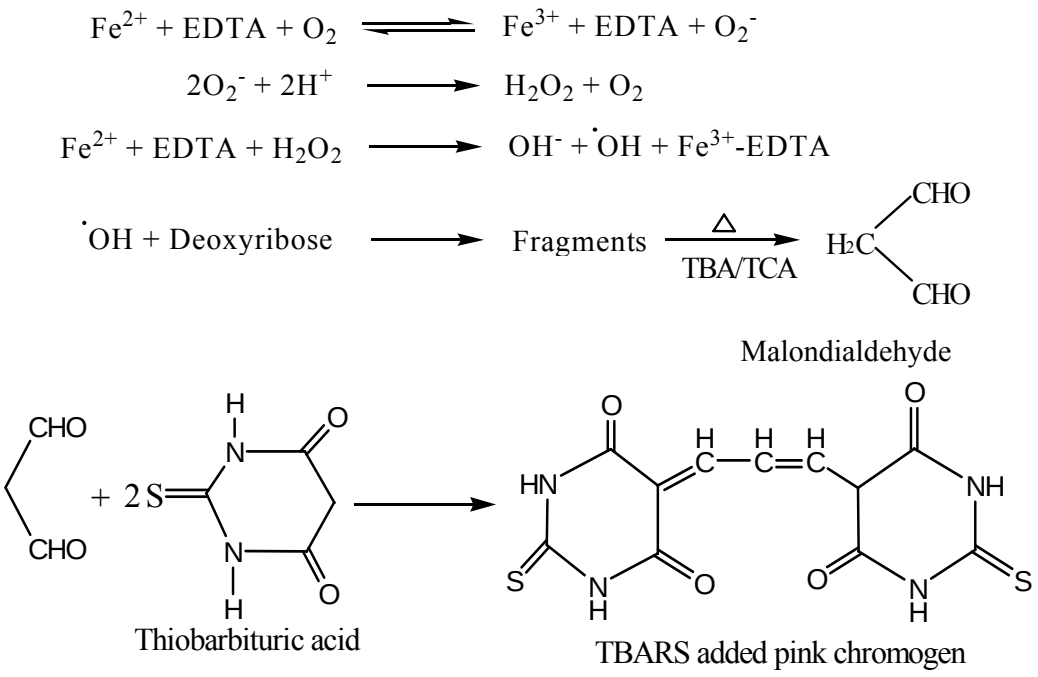
Accurately 13.5 mg of each of the extracts and the standards, ascorbic acid and rutin were weighed separately and dissolved in 2 ml of freshly distilled DMSO. These solutions were serially diluted with DMSO to obtain lower dilutions.

4.9.3.2. Procedure

Accurately 54.8 mg of ABTS was weighed and dissolved in 50 ml of distilled water (2 mM). Potassium persulphate (17 mM, 0.3 ml) was then added. The reaction mixture was left to stand at room temperature overnight in dark before usage. To 0.2 ml of various concentrations of the extracts or standards, 1.0 ml of distilled DMSO and 0.16 ml of ABTS solution were added to make the final volume to 1.36 ml. Absorbance was measured after 20 min at 734 nm (Re et al., 1999).

4.9.4. Scavenging of hydroxyl radical by deoxyribose method

The sugar deoxyribose (2-deoxy-D-ribose) is degraded on exposure to hydroxyl radical generated by irradiation or by Fenton systems. If the resulting complex mixture of products was heated under acidic conditions, malondialdehyde (MDA) is formed and may be detected by its ability to react with thiobarbituric acid (TBA) to form a pink chromogen (Barry et al., 1987).



4.9.4.1. Preparation of extract and standard solutions

Accurately 16 mg of each of the extracts and standard BHA were weighed and separately dissolved in 2 ml of freshly distilled DMSO. These solutions were serially diluted with DMSO to obtain lower dilutions.

4.9.4.2. Procedure

Various concentrations of the extracts or standard in DMSO (0.2 ml) were added to the reaction mixture containing deoxyribose (3 mM, 0.2 ml), ferric chloride (0.1 mM, 0.2 ml), EDTA (0.1 mM, 0.2 ml), ascorbic acid (0.1 mM, 0.2 ml) and hydrogen peroxide (2 mM, 0.2 ml) in phosphate buffer (pH 7.4, 20 mM) to give a total volume of 1.2 ml. The solutions were then incubated for 30 min at 37 ºC. After incubation, ice-cold trichloro acetic acid (0.2 ml, 15% w/v) and thiobarbituric acid (0.2 ml, 1% w/v) in 0.25N HCl were added. The reaction mixture was kept in a boiling water bath for 30 min, cooled and the absorbance was measured at 532 nm (Halliwell et al., 1987).

4.9.5. Scavenging of hydroxyl radical by p-NDA method

Hydroxyl radical was measured by the inhibition of p-nitrosodimethyl aniline (p-NDA) bleaching (Elizabeth and Rao, 1990) by hydroxyl radical. Hydroxyl radical is generated through Fenton reaction. In this reaction, iron-EDTA complex reacts with hydrogen peroxide in presence of ascorbic acid to generate hydroxyl radical, which can bleach p-NDA specifically. Hydroxyl radical shows scavenging activity by inhibition of bleaching and percentage of scavenging as absorbance is measured at 440 nm.

$$Fe^{3+} + EDTA \longrightarrow Fe^{3+}\text{-EDTA complex}$$

$$Fe^{3+}\text{-EDTA complex} + \text{Ascorbate} \longrightarrow Fe^{2+}\text{-EDTA complex} + \text{Oxidized Ascorbate}$$

$$Fe^{2+}\text{-EDTA complex} + H_2O_2 \longrightarrow Fe^{3+}\text{-EDTA} + OH^{\cdot} + OH^{-}$$

$$(Fe^{3+} + O_2^{-} \longrightarrow Fe^{2+} + O_2)$$

4.9.5.1. Preparation of extract and standard solutions

Accurately 30 mg of each of the extracts and the standard rutin were weighed and dissolved separately in 5 ml of freshly distilled DMSO. These solutions were serially diluted with DMSO to obtain lower dilutions.

4.9.5.2. Procedure

Various concentrations of the extracts or standard in distilled DMSO (0.5 ml) were added to a solution mixture containing ferric chloride (0.1 mM, 0.5 ml), EDTA (0.1 mM, 0.5 ml), ascorbic acid (0.1 mM, 0.5 ml), hydrogen peroxide (2 mM, 0.5 ml) and p-NDA (0.01 mM, 0.5 ml) in phosphate buffer (pH 7.4, 20 mM), to produce a final volume of 3 ml. Sample blank was prepared by adding 0.5 ml of sample and 2.5 ml of phosphate buffer. Absorbance was measured at 440 nm (Elizabeth and Rao, 1990).

4.9.6. Scavenging of hydrogen peroxide

Hydrogen peroxide is generated *in vivo* by several oxidase enzymes. There is increasing evidence that hydrogen peroxide, either directly or indirectly via its reduction product hydroxyl radical ($OH^{\bullet}$) causes severe damage to biological systems. In this method, when a scavenger is incubated with hydrogen peroxide, the decay or loss of hydrogen peroxide can be measured spectrophotometrically at 230 nm (Jayaprakasha et al., 2004).

4.9.6.1. Preparation of extract and standard solutions

Accurately 30 mg of each of the extracts and the standard rutin were weighed and dissolved separately in 5 ml of methanol. These solutions were serially diluted with methanol to obtain lower dilutions.

4.9.6.2. Procedure

A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffered saline (PBS, pH 7.4). Various concentrations of 1 ml of the extracts or standards in methanol were added to 2 ml of hydrogen peroxide solution in PBS. The absorbance was measured at 230 nm after 10 min.

4.9.7. Evaluation of Total Antioxidant capacity of the extracts

The total antioxidant capacity was determined by phosphomolybdenum method and is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compounds and the formation of a green Mo (V) complex which has the maximal absorption at 695 nm.

4.9.7.1. Preparation of test and standard solutions

Weighed accurately 55 mg of each extracts and the standard, ascorbic acid and dissolved in 5 ml of DMSO. The lower dilutions were made serially with DMSO.

4.9.7.2. Procedure

An aliquot of 0.1 ml of the sample solution containing a reducing species in DMSO was combined in an Eppendorff tube with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated in water bath at 95 °C for 90 min. The samples were cooled to room temperature, and the absorbance of each solution was measured at 695 nm. The total antioxidant capacity was expressed as mM equivalent of ascorbic acid (Huong et al.,1998).

4.10. *IN VITRO* CYTOTOXICITY STUDIES

Drug development programmes involve pre-clinical screening of a vast numbers of chemicals for their specific and non-specific cytotoxicity against many types of cells. Use of *in vitro* assay systems for the screening of potential anticancer agents has been a common practice almost since the beginning of cancer chemotherapy in 1946. The National Cancer Institute now routinely measures the growth inhibitory properties of every compound under test against a panel of 60 human tumor cell lines which are representative of major human tumor types. There are a number of advantages in *in vitro* testing, using cell cultures which include analysis of species specificity, feasibility of using only small amounts of test substances, and facility to do mechanistic studies. A novel anticancer drug should possess cytotoxicity at low concentrations against cancerous cell lines and should be safe against normal cell lines even at higher concentrations. All the extracts were tested for cytotoxicity by MTT and SRB assays.

4.10.1. Preparation of test solutions

Each extract was weighed separately, dissolved in distilled dimethyl sulphoxide (DMSO) and the volume was made up to 10 ml with MEM/DMEM, pH 7.4, supplemented with 2% inactivated FBS/NBCS (maintenance medium) to obtain a stock solution of 1 mg/ml concentration, sterilized by filtration and stored at -20°C till use. Serial dilution of two folds of the extracts was prepared from the stock solution to obtain lower concentrations.

4.10.2. Cell lines and culture medium

Vero (normal African Green Monkey Kidney), HEp-2 (human laryngeal epithelial carcinoma) cell cultures were procured from National Centre for Cell Sciences (NCCS), Pune, India., and HeLa (Epithelial human cervix cancer) from V.P. Chest Institute, New Delhi, India and Stock cells of all cell lines were cultured in DMEM supplemented with 10% inactivated FBS/NBCS, penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in a humidified atmosphere of 5% CO_2 at 37 °C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 flat bottles and all experiments were carried out in either 96 or 6 well microtitre plates.

4.10.3. Determination of mitochondrial synthesis by MTT assay

The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The cleavage of MTT to a blue formazan derivative by living cells is clearly a very effective principle on which the assay is based.

The principle involved is the cleavage of tetrazolium salt, 3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells is known to be proportional to the extent of formazan production by the cells used (Francis and Rita, 1986).

4.10.3.1. Procedure

The monolayer cell culture was trypsinized and the cell count adjusted to 1.0 x 10^5 cells/ml using MEM/DMEM medium containing 10% NBCS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, the monolayer washed once with medium. Different extracts (100 µl each) were added to the cells in microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO_2 atmosphere, microscopic examination was carried out and observations were noted every 24 h. After 72 h, the extract solutions in the wells were discarded and 50 µl of MTT in MEM – PR (Minimum essential medium without phenol red) was added to each well. The plates were gently shaken and incubated for 3 h at 37°C in 5% CO_2 atmosphere. The supernatant was removed, 50 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at the wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and CTC_{50} (concentration of drug or test extract needed to inhibit cell growth by 50%) values were generated from the dose-response curves for each cell line.

$$\% \text{ Growth Inhibition} = 100 - \left[\frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} X 100 \right]$$

The pattern of all the cell lines as a group is used to rank compounds as toxic or non-toxic.

4.10.4. Determination of total cell protein content by Sulforhodamine B (SRB) assay

SRB is a bright pink aminoxanthine dye with two sulfonic groups. Under mild acidic conditions, SRB binds to protein basic amino acid residues in trichloro acetic acid (TCA) fixed cells to provide a sensitive index of cellular protein content that is linear over a cell density range of at least two orders of magnitude.

Color development in SRB assay is rapid, stable and visible. The developed color can be measured over a broad range of visible wavelength in either a spectrophotometer or a 96 well plate reader. When TCA-fixed and SRB stained samples are air-dried, they can be stored indefinitely without deterioration (Philip et al., 1990).

4.10.4.1. Procedure

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using medium containing 10% new born calf serum. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different extracts were added to the cells in microtitre plates. The plates were then incubated at 37 ^oC for 3 days in 5% CO_2 atmosphere, microscopic examination was carried out and observations were

recorded every 24 h. After 72 h, 25 µl of 50% trichloro acetic acid was added to the wells gently in such a way that it forms a thin layer over the extract to form an over all concentration of 10%. The plates were incubated at 4 ^{o}C for 1 h. The plates were flicked and washed five times with water to remove traces of medium, extract and serum, and air-dried. They were stained with SRB for 30 min. The unbound dye was then removed by rapidly washing four times with 1% acetic acid. The plates were then air-dried. Tris base (10 mM, 100 µl) was then added to the wells to solubilise the dye. The plates were shaken vigorously for 5 min. The absorbance was measured using microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the same formula used for MTT assay and the CTC_{50} values were calculated.

4.11. PHARMACOLOGICAL SCREENING OF CRUDE METHANOL EXTRACTS

4.11.1. Animals

Wistar albino rats weighing 180-220 g were obtained from the animal house of J. S. S. College of Pharmacy, Ootacamund. All the animals were kept under standard laboratory conditions with 12 ± 1 h light-dark cycle. Animals were provided with standard rat feed (Sai Durga Feeds and Foods, Bangalore, India) and water *ad libitum.*

4.11.2. Acute toxicity studies of the crude methanol extracts

An acute oral toxicity study in rats was carried out as per OECD-423 guidelines. A maximum dose of 2000 mg/kg bw of the crude methanol extracts of flower, stem, root and leaves were administered orally to various groups of rats containing ten in each group. The animals were observed for mortality, clinical signs and body weight changes daily for a period of 15 days and at the end of the study period, all the animals were subjected to gross necropsy.

4.12. ACUTE ANTI-INFLAMMATORY ACTIVITY BY THREE DIFFERENT MODELS

The crude methanol extracts of flower, stem, root and leaves showed potent activity in most of the *in vitro* antioxidant methods. Hence, in the present study we were interested to screen these extracts for *in vivo* anti-inflammatory and analgesic studies.

The crude methanol extracts of flower, stem, root and leaves were screened for acute anti-inflammatory studies using standard experimental models. Carrageenan-induced paw edema is a standard and most commonly used technique to screen the anti-inflammatory activity. It is expected that after tissue injury, the animal will display spontaneous pain behaviour. This peripheral hypersensitivity or pain perception can be explained on the basis of local release of various inflammatory mediators i.e. bradykinin, prostaglandins or cytokines which can activate and sensitize the peripheral nerve endings. The most widely used primary test to screen new anti-inflammatory agents measures the ability

of a compound to reduce local edema induced in the rat paw by injection of an irritant agent. This edema depends on the participation of kinins and polymorphonuclear leukocytes with their proinflammatory factors including prostaglandins. The development of edema in the paw of the rat after the injection of carrageenan has been described as a biphasic event. The initial phase, observed around 1 h, is attributed to the release of histamine and serotonin; the second, accelerating phase of swelling is due to the release of prostaglandin-like substances. (Winter et al., 1962).

It is well known that inhibition of formalin-induced edema in rats is one of the most suitable test procedures to screen anti-arthritic and anti-inflammatory agents as it closely resembles human arthritis. Histamine plays an important role in the effector phase of delayed-type hypersensitivity, which has been known to be involved in many inflammation disorders such as hepatitis and arthritis.

4.12.1. Preparation of Extract

The crude methanol extracts of flower, stem, root and leaves (1 g) were suspended separately in 0.5% sodium CMC. These suspensions were administered orally to the animals with the help of intragastric catheter. Based on the acute toxicity studies, the minimum dose level of extracts at 100 and 200 mg/kg bw were selected.

4.12.2. Preparation of standard and phlogistic agents

Ibuprofen (100 mg) was suspended in 10 ml of 0.5% w/v sodium CMC and administered orally to the animals with the help of an intragastric catheter at the

dose of 100 mg/kg body weight. Suspensions or solutions of carrageenin, formalin and histamine were prepared separately in phosphate buffer solution and used for inducing inflammation.

4.12.3. Standard drug and extract administration

The animals were randomly divided into ten groups of six animals each. Group I received 0.5% sodium CMC at the dose of 2 ml/kg bw. Group II served as positive control and was treated with ibuprofen at 100 mg/kg bw. Groups III and IV were treated with crude methanol flower extract at 100 and 200 mg/kg bw, respectively. Groups V and VI were treated with crude methanol root extract at 100 and 200 mg/kg bw, respectively. Groups VII and VIII were treated with crude methanol stem extract at 100 and 200 mg/kg bw, respectively. Groups IX and X were treated with crude methanol leaves extract at 100 and 200 mg/kg bw, respectively. For all experiments, the same protocol of treatment was followed.

4.12.4. Procedure

The initial hind paw volume of rats was determined volumetrically by using a plethysmometer (Kulkarni 1999; Vasudevan et al., 2006). In separate sets of rats, a solution of carrageenan in saline (1%, 0.1 ml/rat), formalin (1%, 0.1 ml/rat) or histamine (0.1%, 0.1 ml/rat) were injected subcutaneously into the right hind paw 30 min after the above treatments. The animals in the control group received the vehicle only. Paw volumes were measured up to 3 h at intervals of 0, 1, 2 and 3 h and percent increase in edema between the control and treated

groups were compared. The percentage inhibition of edema was calculated by using the following formula (Arul et al., 2005).

$$\text{Percentage inhibition} = \frac{(Vt - Vo)\ \text{control} - (Vt - Vo)\ \text{treated}}{(Vt - Vo)\ \text{control}} X 100$$

4.12.5. Statistical analysis

Data are expressed as mean ± S E M. One way ANOVA was applied for the analysis of the results. Subsequently, multiple comparisons were made using the Dunnet's multiple rang test; $p < 0.05$ was considered as significant.

4.13. SUB CHRONIC ANTI-INFLAMMATORY ACTIVITY

4.13.1. Cotton pellet-induced granuloma

Among the extracts screened for acute anti-inflammatory study, the crude methanol flower extract which showed maximum activity was screened for sub chronic anti-inflammatory studies by cotton pellet granuloma model. The cotton pellet granuloma is the model of chronic inflammation and the dry weight has been shown to correlate with the amount of granuloma matous tissue formed (Swingle and Shideman, 1972).

Inflammation was induced by implanting sterile cotton pellets weighing 20.0 ± 1.0 mg in the groin region of the rat under light ether anaesthesia. The animals were randomly divided into five groups of six animals each Group I was control and given only vehicle (saline). Group II was treated with standard Ibuprofen (100 mg/kg bw). Groups III, IV and V were treated with crude methanol flower

extract at three dose levels (100,150, and 200 mg/kg bw) orally for 7 days. All the animals were sacrificed on the eighth day with an over dose of ether. The pellet and the surrounding granuloma were dissected out carefully and dried overnight in an oven at 60 ºC to a constant weight. The weight of the granuloma tissue was obtained by determining the difference between the initial (20 mg) and the final weight of the cotton pellet with its attached granulomatous tissue. The mean weight of the granuloma tissue formed in each group and the percentage inhibition were determined. The statistical analysis as described in 4.12.5 was carried out.

4.14. ANALGESIC ACTIVITY BY HOT PLATE AND TAIL IMMERSION METHODS

The crude methanol extracts of flower, stem, root, leaves and standard ibuprofen (1 g) were suspended separately in 0.5% sodium CMC. These suspensions were administered orally to the animals with the help of intragastric catheter, at the doses of 100 and 200 mg/kg bw doses for extracts and 100 mg/kg bw for standard, respectively.

The animals were randomly divided into ten groups of six animals each. Group I was control and received 0.5% sodium CMC at the dose of 2 ml/kg bw. Group II served as positive control and was treated with ibuprofen at 100 mg/kg bw. Groups III and IV were treated with crude methanol flower extract at 100 and 200 mg/kg bw, respectively. Groups V and VI were treated with crude methanol

root extract at 100 and 200 mg/kg bw, respectively. Groups VII and VIII were treated with crude methanol stem extract at 100 and 200 mg/kg bw, respectively. Groups IX and X were treated with crude methanol leaves extract at 100 and 200 mg/kg bw, respectively. All these treatments were given orally 30 min prior to the test.

4.14.1. Hot plate method

In the Eddy's hot plate method (Kulkarni, 1999), the rats were treated and placed on Eddy's hot plate kept at a temperature of 55 ± 0.5 ºC. A cut off period of 15 sec was observed to avoid damage to the paw. Reaction time and the type of response were noted using a stopwatch. The response is in the form of jumping, withdrawal of the paws or licking of the paws. The latency was recorded before and after 0, 30, 60, 90 and 120 min following the oral administration of the treatments. The percentage protection was calculated using the formula.

$$\text{Protection (\%)} = (t/c\text{-}1) \times 100,$$

Where, t = reaction time of treated group and c = reaction time of control group.

4.14.2. Tail immersion method

In the tail immersion method, the tails of the rats were cleaned with spirit, 5 cm of the end of the rat tail was immersed in warm water maintained at 55 ± 0.5 ºC. The tail withdrawal reflex was recorded before and after 0, 1, 2 and 3 h following the treatments. The percent protection was calculated by using the formula mentioned above. The statistical analysis as described in 4.12.5 was carried out.

4.15. *IN VIVO* ANTIOXIDANT AND HEPATOPROTECTIVE ACTIVITIES OF CRUDE METHANOL FLOWER EXTRACT

Liver is an important organ actively involved in the metabolism, detoxification and secretory functions in the body and its disorders are numerous with no effective remedies. Herbs are known to play a vital role in the management of various liver disorders. Ayurveda, the ancient system of Indian medicine, identified liver diseases and recommended a number of herbal remedies (Chatterjee, 2000). Carbon tetrachloride (CCl_4) induced hepatic injury model was used for hepatoprotective drug screening. The principle causes of carbon tetrachloride induced hepatic damage are free radical mediated lipid peroxidation leading to disruption of biomembrane, dysfunction of cells and tissues, decreased levels of antioxidant enzymes and generation of free radicals (Castro et al., 1974; Poli, 1993). The antioxidant activity involving inhibition of the generation of free radicals is important in providing protection against hepatic damage.

Several plants have been reported for their hepatoprotective property by improving antioxidant status. Among them silymarin, a plant derived medicine obtained from the fruits of *Silybum marianum* is approved for use in liver cirrhosis and alcoholic liver diseases (Sallar et al., 2001). Among the extracts tested for *in vivo* anti-inflammatory and analgesic activities, the crude methanol flower extract showed potent activity. Hence, it was screened for evaluating *in vivo* antioxidant and hepatoprotective properties.

4.15.1. Preparation of extracts and standard

The crude methanol flower extract suspension were prepared in 0.3% sodium carboxy methyl cellulose (CMC) in distilled water. Standard silymarin (100 mg/ml) suspension was also prepared in 0.3% sodium CMC. The suspensions were administered orally to the animals with the help of an intragastric catheter.

4.15.2. Procedure

The rats were divided into six groups containing six animals in each group. Group I was served as control and group II as CCl_4 treated. Both these groups were received sodium CMC (0.3%, 5 ml/kg bw). Groups III, IV and V animals were treated with crude methanol flower extract at the dose levels of 100, 150 and 200 mg/kg bw. Group VI was treated with the standard drug silymarin at 100 mg/kg bw. All these treatments were given orally for eight days. On the last day of the treatment, all the animals except the normal group I and the extract alone treated group II were received a single dose of CCl_4 in liquid paraffin (1:1), 1 ml/kg bw intraperitoneally after 1 h of the vehicle, extract or standard treatments.

On the ninth day, the animals were anesthetized by anesthetic ether and blood was collected from the abdominal artery and kept for 30 min at 4 ºC. Serum was separated by centrifugation at 2500 rpm for 15 min at 4 ºC and used for the biochemical estimations. Serum marker enzymes such as ASAT, ALAT, ALP, TGL, CR, TP, TC, TB and albumin were measured in an autoanalyzer using

Ecoline kits. Superoxide dismutase (SOD, Misra and Fridovich, 1972), catalase (CAT, Beers and Sizer, 1952) and thiobarbituric acid reactive substances (TBARS, Ohkawa et al., 1979) were measured spectrophotometrically.

After the collection of blood samples, the liver and kidney were excised, rinsed in ice-cold normal saline, followed by cold 0.15 M potassium chloride (pH 7.4) and blotted dry. A 10% w/v homogenate was prepared in 0.15 M potassium chloride buffer with Elvenjan homogenizer fitted with Teflon plunger and centrifuged at 2500 rpm for 15 min at 4 ºC. The supernatants were used for the estimation of CAT, SOD and TBARS in both liver and kidney. A portion of the liver and kidney tissues were fixed in 10% formalin, cut into 5 µm thick sections and stained using heamatoxylin-eosin and histopathological observations were made.

4.15.3. Estimation of antioxidant parameters

4.15.3.1. Estimation of CAT

CAT (EC 1.11.1.6) is an enzyme present in the cells of plants, animals and aerobic (oxygen requiring) bacteria (Mates et al., 1999). Catalase is located in a cell organelle called the peroxisome. In animals, catalase is present in all major body organs. The role of catalase is to scavenge hydrogen peroxide and prevent oxidative damage in the cell. Catalase is a heme containing protein that can convert hydrogen peroxide to water and oxygen in a two-step reaction cycle. In the first step, one molecule of hydrogen peroxide is converted to water. The

catalytic cycle begins with the oxidation of the ferric heme by hydrogen peroxide to form the ferryl-oxo porphyrin/protein radical intermediate (compound 1). The catalase cycle is completed by the reduction of compound 1 to the ferric enzyme by hydrogen peroxide, resulting in production of molecular oxygen (Li and Goodwin, 1999).

4.15.3.2. Chemicals and reagents

4.15.3.2.1. Hydrogen peroxide (7.5 mM)

1.043 ml of 30% w/w H_2O_2 was made up to 100 ml with sodium chloride and EDTA solution (9 g of NaCl and 29.22 mg of EDTA dissolved in 1 L distilled water).

4.15.3.2.2. Potassium phosphate buffer (65 mM, pH 7.8)

2.2 g of potassium dihydrogen phosphate and 11.32 g of dipotassium hydrogen phosphate were dissolved in 250 ml and 1 L distilled water, respectively and mixed together. The pH was adjusted to 7.8 with KH_2PO_4.

4.15.3.2.3. Sucrose Solution

10.95 g of sucrose was dissolved in 100 ml of distilled water.

4.15.3.3. Procedure

2.25 ml of potassium phosphate buffer (65 mM, pH 7.8) and 100 µl of the serum or tissue homogenate or sucrose (0.32 M) were incubated at 25 °C for 30 min. H_2O_2 (0.65 ml, 75 mM) was added to initiate the reaction. The change in absorption at 240 nm was measured for 2-3 min, and dy/dx for 1 min for each

assay was calculated and the results were expressed as CAT units/mg of tissue (Beers and Seizer, 1952).

4.15.4. Estimation of SOD

One of the most effective intracellular enzymatic antioxidants is superoxide dismutase (SOD) (EC 1.15.1.1). Superoxide dismutase is the antioxidant enzyme that catalyzes the dismutation of $O_2^{\bullet -}$ (free radical) to O_2 and to the less reactive species H_2O_2. While this enzyme was isolated as early as 1939, it was only in 1969 that McCord and Fridovich proved the antioxidant activity of SOD (Mc Cord and Fridovich., 1969).

Superoxide dismutase exists in several isoforms, differing in the nature of active metal centre and amino acid constituency, as well as their number of subunits, cofactors and other features. In humans there are three forms of SOD: cytosolic Cu, Zn-SOD and mitochondrial Mn-SOD, and extracellular SOD (EC-SOD) (Landis and Tower, 2005). SOD destroys $O_2^{\bullet -}$ with remarkably high reaction rates, by successive oxidation and reduction of the transition metal ion at the active site in a "Ping-Pong" type mechanism (Mates et al., 1999).

Under physiological conditions, a balance exists between the level of reactive oxygen species (ROS) produced during normal cellular metabolism and the level of endogenous antioxidants, which serve to protect tissues from oxidative damage. Disruptions of this balance (either through increased production of ROS or decreased levels of antioxidants) produce a condition known as oxidative stress and leads to a variety of pathological conditions. To protect against

oxidative damage, organisms have developed a variety of antioxidant defenses that include metal sequestering proteins, use of compounds such as vitamin C, E and specialized antioxidant enzymes. One family of antioxidant enzymes, the superoxide dismutases (SOD), function to remove damaging ROS from the cellular environment by catalyzing the dismutation of two superoxide radicals to hydrogen peroxide and oxygen (Misra and Fridovich, 1972).

$$O_2^{-\cdot} + O_2^{-\cdot} \xrightarrow[2\ H^+]{SOD} O_2 + H_2O_2$$

SOD measurement was, therefore, carried out for its ability to inhibit spontaneous oxidation of epinephrine to adrenochrome.

4.15.4.1. Chemicals and reagents

4.15.4.1.1. Sodium carbonate buffer (0.05 M, pH 10.2)

Sodium carbonate (5.3 g) and sodium bicarbonate (1.2 g) were dissolved separately in 1 L of distilled water, which served as a stock solution. Buffer was prepared by mixing 64 ml of sodium carbonate and 70 ml of sodium bicarbonate solutions. The pH of the buffer was adjusted to 10.2 using the above stock solution accordingly.

4.15.4.1.2. Adrenaline (9 mM)

Adrenaline (0.038) was dissolved in distilled water and the final volume was made up to 10 ml with distilled water containing a drop of concentrated HCl (to bring pH down to 2).

Adrenaline being sensitive, the vial was kept covered with aluminum foil at all times.

4.15.4.1.3. Sucrose (0.32 M) solution

Sucrose (10.96 g) was dissolved in distilled water and the volume was made up to 100 ml.

4.15.4.2. Procedure

Sodium carbonate buffer (2.8 ml, 0.05 mM) and 0.1 ml of serum or tissue homogenate or sucrose (blank) was incubated at 30 ºC for 45 min. The absorbance was then adjusted to zero to sample. The reaction was then initiated by adding 10 µl of adrenaline solution (9 mM). The change in absorbance was recorded at 480 nm for 8-12 min. The temperature was maintained at 30ºC throughout the assay. Similarly, SOD calibration curve was prepared by taking 10 unit/ml as standard solution. One unit of SOD produced approximately 50% inhibition of auto-oxidation of adrenaline. The results were expressed as unit (U) of SOD activity/mg of tissue (Saggu et al., 1989).

4.15.5. Estimation of TBARS

Lipid peroxidation is commonly regarded as a deleterious process (Sevanian and Ursini, 2000), leading to structural modification of complex lipid protein assemblies, such as biomembranes and lipoproteins, and is usually associated with cellular malfunction. During lipid peroxidation, a polar oxygen moiety is introduced into the hydrophobic tails of unsaturated fatty acids. This process is

of dual consequence: the presence of hydroperoxy group disturbs the hydrophobic lipid/lipid and lipid/protein interactions, which leads to structural alterations of biomembranes and lipoproteins; hydroperoxy lipids are sources for the formation of free radicals. When free radicals are generated, they can attack polyunsaturated fatty acids in cell membrane leading to a chain of chemical reactions called lipid peroxidation. As the fatty acid is broken down, the hydrocarbon gases and aldehyde are formed. The most common method used to assess malondialdehyde (MDA) is the thiobarbituric acid (TBARS) assay (Halliwell and Chirico, 1993).

4.15.5.1. Chemicals and reagents

4.15.5.1.1. Thiobarbituric acid solution

Thiobarbituric acid (0.8 g) was dissolved in distilled water and the volume was made up to 100 ml. The pH was adjusted to 7.4 with 1 N NaOH/0.1 N HCl solutions.

4.15.5.1.2. Acetic acid solution

Acetic acid (20 ml) was dissolved in distilled water and the volume was made up to 100 ml with distilled water. The pH was adjusted to 3.5 with 1 N NaOH/0.1 N HCl solution.

4.15.5.1.3. Sodium lauryl sulfate solution

Sodium lauryl sulfate (8.1 g) was dissolved in distilled water and the volume was made up to 100 ml with distilled water.

4.15.5.2. Procedure

To 1 ml of serum or tissue homogenate, 0.2 ml of sodium lauryl sulfate solution (8.1%), 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of thiobarbituric acid solution (0.8% w/v, pH 7.4) were added. This incubation mixture was made up to 5.0 ml with double distilled water and then heated in boiling water bath for 30 min. After cooling, the red chromogen was obtained and centrifuged at 4000 rpm for 10 min. The supernatant was taken and its absorbance was measured at 532 nm. 1, 1, 3, 3 - Tetraethoxy propane (TEP) was used as external standard. The results were expressed as nM of MDA/mg of wet tissue or ml of serum using molar extension co-efficient of the chromophore (1.56×10^5 M^{-1} cm^{-1}). Similarly, the calibration curve was prepared for TEP and the results were expressed as nM of MDA/mg of tissue (Ohkawa et al., 1979).

$$\text{nM of MDA/mg of tissue or ml of serum} = \frac{\text{OD} \times \text{Volume of homogenate} \times 100}{.56 \times 105 \times \text{Volume of extract taken}} X 10^3$$

4.15.6. Estimation of hepatoprotective parameters

Biochemical estimations in serum were carried out by using Ecoline diagnostic kits.

4.15.6.1. Assay of ASAT

ASAT catalyzes the following reactions:

$$\text{2-Oxoglutarate + L-aspartate} \xrightleftharpoons{\text{ASAT}} \text{Glutamate + Oxaloacetate}$$

$$\text{Oxaloacetate + NaDH + } H^+ \xrightleftharpoons{\text{MD}} \text{Malate + } NAD^+$$

Where, MD is malatedehydrogenase. The rate of NADH consumption was measured spectrophotometrically at 340 nm and is directly proportional to the ASAT activity in the sample. ASAT level was expressed as U/L.

4.15.6.1.1. Procedure

Reagents 1 and 2 of Ecoline diagnostic kit for ASAT were mixed at the ratio of 4:1 and the temperature was maintained at 30 ºC. To 50 µl of the sample, 0.5 ml of the reagent solution was added and mixed. After 1 min, the decrease in absorbance was measured every min for 3 min at 340 nm.

Enzyme activity [U/I] = (Δ A/ min) x 2143, Where, Δ A is the decrease in absorbance per min.

4.15.6.2. Assay of ALAT

ALAT catalyzes the following reactions:

$$\text{2-Oxoglutarate + L-Alanine} \xrightarrow{\text{ALAT}} \text{Glutamate + Pyruvate}$$

$$\text{Pyruvate + NaDH + } H^+ \xrightarrow{\text{LDH}} \text{Lactate + } NAD^+$$

where, LDH is lactate dehydrogenase. The rate of NADH consumption was measured spectrophotometrically at 340 nm and this is directly proportional to ALAT activity in the sample. ALAT level was expressed as U/L.

4.15.6.2.1. Procedure

Reagents 1 and 2 of Ecoline diagnostic kit for ALAT were mixed at the ratio of 4:1 and the temperature was maintained at 30 ºC. To 50 µl of the sample, 0.5 ml of the reagent solution was added and mixed. After 1 min, the decrease in absorbance was measured every min for 3 min at 340 nm.

Enzyme activity [U/I] = (Δ A/ min) x 2143, where, Δ A is the decrease in absorbance per min.

4.15.6.3. Assay of ALP

ALP catalyzes by the following reaction:

4-Nitrophenyl phosphate + H_2O $\xrightarrow[\text{ALP}]{}$ Phosphate + 4-Nitrophenolate

The rate of increase in 4-nitrophenolate was determined spectrophotometrically at 405 nm and this is directly proportional to the ALP activity in the sample. Alkaline phosphatase level in serum, liver and kidney tissue homogenates was expressed as U/L.

4.15.6.3.1. Procedure

Reagents 1 and 2 of Ecoline diagnostic kit for ALP were mixed at the ratio of 4:1 and the temperature was maintained thermostat at 30 ºC. To 20 µl of the sample, 1 ml of the reagent solution was added and mixed. After 1 min, the increase in absorbance was measured every min for 3 min at 405 nm.

Enzyme activity [U/I] = (Δ A/ min) x 2754, where, Δ A is the increase in absorbance per min.

4.15.6.4. Estimation of TB

The total bilirubin was determined by coupling with diazotized sulfanilic acid after the addition of caffeine, sodium benzoate and sodium acetate. Bilirubin reacts with diazotized sulfanilic acid and forms blue azobilirubin dye in alkaline Fehling's solution II. The absorbance is measured at 578 nm. The concentration of total bilirubin was expressed as mg %.

4.15.6.4.1. Procedure

Reagent 1 and 2 were mixed at the ratio of 4:1. The mixture, known as diazo solution, was maintained at 30 ºC. To 50 µl of the sample, 50 µl of the diazo solution and 250 µl of acceleration solution (mixture of caffeine 5%, sodium benzoate 7.5% and sodium acetate 12.5%) were added and mixed. The reaction mixture was maintained at room temperature. After 1 h, 250 µl of alkaline Fehling's solution II was added and the reaction mixture was incubated at room temperature for 5 min. The absorbance of the sample and standard were measured against the reagent blank, after 5 min at 578 nm.

Total Bilirubin (mg %) = $(A_{sample} - A_{blank})$ x 27.78,

where, A_{sample} is absorbance of sample and A_{blank} is absorbance of the blank.

4.15.6.5. Estimation of TGL

Triglycerides catalyze the following reactions;

$$\text{Triglyceride} \xrightarrow{\text{Lipase}} \text{Glycerol + Fatty Acid}$$

$$\text{Glycerol + ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-Phosphate + ADP}$$

$$\text{Glycerol-3-Phosphate + } O_2 \xrightarrow{\text{GPO}} \text{Dihydroxy acetone phosphate + } H_2O_2$$

$$2\,H_2O_2 + \text{Aminoantipyrine + 4 -Chlorophenol} \xrightarrow{\text{POD}} \text{Chinonimine + } 4H_2O$$

Where, GK is glycerokinase, GPO is glycerol-3-phosphate oxidase and POD is peroxidase. The absorbance of the sample and standard were measured against the blank at 546 nm. The triglyceride levels were expressed as mg/dL.

4.15.6.5.1. Procedure

To 0.10 ml of sample or standard triglyceride (supplied by Ecoline diagnostic kit), 1.00 ml of the reagent was added, mixed well and incubated for 10 min at 37 ºC. The absorbance of sample and standard were measured against reagent blank within 60 min at 546 nm.

Triglycerides [mg/dL] = ($A_{sample}/A_{standard}$) x Concentration of standard

4.15.6.6. Estimation of TC

Cholesterol and its esters are released from lipoproteins by detergents. Cholesterol esterase hydrolyzes the esters. In the subsequent enzymatic oxidation by cholesterol oxidase, H_2O_2 is liberated. This is converted into the colored quinoneimine with 4-aminoantipyrine and phenol catalyzed by peroxidase by following reaction;

$$\text{Cholesterol ester} + H_2O \xrightarrow{CHE} \text{Cholesterol} + \text{Fatty Acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{CHO} \text{Cholesterol-3-one} + H_2O_2$$

$$2H_2O_2 + \text{Amino antipyrine} + \text{Phenol} \xrightarrow{POD} \text{Quinoneimine} + 4\ H_2O_2$$

Where, CHE is cholesterol esterase, CHO is cholesterol oxidase and POD is peroxidase. The absorbance of the sample and standard were measured against the reagent blank at 546 nm. Cholesterol level was expressed as mg/dL.

4.15.6.6.1. Procedure

To 0.10 ml of the sample or standard cholesterol (supplied by Ecoline diagnostic kit), 1.00 ml of the reagent was added, mixed well and incubated for 5 min at 37ºC and the absorbance of sample and standard were measured at 546 nm within 60 min.

Cholesterol [mg/dL] = ($A_{sample}/A_{standard}$) x Concentration of standard

4.15.6.7. Estimation of TP

Proteins and peptides produce a violet colored complex with copper ions in an alkaline solution. This reaction is called as the Biuret reaction, is particularly easy to carry out giving reproducible results, which are in good agreement with Kjeldahl method. The absorbance of the colored complex is directly proportional to the protein concentration in sample materials. The absorbance of the sample and standard total protein were measured against the Biuret reagent and the absorbance of the blank against distilled water at 546 nm. Total protein level was expressed as g/dL.

4.15.6.7.1. Procedure

Reagents 1 and 2 of Ecoline diagnostic kit for assay of total protein were mixed at the ratio of 4:1 and the temperature was maintained always at 37ºC. To 0.10 ml of sample/standard protein (supplied by Ecoline diagnostic kit), 1.00 ml of the reagent was add2ed, mixed well and incubated for 5 min, and the absorbance of the sample and standard were measured within 60 min.

Total Protein [g/dL] = ($A_{sample}/A_{standard}$) x Concentration of standard

4.15.6.8. Estimation of CR

Creatinine forms a yellow-orange compound in alkaline solution with picric acid. At the low picric acid concentration used in this method, a precipitation of protein does not take place. The concentration of the dyestuff formed over a certain reaction time is a measure of the creatinine concentration. As a result of the rapid reaction between creatinine and picric acid, later secondary reaction does not cause interference. This method, thus distinguishes itself by its high specificity.

4.15.6.8.1. Procedure

Buffer solution and picric acid solution from E-Merck diagnostic kit for creatinine were mixed in the ratio of 1:1 and incubated for about 10 min before use. To 1 ml of this reagent solution, 0.20 ml of sample/standard was added, and mixed well. The absorbance was measured exactly after 1 min and 5 min at 492 nm. The concentration of creatinine was calculated by using the following formula.

$$\text{Creatinine concentration (mg/dl)} = \frac{A_2 - A_1}{ASt_1 - ASt_2}$$

A_1 and A_2 = absorbance of sample after 1 and 5 min

ASt_1 and ASt_2 = absorbance of standard after 1 and 5 min.

4.15.6.9. Estimation of albumin

Albumin forms blue-green complex with bromocresol green at slightly acidic pH, which was measured spectrophotometrically. The absorbance of the sample nd standard was measured against blank at 540 nm. Albumin levels in serum and liver and kidney tissue homogenates were expressed as g/dl.

4.15.6.9.1. Procedure

To 1 ml of the reagent solution from E-Merck diagnostic kit for albumin, 100 µl of the sample or standard was added, mixed well and incubated at 37 ºC for 10 min. The absorbance of the sample and standard was measured against the reagent blank solution within 60 min. the concentration of albumin was calculated by using the formula, Albumin (mg/dl) = (As/Astd) x Concentration of standard, where As = Absorbance of sample and Astd = Absorbance of standard.

4.15.7. Histopathological studies

On the 9th day of the experiment, from all the animals liver and kidney were dissected out, cleared off their surrounding tissues and washed with normal saline. Initially, the materials were fixed in 10% buffered neutral formalin for 48 h and then with bovine solution for 6 h. They were processed for paraffin embedding. Paraffin blocks were made and sections were taken at 5 µm thickness using microtome, and placed on microscope slides. These sections were stained with heamotoxylin and alcoholic eosin. The sections were examined microscopically for the evaluation of histopathological changes (Gupta et al., 2004).

4.15.8. Statistical analysis

The significance of the *in vivo* data was analyzed by one-way ANOVA, followed by Tukey-Kramer multiple comparison tests and $P<0.05$ was considered as statistically significant.

5.1. MACROSCOPIC FEATURES

The plant is profusely branched, compared to shrub. Young stem is angular with four ridges, the leaves are elliptic-lanceolate to obovate, 5 cm long and 3 cm wide; lamina is thick and coriaceous, dark green and scalerid above, reticulate and white villous on aseoles below; leaf margin are crenate-serrate; petiole is 1 cm long. Inflorescence is terminal or axillary, branched, unbranched spike. Bract and bracteoles are prominent, leafy; calyx has 5 sepals, gamosepalous, lobes are linear and lanceolate. Corolla is pale-blue to manne with 5 petals, gamopetalous, biloubed. Stamens: two, epipetalous, anthers dithecous, lobes parallel. Ovary is superior, bicarpellary, syncarpous, two ovules in each carpel. Seeds are densely hairy.

5.2. MICROSCOPIC FEATURES

5.2.1. Leaf The leaf has thick, prominantly projecting midrib and lateral veins. The mid rib is 750 µm in vertical planee and 800 µm in horizontal plane. The mid rib has shallow median depression on the adaxial region; this part of the midrib has collenchymatous cells. The abaxial part has uneven and undulate outline; the epidermal layer is thin and continuous comprising of small squarish, thick walled cells. The ground tissue is parenchymatous; the cells are wide, angular, thin walled and compact. The vascular strend is small, single and consist of about eight parallel uniseriate rows of narrow xylem elements and wide thin phloem elements.

The lateral veins is bulbous and prominantly protrudes on the abaxial side of the lamina. It is 650 µm vertically and 500 µm horizontally. It has narrow, small celled epidermal layer and wide, angular copact parenchymatous groud tissue. The vascular strand is small, single and arc-shaped; it has four five short, thin rows of xylem elements and deep bowl-shaped line of phloem elements (Fig. 2).

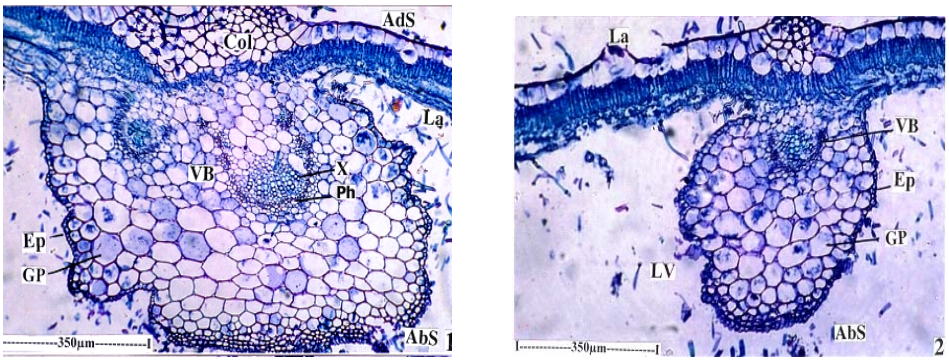


Fig. 2. Microscopic features of leaves of *S. kunthianus*

5.2.2. Lamina

The lamina is glabarous and smooth on the adaxial side and densely hairy on the abaxial side. The adaxial epidermis is quite wide; the cells are vertically oblong with thin anticlinal walls and thick cuticle on the outer tangential walls. The epidermis is 40 µm thick. Some of the epidermal cells are further dilated into wide circular or horizontally elongated lithocysts possessing single, narrowly cylindrical rod like calcium carbonate cystolith. They are 170 µm long and 120 µm thick.

The abaxial epidermis is narrow with cylindrical cells. It bears dense covering type epidermal trichomes and less frequantly glandular trichomes. The glandular trichomes have small epidermal basal cell, short, narrow stalk cell and capitate

type secretory body cells. They have four or eight cells and possess dense cytoplasam and prominent nuclei. The glandular trichomes are 30-40 µm in height and 20-30 µm wide. They occur both on the adaxial. and abaxial side of the lamina. The mesophyll consists of cylindrical compact, narrow pillars of palisade cells which are 50 µm in height. The spongy parenchyma zone is narrow comprising of three or four spherical or lobed cells (Fig. 3).

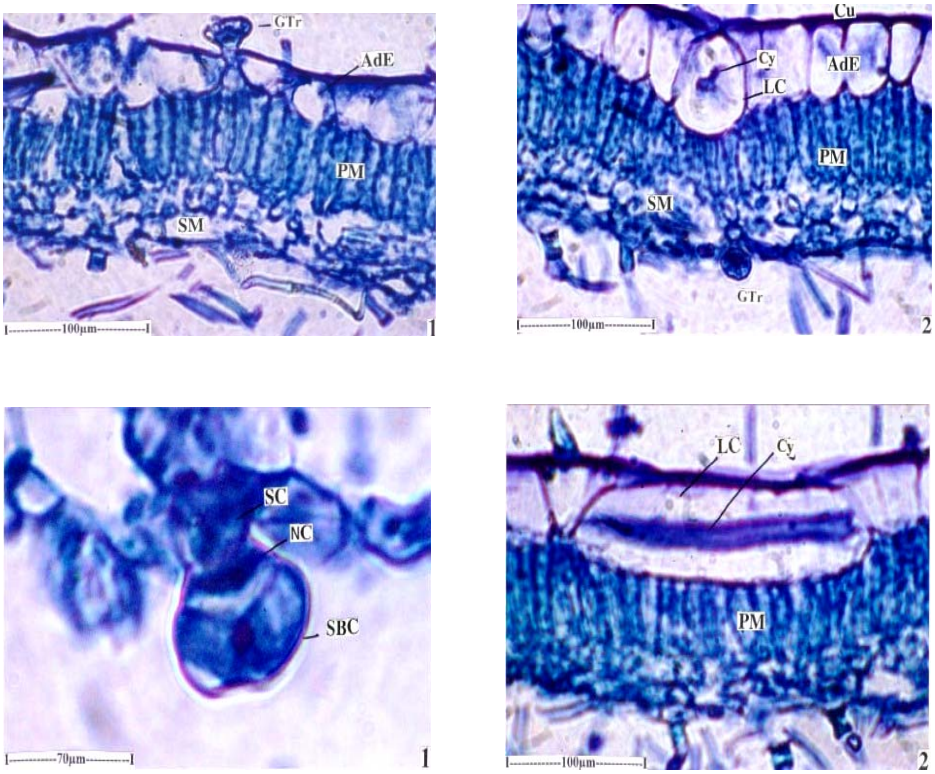


Fig. 3. Microscopic features of lamina of *S. kunthianus*

5.2.3. Petiole

The petiole is wide pot-shaped in sectional view with flat adaxial side, short thick lateral wings on the adaxial part. The epidermal cell layer is thin with small cubical cells. Linear to the epidermal layer are two or three layer of collenchyma

at the adaxial side and six or seven layers of collenchyma at the abaxial side. The ground tissue is parenchymatous, homogeneous and the cells are circular to angular, thin walled and compact.

These is a single, wide, bowl-shaped vascular strand which consists of numerous, thin parallel files of angular, thick walled xylem elements lying on the outer part of the xylem. The petiole is 1.2 mm in vertical and 2.3 mm in horizontal planes. The vascular strand is 150 µm thick (Fig. 4).

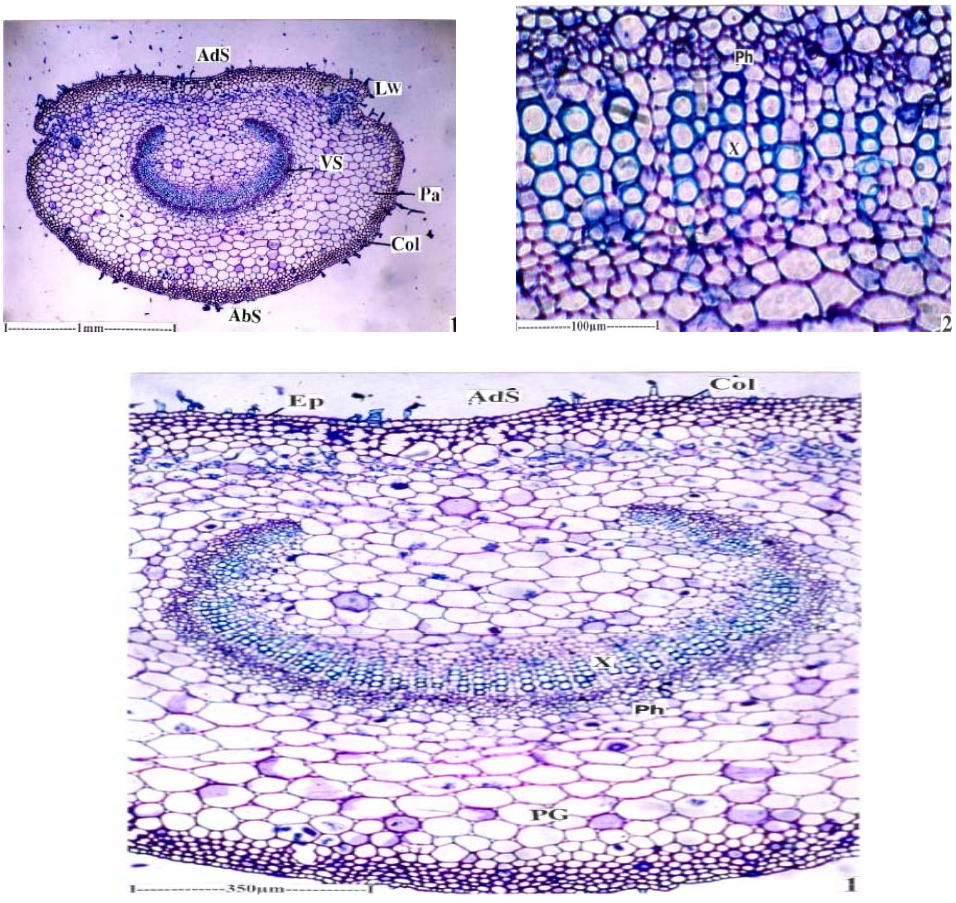


Fig. 4. Microscopic features of petiole of *S. kunthianus*

5.2.4. Stem

The young stem is some what four angled measuring about 3 mm thick. It has thin epidermal layer of small squarish cells bearing dense trichomes. The cortex is 250 µm wide and is differentiated into outer zone of parenchymatous cells. A thin median layer of cells in the cortex is chloenchymatous.

The vascular cylinder is thin, hollow and four-angled. It consists of short, narrow, circular radical files of xylem elements and xylem fibres. All along the outer part of the xylem cylinder occurs the phloem zone which has sieve elements and parenchyma cells. The vascular cylinder is 250 µm thick. The pith is homogeneous and parenchymatous with thin walled compact angular parenchyma tissue (Fig. 5).

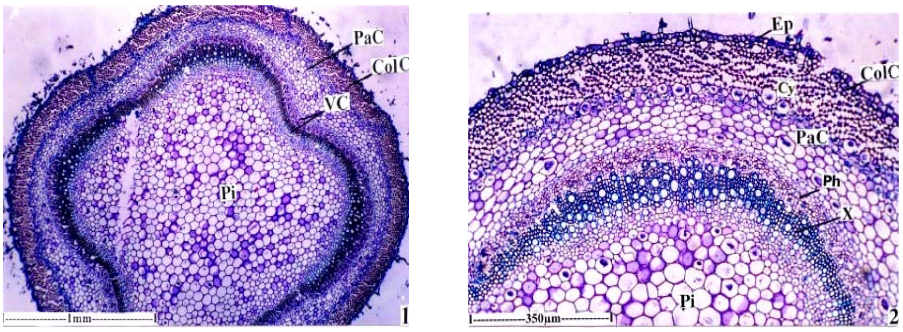


Fig. 5. Microscopic features of stem of *S. kunthianus*

5.2.5. Thick stem

The old and thick stem becomes circular in cross-sectional outline. It has cortex of 250 µm wide, narrow continuous zone of phloem of 50 µm wide and thick and dense secondary xylem (350 µm wide) enclosing a wide central pith.

The cortex is homogeneous having ellipitical tangentially stretched parenchyma cells. Secondary phloem has wide angular sieve elements and phloem parenchyma. Secondary xylem has narrow, circular thick walled vessels; they are solitary and diffusely distributed. The vessels are up to 20 µm wide. Xylem fibers are angular in sectional view, thick walled and lignified; they occur in regular radial rows. Xylem rays are one cell thick and are straight. The rays cell are also thick walled and lignified (Fig. 6).

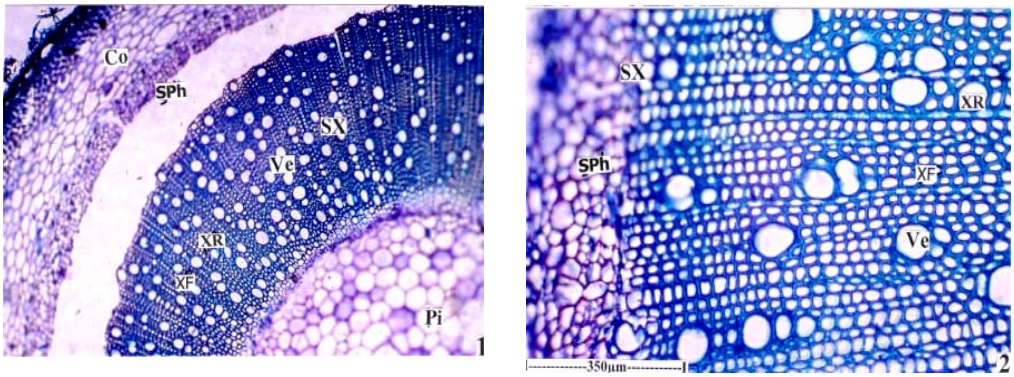


Fig. 6. Microscopic features of thick stem of *S. kunthianus*

5.2.6. Root

The root has rough and uneven outer surface. Periderm is seen at discontinuous places and it is not continuous. The cortex has outer zone of circular, wide, less compact parenchyma cells; the liner zone of cortex has wide gaps of empty chambers and the cells further towards interior are nitaet, elliptical and compact. Secondary phloem is narrow and continuous. It has large, radial files of phloem elements (Fig. 7).

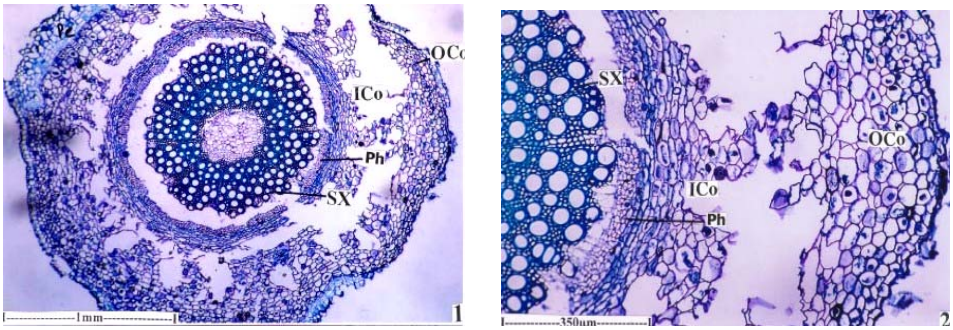


Fig. 7. Microscopic features of root of *S. kunthianus*

5.2.7. Secondary xylem

Secondary xylem has dense, diffuse wide, circular vessels distributed among thick walled fibers. The diameter of the vessels increase gradually towards the periphery and it measures 40 µm wide. The pith is narrow and nitaet. It is parenchymatous, comprises of thin walled circular compact cells (Fig. 8).

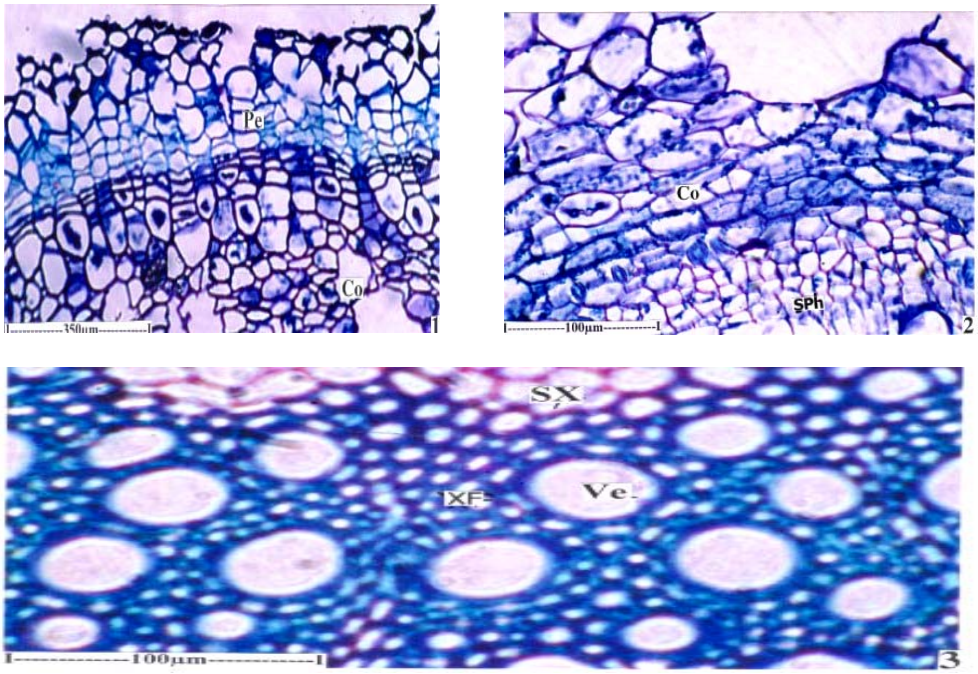


Fig. 8. Microscopic features of xylem and pith of *S. kunthianus*

5.2.8. Venation

The secondary and tertiary veins are thin and less prominent. The venation system is obscured blue to dense outgrowth of the epidermal trichomes. The vein-islets are wide and polygonal in outline. Distinct vein-terminations are not evident (Fig. 9).

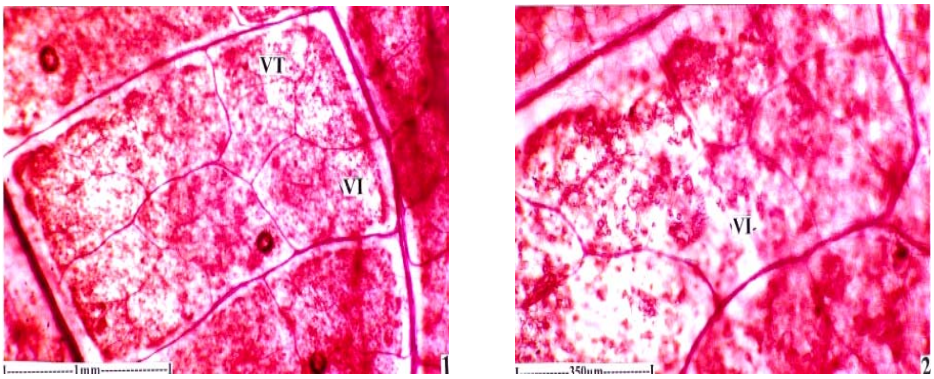


Fig. 9. Microscopic features of vein of *S. kunthianus*

5.3. POWDER MICROSCOPY

The powder exhibits fibers and vessel elements. The vessel elements are long, narrowly cylindrical and have long or short tails. The elements are 600-650 µm long. They have simple, oblique perforatious at the ends. The lateral walls have wide, circular, alternate pits.

The fibers are liberiforus type, having thick lignified walls. They are narrow with pointed ends and narrow lumens. The lumen is 10 µm in wide. These are also septate fibers, which have wide lumen and thin septa; cell inclusions are often seen in the septate fibers. The septate fibers are 20 µm wide.

Cystoliths are frequently seen in the powder. They are narrow, elongated, and blunt at one end and pointed at other. The surface is warty. The cytolith is 60 µm thick and 700 µm long (Fig. 10).

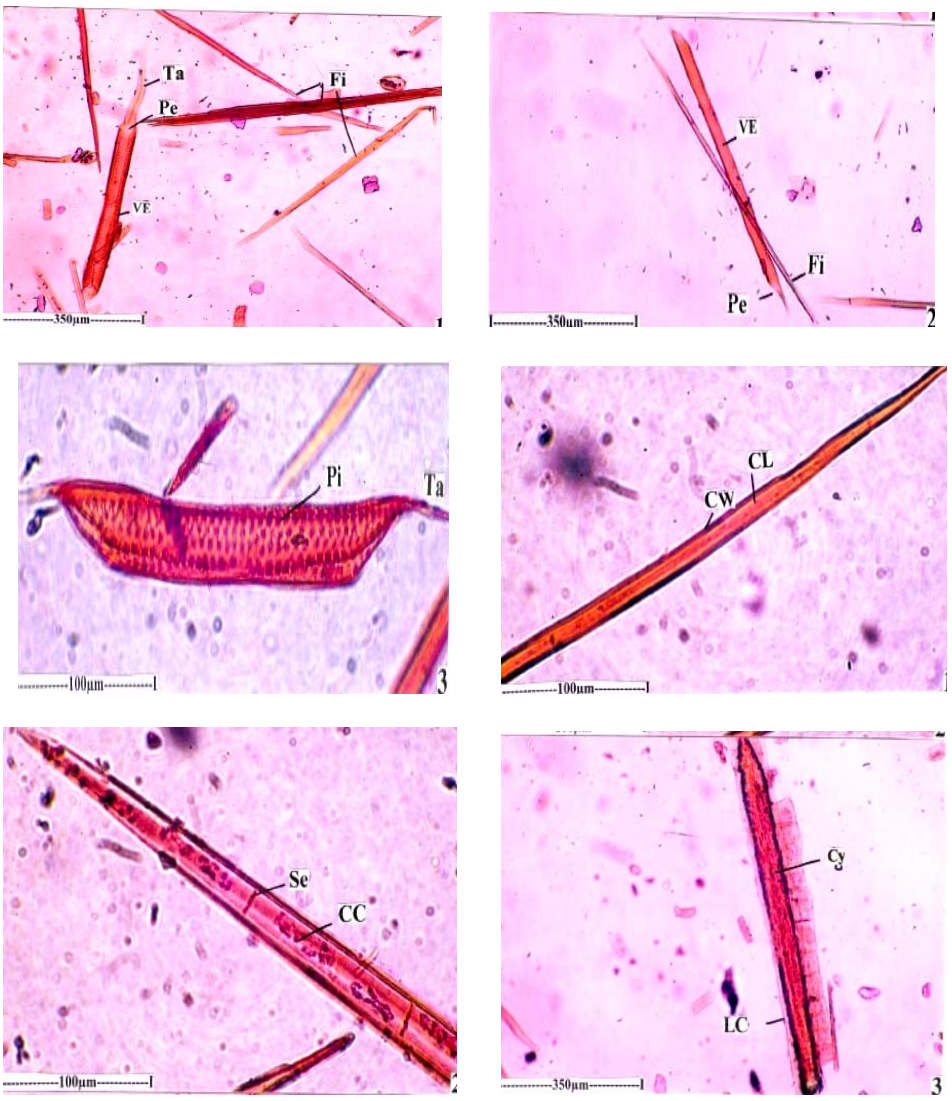


Fig. 10. Powder microscopy of *S. kunthianus*

5.4. PHYSICOCHEMICAL CONSTANT

The percentage yield of different types of ash and extractive values of flowers, root, stem and leaves of *S. kunthianus* powdered material are given in the Table 7. The total ash, water soluble ash, sulphated ash and alcohol soluble extractive values of the leaves of *S. kunthianus* were found to be high when compared to the other plant materials. The acid insoluble ash was found to be high in root and water soluble extractive value in flowers.

Table 7. Physicochemical constants of *S. kunthianus*

S. No	Plant materials	Ash values (% w/w)				Extractive values % w/w	
		Total ash	Acid Insoluble ash	Water Soluble ash	Sulphated ash	Alcohol soluble	Water soluble
1	Flower	6.61 ± 0.03	0.08 ± 0.01	1.52 ± 0.14	0.12 ± 0.01	1.02 ± 0.05	10.32 ± 0.08
2	Root	5.10 ± 0.13	2.06 ± 0.02	0.76 ± 0.05	0.23 ± 0.02	1.09 ± 0.04	2.30 ± 0.06
3	Stem	2.99 ± 0.02	1.40 ± 0.02	0.38 ± 0.04	1.11 ± 0.06	0.73 ± 0.05	5.14 ± 0.09
4	Leaf	20.49 ± 0.31	1.06 ± 0.03	4.05 ± 0.10	3.22 ± 0.09	1.64 ± 0.12	3.43 ± 0.03

Average of three determinations, mean ± SEM.

5.5. PRELIMINARY PHYTOCHEMICAL STUDIES

In the Preliminary phytochemical studies, the successive petroleum ether and chloroform extracts of stem and root were found to contain phenolics, steroids and triterpenoids. The successive ethyl acetate and methanol extracts of stem were found to contain flavonoids, glycosides and phenolics. These two solvent extracts in root showed the presence of glycosides, phenolics, and saponins. The macerated methanolic leaves extract showed the presence of glycosides,

phenolics, steroids, triterpenoids and tannins. Along with these alkaloids, flavonoids, saponins and tannins were found to be present in macerated and crude methanol extracts of the flowers. The crude methanol extracts of the stem and the leaves contain glycosides, phenolics, steroids and triterpenoids. The crude methanol extract of the root showed the presence of flavonoids, glycosides and phenolics.

The total phenol content of the crude methanol extract of the flowers was found to be high among all the extracts i.e. 173.90 ± 1.67. The ethyl acetate extract of the successive root extract, crude methanol extract of the stem and macerated methanol extract of the flowers showed the phenol content as 131.40 ± 0.98, 128. 50 ± 0.97 and 125. 60 ± 1.93. The total phenol content in the petroleum ether extract of the stem and methanol extracts of the leaves were found to be the lowest (Tables 8 and 9).

Table 8. Percentage yield and qualitative phytochemical analysis of successive extracts of stem and root of *S. kunthianus*

Extract	Nature	% Yield	Phytoconstituents								
			Alkaloids	Flavonoids	Glycosides	Phenolics	Saponins	Steroids	Triterpenoids	Tannins	Total Phenol mg/g[a,b]
Successive stem extracts											
Petroleum ether	Greenish yellow powder	3.00	-	-	-	+	-	+	+	-	14.66 ± 0.03
Chloroform	Dark green sticky	0.95	-	-	-	+	-	+	+	-	36.68 ± 1.95
Ethyl acetate	Dark green sticky	0.60	-	+	+	+	-	-	-	-	29.50 ± 0.98
Methanol	Greenish black sticky	2.95	-	+	+	+	-	-	-	+	106.30 ± 0.38
Successive root extracts											
Petroleum ether	Yellowish white powder	3.20	-	-	-	+	-	+	+	-	21.25 ± 0.97
Chloroform	Pale brown sticky	1.10	-	-	-	+	+	+	+	-	56.04 ± 1.02
Ethyl acetate	Reddish brown sticky	0.90	-	-	+	+	+	-	+	-	131.40 ± 0.98
Methanol	Brown semi solid	2.42	-	-	+	+	+	-	-	-	102.40 ± 1.92

[a]Average of six determinations, mean ± SEM , expressed as [b]gallic acid, equivalent in mg/g of the extract
+ : present , - : absent

Table 9. Percentage yield and qualitative phytochemical analysis of methanol extracts of *S. kunthianus*

Extract	Nature	% Yield	Phytoconstituents								
			Alkaloids	Flavonoids	Glycosides	Phenolics	Saponins	Steroids	Triterpenoids	Tannins	Total Phenol mg/g[a,b]
Macerated leaves extract	Greenish black sticky	4.60	-	-	+	+	-	+	+	+	16.44 ± 0.44
Macerated flower extract	Reddish brown sticky	3.05	+	+	+	+	+	+	-	+	125.60 ± 1.93
Crude flower extract	Dark reddish brown sticky	19.60	+	+	+	+	+	+	+	+	173.90 ± 1.67
Crude stem extract	Dark green sticky	9.85	-	-	+	+	-	+	+	-	128.50 ± 0.97
Crude root extract	Pale brown semi solid	4.80	-	+	+	+	-	-	-	-	106.20 ± 0.97
Crude leaves extract	Dark greenish black	12.80	-	-	+	+	-	+	+	+	15.11 ± 0.22

[a]Average of six determinations, mean ± SEM , expressed as [b]gallic acid, equivalent in mg/g of the extract.

+ : present, - : absent

5.6. ISOLATION OF PHYTOCONSTITUENTS

5.6.1. Column chromatography of Petroleum ether root and stem extract

5.6.1.1. Compounds 1 and 3 (GS 1, PES 1)

5.6.1.1.1. Homogeneity

The homogeneity of compounds GS 1 and PES 1 were proved by a single spot on TLC using silica gel G as adsorbent and solvent mixtures of different polarities as developers. The solvent systems used and the corresponding R_f values are given in Table 10.

Table 10. TLC profile of compounds GS1 and PES 1

Mobile Phase	Solvent ratio	R_f value
Chloroform: ethyl acetate	68:32	0.57
Toluene: ethyl acetate	82:18	0.49
Petroleum ether: acetone	85:15	0.30
Dichloromethane: methanol	95:05	0.65

5.6.1.1.2. Color Reaction

The compound exhibited positive response for anisaldehyde-sulfuric acid reagent indicating its triterpenoid nature.

5.6.1.1.3. Melting point of compound

The melting point of compound was found to be 213 ºC.

5.6.1.1.4. Spectral studies

The ^1H NMR spectra displayed a one proton multiplet at δ 3.20 is assignable to H-3 on the basis of biogenetic analogy (Fig. 11). The $^1H/^1H$-

COSY experiment exhibited the coupling between H-3 with H-2α (1.59 ppm) and H-2β (1.30 ppm) indicating that the hydroxyl group is at C-3 (Fig. 12). The seven methyl groups resonated at δ 1.70 (H-30), 1.01 (H-28), 0.95 (H-26), 0.93 (H-23), 0.81 (H-27), 0.77 (H-24), and 0.75 (H-25). The presence of two doublets at δ 4.60 and 4.70 each integrating for one proton was attributed to methylene group at C-20. The multiplet at δ 2.40 for one proton is assigned to H-18. The assignments were made based on the HSQC experiments (Fig. 14).

The ^{13}C NMR and INEPT experiments showed the presence of 30 carbon atoms consisting of six quaternary carbons, six methine, eleven methylene carbons and seven methyl groups (Fig. 13). The signals at δ 109.31 and 150.98 indicates the presence of a C=C group out of which one is a quaternary carbon and the other is a carbon of a vinyl methylene group. The signal at δ 79.04 is attributed to C-3. The signals at δ 27.95, 19.31, 18.00, 16.17, 16.05, 15.43 and 15.29 were due to the presence of seven methyl groups in the compound. The other signals in accordance with the proposed structure of the compound which was identified as lupeol (Fig. 15).

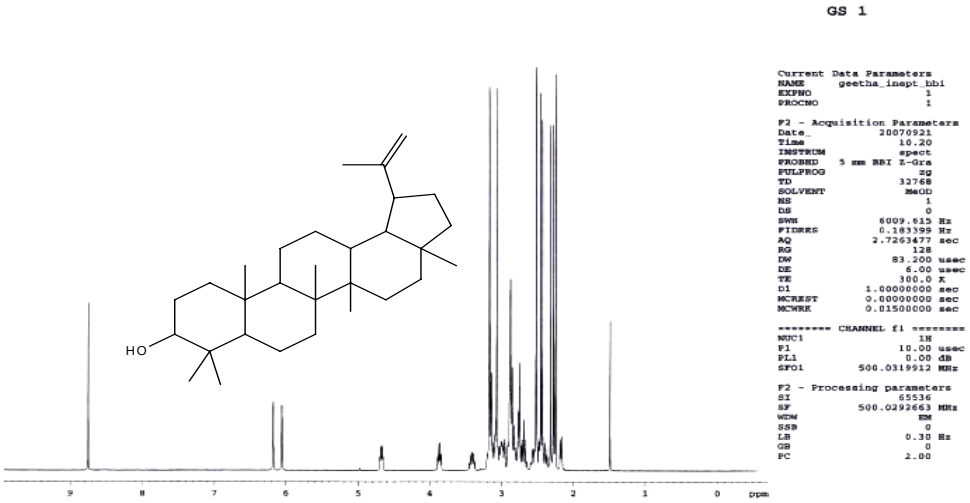


Fig. 11. ^{1}H NMR of compound GS 1

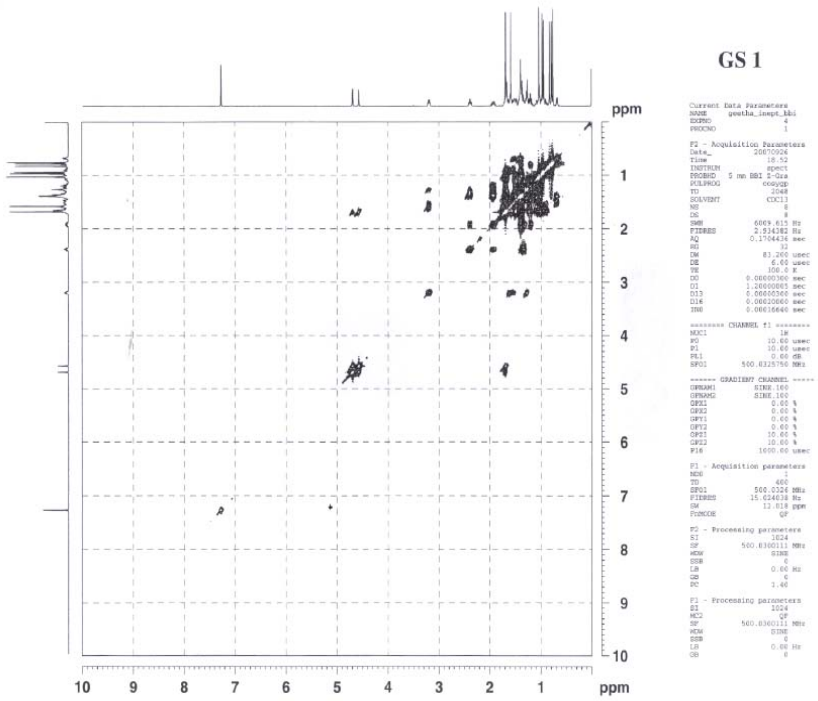


Fig. 12. Homocosy of compound GS 1

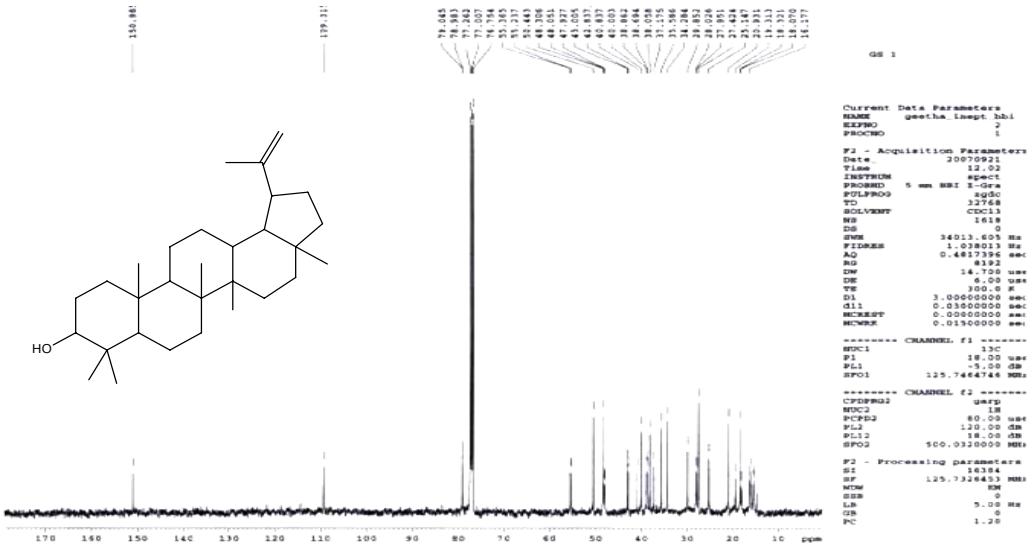


Fig. 13. ^{13}C NMR of compound GS 1

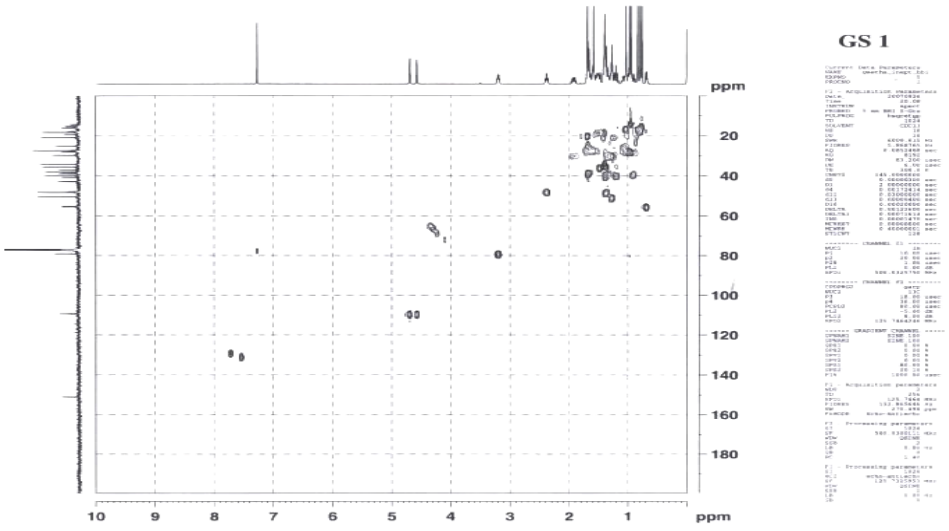


Fig. 14. Heterocosy of compound GS 1

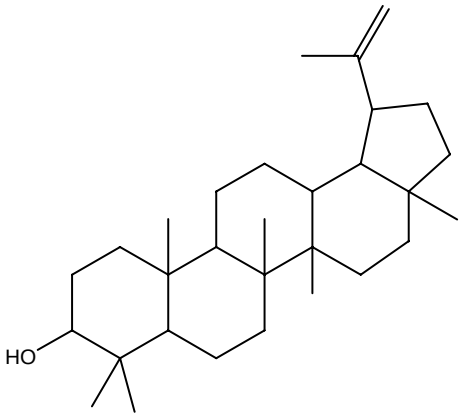


Fig. 15. GS 1 and PES 1 (Lupeol)

5.6.1.2. Compound 2 (GS 2)

5.6.1.2.1. Homogeneity

The homogeneity of compound GS 2 was proved by a single spot on TLC using silica gel G as adsorbent and solvent mixtures of different polarities as developers. The solvent systems used and the corresponding R_f values are given in Table 11.

Table 11. TLC profile of compound GS 2

Mobile Phase	Solvent ratio	R_f value
Chloroform: ethyl acetate	68:32	0.85
Toluene: ethyl acetate	82:18	0.78
Petroleum ether: acetone	85:15	0.62
Dichloromethane: methanol	95:05	0.70

5.6.1.2.2. Color reaction

The compound exhibited positive response for anisaldehyde-sulfuric acid reagent indicating its triterpenoid nature.

5.6.1.2.3. Melting point of compound

The melting point of compound was found to be 252 ºC.

5.6.1.2.4. Spectral studies

The ^{1}H NMR spectra displayed a one proton multiplet at δ 3.19 is assignable to H-3 on the basis of biogenetic analogy. The six methyl groups resonated at δ 1.68 (H-30), 1.02 (H-26), 0.98 (H-23), 0.97 (H-27), 0.82 (H-24) and 0.76 (H-25). The presence of two doublets at δ 4.60 and 4.70 each integrating for one

proton were attributed to methylene group at C-20. The multiplet at δ 2.40 for one proton is assigned to H-18. The pair of doublet signals at δ 3.80 and 3.32 each integrating for one proton was assigned to H-28 (Fig. 16).

The ^{13}C NMR and INEPT experiments showed the presence of 30 carbon atoms consisting of six quaternary carbons, six methine, twelve methylene carbons and six methyl groups. The signals at δ 109.68 and 150.98 indicates the presence of a C=C group out of which one is a quaternary carbon and the other is a carbon of a vinyl methylene group. The signal at δ 78.98 is attributed to C-3. The signals at δ 27.97, 19.07, 16.10, 15.97, 15.35 and at δ 14.75 were due to the presence of six methyl groups in the compound (Fig. 17 and 18). The signal at δ 60.56 is due to a hydroxy methylene carbon atom (C-28). The other signals are in accordance with the proposed structure of the compound which was identified as betulin (Fig. 19).

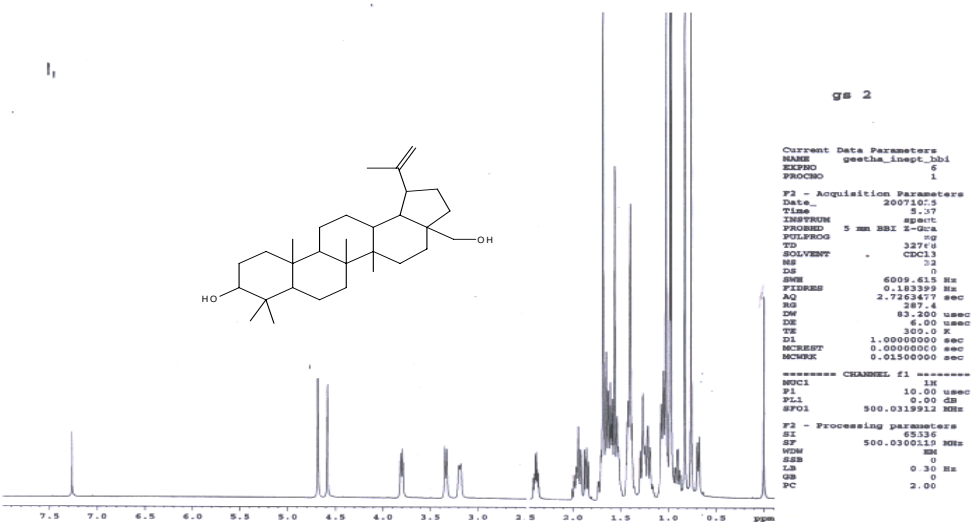


Fig. 16. ^{1}H NMR of compound GS 2

Fig. 17. ^{13}C NMR of compound GS 2

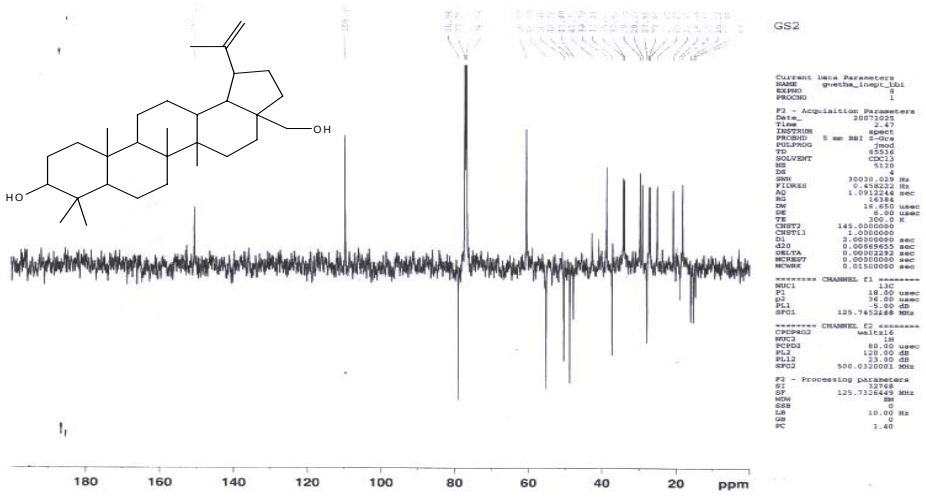


Fig. 18. DEPT of compound GS 2

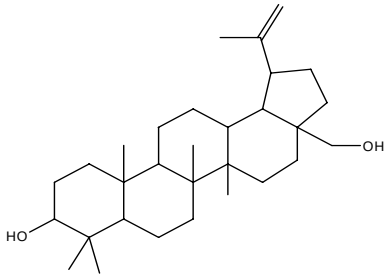


Fig. 19. GS 2, Betulin

5.6.1.3. Column chromatography of chloroform stem extract

5.6.1.3.1. Compound 4 (SC 1)

5.6.1.3.1.1. Homogeneity

The homogeneity of compound SC1 was proved by a single spot on TLC using silica gel G as adsorbent and solvent mixtures of different polarities as developers. The solvent systems used and the corresponding R_f values are given in Table 12.

Table 12. TLC profile of compound SC1

Mobile Phase	Solvent ratio	R_f value
Petroleum ether: ethyl acetate	90:10	0.47
Chloroform: methanol	98:02	0.69
Petroleum ether: acetone	95:05	0.30
n-hexane: ethyl acetate	80:20	0.51

5.6.1.3.1.2. Melting point of compound

The melting point of compound was found to be 118 ºC.

5.6.1.3.1.3. Spectral studies

In its ^{1}H NMR it showed a triplet at δ 0.93 indicating a methyl group which is adjacent to a methylene group. There is a strong singlet at δ 1.29 indicating the presence of a long chain methylene groups. The singlets at δ 2.10 and 2.20 indicate the presence of four methylene groups attached to four carbonyl groups. The strong singlet at δ 2.70 for 24 protons shows the presence of eight methyl groups attached to nitrogen atoms (Fig. 20).

The ^{13}C NMR spectrum showed a signal at δ 13.99 indicating the presence of only one methyl group. The signal at δ 29.55 supports above interpretations that the compound contains long chain methylene carbon atoms. The strong signal at δ 40.91 confirms the presence of methyl groups attached to nitrogen atom and all the methyl groups are in the same environment (Fig. 21).

The above data suggests that the compound may contain four N, N-dimethyl acetamide groups ($-CH_2-CO-N\ (CH_3)_2$) attached to two different methylene groups as shown below.

$$(CH_3)_2N-CO-CH_2-C-\ (-CH_2-CO-N\ (CH_3)_2)$$

Since all similar groups are in the same environment these groups should be attached to one carbon atom.

$$-CH-\ [C-\ (-CH_2-CO-N\ (CH_3)_2)_2]_2$$

Two other valencies of this carbon atom have been satisfied by the long chain methylene groups.

The Mass spectrum indicates that the base peak at m/z 83.44 suggests a cyclohexane ring system. After that successive peaks differ by a mass unit of 14. The m/z 97.00, 111.29, 125.18, 139.07, 153.00, 166.85, 180.76, 194.70, 208.00, 222.50, 236.41, 250.40, 264.35, 278.18, 292.15, 306.08, 320.02, 334.93 and at 348.93 strongly supporting that the compound contains a long chain methylene groups (Fig. 22).

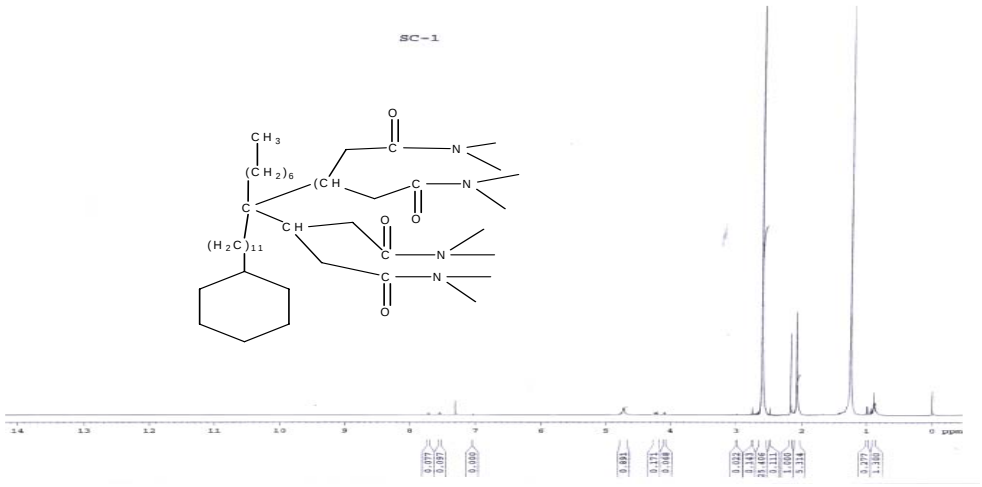


Fig. 20. ^{1}H NMR spectrum of compound SC 1

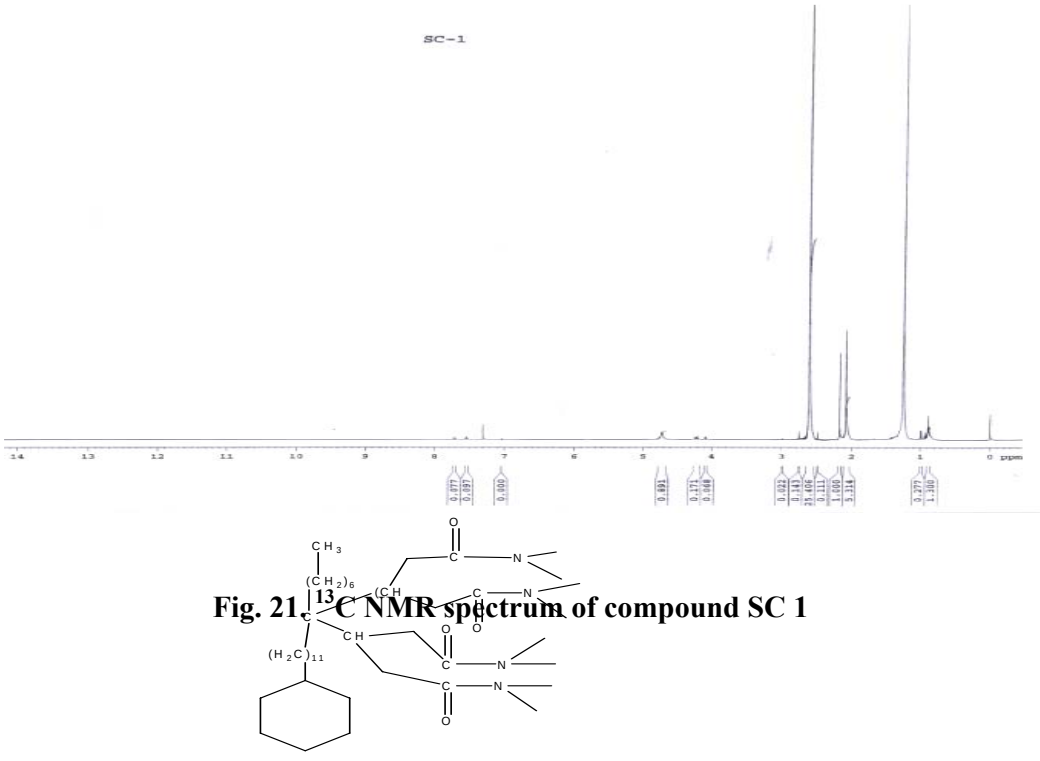


Fig. 21. ^{13}C NMR spectrum of compound SC 1

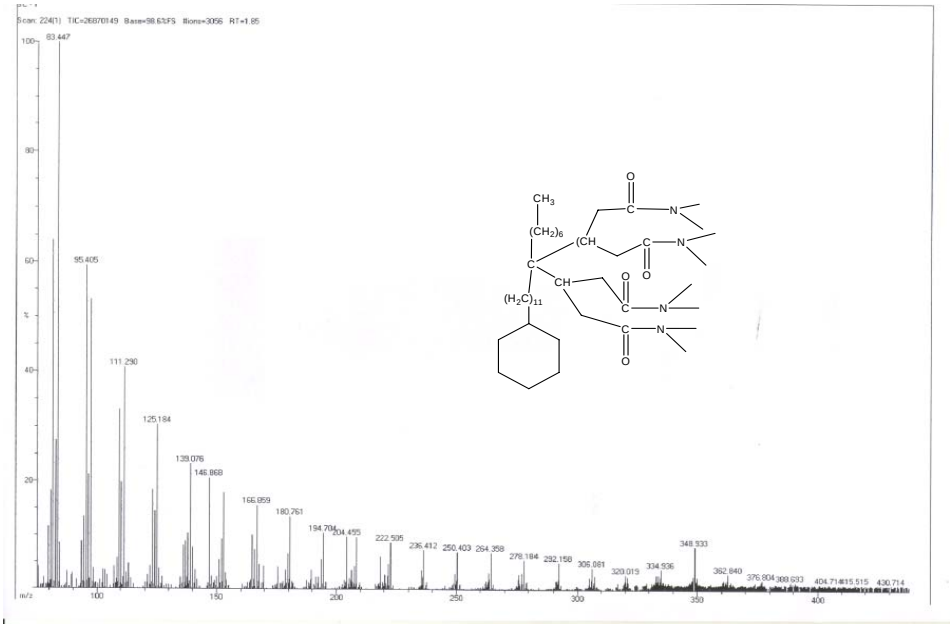


Fig 22. Mass spectrum of compound SC 1

Based on the above facts the structure was proposed as below (Fig. 23).

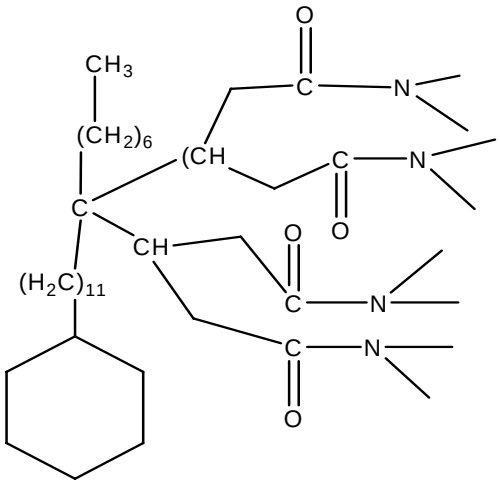


Fig. 23. (SC 1) 3,5-bis (dimethyl carbamoyl) methyl-4- (11-cyclo hexylundecyl)- 4-heptyl- N^1,N^1,N^7,N^7tetra methyl heptane diamide

5.6.1.4. Column chromatography of macerated methanol flower extract

5.6.1.4.1. Compound 5 (GSMES 3)

5.6.1.4.1.1. Homogeneity

The homogeneity of compound GSMES 3 was proved by a single spot on TLC using silica gel G as adsorbent and solvent mixtures of different polarities as developers. The solvent systems used and the corresponding R_f values are given in Table 13.

Table 13. TLC profile of compound GSMES 3

Mobile Phase	Solvent ratio	R_f value
Petroleum ether: chloroform	70:30	0.32
n-hexane: acetone	75:25	0.71
Petroleum ether: ethyl acetate	88:12	0.52
Toluene: ethyl acetate	60:40	0.82

5.6.1.4.1.2. Color Reaction

The compound exhibited positive response for anisaldehyde-sulfuric acid reagent indicating its triterpenoid nature.

5.6.1.4.1. 3. Melting point of compound: The melting point of compound was found to be 180 ºC.

5.6.1.4.1.4. Spectral studies

The ^{1}H NMR spectra exhibited two doublets at δ 0.85 and 0.93 (each 3H, H-29 and H-30), and six singlets at δ 1.25 (3H, H-23), 1.16 (3H, H-26), 0.86 (3H, H-27), 0.84 (3H, H-28), 0.82 (3H, H-25) and 0.70 (3H, H-24) indicating the

presence of eight methyl groups in the compound. The multiplet signal at δ 5.35 (1H, H-12) indicates the presence of a double bond wherein one of the carbon is tetrasubstituted. The signal at δ 3.52 (1H, m, H-3) is characteristic of a proton under hydroxy function is also present in the compound (Fig. 24). These facts suggest the presence of a triterpenoid nucleus.

The ^{13}C NMR spectrum exhibited 30 signals indicating the presence of 30 carbon atoms and supports the presence of a triterpenoid. The signals at δ 121.79 and 139.79 indicate the presence of one double bond. The signal at δ 71.81 shows the presence of a carbon atom connected to a hydroxy group. The hydroxyl group is placed in the C-3 position for biogenetic considerations. The signals at 29.68, 11.96, 11.94, 18.99, 21.07, 25.39, 19.38 and 24.29 were safely attributed to the methyl groups of the triterpenoid moiety (Fig. 25). On comparison of the signals with the known triterpenoids, it matched with the signals of α-amyrin (Fig. 26).

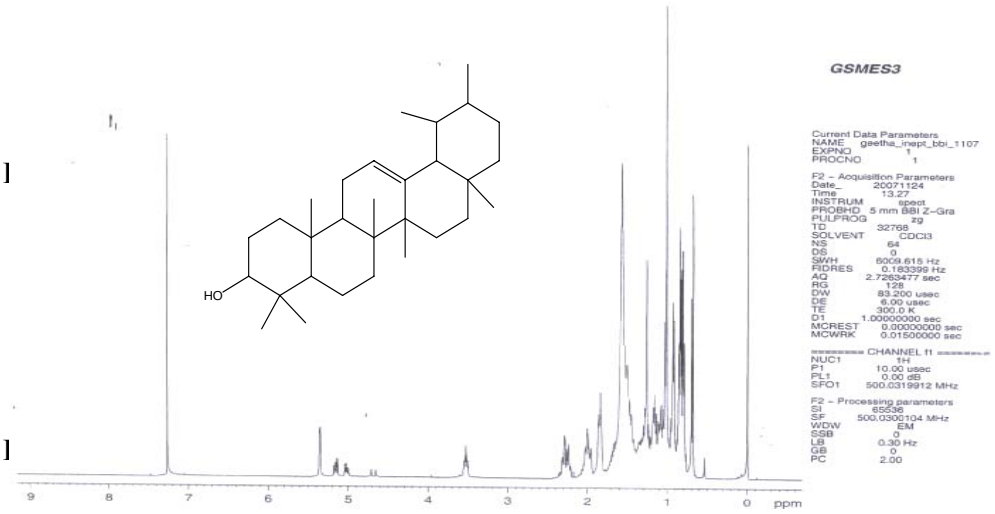


Fig. 24. ^{1}H NMR of compound GSMES3

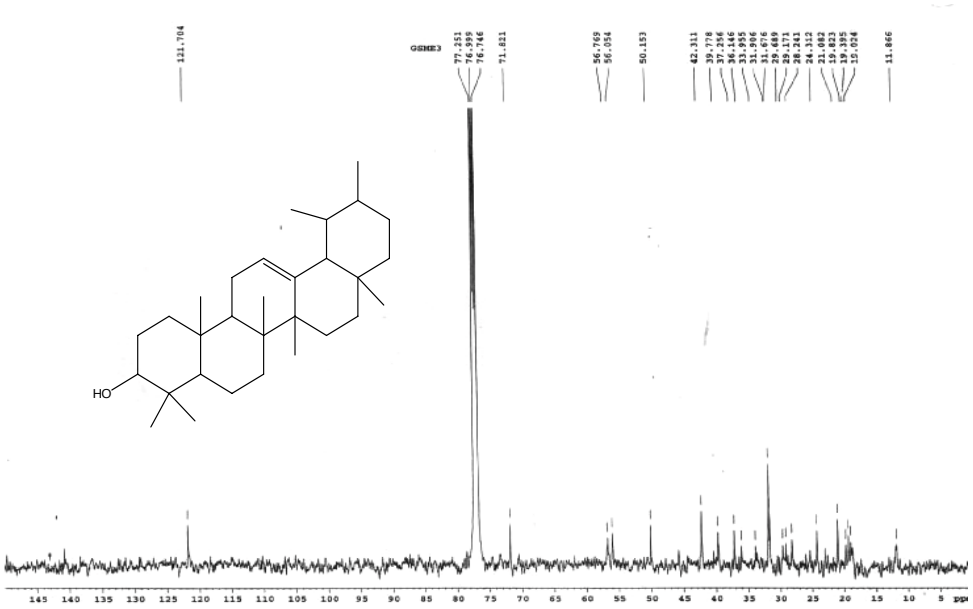


Fig. 25. ^{13}C NMR of compound GSMES3

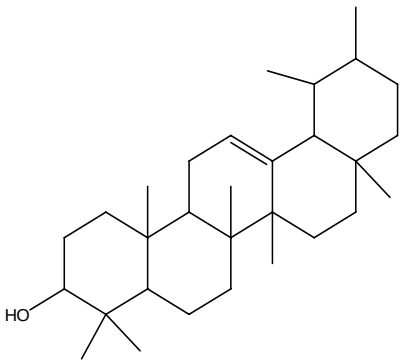


Fig. 26. (GSMES 3) α-amyrin

5.6.1.4.2. Compound 6 (G 1)

5.6.1.4.2.1. Homogeneity

The homogeneity of compound G 1 was proved by a single spot on TLC using silica gel G as adsorbent and solvent mixtures of different polarities as developers. The solvent systems used and the corresponding R_f values are given in Table 14.

Table 14. TLC profile of compound G 1

Mobile Phase	Solvent ratio	R_f value
Chloroform: ethyl acetate	10:90	0.42
Toluene: ethyl acetate	20:80	0.61
Petroleum ether: acetone	65:35	0.80

5.6.1.4.2.2. Melting point of compound

The melting point of compound was found to be 132 ºC.

5.6.1.4.2.3. Spectral studies

The ^{1}H NMR spectrum showed two doublet integrating for two protons each at δ 3.88 (H-2α, H-6α), and at 3.61 (H-2β, H-6β). It also exhibited a complex multiplet of signals centered at δ 3.69 for three protons (H-3, H-4 and H-5). The downfield signal of the H-4 may be due to the intramolecular hydrogen bonding of the amino and hydroxyl group protons with each other (Fig. 27).

In the ^{1}H/^{1}H COSY spectrum the H-2α and H-6α protons showed cross peaks with H-2β and H-6β respectively showing that they mutually couple with each other. Cross peaks were observed between the H-3, H-4 and H-5 protons with

the H-2 and H-6 protons showing that they couple with each other and thereby suggesting that they are in adjacent positions (Fig. 28).

The ^{13}C NMR spectrum exhibited only three signals indicating a highly symmetrical environment in the molecule. The signals appeared at δ 63.30, 69.39 and 70.98 suggesting that all the carbon atoms are under hetero atoms (N or O). The intensity of the peak at δ 63.30 is more than the other two peaks indicating the presence of more than one carbon atom in that environment (Fig. 29). Further the DEPT 135 spectrum of this compound showed that only one peak at δ 63.30 (methine carbons) is in the positive side and the other two (methylene carbons) are in the negative side (Fig. 30).

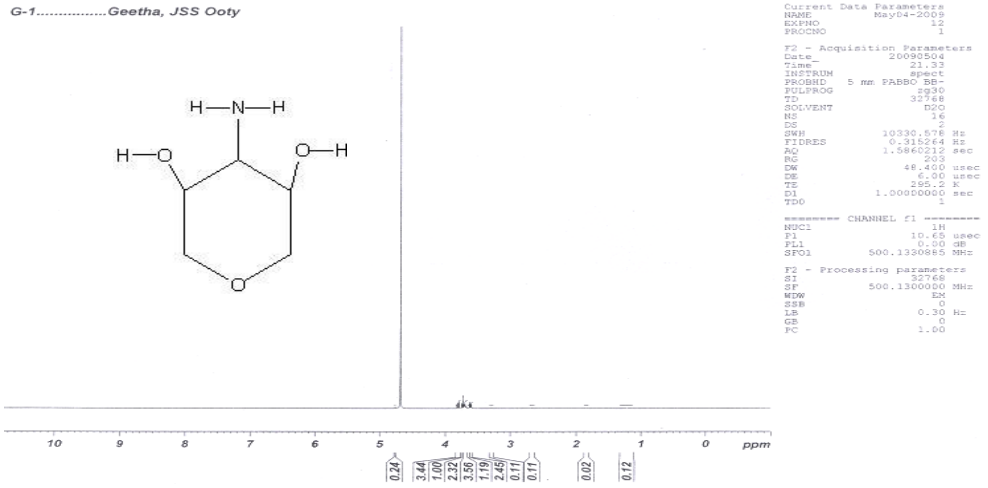


Fig. 27. ^{1}H NMR of compound G 1

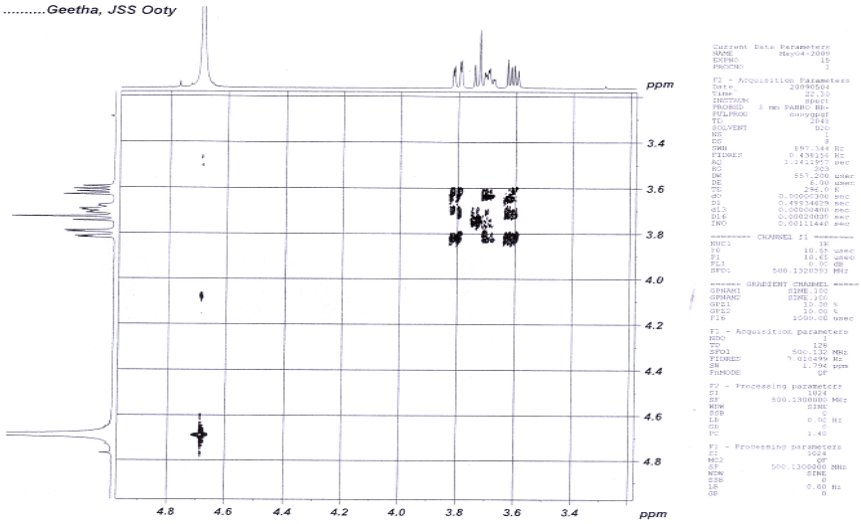


Fig. 28. Homocosy of G 1

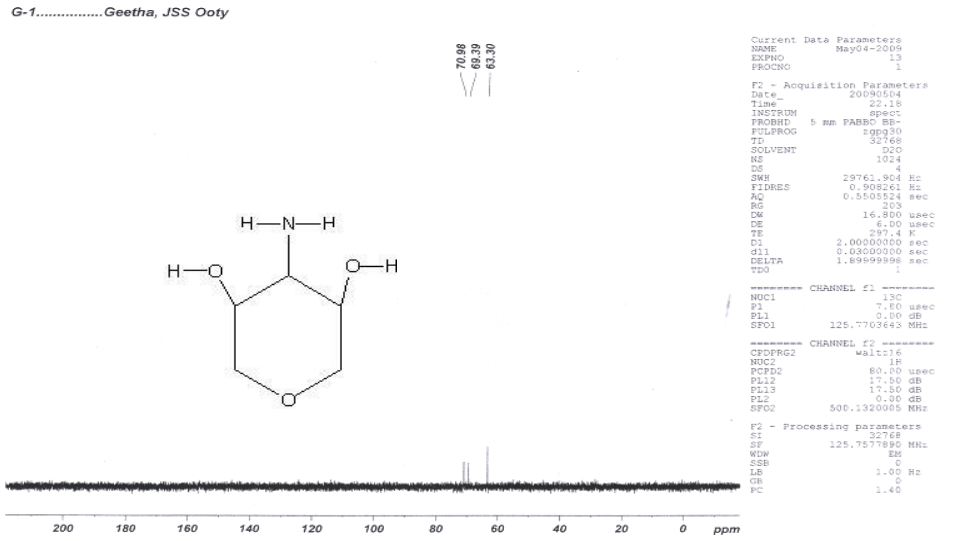


Fig. 29. ^{13}C NMR of compound G 1

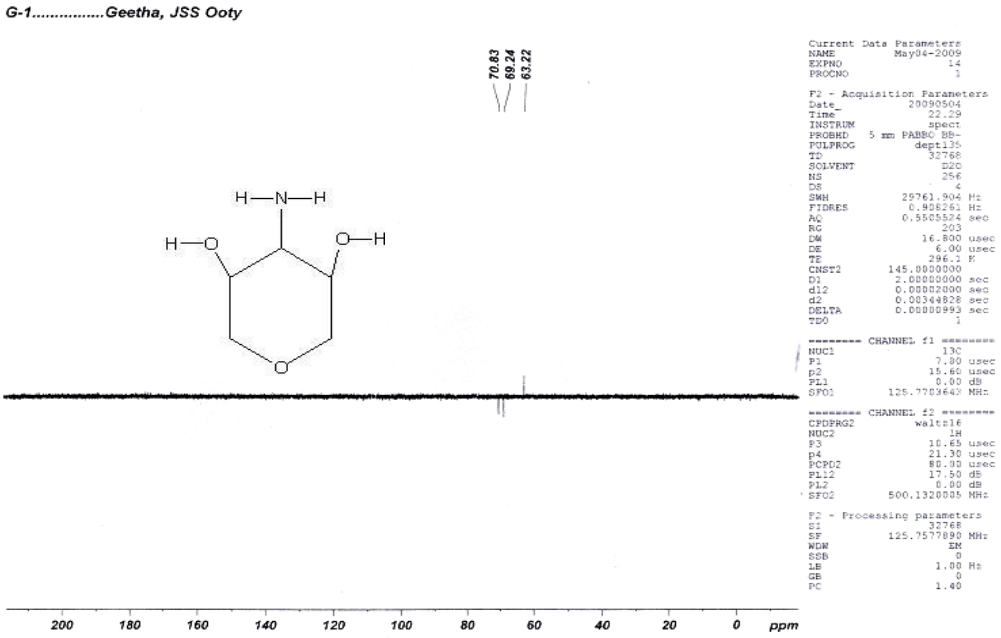


Fig. 30. DEPT of compound G 1

Based on the above facts the structure was proposed as below (Fig. 31).

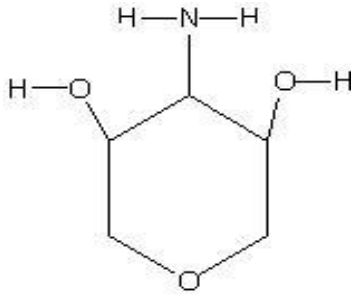


Fig. 31. (G 1) 4-amino-tetrahydro-2H-pyran-3,5-diol

5.6.1.5. Column chromatography of crude methanol flower extract

5.6.1.5.1. Compound 7 (CMF 1)

5.6.1.5.1.1. Homogeneity

The homogeneity of compound CMF 1 was proved by a single spot on TLC using silica gel G as adsorbent and solvent mixtures of different polarities as developers. The solvent systems used and the corresponding R_f values are given in Table 15.

Table 15. TLC profile of compound CMF1

Mobile Phase	Solvent ratio	R_f value
Petroleum ether: chloroform	60:40	0.52
Petroleum ether: ethyl acetate	80:20	0.74
n-hexane: ethyl acetate	65:35	0.47

5.6.1.5.1.2. Color reaction

The isolated compound exhibited positive response for Libermann-Buchard's test and Salkowski test, indicating its steroidal nature.

5.6.1.5.1.3. Melting point of compound CMF1

The melting point of compound CMF 1 was found to be 138 ºC.

5.6.1.5.1.4. Spectral studies

^{1}H and ^{13}C NMR: Its NMR spectrum displayed signals for two tertiary methyl groups at δ 0.70 (s, 3H, C-18) and δ 1.01(s, 3H, C-19, Fig. 32). Three secondary methyl groups at δ 0.94 (d, 3H, Me-21), 0.82 (d, 3H, Me-26), 0.85 (d, 3H, Me-27) and a primary methyl group at δ 0.88 (t, 3H, Me-29). The broad multiplet

signal at δ 5.35 (1H, m) was attributed to H-6 and the hydroxy methine proton signal at δ 3.52 (1H, m) was attributed to H-3 for biogenetic considerations.

^{1}H NMR, δ (ppm): 0.70 (s, 3H, C-18). 0.82 (d, 3H, J = 6.4 Hz, Me-26), 0.85 (d, 3H, J = 6.4 H_Z, Me- 27), 0.88 (t, 3H, J =7.5 Hz, Me-29), 0.94 (d, 3H, J = 6.5 Hz, Me-21), 1.01 (s, 3H, C-19), 3.52 (m, 1H, C-3), 5.35 (m, 1H, C-6) (Fig. 32 and 33). The ^{1}H NMR and ^{13}C NMR values are in agreement with β-sitosterol.

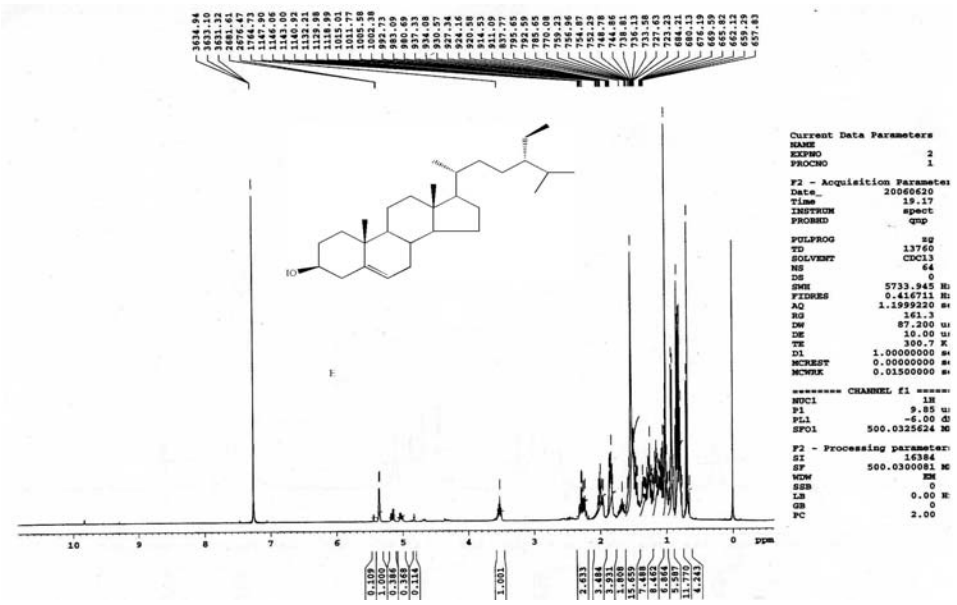


Fig. 32. ^{1}H NMR spectrum of compound CMF 1

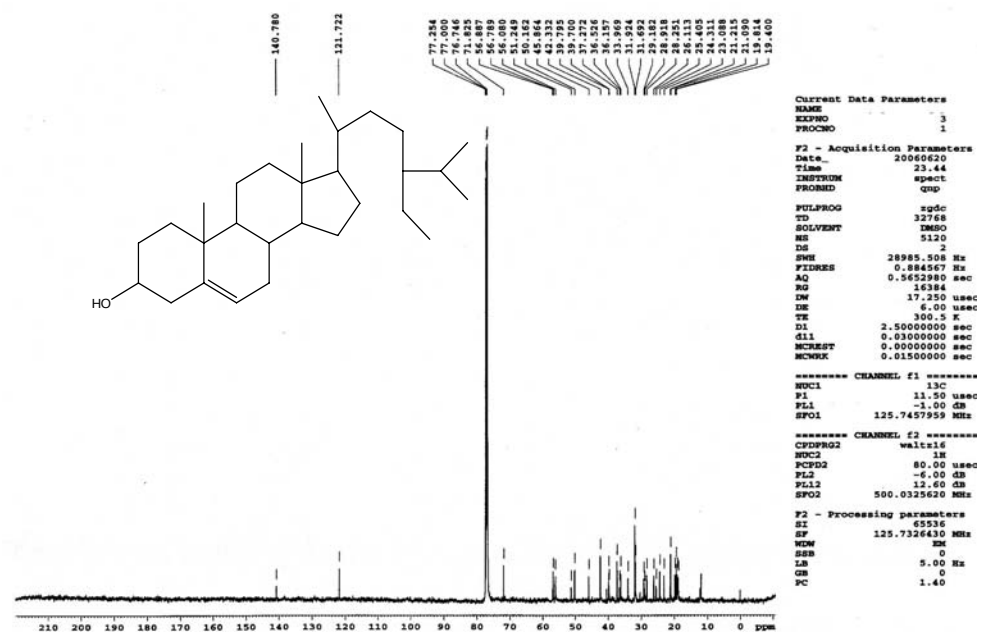


Fig. 33. ^{13}C NMR spectrum of compound CMF 1

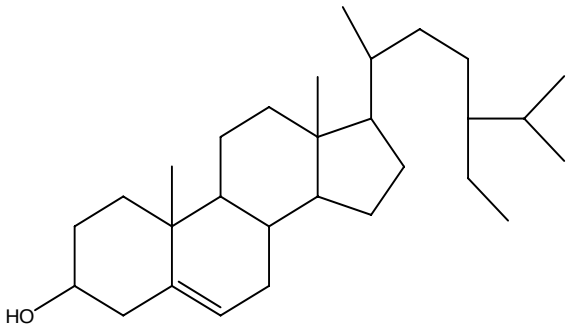


Fig. 34. (CMF 1) β- sitosterol

5.6.1.5.2. Compound 8 (CMF 2)

5.6.1.5.2.1. Homogeneity

The homogeneity of compound CMF 2 was proved by a single spot on TLC using silica gel G as adsorbent and solvent mixtures of different polarities as developers. The solvent systems used and the corresponding R_f values are given in Table 16.

Table 16. TLC profile of compound CMF 2

Mobile Phase	Solvent ratio	R_f value
Dichloro methane: acetone	82:18	0.66
n-hexane: ethyl acetate	50:20	0.57
Chloroform: ethanol	90:10	0.46

5.6.1.5.2.2. Melting point of compound CMF 2

The melting point of compound CMF 2 was found to be 87 ºC.

5.6.1.5.2.3. Spectral studies

The ^{1}H NMR exhibits a multiplet at δ 5.3 for one proton indicating that one of the carbon atoms is tetra substituted. The ^{13}C NMR spectra of the compound CMF 2 exhibits two signals at δ 118.21 and 142.58 (C-11, C-12) in the unsaturated region of the spectrum indicating that there is only one double bond in the compound. In ^{13}C NMR it exhibits signals at δ 75.21 (C-3) and 61.19 (C-24) indicating two carbon atoms attached to oxygen function. The former signal at δ 75.21 appears to be a signal of a methine carbon which is esterified.

Accordingly, the carbonyl carbon appears at δ 173.76. Consistently, its PMR signal appears as a multiplet at downfield region δ 4.0 for one proton. The latter signal at δ 61.19 appears to be of a hydroxy methyl signal. In the PMR spectrum corresponding methylene protons appears in the downfield region at δ 4.8 (m, 1H, H-24) and 4.5 (m, 1H, H-24) each for one proton. This signal can go down only if the hydroxyl group is hydrogen bonded with the carbonyl group. Due to hydrogen bonding it will reduce the electron density in the C-24 carbon and the signals go downfield. This suggests that the carbonyl group and the hydroxy methyl group are nearby each other. There is no signal in between δ 40 and 60 in the ^{13}C NMR. The presence of a methyl group in the C-5 position makes the carbon C-4 electron rich and the signal moves towards up field region and appears at δ 39.98. For similar reasons the long hydrocarbon chain which contains the double bond is connected to C-6. Since there is no other functional group is found in the compound the methyl group at C-11 makes it tetra substituted. To avoid the signals of C-10 and C-13 to go downfield region methyl groups were placed at C-9 and C-13 (Fig. 35 and 36). All the other signals are in accordance with the structure. The mass spectrum also confirms the long chain hydro carbon (Fig. 37).

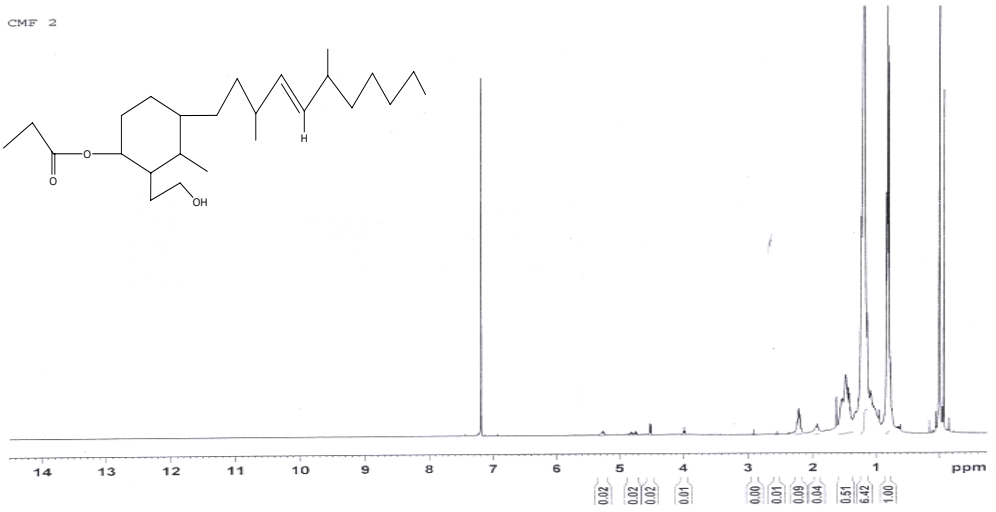


Fig. 35. ^{1}H NMR spectrum of compound CMF 2

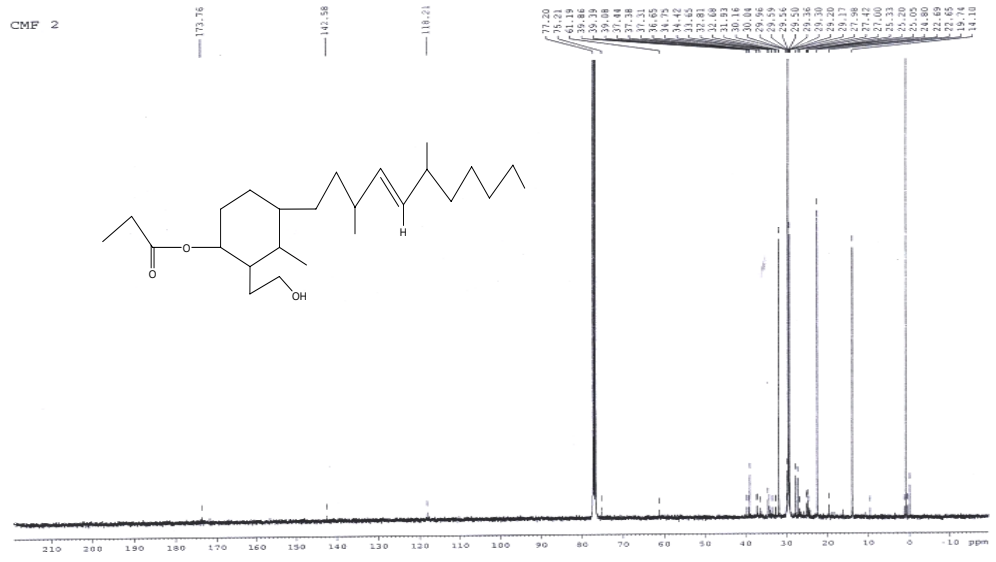


Fig. 36. ^{13}C NMR spectrum of compound CMF 2

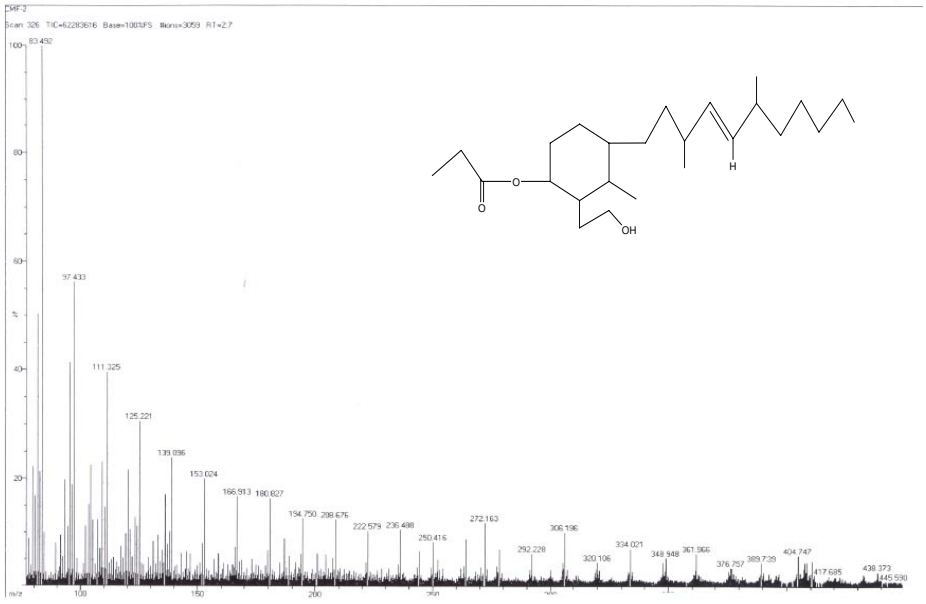


Fig. 37. Mass spectrum of compound CMF 2

Based on the above facts the structure was proposed as below (Fig. 38).

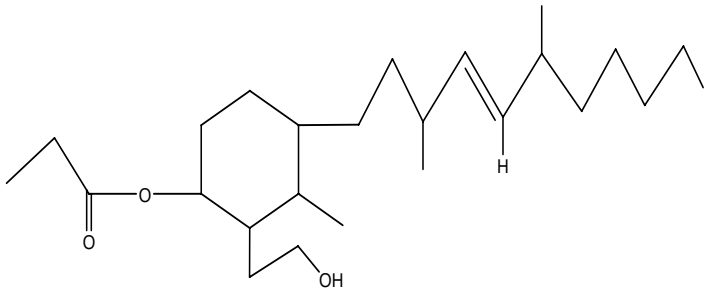


Fig. 38. (CMF 2) 2-(2-hydroxyethyl)-3-methyl-4-[(E)-3, 4, 6-trimethyl undec-4-ethyl] cyclohexyl propionate

5.6.1.5.3. Compound 9 (CMF 5)

5.6.1.5.3.1. Homogeneity

The homogeneity of compound CMF 5 was proved by a single spot on TLC using silica gel G as adsorbent and solvent mixtures of different polarities as developers. The solvent systems used and the corresponding R_f values are given in Table 17.

Table 17. TLC profile of compound CMF 5

Mobile Phase	Solvent ratio	R_f value
Chloroform: methanol: water	50:45:5	0.72
Ethyl acetate: methanol	70:30	0.63
Chloroform: methanol: formic acid	60:38:2	0.47

5.6.1.5.3.2. Melting point of compound CMF5

The melting point of compound CMF 5 was found to be 256 ºC.

5.6.1.5.3.3. Spectral studies

The ^{1}H NMR spectrum exhibited a triplet at δ 1.10 and a methylene signal at δ 1.80 showing the presence of the aliphatic short chain system. The strong signal at δ 2.50 for twelve protons suggests the presence of four methoxy groups. The signals at δ 7.20 (1H, s, H-5'), 6.41 and 6.34 (d, 2H, H-2', H-3'), 6.20 (1H, s, H-6) and at δ 6.07 (1H, s, H-8) supports the presence of the flavanoid moiety. The signals at δ 5.20 (1H) and 4.70 (2H) showed the presence of three anomeric

protons. The complex of signals between δ 3.29 and 3.89 exhibits the presence of three glucose units (Fig. 39).

The ^{13}C NMR spectrum exhibited two signals at δ 182.32 and 175.15 showing the presence of two carbonyl groups. The signals at δ 156.84, 159.57 (each for two carbon atoms), 161.80 and 164.47 indicating the presence of phenolic carbon atoms and the signals at 115.51, 120.88, 128.05, 102.58, 97.55 and 95.20 suggests the presence of a flavanoid nucleus. The signals at 102.31, 99.98 and 94.93 were characteristic of anomeric carbon atoms. In addition to this a couple of signals between 60.66 and 80.55 indicate the presence of three glycosidic moieties. The signals at δ 53.31 (for three carbons) and 59.16 suggest the presence of four methoxy groups. One of the carbonyl group at δ 182.32 may be assigned to the flavanoid nucleus. Apart from this the signals at δ 10.52, 33.10, 38.10 and 42.18 along with unassigned carbonyl signal indicates the presence of a pentanoic acid moiety (Fig. 40 and 41).

The downfield signal at δ 128.04 shows that C-3 is substituted with oxygen function. It is probable that the glucoside moieties are joined one after another at C-3. Absence of phenolic protons in the ^{1}H NMR spectrum and presence of methoxy protons indicate that all the phenolic hydroxyl groups are substituted with methoxy groups. Since all the positions are satisfied with protons and methoxy groups, the probability of the attachment of the pentanoic acid group may not be possible in the flavanoid nucleus. It can be safely placed at the end of the glucosidic moiety.

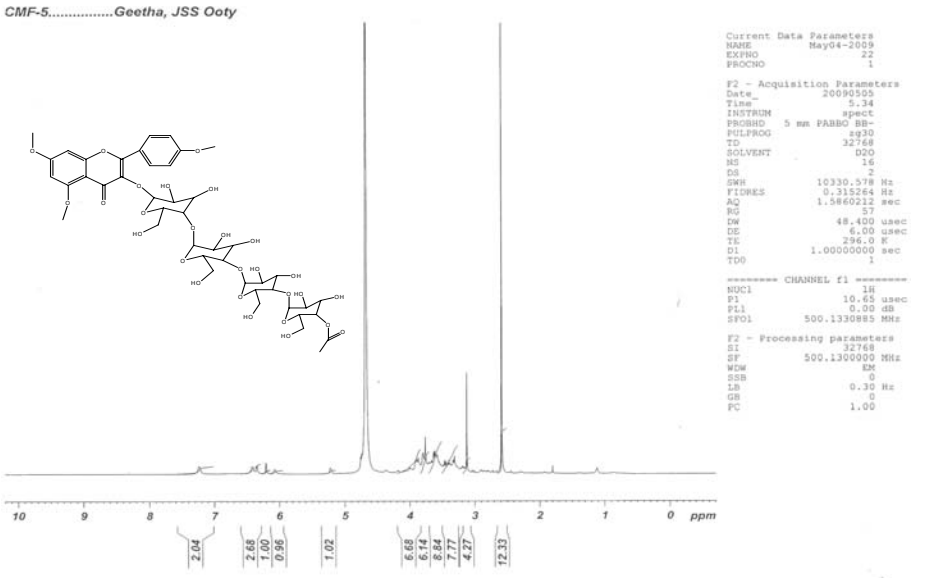


Fig. 39. ^{1}H NMR spectrum of compound CMF 5

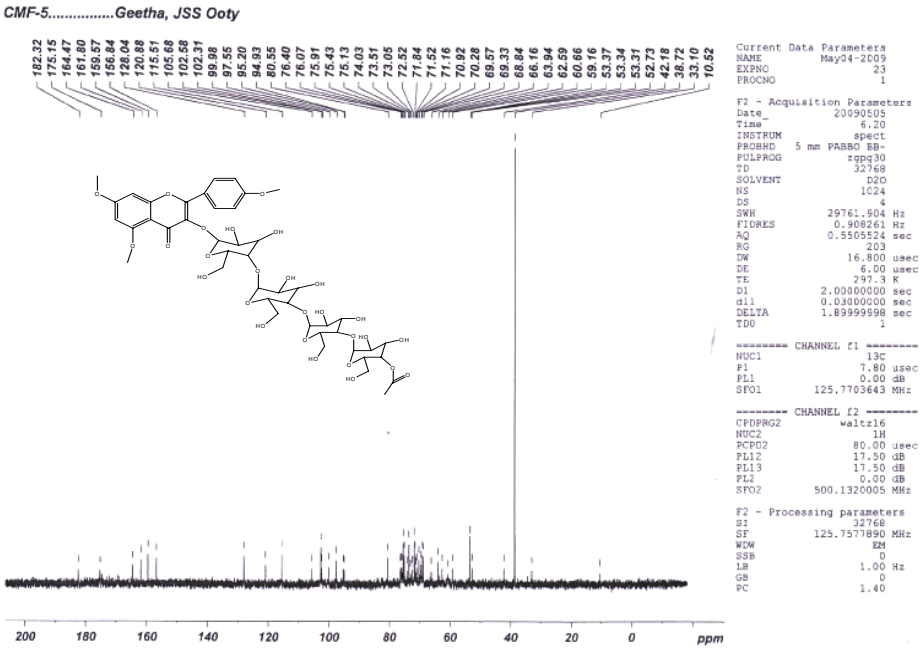


Fig. 40. ^{13}C NMR spectrum of compound CMF 5

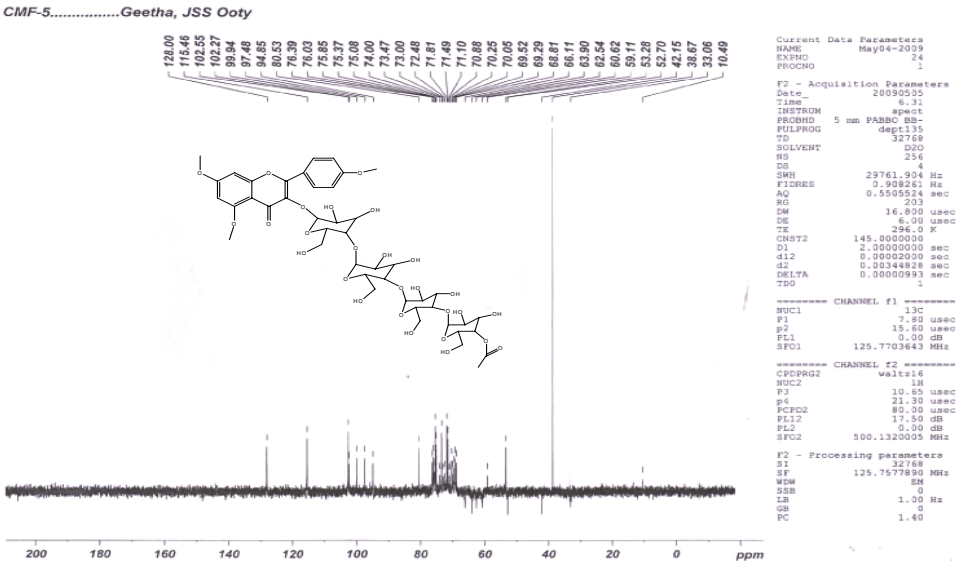


Fig. 41. DEPT of compound CMF 5

Hence, the structure was proposed as below (Fig. 42).

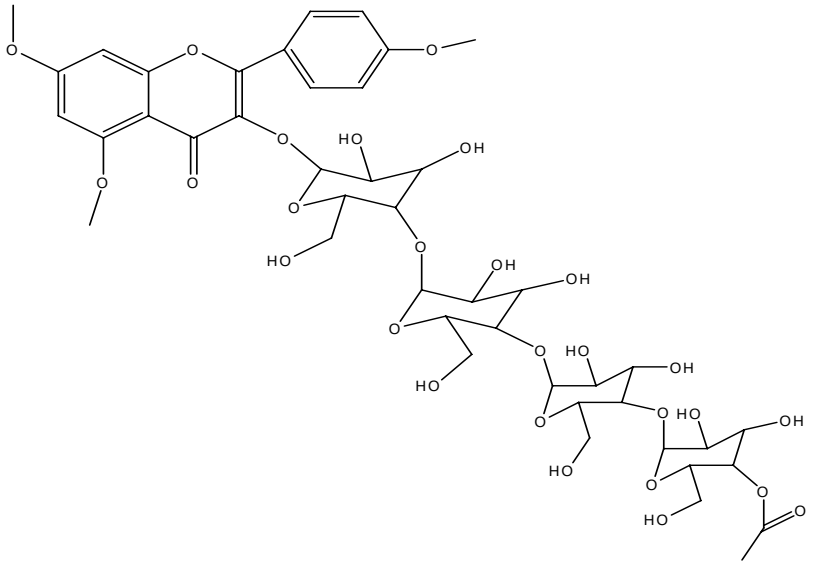


Fig. 42. (CMF 5) Flavone glycoside

5.6.1.6. Solvent solvent extraction of macerated methanol leaves extract

5.6.1.6.1. Compound 10 (ML 1)

5.6.1.6.1.1. Homogeneity

The homogeneity of compound ML 1 was proved by a single spot on TLC using silica gel G as adsorbent and solvent mixtures of different polarities as developers. The solvent systems used and the corresponding R_f values are given in Table 18.

Table 18. TLC profile of compound ML 1

Mobile Phase	Solvent ratio	R_f value
n-hexane: ethyl acetate	95:05	0.52
Petroleum ether: chloroform	60:40	0.74
Petroleum ether: ethyl acetate	75:25	0.64

5.6.1.6.1.2. Melting point of compound ML 1

The melting point of compound ML 1 was found to 136 ºC.

5.6.1.6.1.3. Spectral studies

The compound ML-1 in its ^{1}H NMR spectrum exhibits a peak at δ 1.20 characteristic of the secondary and tertiary methyl groups. The strong singlet at δ 1.60 is characteristic of methylene protons in a ring system. The signal at δ 2.2 is due to the methylene groups attached to a carbonyl group (Fig. 43).

The ^{13}C NMR spectrum exhibits a peak at δ 205 indicating the presence of a carbonyl group. The signal at δ 30 shows the presence of many methylene groups inside a ring system (Fig. 44).

The mass spectrum a molecular ion peak at 262.173 (calculated 262.23) indicating a molecular formula of $C_{18}H_{30}O$. Further the possible fragments were indicated in the scheme (Fig. 45).

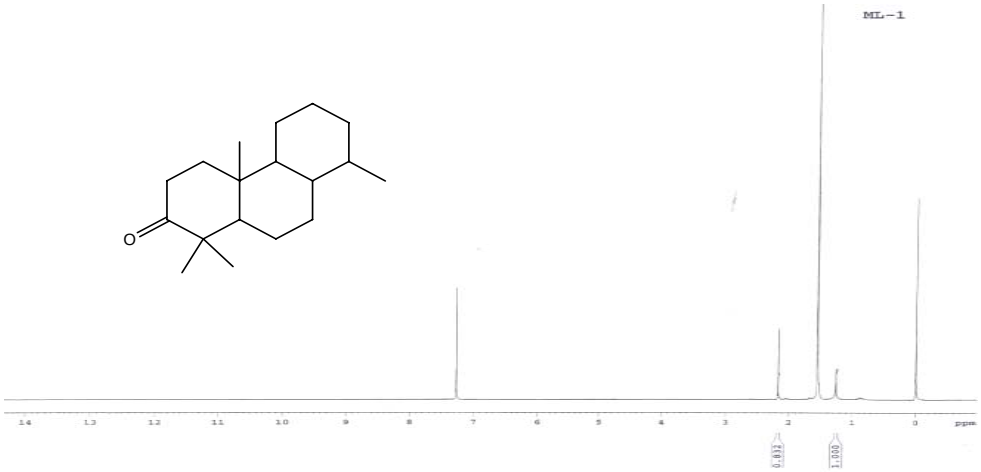


Fig. 43. ^{1}H NMR of compound ML1

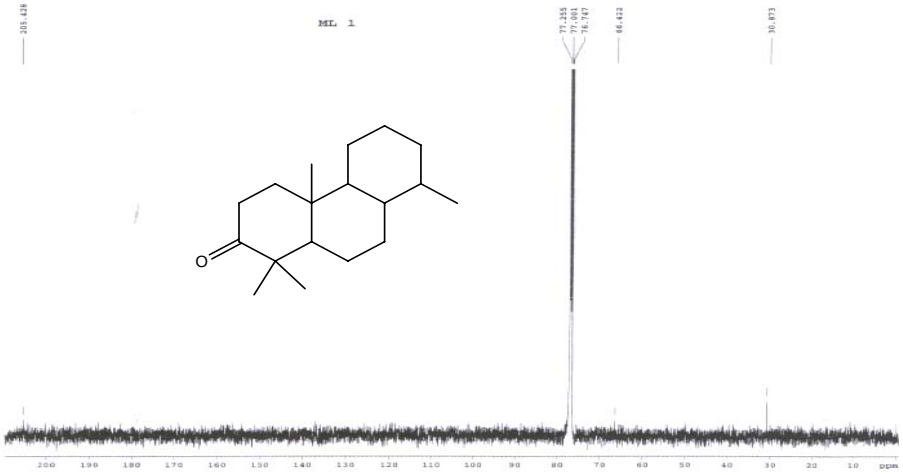


Fig. 44. ^{13}C NMR of compound ML1

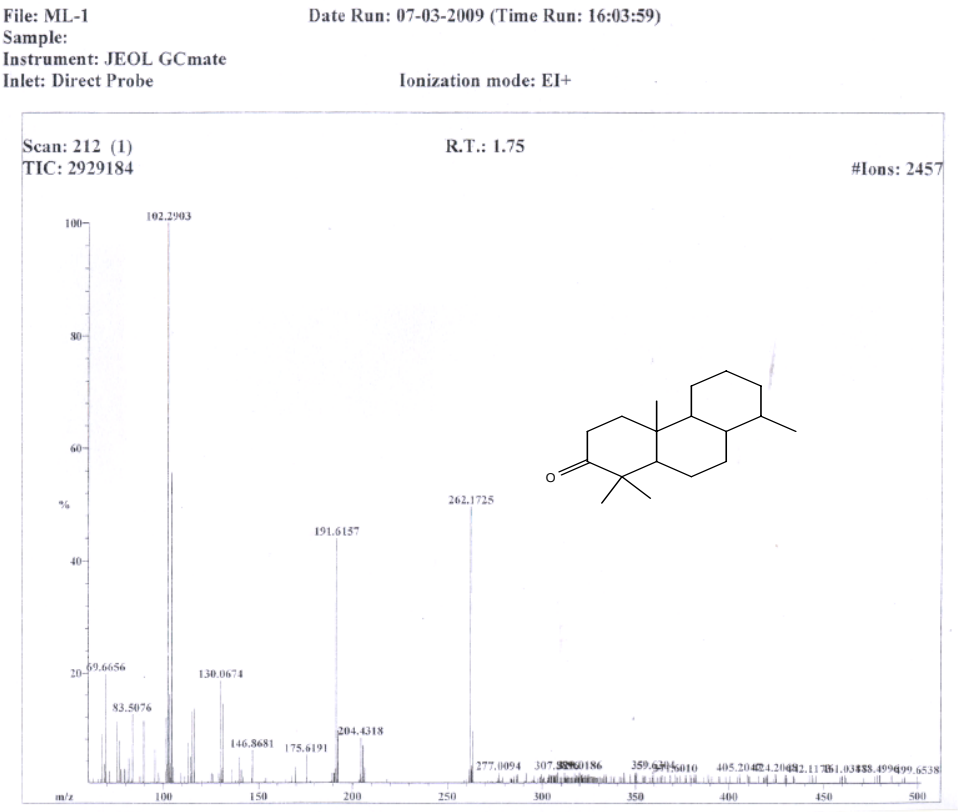


Fig. 45. Mass spectrum of compound ML 1

Based on the above facts the structure was proposed as below (Fig. 46).

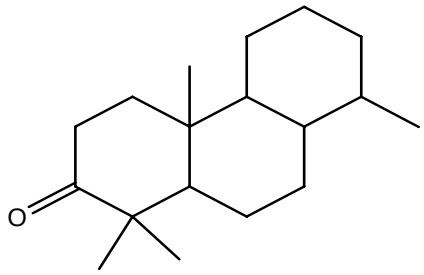


Fig. 46. (ML 1) Decahydro-1, 1, 4a, 8-tetramethyl phenanthren-2

(1H, 3H, 4b H)-one

5.7. *IN VITRO* ANTIOXIDANT ACTIVITY

Among the four successive extracts of stem, the ethyl acetate extract showed potent activity. The IC_{50} values in the DPPH and ABTS method were found to be 19.97 ± 1.30 and 20.97 ± 1.74 µg/ml, respectively. However, the extract was found to be less active than the standards used in all the seven methods (Table 19). Similarly, the ethyl acetate extract of the root showed better antioxidant activity among the four successive extracts. The IC_{50} value in the ABTS method was found to be 4.36 ± 0.38 µg/ml which was found to be less than the standard ascorbic acid (10.92 ± 0.08 µg/ml) and more than the standard rutin (0.71 ± 0.02 µg/ml). However, in all the other methods the extracts showed high IC_{50} values than the standards used indicating its less potency (Table 20).

Among the six methanol extracts, the macerated leaves, crude flowers, stem and root extracts showed potent antioxidant activity in the ABTS method. The IC_{50} values were found to be lower than the standard ascorbic acid indicating their potent activity than the standard ascorbic acid. However, the IC_{50} values were found to be high compared to standard rutin indicating their lesser potency. The crude flower extract also exhibited low IC_{50} values lower than the standards used in p-NDA, deoxy ribose and H_2O_2 methods. The total antioxidant capacity was also found to be higher than the standard α-tocopherol. The crude stem, root and leaves extract also showed low IC_{50} values in H_2O_2 method which are comparable to the IC_{50} values of the standards used. Similar results were observed in the total anti oxidant capacity method. Except this, in all the other methods all the extracts showed less activity than the standards used (Table 21).

Table 19. *In vitro* antioxidant activity of successive stem extracts of *S. kunthianus*

Extracts/ Standards	IC_{50} values ± SEM (µg/ml)[a] by methods						Total antioxidant capacity[b]
	DPPH	ABTS	Nitric oxide	p-NDA	Deoxy Ribose	H_2O_2	
Petroleum ether	>1000	41.03 ± 1.06	>1000	>1000	>1000	242.80 ± 3.41	0.73 ± 0.02
Chloroform	221.70 ± 7.42	40.32 ± 5.10	>1000	>1000	300.30 ± 16.38	>1000	0.85 ± 0.02
Ethyl acetate	19.97 ± 1.30	20.97 ± 1.74	422.40 ± 8.04	193.60 ± 1.46	319.70 ± 10.07	115.40 ± 0.98	2.38 ± 0.03
Methanol	366.70 ± 20.54	26.09 ± 0.80	>1000	340.20 ± 5.17	>1000	117.70 ± 3.20	1.34 ± 0.02
Ascorbic acid	2.71 ± 0.03	10.92 ± 0.08	-	-	-	-	-
Rutin	8.91 ± 0.15	0.71 ± 0.02	67.30 ± 2.81	211.42 ± 3.47	-	38.90 ± 0.18	-
BHA	-	-	-	-	83.24 ± 3.86	25.82 ± 1.91	-
α- tocopherol	-	-	-	-	-	-	3.38 ± 0.08

[a]Average of six determinations, mean ± SEM. [b]The total antioxidant capacity expressed as mM equivalent of ascorbic acid.

Table 20. *In vitro* antioxidant activity of successive root extracts of *S. kunthianus*

Extracts/ Standards	IC_{50} values ± SEM (µg/ml)[a] by methods						Total antioxidant capacity[b]
	DPPH	ABTS	Nitric oxide	p-NDA	Deoxy Ribose	H_2O_2	
Petroleum ether	>1000	42.32 ± 2.12	>1000	>1000	>1000	>1000	0.66 ± 0.01
Chloroform	>1000	28.83 ± 0.40	>1000	282.70 ± 4.38	581.70 ± 12.36	123.80 ± 0.90	2.25 ± 0.04
Ethyl acetate	92.52 ± 2.17	4.36 ± 0.38	217.82 ± 5.02	230.30 ± 5.73	224.10 ± 5.58	58.10 ± 0.98	2.99 ± 0.01
Methanol	163.40 ± 3.74	13.24 ± 0.31	825.70 ± 19.51	234.50 ± 5.30	594.30 ± 0.62	113.70 ± 2.27	2.64 ± 0.05
Ascorbic acid	2.71 ± 0.03	10.92 ± 0.08	-	-	-	-	-
Rutin	8.91 ± 0.15	0.71 ± 0.02	67.30 ± 2.81	211.42 ± 3.47	-	38.90 ± 0.18	-
BHA	-	-	-	-	83.24 ± 3.86	25.82 ± 1.91	-
α- tocopherol	-	-	-	-	-	-	3.38 ± 0.08

[a]Average of six determinations, mean ± SEM. [b]The total antioxidant capacity expressed as mM equivalent of ascorbic acid.

Extracts/ Standards	IC_{50} values ± SEM (µg/ml)[a] by methods	Total

Table 21. ***In vitro*** **antioxidant activity of methanolic extracts of** ***S. kunthianus***

	DPPH	**ABTS**	**Nitric Oxide**	**p-NDA**	**Deoxy Ribose**	**H_2O_2**	**antioxidant capacity[b]**
Macerated leaves extract	>1000	6.33 ± 0.34	641.50 ± 17.00	>1000	143.60 ± 4.66	243.20 ± 1.68	1.00 ± 0.02
Macerated flower extract	>1000	27.43 ± 2.33	>1000	>1000	>1000	72.58 ± 1.44	2.46 ± 0.02
Crude flower extract	58.53 ± 0.92	6.81 ± 0.19	387.60 ± 18.89	56.66 ± 0.94	42.50 ± 3.33	20.78 ± 0.44	5.27 ± 0.02
Crude stem extract	50.22 ± 1.40	7.18 ± 0.12	>1000	226.50 ± 0.94	151.90 ± 10.53	29.15 ± 0.20	3.76 ± 0.03
Crude root extract	57.59 ± 1.00	6.93 ± 0.16	457.70 ± 3.93	231.10 ± 3.44	188.80 ± 25.97	36.46 ± 0.73	3.26 ± 0.04
Crude leaves extract	96.10 ± 1.19	54.03 ± 2.04	>1000	>1000	621.70 ± 18.69	42.46 ± 2.94	2.74 ± 0.02
Ascorbic acid	2.71 ± 0.03	10.92 ± 0.08	-	-	-	-	-
Rutin	8.91 ± 0.15	0.71 ± 0.02	67.30 ± 2.81	211.42 ± 3.47	-	38.90 ± 0.18	-
BHA	-	-	-	-	83.24 ± 3.86	25.82 ± 1.91	-
α- tocopherol	-	-	-	-	-	-	3.38 ± 0.08

[a]Average of six determinations, mean ± SEM. [b]The total antioxidant capacity expressed as mM equivalent of ascorbic acid.

5.8. *IN VITRO* CYTOTOXIC STUDIES

In the cytotoxicity studies, all the extracts of *S. kunthianus* shown CTC_{50} values >100 µg/ml against Hep-2 and HeLa cell lines indicating their weak activity. There is no cytotoxic effect with normal Vero cell lines indicating their safety.

5.9. PHARMACOLOGICAL SCREENING OF CRUDE EXTRACTS

5.9.1. Acute toxicity study of crude methanol extracts of *S. kunthianus*

In the acute toxicity studies, the crude methanol extracts of flower, stem, root and leaves were found to be non toxic and no mortality was observed up to 15 days when administered separately as a single oral dose of up to 2000 mg/kg bw and there were no gross findings of necropsy.

5.9.2. *In vivo* anti-inflammatory activity

All the crude methanol extracts were screened for acute anti-inflammatory studies using standard experimental models.

The effect of the crude methanol extracts of flower, stem, root and leaves and standard ibuprofen on carrageenan, formalin and histamine induced paw edema at different time intervals are depicted in Tables 22-24. Oral administration of all the extracts at 100 and 200 mg/kg bw produced a significant and dose dependent inhibition paw edema after 1 to 3 h of treatment when compared to control in carrageenan, formalin and histamine induced paw oedema methods. However, the values were lesser than the standard ibuprofen when tested at 100 mg/kg bw.

Among all the extracts screened for acute anti-inflammatory studies using carrageenan, formalin and histamine induced methods, the crude methanol flower extract showed a maximum paw edema inhibition in a dose dependent manner. In carrageenan induced method, the percentage inhibitions of crude methanol flower extract at 100 and 200 mg/kg bw after 1 to 3 h were 24.55 ± 1.50 to 42.70 ± 0.82 and 45.50 ± 1.25 to 58.90 ± 0.94, respectively. In formalin induced paw edema, the percentage inhibitions of crude methanol flower extract at 100 and 200 mg/kg bw after 1 to 3 h were 21.70 ± 1.17 to 40.50 ± 0.36 and 30.82 ± 0.63 to 50.76 ± 0.66, respectively. In the histamine induced paw edema, the percentage inhibitions of crude methanol flower extract at 100 and 200 mg/kg bw after 1 to 3 h were found to be 19.92 ± 0.68 to 33.54 ± 0.61 and 30.10 ± 0.67 to 52.99 ± 0.63, respectively (Tables 22-24). Hence, the crude methanol flower extract was screened for sub chronic anti-inflammatory studies using cotton pellet granuloma model.

In the cotton pellet method, crude methanol flower extract showed a dose dependent reduction in both the transudative weight and granuloma tissue formation which was found to be significant ($p<0.001$), when compared to control. The percentage inhibition based on dry weight of the granuloma was found to be 33.35 ± 0.94, 45.60 ± 1.50 and 53.10 ± 1.31 for the three doses 100, 150 and 200 mg/kg bw, respectively. However, anti-inflammatory activity of crude methanol flower extract was less than the standard ibuprofen at 100 mg/kg bw (Table 25).

5.9.3. Analgesic activity by hot plate and tail immersion methods

In the analgesic activity by hot plate method, the crude methanol extract of flower, root, stem and leaves increased their response time dose dependently and significantly, when tested at 100 and 200 mg/kg bw doses. Among the four extracts the flower extract was found to be more potent. The percentage protection at 100 mg/kg bw during 30 to 90 min durations ranged between 60.96 to 173.10%. These values at 200 mg/kg bw were found to be 112.92 to 233.30%. The maximum activity was found to be at 90 min. the leaves extract was found to be the least potent. However, the standard ibuprofen at 100 mg/kg bw produced better activity at all the durations than the extracts (Table 26).

In the tail immersiom method, all the extracts at both the doses showed significant and dose dependant protection indicating their analgesic activity. The crude methanol flower extract at 200 mg/kg bw showed the maximum activity. The percentage protection at 1, 2 and 3 h was found to be 104.55, 180.21 and 250.76, respectively. However, the standard ibuprofen at 100 mg/kg bw exhibited better results than the extracts. The leaves extract was found to be the least active (Table 27).

Table 22. Anti-inflammatory activity of crude methanol extracts of *S. kunthianus* in carrageenan induced paw oedema method

Treatments	Dose (mg/kg)	1 h		2 h		3 h	
		Paw volume (ml)	% Inhibition	Paw volume (ml)	% Inhibition	Paw volume (ml)	% Inhibition
Control	-	0.34 ± 0.00	-	0.59 ± 0.00	-	0.73 ± 0.00	-
Ibuprofen	100	0.19 ± 0.01[a]	43.79 ± 1.71	0.20 ± 0.01[b]	65.62 ± 0.81	0.21 ± 0.01[b]	71.69 ± 0.84
Crude flower extract	100	0.26 ± 0.01[a]	24.55 ± 1.50	0.36 ± 0.00[b]	38.10 ± 0.72	0.42 ± 0.00[b]	42.70 ± 0.82
	200	0.18 ± 0.00[a]	45.50 ± 1.25	0.26 ± 0.00[b]	56.30 ± 0.84	0.30 ± 0.01[b]	58.90 ± 0.94
Crude root extract	100	0.27 ± 0.01[a]	21.10 ± 1.70	0.43 ± 0.00[b]	27.27 ± 0.36	0.45 ± 0.01[b]	38.59 ±1.14
	200	0.23 ± 0.01[a]	33.43 ± 1.70	0.34 ± 0.01[b]	42.32 ± 1.02	0.39 ± 0.00[b]	47.04 ± 0.46
Crude stem extract	100	0.27 ± 0.01[a]	20.61 ± 1.41	0.44 ± 0.00[b]	24.43 ± 0.57	0.50 ± 0.00[b]	31.74 ± 1.08
	200	0.22 ± 0.01[a]	35.41 ± 2.69	0.39 ± 0.00[b]	34.37 ± 0.73	0.45 ± 0.00[b]	38.59 ± 0.82
Crude leaves extract	100	0.28 ± 0.01[a]	16.67 ± 1.78	0.47 ± 0.00[b]	19.88 ± 1.46	0.51 ± 0.01[b]	29.91 ± 0.65
	200	0.25 ± 0.00[a]	26.53 ± 0.91	0.41 ± 0.01[b]	30.39 ± 0.81	0.42 ± 0.01[b]	42.24 ± 0.65

Values are given as mean ± S.E.M. for groups of six animals each; values are statistically significant at [a]$p<0.01$ and [b]$p< 0.001$. Control Vs treated groups.

Table 23. Anti-inflammatory activity of crude methanol extracts of *S. kunthianus* in formalin induced paw edema method

Treatments	Dose (mg/kg)	1 h		2 h		3 h	
		Paw volume (ml)	% Inhibition	Paw volume (ml)	% Inhibition	Paw volume (ml)	% Inhibition
Control	-	0.53 ± 0.00	-	0.74 ± 0.01	-	0.86 ± 0.01	-
Ibuprofen	100	0.33 ± 0.00[a]	38.65 ± 0.81	0.33 ± 0.00[a]	55.40 ± 0.49	0.33 ± 0.00[a]	62.02 ± 0.49
Crude flower extract	100	0.40 ± 0.01[a]	21.70 ± 1.17	0.50 ± 0.00[a]	31.96 ± 0.58	0.51 ± 0.00[a]	40.50 ± 0.36
	200	0.37 ± 0.00[a]	30.82 ± 0.63	0.41 ± 0.01[a]	44.14 ± 0.67	0.42 ± 0.01[a]	50.76 ± 0.66
Crude root extract	100	0.45 ± 0.00[a]	15.09 ± 0.69	0.56 ± 0.00[a]	24.32 ± 0.49	0.56 ± 0.01[a]	34.49 ± 0.71
	200	0.39 ± 0.00[a]	26.73 ± 0.76	0.46 ± 0.00[a]	37.83 ± 0.49	0.48 ± 0.00[a]	44.18 ± 0.42
Crude stem extract	100	0.50 ± 0.00	05.97 ± 0.58	0.65 ± 0.01[a]	12.39 ± 0.64	0.67 ± 0.01[a]	21.71 ± 0.58
	200	0.46 ± 0.01[a]	13.83 ± 1.05	0.61 ± 0.00[a]	18.24 ± 0.58	0.58 ± 0.01[a]	32.17 ± 0.65
Crude leaves extract	100	0.49 ± 0.00	08.17 ± 0.63	0.60 ± 0.01[a]	18.46 ± 0.67	0.61 ± 0.00[a]	29.06 ± 0.43
	200	0.45 ± 0.00[a]	16.05 ± 0.82	0.51 ± 0.01[a]	31.53 ± 0.75	0.54 ± 0.00[a]	37.59 ± 0.49

Values are given as mean ± S.E.M. for groups of six animals each; values are statistically significant at [a]$p < 0.001$. Control Vs treated groups.

Table 24. Anti -inflammatory activity of crude methanol extracts of *S. kunthianus* in histamine induced paw edema method

Treatments	Dose (mg/kg)	1 h		2 h		3 h	
		Paw volume (ml)	% Inhibition	Paw volume (ml)	% Inhibition	Paw volume (ml)	% Inhibition
Control	-	0.46 ± 0.01	-	0.65 ± 0.01	-	0.78 ± 0.00	-
Ibuprofen	100	0.28 ± 0.00[a]	39.48 ± 0.67	0.29 ± 0.00[a]	55.13 ± 0.47	0.29 ± 0.00[a]	62.39 ± 0.43
Crude flower extract	100	0.37 ± 0.00[a]	19.92 ± 0.68	0.47 ± 0.00[a]	27.18 ± 0.50	0.52 ± 0.00[a]	33.54 ± 0.61
	200	0.32 ± 0.00[a]	30.10 ± 0.67	0.37 ± 0.00[a]	43.90 ± 0.66	0.37 ± 0.01[a]	52.99 ± 0.63
Crude root extract	100	0.41 ± 0.00	11.23 ± 0.67	0.51 ± 0.00[a]	21.50 ± 0.56	0.58 ± 0.00[a]	25.40 ± 0.39
	200	0.36 ± 0.00[a]	22.46 ± 0.72	0.43 ± 0.01[a]	34.36 ± 0.86	0.43 ± 0.00[a]	45.08 ± 0.51
Crude stem extract	100	0.43 ± 0.00	6.52 ± 0.80	0.55 ± 0.01[a]	15.13 ± 0.73	0.61 ± 0.01[a]	20.10 ± 0.43
	200	0.39 ± 0.01[a]	14.50 ± 1.07	0.47 ± 0.00[a]	27.69 ± 0.56	0.47 ± 0.00[a]	39.50 ± 0.52
Crude leaves extract	100	0.43 ± 0.00	7.60 ± 0.49	0.55 ± 0.01[a]	15.10 ± 1.22	0.61 ± 0.00[a]	22.01 ± 0.40
	200	0.38 ± 0.01[a]	17.6 ± 1.04	0.45 ± 0.00[a]	30.51 ± 0.62	0.47 ± 0.00[a]	40.39 ± 0.55

Values are given as mean ± S.E.M. for groups of six animals each; values are statistically significant at [a]$p < 0.001$. Control Vs treated groups.

Table 25. Anti-inflammatory activity of crude methanol flower extract of *S. kunthianus* in cotton pellet granuloma method

Treatments	Dose (mg/kg)	Weight of granuloma (mgs)		% Inhibition (based on dry weight)
		Wet weight	Dry weight	
Control	-	190.60 ± 0.99	94.57 ± 0.57	-
Ibuprofen	100	63.65 ± 1.29[a]	32.25 ± 0.58[a]	65.91 ± 0.61
Crude methanol flower extract	100	120.50 ± 3.47[a]	63.08 ± 0.89[a]	33.35 ± 0.94
	150	109.70 ± 3.50[a]	51.47 ± 1.40[a]	45.60 ± 1.50
	200	91.25 ± 2.92[a]	43.48 ± 0.75[a]	53.10 ± 1.31

Values are given as mean ± S.E.M. for groups of six animals each; values are statistically Significant at [a]$p<0.001$. Control Vs treated groups.

Table 26. Analgesic activity of crude methanol extracts of *S. kunthianus* in hot plate method

Treatments	Dose (mg/kg)	Latency period in sec (% protection)				
		0 min	30 min	60 min	90 min	120 min
Control	-	3.67 ± 0.03	3.56 ± 0.03	3.54 ± 0.04	3.42 ± 0.01	3.66 ± 0.02
Ibuprofen	100	3.92 ± 0.03	10.66 ± 0.08[b] (199.43)	12.13 ± 0.02[b] (242.43)	13.25 ± 0.07[b] (287.43)	13.65 ± 0.04[b] (272.95)
Crude flower extract	100	3.61 ± 0.03	5.73 ± 0.05[b] (60.96)	8.34 ± 0.06[b] (135.59)	9.34 ± 0.07[b] (173.1)	9.86 ± 0.02[b] (169.4)
	200	3.56 ± 0.03	7.58 ± 0.09[b] (112.92)	10.41 ± 0.12[b] (194.07)	11.40 ± 0.07[b] (233.3)	11.74 ± 0.08[b] (220.77)
Crude root extract	100	3.55 ± 0.07	4.88 ± 0.02[b] (37.08)	6.21 ± 0.04[b] (75.42)	7.37 ± 0.03[b] (115.5)	7.85 ± 0.02[b] (114.48)
	200	3.51 ± 0.44	6.46 ± 0.11[b] (84.57)	9.66 ± 0.07[b] (172.88)	10.06 ± 0.05[b] (194.15)	10.63 ± 0.05[b] (190.44)
Crude stem extract	100	3.50 ± 0.04	4.08 ± 0.04[b] (14.61)	5.75 ± 0.04[b] (62.43)	6.70 ± 0.03[b] (95.91)	7.02 ± 0.02[b] (91.80)
	200	3.66 ± 0.44	5.59 ± 0.05[b] (57.02)	8.04 ± 0.03[b] (127.12)	8.35 ± 0.03[b] (144.15)	9.24 ± 0.06[b] (152.46)
Crude leaves extract	100	3.58 ± 0.04	3.96 ± 0.04[a] (11.24)	5.26 ± 0.04[b] (48.59)	6.13 ± 0.02[b] (79.24)	6.62 ± 0.04[b] (80.87)
	200	3.64 ± 0.05	5.29 ± 0.03[b] (48.6)	7.13 ± 0.03[b] (101.41)	7.81 ± 0.05[b] (128.36)	8.08 ± 0.02[b] (120.77)

Values are given as mean ± S.E.M. for groups of six animals each; values are statistically significant at [a]$p<0.01$ and [b]$p< 0.001$. Control Vs treated groups.

Table 27. Analgesic activity of crude methanol extracts of *S. kunthianus* in tail immersion method

Treatments	Dose (mg/kg)	Latency period in sec (% protection)			
		0 h	1 h	2 h	3 h
Control	-	1.88 ± 0.06	1.98 ± 0.09	1.92 ± 0.07	1.97 ± 0.07
Ibuprofen	100	1.98 ± 0.10	6.12 ± 0.09[b] (209.09)	7.18 ± 0.07[b] (273.96)	8.10 ± 0.08[b] (311.17)
Crude flower extract	100	2.05 ± 0.08	3.08 ± 0.08[b] (55.55)	4.55 ± 0.06[b] (136.98)	5.30 ± 0.11[b] (136.98)
	200	2.05 ± 0.08	4.05 ± 0.06[b] (104.55)	5.38 ± 0.05[b] (180.21)	6.91 ± 0.08[b] (250.76)
Crude root extract	100	1.97 ± 0.07	2.56 ± 0.08[b] (29.29)	3.66 ± 0.07[b] (90.63)	4.18 ± 0.06[b] (112.18)
	200	2.00 ± 0.06	3.10 ± 0.05[b] (56.57)	4.15 ± 0.04[b] (116.15)	5.00 ± 0.06[b] (153.81)
Crude stem extract	100	1.90 ± 0.07	2.30 ± 0.05[a] (16.16)	2.90 ± 0.04[b] (51.04)	3.30 ± 0.04[b] (67.51)
	200	1.90 ± 0.06	2.80 ± 0.07[b] (141.41)	3.50 ± 0.03[b] (182.29)	4.10 ± 0.04[b] (208.12)
Crude leaves extract	100	1.98 ± 0.05	2.50 ± 0.04[b] (26.26)	2.88 ± 0.06[b] (50.0)	3.40 ± 0.05[b] (72.59)
	200	1.90 ± 0.06	2.90 ± 0.04[b] (46.46)	3.50 ± 0.08[b] (82.29)	4.50 ± 0.06[b] (128.43)

Values are given as mean ± S.E.M. for groups of six animals each; values are statistically significant at [a]p<0.01 and [b]p<0.001. Control Vs treated groups.

5.9.4. Antioxidant and Hepatoprotective effect of crude methanol flower extract against CCl_4 induced liver injury in rats

The serum levels of hepatic enzymes, such as ASAT, ALAT, ALP and TGL, TC, CR, TB and TBARS used as biochemical parameters for evaluation of early hepatic injury were significantly elevated and the levels of TP, albumin, CAT and SOD were significantly decreased in the CCl_4 treated animals.

The administration of the crude methanol flower extract at 100, 150 and 200 mg/kg bw dose to CCl_4 intoxicated rats significantly inhibited the increased levels of ASAT, ALAT, ALP, TGL, TC, TB and TBARS towards the control when compared to CCl_4 treated animals. There was a significant increase in the levels of CR, TP and albumin towards the control. The high dose at 200 mg/kg bw also exhibited a significant reversal of all the changes caused by CCl_4 administration towards the control (Table 28). However, the standard silymarin treatment at 100 mg/kg bw dose showed better result than crude methanol flower extract.

The CCl_4 treatment caused a significant decrease in the levels of antioxidant enzymes CAT, SOD, and a significant increase in the levels of TBARS in serum, liver and kidney when compared to control. The treatment with the extract at 100 mg/kg bw to CCl_4 intoxicated rats exhibited a significant reversal of CAT, SOD and TBARS in serum, liver and kidney towards the normal when compared to CCl_4 treated. The high dose of extract, 200 mg/kg bw also produced significant changes in all these biochemical parameters when compared to CCl_4 treated. The standard silymarin at 100 mg/kg bw also produced similar significant results, except the TBARS in kidney (Table 29). The extract treatment at 200 mg/kg bw

was found to be almost similar to the standard silymarin in reversing most of the biochemical parameters towards the control.

Histopathological examination of liver sections of normal animals showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces and a central vein (Fig. 47a). The liver sections of the rats of CCl_4 treated group showed disarrangement of normal hepatic cells with high degree of damage, characterized by the centrilobular necrosis, focal necrosis and bile duct proliferation (Fig. 47b). The sections of the rats treated with crude methanol flower extract at 100, 150 and 200 mg/kg bw and intoxicated with CCl_4 exhibited less centrilobular necrosis and bile duct proliferation compared to the CCl_4 control (Fig. 47c-e). Dose dependent results were observed and the high dose (200 mg/kg) showed better activity. The standard silymarin at 100 mg/kg bw and CCl_4 treated animals showed almost normal architecture of the liver with few centrilobular fatty changes and bile duct proliferation (Fig. 47f).

Kidney sections of CCl_4 control group showed high degree of tubulointerstitial nephritis compared to normal animals showed normal histological appearance (Fig. 48 b). The sections of the animals belonging to the crude methanol flower extract at all the doses and the standard silymarin treatment groups showed normal histological appearance (Fig. 48 c-f). These findings clearly indicate that the liver and kidney tissues, which were damaged by CCl_4 intoxication showed recovery with the crude methanol flower extract and silymarin treatments.

Table 28. Effect of crude methanol flower extract on biochemical parameter in CCl_4 induced toxicity in rats

Treatments	Dose mg/kg	ASAT	ALAT	ALP	TP	TGL	TC	CR	TB	Albumin
Control	-	97.17 ± 3.27	82.00 ± 2.21	156.50 ± 2.98	6.67 ± 0.21	85.83 ± 2.21	105.30 ± 3.40	0.70 ± 0.04	0.60 ± 0.04	4.32 ± 0.14
CCl_4 treated		313.70 ± 3.99[c]	230.80 ± 3.30[c]	521.30 ± 3.24[c]	2.50 ± 0.22[c]	204.20 ± 3.08[c]	237.70 ± 3.28[c]	4.03 ± 0.15[c]	1.75 ± 0.04[c]	2.48 ± 0.12[c]
Silymarin	100	109.20 ± 2.82[a]	93.33 ± 2.25[a]	177.80 ± 3.02[a]	6.50 ± 0.22[a]	107.50 ± 2.41[a]	125.50 ± 3.64[a]	1.05 ± 0.08[a]	0.80 ± 0.04[a]	4.02 ± 0.05[a]
Crude methanol flower extract + CCl_4	100	177.50 ± 3.30[a]	166.30 ± 3.45[a]	255.00 ± 4.02[a]	5.50 ± 0.22[a]	175.70 ± 2.95[a]	182.50 ± 2.96[a]	2.87 ± 0.07[a]	1.45 ± 0.04[a]	3.12 ± 0.06 [b]
	150	152.00 ± 3.14[a]	138.50 ± 2.88[a]	230.70 ± 4.21[a]	6.12 ± 0.17[a]	142.00 ± 2.99[a]	153.30 ± 1.94[a]	1.67 ± 0.10[a]	1.22 ± 0.03[a]	3.55 ± 0.15[a]
	200	126.80 ± 1.91[a]	108.70 ± 2.96[a]	201.70 ± 4.89[a]	6.67 ± 0.21[a]	121.50 ± 2.26[a]	134.00 ± 2.60[a]	1.17 ± 0.09[a]	0.83 ± 0.03[a]	4.18 ± 0.09[a]

Values are given as mean ± S.E.M. for groups of six animals each; values are statistically significant at [b]$p<0.01$, [a]$p< 0.001$. CCl_4 Vs treated groups. [c]$p<0.001$ between CCl_4 treated Vs control.

Table 29. Effect of crude methanol flower extract of *S. kunthianus* on SOD, Catalase and TBARS in CCl_4 induced toxicity in rats

Treatments	Dose (mg/kg)	SOD (Unit/min/mg of tissue)			Catalase (IU/min/mg of tissue)			LPO (n mole of MDA/mg of tissue)		
		Serum	Liver	Kidney	Serum	Liver	Kidney	Serum	Liver	Kidney
Control	-	0.36 ± 0.01	0.38 ± 0.01	0.29 ± 0.01	1.35 ± 0.03	4.12 ± 0.05	1.26 ± 0.02	3.49 ± 0.09	4.48 ± 0.06	5.64 ± 0.05
CCl_4 treated	-	0.17 ± 0.01[c]	0.24 ± 0.01[c]	0.12 ± 0.00[c]	0.36 ± 0.01[c]	2.27 ± 0.04[c]	0.93 ± 0.02[c]	4.50 ± 0.07[c]	6.77 ± 0.06[c]	7.20 ± 0.04[c]
Silymarin	100	0.32 ± 0.01[a]	0.34 ± 0.01[a]	0.27 ± 0.00[a]	1.41 ± 0.01[a]	3.72 ± 0.07[a]	1.12 ± 0.02[a]	3.27 ± 0.06[a]	4.74 ± 0.06[a]	5.80 ± 0.04[a]
Crude methanol flower extract + CCl_4	100	0.22 ± 0.01[a]	0.28 ± 0.00[b]	0.18 ± 0.00[a]	1.06 ± 0.04[a]	2.85 ± 0.05[a]	0.97 ± 0.02	4.03 ± 0.03[a]	5.78 ± 0.03[a]	6.91 ± 0.03[a]
	150	0.25 ± 0.01[a]	0.31 ± 0.00[a]	0.22 ± 0.00[a]	1.23 ± 0.02[a]	3.30 ± 0.06[a]	1.10 ± 0.02[a]	3.55 ± 0.02[a]	5.13 ± 0.04[a]	6.08 ± 0.02[a]
	200	0.31 ± 0.01[a]	0.33 ± 0.01[a]	0.25 ± 0.01[a]	1.29 ± 0.02[a]	3.58 ± 0.05[a]	1.22 ± 0.01[a]	3.42 ± 0.04[a]	4.89 ± 0.04[a]	5.89 ± 0.02[a]

Values are given as mean ± S.E.M. for groups of six animals each; values are statistically significant at [b]$p<0.05$, [a]$p<0.001$. CCl_4 Vs treated groups. [c]$p<0.001$ between CCl_4 treated Vs control.

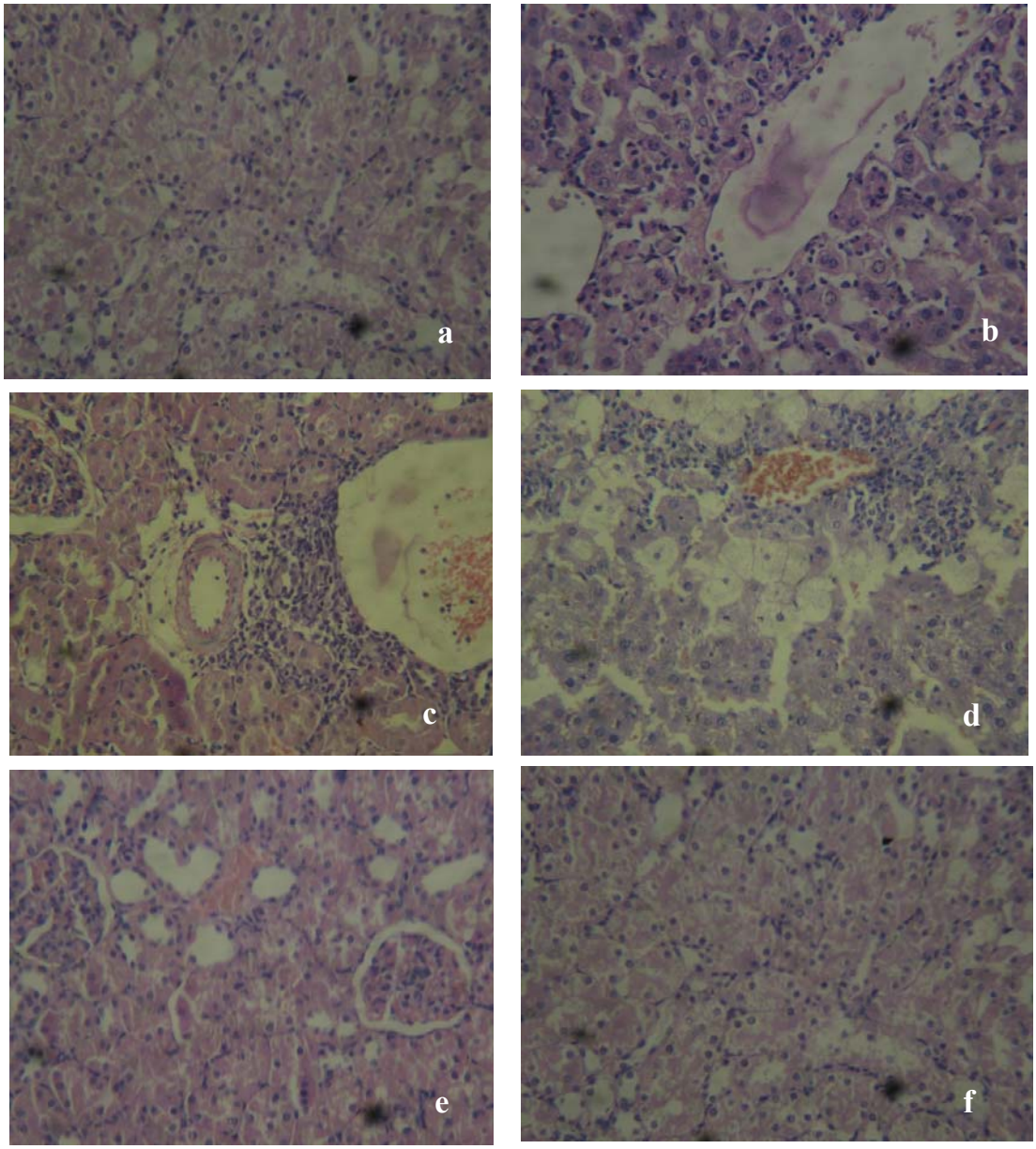


Fig. 47. Histopathological changes occurred in the liver after CCl_4 intoxication and prevention by the treatment with crude methanol flower extract (hematoxylin and eosin, 400x). (a) Control (b) CCl_4 treated (c) Crude methanol flower extract (100 mg/kg) + CCl_4 (d) Crude methanol flower extract (150 mg/kg) + CCl_4 (e) Crude methanol flower extract (200 mg/kg) + CCl_4 (f) Silymarin (100 mg/kg) + CCl_4.

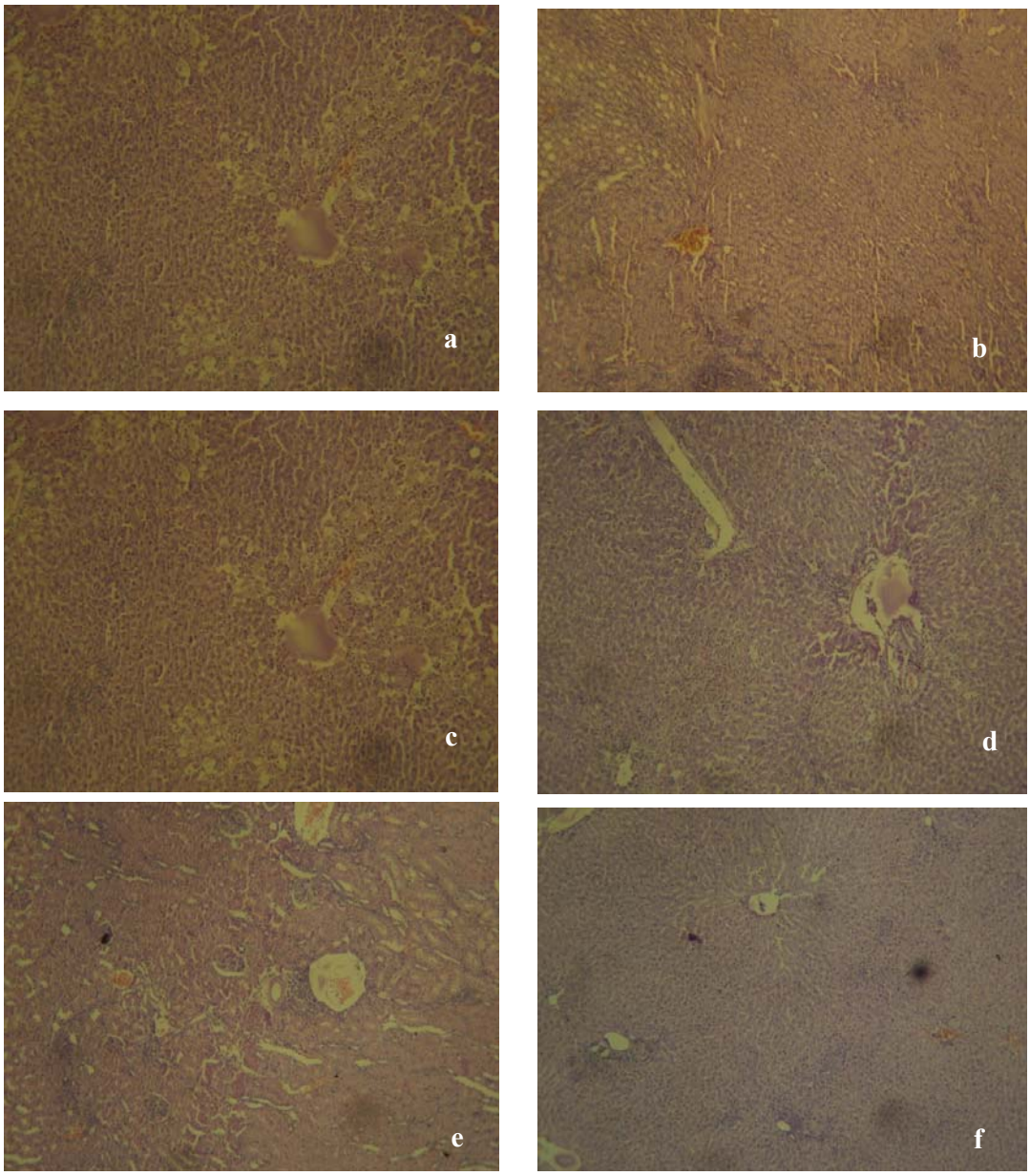


Fig. 48. Histopathological changes occurred in the kidney after CCl_4 intoxication and prevention by the treatment with crude methanol flower extract (hematoxylin and eosin, 400x). (a) Control (b) CCl_4 treated (c) Crude methanol flower extract (100 mg/kg) + CCl_4 (d) Crude methanol flower extract (150 mg/kg) + CCl_4 (e) Crude methanol flower extract (200 mg/kg) + CCl_4 (f) Silymarin (100 mg/kg) + CCl_4.

Plants have been an integral part of the ancient culture of India, China and Egypt as medicine, and their importance even dates back to the Neanderthal period (Saklani and Kutty, 2008). Terrestrial plants, especially higher plants, have a long history of use in the treatment of human diseases. Historical experiences with plants as therapeutic tools have helped to introduce effective, safe and novel chemical entities in modern medicine. Biologically active plant-derived chemicals can be expected to play an increasingly significant role in the commercial development of new drugs, especially when we consider that most species of higher plants have never been described, much less surveyed for chemical or biologically active constituents, and new sources of commercially valuable materials remain to be discovered (Balandrin et al., 1985). In spite of the current preoccupation with synthetic chemistry as a vehicle to discover and manufacture drugs, the potential of plants to disease treatment and prevention is still enormous (Raskin et al., 2002). Hence, in the present study we were interested in natural products research.

Species of *Strobilanthes* grow wild on the Nilgiri ranges between 6000 to 7000 feet. The genus *Strobilanthes* is known for its various biological activities (Sunarto, 1977; Kusumoto et al., 1992; Ismail et al., 2000; Jaksa et al., 2004; Rahmat et al., 2006; Nair et al., 1988). *S. kunthianus* (Neelakurinji) is a shrub in the grasslands of Western Ghats in India. The Nilgiris, which literally means the blue mountains got its name from the purplish blue flowers of Neelakurinji that blossoms gregariously once in twelve years. Lupeol has been isolated from the whole plant. Except this study, so far no phytochemical investigations and

biological activities were carried out. Hence, in the present study we were interested to carry out detailed phytochemical and biological investigations in *S. kunthianus*.

Quality and reproduction of the total spectrum of constituents of the herbal drugs are extremely important. The herbal raw material often show a natural variability due to many external influences such as climate, soil quality, harvesting and drying conditions, with the consequence that the qualitative and quantitative composition varies from batch to batch from harvest to harvest, in particular. Plant identity can be achieved by macro and microscopical examination. Microscopical evaluation is indispensable in the initial identification of herbs, as well as in identifying small fragments of crude or powdered herbs and detection of foreign matter and adulterants. Hence, pharmacognostical studies were carried out with a focus on bringing out diagnostic character will be of immense help in proper identification, which play an important role in the standardization of plant materials. A detailed study of microscopic characters of all the parts viz leaves, flower, stem and root along with the photographs of the special characters taken during the study serves in identifying the plant. The microscopic features of leaves, lamina, petiole, stem, root, xylem, pith and vein islet were studied. The powder microscopy was also studied.

Determination of physicochemical constants is important for the purpose of evaluation of crude drugs. The total ash, water soluble ash, sulphated ash and alcohol soluble extractive values of the leaves of *S. kunthianus* were found to be

high when compared to the other plant materials. The acid insoluble ash was found to be high in root and water soluble extractive value in flowers.

Preliminary phytochemical analysis is used for the purpose of evaluation of crude drugs. The four successive extracts of root and stem and six methanol extracts were tested for their phytoconstituents. Phenolics, steroids and triterpenoids were found in successive petroleum ether and chloroform extracts of stem and root. Glycosides and phenolics were found in ethyl acetate and methanol stem and root extracts and all the methanol extracts. Phenols are responsible for the majority of the oxygen capacity in most plant derived products (Aniya et al., 2005). With few exceptions such as carotene, the antioxidants in food are phenols. Hence the total phenol content of all the extracts was estimated. The crude methanol flower extract was found to contain very high total phenol content among all the extracts.

Isolation of natural products from plant constitutes a chemical research project with potentially significant benefits to mankind. Column chromatography in phytochemistry is a method to purify individual chemical compounds from mixtures of compounds. In order to isolate novel bioactive compounds from extracts column chromatography, studies of six extracts were carried out. The purity of isolated compound was established by TLC and characterization was done by spectral analysis viz. NMR and mass spectra.

From the spectral data the compounds isolated and identified from successive petroleum ether root extract were lupeol and betulin. Lupeol was also isolated from petroleum ether stem extract. 3,5-bis (dimethyl carbamoyl) methyl-4- (11-

cyclo hexylundecyl)-4-heptyl- N^1,N^1,N^7,N^7tetra methyl heptane diamide was isolated from chloroform stem extract. α-Amyrin and 4-amino-tetrahydro-2H-pyran-3,5-diol were isolated from macerated methanol flower extract. β-Sitosterol, 2-(2-hydroxyethyl)-3-methyl-4-[(E)-3, 4, 6-trimethyl undec-4-ethyl] cyclohexyl propionate and a flavone glycoside were isolated from crude methanol flower extract. Decahydro-1, 1, 4a, 8-tetramethyl phenanthren-2 (1H, 3H, 4b H)-one was isolated from macerated methanol leaves extract. Except lupeol all the other compounds were isolated for the first time from *S. kunthianus.*

Majority of the diseases/disorders are mainly linked to oxidative stress due to free radicals (Gutteridgde, 1995). Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism (Tiwari, 2001). The most common reactive oxygen species (ROS) include superoxide ($O_2^{\cdot}$) anion, hydrogen peroxide (H_2O_2), peroxyl ($ROO^{\cdot}$) radicals and reactive hydroxyl ($OH^{\cdot}$) radicals. The nitrogen derived free radicals are nitric oxide ($NO^{\cdot}$) and peroxynitrite anion ($ONOO^{\cdot}$). ROS have been implicated in over a hundreds of diseases states which range from arthritis and connective tissue disorders to carcinogenesis, aging, physical injury, infection and acquired immunodeficiency syndrome (Joyce, 1987). In treatment of these diseases, antioxidant therapy has gained an immense importance. Current research is now directed towards finding naturally occurring antioxidants of plant origin. Antioxidants have been reported to prevent oxidative damage by free radical and ROS, and may prevent the occurrence of disease, cancer and aging. It can interfere with the oxidation process by reacting with free radicals, chelating, catalytic metals, and also by acting as oxygen scavengers (Buyukokuroglu et al., 2001; Shahidi and Wanasundara,

1992). Plant and plant products are being used as a source of medicine since long. The medicinal properties of plants have been investigated in the recent scientific developments. throughout the world, due to their potent antioxidant activities, no side effects and economic viability (Auudy et al., 2003). Flavonoids and phenolic compounds widely distributed in plants which have been reported to exert multiple biological effect, including antioxidant, free radical scavenging abilities, anti-inflammatory, anticarcinogenic. etc. (Miller, 1996). They were also suggested to be a potential iron chelator (Boyer et al., 1988; Havsteen, 1983). Novel natural antioxidants from some plants have been extensively studied in the past few years for their antioxidant and radical scavenging properties. Hence, all the extracts were screened for *in vitro* antioxidant activity using different methods.

Among the four successive extracts, the ethyl acetate extract of stem showed potent activity in ABTS and DPPH methods, root extract in ABTS method. The root extract showed better activity than the standard ascorbic acid. All the other extracts showed weak to moderate activities in the other tested methods. Among the methanol extracts, the macerated leaves, crude flowers, stem and root showed potent activity in ABTS method and the results were better than the standard ascorbic acid. In the H_2O_2 and total antioxidant capacity methods, all the crude extracts showed potent activity than the standard used. The crude flower extract was found to show potent activity in p-NDA and deoxyribose methods. However, the efficiency of each extract differs against free radicals depending on the particular assay methodology, reflecting their complexity of the mechanisms and diversity in the chemical nature of phytoconstituents present.

Many natural products have served as anticancer agents in the treatment and also as lead compounds for further research. All the extracts were tested for their cytotoxic activity against Vero, Hep-2 and HeLa cell lines showed weak activity.

A large number of phenolic compounds are known to possess antioxidant activity (Badami et al., 2003; Le et al., 2004). The total phenol content of most of the crude extracts was found to be higher. Hence, potent *in vitro* antioxidant activity of these extracts might be due to the presence of higher amount of phenolics. Based on these results, in the present study crude methanol extracts of flowers, stem, root and leaves were selected for *in vivo* anti-inflammatory and analgesic activities.

It is well known that carrageenan induced paw edema is characterized by biphasic event with involvement of different inflammatory mediators. In the first phase, chemical mediators such as histamine and serotonin play role, while in second phase kinin and prostaglandins are involved. Formalin induced paw edema in rats is one of the most suitable test to screen antiarthritic and anti-inflammatory agents as it closely resembles human arthritis (Greenwald, 2000). Injection of formalin subcutaneously in to rat hind paw produces localized inflammation and pain. Thus formalin induced model is used for evaluating antiproliferative activity. Histamine is one of the important inflammation mediators and it is a potent vasodilators substance and increases the vascular permeability (Linardi et al., 2002; Cuman et al., 2001).

The crude methanol extracts of flower, root, stem and leaves have been exhibited potent anti-inflammatory activity in carrageenan, formalin and histamine induced paw edema methods at 100 and 200 mg/kg bw doses given orally. Among these crude extracts, the crude methanol flower extract showed better activity from 1-3 h of the treatment in all these methods. This may be due to the inhibition of inflammation mediators such as prostaglandins, kinin, histamine etc. However, the standard ibuprofen showed potent activity than the extracts in all the methods. Hence, in the present study the crude methanol flower extract was selected to screen for subchronic anti-inflammatory studies.

The cotton pellet granuloma method has been widely employed to assess the tranudative, exudative and proliferative components of chronic inflammation.The fluid absorbed by the pellet greatly influences the wet weight of the granuloma and the dry weight correlates well with the amount of granulomatous tissue formed (Swingle and Shideman, 1972). Monocyte infiltration and fibroblast proliferation rather than neutrofil infiltration and exudation take place in chronic inflammation (Dunne, 1990). Our results revealed that the crude methanol flower extract at all the doses significantly decreased both wet and dry weights of granuloma compared to control group in a dose dependent manner. This may be due to the ability of crude methanol flower extract in reducing the number of fibroblasts and synthesis of collagen and mucopolysaccharide, which are natural proliferative agents of granulation of tissue formation.

Injury of the cells of the mucous membrane may provoke the production of active oxygen species such as nitrogen monoxide and superoxide anion radicals by macrophages and neutrophiles permitting into them. Active oxygen species can directly injure the surrounding cells and extracellular matrices, such as hyaluronic acid and produce lipid peroxides and metabolites of arachidonic acid (Oyanagui, 1983). Superoxide anion radicals and hydrogen peroxide have important roles in the inflammatory process (Shingu et al., 1982). Antioxidants, such as SOD and tea catechins are known to suppress inflammation in a rat arthritis model (Yoshikawa et al., 1985). Hence, the observed anti-inflammatory activity may be due to antioxidant activity of crude methanol flower extract. The literature indicates other species like *S. callosus* and *S. ixiocephala* were used in the treatment of inflammation. Our results prove the anti-inflammatory activity of another plant of the same species. The precise mechanism through which the extract exerts its antiedematogenic effects needs to be investigated.

In the present study analgesic activity of crude methanol extracts were evaluated by hot plate and tail immersion methods. These methods have selectivity for opioid derived centrally mediated analgesics (Winter et al., 1962). Animals treated with crude methanol extracts orally at 100 and 200 mg/kg bw doses exhibited significant increase in the hot plate reaction time and tail withdrawal reflex to hot water in rats. In both these methods, the effect of crude methanol flower extract at both the doses was found to be better than other extracts. However, the standard ibuprofen at 100 mg/kg bw showed maximum percentage protection in both the methods. Hence, the extracts tested may possess central antinociceptive actions involving opioid like receptor mediations.

Liver regulates many important metabolic functions. Hepatic injury is associated with distortion of these metabolic functions (Wolf, 1999). In absence of a reliable liver protective drug in the modern system of medicine, a number of medicinal preparations in Ayurveda, the Indian system of medicine, are recommended for the treatment of liver disorders (Chatterjee, 2000). Natural remedies from medicinal plants are considered to be effective and safe alternative treatments for hepatotoxicity.

Since the changes associated with CCl_4 induced liver damage are similar to that of acute viral hepatitis (Suja et al., 2004), CCl_4 mediated hepatotoxicity was chosen as the experimental model. The ability of a hepatoprotective drug to reduce the injurious effects or to preserve the normal hepatic physiological mechanisms, which have been disturbed by a hepatotoxin, is the index of its protective effects (Yadav and Dixit, 2003). The hepatotoxicity induced by CCl_4 is due to its metabolite $CCl_3^{\cdot}$, a free radical that alkylates cellular proteins and other macromolecules with a simultaneous attack on polyunsaturated fatty acids, in the presence of oxygen, to produce lipid peroxides, leading to liver damage (Bishayee et al., 1995).

Hepatic cells participate in a variety of metabolic activities and contain a host of enzymes. In tissues, Asparate amino transferase (ASAT) and Alanine amino transferase (ALAT) are found in higher concentrations in cytoplasm and ASAT in particular also exists in mitochondria. In liver injury, the transport function of the hepatocytes is disturbed, resulting in the leakage of plasma membrane, thereby

causing an increased enzyme level in serum, and soluble enzymes like ASAT will also be similarly released. The elevated activities of ASAT and ALAT in serum are indicative of cellular leakage and loss of functional integrity of cell membranes in liver (Rajesh and Latha, 2004). Administration of CCl_4 significantly raised the serum level of enzymes like ASAT and ALAT in rats. Oral administration of crude methanol flower extract at a dose of 200 mg/kg bw caused a decrease in the activity of the above enzymes, which may be a consequence of the stabilization of plasma membrane as well as repair of hepatic tissue damage caused by CCl_4.

The activity of serum alkaline phosphatase (ALP) was also elevated during CCl_4 administration. ALP is excreted normally via bile by the liver. In liver injury due to hepatotoxin, there is a defective excretion of bile by the liver which is reflected in their increased levels in serum. Hyperbilirubinaemia is a very sensitive test to substantiate the functional integrity of the liver and severity of necrosis which increases the binding, conjugation and excretory capacity of hepatocytes that is proportional to the erythrocyte degeneration rate (Singh et al., 1998). Suppression of activity of ALP in serum of rats treated with crude methanol flower extract, suggest to stabilize biliary dysfunction of rat liver during chronic injury with CCl_4.

The TP levels including albumin levels will be depressed in hepatotoxic conditions due to defective protein biosynthesis in liver (Clawson, 1989). The CCl_4 intoxication causes disruption and disassociation of polyribosomes on

endoplasmic reticulum and thereby reducing the biosynthesis of protein. The pretreatment of crude methanol flower extract restored the proteins synthesis by protecting the polyribosomes.

Hepatocellular damage causes a modest hypertriglyceridemia (Glickmann and Sebasin, 1982), which is due to the biochemical changes inferring with the transport of triglycerides out of liver. The same is evidenced in the CCl_4 induced rats. A significant increase in cholesterol was observed in CCl_4 induced rats, which may be due to the inability of the diseased liver to remove cholesterol from circulation. The pretreatment with the crude methanol flower extract caused a significant reversal of the altered TGL and TC levels towards the normal in dose dependent manner.

A high concentration TB in serum is an indication for increased erythrocyte degeneration rate (Singh et al., 1998). Due to the liver injury caused by the hepatotoxin, there is a defective excretion of bile by the liver which is reflected in their increased levels in serum (Rao, 1973). The pretreatment of crude methanol flower extract at 200 mg/kg bw effectively reduced the serum TB level towards the control indicating the increased hepatic capacity.

Hypoalbuminemia is caused by liver diseases and abnormally high levels of CR indicate possible malfunction or failure of the kidneys. A significant reversal was observed by the extract treatment towards the normal when compared to CCl_4 treated animals indicating the protection of those organs by the extract. It has been suggested that glycogen serves as an energy buffer capable of providing rapid and

short-term energy (Brown, 2004). The elevation of depressed glycogen stores by the crude methanol flower extract in CCl_4 treated rats may be attributed to either an inhibition of hepatic glucose output improvement in plasma insulin levels or by synthetase responsible for the incorporation of glucose moieties into pre-existing glycogen chains.

Antioxidant activity or the inhibition of the generation of free radicals is important in the protection against CCl_4 induced hepatopathy (Castro et al., 1974). The body has an effective defense mechanism to prevent and neutralize the free radical induced damage. This is proficient by a set of endogenous antioxidant enzymes such as SOD, and catalase. These enzymes constitute a mutually supportive team of defense against ROS. In CCl_4 induced hepatotoxicity, the balance between ROS production and these antioxidant defenses may be lost, 'oxidative stress' results, which through a series of events deregulates the cellular functions leading to hepatic necrosis. The reduced activities of SOD and catalase observed point out the hepatic damage in the rats administered with CCl_4 but the treated with 100, 150 and 200 mg/kg of the crude methanol flower extract showed significant increase in the level of these enzymes, which indicates the *in vivo* antioxidant activity of the extract.

The level of lipid peroxide is a measure of membrane damage and alterations in structure and function of cellular membranes. In the present study, elevation of lipid peroxidation in the liver of rats treated with CCl_4 was observed. The increase in MDA levels in liver suggests enhanced lipid peroxidation leading to tissue

damage and failure of antioxidant defense mechanisms to prevent the formation of excessive free radicals (Ashok Shenoy et al., 2001). Treatment with crude methanol flower extract significantly reversed these changes. Hence, it is possible that the mechanism of hepatoprotection of the extract may be due to its antioxidant action.

The normal architecture of liver was completely lost in rats treated with CCl_4 with the appearance of vacuolated hepatocytes and degenerated nuclei. Vacuolization, fatty changes and necrosis of hepatocytes were severe in the centrilobular region. CCl_4 poisoning led to excessive formation of deposition of connective tissue and development of scars. Minimal disruption of the structure of the hepatocyte was observed in liver tissue of rats intoxicated with CCl_4 and treated with crude methanol flower extract. Kidney sections of CCl_4 showed a high degree of tubulointerstitial nephritis compared to control. The crude methanol extract treated kidney tissue showed almost normal histological appearance indicating its hepatoprotective activity. These results compliment the result of biochemical estimations, where a reversal of effects of CCl_4 towards the normal was observed.

- The herbal medicines are the major remedy in traditional medicinal system and are being used in medicinal practice for thousands of years. These have made a great contribution in maintaining human health. The practice continues even today because of the biomedical benefits in many parts of the world. There is phenomenal increase in the demand for the herbal medicines especially for those which have been scientifically validated. The alarming rapid rate of species extension which we are currently witnessing due in part to habitat destruction, lends certain urgency to the quest for plant derived drugs. Natural product research continues to be an important part of the drug discovery process. The main advantage of natural products as a source of lead compounds is the tremendous molecular diversity found in nature. Hence, we were interested in carrying out natural products research.
- Most of the species of *Strobilanthes* flower only in long intervals, such as between six and twelve years and in some, even at longer intervals like 35 years. One of the plant best known species *S. Kunthianus* (Kurinji), flowers once in twelve years. Interesting results of gregarious flowering of *Strobilanthes* increases bees and its vicinity during the flowering period. Immense quantities of honey become available and the rockbees in common hill visit the plant. However, so far there is no biological studies have been carried out on this special plant. So far only Lupeol has been isolated from the whole plant. Hence in the present study, a detailed evaluation of phytochemical and biological studies such as antioxidant, anti-inflammatory, analgesic and hepatoprotective activities were carried out.

- The whole plant of *S. kunthianus* was collected from Thalaikuntha region, near Ootacamund, Nilgiris district, Tamilnadu, India. The plant was identified and authenticated at Botanical Survey of India, Coimbatore.
- Pharmacognostical evaluation was carried out in order to establish the identity and to standardize the plant. Morphological and microscopical characters of leaves, flowers, stem and root were studied. Physicochemical parameters like total ash, acid insoluble ash, water soluble ash and sulphated ash values and extractive values like alcohol soluble and water soluble were determined.
- The stem and root of *S. kunthianus* were successively extracted with petroleum ether, chloroform, ethyl acetate and methanol. The flowers, stem, leaves and root were also extracted with methanol to obtain crude extracts by using Soxhlet method. The leaves and flowers were also subjected for cold methanol maceration. All the fourteen extracts were concentrated to dryness under reduced pressure and controlled temperature and the yields were noted. Preliminary phytochemical analysis was carried out. Glycosides and phenolics were found in successive ethyl acetate and methanol stem and root extracts and all the crude methanol extracts. The total phenol content was estimated by Folin Ciocalteau method in all the fourteen extracts. The crude methanol flower extract was found to contain very high total phenol content among all the extracts.
- The successive petroleum ether root and stem extracts, chloroform stem extract, macerated and crude methanol flower extracts were subjected to

column chromatography for the isolation of constituents. The macerated methanol leaves extract was subjected to solvent-solvent extraction method to isolate phytoconstituents. Nine compounds were isolated and their purity was confirmed by TLC using different solvent systems. Based on the spectral analysis such as IR, NMR and Mass spectral data, the compounds were characterized. The compounds isolated from the petroleum ether root extract were lupeol (GS 1) and betulin (GS 2). Lupeol (PES 1) was also isolated from petroleum ether stem extract. From chloroform stem extract, 3, 5-bis (dimethyl carbamoyl) methyl-4- (11-cyclo hexyl undecyl)- 4-heptyl-N^1,N^1,N^7,N^7 tetra methyl heptane diamide (SC 1) was isolated. The compounds α-amyrin (GSMES 3) and 1, 4-amino-tetrahydro-2H-pyran-3, 5-diol (G 1) were isolated from macerated methanol flower extract. From crude methanol flower extract, compounds β-sitosterol (CMF 1), 2, 2-(2-hydroxyethyl)-3-methyl-4-[(E)-3, 4, 6-trimethyl undec-4-ethyl] cyclohexyl propionate (CMF 2) and flavone glycoside (CMF 5) were isolated. From macerated methanol leaves extract, the compound decahydro-1, 1, 4a, 8-tetramethyl phenanthren-2 (1H, 3H, 4b H)-one (ML 1) was isolated. Except lupeol, all the other eight compounds were isolated for the first time from the plant.

- During the last two decades, there has been a growing interest in studies that are concerned with prevention of uncontrolled oxidative process leading to various diseases in living system. Several studies have shown the role of oxidative stress in the causation and progression of various diseases

including atherosclerosis, carcinogenesis, neurodegenerative diseases, radiation damage, aging and various other pathological effects. Hence, in the present study, all the successive extracts of stem and root and six methanol extracts were screened for *in vitro* antioxidant activity using seven different methods.

- Among the tested extracts, the ethyl acetate extracts of root and stem showed potent *in vitro* antioxidant activity, when compared to ascorbic acid in ABTS method. These extracts showed better activity than other successive extracts of root and stem. In case of methanol extracts, the crude extracts showed good antioxidant activity in ABTS, H_2O_2, and total antioxidant capacity methods. The crude methanol flower extract was found to be the most potent among the methanol extracts.
- Many natural products have served as anticancer agents in the treatment and also has lead compounds for further research. All the extracts were tested for their cytotoxic activity against Hep-2 and HeLa cell lines. But weak activity in the tested cell lines indicated their safety inactivity towards cancer cell lines.
- Free radical and reactive oxygen species are well known inducers of cellular and tissue pathogenesis leading to several human diseases such as cancer, inflammatory disorders, hepatitis, diabetes mellitus, as well as in aging process. Many plant species possessing antioxidant activities were served as protective agents against these diseases. The crude methanol extracts of flower, stem, root and leaves showed potent *in vitro* antioxidant activity. Hence, in the present study, crude methanol extracts were selected for *in vivo*

anti-inflammatory and analgesic studies. In the acute toxicity studies, all the four crude methanol extracts were found to be non toxic and no mortality was observed up to 15 days when given as single oral dose of 2000 mg/kg bw with no gross necropsy findings.

- *In vivo* acute anti-inflammatory activity was done with carrageenan, formalin and histamine induced paw edema methods and analgesic activity by hot plate and tail immersion methods for all the crude extracs at two different dose levels. The crude methanol flower extract treatment showed the maximum activity in all the methods among the tested extracts. However, standard ibuprofen showed potent activity than the extracts. Based on these results, crude methanol flower extract was selected for sub chronic anti-inflammatory studies using cotton pellet method. The extract was found to be active indicating its potency. Hence, this extract was subjected for *in vivo* hepatoprotective and antioxidant activities.
- The crude methanol flower extract was tested against CCl_4 induced liver injury in rats at 100, 150 and 200 mg/kg bw doses. The administration of CCl_4 caused a significant increase in the levels of ASAT, ALAT, ALP, TGL, TC, CR, TB and TBARS and decrease in the levels of TP, Albumin, CAT and SOD in serum. A significant restoration of these values towards the normal was observed in all the doses of the extract treatment. Similar results were observed for CAT, SOD and TBARS in both liver and kidney tissues. These results indicated strong antioxidant and hepatoprotective nature of crude methanol flower extract. The histopathological examination of liver and kidney tissues also confirmed these activities.

- In conclusion, the present work provides the pharmacognostical and phytochemical evaluation profiles to identify the plant. From the extracts by column chromatographic studies, eight compounds were isolated for the first time from this plant. The *in vitro* antioxidant and cytotoxicity studies of the extracts were established by using several standard methods. The *in vivo* anti-inflammatory and analgesic activities were established for crude methanol extracts in different models. The antioxidant and hepatoprotective properties were established for crude methanol flower extract. Biochemical and histological evidences were used to prove the activities. Hence, the present study provides a proof for the strong chemotaxonomical relationship and similarity of the biological property with the other species of *Strobilanthes.*

Objectives achieved

1. Pharmacognostical evaluation profile to identify the plant *S. kunthianus* was established.
2. Eight phytoconstituents were isolated for the first time from this plant.
3. Potent extracts were selected from seven standard *in vitro* antioxidant activity methods and subjected to *in vivo* studies.
4. *In vivo* anti-inflammatory, analgesic, antioxidant and hepatoprotective activities of the crude methanol flower extract of *S. kunthianus* was proved.

Scope for further research

There is further scope for research in isolating the other phytoconstituents and to carry out the other biological properties of the extracts. There is also a need to establish the mechanism of the observed activities.

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PAPERS COMMUNICATED FOR PUBLICATION

1. **Geetha B,** Badami S. Pharmacognostical studies of *Strobilanthes kunthianus*. (Communicated to Journal of **Natural Products Research and Resources**).

2. **Geetha B,** Badami S. *In vitro* antioxidant activity of *Strobilanthes kunthianus* (Communicated to **Indian Drugs**).

3. **Geetha B,** Mahendran S, Badami S, Maithili V. Analgesic and anti-inflammatory activities of *Strobilanthes kunthianus* (Communicated to **Indian Journal of Pharmacology**).

4. **Geetha B,** Mahendran S, Badami S, Maithili V. Antioxidant and hepatoprotective activities of *Strobilanthes kunthianus* (Communicated to Indian Journal of Experimental Biology**).**

5. **Geetha B,** Ravi S, Badami S. Phytoconstituents from *Strobilanthes kunthianus* (Communicated to **Indian Journal of Heterocyclic Chemistry**).