STRUCTURE ACTIVITY RELATIONSHIP OF BIOACTIVE COMPOUNDS FROM MEDICINAL PLANTS

Thesis submitted to

The Tamil Nadu Dr. M. G. R. Medical University, Chennai, for the fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Submitted by

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Under the Guidance of

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This is to certify that the thesis entitled **"Structure activity relationship of bioactive compounds from medicinal plants"** submitted by **Mr. S. Mahendran**, to The Tamilnadu Dr. M. G. R. Medical University, Chennai, for the award of the Degree of Doctor of Philosophy in Pharmaceutical Sciences, is based on the research work carried out by him 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 other research degree, of this or any other University, previously.

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DECLARATION

I hereby declare that the thesis entitled **"Structure activity relationship of bioactive compounds from 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.

(S. Mahendran)

Dedicated to
My Parents
&
Teachers

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(S. Mahendran)

CONTENTS

1.1. Drug discovery from medicinal plants

Herbal medicine is the oldest form of healthcare known to mankind. Herbs had been used by all cultures throughout history. It was an integral part of the development of modern civilization. Primitive man observed and appreciated the great diversity of plants available to him. The plants provided food, clothing, shelter and medicine. Much of the medicinal use of plants seems to have been developed through observations of wild animals, and by trial and error. As time went on, each tribe added the medicinal power of herbs in their area to its knowledge base. They methodically collected information on herbs and developed well-defined herbal pharmacopoeias. Indeed, well into the 20th century much of the pharmacopoeia of scientific medicine was derived from the herbal lore of native peoples.

The World Health Organization (WHO) estimated that 80% of the populations of developing countries rely on traditional medicines, mostly plant drugs, for their primary health care needs. Also, modern pharmacopoeias still contain at least 25% drugs derived from plants and many others which are synthetic analogues built on prototype compounds isolated from plants. In both 2001 and 2002, approximately one quarter of the best selling drugs worldwide were natural products or derived from natural products [1]. Demand for medicinal plant is increasing in both developing and developed countries due to growing recognition of natural products, being non-narcotic, having no side-effects, easily available at affordable prices and sometime the only source of health care available to the poor. Medicinal plant sector has traditionally occupied an important position in the socio cultural, spiritual and medicinal arena of rural and tribal lives of India.

Medicinal plants account for around 50% of all the higher flowering plant species of India. Millions of rural households use medicinal plants in a self-help mode. Over one and a half million practitioners of the Indian System of Medicine in the oral and codified streams use medicinal plants in preventive, promotive and curative applications. There are estimated to be over 7800 manufacturing units in India. In recent years, the growing demand for herbal products has led to a quantum jump in volume of plant materials traded within and across the countries. An estimate of the EXIM Bank puts the international market of medicinal plants related trade at US $ 60 billion per year growing at the rate of 7% only. According to an all India ethno biological survey carried out by the Ministry of Environment & Forests, Government of India, there are over 8000 species of plants being used by the people of India. Though India has a rich biodiversity, the growing demand is putting a heavy strain on the existing resources. While the demand for medicinal plants is growing, some of them are increasingly being threatened in their natural habitat. For meeting the future needs, cultivation of medicinal plant has to be encouraged.

Substances derived from the plants remain the basis for a large proportion of the commercial medications used today for the treatment of heart disease, high blood pressure, body pain, asthma and other problems. For example, ephedra is a herb used in Traditional Chinese Medicine for more than two thousand years to treat asthma and other respiratory problems. Ephedrine, the active ingredient in ephedra, is used in the commercial pharmaceutical preparations for the relief of asthma symptoms and other respiratory problems. It helps the patient to breathe more easily. Another example of the use of herbal preparation in modern medicine is the foxglove plant. This herb had been in

use since 1775. At present, the powdered leaf of this plant is known as the cardiac stimulant digitalis to the millions of heart patients it keeps alive worldwide.

The complete synthesis of natural products has been an area of interest for a long time, and the efforts to produce man-made natural products has provided significant challenge and learning opportunity over the years. Because of the widespread chemical diversity that can be found in natural products, an ever-expanding collection of fascinating natural product compounds will continue to be presented to chemists for synthesis. If one compares the chemical diversity of man-made synthetic products with the chemical diversity of natural product compounds, it quickly becomes apparent that there are significant qualitative differences between synthetics and natural products. Natural product compounds contain more alcoholic and ether groups, while pure synthetic compounds possess more aromatics, amines, and amides. If one looks at group combinations, there are higher percentages of alcohol/ether, alcohol/ester, arene/alcohol, arene, alcohol, or ether functionalities in natural product compounds when compared to the synthetic compounds. However, pure man-made synthetic compounds are found to have combinations such as arene/amine, amine/amide, or amine/arene/amide in higher frequencies than natural product type of compounds.

Finally, natural product compounds are found to more commonly possess bridgehead atoms and contain a greater number of rotatable bonds per molecule, chiral centers per molecule, and rings per molecule than pure man-made synthetic compounds. The importance of these differences is that they reveal and emphasize the complementarity of natural product compounds as a group with man-made synthetic compounds. Despite all

of the knowledge and achievements that have been gained and the advances that have been made, classical synthetic organic chemistry will not alone unlock and open the potential of natural products to the pharmaceutical market place. Instead, the future lies in the synergistic union of classical organic chemistry with microbiology, biochemistry, combinatorial chemistry, and other fields to provide new synthetic strategies to generate natural product based drugs.

The engineering of polyketide synthases has thus far been the central point of this activity and led to the production of several erythromycin analogs. The end result of such research activity will be the development of more rational and faster methods of production of new compounds for the development of therapeutic agents from natural products. The research on and screening of natural products today is focusing on many different therapeutic indications. Fermentation broths and plant extracts have done well in delivering leads and genomics and molecular biology have done well in delivering targets. Regardless of the type of compounds involved, improved efficiencies in the design and synthesis of natural product based agonists and antagonists will be key to a realization of the full potential of natural products as drugs.

The extensive advances and development of science of phytopharmaceuticals and hopes for remedies in chronic diseases generated new enthusiasm in the research workers to develop herbal medicines and remedies continue to be demanded by the public. Approximately, 130 pure chemical substances were isolated from some 100 species of higher plants and are used in medicines throughout the world. The major achievement in this field was the discovery of vincristine and vinblastine in the *Madagascars periwinkle*

by researchers at Eli Lilly and Company, USA, and it made marked differences in the treatment of cancer. Morphine, digitoxin, ergotamine and quinine are some of the other excellent drugs obtained from different plant species [2].

The natural plant products often serve as chemical models or prototypes for the design and total synthesis of new drug entities. The concept of drug design of some of the synthetic molecules has emerged out of their quantitative structural activity relationship (QSAR) in terms of biodynamic constituents. For example, the *Belladonna* alkaloids (atropine), quinine, cocaine, opiates (morphine and codeine) and salicylic acid have been served as models for design and synthesis of anticholinergics, antimalarials, benzocaine, procaine and other local anesthetics and aspirin, respectively [3].

Virtually, more than 13,000 plants have been studied during the last five years period. About 9.5% of the new structures obtained from higher plants were tested for pharmacological effects. Efforts have been made in last three decades for the development of technical know-how for extraction of phytochemicals on commercial scale by pharmaceutical and chemical industries and research institutions throughout the world. As a result of modern isolation techniques and pharmacological testing procedures, new plant drugs usually find their road into the medicines as purified substances [4].

Approximately, one third of the top selling drugs in the world are natural products or their derivatives [5]. Of the 20 best selling non-protein drugs in 1999, nine were either derived from or developed as the result of leads generated by natural products. Some of these viz., simvastatin, lovastatin, enalapril, pravastatin, atorvastatin, augmentin,

ciprofloxacin, clarithromycin and cyclosporin had a combined annual sale of > US $ 16 billion [6].

Some examples of successful drugs derived from plants are briefly described here. Arteether is a potent anti-malarial drug and is derived from artemisinin, a sesquiterpene lactone isolated from *Artemisia annua* L. (Asteraceae), a plant used in traditional Chinese medicine. Galanthamine is a natural product discovered through an ethnobotanical lead and first isolated from *Galanthus woronowii* L. (Amaryllidaceae) in Russia. Galanthamine is approved for the treatment of Alzheimer's disease, slowing the process of neurological degeneration by inhibiting acetylcholine esterase as well as binding to and modulating the nicotinic acetylcholine receptor. Tiotropium has been released recently in the US for treatment of chronic obstructive pulmonary disease. Tiotropium is an inhaled anticholinergic bronchodilator, based on ipratropium, a derivative of atropine, isolated from *Atropa belladonna* L. (Solanaceae) and other members of the Solanaceae family. Morphine-6-glucuronide is a metabolite of morphine from *Papaver somniferum* L. (Papaveraceae), reported as an alternative pain medication with fewer side effects than morphine. Exatecan is an analogue of camptothecin isolated from *Camptotheca acuminata* D. (Nyssaceae) and being developed as an anticancer agent. Vinflunine is a modification of vinblastine from *Catharanthus roseus* G. (Apocynaceae) for use as an anticancer agent with improved efficacy. Compounds (Morphine-6-glucuronide, Exatecan and Vinflunine) all are in phase III clinical trials. Thus, from these three examples, it is evident that modifications of existing natural products can lead to NCEs and possible drug leads, from medicinal plants.

1.2. Challenges in drug discovery from medicinal plants

Despite the evident successes of drug discovery from medicinal plants, future endeavors face many challenges. Pharmacognosists, phytochemists and other natural product scientists will need to continuously improve the quality and quantity of compounds that enter the drug development phase to keep pace with other drug discovery efforts [1]. The process of drug discovery has been estimated to take an average of 10 years upwards [7] and cost more than 800 million dollars [8]. Much of this time and money is spent on the numerous leads that are discarded during the drug discovery process. In fact, it has been estimated that only one in 5000 lead compounds will successfully advance through clinical trials and be approved for use. Lead identification is the first step in a lengthy drug development process (Fig. 1). Lead optimization (involving medicinal and combinatorial chemistry), lead development (including pharmacology, toxicology, pharmacokinetics, ADME (absorption, distribution, metabolism and excretion), drug delivery and clinical trials all take a considerable length of time.

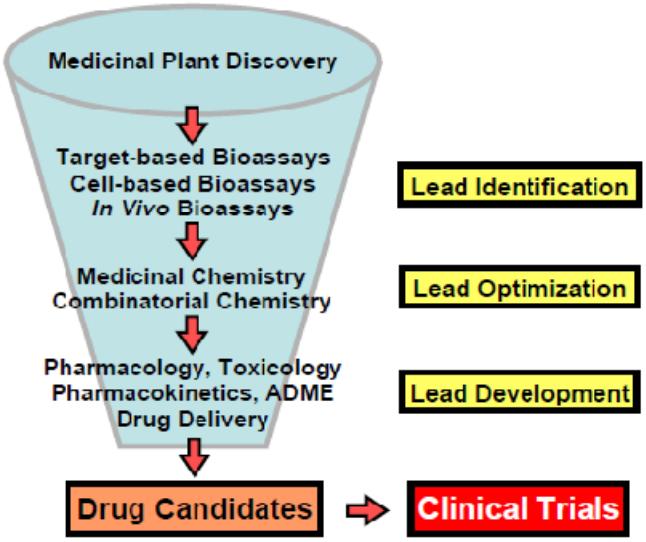


Fig. 1. Schematic of typical medicinal plant drug discovery and development

Embelia ribes and *Mangifera indica* are the two plants used very widely in traditional medicine. Many phytoconstituents were isolated from these two plants and biological studies were conducted on both the plant extracts and phtoconstituents.

Embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone) is a naturally occurring alkyl substituted hydroxy benzoquinone and a major constituent of *Embelia ribes* Burm. (Family: Myrsinaceae). The plant is indicated in traditional medicine for the treatment of various diseases. The fruit is bitter in taste, good appetizer, cures tumors, ascites, bronchitis, jaundice, brain tonic, mental disorders, dyspnoea, diseases of the heart, urinary discharges, scorpion-sting, snake-bite and tooth ache [9,10]. It has been reported to possess antioxidant properties in diabetic animals [11] and anti-inflammatory to relieve rheumatism and fever [12]. Embelin showed antifertility [13], anti-implantation [14], antitumour, anti-inflammatory, analgesic [15], antioxidant [16], hepatoprotective [17], wound healing [18] and antibacterial [19] activities.

Mangiferin (1,3,6,7-tetrahydroxyxanthone-C2-β-D-glucoside) is a pharmacologically active phytochemical present in large amounts in the bark, fruits, roots and leaves of *Mangifera indica* (family: Anacardiaceae). The plant is indicated in traditional medicine for the treatment of various diseases. The root, bark, leaves and flowers are astringent, refrigerant, styptic, vulnerary, laxative, cardiotonic, haemoptysis, haemorrhages, constipating and diabetes [20,21]. Mangiferin showed antidiabetic [22], anticancer [23,24], antioxidant [25], anti-inflammatory [26], antiviral [27] and antimicrobial [28] activities.

Except these studies so far no other biological studies have been carried out. Hence, in the present study embelin and mangiferin isolated from *Embelia ribes* and *Mangifera indica,* respectively were selected for structure activity relationship studies.

1.3. Structural modification of bioactive compounds

Quinonic compounds are ubiquitous in nature. They are implicated in numerous cellular functions and are involved in mechanisms of electron and hydrogen transfers. Quinones form a large class of antitumor agents approved for clinical use, and many other antitumor quinones are in different stages of clinical and preclinical development [29]. The efficiency of the quinonic compounds in inhibiting cancer cell growth is believed to stem from their participation in key cellular redox mechanisms with consequent generation of highly reactive oxygen species (ROS). The ROS turn out to modify and degrade nucleic acids and proteins within the cells [30].

One of the most simple 1,4-benzoquinonic compound isolated from natural sources is embelin. Padmanabha Rao and Venkateswarlu [31], reported a condensation reaction of embelin and various primary amines to afford di-imines. Gupta et al. [32] reported analgesic and anti-inflammatory properties of embelin di-imine and di-salt derivatives. It represents a promising lead compound for designing a new class of analgesic and anti-inflammatory agents. These antecedents justify the interest in the construction of newer embelin derivatives by using primary amines.

Various reports established that the C-2 position of the ninhydrin is more reactive towards nitrogen, sulfur, oxygen and carbon based nucleophiles [33]. The electrophilic character of the C-2 position allows for the formation of condensation products with

various aromatic substrates [34]. It has also been reported that ninhydrin reacts with phenol at ortho position to –OH group in high yield [35]. The acetic acid catalyzed condensation of ninhydrin with phenols, aromatic substances (excluding ethers) have been studied. It was reported that amino, C-alkyl, hydroxy and alkoxy phenols react with ninhydrin to produce monoarylated products and the mechanism of the reaction has been postulated [33,34]. The unique reactivity of phenols with ninhydrin led us to examine the usefulness of the ninhydrin reaction for chemical modification of embelin aiming to synthesize new types of biological agents.

Bhatia et al. [36] reported acetylation and methylation of mangiferin. Further, Hu et al. [37] synthesized a series of substitutional benzyl mangiferin derivatives. However, literature showed that detailed pharmacological investigation of mangiferin derivatives was not reported. Hence, the structural modification of mangiferin was taken up for the development of new pharmacologically active compounds.

1.4. Free radicals and reactive oxygen species

The recent growth in knowledge of free radicals in biology is producing a medical revolution that promises a new age of health and disease management [38]. Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons. The presence of unpaired electrons usually confers a considerable degree of reactivity upon a free radical. In the last two decades, there has been an explosive interest in the role of oxygen-free radicals, more generally known as “reactive oxygen species,” (ROS) and of “reactive nitrogen species” (RNS) in experimental and clinical medicine [39].

ROS/RNS are known to play a dual role in biological systems, since they can be either harmful or beneficial to living systems [40]. Beneficial effects of ROS involve physiological roles in cellular responses to noxia, as for example in defense against infectious agents and in the function of a number of cellular signalling systems. One further beneficial example of ROS at low concentrations is the induction of a mitogenic response. In contrast, at high concentrations, ROS can be important mediators of damage to cell structures, including lipids and membranes, proteins and nucleic acids [41].

1.4.1. Reactive oxygen species and biochemical consequences

Oxygen radical is capable of reversibly or irreversibly damaging compounds of all biochemical classes, including nucleic acids, proteins, lipids, carbohydrates and connective tissue macromolecules. ROS are formed through a variety of events and pathways. It has been estimated that one human cell is exposed to approximately 1.5×10^5 oxidative hits a day from hydroxyl radicals and other such reactive species [42]. The hydroxyl radical is known to react with all components of the DNA molecule, damaging both the purine and pyrimidine bases and also the deoxyribose backbone [43]. Permanent modification of genetic material resulting from these “oxidative damage” incidents represents the first step involved in mutagenesis, carcinogenesis and ageing. In fact, as is well established, in various cancer tissues free radical-mediated DNA damage has occurred.

It is known that metal-induced generation of oxygen radicals results in the attack of not only DNA in the cell nucleus, but also other cellular components involving

polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation [44,45].

Mechanisms involved in the oxidation of proteins by ROS were elucidated by studies in which amino acids, simple peptides and proteins were exposed to ionizing radiations under conditions where hydroxyl radicals or a mixture of hydroxyl/superoxide radicals are formed [46]. The results of these studies demonstrated that reactions with hydroxyl radicals lead to abstraction of a hydrogen atom from the protein polypeptide backbone to form a carbon-centered radical, which under aerobic conditions reacts readily with dioxygen to form peroxyl radicals [47]. The free radical-related damage to DNA, to proteins and to lipids has been proposed to play a key role in the development of age-dependent diseases such as cancer, arteriosclerosis, arthritis, neurodegenerative disorders and other conditions [39].

1.4.2. Antioxidant defense mechanisms

The harmful effects of ROS and RNS are balanced by the antioxidant action of non-enzymatic antioxidants in addition to antioxidant enzymes [48]. These antioxidant defenses are extremely important as they represent the direct removal of free radicals (pro-oxidants), thus providing maximal protection for biological sites.

The most efficient enzymatic antioxidants involve superoxide dismutase, catalase and glutathione peroxidase [49]. Non-enzymatic antioxidants involve vitamin C, vitamin E, carotenoids, thiol antioxidants (glutathione, thioredoxin and lipoic acid), natural flavonoids, a hormonal product of the pineal gland, melatonin and other compounds [50]. As plants produce a lot of antioxidants to control the oxidative stress caused by sunbeams

and oxygen, they can represent a source of new compounds with antioxidant activity. Hence, in the present study, derived compounds of embelin and mangiferin were tested for *in vitro* antioxidant activity using standard methods.

1.4.3. Role of antioxidants in analgesic and anti-inflammatory studies

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 [51]. 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 [52]. Free radicals also can damage membranes, proteins, enzymes and DNA, increasing the risk of diseases such as cancer, Alzheimer's, Parkinson's, angiocardiopathy, arthritis, asthma, diabetes, and degenerative eye diseases [53]. Natural products, natural products derivatives, synthetic compounds with natural products-derived pharmacophore and synthetic compounds designed from natural products are played an important role to manage pathological conditions of those diseases caused by free radicals [54]. Hence, in the present study, the potent antioxidant compounds were tested for *in vivo* analgesic and anti-inflammatory studies.

1.5. Anticonvulsant activity

Epilepsy is one of the most common serious neurological conditions and is characterized by recurrent unprovoked epileptic seizures. These seizures are transient signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain. Incidence of epilepsy in developed countries is approximately 50 per 100,000 while that

of developing countries is 100 per 100,000. The entire currently available antiepileptic drugs are synthetic molecules associated with side effects and approximately 30% of the patients continue to have seizures with this therapy [55]. Several natural products including quinone derivatives, which are considered to possess better safety and efficacy profile, are known for their anticonvulsant effects [56]. However, embelin, a benzo quinone has not been tested so far for anticonvulsant properties, though the plant is traditionally used against such disorders [10]. In the present study, we investigated the anticonvulsant activity of embelin by using maximal electroshock (MES), pentylenetetrazole (PTZ) induced seizures along with locomotor activity.

1.6. Antidiabetic activity

Diabetes is a major endocrine disorder causing morbidity and mortality worldwide. The problem of diabetes is particularly relevant to India, as several studies have clearly documented an increased ethnic susceptibility to diabetes in-migrant Asian Indians [57]. Recent epidemiological studies have pointed to the growing epidemic of diabetes in India [58]. Indeed, according to the recent Diabetes Atlas produced by the International Diabetes Federation (IDF), India is home to the largest number of people with diabetes in the world, 40.9 million diabetic subjects in 2007, and these numbers are predicted to increase to 69.9 million by 2025 [59].

Plants have always been an exemplary source of drugs and many of the currently available drugs have been derived directly or indirectly from them. The ethnobotanical information reports about 800 plants that may possess anti-diabetic potential [60]. Several herbs have shown anti-diabetic activity when assessed using presently available

experimental techniques [61]. Wide arrays of plant derived active principles representing numerous chemical compounds have demonstrated activity consistent with their possible use in the treatment of non-insulin-dependent diabetes mellitus [62].

In a preliminary study, Tripathi [63], reported the antihyperglycaemic activity of the decoction of *Embelia ribes* in glucose-fed albino rabbits. Further, Bhandari et al. [64] reported the ethanolic extract of the plant in diabetic dyslipidemia and protection from lipid peroxidation in tissues in streptozotocin-induced diabetes in rats. The effect of chronic oral treatment with ethanolic extract of the plant on the basal level of some key serum, tissue antioxidants, blood pressure and glycosylated haemoglobin was investigated in streptozotocin-induced oxidative damage in rats [11,65].

However, embelin has not been tested so far for its antidiabetic properties. Hence, in the present study embelin was evaluated for antidiabetic effect against alloxan induced diabetes in rats.

1.7. Inflammatory bowel disease

Inflammatory bowel disease is a group of multifactorial intestinal disorders of unknown etiology including Crohn's disease and ulcerative colitis. Both Crohn's disease and ulcerative colitis are common in Western populations and in urban rather than rural areas. Patients with these diseases are 10-20 times more likely to get cancer of the colon or bowel cancer [66]. The therapeutic strategy for treating inflammatory bowel disease now focuses on the use of anti-inflammatory agents [67]. Glucocorticoids and aminosalicylates have been used for the treatment of inflammatory bowel disease, but their side effects remain a major clinical problem. Plant remedies play an important role

in the therapy of many inflammatory disease conditions including inflammatory bowel disease [68,69].

A growing body of literature suggests that inflammatory bowel disease results from a dysregulated immune response to normal bacterial antigens. This uncontrolled immune system activation results in the sustained overproduction of reactive metabolites of oxygen and nitrogen. Some of the intestinal and/or colonic injury and dysfunction observed in inflammatory bowel disease is due to elaboration of these reactive species. In many studies, it has been reported that antioxidants showed beneficial effects on experimental colitis [70]. Quinone derivatives from plants are known to possess protective effects against colitis induced by acetic acid [68].

However, so far embelin was not tested for its protective action against inflammatory bowel disease though it is reported to possess potent antioxidant [16] and anti-inflammatory [15] activities. Various animal models of experimental colitis to screen drugs effective against inflammatory bowel disease have been established and acetic acid induced colitis is an animal model that mimics some of the acute inflammatory responses seen in ulcerative colitis [71]. Hence, the present study was aimed to assess the protective effect of embelin in the rat model of colitis induced by acetic acid.

1.8. Neuroprotective activity

Stroke is one of the leading causes of death and the principal cause of adult disability in the world. However, there is currently no effective treatment [72] and two major approaches have been developed for the treatment of ischemic stroke. One approach is to reperfuse with thrombolytic drugs to dissolve the clot and the second approach is to

protect against neuronal cell death by interfering with the biochemical cascade of events using neuroprotective agents [73]. Many antioxidants are reported to reduce ROS mediated reactions and rescue neurons from ischemia-reperfusion-induced neural loss in animal models of cerebral ischemia [74]. Many natural products have been studied for their neuroprotective effects [75]. In a recent study, *Embelia ribes* was reported as an important herbal drug for the treatment of cerebral ischemia [76]. The ethanolic extract of *Embelia ribes* was reported to possess protective effect against methionine induced hyperhomocysteinemia and oxidative stress in rat brain [77]. Moreover, thymoquinone, a substituted benzoquinone has also been reported to protect the neurons against ischemic reperfusion injury in rats [75].

However, no work has been carried out to evaluate the neuroprotective effect of embelin, though the plant and similar structural analogues have been reported as neuroprotectives. Hence, the present study was also aimed to investigate the effect of embelin on global cerebral ischemia reperfusion induced neuronal damage in rats.

Plants have always played an important role for the mankind, especially as food and medicine. The increased use of herbal medicines in developed countries is mainly due to the failure of modern medicine in providing effective treatment for chronic diseases and emergence of multi-drug resistant bacteria and parasites. These include various diseases such as cancer, HIV/AIDS, diabetes, hepatitis, allergies and mental disorders. The adverse effects of chemical drugs, questioning of the approaches and assumptions of allopathic medicine, their increasing costs and greater public access to information on safety and efficacy of medicinal plants has also led to an increased interest in medicinal plants [78]. Numerous methods have been utilized to acquire compounds for drug discovery, including isolation from plants and other natural sources, synthetic chemistry, combinatorial chemistry and molecular modeling [79,80].

The overwhelming concern today in the pharmaceutical industry is to improve the ability to find new drugs and to accelerate the speed with which new drugs are discovered and developed. This will only be successfully accomplished if the procedures for drug target elucidation and lead compound identification and optimization are themselves optimized. Structure activity studies on leads generated from natural product sources combined with computerized graphic model building will become increasingly more prevalent. Such activity in turn will result in the discovery of molecules with optimal activity, improved bioavailability, fewer side effects, and very desirable therapeutic indices. Hence, in the present study, embelin and mangiferin were used as lead compounds for chemical modification to obtain better active compounds.

ROS and free radicals which are formed in the body as a consequence of normal metabolic reactions, exposure to ionizing radiation and by the influence of many xenobiotics are indicated in the causation of several diseases like cancer, heart diseases, aging etc. Antioxidants, which can scavenge free radicals, have an important role in biological systems. Several antioxidants of plant origin are experimentally proved and used as effective protective agents against free radical mediated toxicity. Both the compounds and their synthetic derivatives were screened for *in vitro* antioxidant activity and the potent compounds were selected for *in vivo* analgesic and anti-inflammatory activities.

Embelia ribes is traditionally being used as brain tonic and for the treatment of mental disorders [10]. Its decoction was reported as antihyperglycemic [63], neuroprotective [76,77] and also used in gastrointestinal ailments [32]. However, embelin a major constituent of *Embelia ribes* has not been tested so far for anticonvulsant, antidiabetic, neuroprotective and the effect in inflammatory bowel disease, though the plant is traditionally used against such disorders. Hence, in the present study, the screening for anticonvulsant, antidiabetic and the effect of embelin in inflammatory bowel disease along with neuroprotective activity was aimed.

Plan of work

1. Collection and authentication of berries of *Embelia ribes* and leaves of *Mangifera indica*.

2. Extraction of plant materials.

3. Isolation of embelin and mangiferin and their characterization.

4. Preparation of synthetic derivatives of embelin and mangiferin and their characterization.

5. *In vitro* antioxidant screening of the synthetic derivatives by using DPPH, scavenging of ABTS radical cation, hydroxyl radical scavenging by deoxyribose, hydroxyl radical scavenging by p-NDA, hydrogen peroxide scavenging, nitric oxide radical inhibition assay and superoxide radical scavenging by alkaline DMSO methods.

6. Based on the *in vitro* antioxidant studies, screening for *in vivo* analgesic and anti-inflammatory activities of the potent compounds.
    - Analgesic activity by Eddy's hotplate, tail immersion and acetic acid induced writhing methods using mice.
    - Anti-inflammatory activity by carrageenan induced paw edema in rats

7. Biological studies of embelin
    - Anticonvulsant activity by MES induced seizure in rats, PTZ induced seizure and locomotor activity in mice.
    - Antidiabetic activity by alloxan induced diabetes in rats.
    - Effect of embelin against IBD by acetic acid induced colitis in rats.
    - Neuroprotective activity of embelin by global cerebral ischemia induced neuronal damage in rats.

3.1. *Embelia ribes*

Botanical Name : *Embelia ribes* Burm

Family : Myrsinaceae

Synonyms

English : Embelia

Hindi : Vayvidamg

Kannada : Vayuvidanga

Malayalam : Vilal

Sanskrit : Vidangah, Vellah

Tamil : Vayuvilamga

Telegu : Vayuvidanamu, Vidanga

3.1.1. Distribution

These climbers are found in throughout India, in areas upto 1500 m elevation in hilly regions.

3.1.2. Parts used

Roots, leaves and fruits (Fig. 2).

3.1.3. Chemical constituents

Embelin (embelic acid), a volatile oil (vilangin), embelinol, embeliaribyl ester, embeliol, 2,5-isobutylanine salts, quercetol, colouring matter, tannins and an alkaloid called christembine [81,82].

3.1.4. Biological properties of *Embelia ribes*

The roots are acrid, astringent, thermogenic and stomachic, and are useful in vitiated conditions of odontalgia, colic, flatulence and dyspepsia. The leaves are astringent, thermogenic, demulcent and depurative, and are useful in pruritus, skin diseases and leprosy. The fruits are acrid, astringent, bitter, thermogenic, anthelmintic, depurative, brain tonic, digestive, carminative, stomachic, diuretic, contraceptive, rejuvenating, alterant, stimulant, alexeteric, laxative, anodyne, vulnerary, febrifuge and tonic, and are useful in vitiated conditions helminthiasis, skin diseases, leprosy, pruritus, nervous dibility, amentia, dyspepsia, flatulence, colic, constipation, strangury, tumours, asthma, bronchitis, dental caries, odontolgia, hemicrania, dyspnoea, cardiopathy, psycopathy, ring worm infestation, fever, emaciation and general debility [9,10].

Fruits of *Embelia ribes* have been shown to possess analgesic, antipyretic [83], antimicrobial [84,85], antifertility [86], anthelmintic [87], neuroprotection [76,77], hepatoprotective [88] and acetylcholineesterase inhibitory [89] activities. In a preliminary study, Tripathi, [63] reported the antihyperglycemic activity of decoction of the *Embelia ribes* fruits in glucose-fed albino rabbits. Further, Bhandari et al. [64] reported the potential of ethanolic extract of *Embelia ribes* in diabetic dyslipidemia and protection from lipid peroxidation in tissues in streptozotocin-induced diabetes in rats. Recently Bhandari et al. [65] have reported the cardioprotective activity of the aqueous extract of *Embelia ribes* in isoproterenol induced myocardial infarction in albino rats.

3.1.5. Biological activities of embelin

Embelin is a naturally occurring alkyl substituted hydroxy benzoquinone and a major constituent of *Embelia ribes*. It has been reported to possess antifertility, anti-implantation, antitumour, anti-inflammatory, analgesic, antioxidant, hepatoprotective, wound healing and antibacterial activities [13-19].

Fig. 2. Different parts of *Embelia ribes*

3.2. *Mangifera indica*

Botanical Name : *Mangifera indica* Linn

Family : Anacardiaceae

Synonyms

English : Mango

Hindi : Am, Amb

Kannada : Mavu

Malayalam : Mavu

Sanskrit : Amrah, Cutah

Tamil : Mamaram, Mankai

Telegu : Mamidi

3.2.1. Distribution

Found in throughout India, in forests upto 1200 m also cultivated.

3.2.2. Parts used

Roots, bark, leaves, flowers, fruits and seed kernel (Fig. 3).

3.2.3. Chemical constituents

The chemical constituents of the different parts of *Mangifera indica* are reviewed by Ross [90] and Scartezzini and Speroni [91]. The bark is reported to contain protocatechic acid, catechin, mangiferin, alanine, glycine, γ-aminobutyric acid, kinic acid, shikimic acid and the tetracyclic triterpenoids cycloart-24-en-3β,26diol, 3-ketodammar-24 (*E*)-en-20S,26-diol, C-24 epimers of cycloart-25 en 3β,24, 27-triol and cycloartan-3β,24,27-triol.

3.2.4. Biological properties of *Mangifera indica*

The roots and bark are astringent, acrid, refrigerant, styptic, antisyphilitic, vulnerary, antiemetic, anti-inflammatory and constipating. They are useful in vitiated conditions of *Pitta,* metorrhagia, colonorrhagia, pneumorrhagia, leucorrhoea, syphilis, wounds, ulcers, vomiting, uteritis, diarrhoea, dysentery, diphtheria and rheumatism. The leaves are astringent, refrigerant, styptic, vulnerary and constipating. They are useful in vitiated conditions of *Kapha* and *Pitta*, hiccough, hyperdipsia, burning sensation, haemorrhages, haemoptysis, haemorrhoides, wounds, ulcers, diarrhoea, dysentery, pharyngopathy and stomatopathy. The ashes of the burnt leaves are useful in burns and scalds. The flowers are astringent, refrigerant, styptic, vulnerary, constipating and haematinic. They are useful in vitiated conditions of *Pitta*, haemorrhages, haemoptysis, wounds, ulcers, anorexia, dyspepsia, dysentery, urethorrhea and vaginopathy. The ripe fruits are refrigerant, sweet, emollient, laxative, cardiotonic, haemostatic, aphrodisiac and tonic. The seed kernel is sweet, acrid, astringent, refrigerant, anthelmintic, constipating, haemostatic, vulnerary and uterine tonic [20,21].

The fruits, barks and leaves of *Mangifera indica* have been reported to possess diverse medicinal properties in the traditional Indian system of medicine, the Ayurveda and are widely used in several medicinal preparations [92]. The plant has been reported as antiviral, antidiabetic, antibacterial, antiamoebic, antidiarrhoeal, immunostimulant, immunomodulatory, analgesic and antiinflammatory properties [26,27,92-98]. The extract of *Mangifera indica* has been reported to be an antioxidant and a potent scavenger of hydroxyl radicals and hypochlorous acid. It has also been found to chelate iron, inhibit lipid peroxidation and DNA damage *in vitro* [99].

3.2.5. Biological activities of mangiferin

Mangiferin was first isolated from *Mangifera indica*. Since its first isolation in 19th century, mangiferin has become the most extensively studied compound for various type of diseases including, analgesic, anti-inflammatory, antidiabetic, antioxidant, antitumor, antimicrobial, antiviral and immunomodulatory activities [22,23,27,28,100-105].

Fig. 3. Different parts of *Mangifera indica*

4.1. Instruments

Autoanalyser: Merck Microlab 200, 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., Thane, India.

Grinding Mill: Junior Grindwell, Chowdhry, J.U.C, Mumbai.

Homogenizer: Elvenjan homogenizer, Remi Motors Ltd., Mumbai.

Melting Point Apparatus: Lab India, Mumbai.

NMR: DDR X – 500 m/z – Bruker Daltonics, Karsruhe, Germany.

Rotary Evaporator: Rotavapor R-205, Buchi Laboratory Equipments, Flawil, Switzerland.

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), 1-chloro-2,4-dinitrobenzene and

pentylenetetrazole (PTZ) were obtained from Sigma Aldrich Co, St Louis, USA. Rutin and p–nitroso dimethyl aniline (p–NDA) were obtained from Acros Organics, New Jersy, USA. Naphthyl ethylene diamine dihydrochloride (NEDD) was obtained from Roch–Light Ltd., Suffolk, UK. Ascorbic acid, nitro blue tetrazolium (NBT), and butylated hydroxy anisole (BHA) were obtained from S.D. Fine Chem, Ltd., Biosar, India. 2-Deoxy–D-ribose and triphenyltetrazolium chloride (TTC) were purchased from Hi-Media Laboratories Ltd., Mumbai. Reduced glutathione (GSH) and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) were obtained from Sisco Research Laboratories Pvt. Ltd., Mumbai. Diazepam was purchased from Ranbaxy Ltd., New Delhi, India. Phenytoin was purchased from Sun Pharma, Mumbai, India. Alloxan monohydrate was purchased from Loba Chemie, Bombay. Aluminium backed high performance thin layer chromatography (HPTLC) plates coated with 0.2 mm layers of silica gel 60 F_{254} plates, Bovine serum albumin (BSA) and assay kits for serum triglycerides (TGL), total cholesterol (TC), total bilirubin (TB), total protein (TP), creatinine (CR), albumin, lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) were obtained from E–Merck Ltd., Mumbai. All other chemicals used were of analytical grade.

4.3. Plant materials

The berries of *Embelia ribes* were purchased from Abirami Botanicals, Tuticorin, Tamilnadu, India and leaves of *Mangifera indica* were collected from Namakkal, Tamilnadu, India and authenticated by Medicinal Plants Survey and Collection Unit, Ootacamund, Tamil Nadu, India, where a voucher has been deposited for further reference.

4.4. Extraction and isolation of embelin

Coarsely powdered berries of *Embelia ribes* (6 kg) were exhaustively extracted with *n*-hexane by cold extraction method (5 x 2 L). After 72 h, the extracts were concentrated to dryness in a rotavapor under reduced pressure and controlled temperature (40-50 ^{o}C). The residue so obtained was subjected to column chromatography over silica gel (100-200 mesh) and elution with benzene yielded an orange coloured powder [106], which on crystallization with ether afforded orange plates of embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone, mp 141-143 ºC, yield 19.5 g, 0.325%). It was found to be homogenous by HPTLC when separated using the solvent system ethyl acetate: benzene (70:30, R_f = 0.53). It was characterized by comparing its melting point, IR, NMR and MS data with literature values [107].

4.5. Structural modification of embelin

4.5.1. Methylation of embelin (2)

Embelin (0.294 g, 1 mmol) was refluxed with dimethylsulphate (0.630 g, 5 mmol) and anhydrous potassium carbonate (2 g) in dry acetone (100 ml) for 30 h. The product (2-hydroxy-5-methoxy-3-undecyl-1,4-benzoquinone) was crystallized from alcohol as deep yellow rectangular tablets [108].

4.5.2. Condensation of embelin with primary amines

4.5.2.1. General procedure

Embelin (0.294 g, 1 mmol) and the primary amine (1 mmol) were boiled under reflux on a water bath at 100 ºC for 2-3 h [31,32]. The cooled reaction mixture was decomposed using excess of ice cold dil. HCl and the product obtained was subjected to column

chromatography over silica gel (100-200 mesh). The elution with petroleum ether and ethyl acetate (90:10) yielded the products **3-17** and **19-25** (Fig. 4).

4.5.3. Synthesis of compound 18

In a beaker, 25 ml of the 10% NaOH and 25 ml of ethanol were taken and stirred continuously for 30 min. To this benzaldehyde (0.106 g, 1 mmol) was added and stirred continuously for another half an hour, compound **17** (0.411 g, 1 mmol) were added at the last. The solution was allowed to stir for 3-4 h at room temperature. After completion of the reaction 100 ml of cold water was added, formed precipitate was filtered, washed three times with 50 ml of water each time to remove sodium hydroxide. Then the precipitate was dried and re-crystallized from ethanol. It designated as compound **18**.

4.6. Synthesis of embelin-ninhydrin adduct

The synthesis of embelin-ninhydrin adduct can be done easily in acidic medium. In acetic acid medium, the ortho-position of phenol reacts selectively with the carbonyl group at the 2-position of ninhydrin followed by acid-catalyzed formation of cyclic hemiketal to form the tetracyclic skeleton (Scheme 1). Embelin (0.617 g, 2.1 mmol) was added to the solution of ninhydrin (0.125 g, 0.7 mmol) in 10 ml acetic acid and the mixture was stirred at 80-90 ˚C for 2-3 h and kept at room temperature for certain period. The reaction mixture was diluted with ethyl acetate (25 ml) and washed with water. The combined organic layers were dried over $MgSO_4$, filtered, concentrated, and purified by column chromatography by using solvent system petroleum ether : ethyl acetate (4:6) to give the mono-adduct of embelin. The product was recrystallized using ethanol to yield pure embelin-ninhydrin adduct (0.415 g, 43.55%).

4.7. Extraction and isolation of mangiferin

Shade-dried and powdered leaves of *Mangifera indica* (1 kg) were soxhleted for 18-20 h with petroleum ether (60–80 °C). The defatted plant material was extracted with ethanol under reflux for 18-20 h. On keeping overnight a solid matter separated out, it was filtered and washed with petroleum ether repeatedly, affording yellow powder of mangiferin [22]. After repeated crystallization of the powder in aqueous ethyl acetate and methanol pale-yellow needle-shaped crystals of mangiferin were obtained (1,3,6,7-tetrahydroxyxanthone-C2-β-D-glucoside, mp 270-272 ºC, yield 4.5 g, 0.45%), and exhibited violet color with ferric chloride. It was found to be homogenous by HPTLC when separated using the solvent system ethyl acetate: methanol: water: formic acid (6:2:1:1, R_f = 0.76). It was characterized by comparing its melting point, IR, NMR and MS data with literature values [109].

4.8. Structural modification of mangiferin

4.8.1. Benzoylation of mangiferin

Mangiferin (0.844 g, 2 mmol), benzoyl chloride (0.560 g, 4 mmol), pyridine (0.474 g, 0.6 mmol) and basic alumina (2 g) were added in a 50 ml beaker [110]. The mixture was stirred to obtain a free flowing powder, at room temperature. Then the temperature was increased upto 70 ºC for 30 min. After cooling to room temperature, the product was extracted with methylene chloride (3 x 15 ml). The combined extracts were washed with water and dried over sodium sulfate. The product obtained after removal of solvent under reduced pressure was crystallized from a suitable solvent (Ethyl acetate-petroleum ether; ethanol), yielded tribenzoyl mangiferin and designated as compound MGN 1 (Fig. 5).

4.8.2. Benzylation of mangiferin

A solution of mangiferin (0.422 g, 1 mmol) in dry DMF (20 ml) was treated with benzyl chloride (0.504 g, 4 mmol) and K_2CO_3 (0.1 g) with stirring at 60 ºC for 10 h [37]. The reaction mixture on evaporation in vacuo gave a residue and was submitted to column chromatography using dichloro methane and methanol as eluent to give tribenzyl mangiferin, designated as MGN 2.

4.8.3. Acetylation of mangiferin

A solution of mangiferin (0.422 g, 1 mmol) in pyridine (0.395 g, 5 mmol) was treated with acetic anhydride (0.306 g, 3 mmol) and then stirred at 40 ºC for 36 h at room temperature [36]. The reaction mixture was poured into ice-cold water and extracted with chloroform soluble fractions. On evaporation in vacuo gave a residue and was submitted to column chromatography using chloroform and methanol as eluent to give octaacetyl mangiferin, designated as MGN 3.

4.8.4. Methylation of Mangiferin

A solution of mangiferin (0.422 g, 1 mmol) in acetone (150 ml) was treated with dimethyl sulphate (0.882 g, 7 mmol) and K_2CO_3 (0.1 g) with stirring at room temperature for seven days [36]. The reaction mixture on evaporation under hood gave a residue and was submitted to column chromatography using chloroform and methanol as eluent to give trimethyl mangiferin, designated as MGN 4.

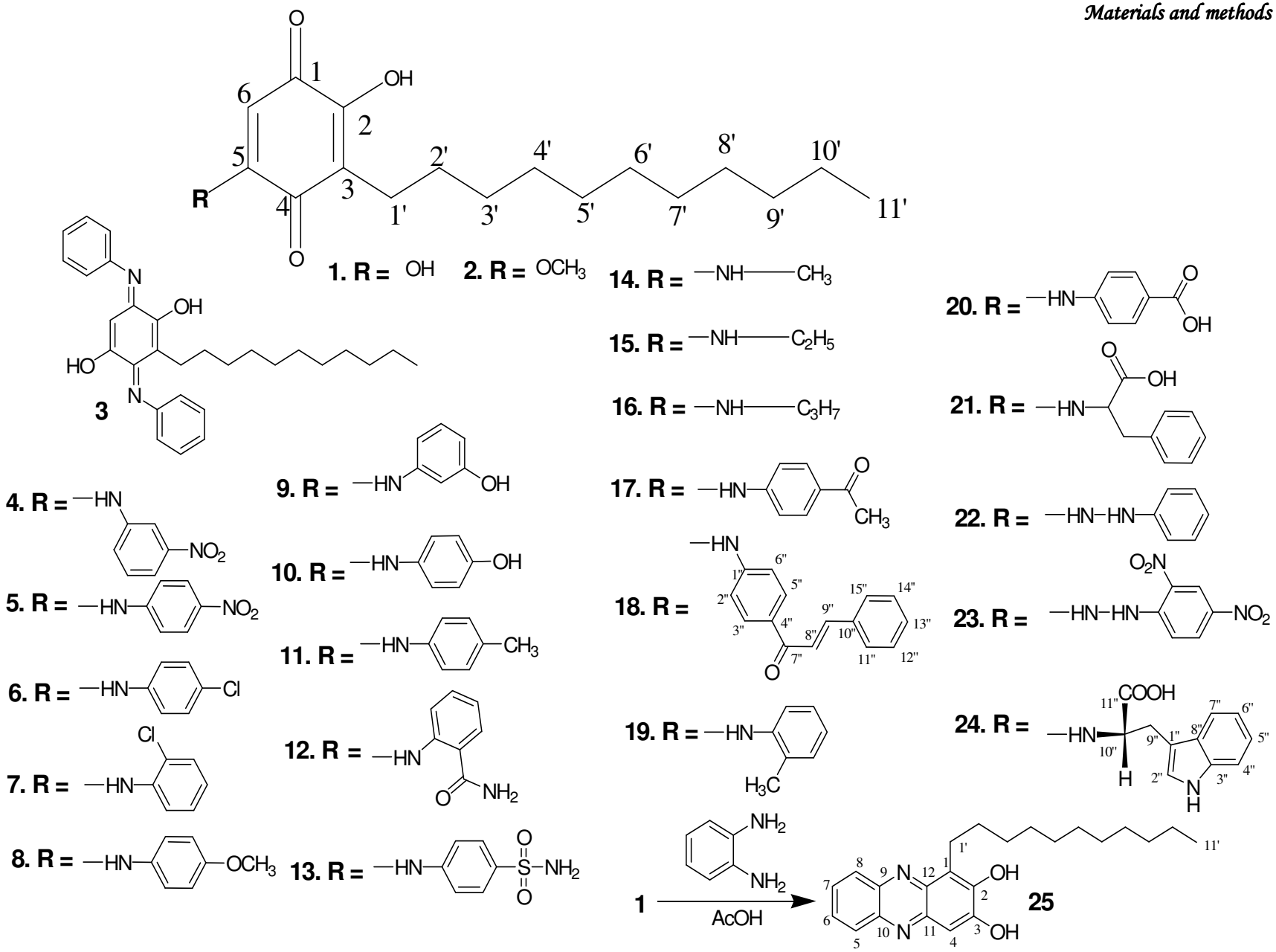
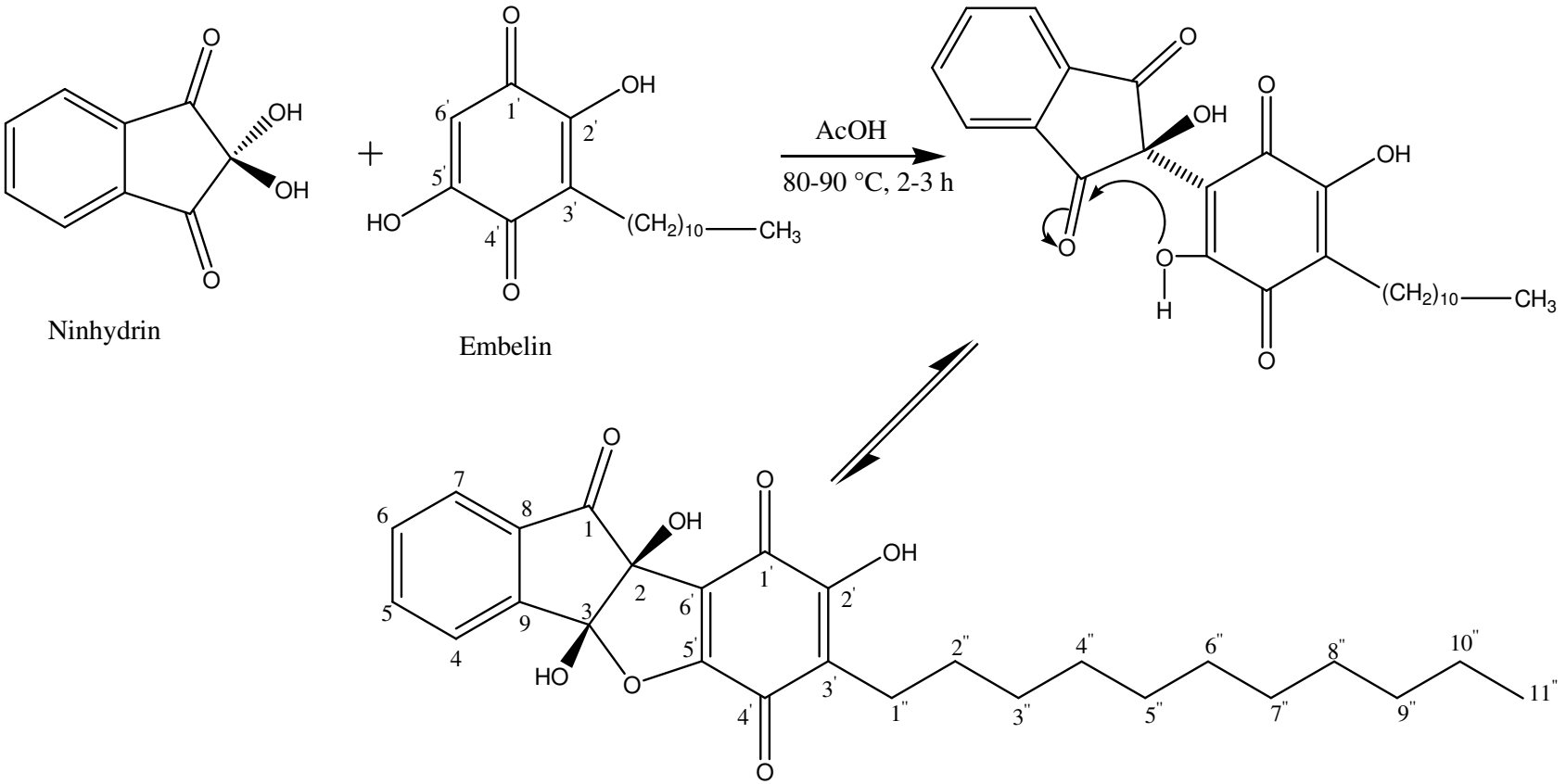
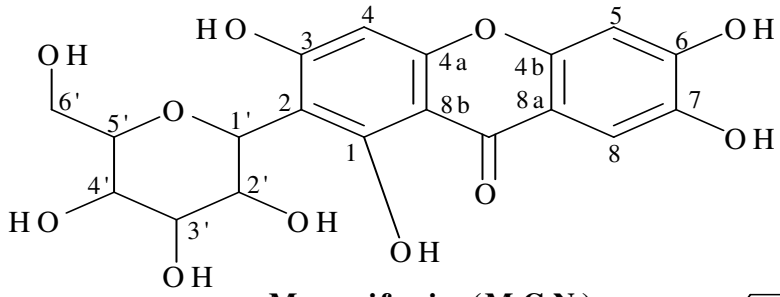


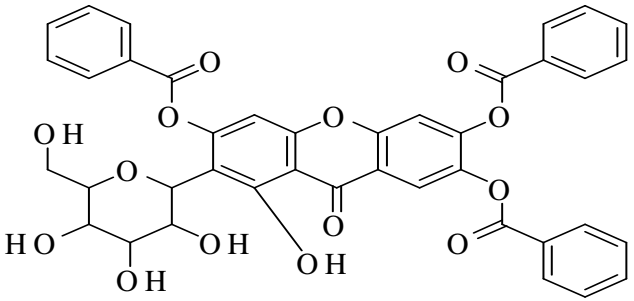
Fig. 4. Structure of embelin and its derivatives



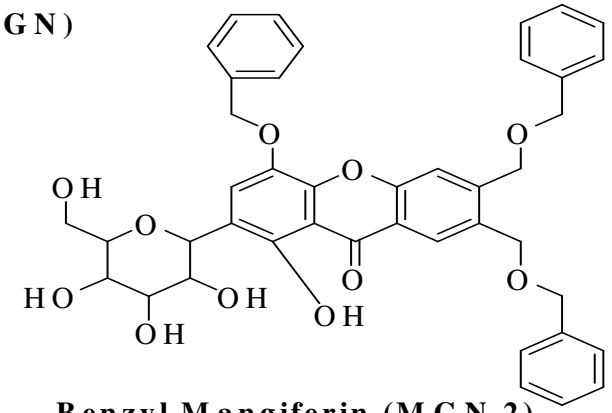
Scheme 1. Synthesis of embelin-ninhydrin adduct



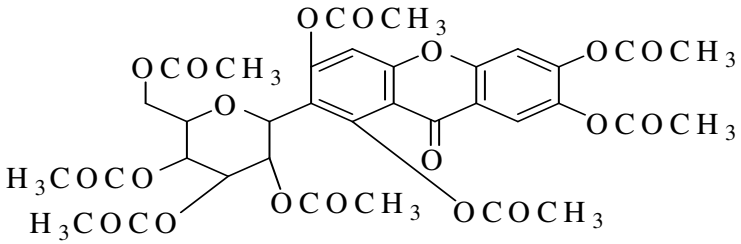
Mangiferin (MGN)



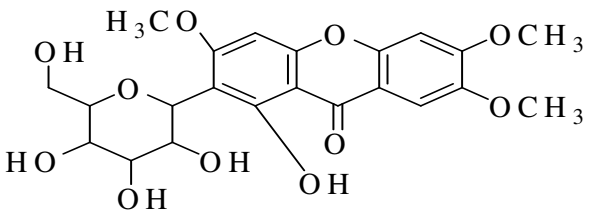
Benzoyl Mangiferin (MGN 1)



Benzyl Mangiferin (MGN 2)



Acetyl Mangiferin (MGN 3)



Methyl Mangiferin (MGN 4)

Fig. 5. Structure of mangiferin and its derivatives

4.9. *In vitro* antioxidant activity

The *in vitro* methods are based on inhibition. Samples are added to a free radical-generating system, inhibition of the free radical action is measured and 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 endpoint that is used for the determination.

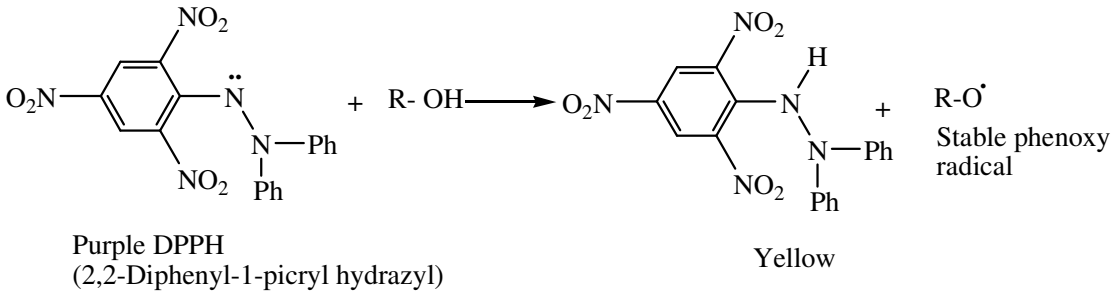
Even though *in vitro* methods provide a useful indication of antioxidant activities, data obtained from *in vitro* methods are difficult to apply to biological systems and do not necessarily predict a similar *in-vivo* antioxidant activity. Important is that all methods developed have strengths and limitations and 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. All the synthesized compounds were tested for *in vitro* antioxidant activity using several standard methods. The final concentration of the synthesized compounds and standard solutions used were 1000, 500, 250, 125, 62.5, 31.25 and 15.625, 7.812 µg/ml etc. The absorbance was measured spectrophotometrically against the corresponding blank solution. The percentage inhibition was calculated by using the following formula.

$$\text{Radical scavenging activity (\%)} = \frac{\text{OD control - OD sample}}{\text{OD control}} \times 100$$

IC_{50}, which is the concentration of the sample required to scavenge 50% of free radicals was 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 hydrogen donor's 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 was measured at 490 nm.



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

Yellow

Reagents

2,2-Diphenly-1-picryl hydrazyl solution (DPPH, 100 µM): Accurately weighed 22 mg of DPPH 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.

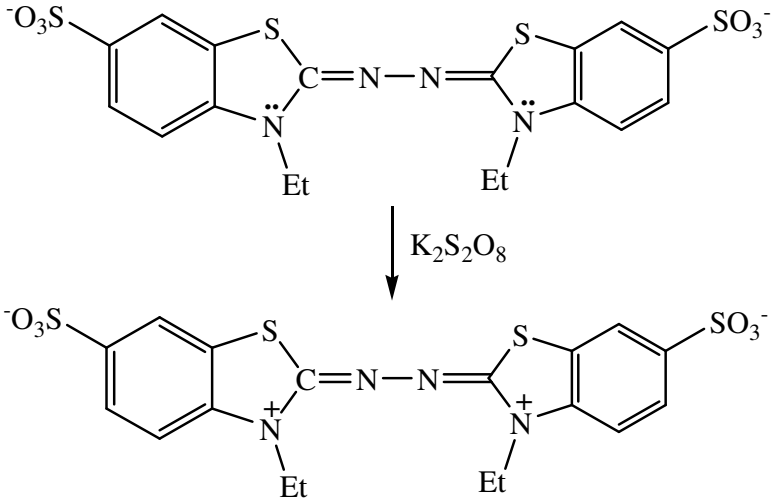
Preparation of sample solutions: Accurately weighed 21 mg of each of the synthesized compounds and dissolved in 1 ml of freshly distilled DMSO separately to obtain solutions of 21 mg/ml concentration. These solutions were serially diluted separately to obtain the lower concentrations.

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

Procedure: The assay was carried out in a 96 well microtitre plate. To 200 µl of DPPH solution, 10 µl of each of synthesized compounds 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 [111], using ELISA reader.

4.9.2. Scavenging of ABTS radical cation assay

ABTS assay is relatively recent one, which involves a more drastic radical, chemically produced and, is often used for screening complex antioxidant mixture such as plant synthesized compounds, beverages and biological fluids. The solubility in both the organic and aqueous media and the stability in a wide pH range raised the interest in the use of $ABTS^{\bullet+}$ for the estimation of the antioxidant activity [112].



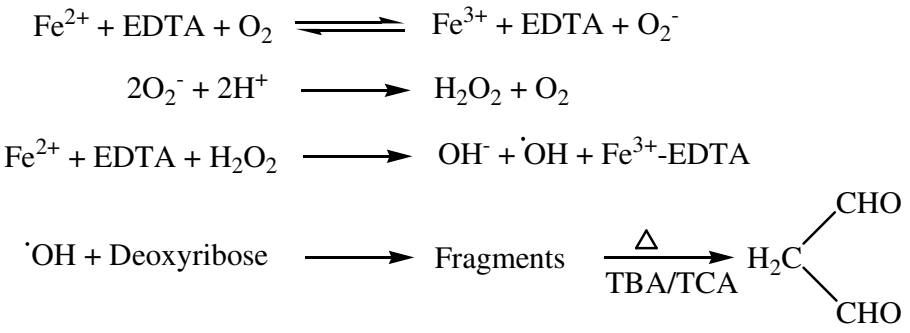
ABTS radical cation

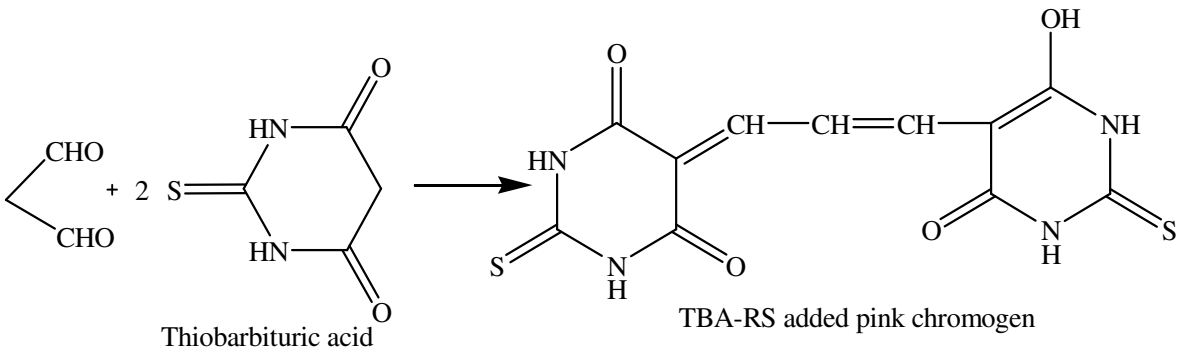
Preparation of sample and standard solutions: Weighed 13.5 mg of each of the synthesized compounds and the standards, ascorbic acid and rutin and separately dissolved in 2 ml of freshly distilled DMSO. These solutions were serially diluted with DMSO to obtain the lower dilutions.

Procedure: Accurately weighed 54.8 mg of ABTS and dissolved in 50 ml of distilled water (2 mM) and potassium persulphate (17 mM, 0.3 ml) was 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 synthesized compounds or standards, added 1.0 ml of distilled DMSO and 0.16 ml of ABTS solution to make the final volume of 1.36 ml. Absorbance was measured after 20 min at 734 nm [113].

4.9.3. 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 is heated under acid conditions, malondialdehyde (MDA) is formed and may be detected by its ability to react with thiobarbituric acid (TBA) to form a pink chromogen [114].





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

Procedure: 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), were added to 0.2 ml of various concentrations of the synthesized compounds or standard in DMSO to give a total volume of 1.2 ml. The solutions were then incubated for 30 min at 37 °C. After incubation, ice-cold trichloro acetic acid (0.2 ml, 15% w/v) and thiobarbituric acid (0.2 ml, 1% w/v), in 0.25 N 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 [114].

4.9.4. Scavenging of hydroxyl radical by p-NDA method

Hydroxyl radical is measured by the inhibition of p-nitrosodimethyl aniline (p-NDA) bleaching [115] 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 scavenger shows scavenging activity by inhibition of bleaching and percentage of scavenging as absorbance is measured at 440 nm.

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

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

$$Fe^{2+}\text{-EDTA complex} + H_2O_2 \longrightarrow Fe^{3+}\text{-EDTA} + H_3O + OH^-$$

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

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

Procedure: To a reaction 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) were added various concentrations of the synthesized compounds or standard (0.5 ml) to give a final volume of 3 ml. Sample blank was prepared by adding 0.5 ml sample and 2.5 ml of phosphate buffer. Absorbance was measured at 440 nm [115].

4.9.5. 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 [116].

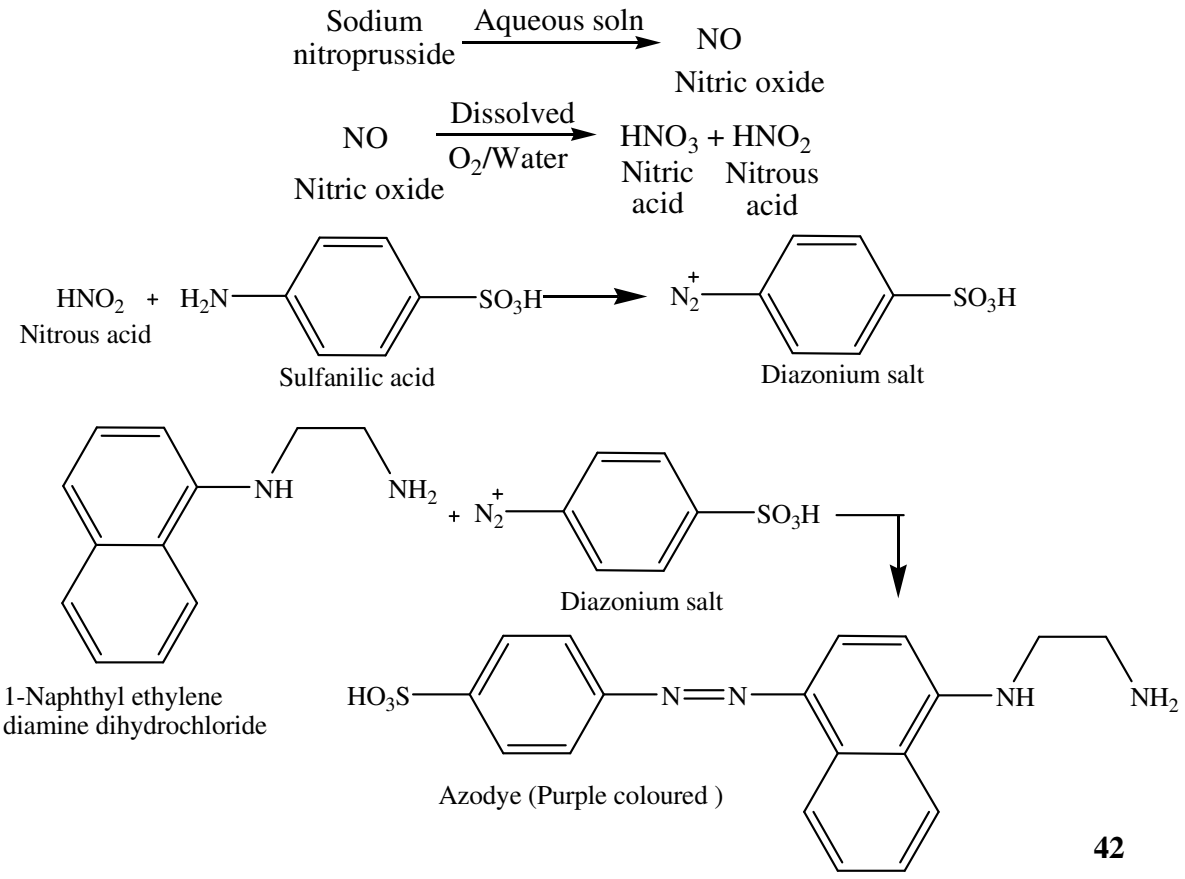
Preparation of sample and standard solutions: Accurately weighed 30 mg of each of the synthesized compounds and the standard and dissolved separately in 5 ml of methanol. These solutions were serially diluted with methanol to obtain the lower dilutions.

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 synthesized

compounds 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.

4.9.6. 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 [117]. In the present investigation, Griess Ilosvay reagent is 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 nitrites 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.



Reagents

1. Sodium nitroprusside solution (10 mM): Weighed accurately 0.30 g of sodium nitroprusside and dissolved in distilled water to make up the volume to 100 ml in a volumetric flask.

2. Naphthyl ethylene diamine dihydrochloride (NEDD, 0.1%): Weighed accurately 0.1 g of NEDD and dissolved in 60 ml of 50% glacial acetic acid by heating and made up the volume to 100 ml with distilled water in a volumetric flask.

3. Sulphanilic acid reagent (0.33% w/v): Weighed accurately 0.33 g of sulphanilic acid and dissolved in 20% glacial acetic acid by heating and made up the volume to 100 ml in a volumetric flask.

*Preparation of sample and standard solutions***:** These solutions were prepared as described in DPPH assay**.**

Procedure: The reaction mixture (6 ml) containing sodium nitroprusside (10 mM, 4 ml), phosphate buffer saline (PBS, pH 7.4, 1 ml) and synthesized compound 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, 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 [118].

4.9.7. Lipid peroxidation inhibitory activity

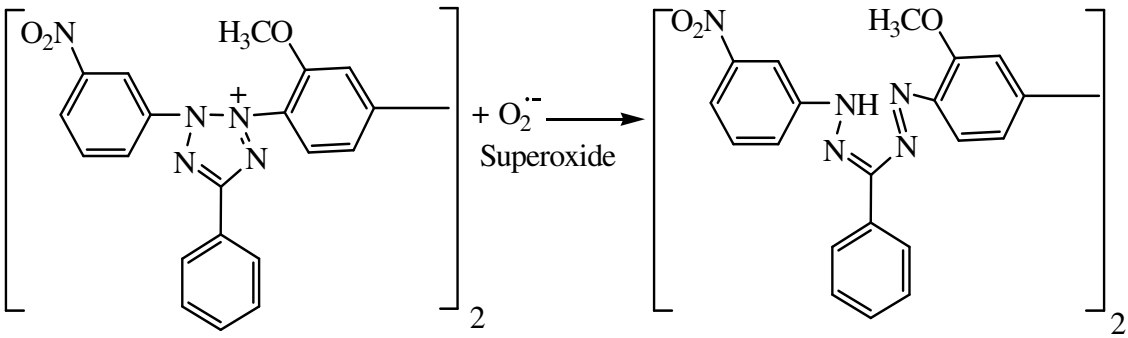
Lipid peroxidation can be initiated by ROS such as hydroxyl radicals by extracting a hydrogen atom from lipids, and forming a conjugated lipid radical. This reacts rapidly with oxygen to form a lipid radical until the chain reaction is terminated. The lipid peroxidation adducts may induce the oxidation of biomolecules such as DNA, proteins and another lipids, resulting in cellular damage. Ability of synthesized compounds to inhibit lipid peroxidation was also investigated in the present investigation.

Preparation of egg lectin: Egg lectin (3 mg/ml) prepared in phosphate buffer (pH 7.4) was sonicated in an ultrasonicator.

Procedure: The test samples (100 µl) of different concentrations were added to 1 ml of liposome mixture. A control was used without test sample. Lipid peroxidation was induced by adding 10 µl $FeCl_3$ (400 mM) and 10 µl L-ascorbic acid (200 mM). After incubation for 1 h at 37^{o}C, the reaction was stopped by adding 2 ml of 0.25 N HCl containing 15% TCA and 0.375% TBA and the reaction mixture was boiled for 15 min, cooled, centrifuged and the absorbance of the supernatant was measured at 532 nm [119].

4.9.8. Scavenging of superoxide radical by alkaline DMSO method

In alkaline DMSO method, superoxide radical is generated by the addition of sodium hydroxide to air saturated dimethyl sulfoxide (DMSO). The generated superoxide remains stable in solution, which reduces nitro blue tetrazolium into formazan dye at room temperature and that can be measured at 560 nm. Superoxide scavenger capable of reacting inhibits the formation of a red dye formazan [115].



Nitroblue tetrazolium

Formazan (Red dye)

Preparation of sample and standard solutions: Accurately weighed 14 mg of each of the synthesized compounds and dissolved separately in 3 ml of freshly distilled DMSO. These solutions were serially diluted with DMSO to obtain the lower dilutions.

Procedure: To the reaction mixture containing 1 ml of alkaline DMSO (1 ml DMSO containing 5 mM NaOH in 0.1 ml water) and 0.3 ml of the synthesized compounds or standards in DMSO at various concentrations, added 0.1 ml of NBT (1 mg/ml) to give a final volume of 1.4 ml. The absorbance was measured at 560 nm [115].

4.10. Pharmacological studies of potent antioxidant compounds

4.10.1. Animals

The animals were obtained from the animal house of JSS College of Pharmacy, Ootacamund, India and Sree Siddaganga College of Pharmacy, Tumkur, India, maintained under standard conditions (12 h light / dark cycle; 25 ± 3 ºC, 45-65% humidity) and had free access to standard rat feed and water *ad libitum*. All the animals were acclimatized to laboratory conditions for a week before commencement of the experiment. The experiments were performed during the light portion between 07:00 - 18:00 h to avoid circadian influences. Animal studies were performed according

to the prescribed guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, India.

4.10.2. Toxicity and dose profile

Different doses of embelin and its semisynthetic derivatives from 2.5 to 20 mg/kg (i.p.) were selected based on the earlier study [32]. However, the LD_{50} of embelin is as high as 2000 mg/kg (p.o.) in rats and mice with no mortality or adverse effects indicating its safety [120]. The minimum dose level of embelin 25 and 50 mg/kg (p.o.) were selected for antidiabetic, neuroprotective and inflammatory bowel studies. Wauthozl et al. [121] reported the LD_{50} of mangiferin as 365 mg/kg (i.p.). Based on this report, in the present study, the minimum dose level of mangiferin and its semisynthetic derivatives (10 and 20 mg/kg, i.p.) were selected.

Three different sets of mice were randomized into twenty six groups, each containing six animals and used in three different models for the evaluation of analgesic activity. Different doses of compounds **1**, **10**, **13** and **21-25**, embelin-ninhydrin adduct, MGN, MGN 1 and MGN 3 were prepared as suspensions in Tween-80 (1% v/v in saline). Group I were treated with Tween-80 (1% v/v in saline) as normal vehicle control. Groups II to XXV were treated with compounds **1**, **10**, **13** and **21-25**, embelin-ninhydrin adduct, MGN, MGN 1 and MGN 3 at 10 and 20 mg/kg, respectively and Group XXVI animals were treated with standard pentazocine at 20 mg/kg. All the treatments were administered intraperitoneally. The experiments were carried out six groups in a day.

4.10.3. Analgesic activity

The method for testing analgesic activity was selected such that both centrally and peripherally mediated effects were investigated. The acetic acid-induced abdominal constriction and the tail immersion methods elucidated peripheral and central activity, respectively. Acetic acid causes an increase in peritoneal fluid levels of prostaglandins (PGE2 and PGF2), involving in part peritoneal receptors and inflammatory pain by inducing capillary permeability. Although the writhing test has poor specificity, it is a very sensitive method of screening antinociceptive effects and with a good correlation between ED_{50} values obtained in animals using this test and analgesic doses in human [122].

4.10.3.1. Eddy's Hot-plate method

In this method heat is used as a source of pain. Animals were individually placed on a hot plate maintained at constant temperature (55 ± 0.5 ºC) and the reaction of animals, such as paw licking or jump is taken as the end point. Analgesics increase the reaction time [123].

Procedure: Mice 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 15, 30 and 45 min following the treatments. The percentage protection was calculated using the formula, protection (%) = (t-n/t) ×100, where, t = reaction time of treated group and n = reaction time of normal group [123].

4.10.3.2. Tail immersion method

In this method also heat is used as a source of pain. The tail of the animals was immersed in warm water maintained at constant temperature (55 ± 0.5 ºC) and the reaction of animals, the tail withdrawal from the heat is taken as the end point. Analgesics increase the reaction time.

Procedure: In this method [124], 5 cm of the end of the mice tail was immersed in warm water maintained at 55 ± 0.5 ºC. The tail withdrawal reflex was recorded before and after 60 min following the treatments. The percentage protection was calculated as per hot plate method.

4.10.3.3. Acetic acid induced writhing method

Painful reactions in animals may be produced by chemicals also. Intraperitoneal injection of acetic acid produces pain reaction which is characterized as writhing response. Constriction of abdomen, turning of trunk (twist) and extention of hind legs are taken as reaction to chemically induced pain. Analgesics, both narcotic and non-narcotic type, inhibit writhing response [123].

Procedure: In the acetic acid induced writhing [123] in mice an intraperitoneal injection of acetic acid (1%, 10 ml/kg) was given 30 min after the treatments. The response is in the form of abdominal contractions, trunk twist and extension of hind limb. The number of writhing in each mouse was counted for 20 min from the injection of acetic acid. The percentage protection was calculated using the formula, protection (%) = (c-t/c) ×100, where, t = reaction time of treated group and c = reaction time of control group.

4.10.4. Anti-inflammatory activity

Inflammation is a tissue-reaction to infection, irritation or foreign substance. It is a part of the host defence mechanism but when it becomes great it is a hopeless condition. Aging is also considered to be an inflammatory response. There are several tissue factors or mechanisms that are known to be involved in the inflammatory response such as release of histamine, bradykinin and prostoglandins. The development of non-steroidal antiinflammatory agents in recent years has contributed a lot in not only overcoming the human suffering such as arthritis but also has helped in understanding the tissue mechanisms of inflammation.

4.10.4.1. Carrageenan induced paw edema in rats

The inflammatory reaction is readily produced in rats in the form of paw edema with the help of irritants. Substances such as carrageenan, formalin, bradykinin, histamine, 5-hydroxytryptamine, mustard, or egg white when injected in the dorsum of the foot of the rats produce acute paw edema within a few minutes of the injection. Carrageenan induced paw edema is the most commonly used method in experimental pharmacology. Carrageenan is a sulphated polysaccharide obtained from sea weed (Rhoodophyceae), and by causing the release of histamine, 5-HT, bradykinin and prostaglandins, it produces inflammation and edema [123].

Procedure: Swiss albino rats (150-200 g) were divided into eight groups with six animals in each group. Group I was served as control and received Tween-80 (1% v/v in saline). Groups II to XXV were received the treatments as described in analgesic activity. Group XXVI was treated as positive control and received standard diclofenac (20 mg/kg). All

the treatments were administered intraperitoneally. The experiments were carried out six groups in a day.

The initial hind paw volume of rats was determined volumetrically by using a plethysmometer [35]. A solution of carrageenan in saline (1%, 0.1 ml/rat) was injected subcutaneously into the right hind paw 30 min after the treatments. The animals in the control group received the vehicle only. Paw volumes were measured up to 6 h at intervals of 30, 60, 120, 180 and 360 min and percent increase in edema between the control and treated groups were compared. The percentage protection was calculated as acetic acid induced writhing method.

4.10.5. Statistical analysis

The values were expressed as mean ± SEM. The statistical analysis was carried out by one way analysis of variance (ANOVA) followed by multiple comparison using the Dunnet's test. P values < 0.05 were considered as significant.

4.11. Anticonvulsant activity of embelin

Different types of epilepsies, i.e. grand mal, petit mal or psychomotor type, can be studied in laboratory animals. The maximal electro-shock (MES) induced convulsions in animals represent grand mal type of epilepsy. Similarly, chemo-convulsions due to pentylenetetrazole which produce clonic type of convulsion resemble petit mal type of convulsions in man. These are the two procedures used to study convulsions, and to test anticonvulsant drugs in laboratory animals.

4.11.1. MES induced seizure model

In MES-convulsions, electroshock is applied through the corneal electrodes. Through optic stimulation cortical excitation is produced. The MES-convulsions are divided into five phases such as (a) tonic flexion, (b) tonic extensor, (c) clonic convulsions, (d) stupor, and (e) recovery or death. A substance is known to possess anticonvulsant property if it reduces or abolishes the extensor phase of MES convulsions.

Procedure: Swiss albino rats (150-200 g) were divided into five groups with six animals in each group. Group I was served as solvent control and received 0.9% w/v of saline (1 ml/100 g). Groups II, III and IV were received embelin 2.5, 5 and 10 mg/kg suspended in 1% Tween-80 (v/v) and Group V treated as positive control was received phenytoin (25 mg/kg). All the treatments were administered intraperitoneally 30 min prior to the electroshock. The electroshock was induced in animals by passing a current of 150 mA for 0.2 sec duration through electroconvulsiometer (INCO, Ambala Pvt. Ltd., India) using corneal electrodes. A drop of electrolyte solution (0.9% sodium chloride) was instilled in the eyes of the animals prior to delivery of electroshock for good electrode contact [125]. Total duration of Hind Limb Tonic Extension (HLTE) was recorded after the delivery of the electroshock for control and experimental groups of animals. The onset of stupor, time taken for death/recovery as well as the percentage of protection against mortality were also recorded [56].

4.11.2. PTZ induced seizure model

Pentylenetetrazole is a central nervous system stimulant. It produces jerky type of clonic convulsions in rats and mice. The convulsive effect of this drug is considered to be

analogous to petit mal type of convulsions in man. Recently pentylenetetrazole has been reported to act through GABA-benzodiazepine receptor mechenisms in the brain. It is widely used as a tool in experimental pharmacology to study the convulsions and anticonvulsant action of drugs.

Procedure: Swiss albino mice (20-25 g) were divided into five groups with six animals in each group. Groups I, II, III and IV were received the treatments as described above. Group V, positive control, was received diazepam (4 mg/kg). All the treatments were administered intraperitoneally 30 min prior to the administration of PTZ (85 mg/kg). Each animal was observed for 1 h by placing in a separate cage. The onset of action showing fore limb and hind limb clonus (clonic and tonic action) and the time taken for death/recovery as well as the percentage of protection against mortality were recorded [56,126].

4.11.3. Locomotor activity

Most of the central nervous system acting drugs influence the locomotor activities in man and animals. The CNS depressant drugs such as barbiturates and alcohol reduce the motor activity while the stimulates such as caffeine and amphetamines increase the activity. In other words, the locomotor activity can be an index of walkfulness (alertness) of mental activity.

The locomotor activity (horizontal activity) can be easily measured using an actophotometer which operates on photoelectric cells which are connected in circuit with a counter. When the beam of light falling on the photocell is cut off by the animal, a

count is recorded. An actophotometer could have either circular or square areana in which the animal moves. Both rats and mice may be used for testing in this equipment.

Procedure: Mice were acclimatized with environment and placed individually in an actophotometer (INCO, Ambala Pvt. Ltd., India) for 10 min and a basal activity score was obtained. Subsequently they were divided into five groups with six animals in each group and all the groups except Group V, were treated as per MES induced seizure model. Group V, positive control was received diazepam (1 mg/kg, i.p). After 30 min, the activity score was recorded [123]. The percentage reduction in locomotor activity was calculated.

4.11.4. Statistical analysis

The values were expressed as mean ± S.E.M. The statistical analysis was carried out by one way analysis of variance (ANOVA) followed by multiple comparison test of Tukey-Kramer. P values < 0.05 were considered as significant.

4.12. Antidiabetic activity of embelin

4.12.1. Alloxan induced diabetes in rats

Alloxan is the most prominent diabetogenic chemical in diabetes research. Alloxan has two distinct pathological effects, it selectively inhibit glucose-induced insulin secretion through specific inhibition of glucokinase, the glucose sensor of the beta cell, and it causes a state of insulin-dependent diabetes through its ability to induce ROS formation, resulting in the selective necrosis of beta cells. These two effects can be assigned to the specific chemical properties of alloxan, the common denominator being selective cellular uptake and accumulation of alloxan by the beta cell.

Procedure: Animals were divided into five groups of six animals each. Group I was served as normal control and received 1% v/v of Tween-80 (1 ml/100 g) in saline. Groups II – V animals were fasted overnight and alloxan monohydrate at 120 mg/kg was administered by intraperitoneal injection to induce diabetes [127]. The fasting blood glucose level was determined after 72 h of alloxan injection. The rats having serum blood glucose levels above 200 mg/dl were used for the study. This is considered as day zero of the study. Group II was served as diabetic control and received 1% v/v of Tween-80 in saline. Groups III and IV were received embelin 25 and 50 mg/kg suspended in 1% Tween-80 (v/v) and Group V treated as positive control was received glibenclamide (10 mg/kg) dissolved in saline. All these treatments were given orally for 21 days. The animals were fasted on days 6, 13 and 20, blood samples were collected on 7, 14 and 21 days by retro-orbital plexus puncture method and fasting serum blood glucose and body weight were measured.

On day 21, blood was collected by cardiac puncture under mild ether anesthesia from overnight fasted rats and serum was separated and analyzed for TGL, TC, TB, TP, CR, albumin, LDH and ALP by autoanalyzer using Ecoline Kits. Very low density lipoprotein (VLDL) was calculated as per Friedevald's equation, VLDL Cholesterol = TGL/5.

After the collection of blood samples, the liver, kidney and pancreas were excised and rinsed in ice-cold normal saline. A portion of the tissues were fixed in 10% formalin, cut into 5 μm thick sections and stained using heamatoxylin-eosin and histopathological observations were made. The statistical analysis as described in 4.11.4 was carried out.

4.12.2. Estimation of antidiabetic parameters

4.12.2.1. *Assay of triglycerides (TGL)*

Triglycerides catalyze the following reactions.

$$\text{Triglycerides} \xrightarrow{\text{Lipase}} \text{Glycerol + Fatty acid}$$

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

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

$$2\ H_2O_2 + \text{ Aminoantipyrine + 4 –Chlorophenol} \xrightarrow{\text{POD}} \text{Chinonimine + 4 } H_2O_2$$

The absorbance of the sample and standard were measured against the blank at 546 nm. Triglyceride levels in serum, liver and kidney tissue homogenates were expressed as mg/dl.

Procedure: To 1 ml of the reaction solution from E-Merck diagnostic kit for triglycerides, 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 triglyceride was calculated using the following formula.

Triglycerides (mg/dl) = (As/Astd) x Concentration of standard

As = absorbance of sample, Astd = absorbance of standard

4.12.2.2. ***Assay of total cholesterol (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 formed. This is converted into a colored quinonimine in a reaction with 4-aminoantipyrine and phenol catalyzed by peroxidase. The absorbance of the sample and standard was measured against the reagent blank at 546 nm. Cholesterol levels in serum and tissue homogenates were expressed as mg/dL.

Procedure: To 1 ml of the reaction solution from E-Merck diagnostic kit for cholesterol, 100 µl of the sample or standard was added, mixed well and incubated at 37 ºC for 5 min. The absorbance of the sample and standard was measured against the reagent blank solution within 45 min. The concentration of cholesterol was calculated by using the following formula.

Total cholesterol (mg/dl) = (As/Astd) x Concentration of standard

As = absorbance of sample, Astd = absorbance of standard

4.12.2.3. ***Assay of total bilirubin (TB)***

Bilirubin reacts with diazotized sulfanilic acid to form a pink colored dye in acidic medium. Bilirubin kit is based on the modified Jendrassik and Grof's method in which caffeine is used as an activator. The total bilirubin reacts with diazotized sulfanilic acid in basic medium in presence of an activator to form pink colored complex, whose absorbance is proportional to total bilirubin concentration. Direct bilirubin, being water soluble reacts with diazo reagent to form a pink colored complex in absence of an

activator. The indirect bilirubin is calculated from difference between the total and direct bilirubin.

Procedure: Diazotization solution (containing sodium nitrite and sulfanilic acid) 50 µl and 250 µl of accelerator solution from E-Merck diagnostic kit for bilirubin was mixed. To this solution, 50 µl of sample/standard was added, mixed well and incubated at room temperature for 10 to 60 min. To this solution, 250 µl of Fehling's solution II was added, mixed well and incubated at room temperature for 5 min. The absorbance of the sample solution (A) was measured against distilled water at 578 nm.

$$\text{Total bilirubin concentration (mg/dl)} = A \times 10.5$$

4.12.2.4. ***Assay of total protein (TP)***

Proteins and peptides, in contrast to other nitrogen containing compounds (e.g. creatinine, urea and uric acid) produce a violet colored complex with copper ions in an alkaline solution. The so called Biuret reaction is particularly easy to carry out giving reproducible results, which are good in agreement with Kjeldahl method. The absorbance of the color complex is directly proportional to the protein concentration in sample materials. The absorbance of the sample and standard was measured against the Biuret reagent and the absorbance of the blank against distilled water at 546 nm. Total protein levels in serum and tissue homogenates were expressed as g/dL.

Procedure: The reagents 1 and 2 from E-Merck diagnostic kit for TP were mixed at the ratio of 4:1, respectively and the temperature was maintained at 37 ºC. To 1 ml of this reaction solution, 0.10 ml of sample or standard was added, mixed well and incubated for 5 min at 37 ºC. The absorbance of the sample and standard was measured against the

reagent blank solution within 60 min. The concentration of total protein was calculated by using the following formula.

Total protein (g/dl) = (As/Astd) x Concentration of standard

As = absorbance of sample, Astd = absorbance of standard

4.12.2.5. ***Assay of creatinine (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.

Procedure: Buffer solution and the 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.

4.12.2.6. ***Assay of albumin***

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

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 was measured against the reagent blank solution within 60 min. the concentration of albumin was calculated by using the following formula.

Albumin (mg/dl) = (As/Astd) x Concentration of standard

As = absorbance of sample, Astd = absorbance of standard

4.12.2.7. ***Assay of lactate dehydrogenase (LDH)***

LDH catalyze the following reactions.

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

The rate of NADH consumption was measured spectrophotometrically at 340 nm and is directly proportional to the LDH activity in the sample. LDH levels in serum and tissue homogenates were expressed as U/L.

Procedure: The reagents 1 and 2 from E-Merck diagnostic kit for LDH were mixed at the ratio of 4:1, respectively and the temperature was maintained at 30 ºC. To 1 ml of this reaction solution, 20 µl of the sample was added and mixed well. The decrease in

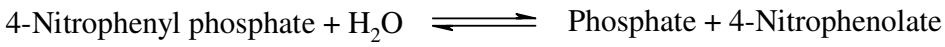
absorbance was measured every min for 3 min at 365 nm. The enzyme activity was calculated by using the following formula.

Enzyme activity (U/l) = (ΔA/min) x F

ΔA = decrease in absorbance, F= factor = 16030

4.12.2.8. ***Assay of alkaline phosphatase (ALP)***

ALP catalyzes the following reaction:



The rate of increase in 4-nitrophenolate was determined spectrophotometrically at 405 nm and is directly proportional to the ALP activity in the sample. ALP levels in serum and tissue homogenates were expressed as U/L.

Procedure: The reagents 1 and 2 from E-Merck diagnostic kit for ALP were mixed at the ratio of 4:1, respectively and the temperature was maintained at 30 ºC. To 1 ml of this reaction solution, 20 µl of the sample was added and mixed well. The increase in absorbance was measured every min for 3 min at 405 nm. The activity was calculated using the following formula.

Enzyme activity (U/l) = (ΔA/min) x F

ΔA = increase in absorbance, F= factor = 2754

4.13. Effect of embelin in inflammatory bowel disease

4.13.1. Acetic acid induced colitis in rats

Epithelial or mucosal necrosis and transient inflammation can be induced by luminal instillation of dilute acetic acid in a dose-responsive fashion [128]. In the original

description of the model [129], 0.5 ml of 10–50% acetic acid diluted with water was instilled into the rectum of the rats. After 10 sec of surface contact, the acidic solution was withdrawn, and the lumen was flushed three times with 0.5 ml saline. In a later modification [130], 1 ml 4% acetic acid (pH 2.3) was slowly infused 5 cm into the rectal lumen of a lightly anesthetized rat. After a 30 sec exposure, excess fluid was withdrawn, and the colon was flushed with 1.5 ml PBS. Many further modifications have been introduced throughout the years, and most subsequent studies have used 15 to 30 sec exposures to 4% or 5% acetic acid in both enema and ascending colon models because higher concentrations induced frequent perforations [128].

The initial injury in this model was a relatively bland epithelial necrosis and edema that variably extended into the lamina propria, submucosa, or external muscle layers, depending of the concentrations and length of exposure of acetic acid. Epithelial injuries were a relatively specific reaction to organic acids because HCl at similar pH did not induce a similar injury [130]. Transient local ischemia might contribute to the acute injury, but neutrophils were apparently not involved in very early phases. Mucosa and submucosal inflammation followed initial injury and was associated with activation of arachidonic acid pathways [128]. Acetic acid induced colitis is an easily inducible model of IBD, and the similarity of the inflammatory mediators profile to IBD suggests that the inflammatory phase bears some resemblance to acute human intestinal inflammation [128]. Treatment with either antioxidant [131] or antianginal [132] drugs improved the macroscopic and microscopic scores of this model.

4.13.2. Induction of colitis

Animals were divided into six groups of six animals each. Group I served as normal control and received the vehicle (sodium CMC, 0.3% w/v). Group II received embelin alone (50 mg/kg). Group III served as colitis control and received only the vehicle (sodium CMC, 0.3% w/v). Group IV and V received embelin at 25 and 50 mg/kg. Group VI was treated with standard sulfasalazine (100 mg/kg, p.o). All these treatments were given for seven days orally by using oral gavage. On the 4^{th} day of the treatment, the animals were fasted overnight with access to water *ad libitum*. On 5^{th} day after 1 h of the above treatments, the animals (Group III, IV, V and VI) were anesthetized by ether inhalation and a polypropylene tube with 2 mm diameter was inserted through the rectum into the colon to a distance of 8 cm. A solution of 2 ml of acetic acid (3%, v/v) in 0.9% saline was instilled into the lumen of the colon and maintained in a supine Trendelenburg position for 30 sec to prevent the leakage of the intracolonic instillate. After 72 h of single dose administration of acetic acid (8^{th} day), clinical activity score was measured and the animals were anaesthetized with ether and blood was collected by retro orbital puncture for biochemical estimations. The animals were sacrificed by cervical dislocation and colon was dissected out. Colon was flushed gently with saline and weighed. It is used for macroscopic scoring, histopathological and biochemical estimations.

4.13.3. Evaluation of the disease

4.13.3.1. Clinical activity score

Colitis was quantified with a clinical score assessing weight loss, stool consistency and bleeding of the colon (measured by guaiac reaction, hemoccult) as described previously [133]. No weight loss was counted as 0 point, weight loss of 1 to 5% as 1 point, 5 to 10%

as 2 points, 10 to 20% as 3 points, and 20% as 4 points. For stool consistency, the stool were collected in a thick paper and observed, 0 points were given for well formed pellets, 2 points for pasty and semiformed stools that did not stick to the anus, and 4 points for liquid stools that did stick to the anus. Bleeding was scored 0 points for no blood in hemoccult, 2 points for positive hemoccult, and 4 points for gross bleeding. These scores were added and divided by 3, forming a total clinical score that ranged from 0.0 (healthy) to 4.0 (maximal activity of colitis).

4.13.3.2. Macroscopic characters

The severity of colitis was evaluated by an independent observer who was blinded to the treatment. For each animal, the distal 10 cm portion of the colon was removed and cut longitudinally, slightly cleaned in physiological saline to remove faecal residues and weighed. Macroscopic inflammation scores were assigned based on clinical features of the colon using the following scoring pattern. No visible change was counted as 0 point, hyperemia at sites as 1 point, lesions having diameter 1 mm or less counted as 2 points, lesions having diameter 2 mm or less (number <5, 5-10 and >10) as 3, 4 and 5 points, respectively and lesions having diameter more than 2 mm (number <5, 5-10, >10) counted as 6, 7 and 8 points, respectively [134].

4.13.3.3. Scoring for rat caecum and colon

Rat caecum and colon (5 cm long) was scored for macroscopic features using following scoring pattern. Score for an individual rat is calculated as the combined score of colon and caecum. No percent area affected was counted as 0 point, 1-5 percent as 1 point, 5-10

as 2 points, 10-25 as 3 points, 25-50 as 4 points, 50-75 as 5 points and 75-100 was counted as 6 points, respectively [134].

4.13.4. Biochemical studies

A portion of colonic tissue (remaining from histopathological study) samples (n = 6) were homogenized in 10% (w/v) of ice-cold potassium phosphate buffer (pH 7.4) using Elvenjan homogenizer (Remi Motors Ltd., Mumbai) and the homogenate was used for the measurement of myeloperoxidase activity (MPO), lipid peroxidation and reduced glutathione (GSH). In addition, serum lactate dehydrogenase (LDH) was also measured using the ERBA diagnostics kit.

4.13.4.1. Assessment of colonic MPO activity

Mucosal scrapings were obtained from the colon for measurement of MPO activity. Tissue samples were assessed biochemically for the neutrophil marker enzyme MPO, according to a previously reported method [135]. The MPO level is a sensitive and specific index of neutrophil infiltration and was used as an index for inflammation.

Procedure: The tissue homogenate was centrifuged at 3500 rpm for 30 min at 4 ºC. The supernatant was discarded. 10 ml of ice-cold 50 mM potassium phosphate buffer (pH 6.0), containing 0.5 % hexadecyltrimethylammonium bromide and 10 mM EDTA was then added to the pellet. It was then subjected to one cycle of freezing, thawing and brief period (15 sec) of sonication. After sonication, solution was centrifuged at 15,000 rpm for 20 min. The MPO activity was measured spectrophotometrically [135]. 0.1 ml of supernatant was combined with 2.9 ml of 50 mM phosphate buffer containing 0.167 mg/ml of O-dianisidine hydrochloride and 0.0005% hydrogen peroxide. The change in

absorbance was measured at 460 nm. One unit of MPO activity is defined as the change in absorbance per min by 1.0 at room temperature, in the final reaction. It has been calculated by using the following formula

MPO activity (U/g) = X/weight of the piece of tissue taken

Where X = 10 x change in absorbance per min/volume of supernatant taken in the final concentration.

4.13.4.2. Measurement of colonic lipid peroxides concentration

Lipid peroxidation is commonly regarded as a deleterious process [136], 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 aldehydes are formed. The most common method used to assess malondialdehyde (MDA) is the thiobarbituric acid (TBARS) assay [137].

Procedure: Thiobarbituric acid reactive substance in the homogenate was estimated by using standard protocol. Briefly, the homogenate was incubated with 15% trichloroacetic acid, 0.375% thiobarbituric acid and 5N HCL at 95 ºC for 15 min, the mixture was

cooled, centrifuged and the absorbance of the supernatant was measured at 532 nm against appropriate blank. The amount of lipid peroxides was determined by using $\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$ and expressed as μmol/g of wet tissue weight [138].

4.13.4.3. Determination of colonic GSH contents

Reduced glutathione in the tissue was determined according to the method of Moron et al., [139]. The acid soluble sulfhydryl groups (non-protein thiols of which more than 93% is reduced glutathione) form a yellow colored complex with dithionitrobenzene (DTNB). The absorbance of the colored complex was measured at 412 nm.

Procedure: The assay is based on the formation of a relatively stable yellow product when sulphydryl groups react with 5,5-dithio-bis-2-nitrobenzoic acid (DTNB). Briefly, proteins were precipitated using 10% TCA, centrifuged and 0.5 ml of the supernatant was mixed with 0.2M phosphate buffer (pH 8.0) and 10 mM DTNB. This mixture was incubated for 10 min and the absorbance was measured at 412 nm against appropriate blank [139]. The results were expressed as nmol/g of wet tissue weight.

4.13.5. Histolopathological study

A portion (2 cm) of the colonic specimen from each rat (n = 6) was fixed in 10% formalin, cut into 5 μm thickness, stained using heamatoxylin-eosin and histopathological observations were made. The stained sections of colon were examined for any inflammatory changes like infiltration of the cells, necrotic foci and damage to tissue structures like payers patches, damage to nucleus, etc. The statistical analysis as described in 4.11.4 was carried out.

4.14. Neuroprotective effect of embelin

4.14.1. Effect of embelin against global cerebral ischemia/reperfusion

Ischemia is defined as diminution of cerebral blood flow to a critical threshold that propagates brain damage involving the entire brain or a selective region. Global cerebral ischemia entails diminution in cerebral blood flow over the entire brain, encountered clinically as sequelae during extracorporeal circulation following cardiac arrest from ventricular fibrillation or asystole that lasts 5 to 10 min. Global ischemia from cardiac arrest results in a predictable pattern of histologic injury in which specific neuronal populations are affected (selective ischemic necrosis). Although reperfusion restores cerebral blood flow, it can lead to secondary brain injury from influx of neutrophils and to increases in ROS, cerebral edema and hemorrhage. Elevated levels of ROS may lead to damage of intracellular proteins and DNA by way of oxidation and by activating a number of pathways that lead to cell death [140].

Animals were divided into five groups of 6-10 rats each. Group I served as sham operated control and received the vehicle (Tween 80, 1% v/v). Group II received embelin alone (50 mg/kg) and were subjected to sham operation. Group III received only the vehicle and served as ischemic control. Groups IV and V received embelin at 25 and 50 mg/kg. All these treatments were given for four days orally by using oral gavage. On 4^{th} day, after 2 h of the above treatments, the animals (Groups III, IV and V) were subjected to cerebral ischemia/reperfusion.

4.14.2. Induction of cerebral ischemia/reperfusion in rats

The animals were treated with atropine sulphate (0.5 mg/kg, i.p.) as preanaesthetic medication and anaesthetized with chloral hydrate (350 mg/kg, i.p.). Both common carotid arteries were exposed over a midline incision, and a dissection was made between the sternocleidomastoid and the sternohyoid muscles parallel to the trachea [141]. Each carotid artery was freed from its adventitial sheath and vagus nerve, which was carefully separated and maintained. The induction of ischemia was performed by occluding bilateral common carotid arteries (BCCA) with clamps for 30 min followed by 24 h reperfusion and the skin was closed with stitches using suture. Sham control and embelin alone treated animals received the same surgical procedures, except BCCA were not occluded. After the completion of reperfusion period the animals were assessed for neurological outcome and then sacrificed, brain was removed and stored at -20 ºC until further use.

4.14.3. Motor performance tests

4.14.3.1. Locomotor activity

The locomotor activity was recorded by using the procedure [123] as described in 4.11.3.

4.14.3.2. Hanging wire test

This task was used to measure griping and forelimb strength of the rats. In this test, animals were suspended by the forelimbs on a wire (45 cm long and 0.3 cm diameter) stretched between two posts 40 cm above a foam pillow. The time (sec) until the animal fell was recorded [142]. The cut off time was taken as 90 sec.

4.14.3.3. Beam walking test

This test was used to evaluate gross vestibulomotor function. The apparatus consisted a narrow rectangular rod of 120 cm in length and a diameter of 2.3 cm, suspended 50 cm above a foam pad. Rats were trained thrice a day for two days before behavioral tests (i.e. prior to BCCA occlusion). The time taken to traverse the beam was recorded [143].

4.14.4. Biochemical estimations

The brains were homogenized in cold phosphate buffered saline (10% w/v) and were centrifuged at 10,000 rpm for 15 min at -4 °C. The supernatant was used for the biochemical analysis.

4.14.4.1. Mesurement of total Protein

The protein content of the brain homogenate was determined by Lowry's method [144] using bovine serum albumin as standard.

4.14.4.2. Measurement of lipid peroxidation

The lipid peroxidation was measured by using the procedure [138] as described in 4.13.4.2 and the results were expressed as nmoles MDA/mg protein.

4.14.4.3. Measurement of total thiols

The total thiol content was determined by the method described by Sedlak and Lindsay, [145], with slight modification. To a mixture of 0.2 ml homogenate and 0.36 ml of buffer, 0.04 ml of 10 mM DTNB and 1.5 ml of methanol were added and mixed well. The mixture was centrifuged at 3500 rpm for 5 min at -4 °C. Then the intensity of yellow

color developed was measured at 412 nm and the results were expressed as nmoles/mg protein.

4.14.4.4. Measurement of glutathione-S-transferase

The glutathione-S-transferase (GST) activity was measured as described by Habig et al. [146]. Briefly, 0.85 ml of phosphate buffer (pH 7.4), 0.05 ml of homogenate and 0.05 ml of 10 mM GSH were added, to which 0.05 ml of 1 mM CDNB was added to initiate the reaction. The rate of formation of GSH-CDNB complex was monitored for 5 min at 340 nm and the results were expressed as nmoles of CDNB conjugate formed/min/mg protein.

4.14.5. Measurement of infarct volume

After 24 h of reperfusion period, rats were sacrificed under deep anesthesia. The brains were rapidly removed and sectioned coronally into 2 mm thick sections. The sections were placed in phosphate buffered saline (pH 7.4) containing 2% of 2,3,5-triphenyltetrazolium chloride (TTC) for 30 min at 37 °C and followed by fixing in 10% neutral buffered formalin for overnight [147]. The images of the stained sections were acquired by scanning with a high resolution scanner. Infarct volume of all the samples was analyzed by using Image J 1.43u software (NIH, USA).

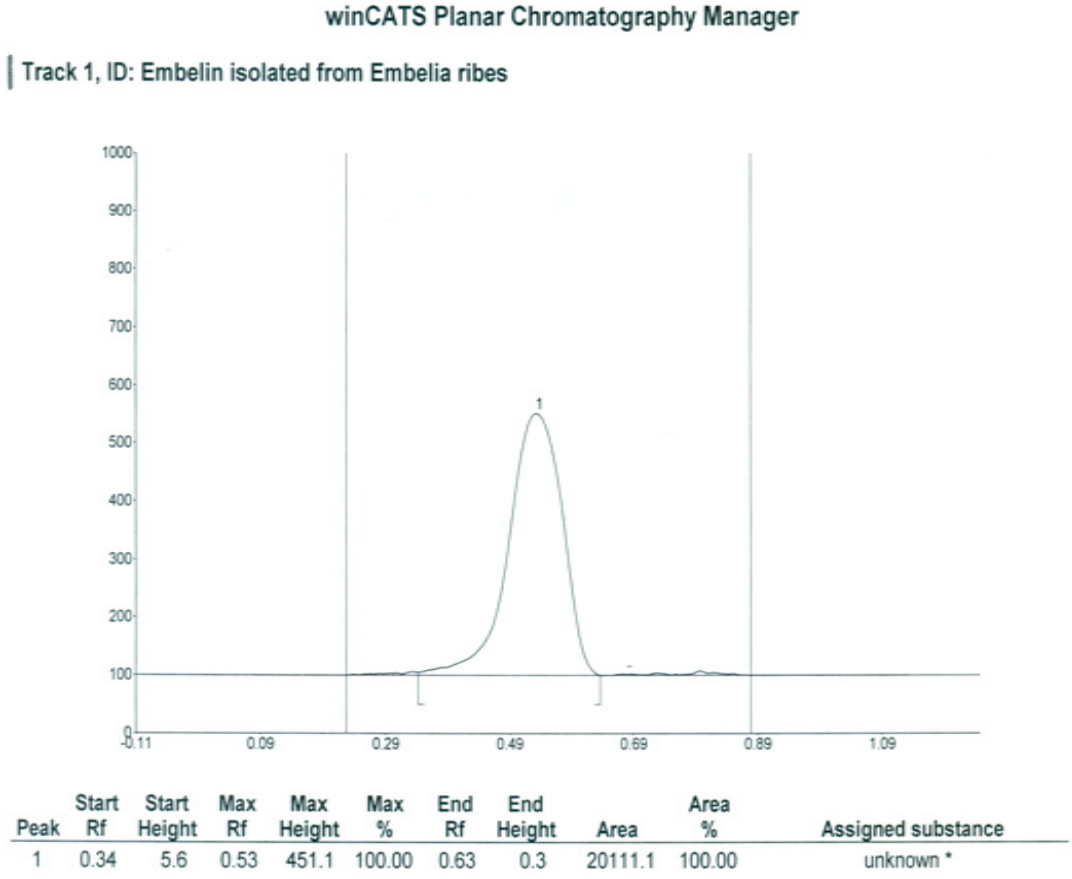
4.14.6. Histopathological studies

The brains were fixed with 10% formalin and embedded in paraffin wax and cut into sections of 5 µm thickness. The sections were stained with haemotoxylin and eosin dye for histopathological observation. The statistical analysis as described in 4.11.4 was carried out.

5. 1. Structural modification of embelin

5.1.1. 2,5-dihydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione, embelin (1)

Embelin was isolated from the berries of *Embelia ribes*, found to be homogenous by HPTLC (Fig. 6) when separated using the solvent system ethyl acetate: benzene (70:30, R_f = 0.53). Obtained as orange plates mp 141-143 ºC; yield 19.5 g, 0.325%; IR υ_{max} (KBr) cm^{-1}: 3308 (O-H), 2920, 2848 (C-H), 1643 (α, β- unsaturated C=O), 1614 (C=C), 1329, 1193 (Fig. 7); ^1H NMR (400 MHz, $CDCl_3$, Fig. 8) δ: 7.68 (s, 2H, -OH), 6.00 (s, 1H, H-6), 2.44 (t, 2H, H-1′), 1.47 (m, 2H, H-2′), 1.25-1.30 (m, 16H, H-3′ to 10′), 0.88 (t, 3H, H-11′); ^{13}C NMR (100 MHz, $CDCl_3$); see Table 1 and Fig. 9; negative ESI-MS (Fig. 10): m/z calculated for 294.18, Found : 293 $[M-H]^{-1}$.



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.34	5.6	0.53	451.1	100.00	0.63	0.3	20111.1	100.00	unknown *

Fig. 6. HPTLC spectrum of embelin (1)

5.1.2. 2-hydroxy-5-methoxy- 3-undecylcyclohexa-2,5-diene-1,4-dione (2)

Obtained as deep yellow rectangular tablets, mp 88-90 ºC; yield 0.273 g, 88.63%; IR υ_{max} (KBr) cm^{-1}: 3352 (O-H), 2918, 2850 (C-H), 1635 (α, β- unsaturated C=O), 1599 (C=C), 1444, 1323, 1207, 1112, 1079, 839, 686 (Fig. 11); ^{1}H NMR (400 MHz, $CDCl_3$, Fig. 12) δ: 7.80 (s, 1H, -OH), 5.80 (s, 1H, H-6), 3.85 (s, 3H, $-OCH_3$), 2.46 (t, 2H, H-1′), 1.56 (m, 2H, H-2′), 1.25-1.30 (m, 16H, H-3′ to 10′), 0.87 (t, 3H, H-11′); ^{13}C NMR (100 MHz, $CDCl_3$) see Table 1 and Fig. 13; negative ESI-MS (Fig. 14): m/z calculated for 308.20, Found: 307 $[M-H]^{-1}$.

5.1.3. 3,6-bis (phenylimino)-2-undecylcyclohexa-1,4-diene-1,4-diol (3)

Obtained as green prisms, mp 192-194 ºC; yield 0.352 g, 79.28%; IR ν_{max} (KBr) cm^{-1}: 3109 (O-H), 2920, 2850 (C-H), 1599 (C=C), 1518 (C=N), 1494, 1435, 1390, 1228, 827, 686 (Fig. 15); ^{1}H NMR (400 MHz, $CDCl_3$, Fig. 16) δ: 7.45 (s, 2H, -OH), 7.19-7.40 (m, 10H, aromatic), 6.07 (s, 1H, H-6), 2.49 (t, 2H, H-1′), 1.52 (m, 2H, H-2′), 1.25-1.30 (m, 16H, H-3′ to 10′), 0.87 (t, 3H, H-11′); ^{13}C NMR (100 MHz, $CDCl_3$) see Table 1and Fig. 17; negative ESI-MS: m/z calculated for 444.28, Found: 444.68 (Fig. 18).

5.1.4. 5-(3-nitrophenylamino)-2-hydroxy-3-undecyl-1,4-benzoquinone (4)

Obtained as reddish brown rectangular prisms, mp 199-201 ºC; yield 0.391 g, 94.44%; IR ν_{max} (KBr) cm^{-1}: 3308 (O-H), 3248 (N-H), 2918, 2850 (C-H), 1635 (α, β- unsaturated C=O), 1616 (C=C), 1504, 1348 (NO_2), 1222, 1111, 829, 732 (Fig. 19); ^{1}H NMR (400 MHz, $CDCl_3$, Fig. 20) δ: 8.12 (t, 1H, H-6″), 8.08 (dt, 1H, H-4″), 8.00 (s, 1H, -NH), 7.80 (s, 1H, -OH), 7.60 (t, 1H, H-5″), 7.58 (t, 1H, H-2″), 6.08 (s, 1H, H-6), 2.47 (t, 2H, H-1′), 1.53 (m, 2H, H-2′), 1.25-1.30 (m, 16H, H-3′ to 10′), 0.88 (t, 3H, H-11′); ^{13}C NMR (100

MHz, $CDCl_3$) see Table 1 and Fig. 21; negative ESI-MS (Fig. 22): m/z calculated for 414.53, Found: 414.

5.1.5. 5-(4-nitrophenylamino)-2-hydroxy-3-undecyl-1,4-benzoquinone (5)

Obtained as brown prisms, mp 168-170 ºC; yield 0.368 g, 88.89%; IR ν_{max} (KBr) cm^{-1}: 3309 (O-H), 3261 (N-H), 2920, 2848 (C-H), 1643 (α, β- unsaturated C=O), 1616 (C=C), 1504, 1352 (NO_2), 1220, 1116, 769, 696 (Fig. 23); ^1H NMR (400 MHz, $CDCl_3$, Fig. 24) δ: 8.30 (d, 2H, H-3″, 5″), 8.15 (s, 1H, -NH), 7.65 (s, 1H, -OH), 7.40 (d, 2H, H-2″, 6″), 6.00 (s, 1H, H-6), 2.40 (t, 2H, H-1′), 1.56 (m, 2H, H-2′), 1.25-1.30 (m, 16H, H-3′ to 10′), 0.86 (t, 3H, H-11′); ^{13}C NMR (100 MHz, $CDCl_3$) see Table 1 and Fig. 25; negative ESI-MS (Fig. 26): m/z calculated for 414.22, Found: 414.68.

5.1.6. 5-(4-chlorophenylamino)-2-hydroxy-3-undecyl-1,4-benzoquinone (6)

Obtained as violet prisms, mp 176-178 ºC; yield 0.330 g, 82.13%; IR ν_{max} (KBr) cm^{-1}: 3319 (O-H), 3240 (N-H), 2920, 2848 (C-H), 1637 (α, β- unsaturated C=O), 1572 (C=C), 1381, 1217, 1172, 817, 707 (Fig. 27); ^1H NMR (400 MHz, $CDCl_3$, Fig. 28) δ: 7.90 (s, 1H, -NH), 7.85 (s, 1H, -OH), 7.40 (d, 2H, H-2″,6″), 7.20 (d, 2H, H-3″, 5″), 5.94 (s, 1H, H-6), 2.42 (t, 2H, H-1′), 1.53 (m, 2H, H-2′), 1.25-1.30 (m, 16H, H-3′ to 10′), 0.89 (t, 3H, H-11′); ^{13}C NMR (100 MHz, $CDCl_3$) see Table 1 and Fig. 29; negative ESI-MS (Fig. 30): m/z calculated for 403.47, Found: 403.

5.1.7. 5-(2-chlorophenylamino)-2-hydroxy-3-undecyl-1,4-benzoquinone (7)

Obtained as dark violet rectangular prisms, mp 122-124 ºC; yield 0.248 g, 61.54%; IR ν_{max} (KBr) cm^{-1}: 3308 (O-H), 3267 (N-H), 2922, 2854 (C-H), 1637 (α, β- unsaturated C=O), 1572 (C=C), 1379, 1213, 1060, 831, 752 (Fig. 31); ^1H NMR (400 MHz, $CDCl_3$,

Fig. 32) δ: 8.20 (s, 1H, -NH), 7.80 (s, 1H, -OH), 7.48 (dd, 1H, H-6″), 7.40 (dd, 1H, H-3″), 7.32 (td, 1H, H-5″), 7.19 (td, 1H, H-4″), 6.00 (s, 1H, H-6), 2.50 (t, 2H, H-1′), 1.50 (m, 2H, H-2′), 1.25-1.30 (m, 16H, H-3′ to 10′), 0.90 (t, 3H, H-11′); ^{13}C NMR (100 MHz, $CDCl_3$) see Table 1 and Fig. 33; negative ESI-MS (Fig. 34): m/z calculated for 403.19, Found: 403.65.

5.1.8. 5-(4-methoxyphenylamino)-2-hydroxy-3-undecyl-1,4-benzoquinone (8)

Obtained as brown prisms, mp 173-175 ºC; yield 0.360 g, 90.22%; IR v_{max} (KBr) cm^{-1}: 3313 (O-H), 3230 (N-H), 2918, 2850 (C-H), 1637 (α, β- unsaturated C=O), 1612, 1572 (C=C), 1519, 1498, 1219, 1031, 821, 711 (Fig. 35); ^{1}H NMR (400 MHz, $CDCl_3$, Fig. 36) δ: 8.05 (s, 1H, -NH), 7.95 (s, 1H, -OH), 7.22 (d, 2H, H-2″, 3″), 6.99 (d, 2H, H-3″, 5″), 5.88 (s, 1H, H-6), 3.87 (s, 3H, -OCH_3), 2.51 (t, 2H, H-1′), 1.59 (m, 2H, H-2′), 1.25-1.30 (m, 16H, H-3′ to 10′), 0.93 (t, 3H, H-11′); ^{13}C NMR (100 MHz, $CDCl_3$) see Table 1 and Fig. 37; negative ESI-MS (Fig. 38): m/z calculated for 399.24, Found: 398 $[M-H]^{-1}$.

5.1.9. 5-(3-hydroxyphenylamino)-2-hydroxy-3-undecyl-1,4- benzoquinone (9)

Obtained as black amorphous solid, mp 170-173 ºC; yield 0.321 g, 83.38%; IR v_{max} (KBr) cm^{-1}: 3317 (O-H), 3240 (N-H), 2918, 2848 (C-H), 1637 (α, β- unsaturated C=O), 1604, 1570 (C=C), 1508, 1381, 1217, 1151, 829, 707 (Fig. 39); ^{1}H NMR (400 MHz, $CDCl_3$, Fig. 40) δ: 10.05 (s, 1H, -OH), 9.55 (s, 1H, -NH), 7.90 (s, 1H, -OH), 6.81 (dd, 1H, H-6″), 6.74 (t, 2H, H-2″, 5″), 6.70 (dd, 1H, H-4″), 6.04 (s, 1H, H-6), 2.44 (t, 2H, H-1′), 1.50 (m, 2H, H-2′), 1.25-1.30 (m, 16H, H-3′ to 10′), 0.89 (t, 3H, H-11′); ^{13}C NMR (100 MHz,

$CDCl_3$) see Table 1 and Fig. 41; negative ESI-MS (Fig. 42): m/z calculated for 385.24, Found: 385.68.

5.1.10. 5-(4-hydroxyphenylamino)-2-hydroxy-3-undecyl-1,4-benzoquinone (10)

Obtained as brown amorphous solid, mp 180-182 ºC; yield 0.277 g, 71.95%; IR v_{max} (KBr) cm^{-1}: 3313 (O-H), 3232 (N-H), 2920, 2848 (C-H), 1635 (α, β- unsaturated C=O), 1614, 1570 (C=C), 1519, 1504, 1383, 1219, 1112, 823, 709 (Fig. 43); ^1H NMR (400 MHz, $CDCl_3$, Fig. 44) δ: 10.58 (s, 1H, -OH), 9.61 (s, 1H, -NH), 7.83 (s, 1H, -OH), 7.12 (d, 2H, H-2″, 6″), 6.81 (d, 2H, H-3″, 5″), 5.80 (s, 1H, H-6), 2.42 (t, 2H, H-1′), 1.46 (m, 2H, H-2′), 1.25-1.30 (m, 16H, H-3′ to 10′), 0.87 (t, 3H, H-11′); ^{13}C NMR (100 MHz, $CDCl_3$) see Table 1 and Fig. 45; negative ESI-MS (Fig. 46): m/z calculated for 385.24, Found: 385.68.

5.1.11. 5-(p-tolylamino)-2-hydroxy-3-undecyl-1,4-benzoquinone (11)

Obtained as deep violet prisms, mp 147-149 ºC; yield 0.350 g, 91.38%; IR v_{max} (KBr) cm^{-1}: 3302 (O-H), 3244 (N-H), 2920, 2848 (C-H), 1641 (α, β- unsaturated C=O), 1608, 1572 (C=C), 1518, 1496, 1384, 1222, 1205, 813, 719 (Fig. 47); ^1H NMR (400 MHz, $CDCl_3$, Fig. 48) δ: 8.45 (s, 1H, -NH), 7.91 (s, 1H, -OH), 7.20 (d, 2H, H-3″, 5″), 7.11 (d, 2H, H-2″, 6″), 5.93 (s, 1H, H-6), 2.44 (t, 2H, H-1′), 2.36 (s, 3H, $-CH_3$), 1.46 (m, 2H, H-2′), 1.25-1.30 (m, 16H, H-3′ to 10′), 0.86 (t, 3H, H-11′); ^{13}C NMR (100 MHz, $CDCl_3$) see Table 1 and Fig. 49); negative ESI-MS (Fig. 50): m/z calculated for 383.25, Found: 383.68.

5.1.12. 2-(4-hydroxy-3,6-dioxo-5-undecylcyclohexa-1,4-dienylamino) benzamide (12)

Obtained as dark brown solid, mp 127-129 ºC; yield 0.327 g, 79.37%; IR v_{max} (KBr) cm^{-1}: 3308 (O-H), 3227 (N-H), 2922, 2850 (C-H), 1649 (α, β- unsaturated C=O), 1614, 1541 (C=C), 1518, 1465, 1431, 1390, 1143, 1120, 756, 594 (Fig. 51); ^{1}H NMR (400 MHz, $CDCl_3$, Fig. 52) δ: 10.79 (s, 2H, $-CONH_2$), 7.90 (s, 1H, -NH), 7.82 (s, 1H, -OH), 7.60 (d, 2H, H-5″, 6″), 7.53 (d, 2H, H-3″, 4″), 6.00 (s, 1H, H-6), 2.46 (t, 2H, H-1′), 1.49 (m, 2H, H-2′), 1.25-1.30 (m, 16H, H-3′ to 10′), 0.87 (t, 3H, H-11′); ^{13}C NMR (100 MHz, $CDCl_3$) see Table 1 and Fig. 53); negative ESI-MS (Fig. 54): m/z calculated for 412.24, Found: 412.82.

5.1.13. 5-(4-sulfonylamine phenylamino)-2-hydroxy-3-undecyl-1,4-benzoquinone (13)

Obtained as black amorphous solid, mp 157-159 ºC; yield 0.409 g, 91.29%; IR v_{max} (KBr) cm^{-1}: 3375, 3277 (NH_2), 3311 (O-H), 3238 (N-H), 2920, 2848 (CH), 1637 (α, β-unsaturated C=O), 1616, 1573 (C=C), 1508, 1465, 1327, 1220, 1163, 1101, 767, 707, 543 (Fig. 55); ^{1}H NMR (400 MHz, $CDCl_3$, Fig. 56) δ: 8.10 (s, 1H, -NH), 7.96 (s, 1H, -OH), 7.31 (d, 2H, H-3″, 5″), 7.21 (d, 2H, H-2″, 6″), 6.59 (s, 2H, $-NH_2$), 5.99 (s, 1H, H-6), 2.42 (t, 2H, H-1′), 1.45 (m, 2H, H-2′), 1.25-1.30 (m, 16H, H-3′ to 10′), 0.88 (t, 3H, H-11′); ^{13}C NMR (100 MHz, DMSO) see Table 1 and Fig. 57; negative ESI-MS (Fig. 58): m/z calculated for 448.58, Found: 448.58.

Table 1. ^{13}C NMR spectrum of embelin and its derivatives

Carbon	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
1	***	182.82	170.10	177.73	***	182.73	182.66	182.94	***	183.29	182.95	182.79	183.16
2	***	161.14	153.76	***	153.64	154.17	153.89	154.61	***	156.66	154.45	153.49	155.98
3	116.99	119.29	113.05	117.10	117.39	116.54	116.81	116.05	114.94	116.19	116.16	117.10	117.22
4	***	181.67	170.10	180.88	181.18	180.45	180.79	179.91	180.59	180.02	180.13	***	181.40
5	***	151.51	153.76	145.30	143.22	145.73	145.14	146.88	154.29	147.48	146.31	145.27	145.33
6	102.15	102.15	84.36	96.15	97.76	94.90	95.59	93.62	94.95	93.94	94.13	96.05	97.19
Chain 1′ to	22.51	22.50	22.69	22.67	22.50	22.68	22.67	22.68	22.68	22.54	22.67	22.51	22.43
10′	to	to	to	to	to	to	to	to	to	to	to	to	to
	31.90	31.89	31.94	31.91	31.89	31.91	31.91	31.91	31.91	31.74	31.91	31.90	31.74
11′	14.09	14.08	14.10	14.09	14.08	14.10	14.09	14.09	14.10	14.41	14.09	14.09	14.40
1″	-	-	137.25	145.30	131.89	131.66	130.51	129.55	138.20	129.27	136.37	132.59	141.63
2″	-	-	123.29	127.96	125.62	129.90	127.85	124.73	109.66	126.00	122.79	128.41	117.85
3″	-	-	129.73	148.78	121.18	123.99	126.76	114.93	154.29	116.37	130.26	124.34	123.21
4″	-	-	127.40	127.96	129.32	135.56	134.17	158.07	113.39	155.89	134.28	138.33	140.32
5″	-	-	129.73	130.68	121.18	123.99	123.20	114.93	130.74	116.37	130.26	132.59	127.38
6″	-	-	123.29	120.45	125.62	129.90	127.73	124.73	114.94	126.00	122.79	122.01	117.22
1‴	-	-	137.25	-	-	-	-	-	-	-	-	-	-
2‴	-	-	123.29	-	-	-	-	-	-	-	-	-	-
3‴	-	-	129.73	-	-	-	-	-	-	-	-	-	-
4‴	-	-	127.40	-	-	-	-	-	-	-	-	-	-
5″	-	-	129.73	-	-	-	-	-	-	-	-	-	-
6‴	-	-	123.29	-	-	-	-	-	-	-	-	-	-
5-OCH_3	-	56.71	-	-	-	-	-	-	-	-	-	-	-
4″-OCH_3	-	-	-	-	-	-	-	55.55	-	-	-	-	-
4″-CH_3	-	-	-	-	-	-	-	-	-	-	20.99	-	-
2″-$CONH_2$	-	-	-	-	-	-	-	-	-	-	-	170.02	-

***Carbon peaks not appeared due to fluxional effect

5.1.14. 5-(methylamino)-2-hydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione (14)

Obtained as pink rectangular prisms, mp 138-140 ºC; 0.271 g, yield 88.27%; IR ν_{max} (KBr) cm^{-1}: 3308 (O-H), 3269 (N-H), 2918, 2850 (C-H), 1651 (α, β- unsaturated C=O), 1577, 1514, 1213 (C-N), 1151 (C-O), 790, 688 (Fig. 59); ^{1}H NMR (500 MHz, DMSO, Fig. 60) δ: 10.49 (s, 1H, -NH), 7.80 (s, 1H, -OH), 5.30 (s, 1H, H-6), 2.73 (s, 3H, 1″-CH_3), 2.49 (t, 2H, H-1′), 1.48 (m, 2H, H-2′), 1.21-1.32 (m, 16H, H-3′ to 10′), 0.84 (t, 3H, H-11′); ^{13}C NMR (100 MHz, DMSO) see Table 2 and Fig. 61; negative ESI-MS (Fig. 62): m/z calculated for 307.21, Found: 306.45.

5.1.15. 5-(ethylamino)-2-hydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione (15)

Obtained as pink solid, mp 132-134 ºC; yield 0.296 g, 92.21%; IR ν_{max} (KBr) cm^{-1}: 3308 (O-H), 3277 (N-H), 2922, 2850 (C-H), 1641 (α, β- unsaturated C=O), 1568, 1504, 1213 (C-N), 1143 (C-O), 763, 704 (Fig. 63); ^{1}H NMR (500 MHz, DMSO, Fig. 64) δ: 10.50 (s, 1H, -NH), 7.68 (s, 1H, -OH), 5.26 (s, 1H, H-6), 3.14 (t, 2H, H-1″), 2.24 (t, 2H, H-1′), 1.32 (m, 2H, H-2′), 1.09 (t, 3H, H-2″), 1.12-1.22 (m, 16H, H-3′ to 10′), 0.84 (t, 3H, H-11′); ^{13}C NMR (100 MHz, DMSO) see Table 2 and Fig. 65; negative ESI-MS (Fig. 66): m/z calculated for 321.23, Found: 320.65.

5.1.16. 5-(butylamino)-2-hydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione (16)

Obtained as pink prisms, mp 145-147 ºC; yield 0.275 g, 78.80%; IR ν_{max} (KBr) cm^{-1}: 3311 (O-H), 3273 (N-H), 2922, 2848 (C-H), 1641 (α, β- unsaturated C=O), 1570, 1508, 1211 (C-N), 1145 (C-O), 767, 704 (Fig. 67); ^{1}H NMR (500 MHz, DMSO, Fig. 68) δ: 10.50 (s, 1H, -NH), 7.70 (s, 1H, -OH), 5.26 (s, 1H, H-6), 3.33 (t, 2H, H-1″), 3.10 (t, 2H, H-2″), 2.24 (t, 2H, H-1′), 1.48 (m, 2H, H-2′), 1.32 (t, 2H, H-3″), 1.22-1.31

(m, 16H, H-3′ to 10′), 0.87 (t, 3H, H-4″), 0.89 (t, 3H, H-11′); ^{13}C NMR (100 MHz, DMSO) see Table 2 and Fig. 69; negative ESI-MS (Fig. 70): m/z calculated for 349.26, Found: 349.58.

5.1.17. 5-(4-acetylphenylamino)-2-hydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione (17)

Obtained as dark brown solid, mp 190-192 ºC; yield 0.350 g, 85.16%; IR ν_{max} (KBr) cm^{-1}: 3308 (O-H), 3238 (N-H), 2920, 2848 (C-H), 1681 (α, β- unsaturated C=O), 1637 (C=O), 1573, 1518, 1220 (C-N), 1176 (C-O), 767, 707 (Fig. 71); ^{1}H NMR (500 MHz, DMSO, Fig. 72) δ: 10.80 (s, 1H, -NH), 9.40 (s, 1H, -OH), 7.98 (t, 2H, H-3″, 5″), 7.51 (d, 2H, H-2″, 6″), 5.97 (s, 1H, H-6), 2.55 (s, 3H, -COCH$_3$), 2.28 (t, 2H, H-1′), 1.39 (m, 2H, H-2′), 1.22-1.33 (m, 16H, H-3′ to 10′), 0.84 (t, 3H, H-11′); ^{13}C NMR (100 MHz, DMSO) see Table 2 and Fig. 73; negative ESI-MS (Fig. 74): m/z calculated for 411.24, Found: 411.

5.1.18. 5-(4-((E)-3-phenylacryloyl)phenylamino)-2-hydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione (18)

Obtained as black prisms, mp 182-184 ºC; yield 0.360 g, 72.14%; IR ν_{max} (KBr) cm^{-1}: 3367 (O-H), 3246 (N-H), 2922, 2850 (C-H), 1654 (α, β- unsaturated C=O), 1597, 1514, 1479, 1219 (C-N), 1176 (C-O), 765, 692 (Fig. 75); ^{1}H NMR (500 MHz, DMSO, Fig. 76) δ: 9.50 (s, 1H, -NH), 9.22 (s, 1H, -OH), 8.17-8.19 (m, 2H, H-11″ and H-15″), 7.97 and 7.71 (m, 2H, CH=CH), 7.89 (d, 2H, H-2″, 6″), 7.49 (d, 2H, H-3″, 5″), 7.45-7.50 (m, 3H, H-12″-14″), 5.79 (s, 1H, H-6), 2.23 (t, 2H, H-1′), 1.30 (m, 2H, H-2′), 1.23 (m, 16H, H-3′

to 10′), 0.84 (t, 3H, H-11′); ^{13}C NMR (100 MHz, DMSO) see Table 2 and Fig. 77; negative ESI-MS (Fig. 78): m/z calculated for 499.27, Found: 498 $[M-H]^{-1}$.

5.1.19. 5-(o-tolylamino)-2-hydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione (19)

Obtained as black prisms, mp 135-137 ºC; yield 0.350, 91.38%; IR v_{max} (KBr) cm^{-1}: 3302 (O-H), 3259 (N-H), 2920, 2852 (C-H), 1637 (α, β- unsaturated C=O), 1566, 1508, 1213 (C-N), 1114 (C-O), 763, 717 (Fig. 79); ^{1}H NMR (500 MHz, DMSO, Fig. 80) δ: 10.62 (s, 1H, -NH), 9.07 (s, 1H, -OH), 7.33 (m, 1H, H-6′′), 7.25 (m, 2H, H-4′′, 5′′), 7.17 (d, 1H, H-3′′), 5.00 (s, 1H, H-6), 2.31 (s, 3H, 2′′-CH_3), 2.15 (t, 2H, H-1′), 1.39 (m, 2H, H-2′), 1.23-1.27 (m, 16H, H-3′ to 10′), 0.84 (t, 3H, H-11′); ^{13}C NMR (100 MHz, DMSO) see Table 2 and Fig. 81; negative ESI-MS (Fig. 82): m/z calculated for 383.25, Found: 383.68.

5.1.20. 4-(4-hydroxy-3,6-dioxo-5-undecylcyclohexa-1,4-dienylamino) benzoic acid (20)

Obtained as violet rectangular prisms, mp 170-172 ºC; yield 0.371 g, 89.83%; IR v_{max} (KBr) cm^{-1}: 3309 (O-H), 3240 (N-H), 2920, 2848 (C-H), 1697 (C=O-OH), 1639 (α, β-unsaturated C=O), 1575, 1518, 1220, 1116, 767, 709 (Fig. 83); ^{1}H NMR (500 MHz, DMSO, Fig. 84) δ: 10.86 (s, 1H,-COOH), 9.72 (s, 1H, -NH), 9.37 (s, 1H, -OH), 7.96 (d, 2H, H-2′′, 6′′), 7.50 (d, 2H, H-3′′, 5′′), 5.96 (s, 1H, H-6), 2.32 (t, 2H, H-1′), 1.40 (m, 2H, H-2′), 1.23-1.27 (m, 16H, H-3′ to 10′), 0.86 (t, 3H, H-11′); ^{13}C NMR (100 MHz, DMSO) see Table 2 and Fig. 85; negative ESI-MS (Fig. 86): m/z calculated for 413.22, Found: 413.76.

5.1.21. 3-(4-hydroxy-3,6-dioxo-5-undecylcyclohexa-1,4-dienylamino)-3-phenyl prop - anoic acid (21)

Obtained as brown solid, mp 147-149 ºC; yield 0.324 g, 73.47%; IR ν_{max} (KBr) cm^{-1}: 3431, 3309 (O-H), 3234 (N-H), 2920, 2848 (C-H), 1618 (α, β- unsaturated C=O), 1585, 1465, 1220 (C-N), 1120 (C-O), 767, 688 (Fig. 87); ^{1}H NMR (500 MHz, DMSO, Fig. 88) δ: 13.05 (s, 1H, -COOH), 10.50 (s, 1H, -NH), 9.25 (s, 1H, -OH), 7.20-7.50 (5H, aromatic), 5.77 (s, 1H, H-6), 5.36 (2H, $-CH_2$ of phenylalanine), 2.49 (1H, -CH of phenylalanine), 2.30 (t, 2H, H-1′), 1.39 (m, 2H, H-2′), 1.22 (m, 16H, H-3′ to 10′), 0.84 (t, 3H, H-11′); ^{13}C NMR (100 MHz, DMSO) see Table 2 and Fig. 89; negative ESI-MS (Fig. 90): m/z calculated for 441.25, Found: 395 $[M-COOH]^{-1}$

5.1.22. 5-(2-phenylhydrazinyl)-2-hydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione (22)

Obtained as brown prisms, mp 171-173 ºC; yield 0.303 g, 78.91%; IR ν_{max} (KBr) cm^{-1}: 3182 (O-H), 3063 (N-H), 2922, 2850 (C-H), 1621 (α, β- unsaturated C=O), 1599, 1581, 1496, 1298 (C-N), 1116 (C-O), 750, 690 (Fig. 91); ^{1}H NMR (500 MHz, DMSO, Fig. 92) δ: 10.03 (s, 2H, -NH), 9.22 (1H, -OH), 7.19-7.50 (5H, aromatic), 5.69 (s, 1H, H-6), 2.32 (t, 2H, H-1′), 1.39 (m, 2H, H-2′), 1.22-1.32 (m, 16H, H-3′ to 10′), 0.84 (t, 3H, H-11′); ^{13}C NMR (100 MHz, DMSO) see Table 2 and Fig. 93; negative ESI-MS (Fig. 94): m/z calculated for 384.24, Found: 382 $[M-H]^{-1}$.

5.1.23. 5-(2-(2,4-dinitrophenyl)hydrazinyl)-2-hydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione (23)

Obtained as brown prisms, mp 162-164 ºC; yield 0.385 g, 81.22%; IR ν_{max} (KBr) cm^{-1}: 3317 (O-H), 3252, 3190 (N-H), 2922, 2850 (C-H), 1614 (α, β- unsaturated C=O), 1593 (C=C), 1465, 1330 (-NO_2), 1195 (C-N), 767, 649 (Fig. 95); ^{1}H NMR (500 MHz, DMSO, Fig. 96) δ: 9.22 (s, 2H, NH), 8.19 (s, 1H, -OH), 7.45-7.97 (3H, aromatic), 5.79 (s, 1H, H-6), 2.23 (t, 2H, H-1′), 1.30 (m, 2H, H-2′), 1.23-1.30 (m, 16H, H-3′ to 10′), 0.84 (t, 3H, H-11′); ^{13}C NMR (100 MHz, DMSO) see Table 2 and Fig. 97; negative ESI-MS (Fig. 98): m/z calculated for 474.21, Found: 473 $[M-H]^{-1}$.

5.1.24. (S)-2-(4-hydroxy-3,6-dioxo-5-undecylcyclohexa-1,4-dienylamino)-3-(1H-indol-3-yl) propanoic acid (24)

Obtained as black powder, mp 152-154 ºC; yield 0.328 g, 68.33%; IR ν_{max} (KBr) cm^{-1}: 3309 (O-H), 3252 (-NH), 2920, 2848 (C-H), 1614 (α, β- unsaturated C=O), 1587 (C=C), 1195 (C-N), 1116 (C-O), 767, 690 (Fig. 99); ^{1}H NMR (500 MHz, DMSO, Fig. 100) δ: 12.10 (s, 1H,-COOH), 8.93 (s, 2H, -NH), 8.31 (s, 1H, -OH), 6.80-7.25 (m, 4H, H-4″,5″,6″ and 7″), 5.77 (s, 1H, H-6), 5.37 (s, 1H, H-2″), 3.82 (s, 1H, H-10″), 2.70 (t, 2H, H-9″), 2.27 (t, 2H, H-1′), 1.51 (m, 2H, H-2′), 1.22-1.33 (m, 16H, H-3′ to 10′), 0.83 (t, 3H, H-11′); ^{13}C NMR (100 MHz, DMSO) see Table 2 and Fig. 101; negative ESI-MS (Fig. 102): m/z calculated for 480.26, Found: 435 $[M-COOH]^{-1}$.

5.1.25. 1-undecylphenazine-2,3-diol (25)

Obtained as golden yellow prisms, mp 180-182 ºC; yield 0.366 g, 90.44%; IR ν_{max} (KBr) cm^{-1}: 3302 (O-H), 2918, 2850 (C-H), 1558 (C=C), 1215 (C-N), 1143, 1120 (C-O), 756,

594 (Fig. 103); ^{1}H NMR (500 MHz, DMSO, Fig. 104) δ: 7.73-8.13 (aromatic H, 4H, H-5,6,7 and 8), 7.17 (s, 2H, H-2 and 3 -OH), 6.30 (s, 1H, H-4), 3.10 (t, 2H, H-1′), 1.60 (m, 2H, H-2′), 1.20-1.40 (m, 16H, H-3′ to 10′), 0.90 (t, 3H, H-11′); ^{13}C NMR (100 MHz, DMSO) see Table 2 and Fig. 105; negative ESI-MS (Fig. 106): m/z calculated for 366.23, Found: 366.82.

5.1.26. Embelin-ninhydrin adduct

Obtained as dark brown solid, mp 178-180 ^{o}C; yield 0.415 g, 43.55%; IR υ_{max} (KBr) cm^{-1}: 3309 (O-H), 2922, 2850 (C-H), 1716 (C=O), 1650 (α, β- unsaturated C=O), 1614 (C=C), 1118 (C-O), 767, 696 (Fig. 107); ^{1}H NMR (500 MHz, DMSO, Fig. 108) δ : 8.05 (s, 1H, 3-OH), 7.94-7.99 (4H, aromatic, H-4 to 7), 7.89 (s, 1H, 2-OH), 7.83 (s, 1H, 2′-OH), 2.29 (t, 2H, H-1″), 1.35 (m, 2H, H-2″), 1.22-1.30 (m, 16H, H-3″ to 10″), 0.84 (t, 3H, H-11″); ^{13}C NMR (100 MHz, DMSO, Fig. 109) δ: 198.53 (C-1), 183.16 (C-1′), 181.40 (C-4′), 155.98 (C-2′), 151.68 (C-5′), 142.20 (C-8), 136.19 (C-5), 130.99 (C-6′), 127.06 (C-7,8), 122.72 (C-6), 117.83 (C-4), 117.22 (C-3′), 104.29 (C-3), 51.29 (C-2), 22.44-31.74 (C-1″to C-10″) and 14.40 (C-11″). Negative ESI-MS (Fig. 110): m/z calculated for 454.20, Found: 453 [M-H]$^{-1}$.

Table 2. ^{13}C NMR of embelin and its derivatives

Carbons	**1**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**
1	***	182.41	182.52	***	***	***	***	183.18	***	***	***	183.16	114.78
2	***	156.81	156.74	***	***	***	***	167.18	***	***	***	156.79	152.00
3	116.99	115.35	115.42	115.38	122.64	114.50	115.97	117.22	115.76	117.82	114.73	115.49	151.52
4	***	178.18	178.29	176.05	***	***	***	181.41	***	***	***	181.40	120.20
5	***	150.19	148.96	***	***	152.13	148.07	155.97	151.52	151.48	147.42	151.68	133.36
6	102.15	91.56	91.48	91.49	93.83	104.31	93.83	97.40	94.61	104.27	105.83	94.62	131.75
Chain 1′ to 10′	22.51 to 31.90	21.92 to 31.27	21.96 to 31.27	21.96 to 31.26	22.56 to 31.76	22.43 to 31.73	22.08 to 31.27	22.41 to 31.74	22.40 to 31.74	22.43 to 31.73	22.39 to 31.75	22.04 to 31.75	22.52 to 31.71
11′	14.09	13.93	13.93	13.93	14.42	14.50	13.94	14.39	14.39	14.39	14.39	14.38	14.38
1″	-	31.27	36.77	41.70	148.07	152.13	135.98	142.73	129.62	142.89	142.24	121.47	-
2″	-	-	12.95	29.42	127.24	114.76	134.22	130.99	128.96	127.73	130.68	129.10	-
3″	-	-	-	19.64	130.03	130.64	126.81	122.72	129.21	127.53	127.56	130.60	-
4″	-	-	-	13.61	127.24	128.26	127.24	145.20	126.28	128.52	142.24	124.62	-
5″	-	-	-	-	130.03	129.89	126.43	117.83	128.70	127.66	126.99	127.07	-
6″	-	-	-	-	127.24	117.35	130.97	127.06	126.28	127.59	127.50	128.22	-
-CH_3	-	-	-	-	-	-	17.31	-	-	-	-	-	-
-COOH	-	-	-	-	-	-	-	197.01	192.00	-	-	-	-
-CH_2, -CH	-	-	-	-	-	-	-	-	36.19, 56.12	-	-	-	-
7″or 7	-	-	-	-	177.44	172.46	-	-		-	-	125.71	130.80
8″ or 8	-	-	-	-	31.76	122.10	-	-	-	-	-	130.02	130.60
9″ or 9	-	-	-	-	-	130.85	-	-	-	-	-	29.99	147.44
10″ or 10	-	-	-	-	-	129.73	-	-	-	-	-	63.32	138.73
11″ or 11	-	-	-	-	-	127.49	-	-	-	-	-	179.31	138.90
12″ or 12	-	-	-	-	-	129.55	-	-	-	-	-	-	133.67
13″, 14″, 15″						126.78, 129.55, 126.78							

***Carbon peaks not appeared due to fluxional effect.

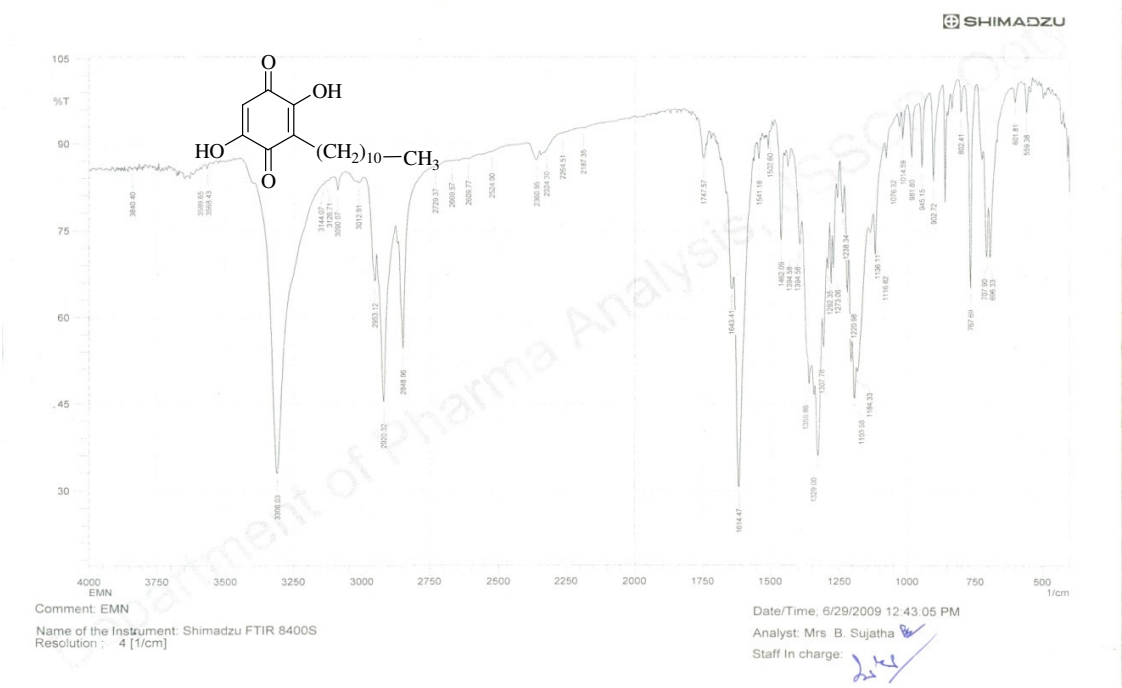


Fig. 7. IR spectrum of compound 1

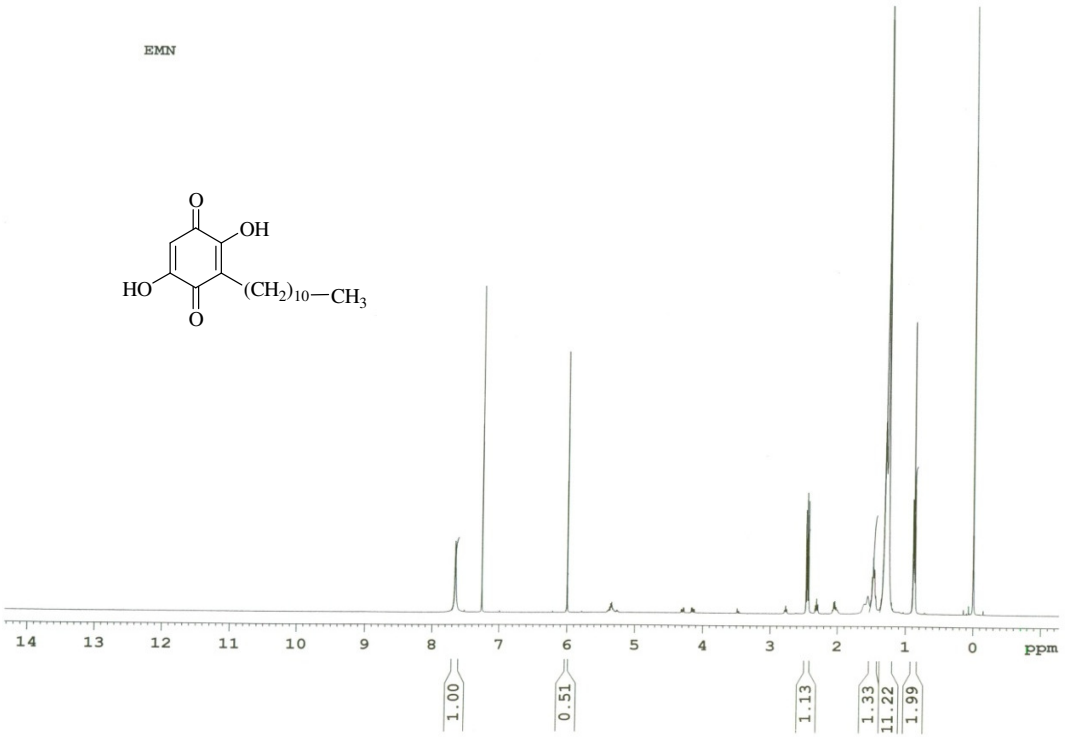


Fig. 8. ^{1}H NMR spectrum of compound 1

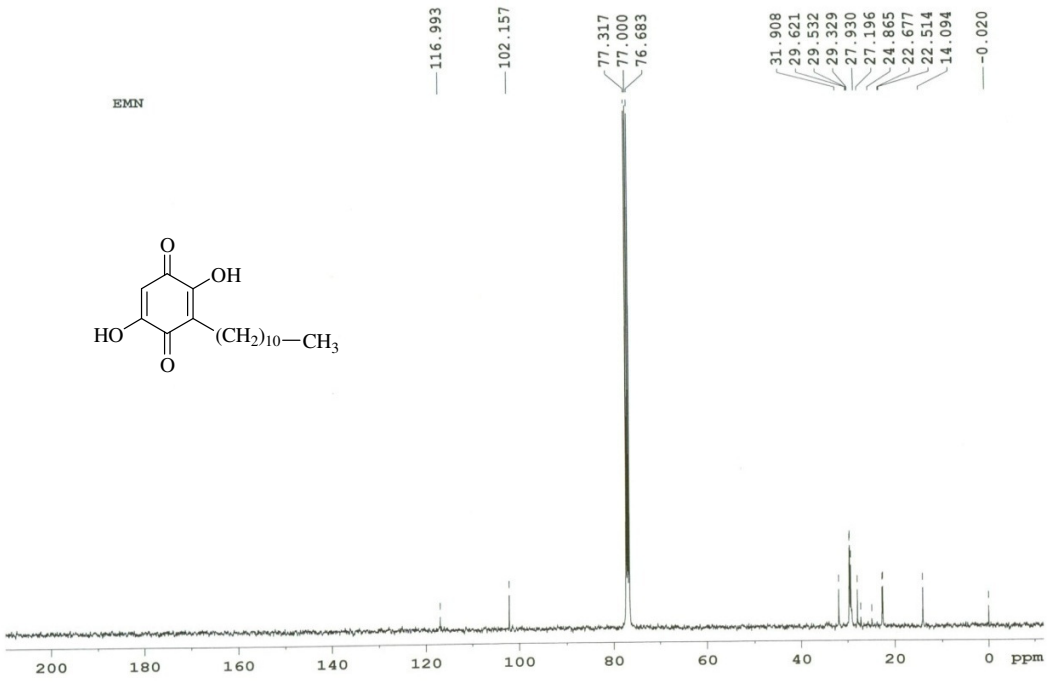


Fig. 9. ^{13}C NMR spectrum of compound 1

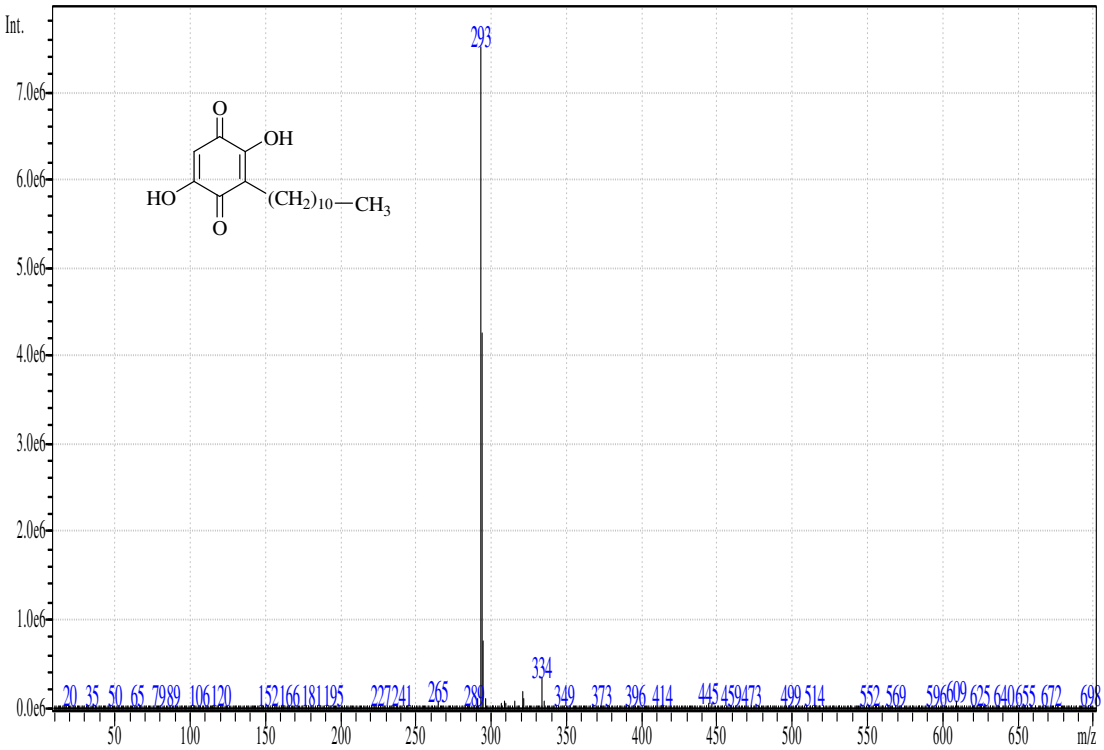


Fig. 10. Mass spectrum of compound 1

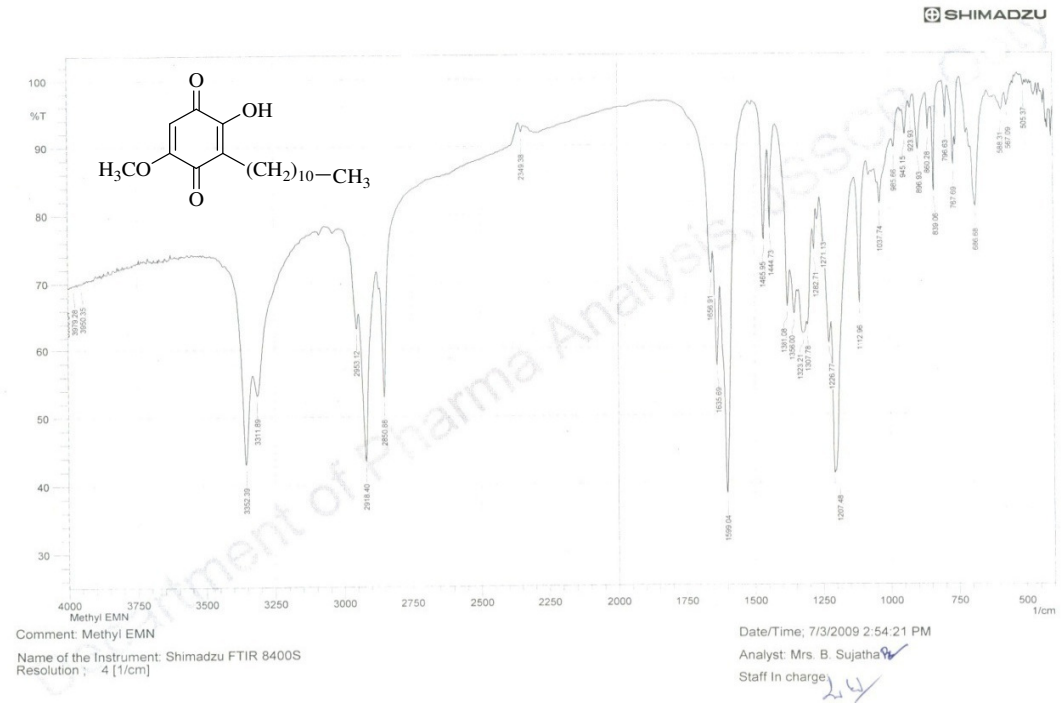


Fig. 11. IR spectrum of compound 2

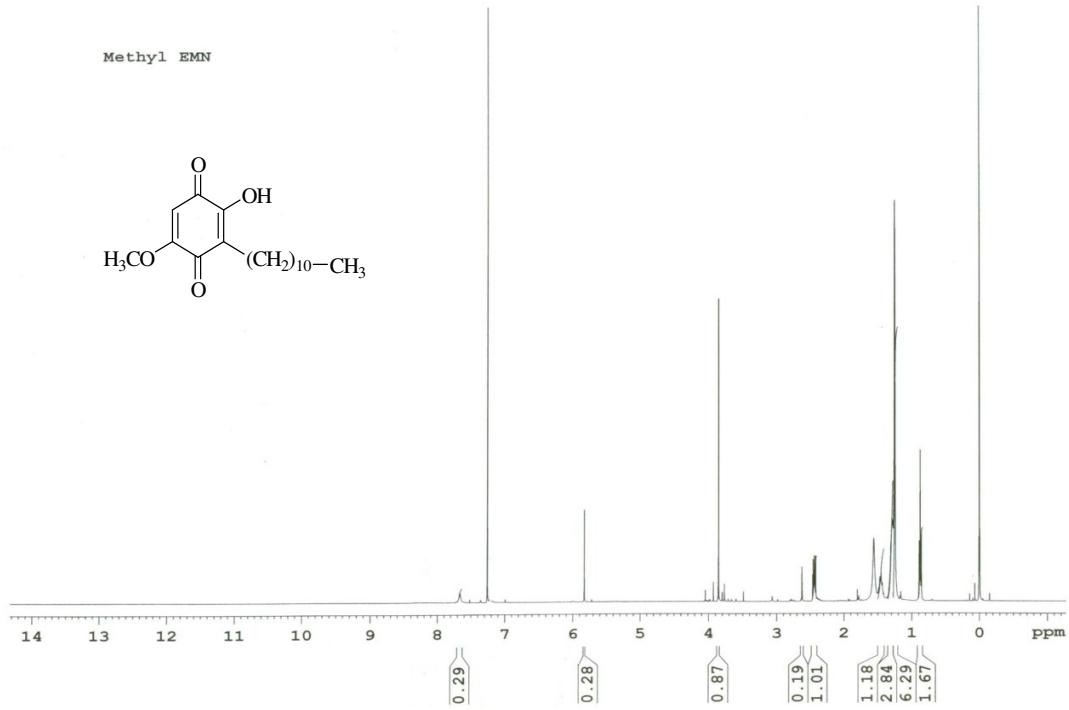


Fig. 12. ^{1}H NMR spectrum of compound 2

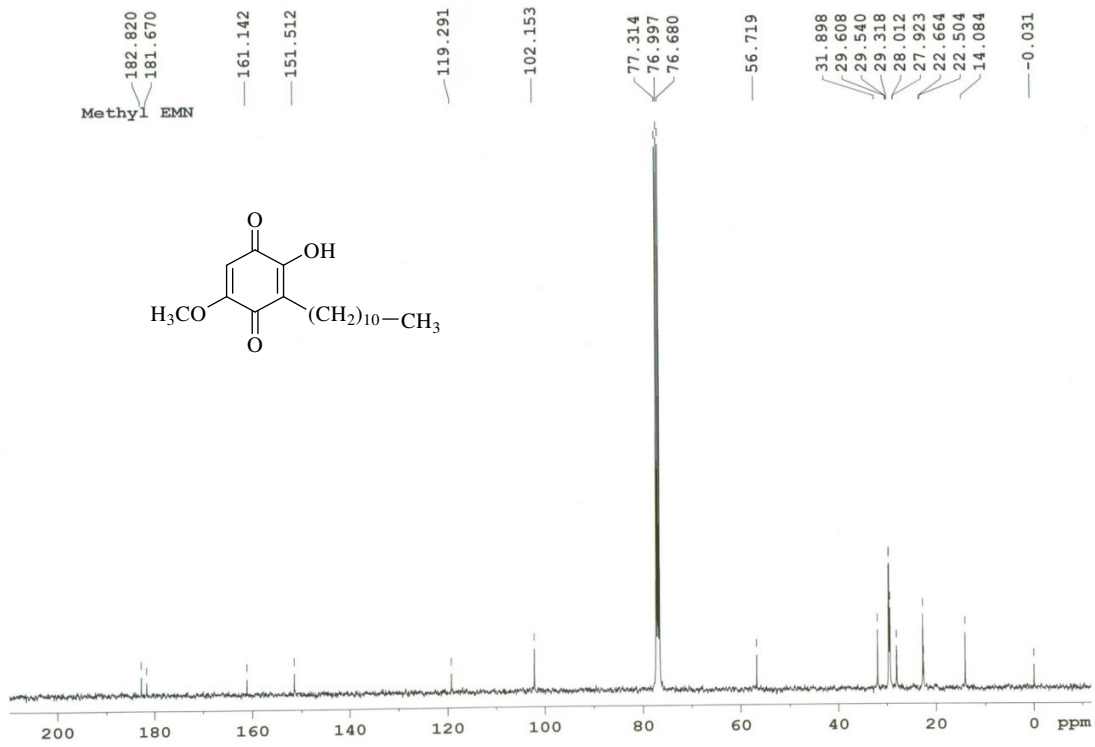


Fig. 13. ^{13}C NMR spectrum of compound 2

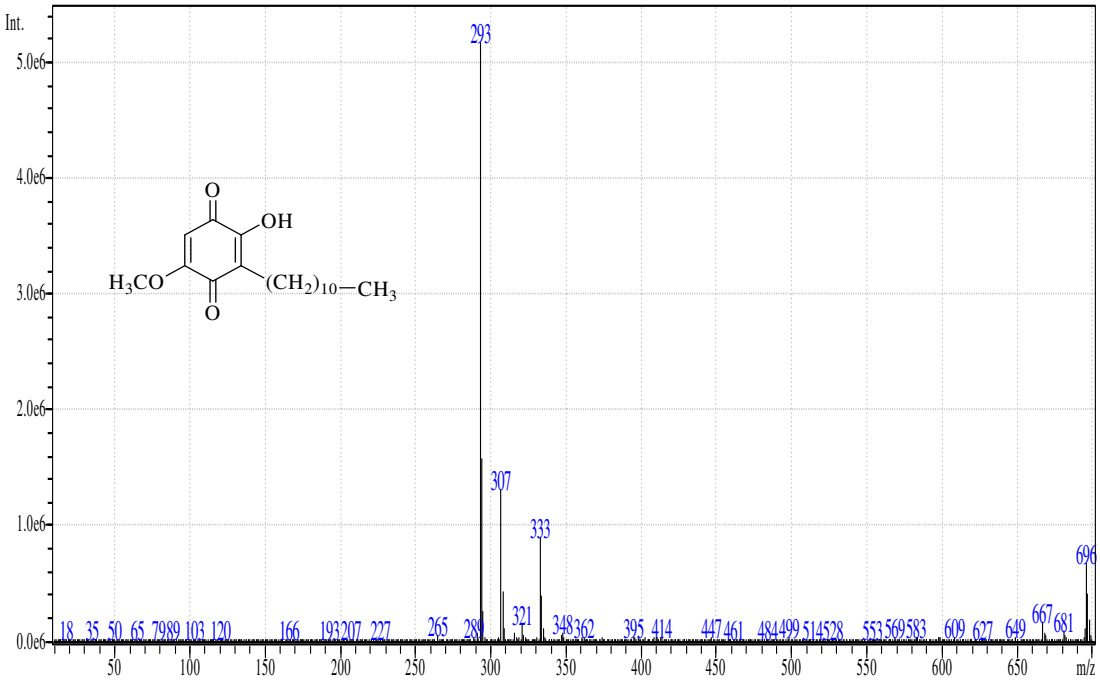


Fig. 14. Mass spectrum of compound 2

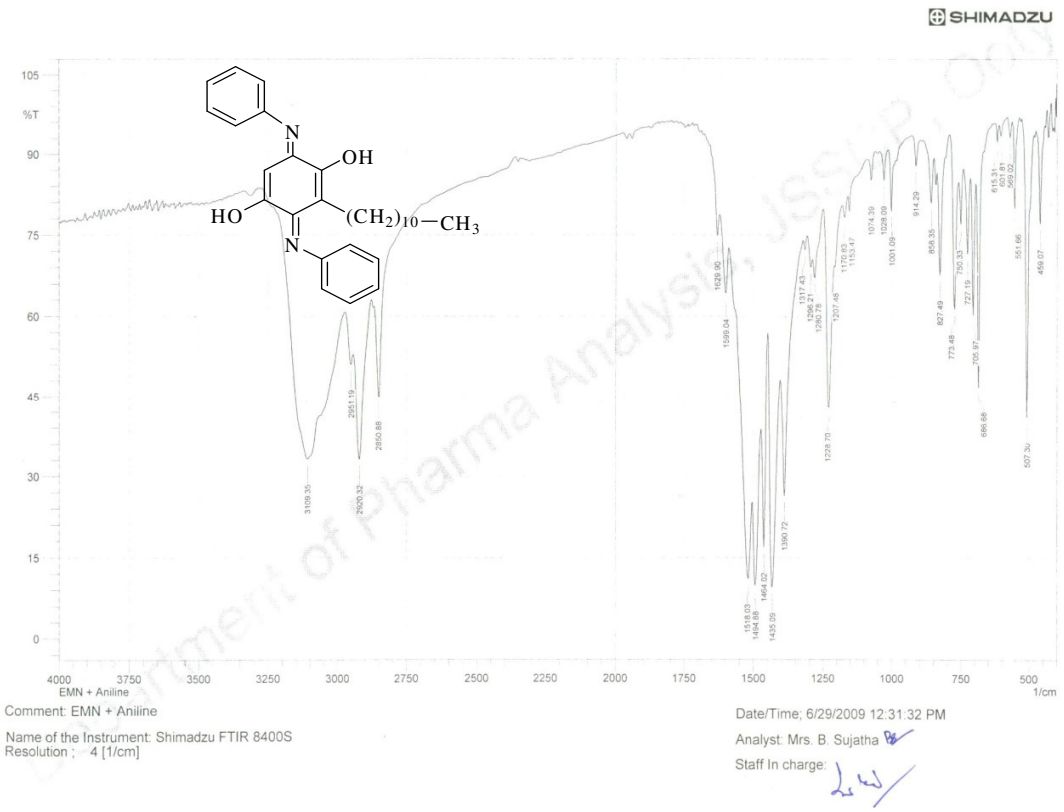


Fig. 15. IR spectrum of compound 3

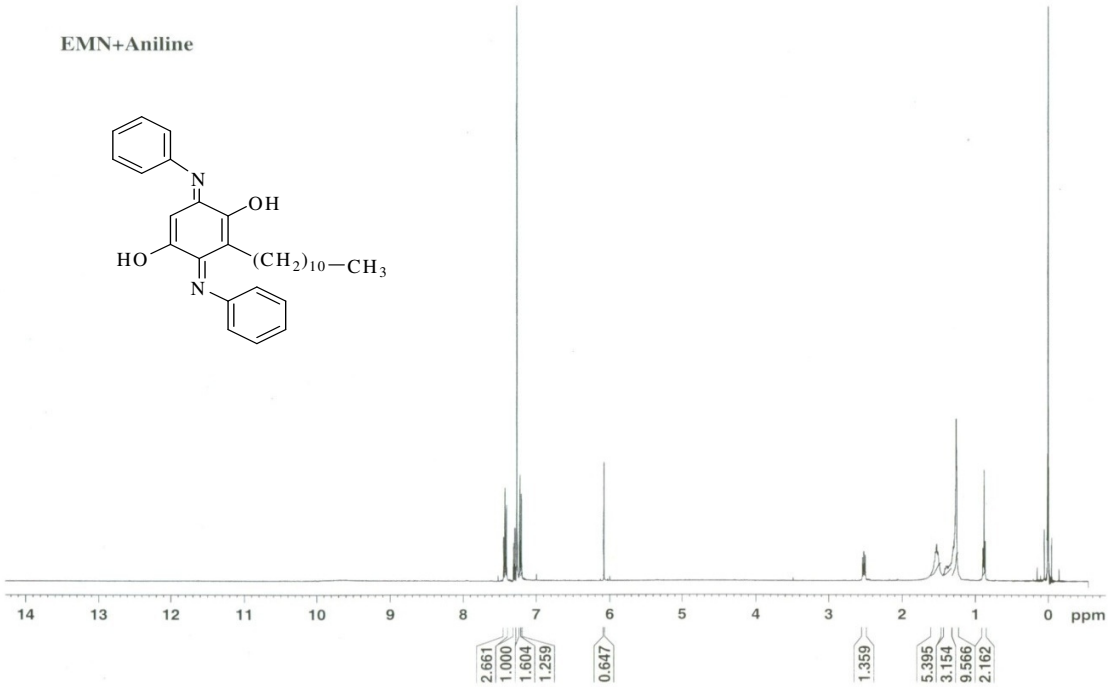


Fig. 16. ^{1}H NMR spectrum of compound 3

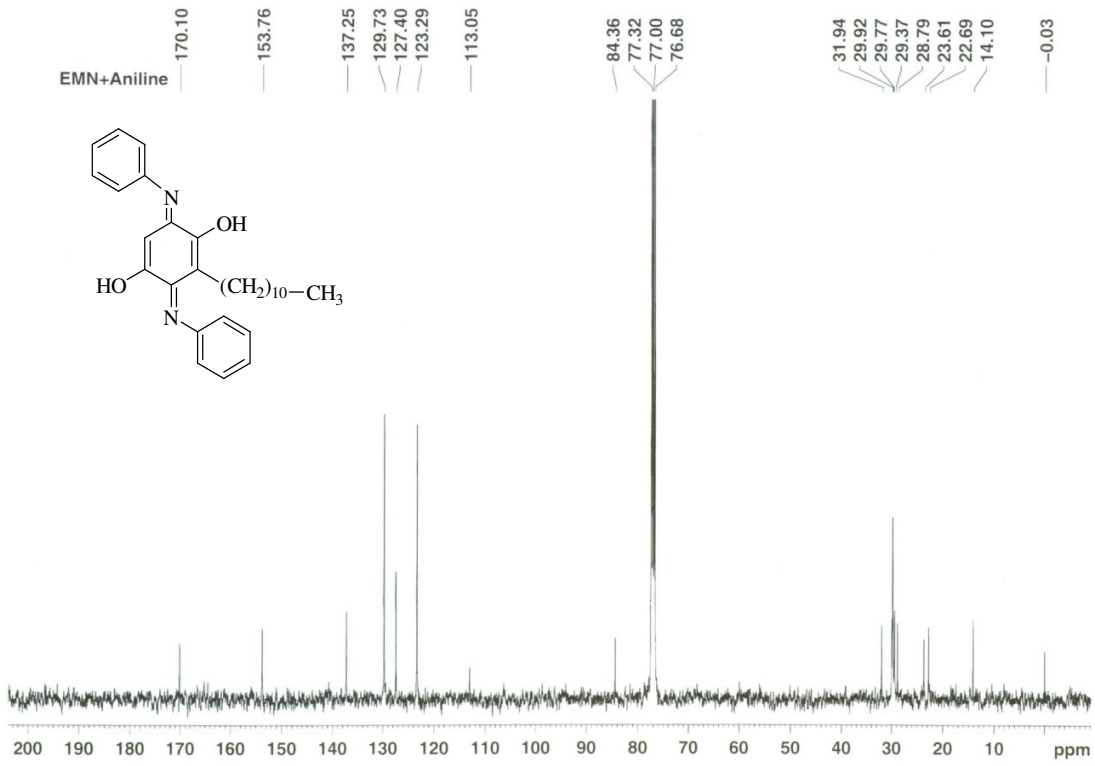


Fig. 17. ^{13}C NMR spectrum of compound 3

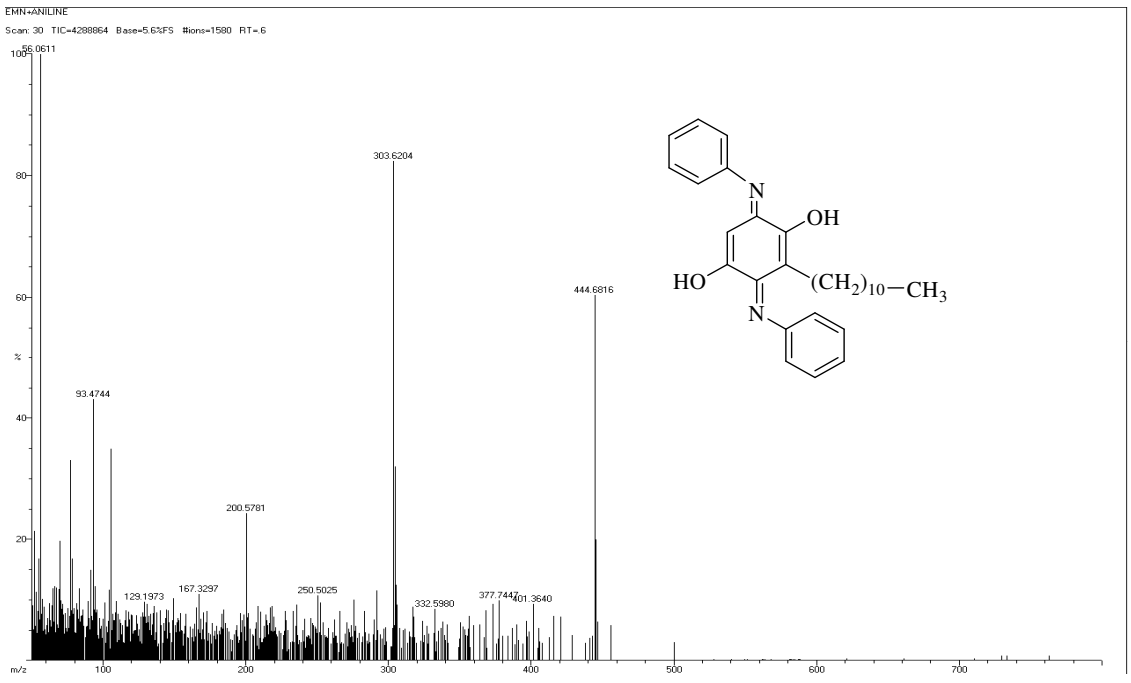


Fig. 18. Mass spectrum of compound 3

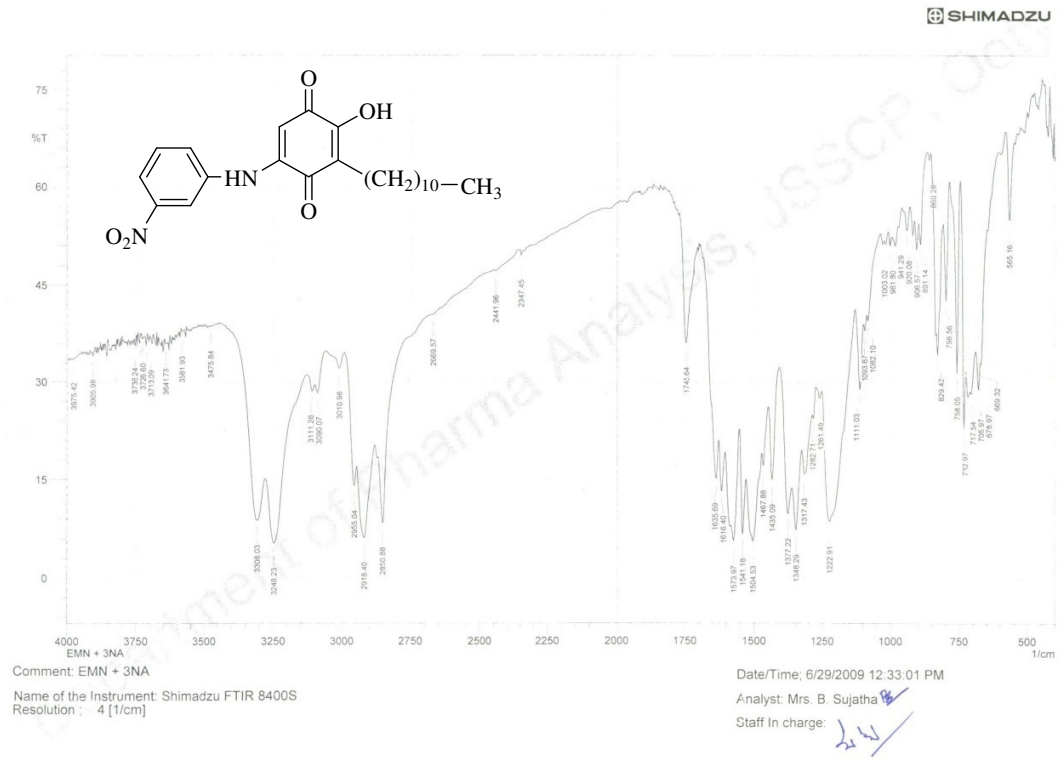


Fig. 19. IR spectrum of compound 4

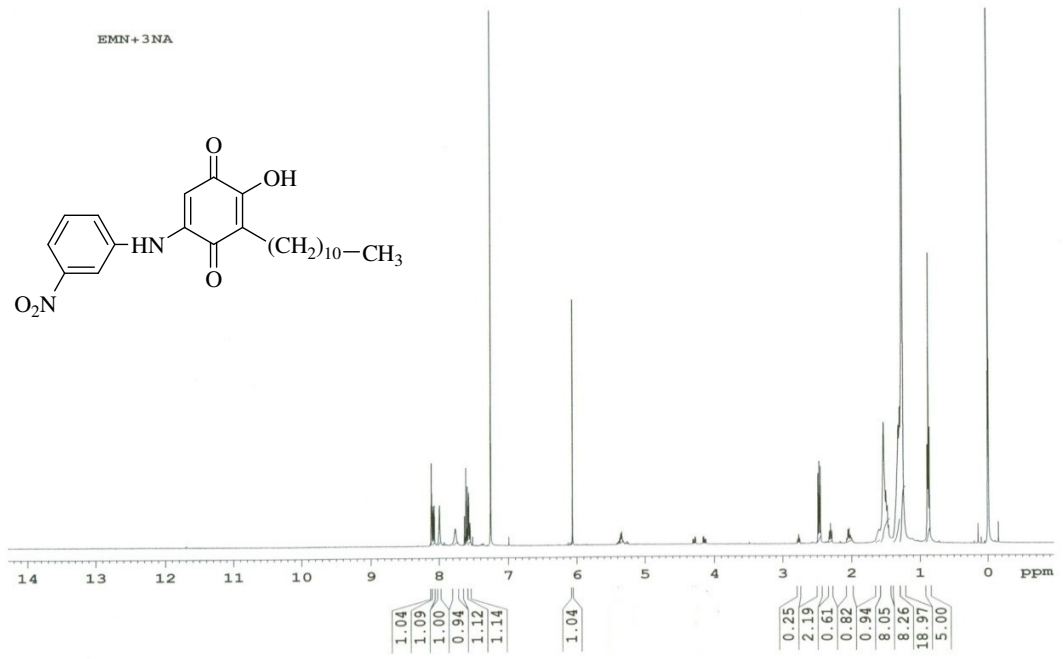


Fig. 20. ^{1}H NMR spectrum of compound 4

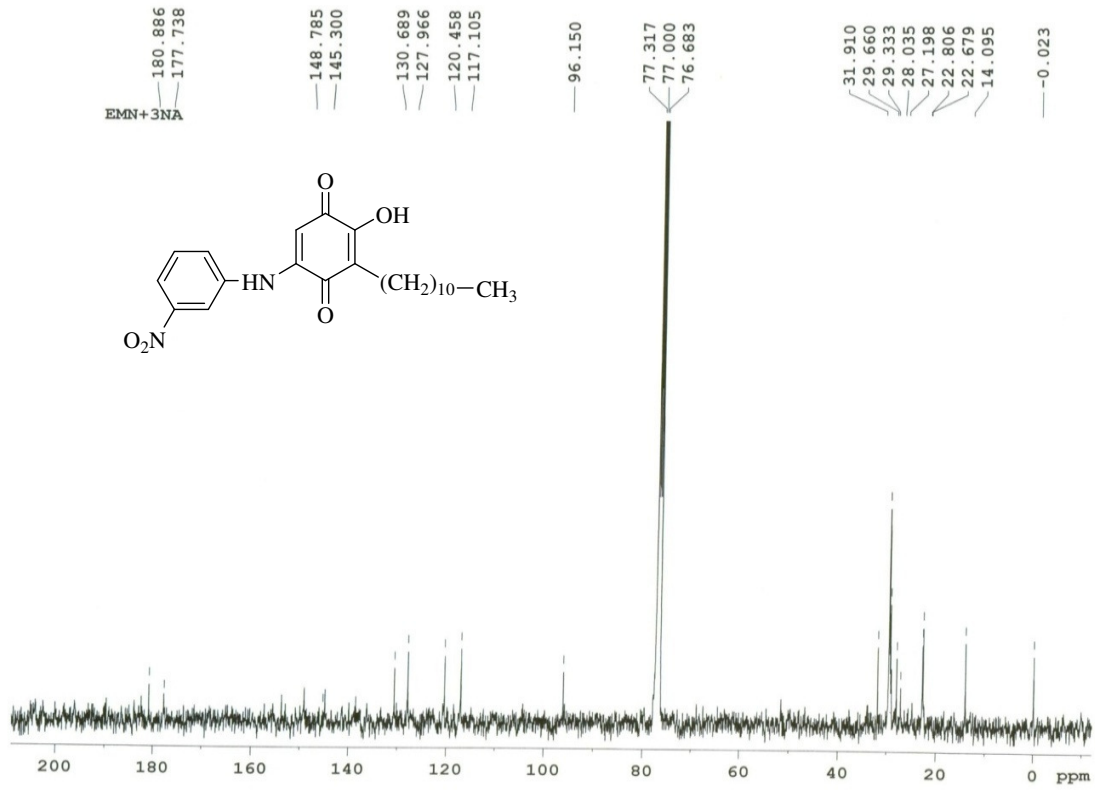


Fig. 21. ^{13}C NMR spectrum of compound 4

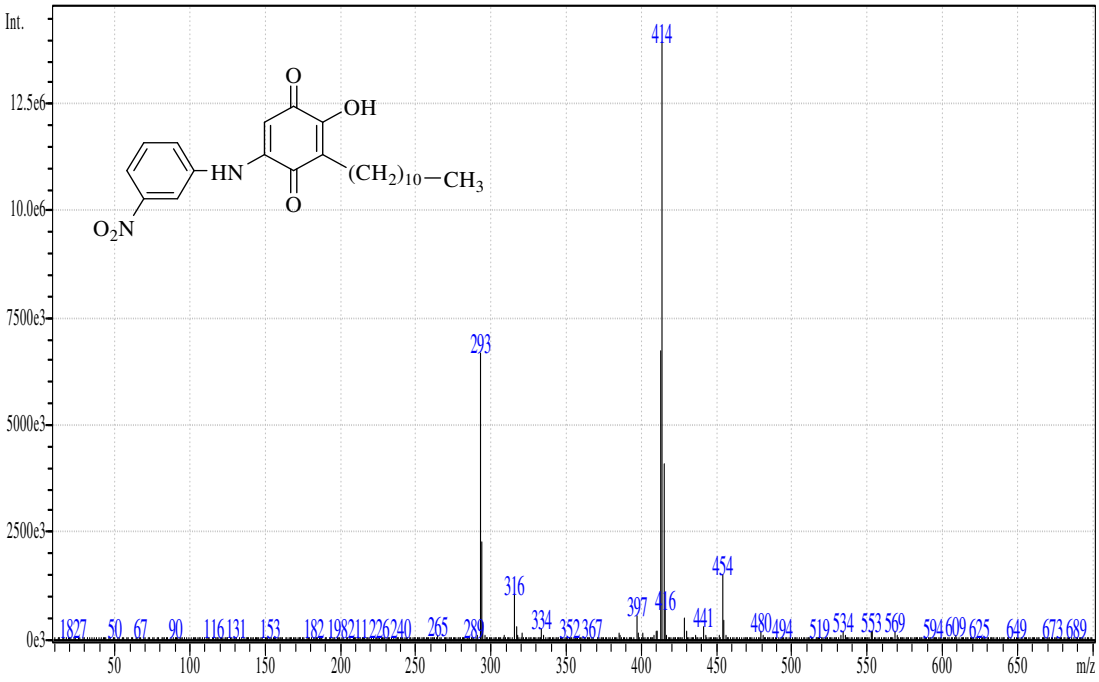


Fig. 22. Mass spectrum of compound 4

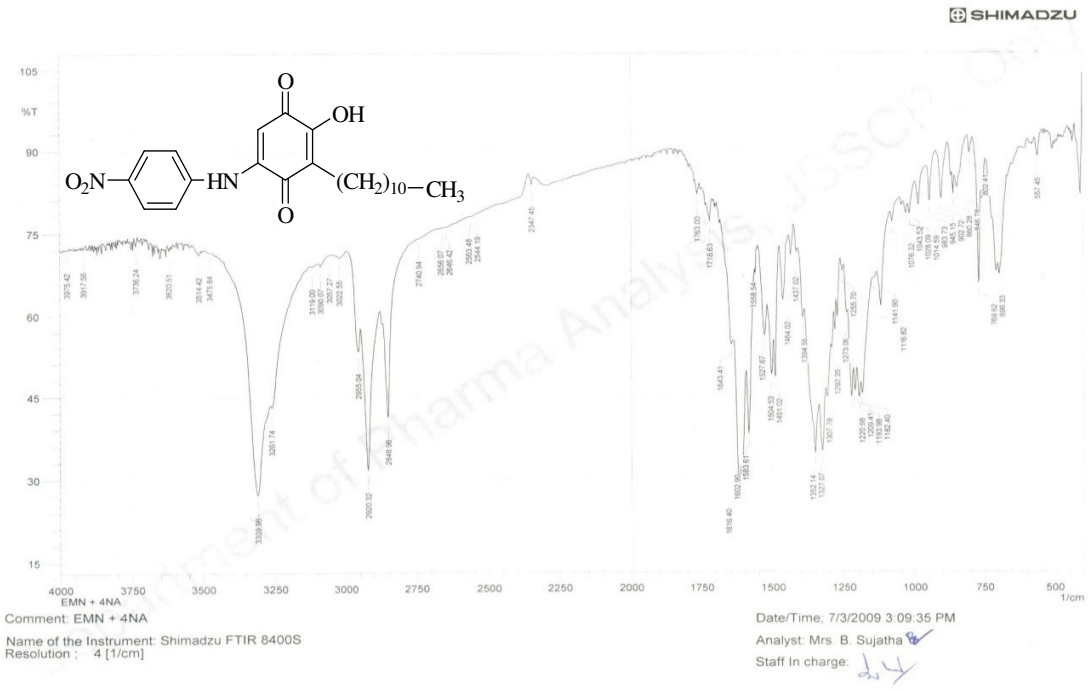


Fig. 23. IR spectrum of compound 5

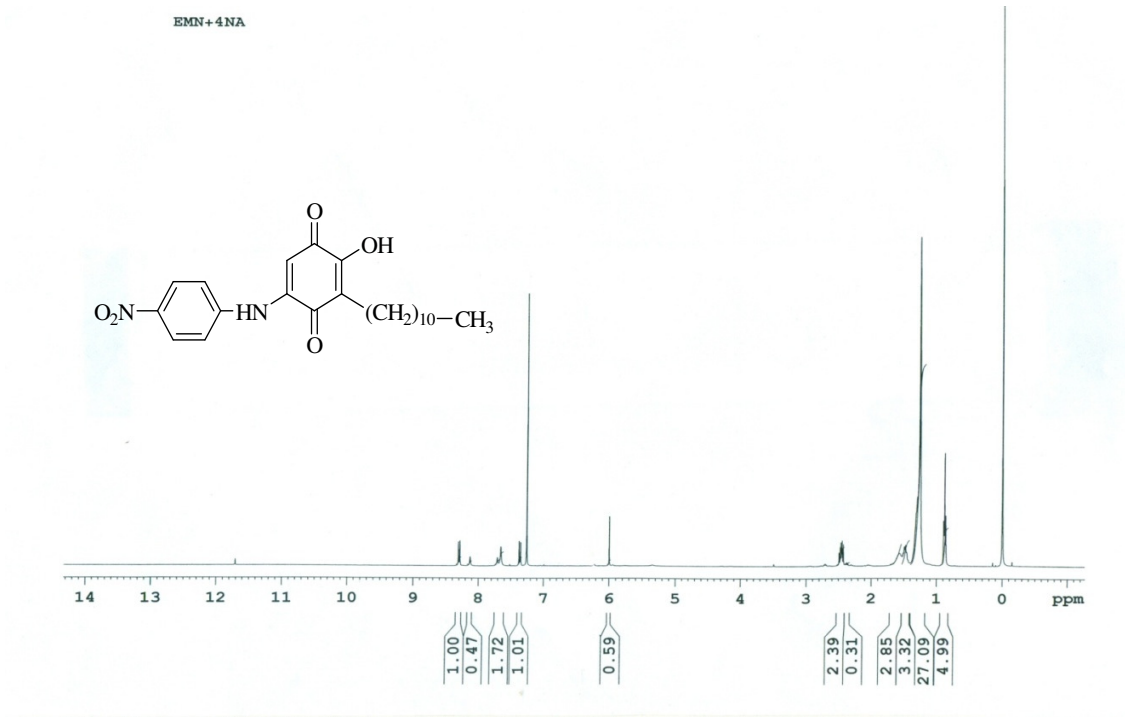


Fig. 24. ^{1}H NMR spectrum of compound 5

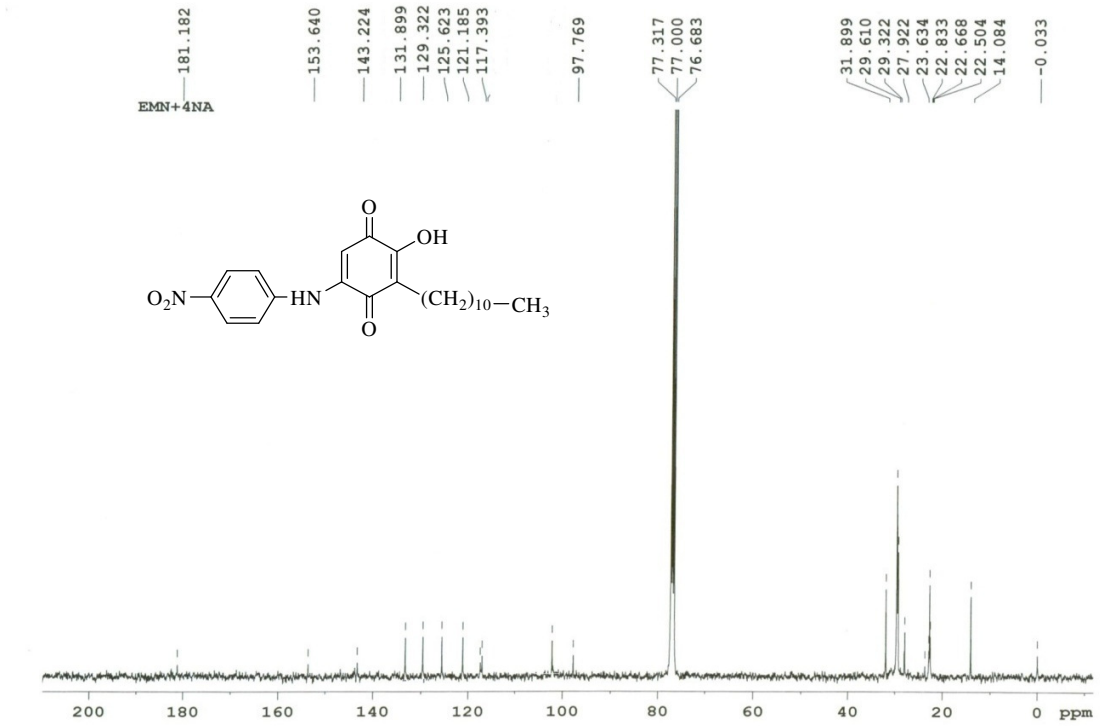


Fig. 25. ^{13}C NMR spectrum of compound 5

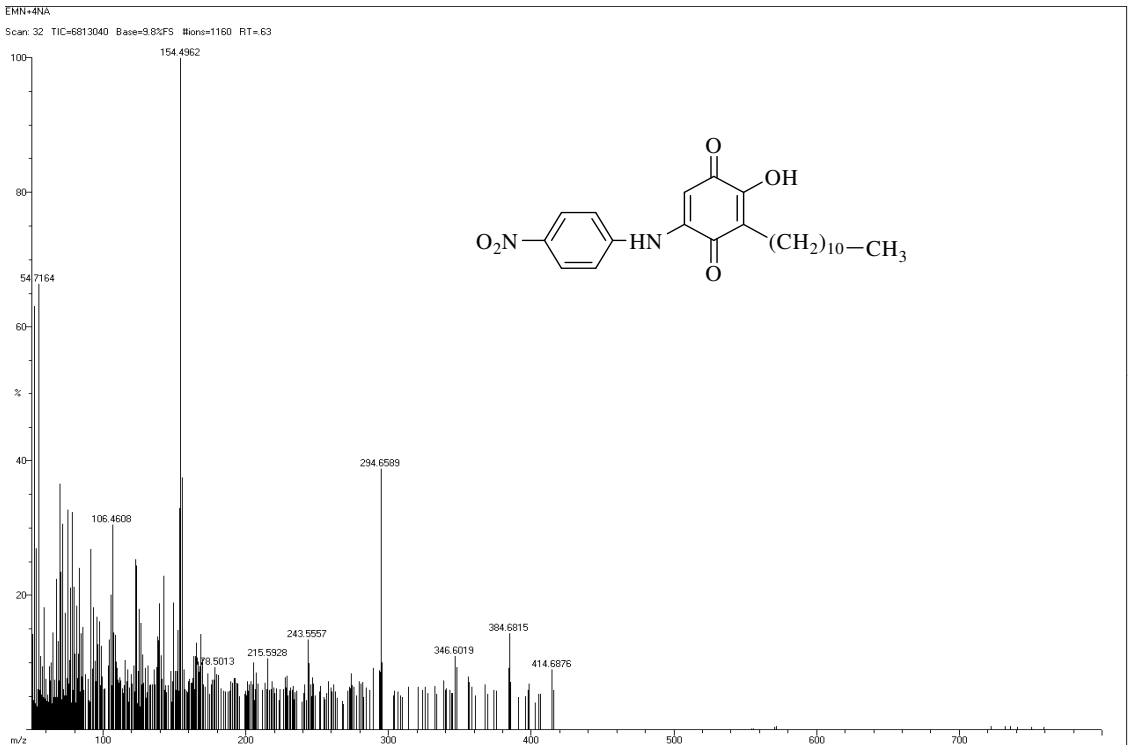


Fig. 26. Mass spectrum of compound 5

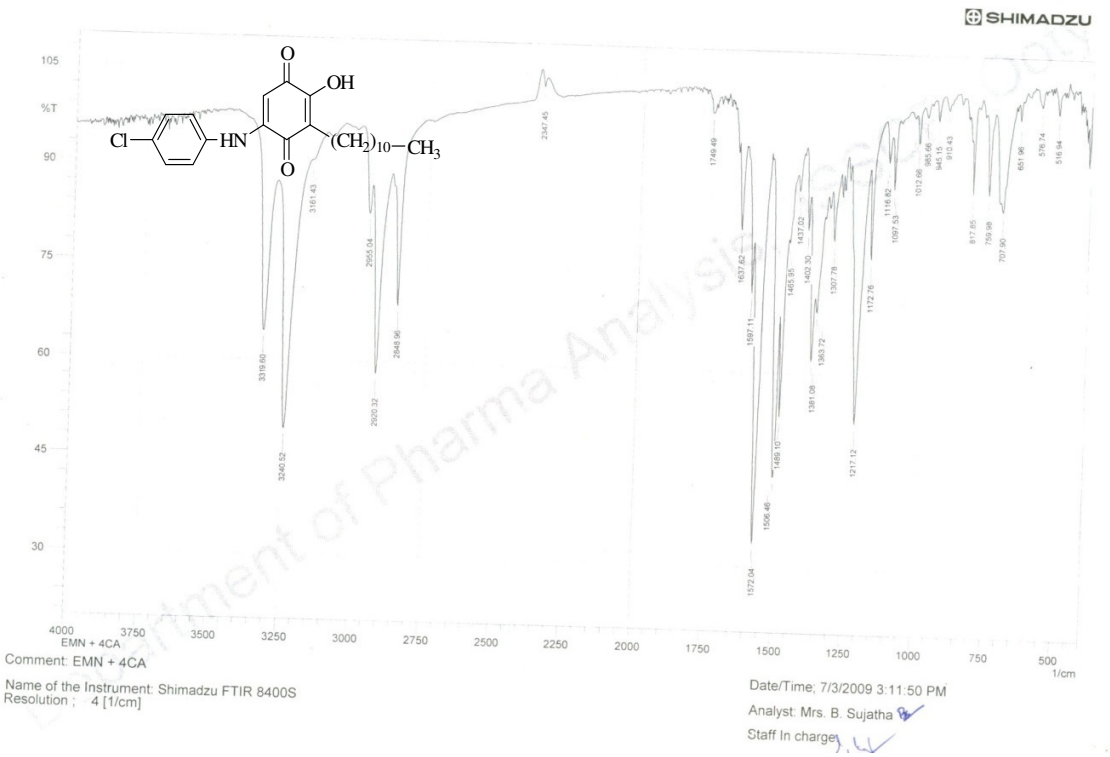


Fig. 27. IR spectrum of compound 6

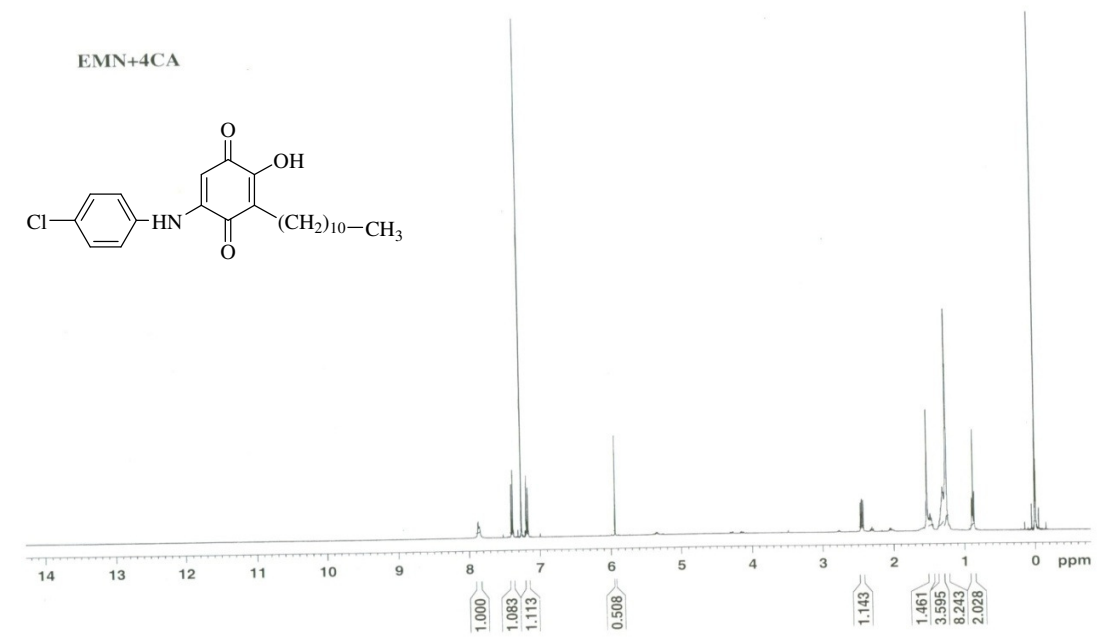


Fig. 28. ^{1}H NMR spectrum of compound 6

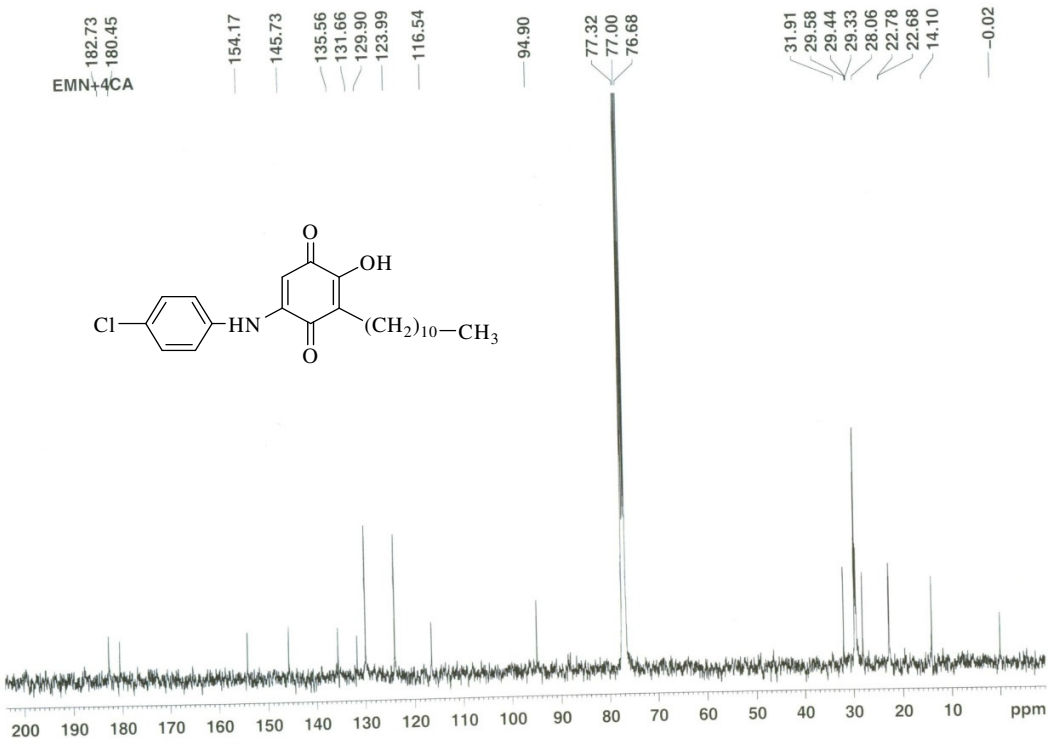


Fig. 29. ^{13}C NMR spectrum of compound 6

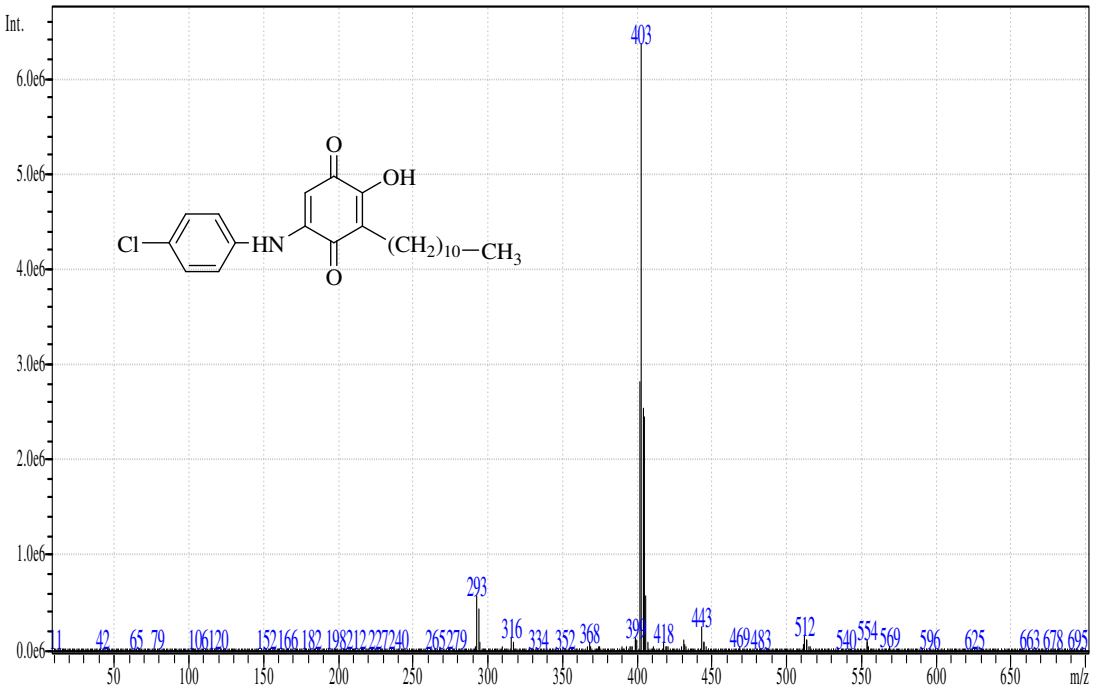


Fig. 30. Mass spectrum of compound 6

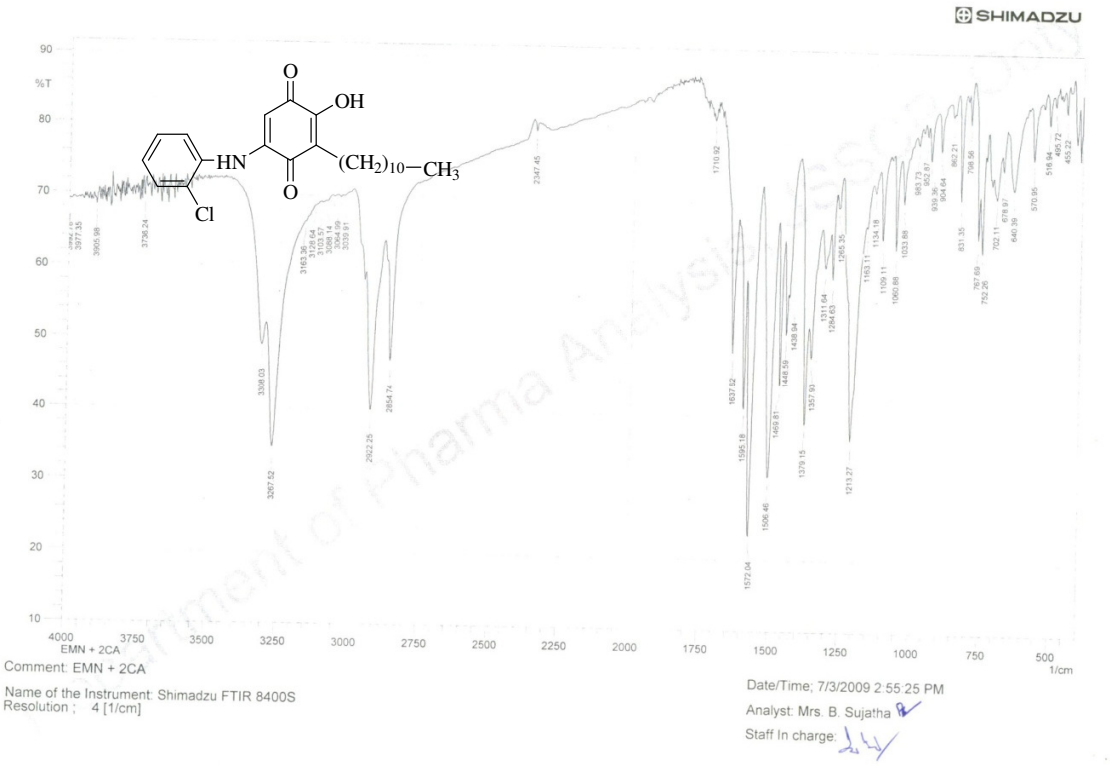


Fig. 31. IR spectrum of compound 7

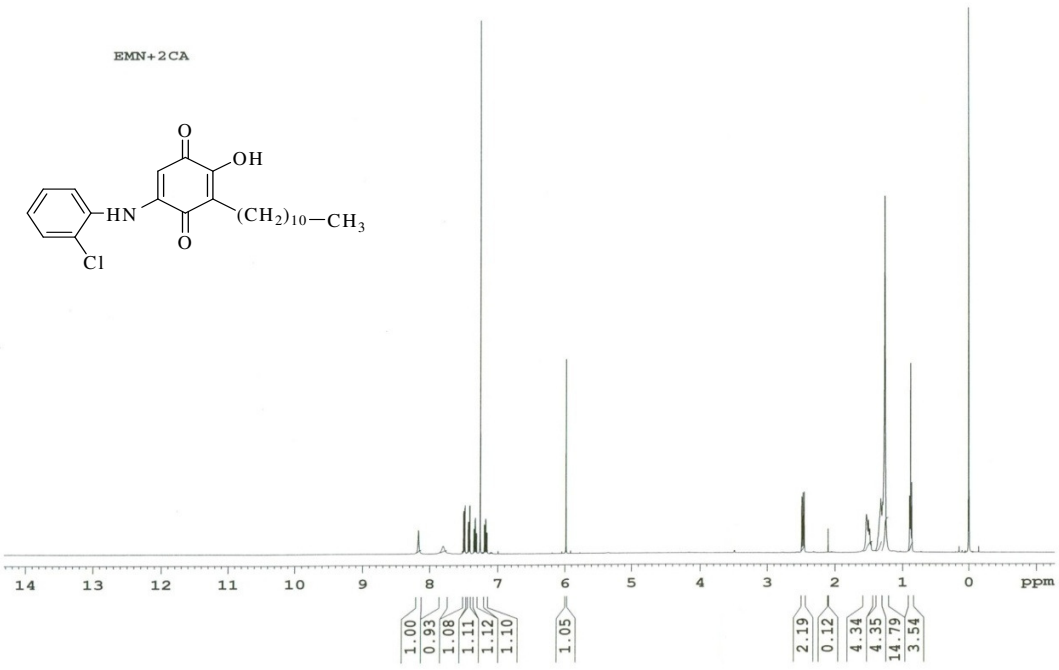


Fig. 32. ^{1}H NMR spectrum of compound 7

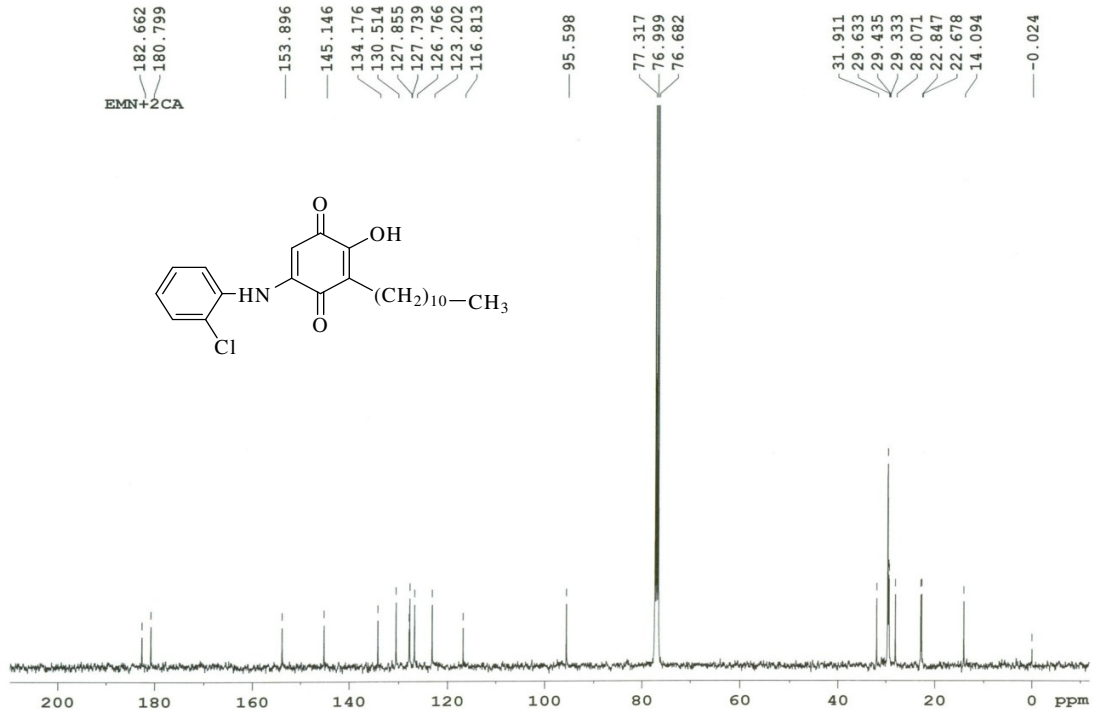


Fig. 33. ^{13}C NMR spectrum of compound 7

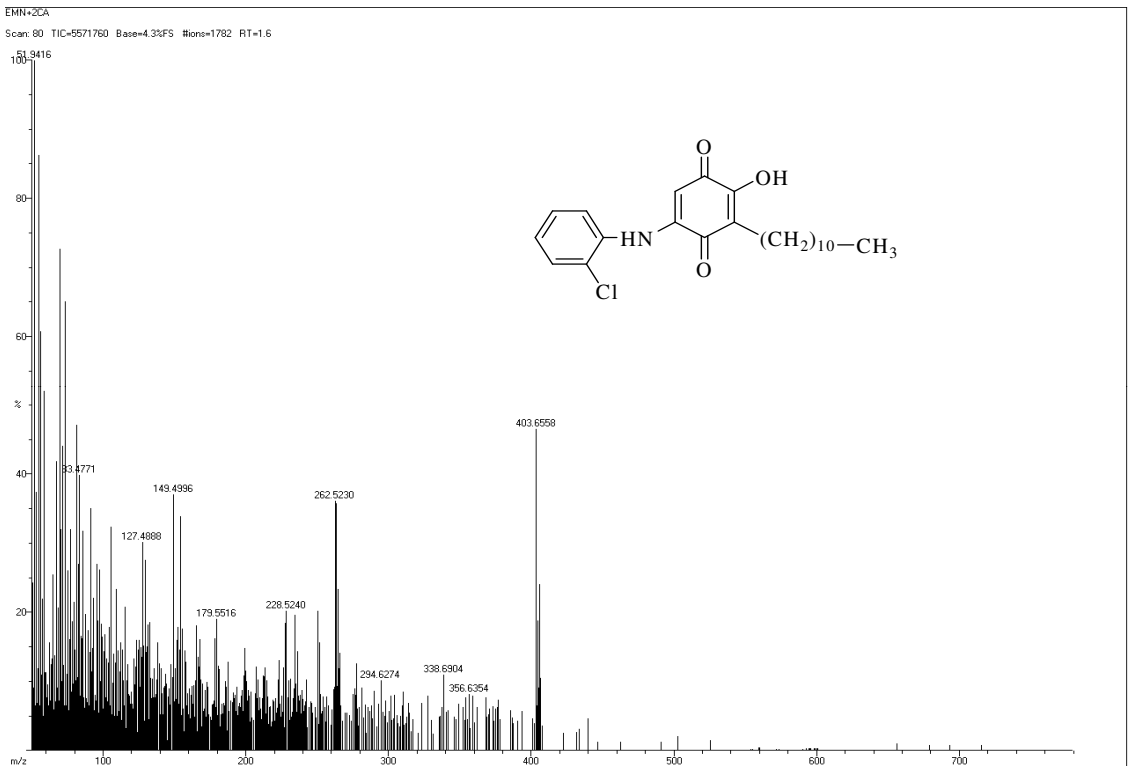


Fig. 34. Mass spectrum of compound 7

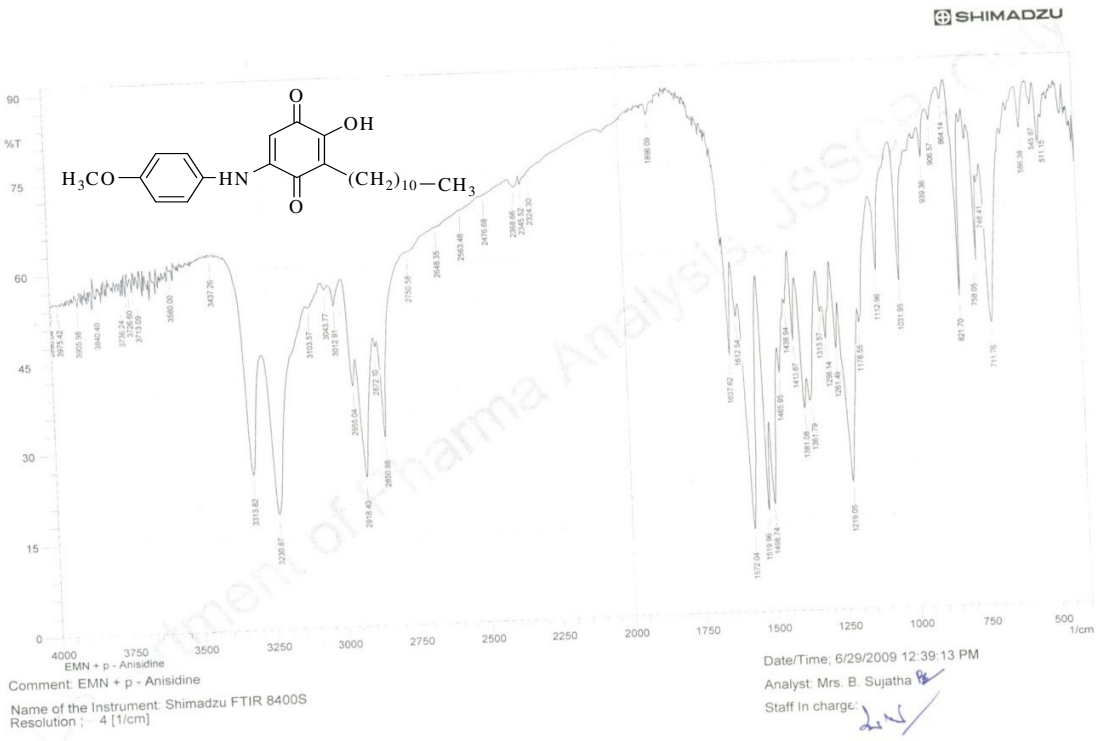


Fig. 35. IR spectrum of compound 8

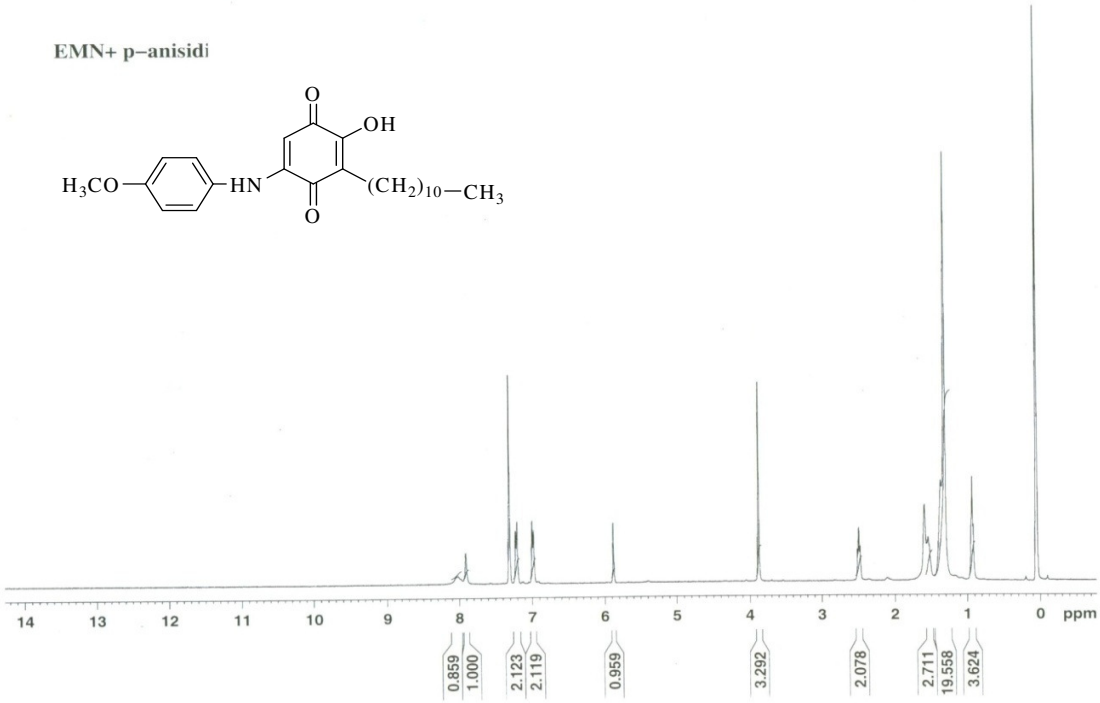


Fig. 36. ^{1}H NMR spectrum of compound 8

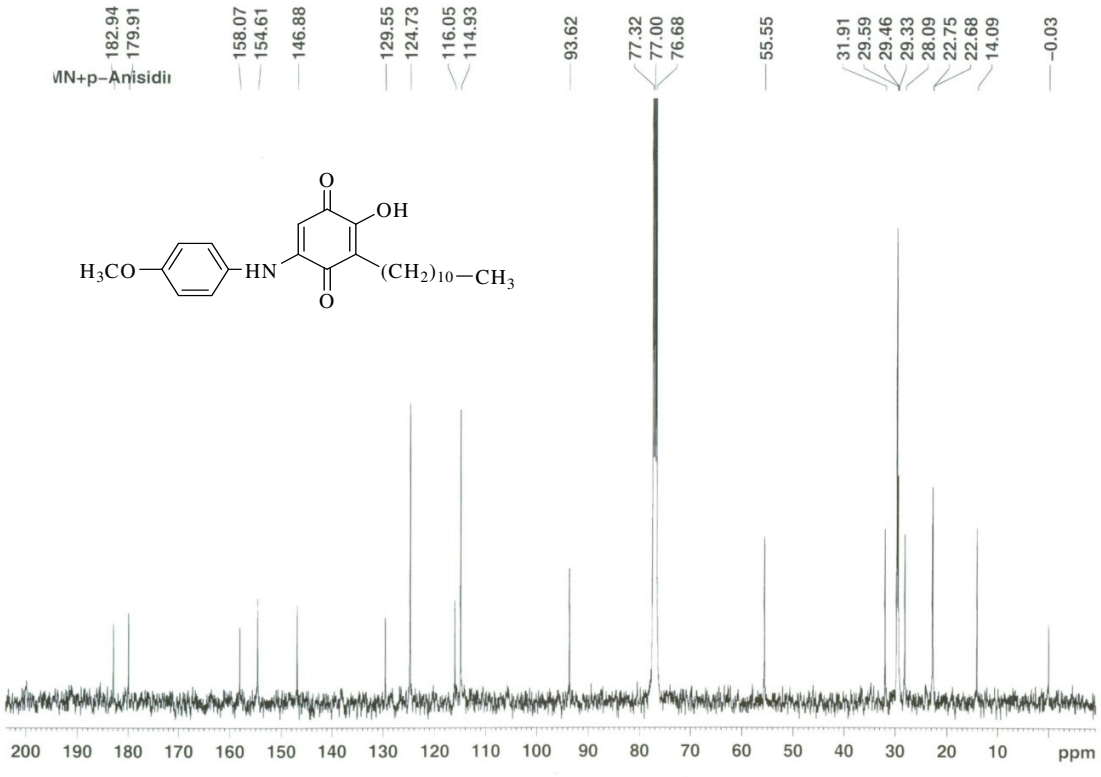


Fig. 37. ^{13}C NMR spectrum of compound 8

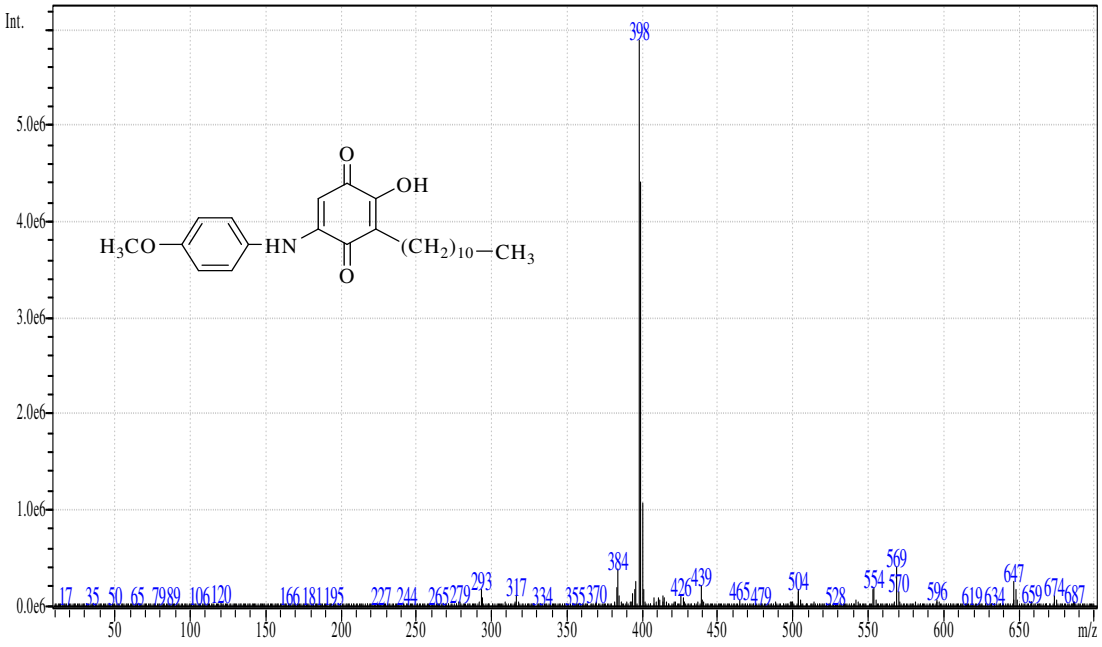


Fig. 38. Mass spectrum of compound 8

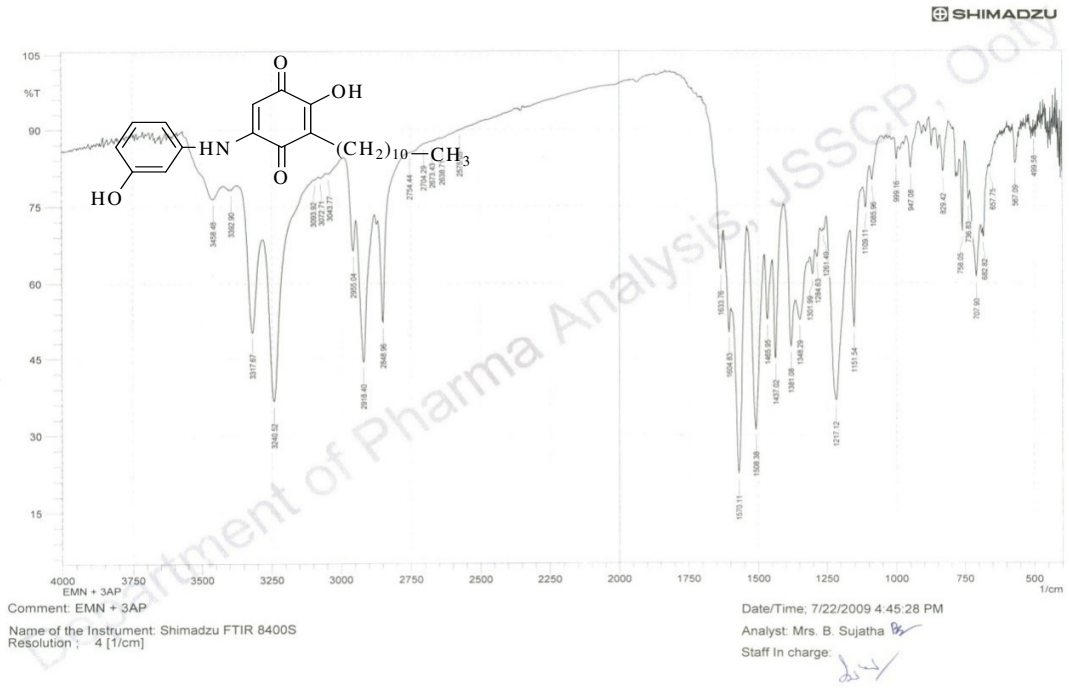


Fig. 39. IR spectrum of compound 9

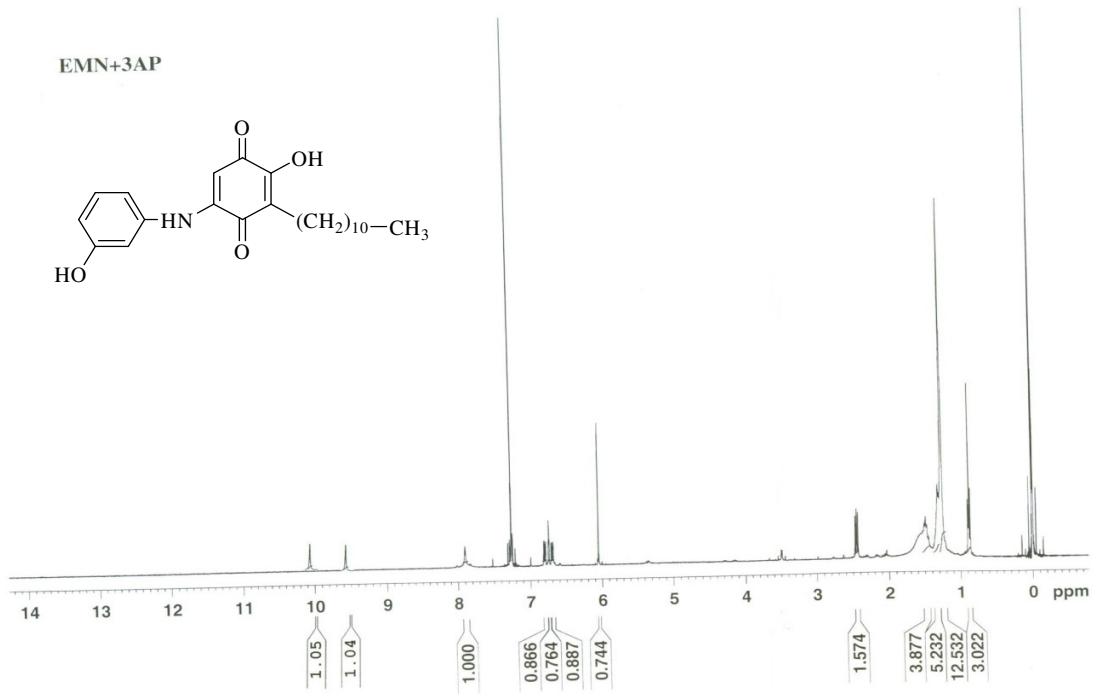


Fig. 40. ^{1}H NMR spectrum of compound 9

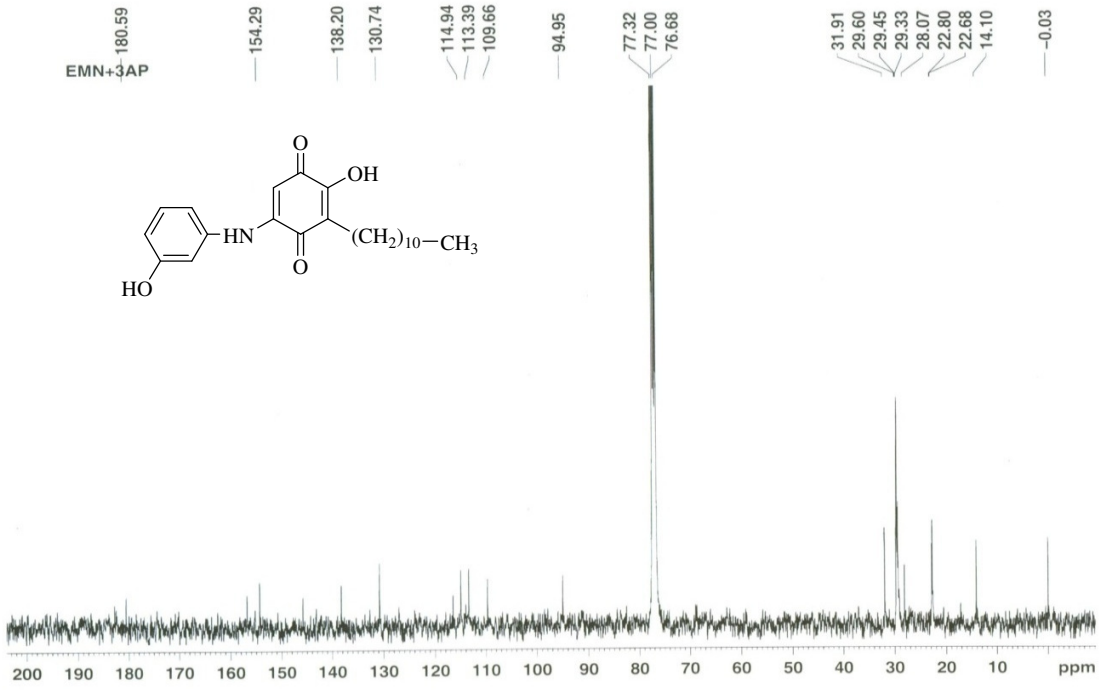


Fig. 41. ^{13}C NMR spectrum of compound 9

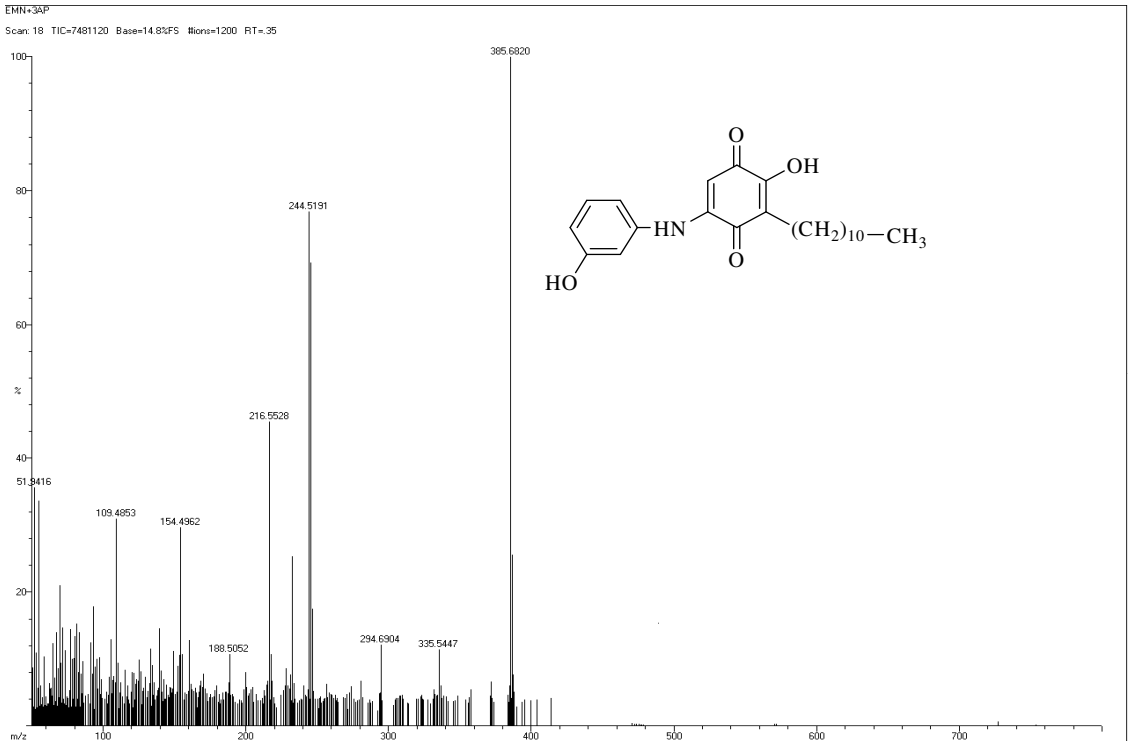


Fig. 42. Mass spectrum of compound 9

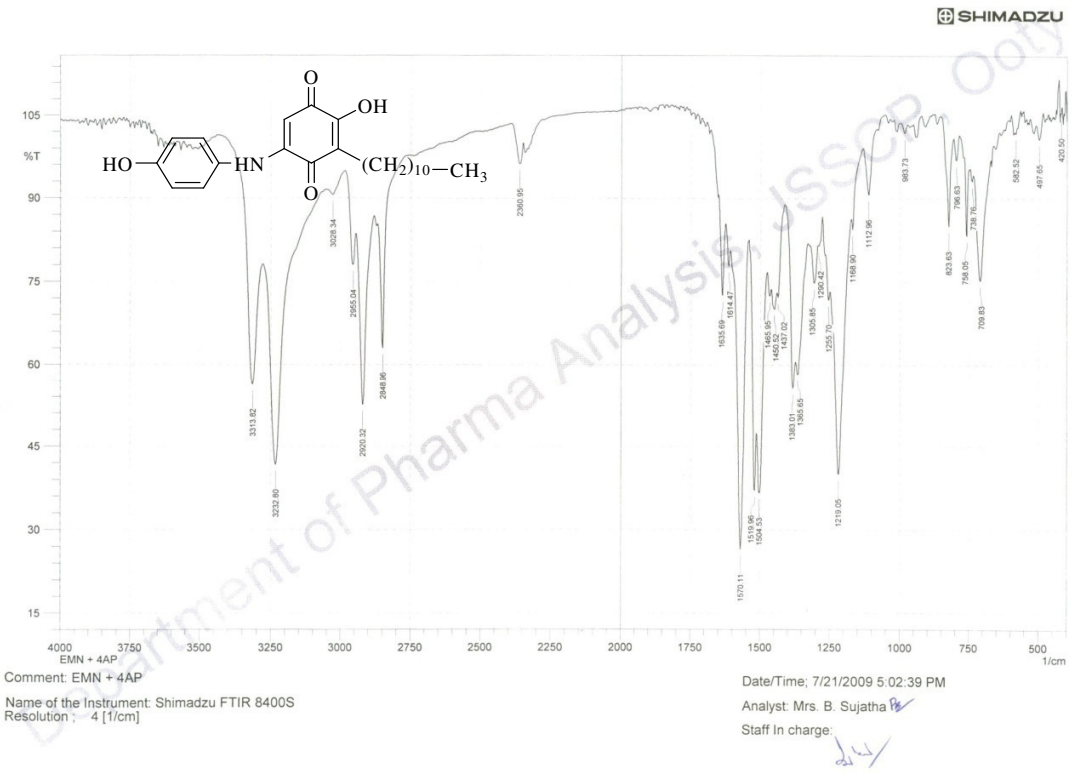


Fig. 43. IR spectrum of compound 10

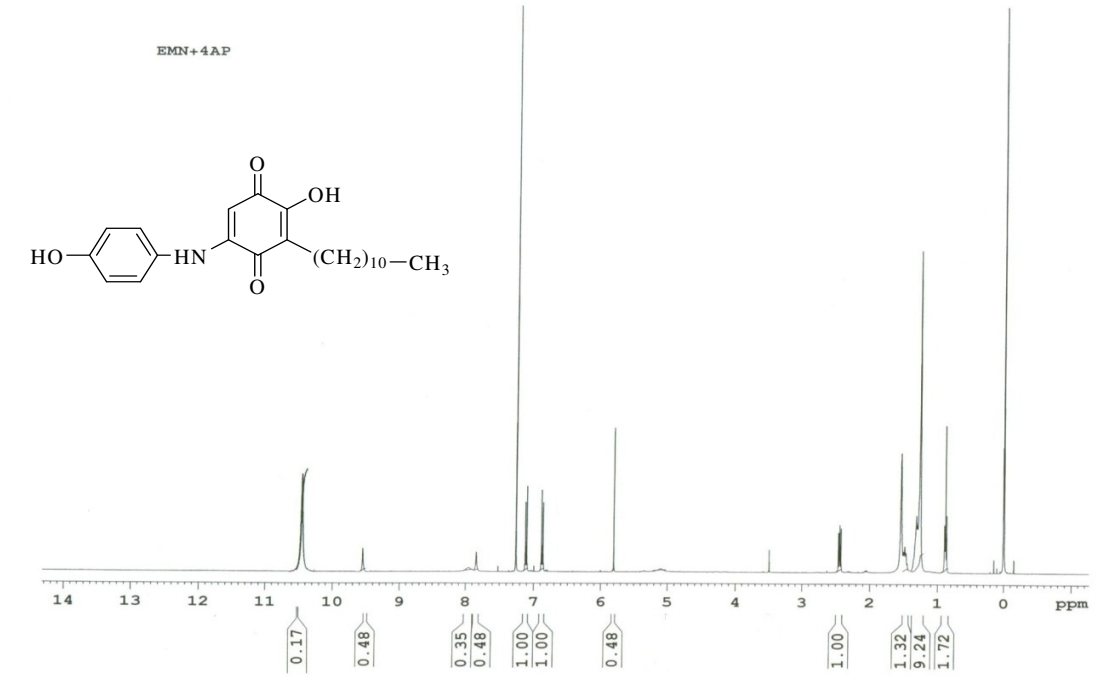


Fig. 44. ^{1}H NMR spectrum of compound 10

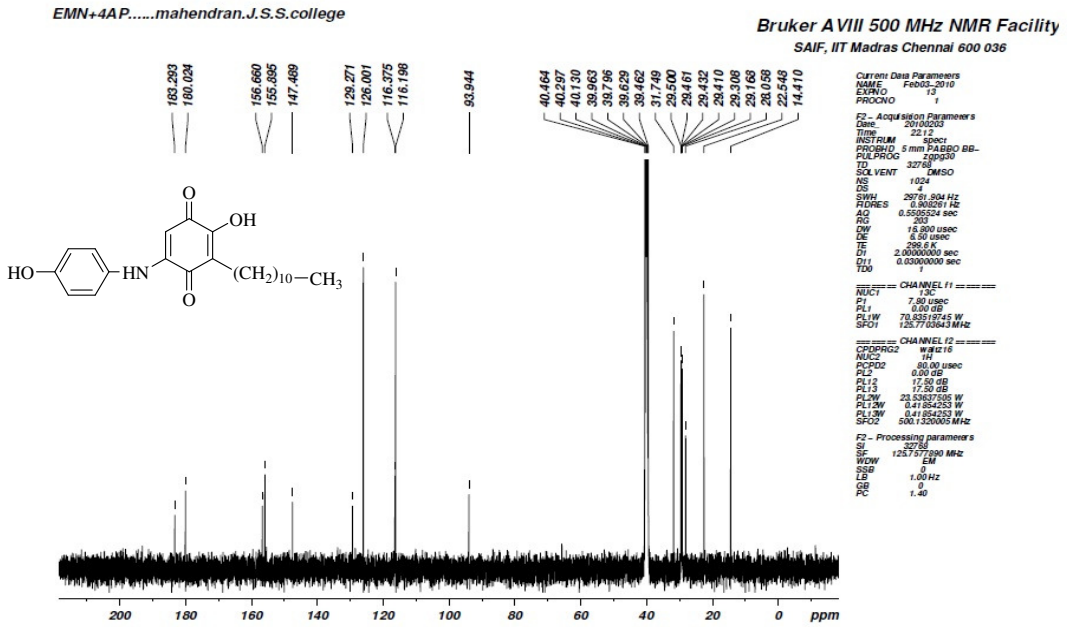


Fig. 45. ^{13}C NMR spectrum of compound 10

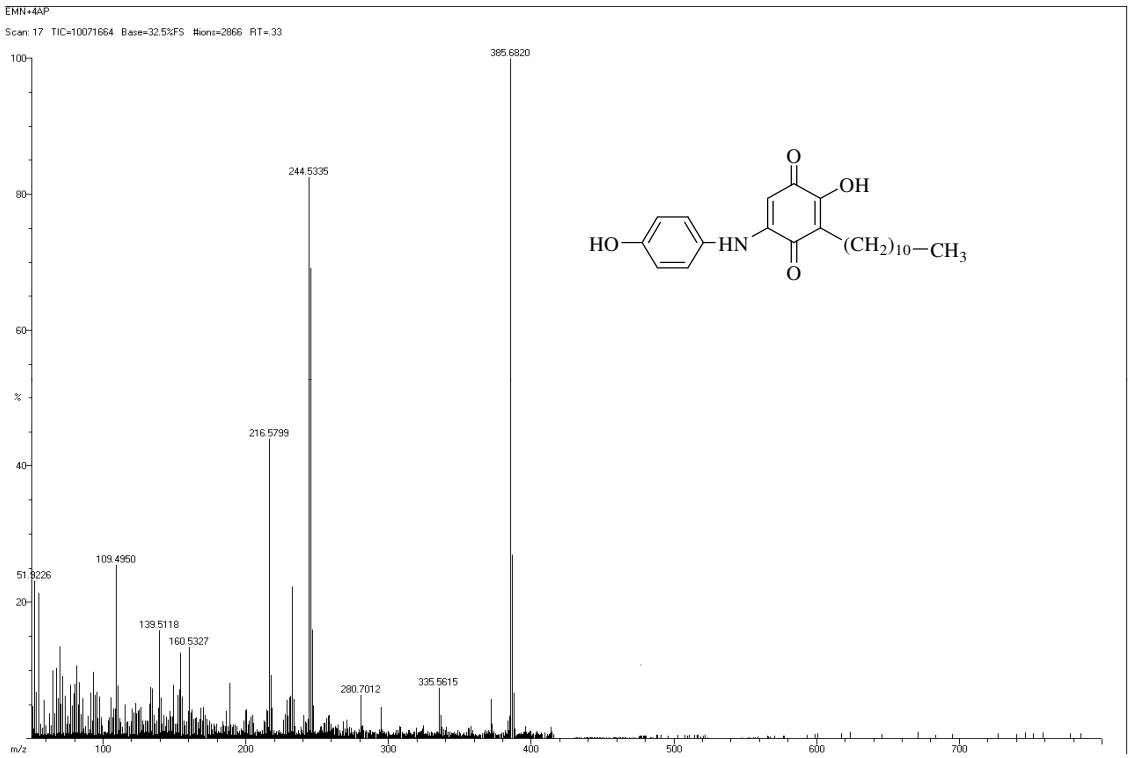


Fig. 46. Mass spectrum of compound 10

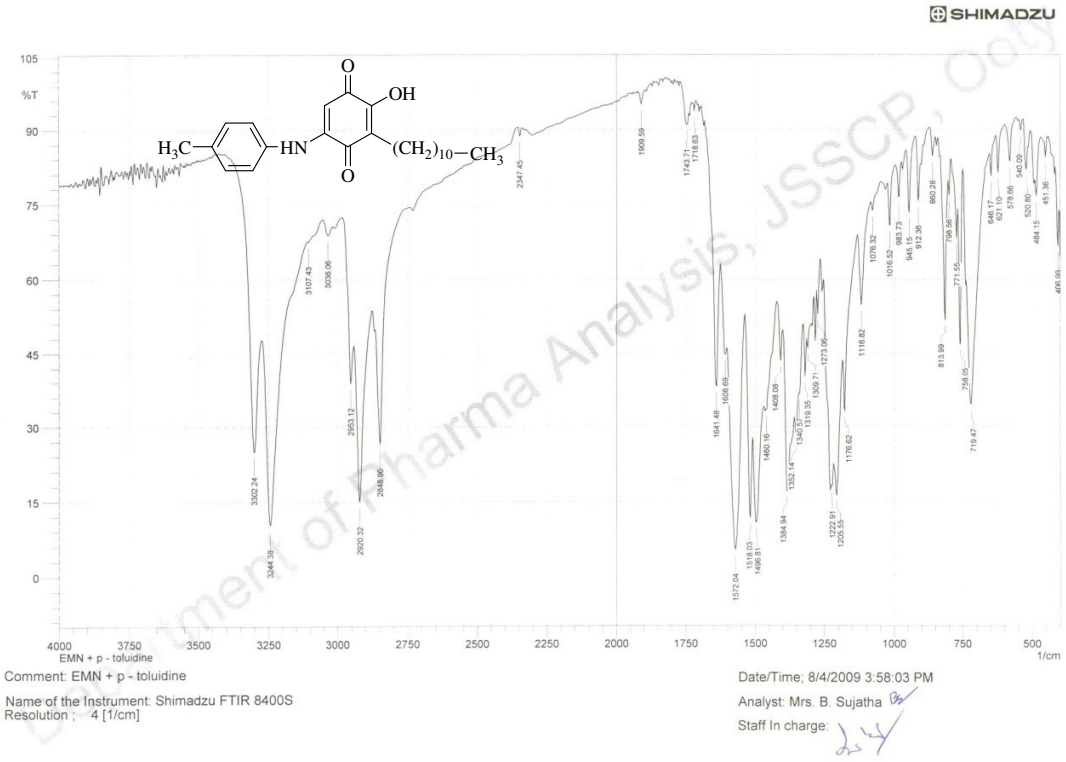


Fig. 47. IR spectrum of compound 11

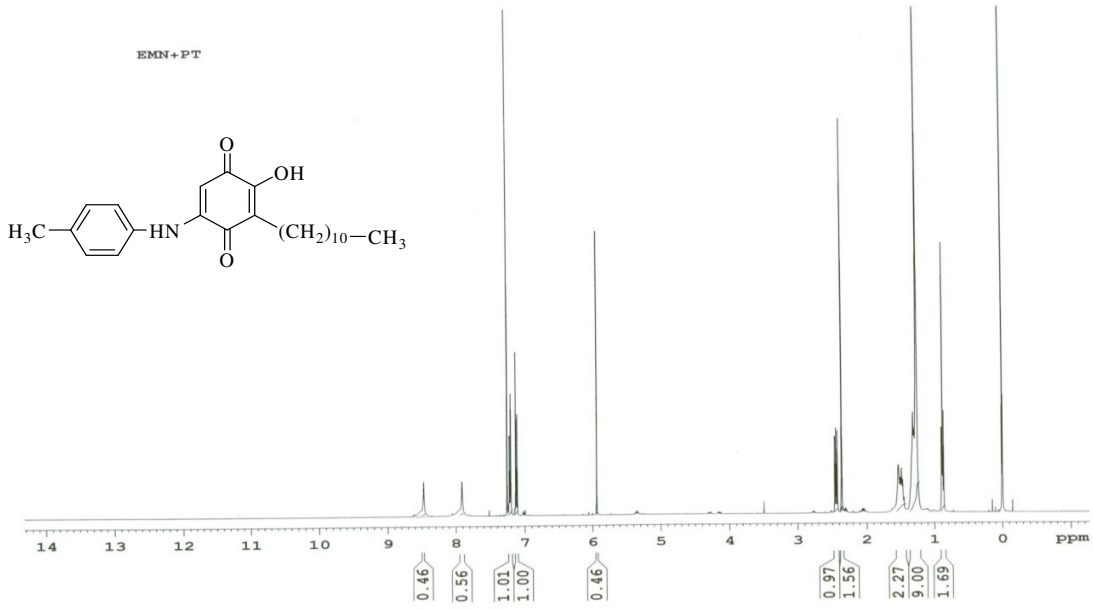


Fig. 48. ^{1}H NMR spectrum of compound 11

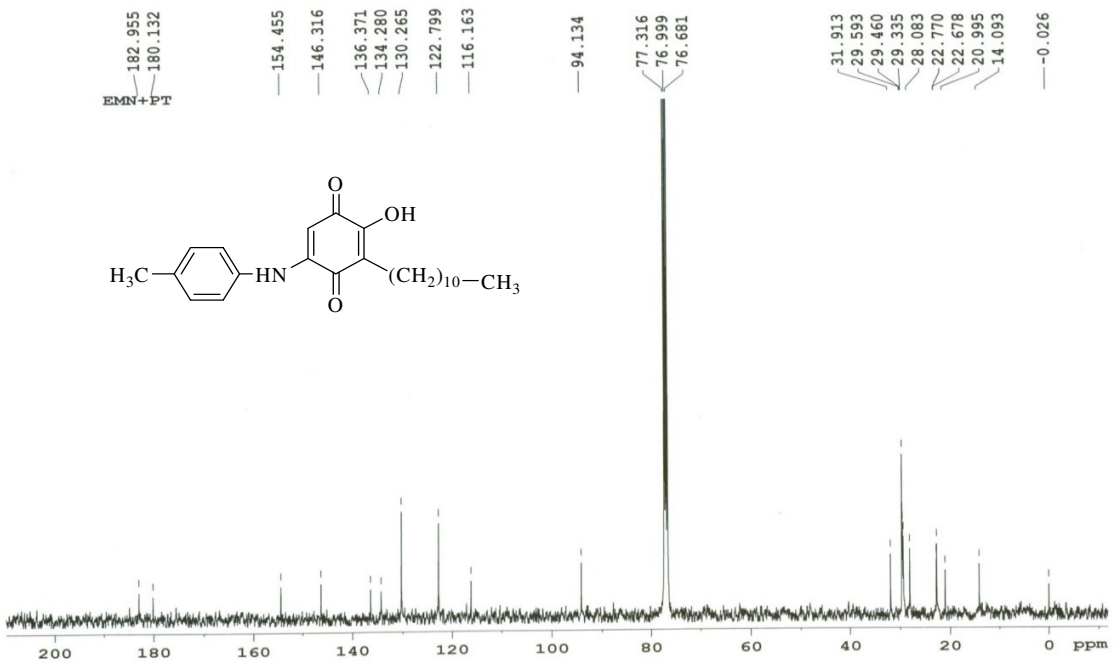


Fig. 49. ^{13}C NMR spectrum of compound 11

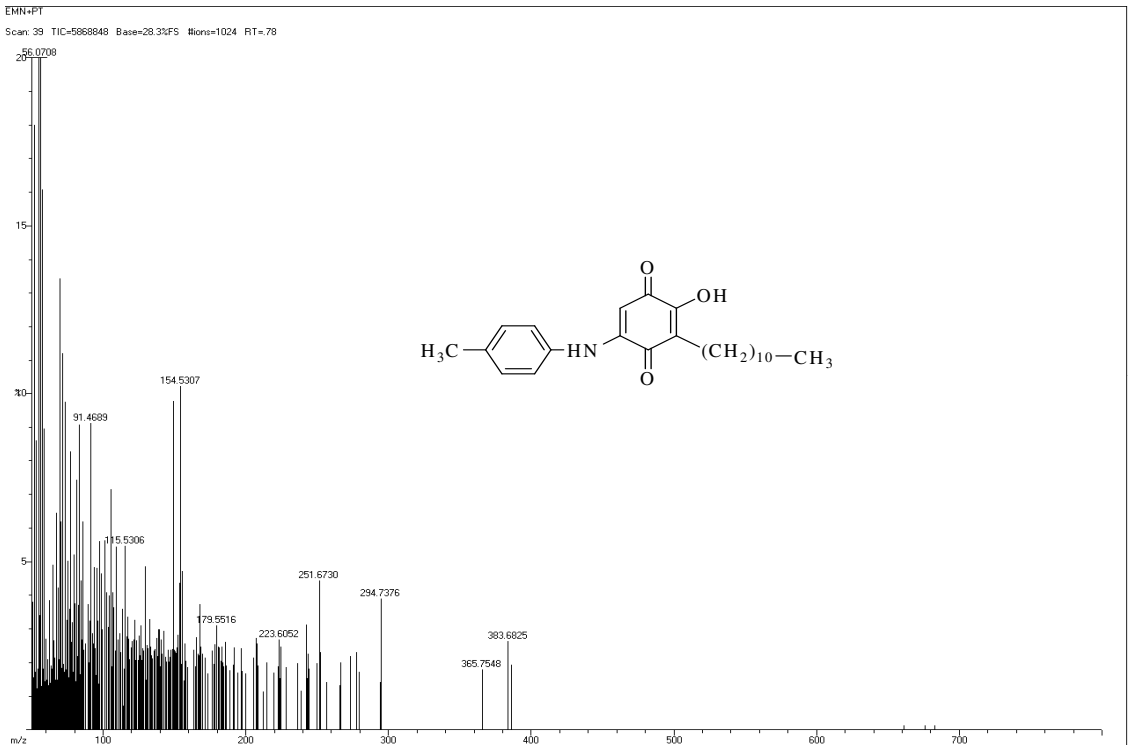


Fig. 50. Mass spectrum of compound 11

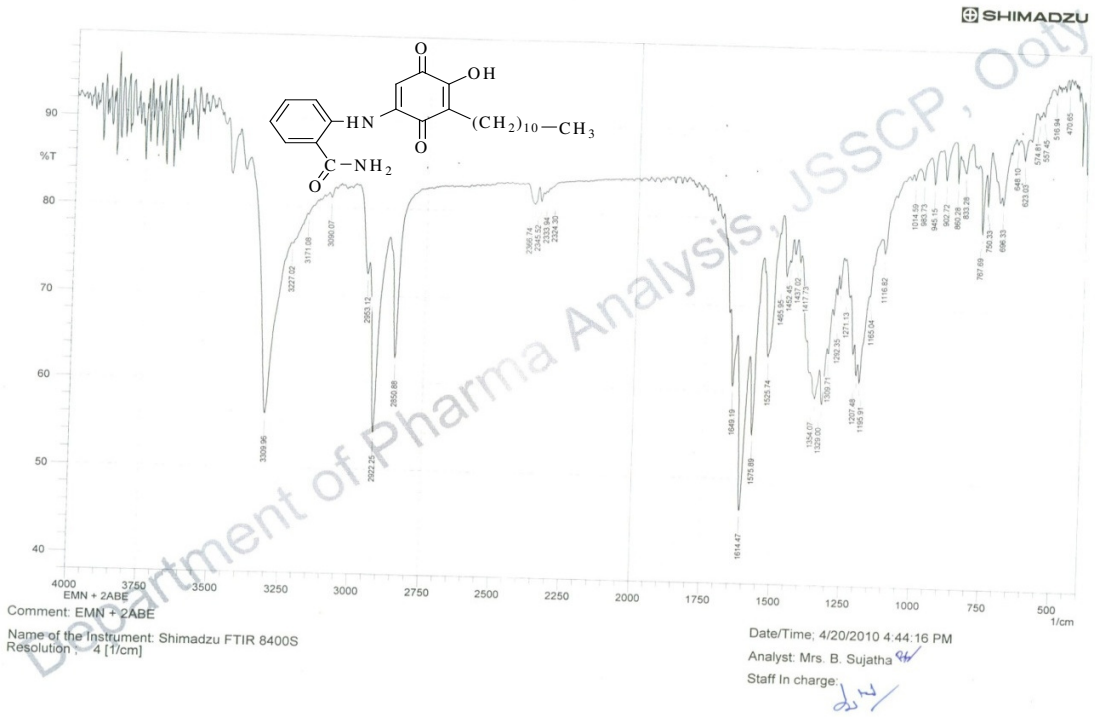


Fig. 51. IR spectrum of compound 12

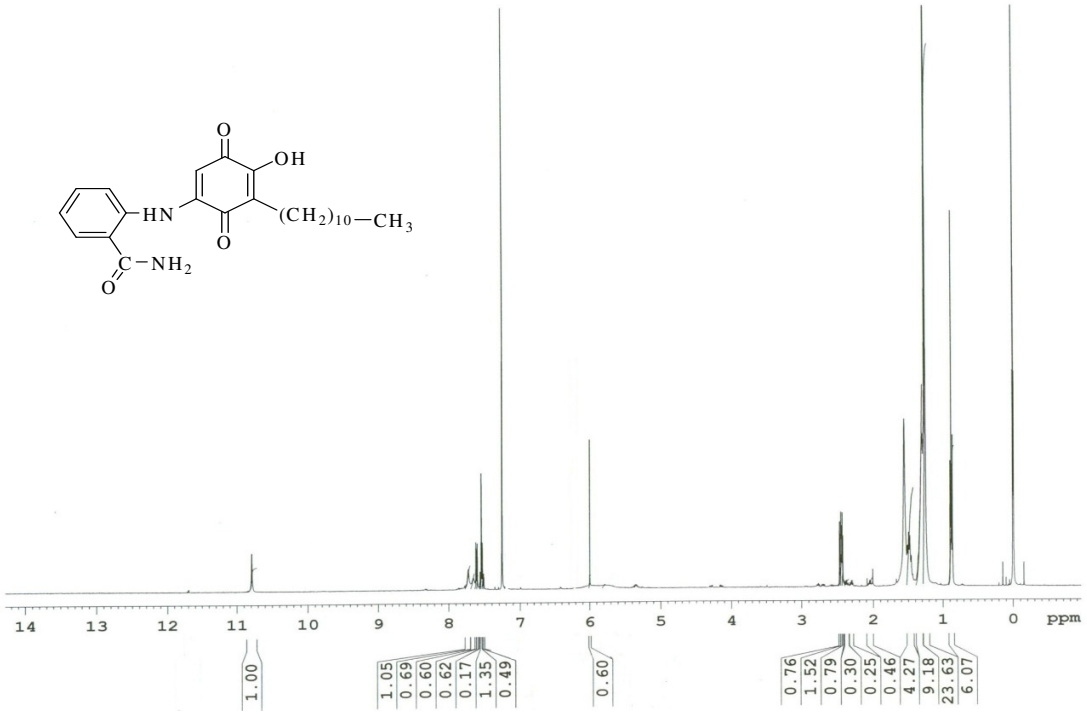


Fig. 52. ^{1}H NMR spectrum of compound 12

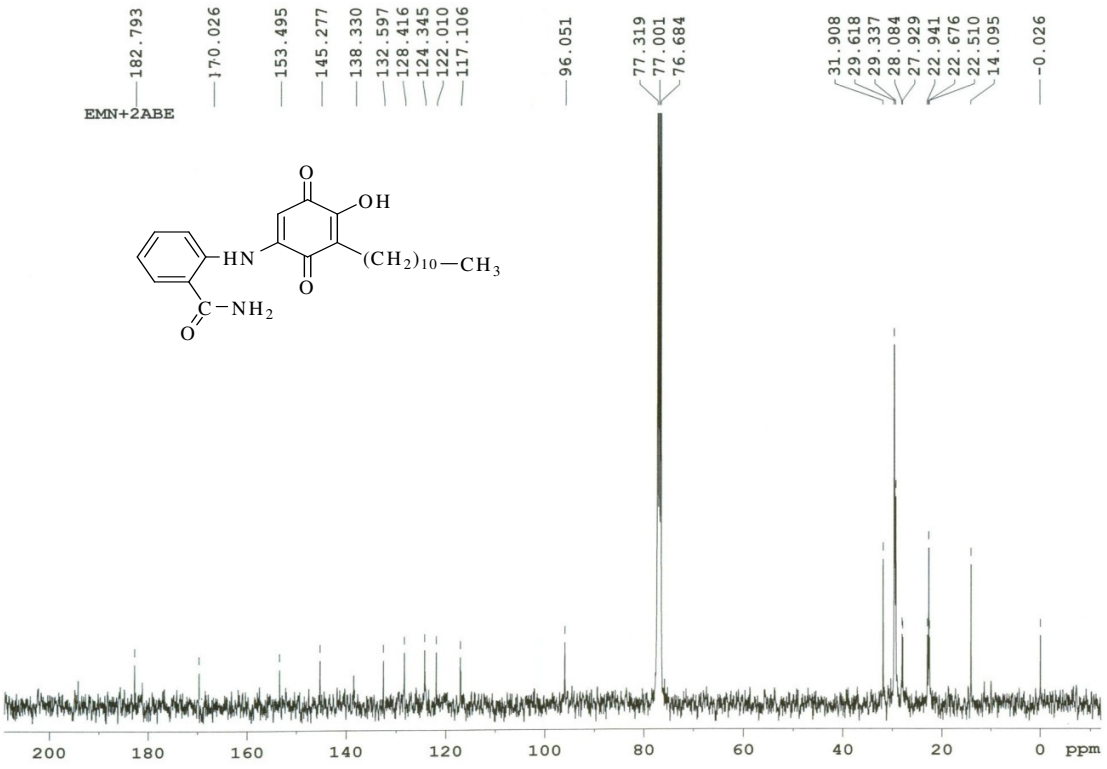


Fig. 53. ^{13}C NMR spectrum of compound 12

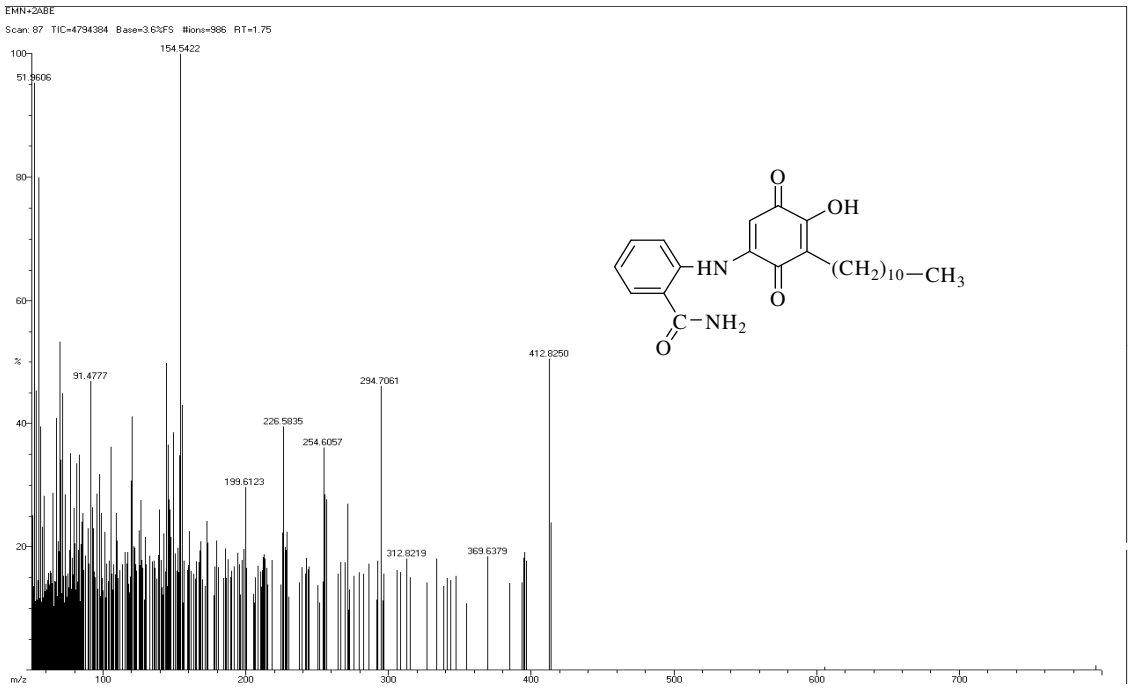


Fig. 54. Mass spectrum of compound 12

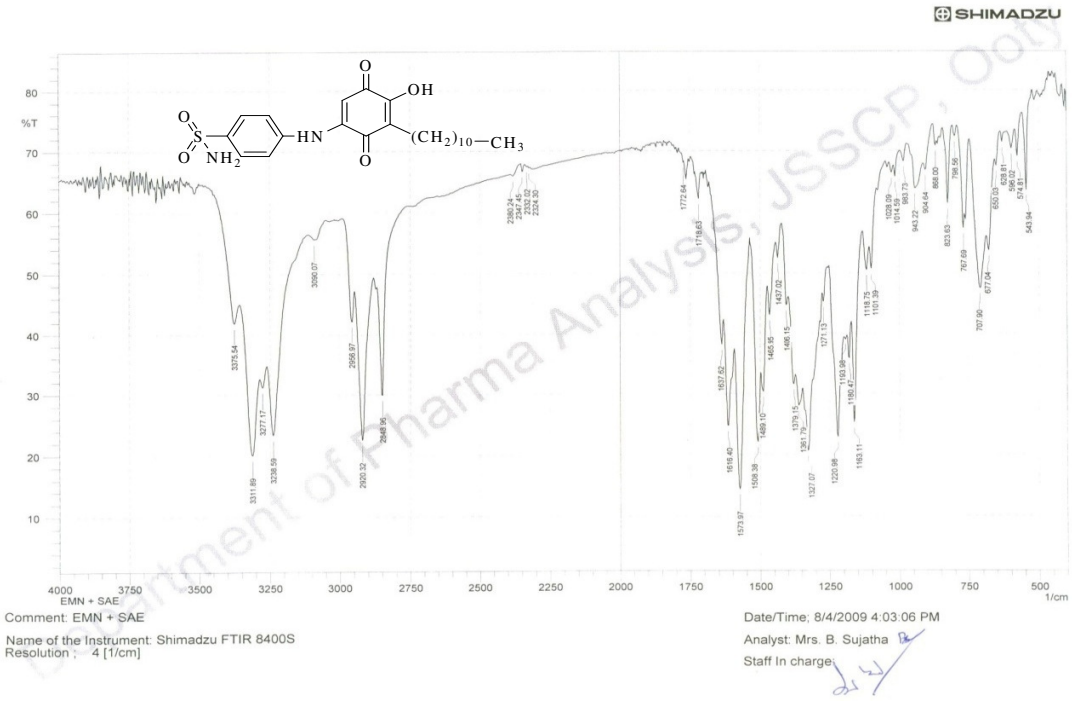


Fig. 55. IR spectrum of compound 13

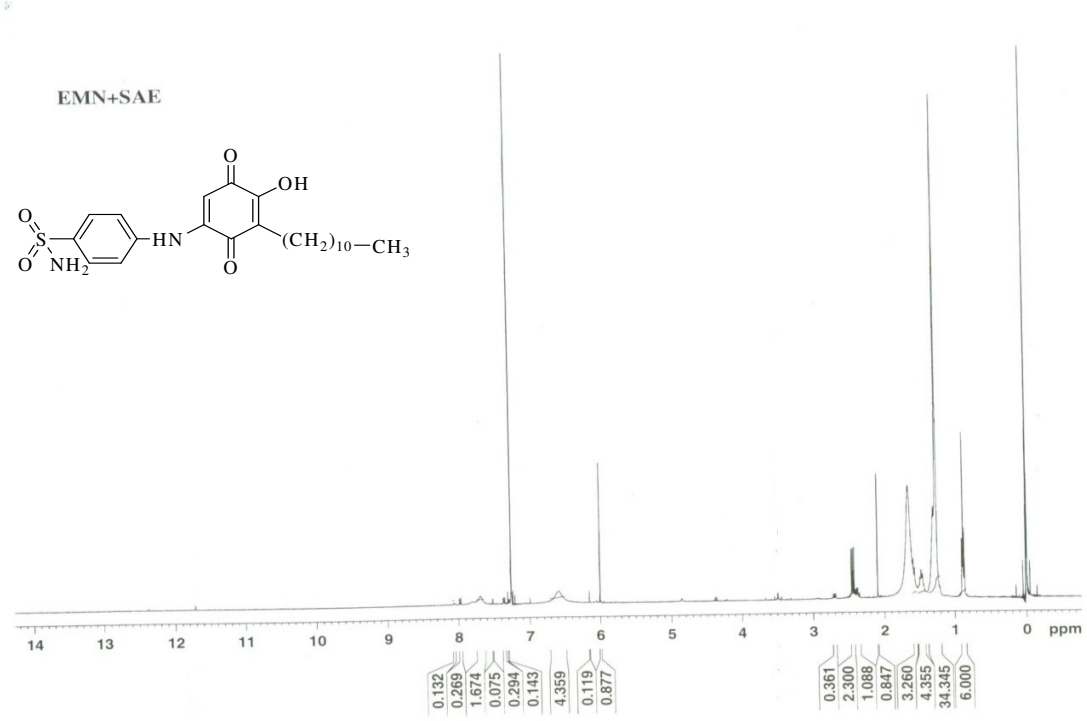


Fig. 56. ^{1}H NMR spectrum of compound 13

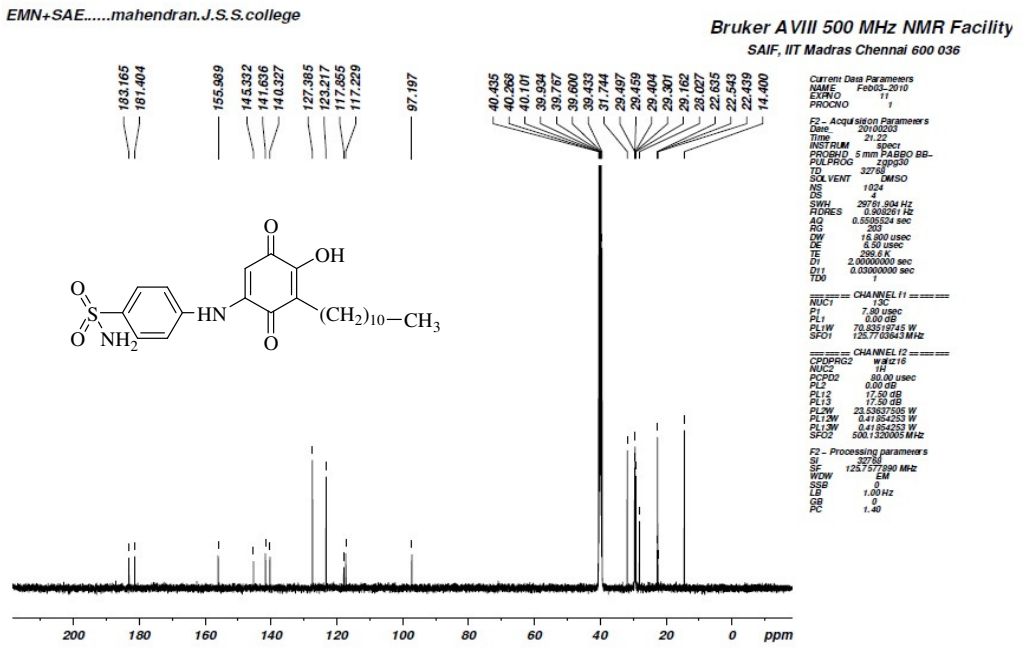


Fig. 57. ^{13}C NMR spectrum of compound 13

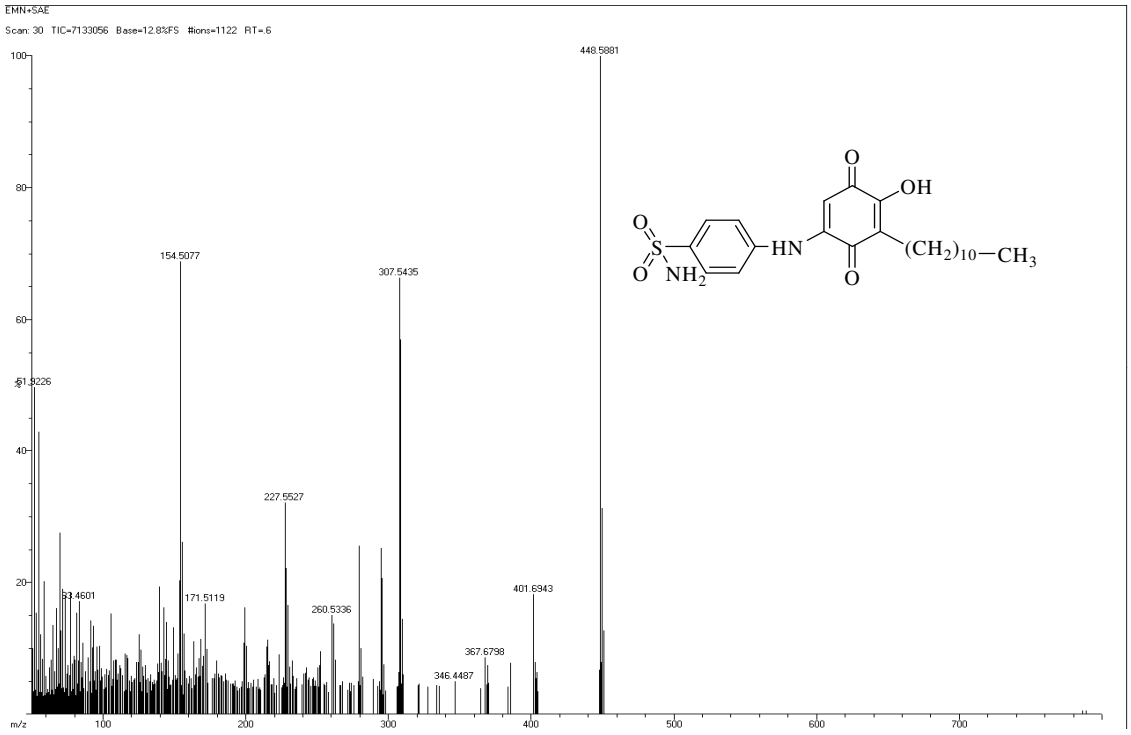


Fig. 58. Mass spectrum of compound 13

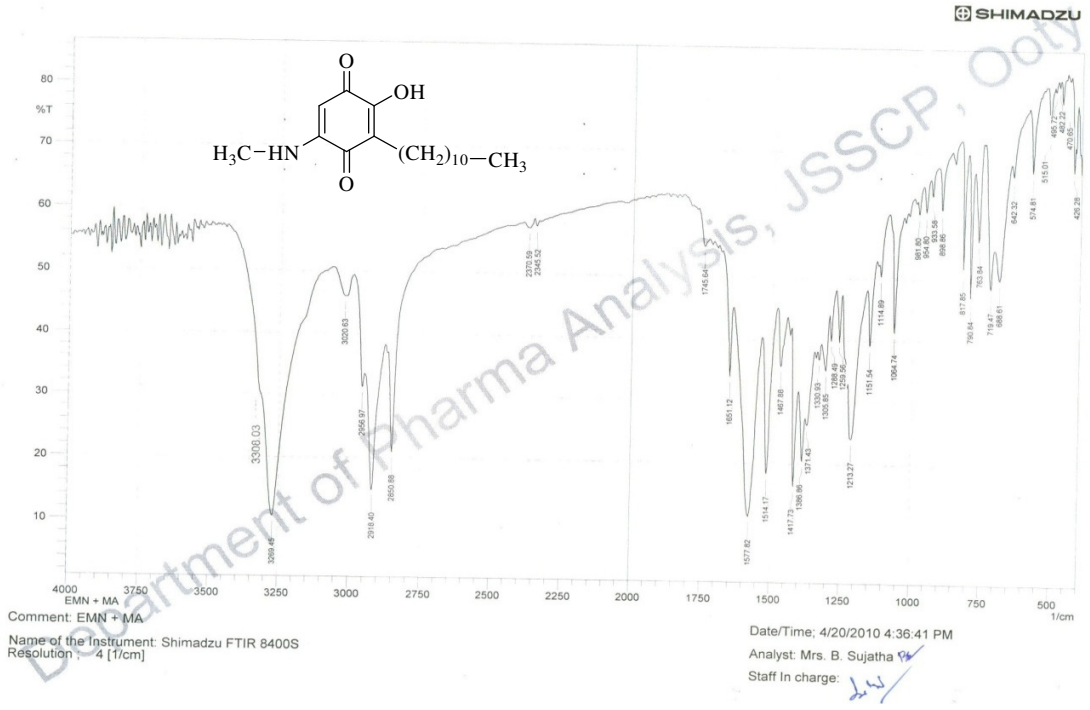


Fig. 59. IR spectrum of compound 14

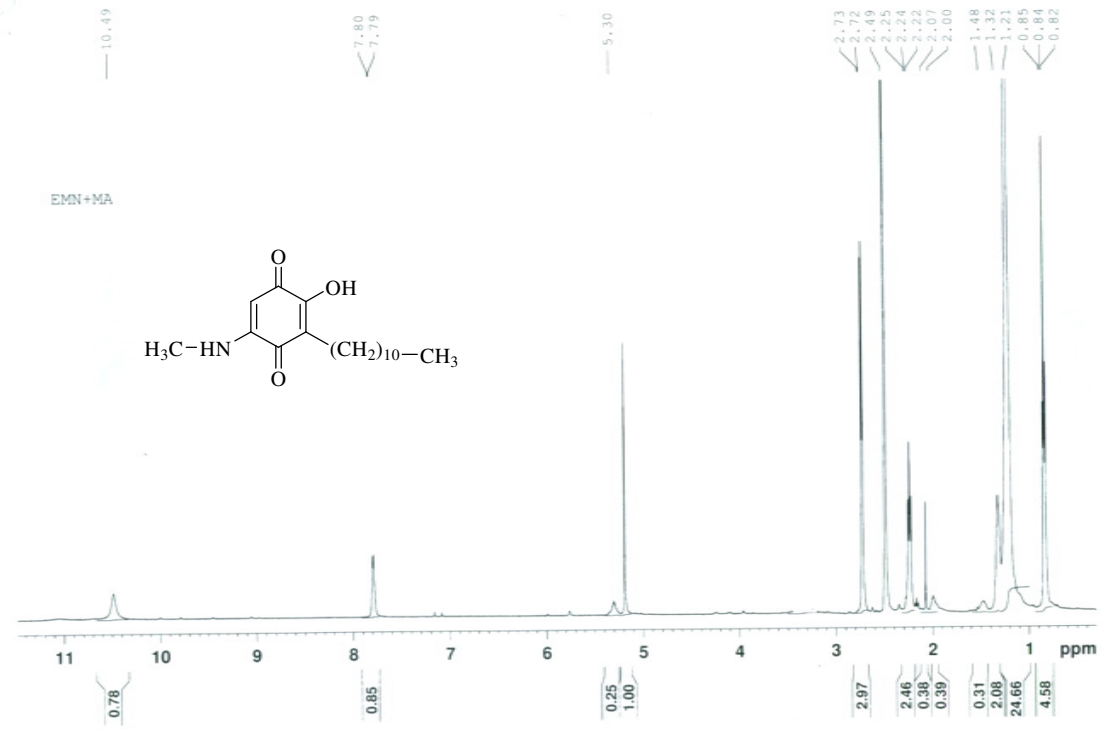


Fig. 60. ^{1}H NMR spectrum of compound 14

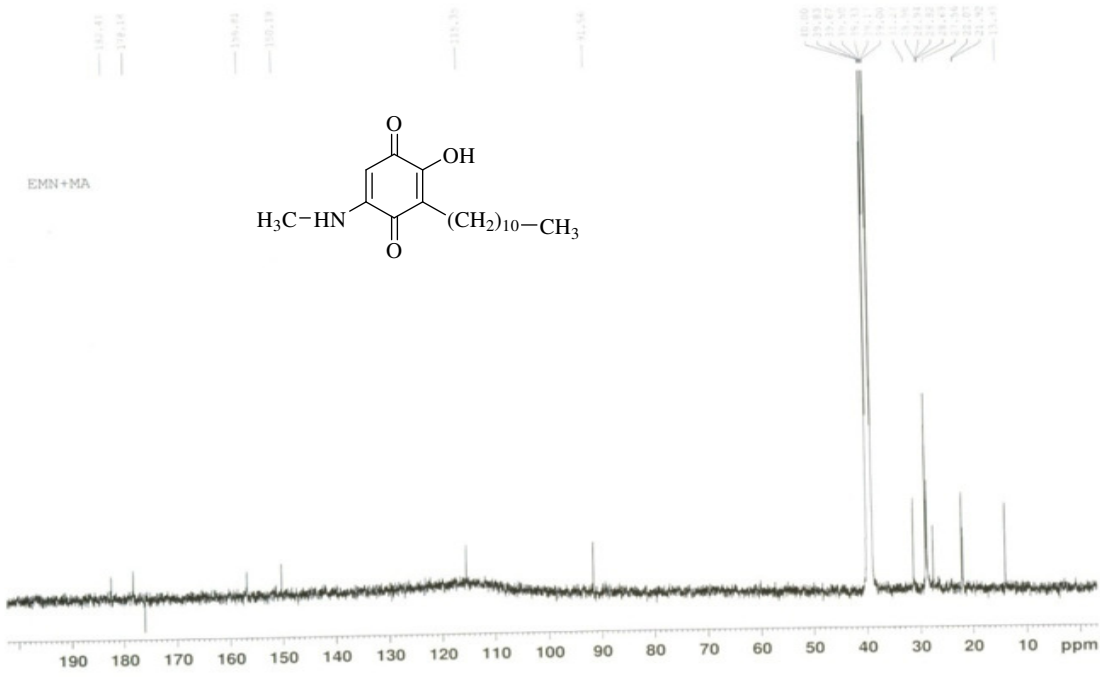


Fig. 61. ^{13}C NMR spectrum of compound 14

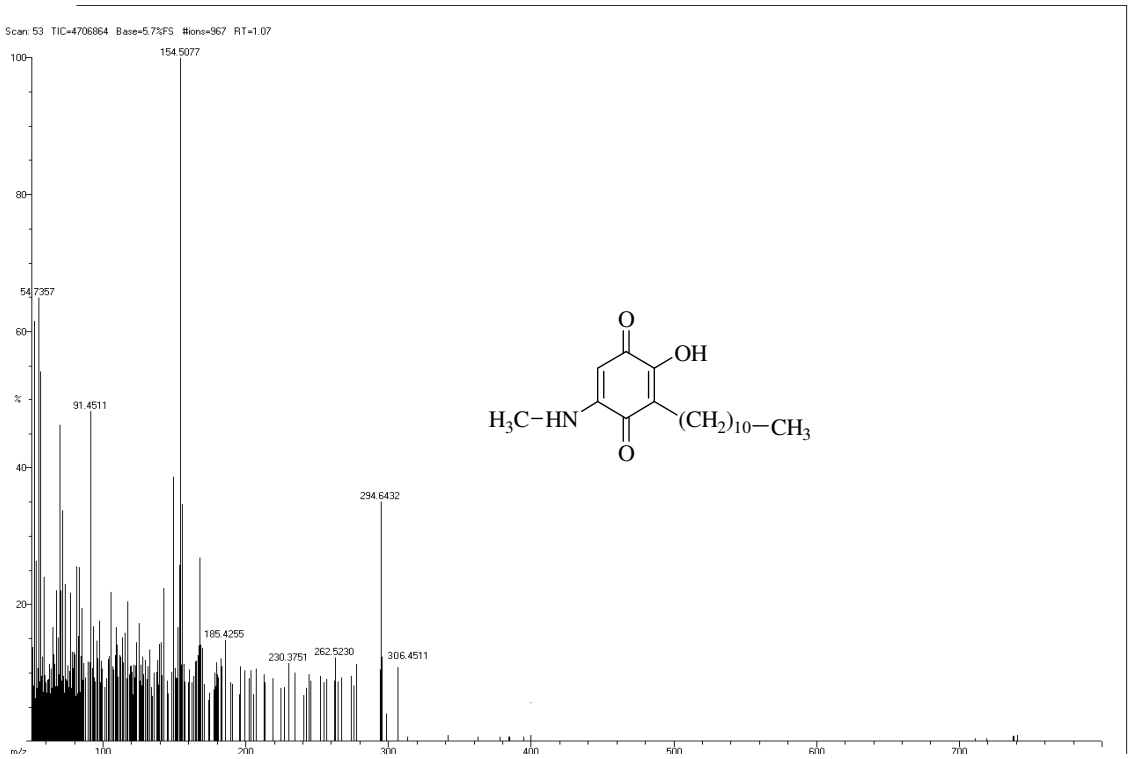


Fig. 62. Mass spectrum of compound 14

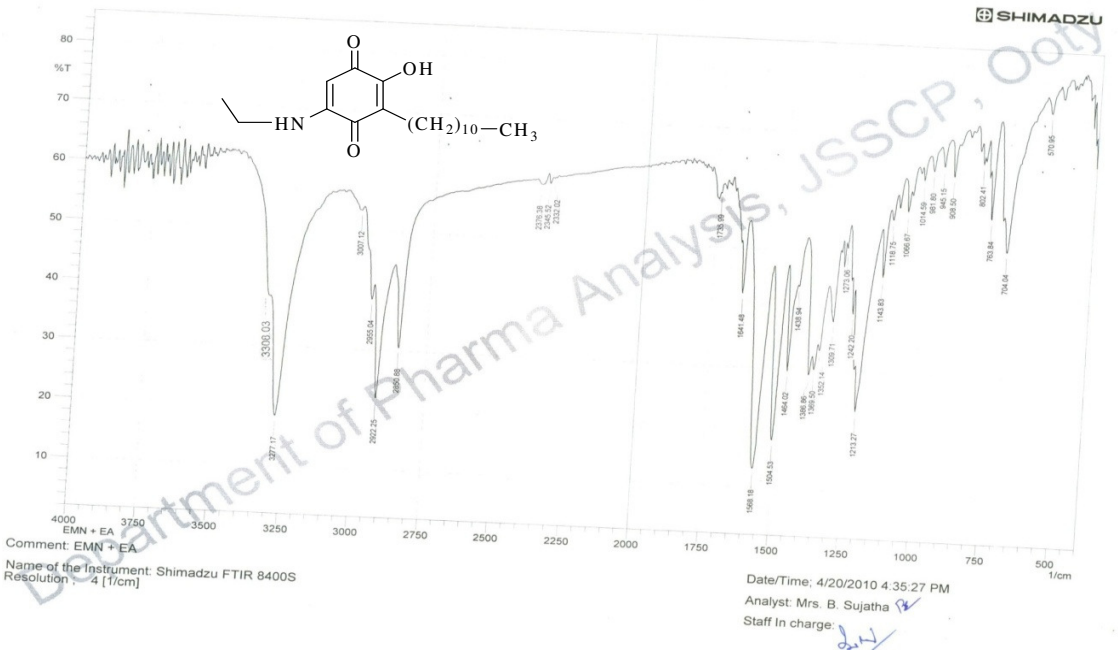


Fig. 63. IR spectrum of compound 15

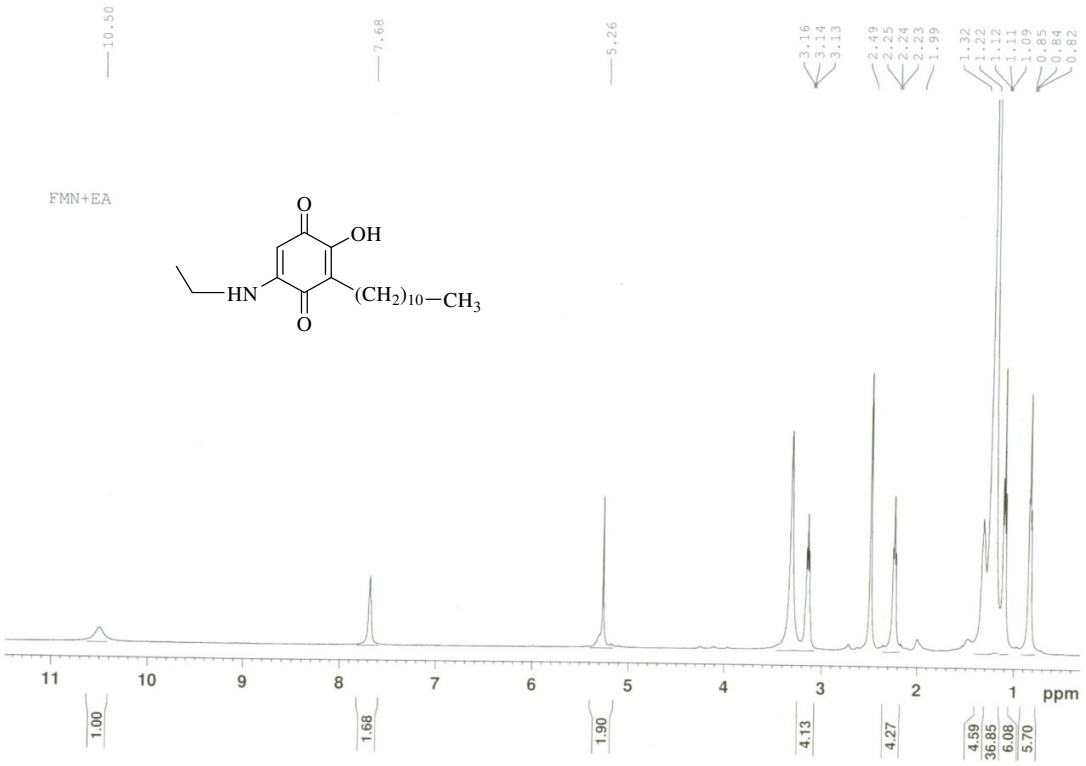


Fig. 64. ^{1}H NMR spectrum of compound 15

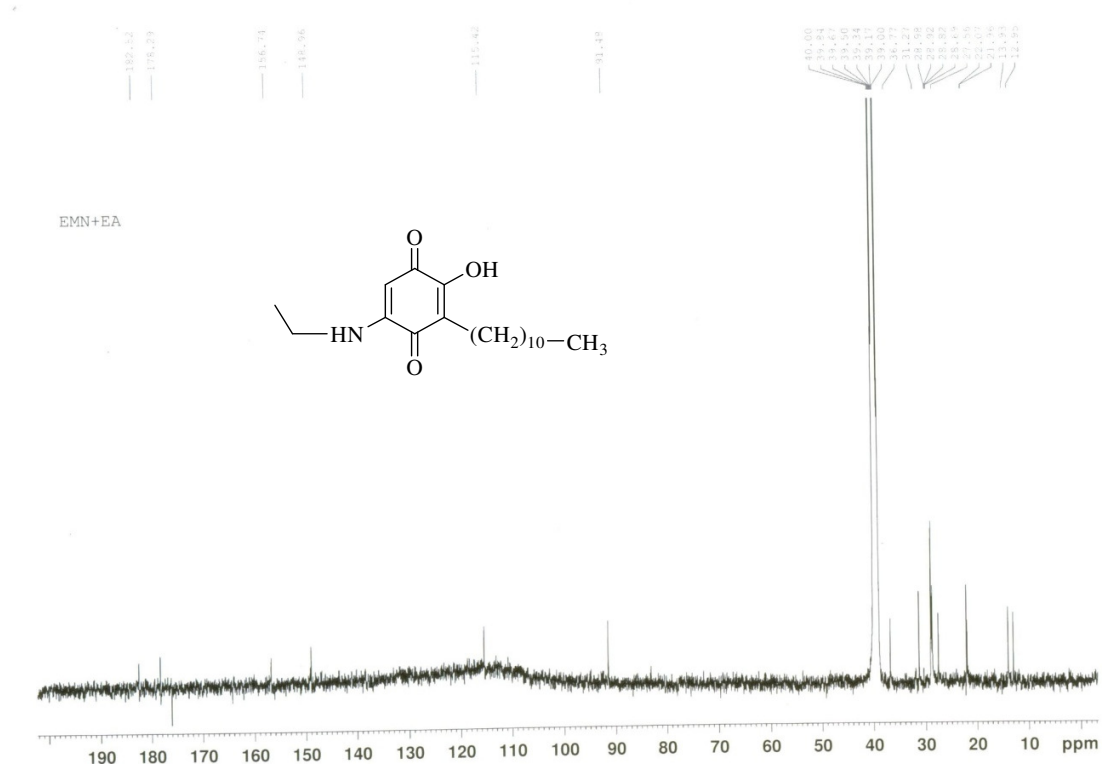


Fig. 65. ^{13}C NMR spectrum of compound 15

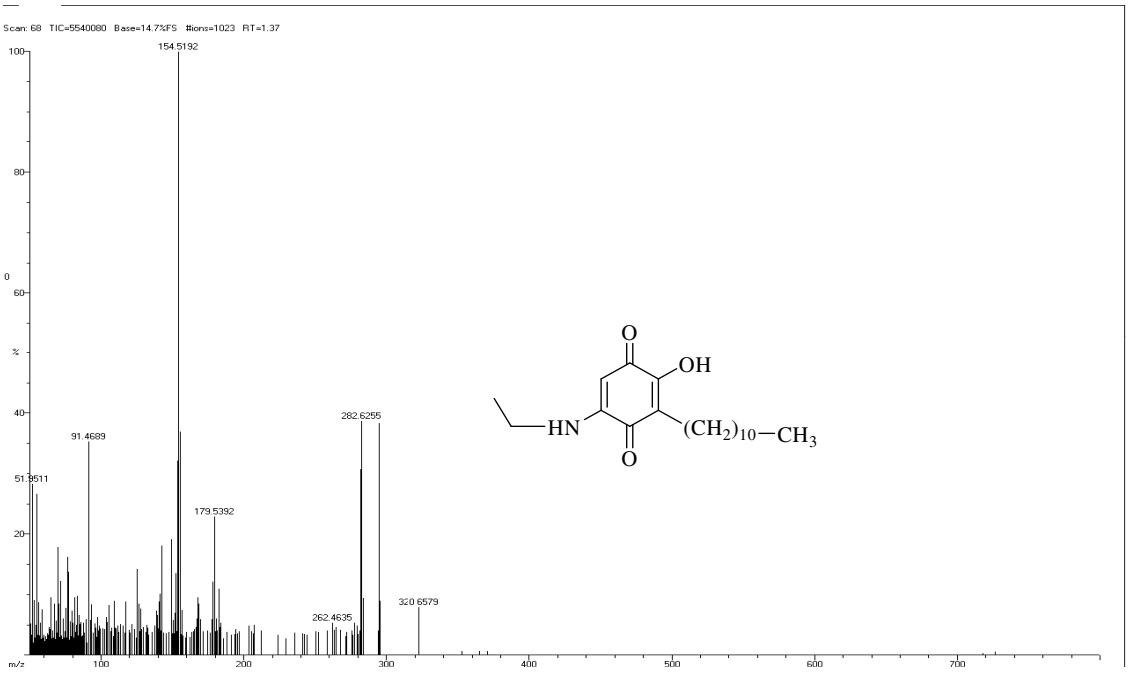


Fig. 66. Mass spectrum of compound 15

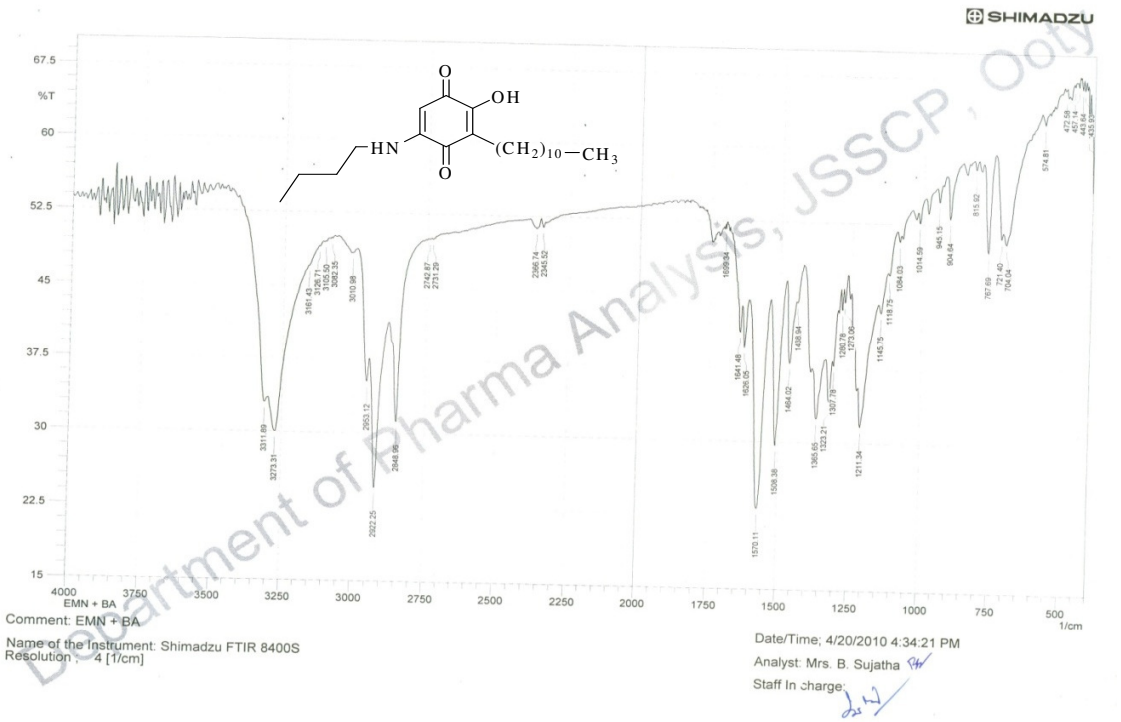


Fig. 67. IR spectrum of compound 16

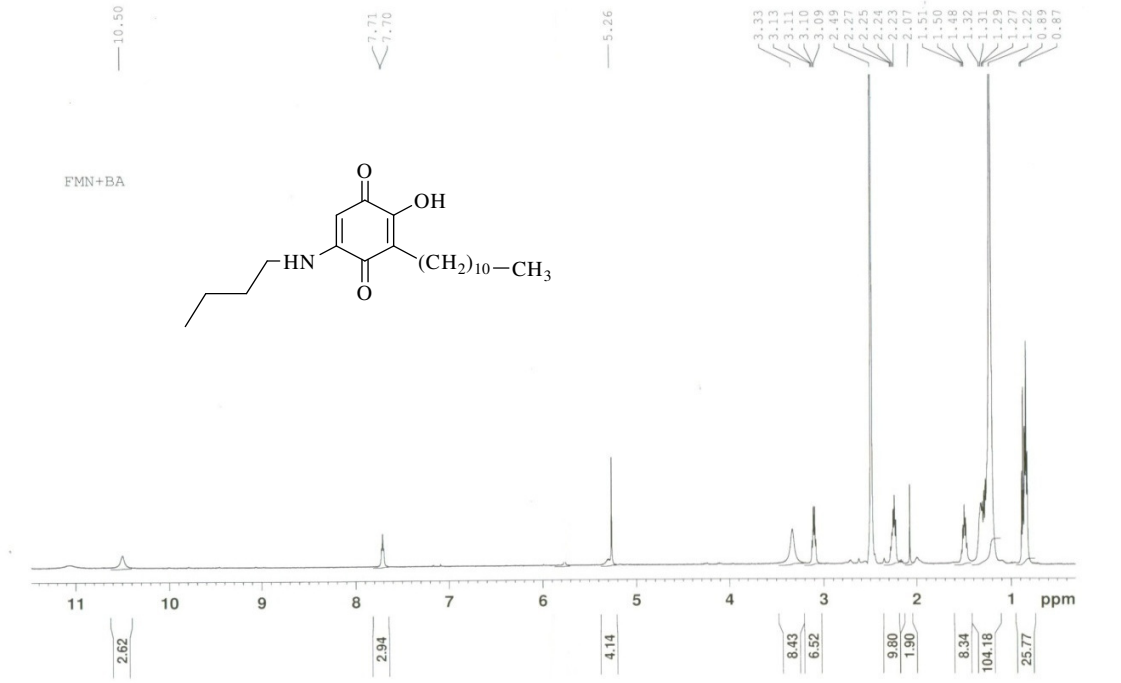


Fig. 68. ^{1}H NMR spectrum of compound 16

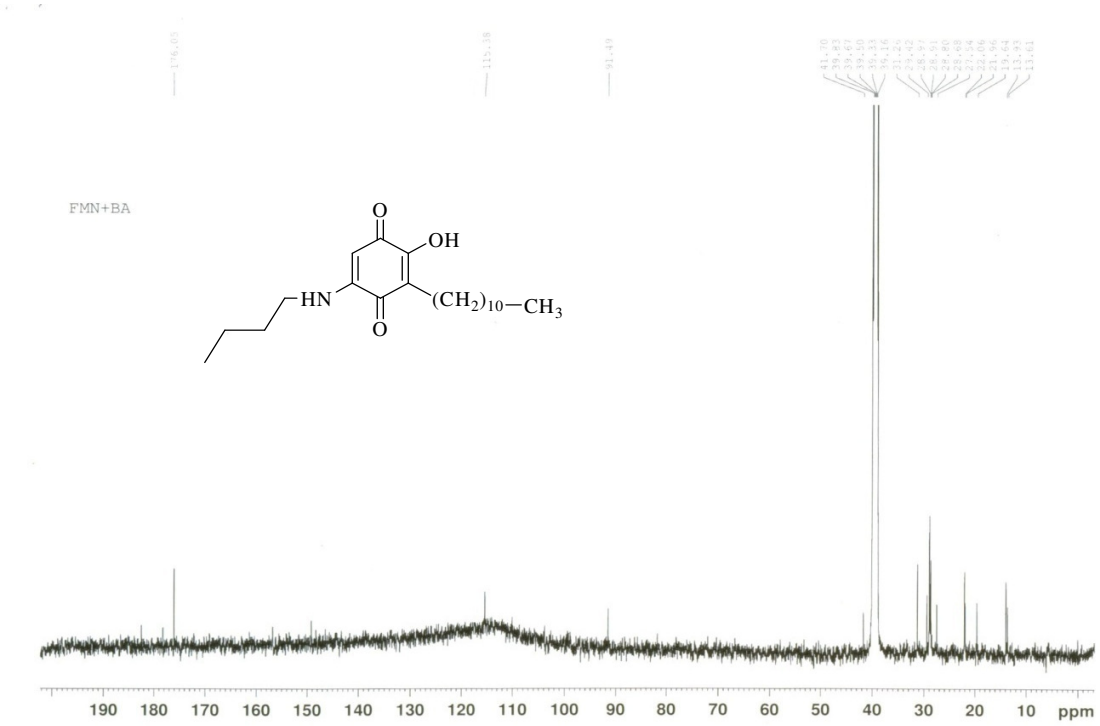


Fig. 69. ^{13}C NMR spectrum of compound 16

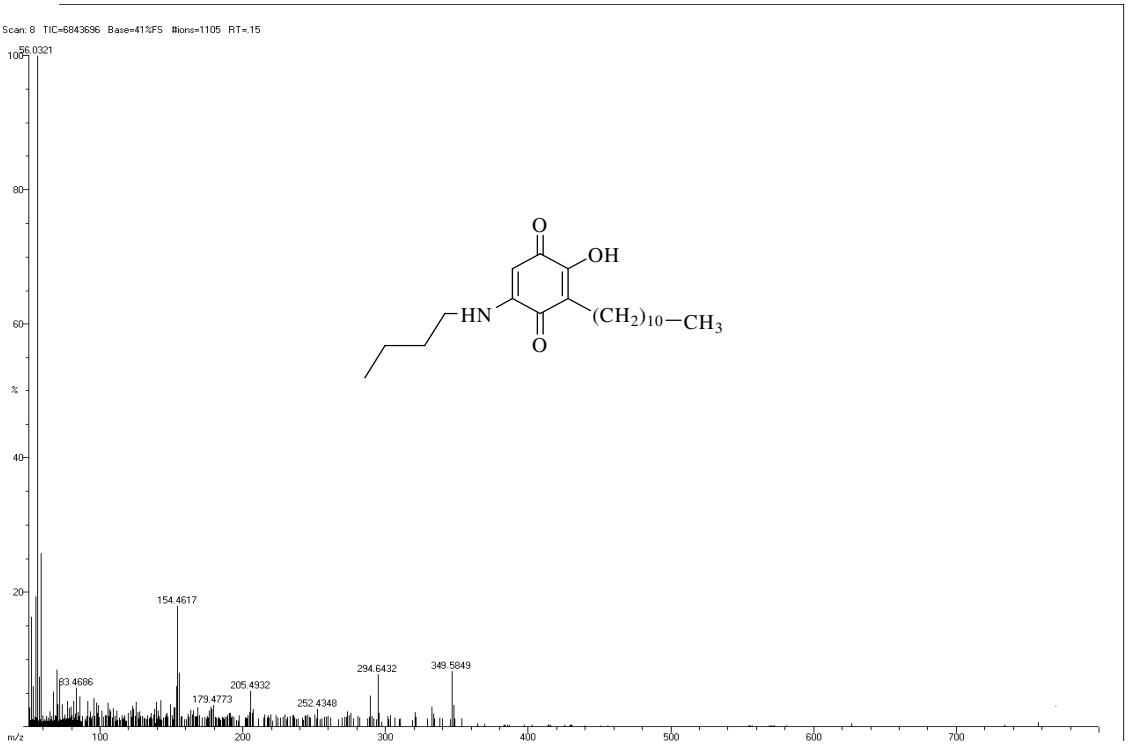


Fig. 70. Mass spectrum of compound 16

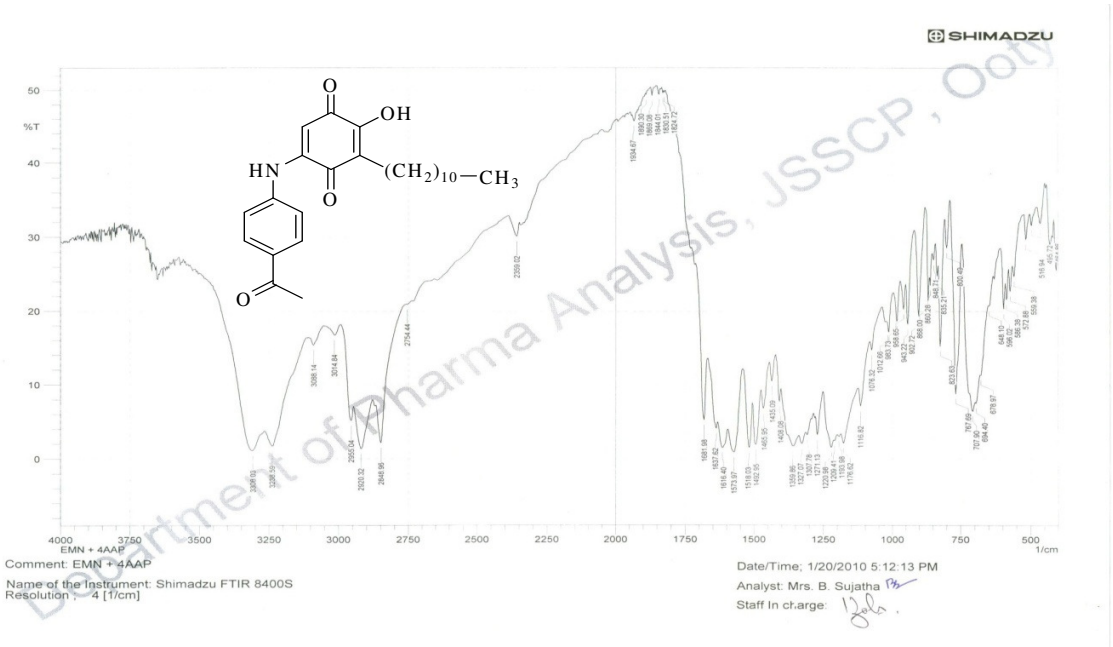


Fig. 71. IR spectrum of compound 17

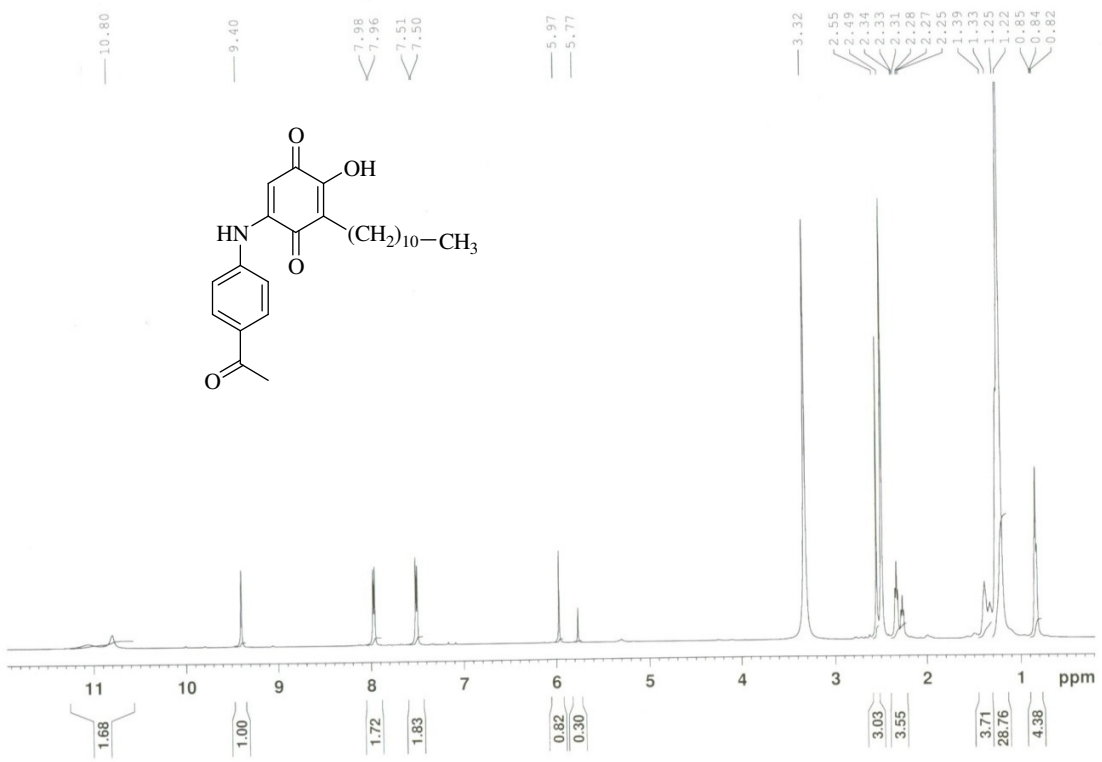


Fig. 72. ^{1}H NMR spectrum of compound 17

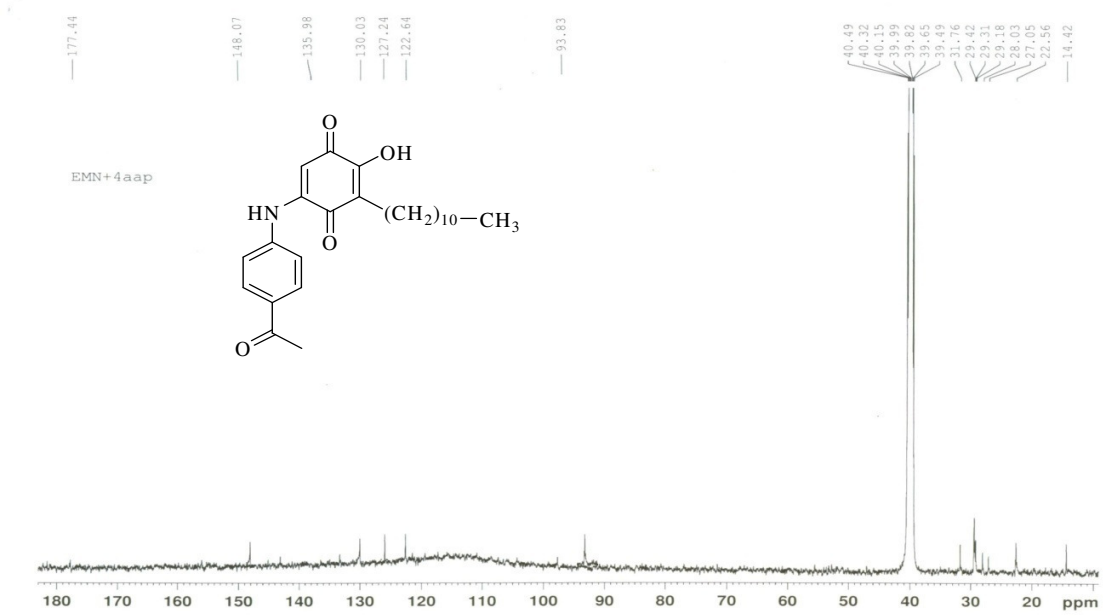


Fig. 73. ^{13}C NMR spectrum of compound 17

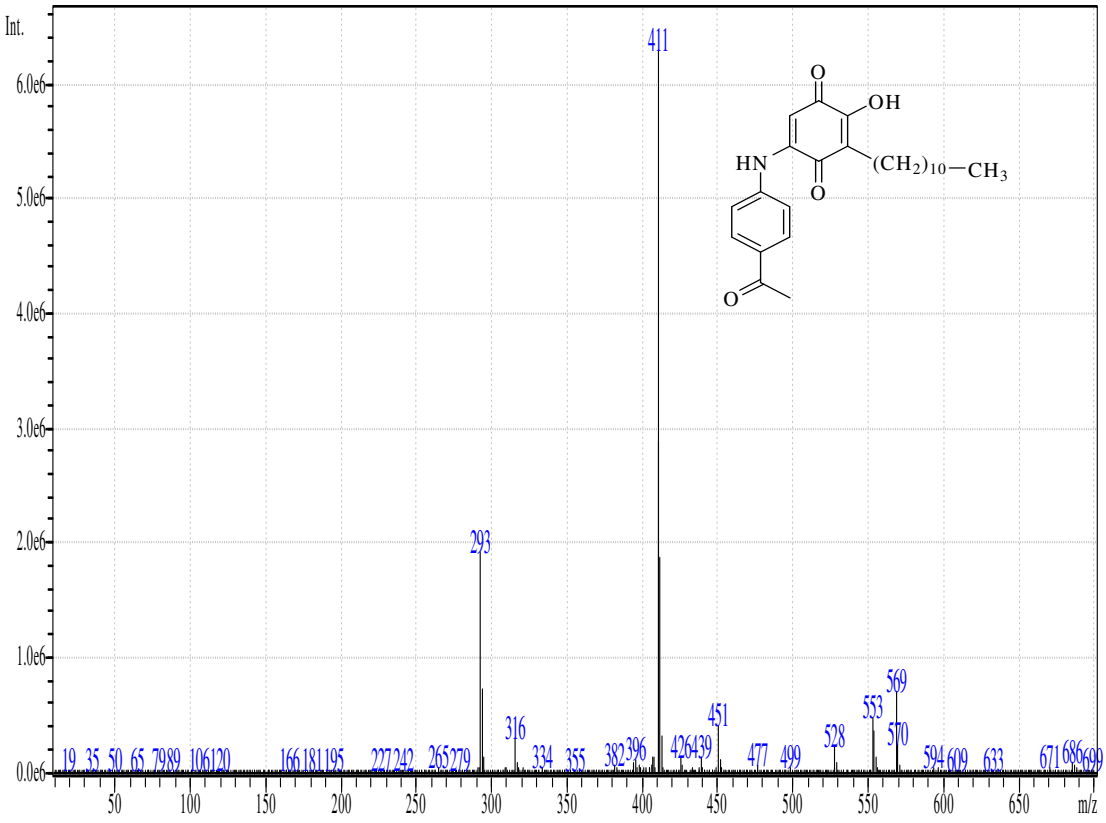


Fig. 74. Mass spectrum of compound 17

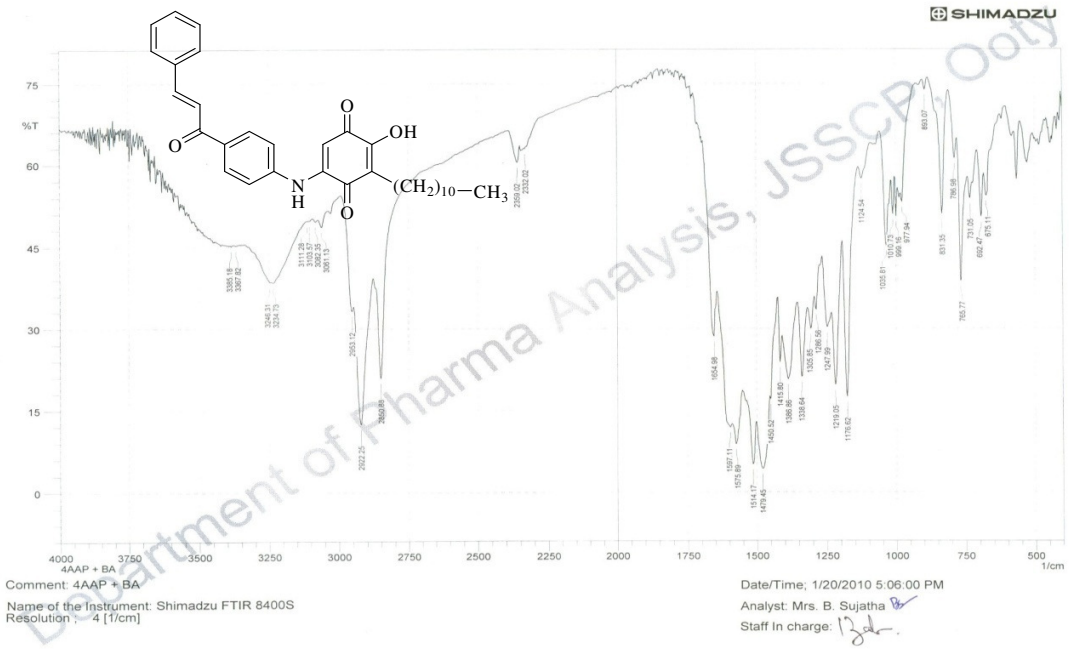


Fig. 75. IR spectrum of compound 18

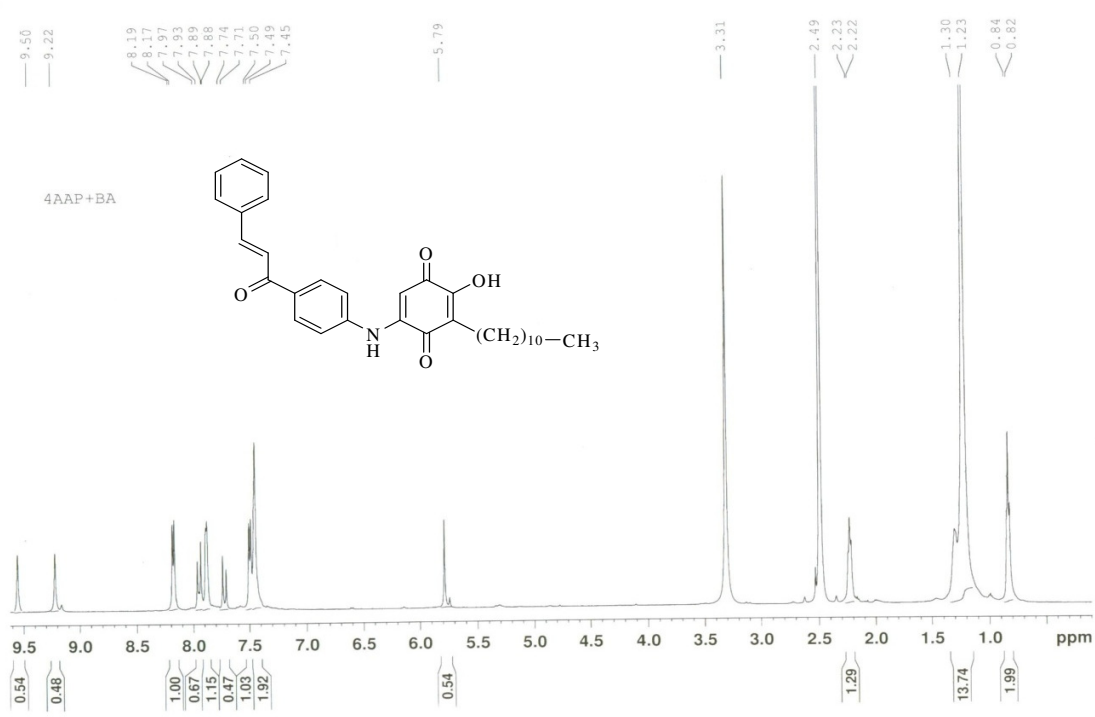


Fig. 76. ^{1}H NMR spectrum of compound 18

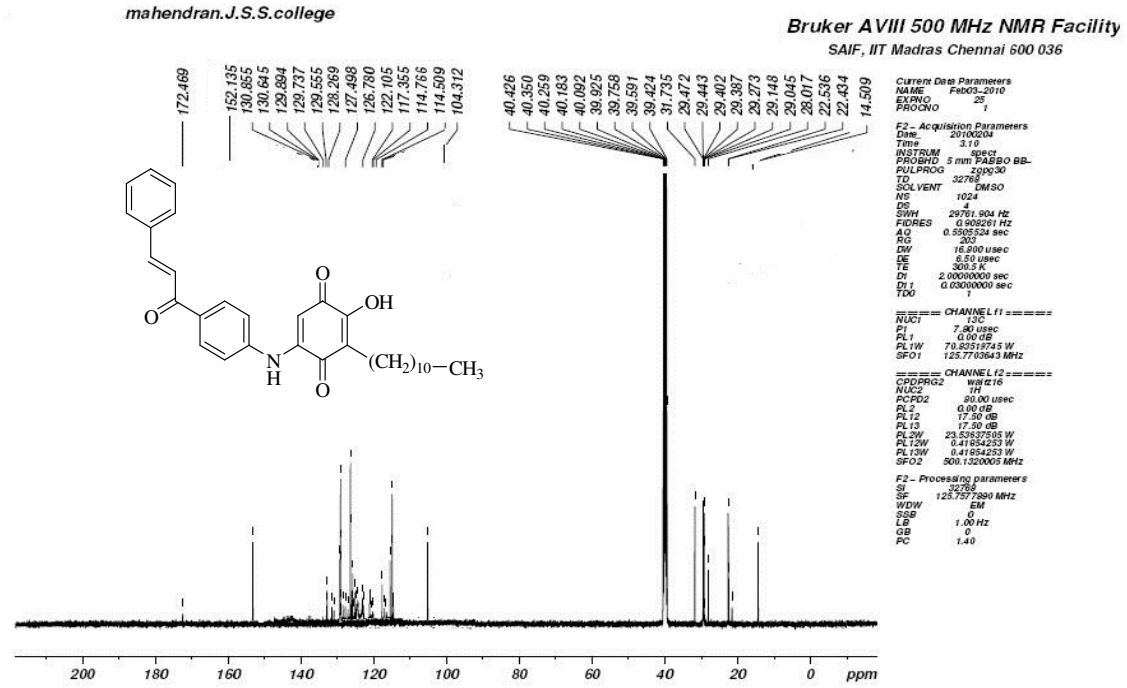


Fig. 77. ^{13}C NMR spectrum of compound 18

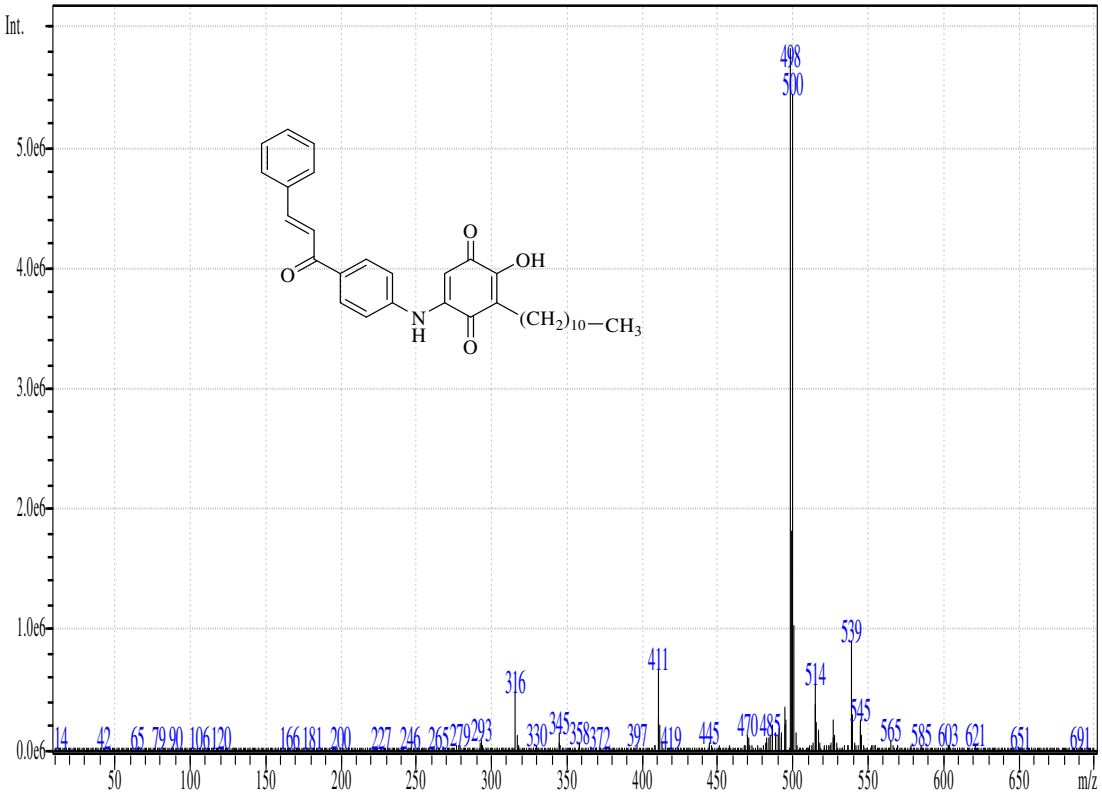


Fig. 78. Mass spectrum of compound 18

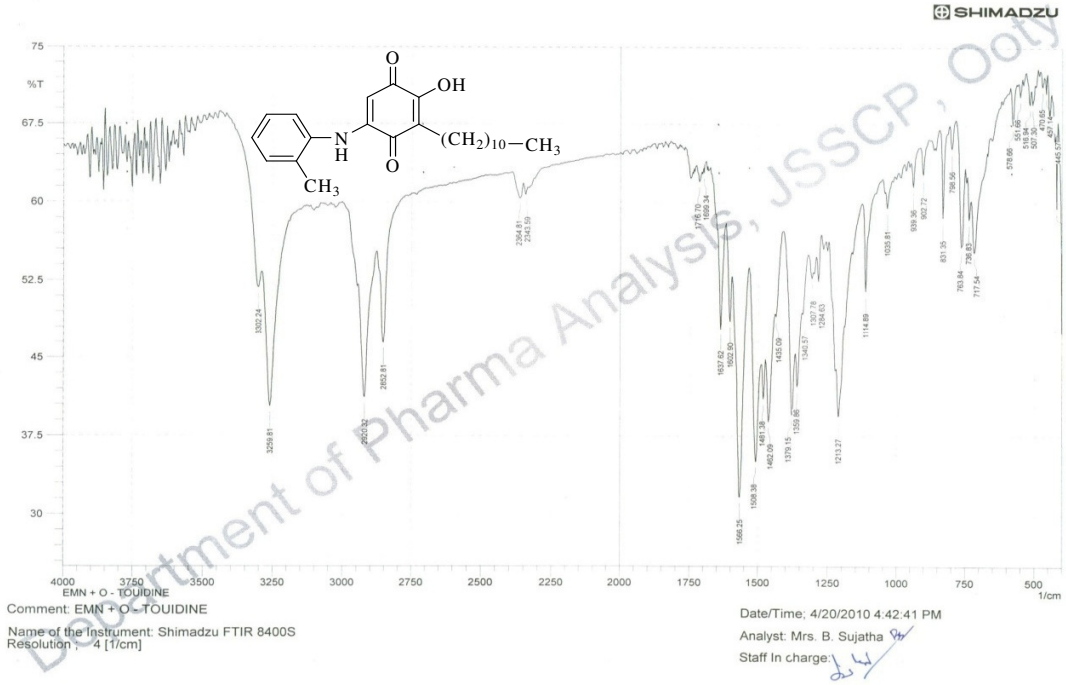


Fig. 79. IR spectrum of compound 19

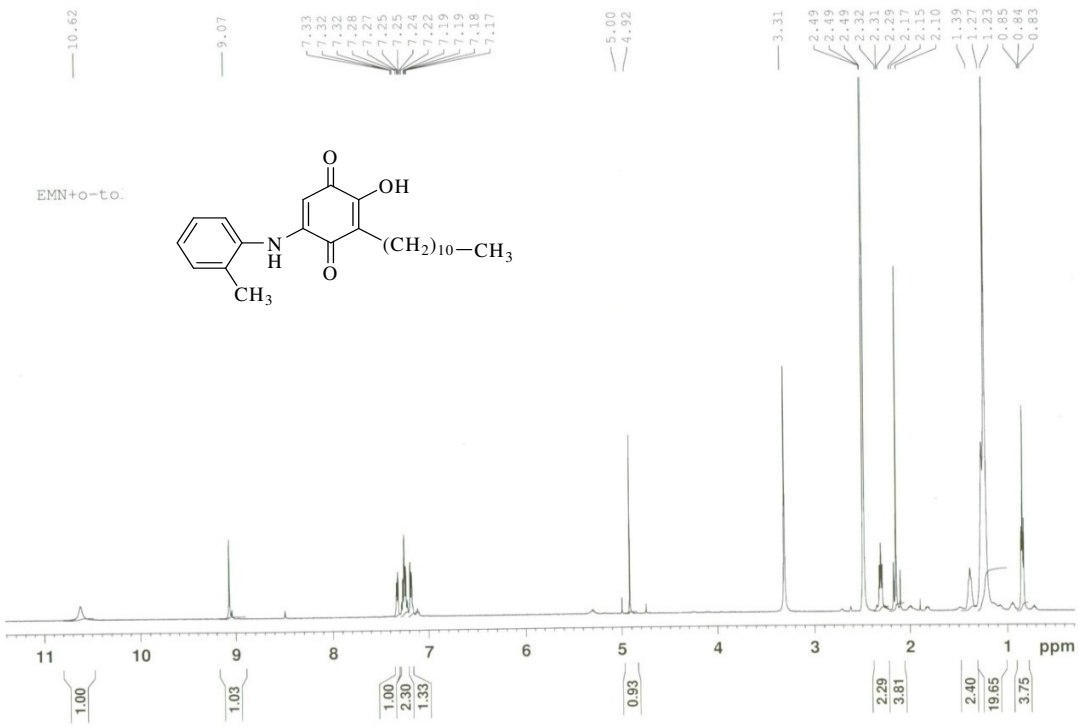


Fig. 80. ^{1}H NMR spectrum of compound 19

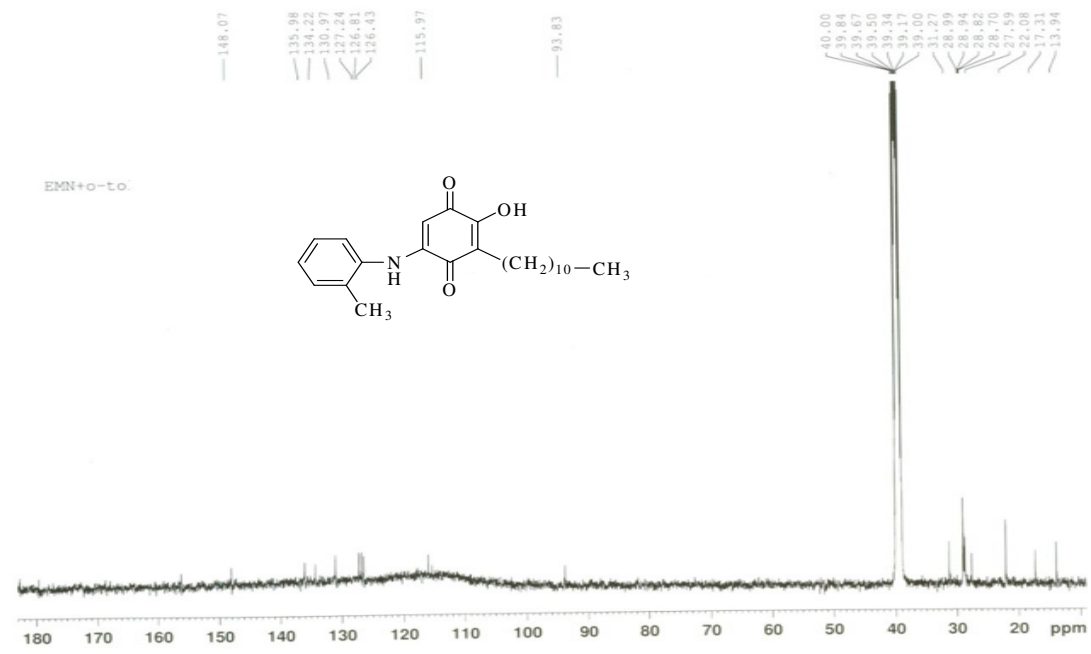


Fig. 81. ^{13}C NMR spectrum of compound 19

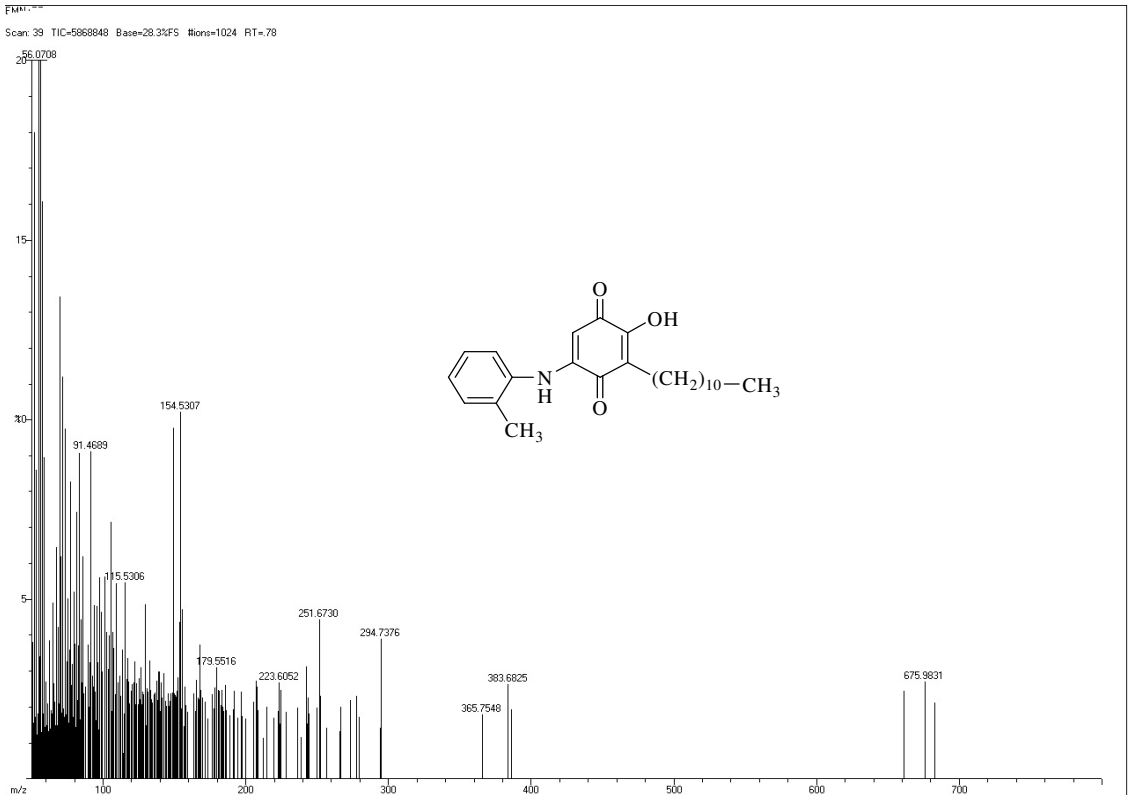


Fig. 82. Mass spectrum of compound 19

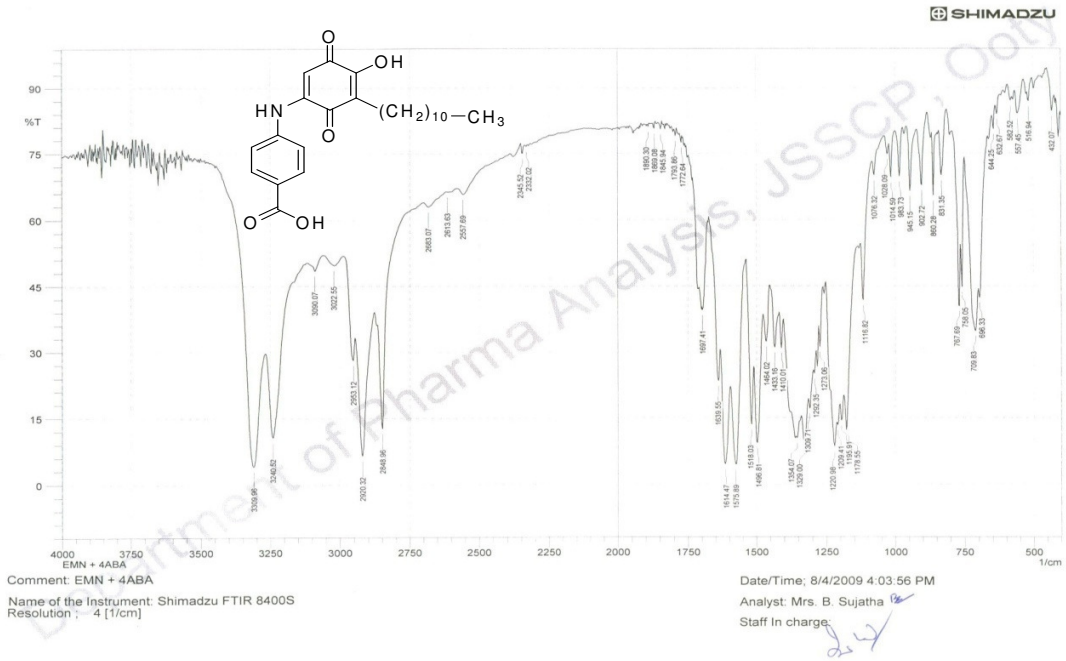


Fig. 83. IR spectrum of compound 20

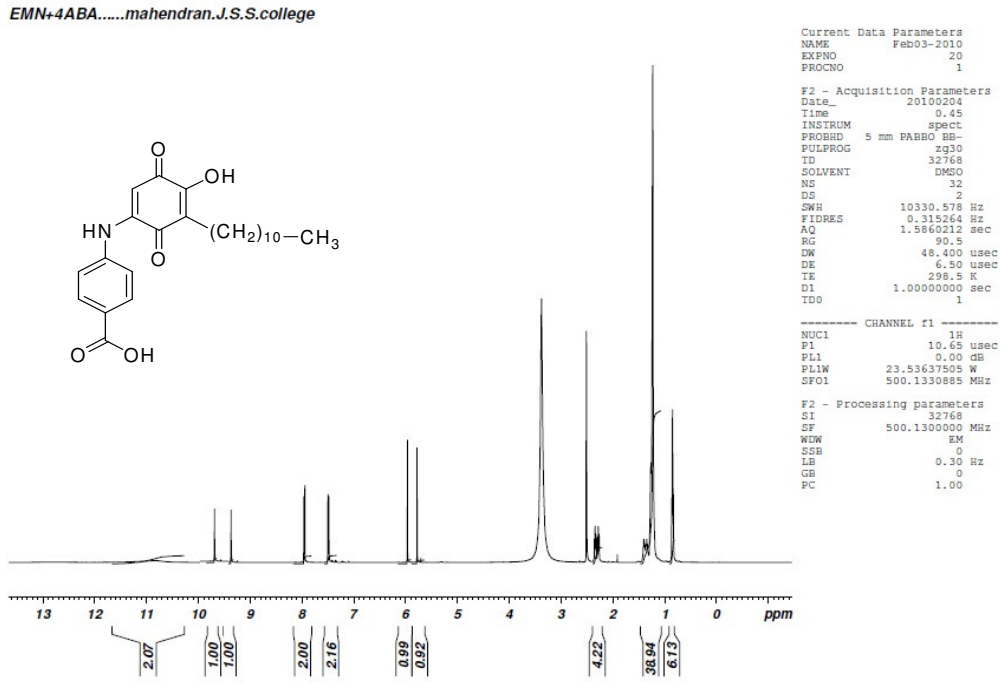


Fig. 84. ^{1}H NMR spectrum of compound 20

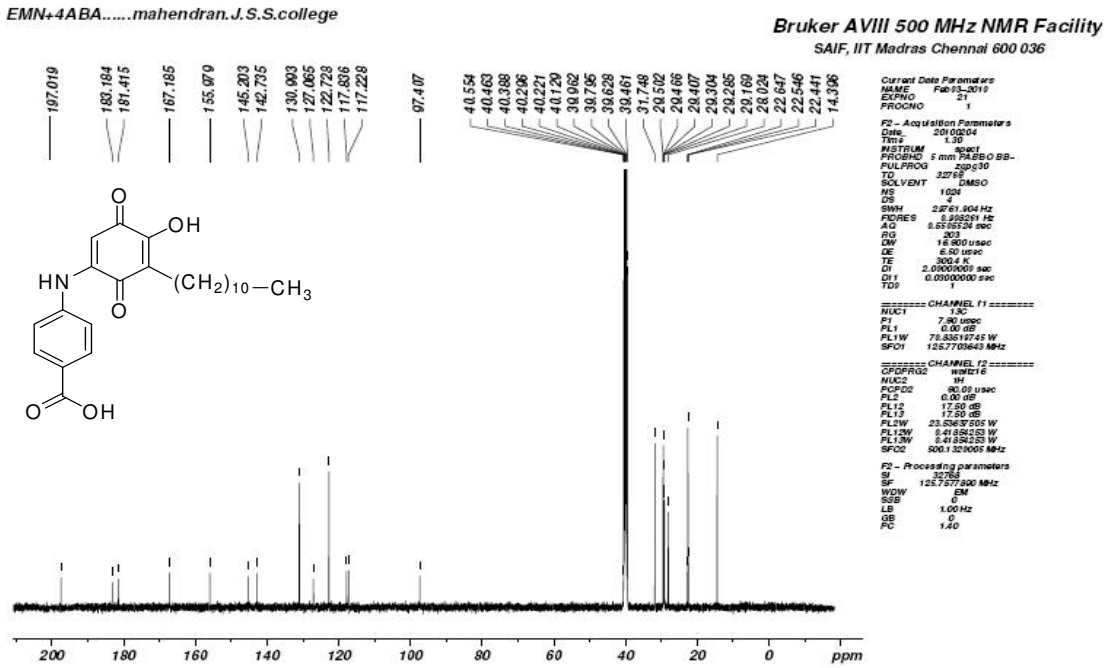


Fig. 85. ^{13}C NMR spectrum of compound 20

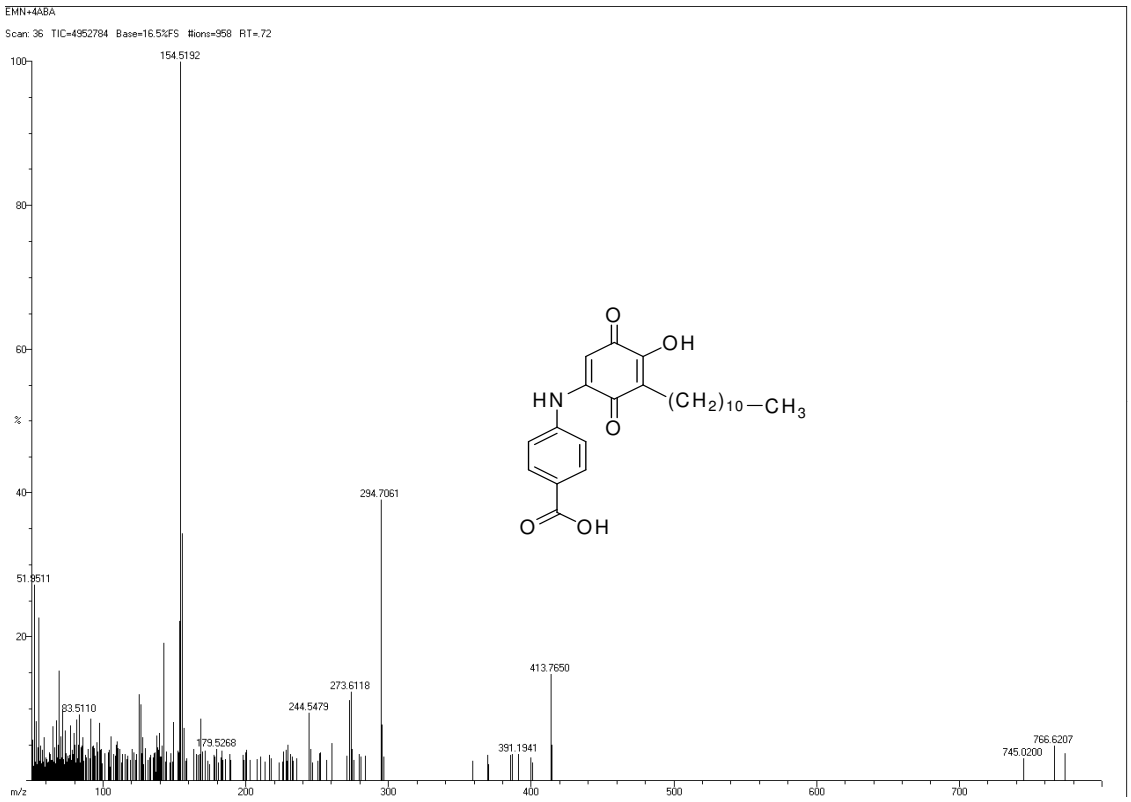


Fig. 86. Mass spectrum of compound 20

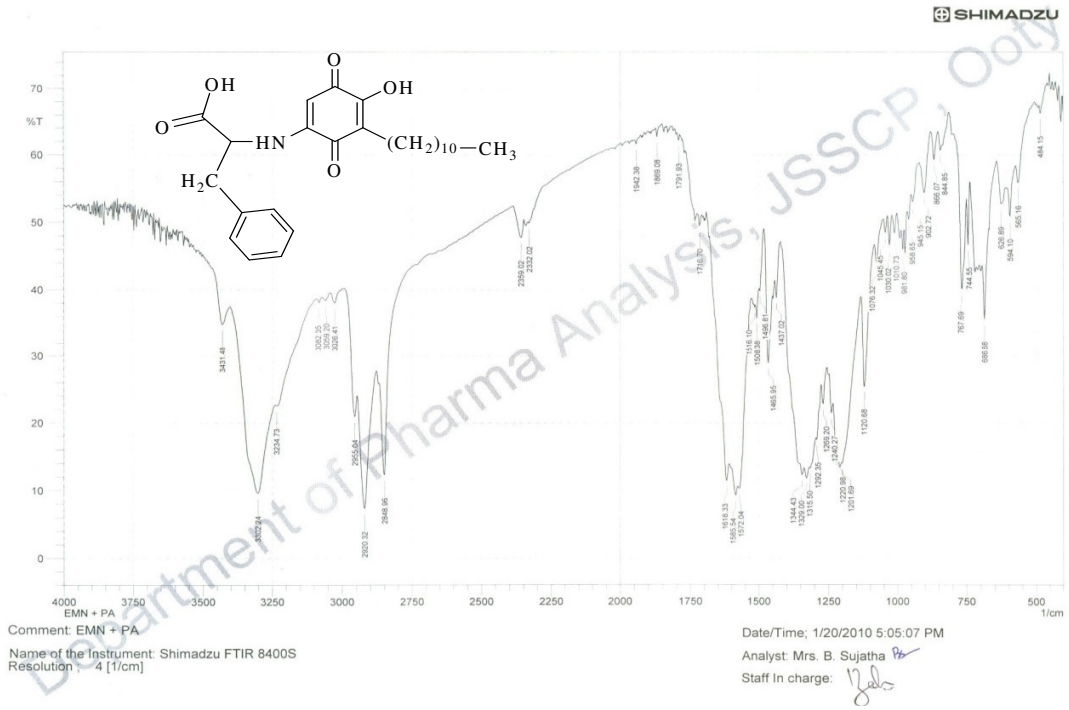


Fig. 87. IR spectrum of compound 21

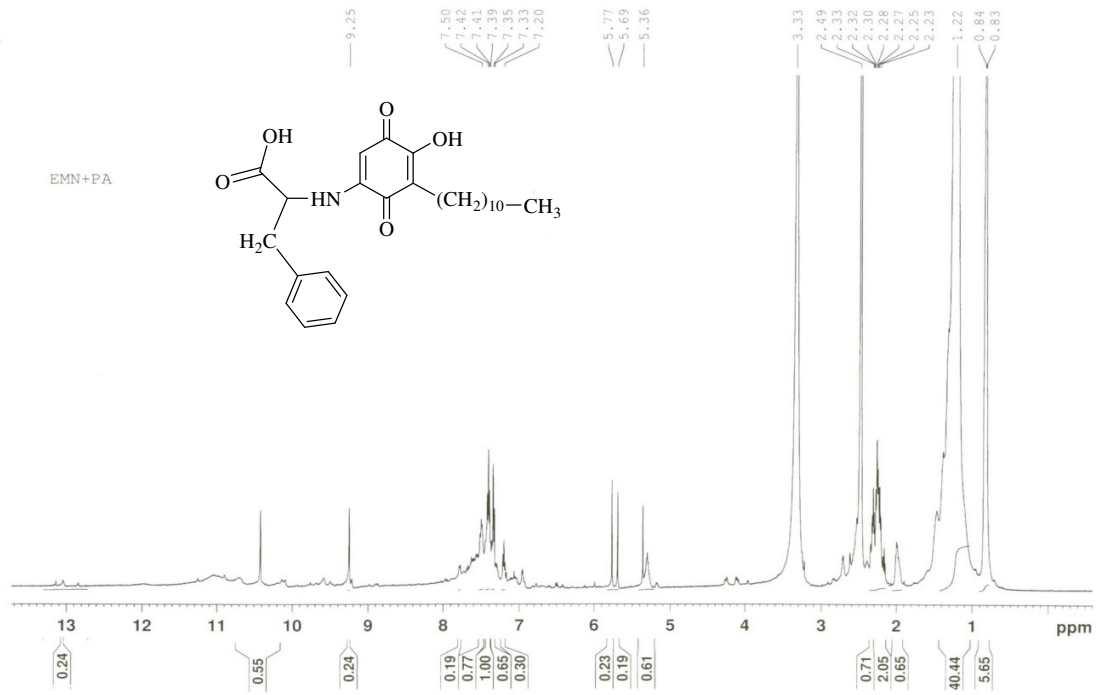


Fig. 88. ^{1}H NMR spectrum of compound 21

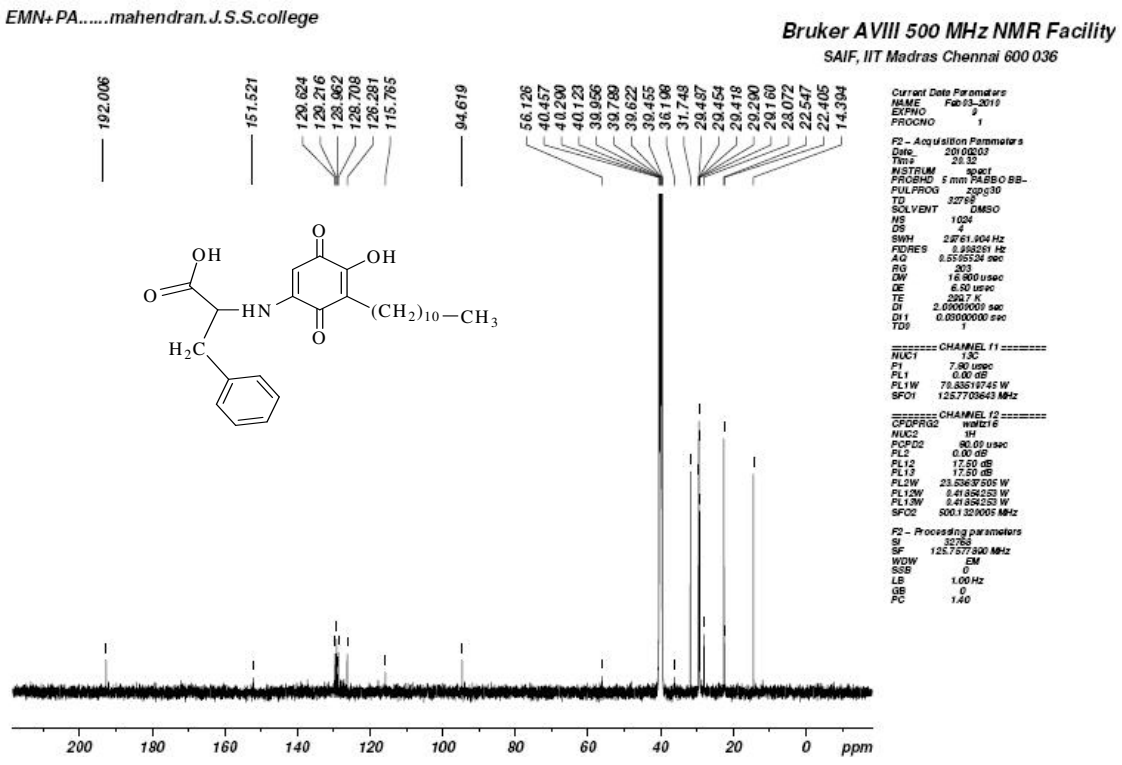


Fig. 89. ^{13}C NMR spectrum of compound 21

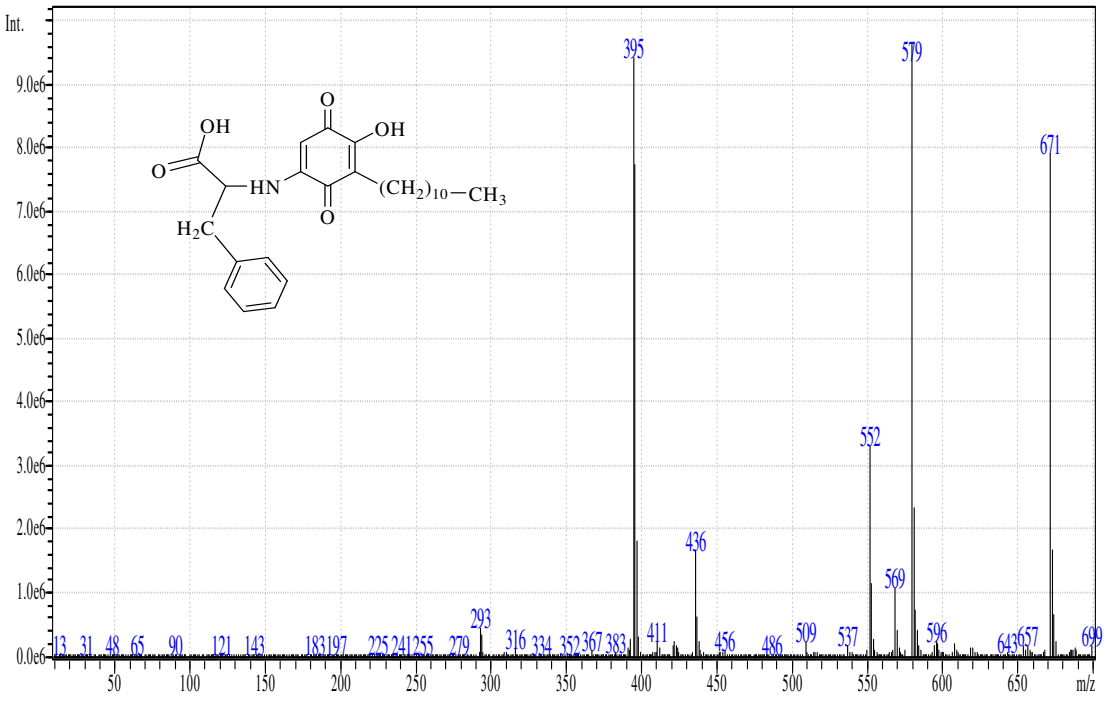


Fig. 90. Mass spectrum of compound 21

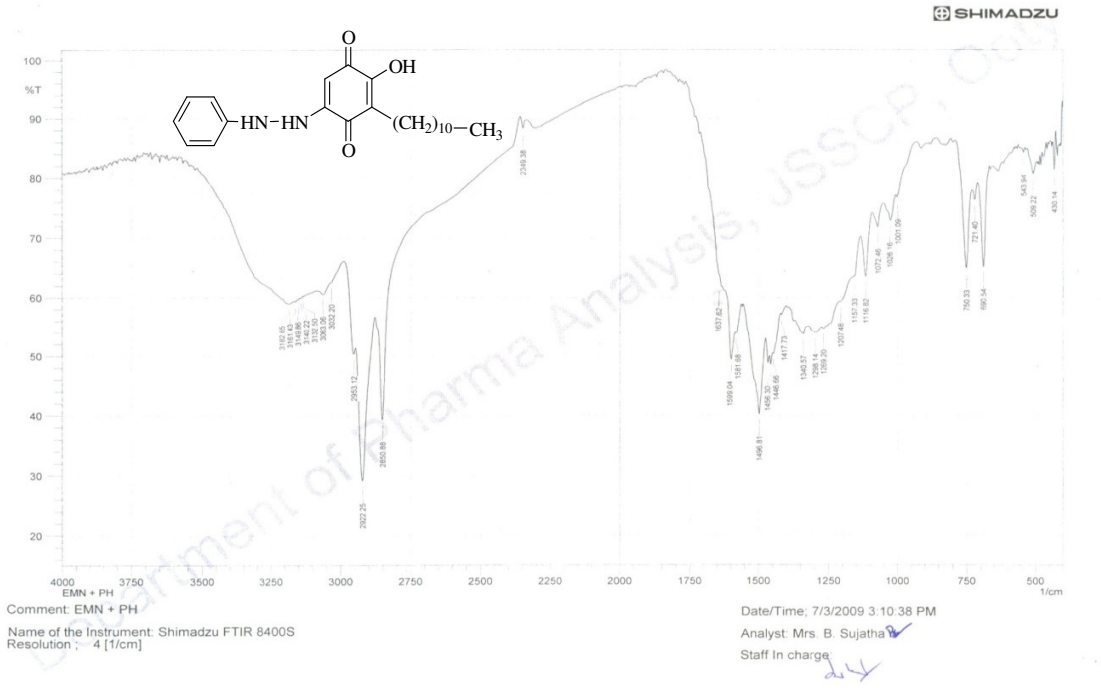


Fig. 91. IR spectrum of compound 22

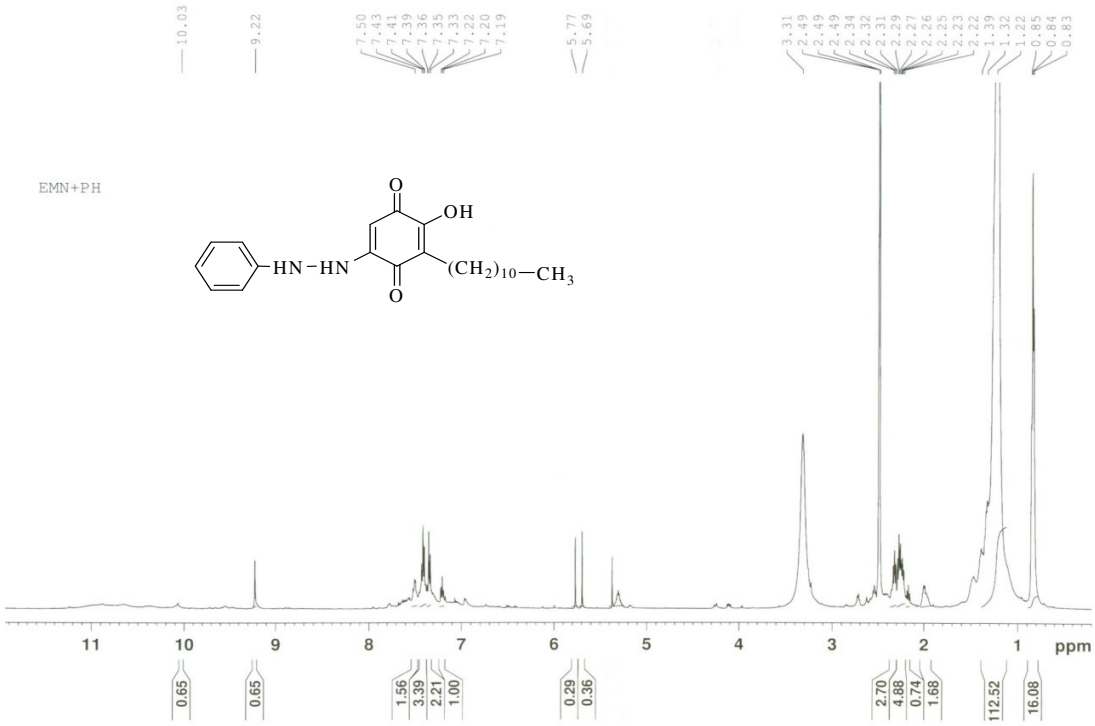


Fig. 92. ^{1}H NMR spectrum of compound 22

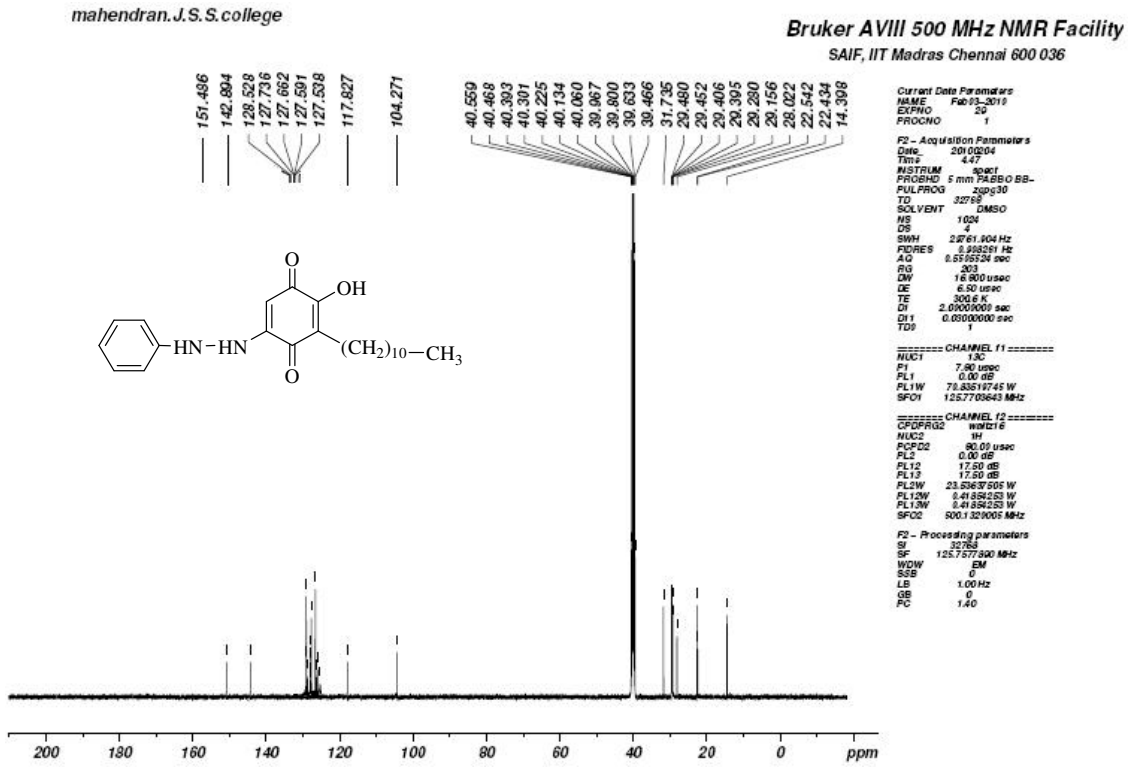


Fig. 93. ^{13}C NMR spectrum of compound 22

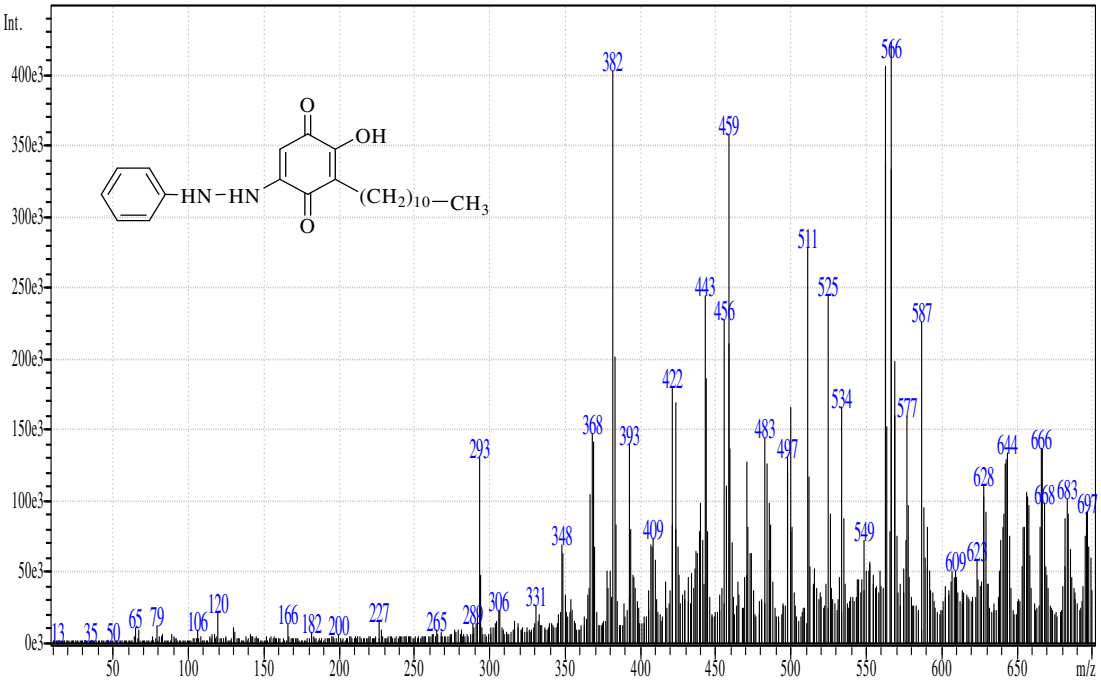


Fig. 94. Mass spectrum of compound 22

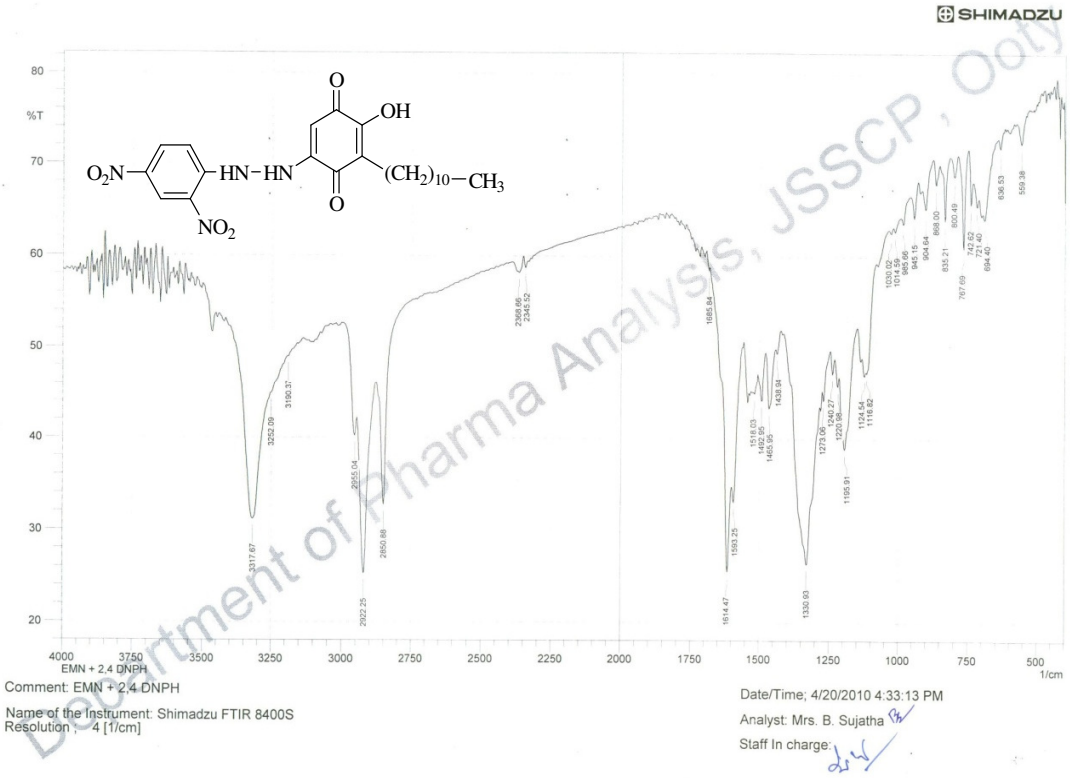


Fig. 95. IR spectrum of compound 23

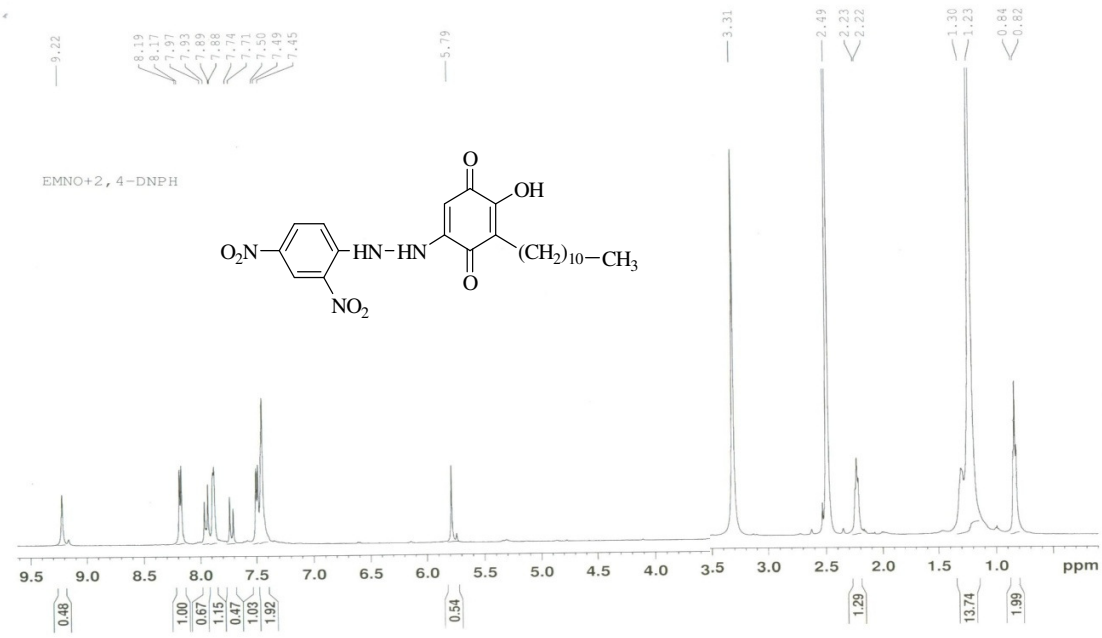


Fig. 96. ^{1}H NMR spectrum of compound 23

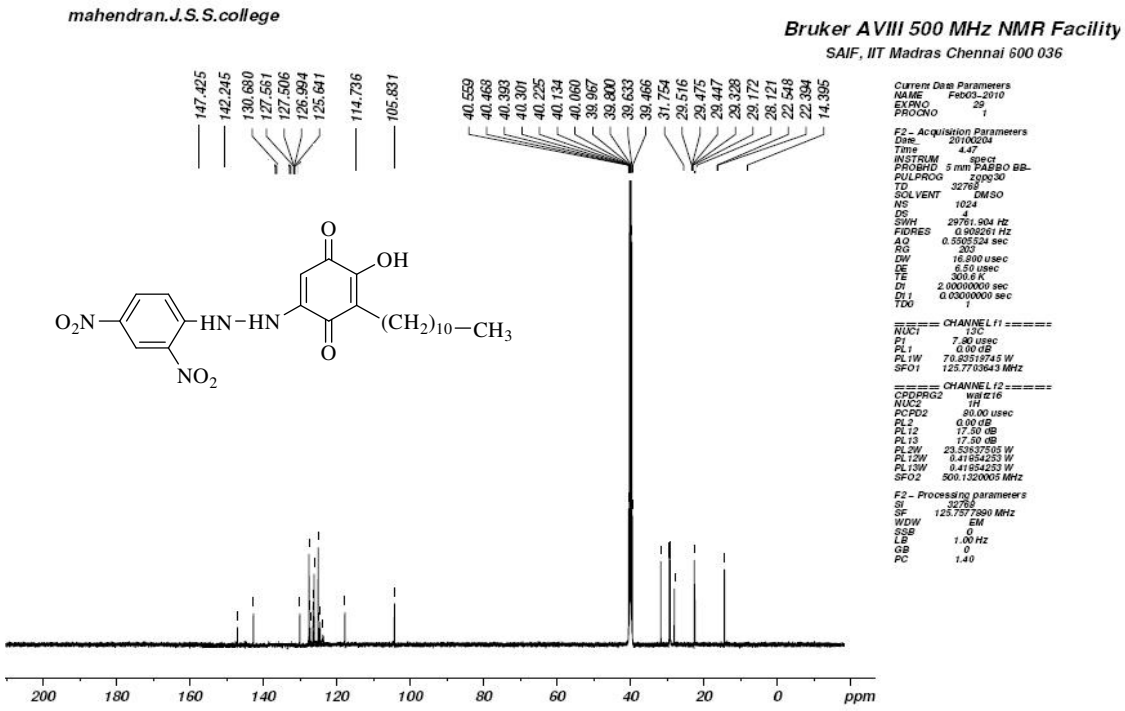


Fig. 97. ^{13}C NMR spectrum of compound 23

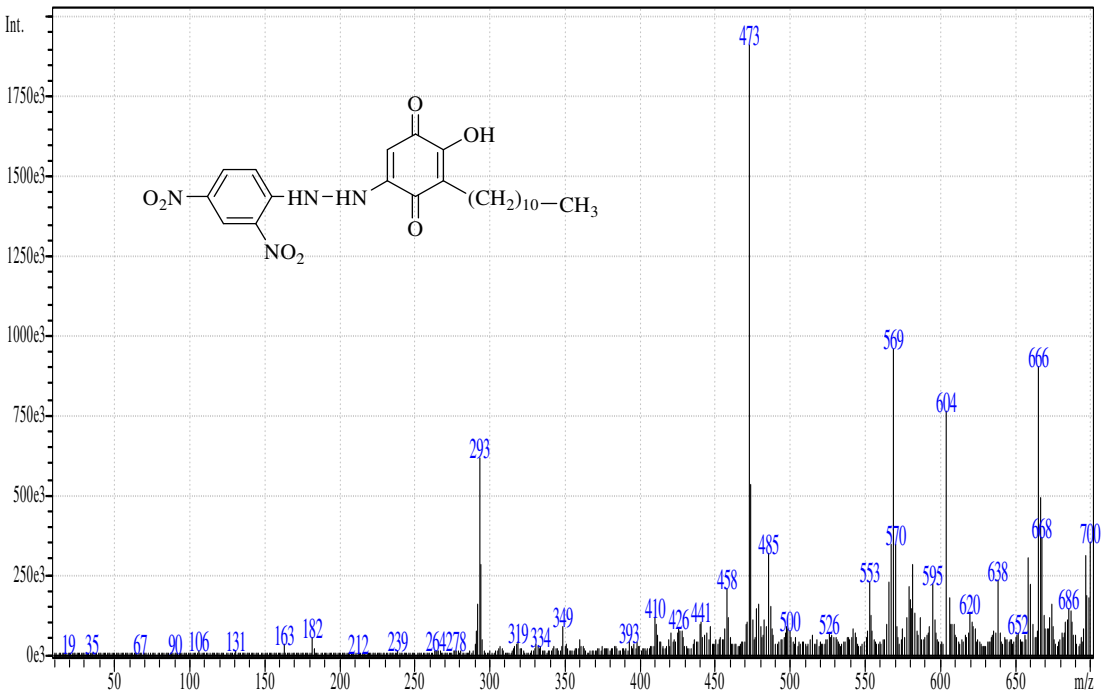


Fig. 98. Mass spectrum of compound 23

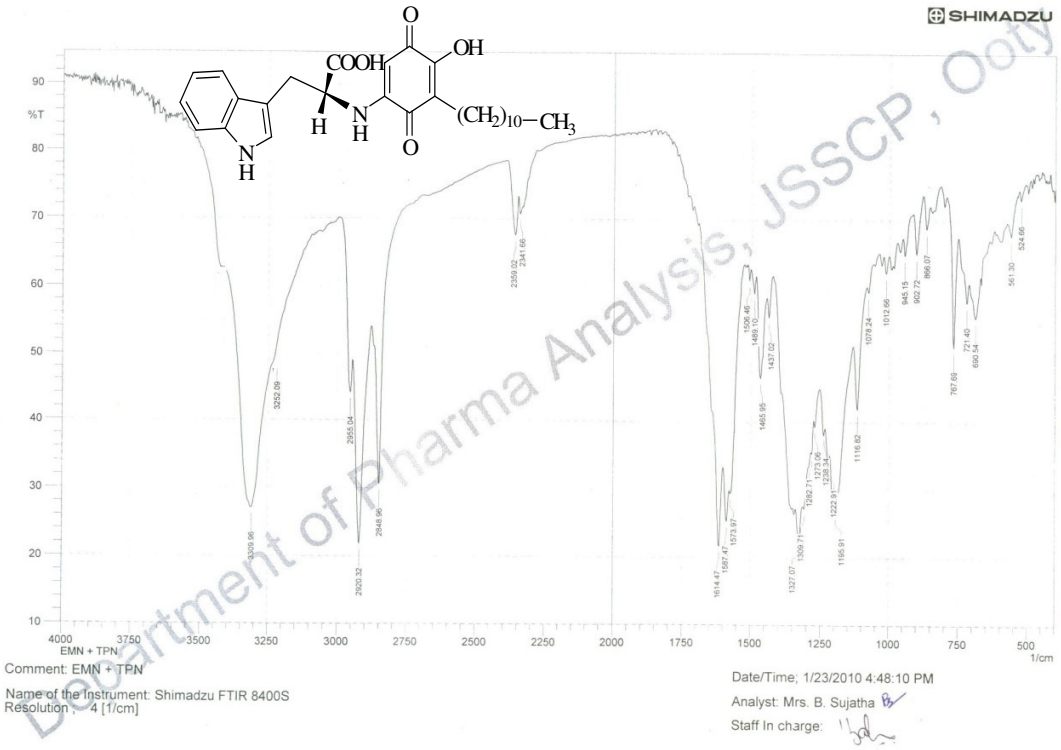


Fig. 99. IR spectrum of compound 24

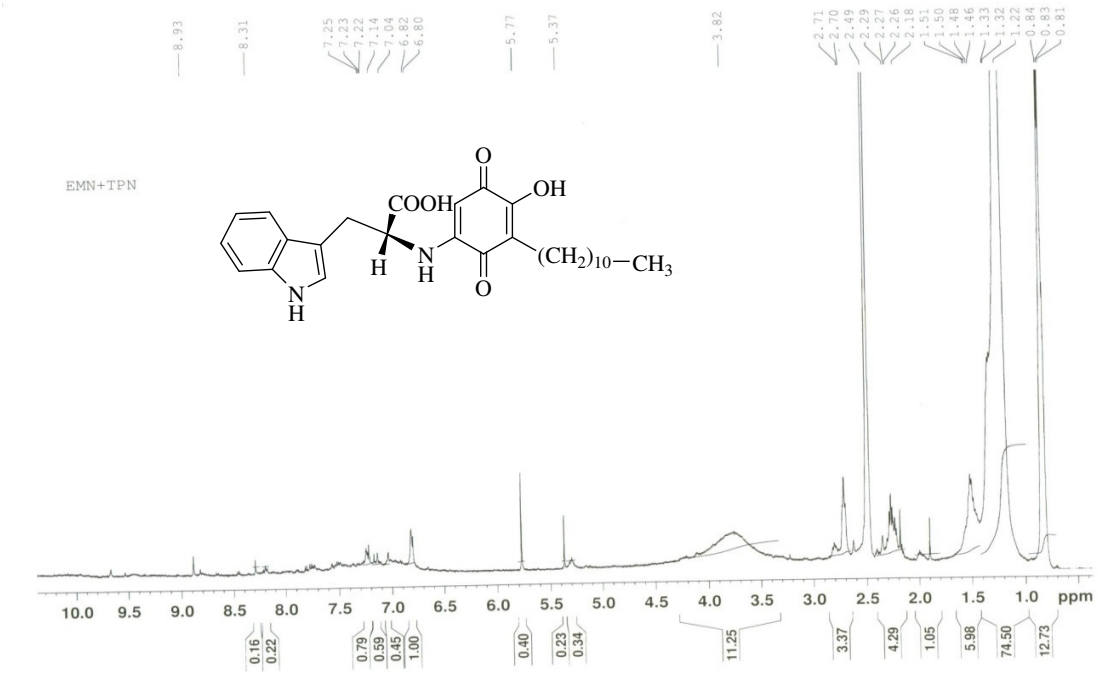


Fig. 100. ^{1}H NMR spectrum of compound 24

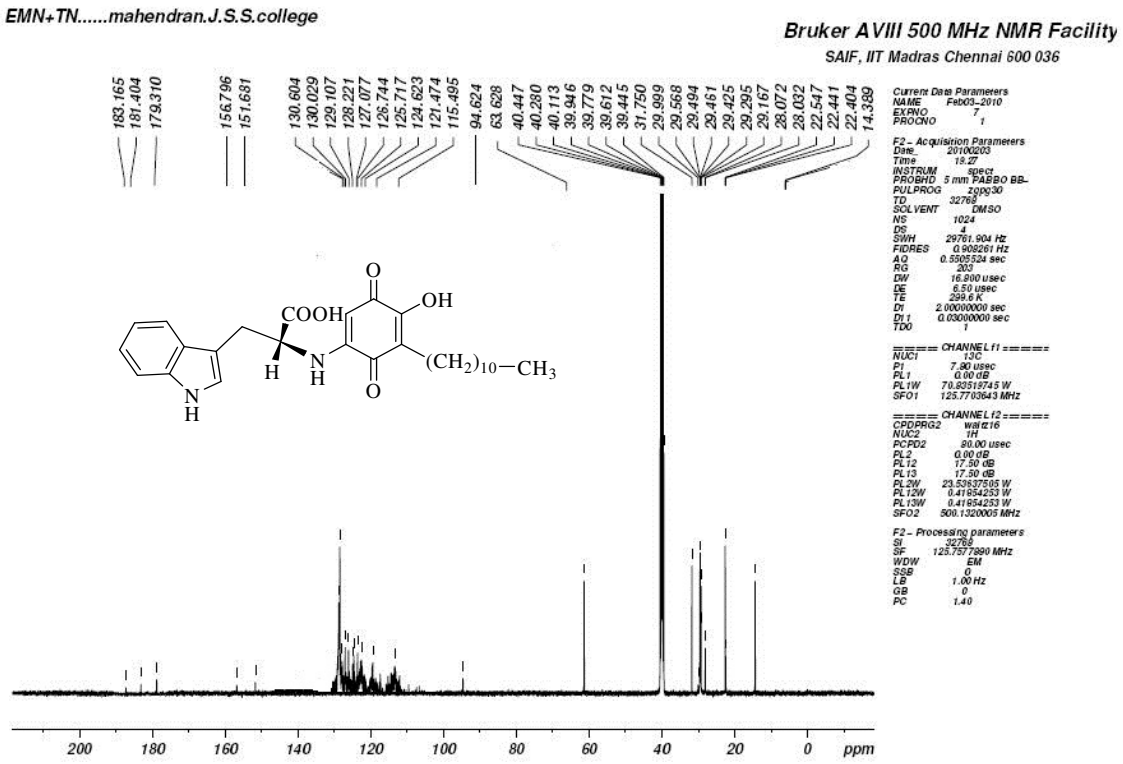


Fig. 101. ^{13}C NMR spectrum of compound 24

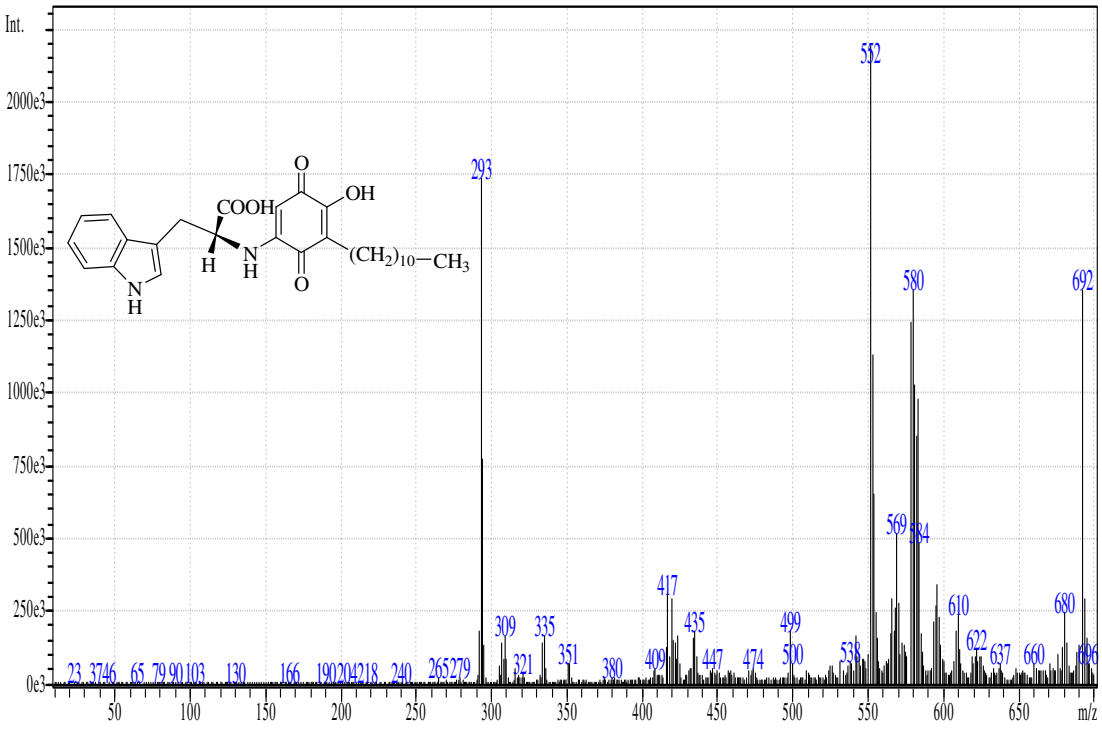


Fig. 102. Mass spectrum of compound 24

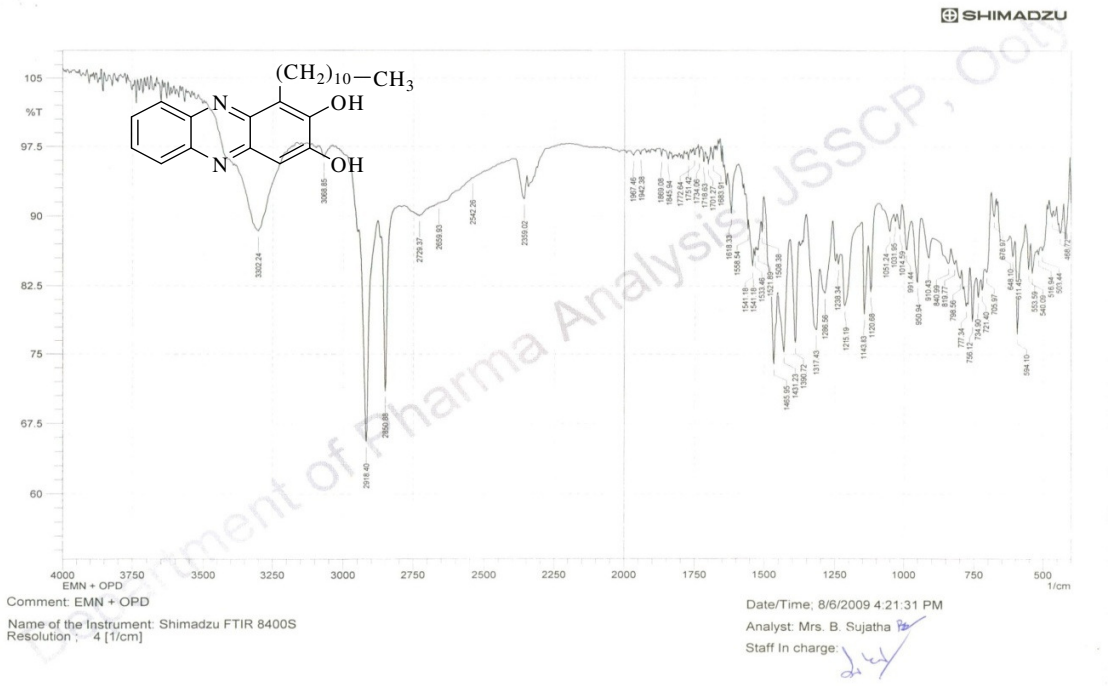


Fig. 103. IR spectrum of compound 25

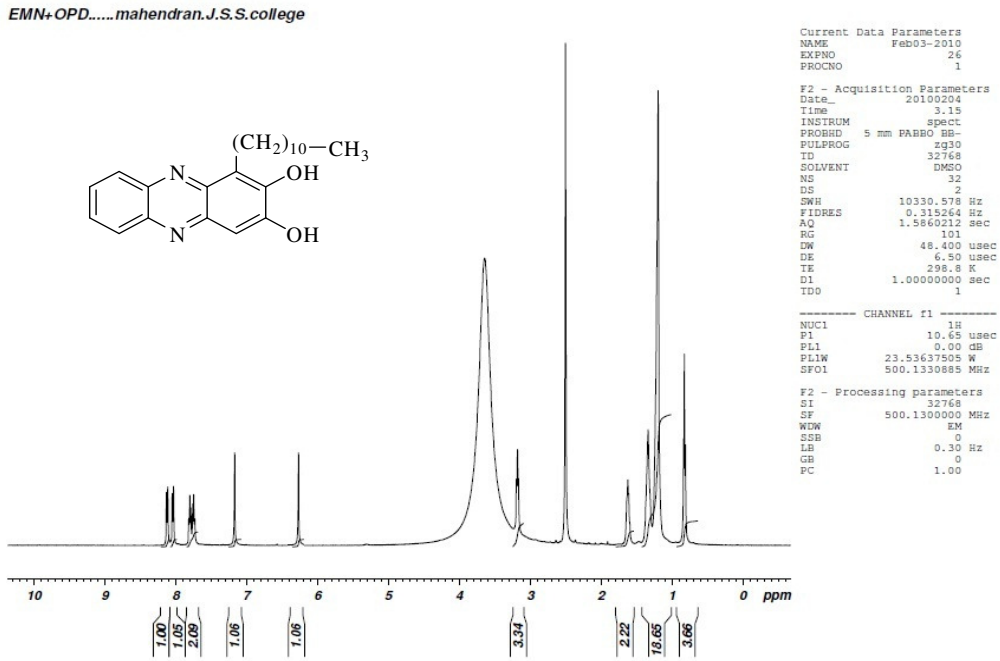


Fig. 104. ^{1}H NMR spectrum of compound 25

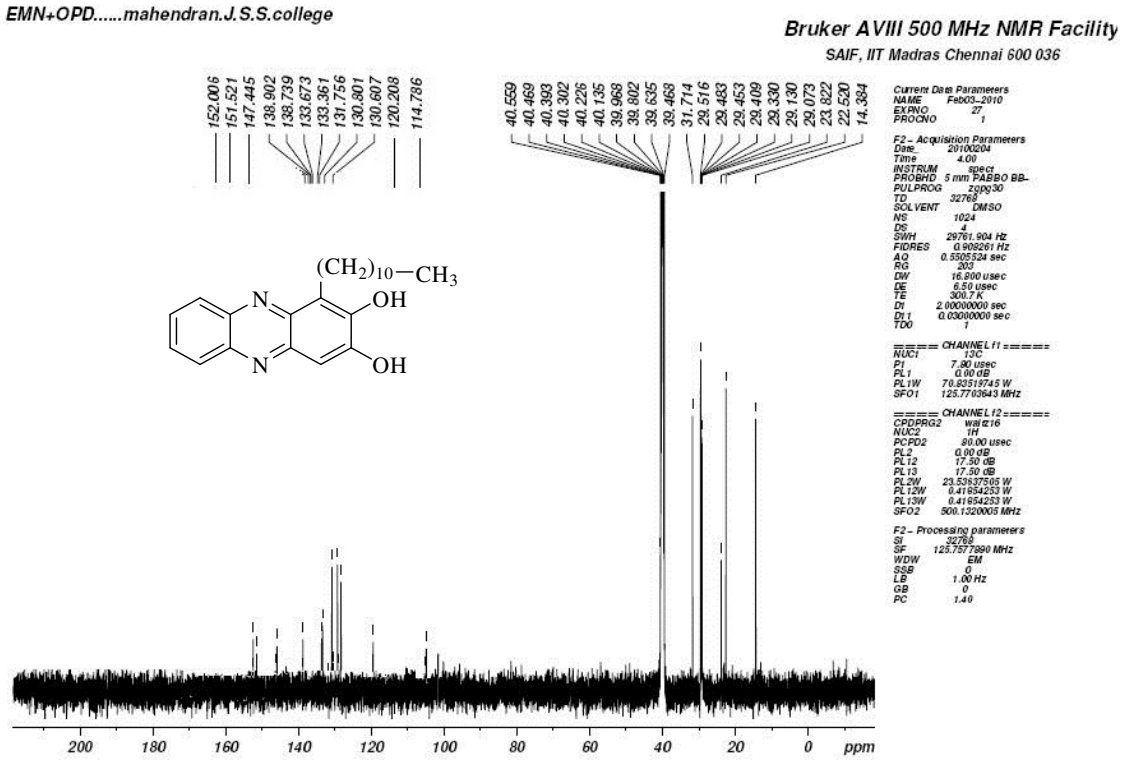


Fig. 105. ^{13}C NMR spectrum of compound 25

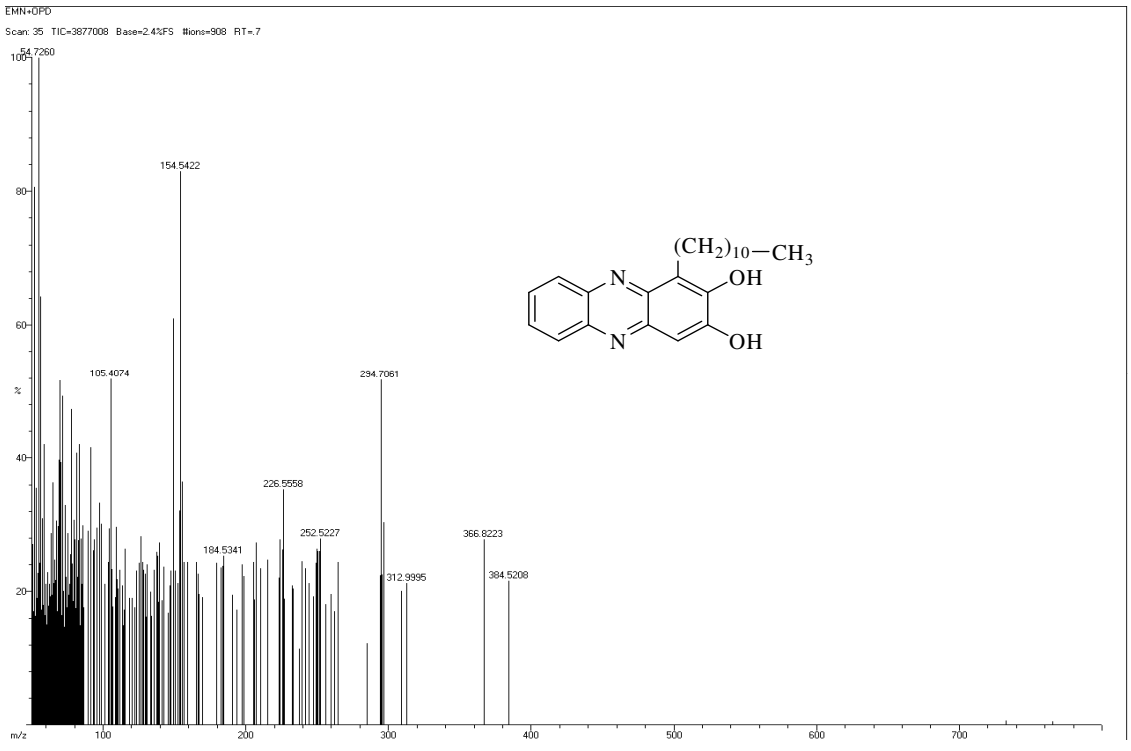


Fig. 106. Mass spectrum of compound 25

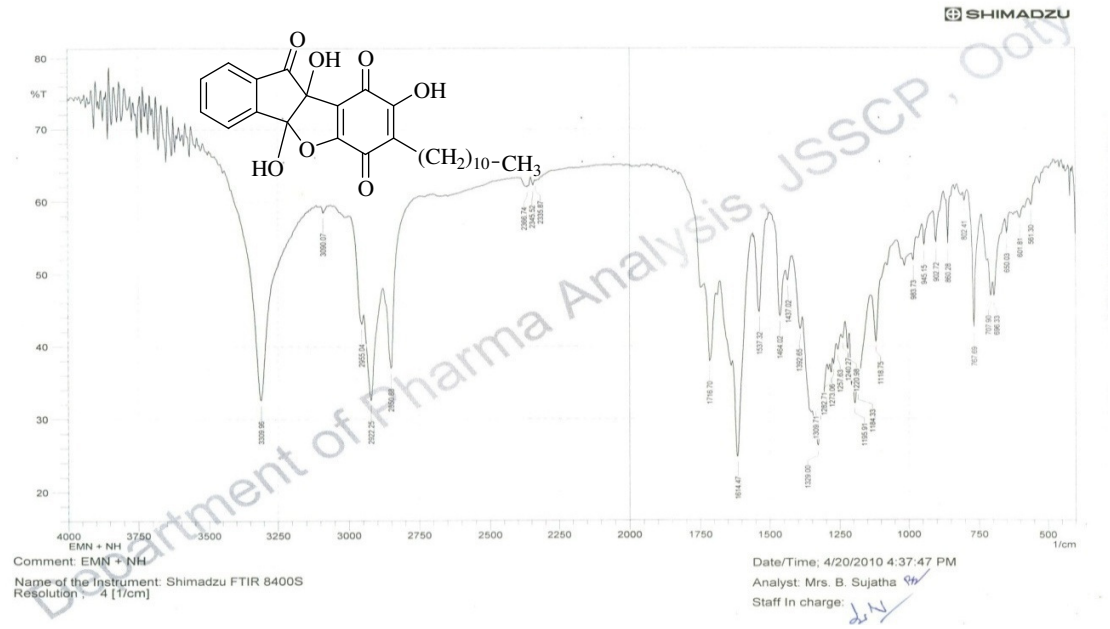


Fig. 107. IR spectrum of embelin-ninhydrin adduct

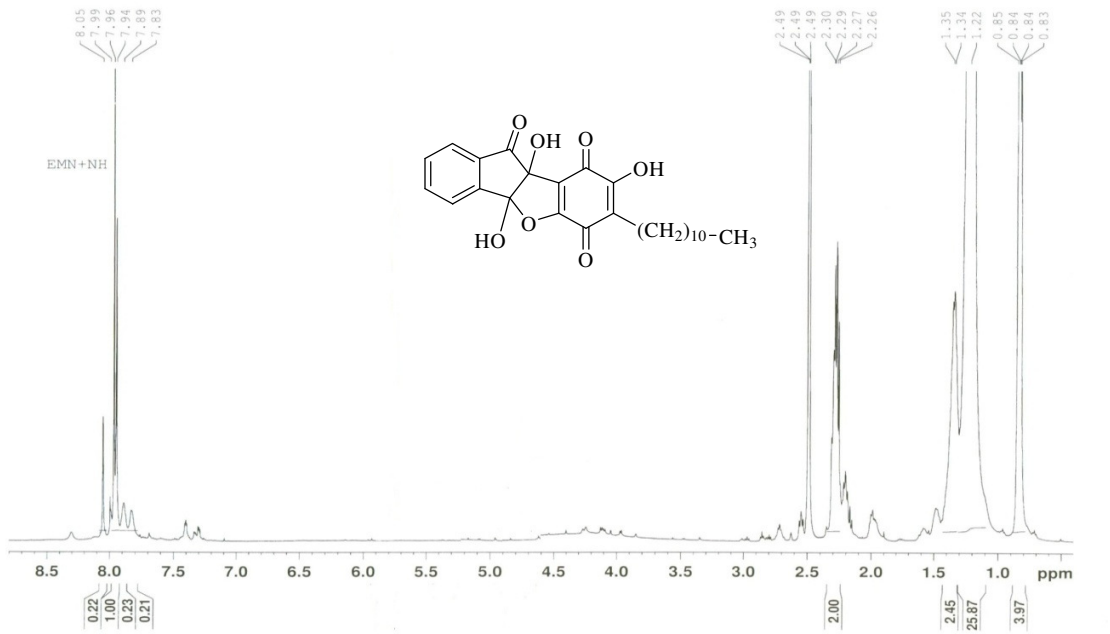


Fig. 108. ^{1}H NMR spectrum of embelin-ninhydrin adduct

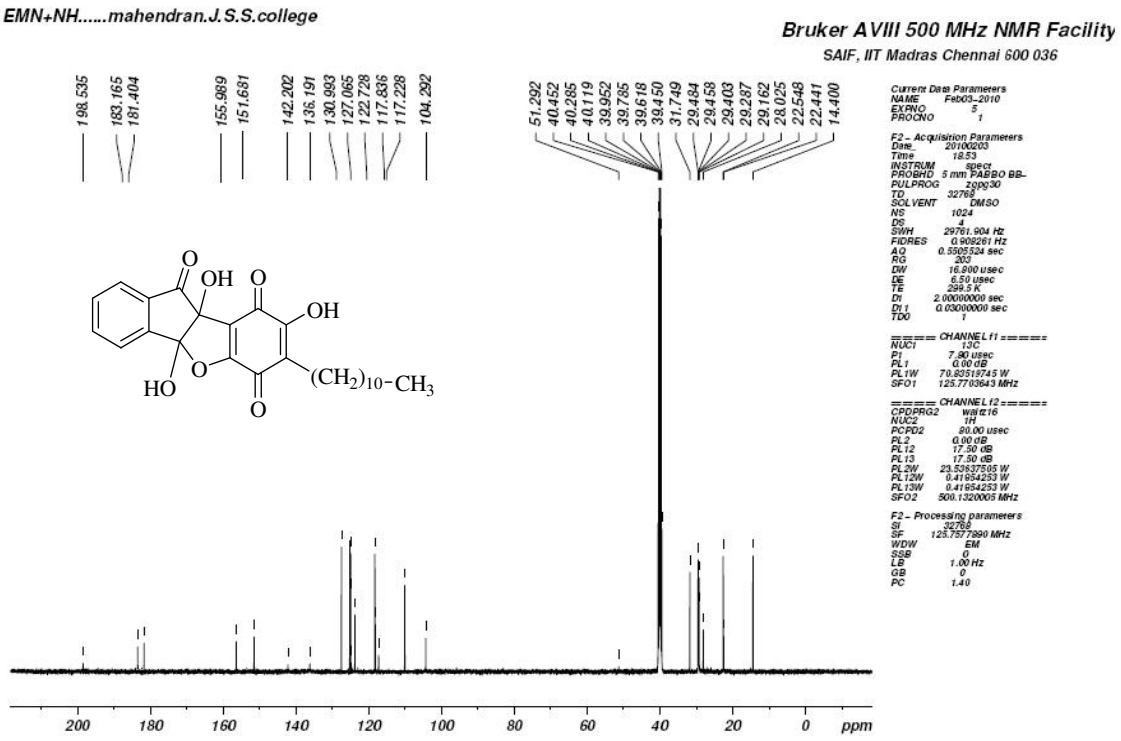


Fig. 109. ^{13}C NMR spectrum of embelin-ninhydrin adduct

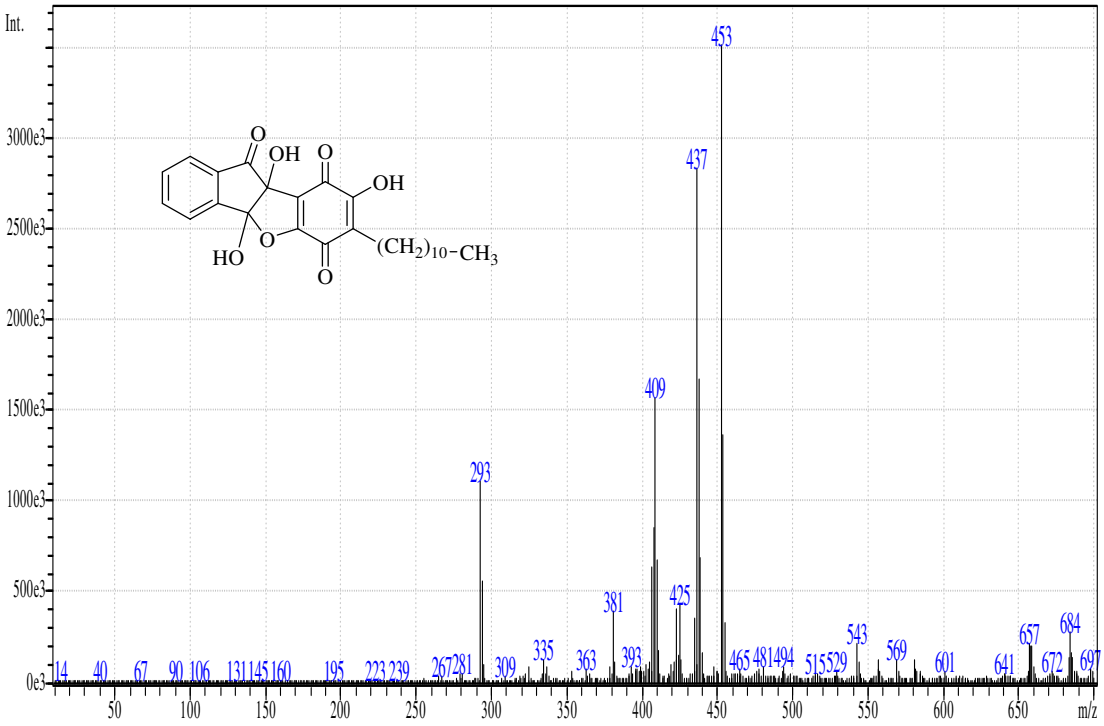


Fig. 110. Mass spectrum of embelin-ninhydrin adduct

5.2. *In vitro* antioxidant screening of embelin and its derivatives

Free radicals play important roles in many physiological and pathological conditions. In general, excess of free radicals caused by the imbalance between free radicals generation and scavenging may contribute to disease development. Hence, in the present study *in vitro* antioxidant activity of the derivatives of embelin was assessed using ABTS and DPPH methods.

Embelin showed potent antioxidant activity with IC_{50} values 0.23 ± 0.04 µg/ml and 27.92 ± 1.73 µg/ml in ABTS and DPPH methods, respectively. Compounds **10**, **21** and **23-25** were showed better activity than embelin in both the methods and compound **13** in ABTS and compound **22** in DPPH methods. Potent antioxidant activity with very low IC_{50} values were obtained for all the compounds in ABTS method. The activity was found to be more than the standard ascorbic acid in all the compounds and more than the standard rutin in compounds **1**, **2**, **5**, **9**, **10**, **12**, **13** and **20-25** (Table 3).

In DPPH method, all the compounds were found to possess higher IC_{50} values than the standards, ascorbic acid and rutin indicating the activity lesser than the standards. However, among all the compounds, **1, 10, 13** and **21-25** were found to possess potent and **2, 5, 8, 9** and **20** moderate antioxidant activities. Our efforts to carryout *in vitro* antioxidant activity using hydroxyl radical (by deoxyribose and p-NDA methods), hydrogen peroxide, nitric oxide, lipid peroxidation and super oxide radical by alkaline DMSO inhibition methods were failed due to the precipitation of the compounds with the reagents.

Table 3. *In vitro* antioxidant activity of embelin derivatives by using ABTS and DPPH methods

Compound	IC_{50} values ± SEM (µg/ml) by methods*		Compound	IC_{50} values ± SEM (µg/ml) by methods*	
	ABTS	DPPH		ABTS	DPPH
1	0.23 ± 0.04	27.92 ± 0.33	**14**	0.60 ± 0.12	84.20 ± 1.27
2	0.50 ± 0.07	97.20 ± 3.59	**15**	0.72 ± 0.13	92.60 ± 3.78
3	2.82 ± 0.32	>250	**16**	0.88 ± 0.20	148.40 ± 4.84
4	1.86 ± 0.25	>250	**17**	1.32 ± 0.18	81.60 ± 3.98
5	0.34 ± 0.11	52.40 ± 2.78	**18**	1.67 ± 0.35	>250
6	3.54 ± 0.53	>250	**19**	0.78 ± 0.11	73.80 ± 4.74
7	2.99 ± 0.22	>250	**20**	0.50 ± 0.08	48.80 ± 2.07
8	1.08 ± 0.13	59.30 ± 1.98	**21**	0.19 ± 0.02	13.98 ± 0.55
9	0.42 ± 0.18	50.70 ± 4.28	**22**	0.28 ± 0.04	24.88 ± 1.25
10	0.18 ± 0.02	25.96 ± 1.73	**23**	0.21 ± 0.02	22.50 ± 0.80
11	1.44 ± 0.28	>250	**24**	0.13 ± 0.04	14.70 ± 0.59
12	0.43 ± 0.12	111.00 ± 3.21	**25**	0.20 ± 0.02	13.60 ± 0.91
13	0.20 ± 0.07	30.40 ± 2.79	Ascorbic acid	11.25 ± 0.49	4.92 ± 0.28
			Rutin	0.52 ± 0.04	8.91 ± 0.10

*Average of three determinations

5.2.1. *In vitro* antioxidant activity of embelin-ninhydrin adduct

The ninhydrin adduct of embelin exhibited potent antioxidant activity better than embelin with IC_{50} value of 21.23 ± 0.21 µg/ml and less than standard ascorbic acid and rutin (4.92 ± 0.28 and 8.91 ± 0.10 µg/ml, respectively) in DPPH method. Therefore, chemical modification of embelin is a promising approach for developing new biological agents.

5.3. Analgesic activity of potent antioxidant compounds of embelin derivatives

Since, embelin exhibited potent antioxidant, analgesic and anti-inflammatory activities, in the present study a comparative evaluation of potent antioxidant compounds for *in vivo* analgesic and anti-inflammatory screening in experimental animals was carried out.

Analgesic activity was assessed by Eddy′s hot-plate, tail immersion and acetic acid induced writhing methods by using standard procedures [123,124]. In the hot plate method of analgesic activity, embelin and its derivatives (**1**, **10**, **13** and **21-25**) exhibited potent activity. The response time observed for all the eight compounds was significantly increased when compared to normal control (Table 4). The activity observed was found to be higher than the standard pentazocine after 15 min for compound **10** at both the doses, and for compound **13** and **25** at 20 mg/kg. Similar results were observed for compounds **10**, **13** and **25** at 20 mg/kg after 30 min. However, the standard pentazocine was found to be better active than all the three compounds during 45 min response. The percentage protection after 45 min for all the compounds ranged between 50.70 to 74.15%. Compounds **10** and **13** at both the doses and compounds **22, 24** and **25** at 20 mg/kg were found to be better active than embelin.

In the tail immersion and acetic acid induced writhing methods, all the compounds (**1**, **10**, **13** and **21-25**) were showed dose dependent and potent analgesic activity. The values were significant for all the compounds at both the doses in the acetic acid induced writhing and for all the compounds except **1**, **21** and **23**-**25** at 10 mg/kg and compounds **22** and **24** at both the doses in tail immersion method (Table 5). Compounds **10**, **13, 21**

Table 4. Analgesic activity of embelin and its derivatives by using hot plate method

Treatment	Latency period, sec (% Protection)

(dose, mg/kg, i.p.)	15 min	30 min	45 min
Normal	2.32 ± 0.25	2.27 ± 0.26	2.43 ± 0.28
1 (10)	3.12 ± 0.22 (25.64)	4.78 ± 0.59 (52.51)	7.28 ± 0.37[b] (66.62)
1 (20)	4.46 ± 0.67[c] (47.98)	6.11 ± 0.96[c] (62.85)	7.39 ± 1.07[b] (67.12)
10 (10)	5.27 ± 0.92[b] (55.98)	6.41 ± 0.86[b] (64.59)	8.27 ± 0.78[a] (70.62)
10 (20)	6.48 ± 0.70[a] (64.20)	8.42 ± 0.84[a] (73.04)	9.40 ± 0.87[a] (74.15)
13 (10)	4.93 ± 0.58[c] (52.94)	6.54 ± 0.64[b] (65.30)	7.70 ± 1.61[b] (68.44)
13 (20)	6.24 ± 0.87[a] (62.82)	7.83 ± 1.25[a] (71.01)	8.86 ± 1.39[a] (72.57)
21 (10)	3.52 ± 0.52 (34.09)	5.41 ± 0.78 (58.04)	5.80 ± 0.56 (58.10)
21 (20)	3.85 ± 0.39 (39.74)	6.73 ± 0.70[b] (66.27)	7.27 ± 0.65[b] (66.57)
22 (10)	4.03 ± 0.40 (42.43)	4.58 ± 0.27 (50.44)	6.10 ± 0.34 (60.16)
22 (20)	4.77 ± 0.51[c] (51.51)	5.87 ± 0.54[c] (61.33)	8.11 ± 0.66[a] (70.03)
23 (10)	3.66 ± 0.41 (36.61)	5.05 ± 0.53 (55.05)	5.49 ± 0.57 (55.73)
23 (20)	3.97 ± 0.41 (41.56)	5.34 ± 0.72 (57.50)	6.34 ± 0.77[c] (61.67)
24 (10)	4.18 ± 0.49 (44.50)	4.58 ± 0.53 (50.44)	4.93 ± 0.61 (50.70)
24 (20)	4.75 ± 0.63[c] (51.16)	6.16 ± 0.86[c] (63.15)	8.11 ± 0.87[a] (70.03)
25 (10)	4.95 ± 0.48[c] (53.13)	5.42 ± 0.72 (58.12)	7.08 ± 1.06[b] (65.68)
25 (20)	6.01 ± 0.57[a] (61.40)	7.55 ± 1.78[a] (69.93)	9.35 ± 1.53[a] (74.01)
Pentazocine (20)	5.11 ± 0.32[b] (54.60)	6.85 ± 0.50[b] (66.86)	10.61 ± 1.14[a] (77.10)

Values are given as mean ± S.E.M. for groups of six animals each, Dunnet′s test; values are statistically significant at [a]P<0.001, [b]P<0.01, [c]P<0.05 between control and treated groups.

and **25** at both the doses and compound **23** at 20 mg/kg were found to be more active than embelin. Compound **13** at 20 mg/kg showed better activity than the standard pentazocine in tail immersion method. In acetic acid induced writhing method, except compounds **21** and **24** at 10 mg/kg, all the other compounds at both the doses almost completely abolished the writhing indicating their potent analgesic activity. The activity was found to be higher than the standard pentazocine at both the doses except compounds **21** and **24** at 10 mg/kg.

5.3.1. Analgesic activity of embelin-ninhydrin adduct

In the hot plate test embelin at 10 and 20 mg/kg produced potent and dose dependent analgesic activity. The percent protection ranged between 25.64 to 67.80 (Fig. 111). The values were found to be significant for 30 and 45 min duration of the lower dose and for all the durations for the higher dose. Embelin-ninhydrin adduct also produced similar results. In the tail immersion method, embelin and it's adduct at 20 mg/kg produced significant analgesic activity. In both these methods, the adduct showed better activity than embelin, and the activity was comparable with standard pentazocine at 20 mg/kg. Embelin and its adduct produced significant protection at both the doses in the acetic acid induced writhing and the activity was found to be better than the standard pentazocine (Table 6). Embelin at 20 mg/kg and the adduct at both the doses abolished the writhing almost completely. The adduct produced better results than embelin.

Table 5. Analgesic activity of embelin and its derivatives by using tail immersion and acetic acid induced writhing methods

Treatment (dose, mg/kg, i.p.)	Tail immersion		Treatment (dose, mg/kg, i.p.)	Acetic acid induced writhing	
	Latency period (sec)	% Protection		No. of writhing	% Protection
Normal	4.39 ± 0.60	-	Control	43.83 ± 2.32	-
1 (10)	5.46 ± 0.35	19.59	**1** (10)	3.00 ± 0.68[a]	93.15
1 (20)	7.84 ± 0.69[c]	44.00	**1** (20)	0.83 ± 0.48[a]	98.11
10 (10)	7.68 ± 0.70[c]	42.84	**10** (10)	3.17 ± 0.48[a]	92.77
10 (20)	9.90 ± 1.10[a]	55.66	**10** (20)	0.17 ± 0.17[a]	99.61
13 (10)	7.72 ± 0.60[c]	43.13	**13** (10)	3.67 ± 0.67[a]	91.63
13 (20)	11.94 ± 1.22[a]	63.23	**13** (20)	0.50 ± 0.34[a]	98.86
21 (10)	6.49 ± 0.98	32.35	**21** (10)	10.33 ± 1.82[a]	76.43
21 (20)	8.69 ± 0.50[b]	49.48	**21** (20)	1.83 ± 0.65[a]	95.82
22 (10)	5.46 ± 0.47	19.59	**22** (10)	5.17 ± 1.99[a]	88.20
22 (20)	7.52 ± 0.68	41.62	**22** (20)	1.33 ± 0.67[a]	96.97
23 (10)	5.85 ± 0.52	24.95	**23** (10)	6.67 ± 1.48[a]	84.78
23 (20)	8.83 ± 0.41[b]	50.58	**23** (20)	2.33 ± 0.71[a]	94.68
24 (10)	5.81 ± 0.96	24.44	**24** (10)	8.67 ± 1.74[a]	80.22
24 (20)	7.55 ± 0.73	41.85	**24** (20)	2.50 ± 1.18[a]	94.30
25 (10)	6.17 ± 0.39	28.85	**25** (10)	2.50 ± 0.85[a]	94.30
25 (20)	9.22 ± 0.72[a]	52.39	**25** (20)	0.67 ± 0.33[a]	98.47
Pentazocine (20)	10.21 ± 1.00[a]	57.00	Pentazocine (20)	7.67 ± 1.45[a]	82.50

Values are given as mean ± S.E.M. for groups of six animals each, Dunnet′s test; values are statistically significant at [a]P<0.001, [b]P<0.01, [c]P<0.05 between control and treated groups.

Table 6. Analgesic activity of embelin and its ninhydrin adduct

Treatment (dose, mg/kg, i.p.)	Hot plate test			Tail immersion	Acetic acid induced writhing
	Latency period (sec)			Latency period (sec)	No. of writhings
	15 min	30 min	45 min		
Control	2.32 ± 0.25	2.27 ± 0.26	2.43 ± 0.27	4.39 ± 0.60	43.83 ± 2.32
Embelin (10)	3.12 ± 0.22	4.78 ± 0.59[c]	7.28 ± 0.37[b]	5.46 ± 0.35	3.00 ± 0.68[a]
Embelin (20)	4.46 ± 0.67[c]	6.11 ± 0.96[a]	7.39 ± 1.07[b]	7.84 ± 0.69[b]	0.83 ± 0.48[a]
Embelin-ninhydrin adduct (10)	3.20 ± 0.35	5.92 ± 0.32[a]	7.53 ± 0.97[a]	7.02 ± 0.67	0.33 ± 0.21[a]
Embelin-ninhydrin adduct (20)	5.25 ± 0.85[b]	7.05 ± 0.52[a]	9.16 ± 0.69[a]	9.73 ± 0.73[a]	0.17 ± 0.17[a]
Pentazocine (20)	5.11 ± 0.32[b]	6.85 ± 0.50[a]	10.61 ± 1.14[a]	10.21 ± 1.00[a]	7.67 ± 1.45[a]

Values are given as mean ± S.E.M. for groups of six animals each, Dunnet′s test; values are statistically significant at [a]P<0.001, [b]P<0.01, [c]P<0.05 between control and treated groups.

5.4. Anti-inflammatory activity of selected embelin derivatives

Anti-inflammatory activity was assessed by carrageenan induced paw edema method using standard procedure [35]. Embelin at 20 mg/kg significantly reduced the paw edema after 120, 180 and 360 min when compared to control (Table 7). All the compounds exhibited significant activity at both the doses after 360 min and compounds **22** and **24** at 20 mg/kg after 120, 180 and 360 min. However, compounds **13, 21** and **25** produced significant activity at 20 mg/kg dose during 30 to 360 min measurements. The percentage protection after 360 min for all the compounds ranged between 15.49 to 41.31% (Table 8). The standard diclofenac at 20 mg/kg also produced similar and better results than the tested samples.

5.4.1. Anti-inflammatory activity of embelin-ninhydrin adduct

In the anti-inflammatory activity, embelin at 20 mg/kg produced significant reduction in the paw volume. The percent protection ranged between 13.50 to 31.92 during 30 to 360 min (Table 9). Similar significant results were obtained for the adduct during the period 120 to 360 min at 10 mg/kg and 60 to 360 min at 20 mg/kg. The activity for the adduct was found to be better than embelin but lower than the standard diclofenac sodium.

Table 7. Anti-inflammatory activity of embelin and its derivatives by carregeenan induced paw edema in rats

Treatment	Paw volume (ml), after min					
(dose, mg/kg, i.p.)	0	30	60	120	180	360
Control	0.84 ± 0.02	1.63 ± 0.04	1.77 ± 0.05	1.90 ± 0.07	1.96 ± 0.07	2.13 ± 0.06
1 (10)	0.90 ± 0.04	1.62 ± 0.05	1.73 ± 0.04	1.84 ± 0.11	1.82 ± 0.03	1.80 ± 0.07[c]
1 (20)	0.80 ± 0.02	1.41 ± 0.06	1.54 ± 0.07	1.62 ± 0.06[c]	1.59 ± 0.05[b]	1.45 ± 0.07[a]
10 (10)	0.96 ± 0.04	1.60 ± 0.12	1.70 ± 0.11	1.84 ± 0.11	1.83 ± 0.11	1.74 ± 0.11[b]
10 (20)	0.89 ± 0.06	1.49 ± 0.03	1.69 ± 0.04	1.74 ± 0.03	1.60 ± 0.07[b]	1.52 ± 0.03[a]
13 (10)	0.85 ± 0.07	1.55 ± 0.08	1.71 ± 0.08	1.73 ± 0.06	1.75 ± 0.13	1.63 ± 0.15[a]
13 (20)	0.92 ± 0.07	1.32 ± 0.07[c]	1.44 ± 0.05[b]	1.62 ± 0.04[c]	1.49 ± 0.05[a]	1.36 ± 0.07[a]
21 (10)	0.83 ± 0.06	1.49 ± 0.06	1.56 ± 0.07	1.74 ± 0.06	1.69 ± 0.04	1.57 ± 0.07[a]
21 (20)	0.95 ± 0.07	1.34 ± 0.06[c]	1.48 ± 0.05[c]	1.65 ± 0.05	1.65 ± 0.05[c]	1.53 ± 0.04[a]
22 (10)	1.05 ± 0.04	1.62 ± 0.04	1.75 ± 0.04	1.76 ± 0.05	1.68 ± 0.09[c]	1.61 ± 0.09[a]
22 (20)	0.95 ± 0.04	1.54 ± 0.07	1.71 ± 0.07	1.64 ± 0.07[c]	1.60 ± 0.04[b]	1.51 ± 0.02[a]
23 (10)	0.97 ± 0.04	1.60 ± 0.10	1.67 ± 0.09	1.86 ± 0.02	1.79 ± 0.04	1.66 ± 0.03[a]
23 (20)	0.99 ± 0.04	1.56 ± 0.06	1.61 ± 0.06	1.81 ± 0.02	1.73 ± 0.03	1.61 ± 0.04[a]
24 (10)	0.91 ± 0.05	1.62 ± 0.06	1.74 ± 0.05	1.78 ± 0.04	1.77 ± 0.04	1.69 ± 0.04[a]
24 (20)	1.00 ± 0.04	1.57 ± 0.07	1.57 ± 0.09	1.55 ± 0.06[b]	1.51 ± 0.06[a]	1.45 ± 0.03[a]
25 (10)	0.88 ± 0.05	1.52 ± 0.11	1.61 ± 0.04	1.70 ± 0.04	1.52 ± 0.09[a]	1.49 ± 0.07[a]
25 (20)	0.90 ± 0.05	1.28 ± 0.06[b]	1.42 ± 0.05[b]	1.47 ± 0.04[a]	1.32 ± 0.05[a]	1.25 ± 0.02[a]
Diclofenac (20)	0.89 ± 0.04	1.22 ± 0.05[b]	1.25 ± 0.05[a]	1.30 ± 0.03[a]	1.16 ± 0.02[a]	1.13 ± 0.03[a]

Values are given as mean ± S.E.M. for groups of six animals each, Dunnet′s test; values are statistically significant at [a]P<0.001, [b]P<0.01, [c]P<0.05 between control and treated groups.

Table 8. Percentage protection of embelin and its derivatives against carrageenan induced paw edema in rats

Treatment (dose, mg/kg, i.p.)	% Protection				
	30 min	**60 min**	**120 min**	**180 min**	**360 min**
1 (10)	0.61	2.26	3.15	7.14	15.49
1 (20)	13.50	12.99	14.74	18.88	31.92
10 (10)	1.84	3.95	3.15	6.63	18.31
10 (20)	8.59	4.50	8.42	18.31	28.64
13 (10)	4.90	3.39	8.95	10.71	23.47
13 (20)	19.02	18.64	14.74	23.98	36.15
21 (10)	8.59	11.86	8.42	13.78	26.29
21 (20)	17.79	16.38	13.16	15.82	28.17
22 (10)	0.61	0.11	7.37	14.29	23.47
22 (20)	5.52	3.39	13.68	18.37	29.11
23 (10)	1.84	5.65	2.11	8.67	22.07
23 (20)	4.30	9.04	4.74	11.73	24.41
24 (10)	0.06	1.70	6.32	9.69	20.67
24 (20)	3.69	11.30	18.42	22.96	31.92
25 (10)	6.75	9.04	10.53	22.45	30.05
25 (20)	21.47	19.77	22.63	32.65	41.31
Diclofenac (20)	25.15	29.38	31.58	40.82	46.94

Values are given as mean ± S.E.M. for groups of six animals each, Dunnet′s test; values are statistically significant at $^{a}P<0.001$, $^{b}P<0.01$, $^{c}P<0.05$ between control and treated groups.

Table 9. Effect of embelin and its ninhydrin adduct on carrageenan induced paw edema

Treatment	Paw volume, ml after min (% Protection)					
(dose, mg/kg, i.p.)	0	30	60	120	180	360
Control	0.84 ± 0.02	1.63 ± 0.04	1.77 ± 0.05	1.90 ± 0.07	1.96 ± 0.07	2.13 ± 0.06
Embelin (10)	0.90 ± 0.04	1.62 ± 0.05	1.73 ± 0.04	1.84 ± 0.11	1.82 ± 0.03	1.80 ± 0.07[b]
		(0.61)	(2.26)	(3.15)	(7.14)	(15.49)
Embelin (20)	0.80 ± 0.02	1.41 ± 0.06[c]	1.54 ± 0.07[c]	1.62 ± 0.06	1.59 ± 0.05[a]	1.45 ± 0.07[a]
		(13.50)	(12.99)	(14.74)	(18.88)	(31.92)
Embelin-ninhydrin	0.93 ± 0.03	1.54 ± 0.04	1.56 ± 0.05	1.51 ± 0.08[b]	1.42 ± 0.10[a]	1.42 ± 0.06[a]
adduct (10)		(5.52)	(11.86)	(20.53)	(27.55)	(33.33)
Embelin-ninhydrin	0.99 ± 0.04	1.50 ± 0.04	1.42 ± 0.06[a]	1.38 ± 0.06[a]	1.36 ± 0.04[a]	1.29 ± 0.03[a]
adduct (20)		(8.55)	(19.77)	(27.37)	(30.61)	(39.44)
Diclofenac (20)	0.89 ± 0.04	1.22 ± 0.05[a]	1.25 ± 0.05[a]	1.30 ± 0.03[a]	1.16 ± 0.02[a]	1.13 ± 0.03[a]
		(25.15)	(29.38)	(31.58)	(40.82)	(46.94)

Values are given as mean ± S.E.M. for groups of six animals each, Dunnet′s test; values are statistically significant at [a]P<0.001, [b]P<0.01, [c]P<0.05 between control and treated groups.

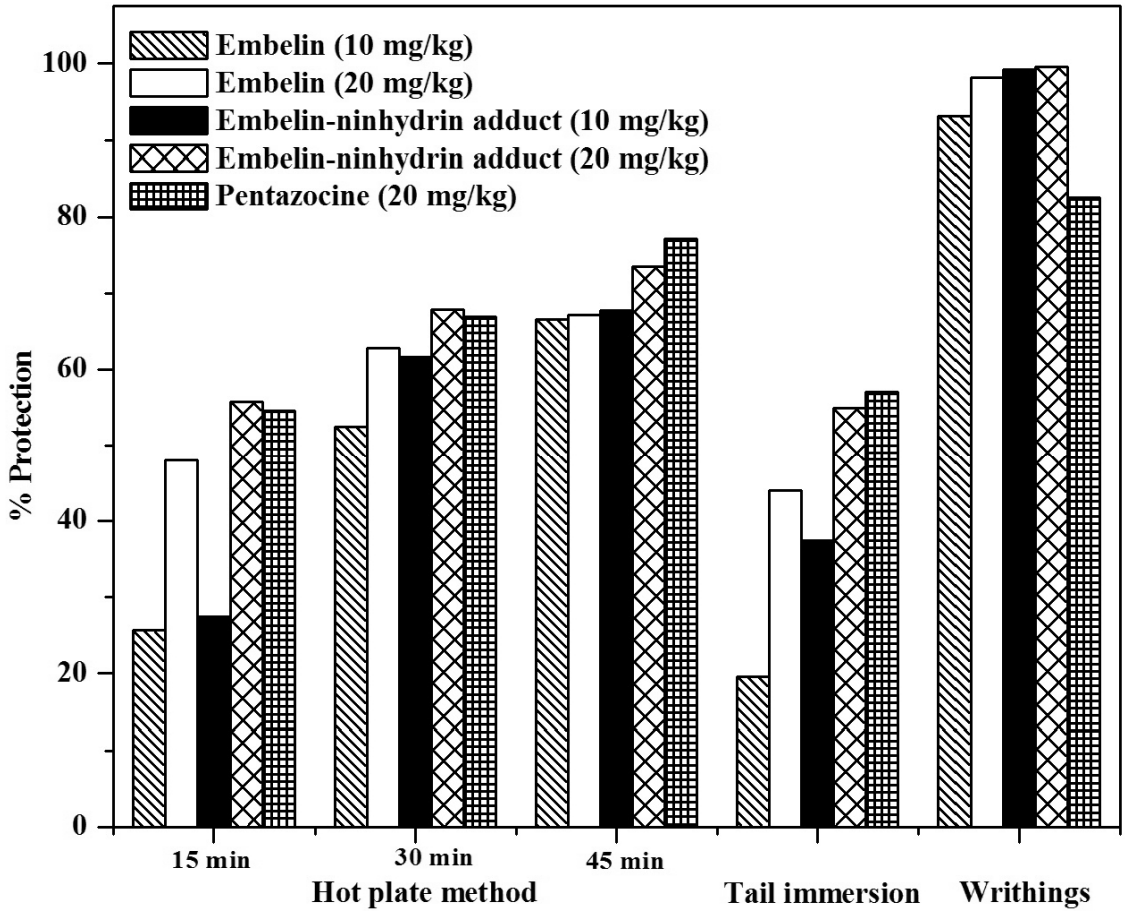


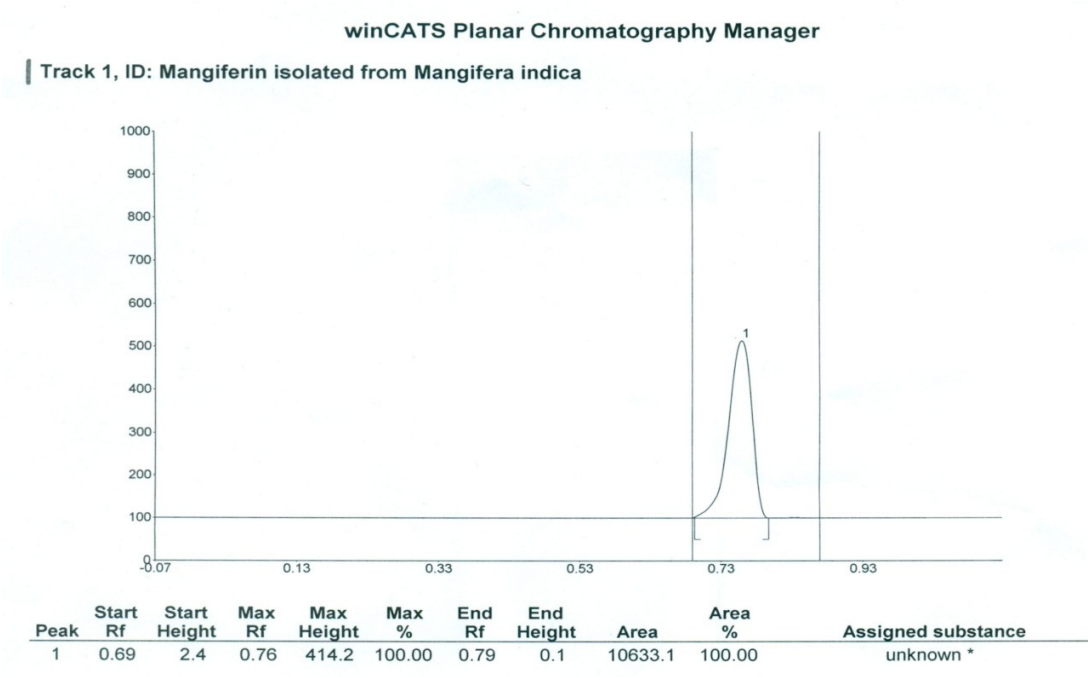
Fig. 111. Percentage protection of embelin and its ninhydrin adduct in various analgesic methods

5.5. Structural modification of mangiferin

5.5.1. 2-b-D-glucopyranosyl-1,3,6,7-tetrahydroxy-9*H*-xanthen-9-one, mangiferin (MGN)

Mangiferin was isolated from the leaves of *Mangifera indica,* found to be homogenous by HPTLC (Fig. 112) when separated using the solvent system ethyl acetate: methanol: water: formic acid (6:2:1:1, R_f = 0.76). Obtained as pale-yellow needle shaped crystals, mp 270-272 ºC; yield 4.5 g, 0.45%; IR υ_{max} (KBr) cm^{-1}: 3367 (OH), 1651 (C=O), 1622 and 1585 (aromatic C=C), 1199 and 1095 (C–O), 1051, 829, 588 (Fig. 113); ^{1}H NMR

(400 MHz, DMSO) δ : see Table 10 and Fig. 114; negative ESI-MS (Fig. 115): m/z calculated for 422.08, Found : 444 for [M +Na – H].



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.69	2.4	0.76	414.2	100.00	0.79	0.1	10633.1	100.00	unknown *

Fig. 112. HPTLC Spectrum of mangiferin (MGN)

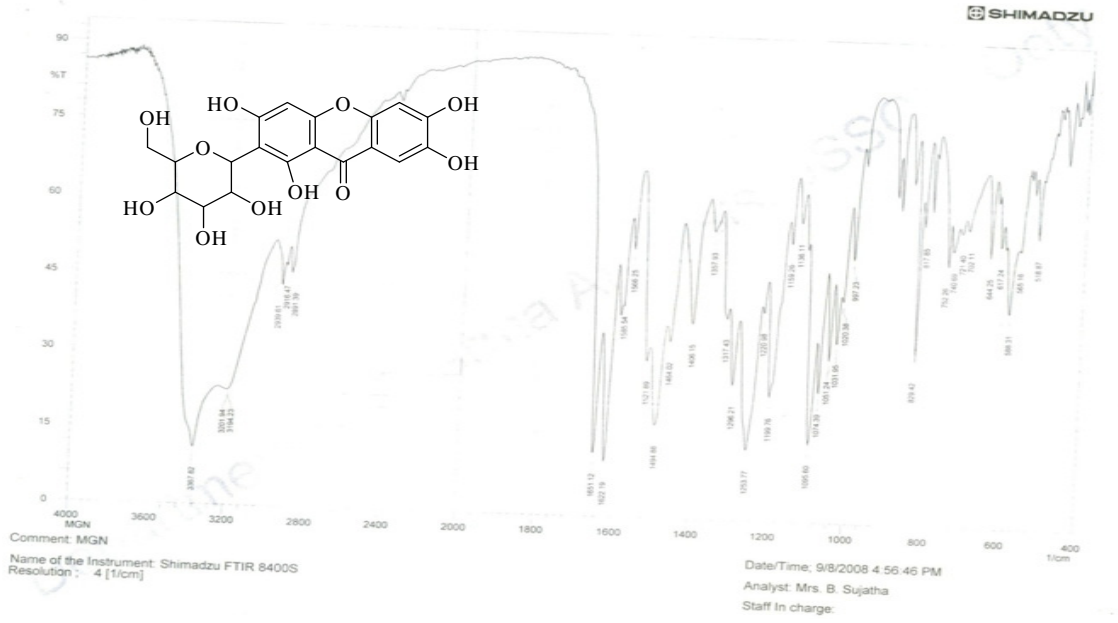


Fig. 113. IR spectrum of mangiferin (MGN)

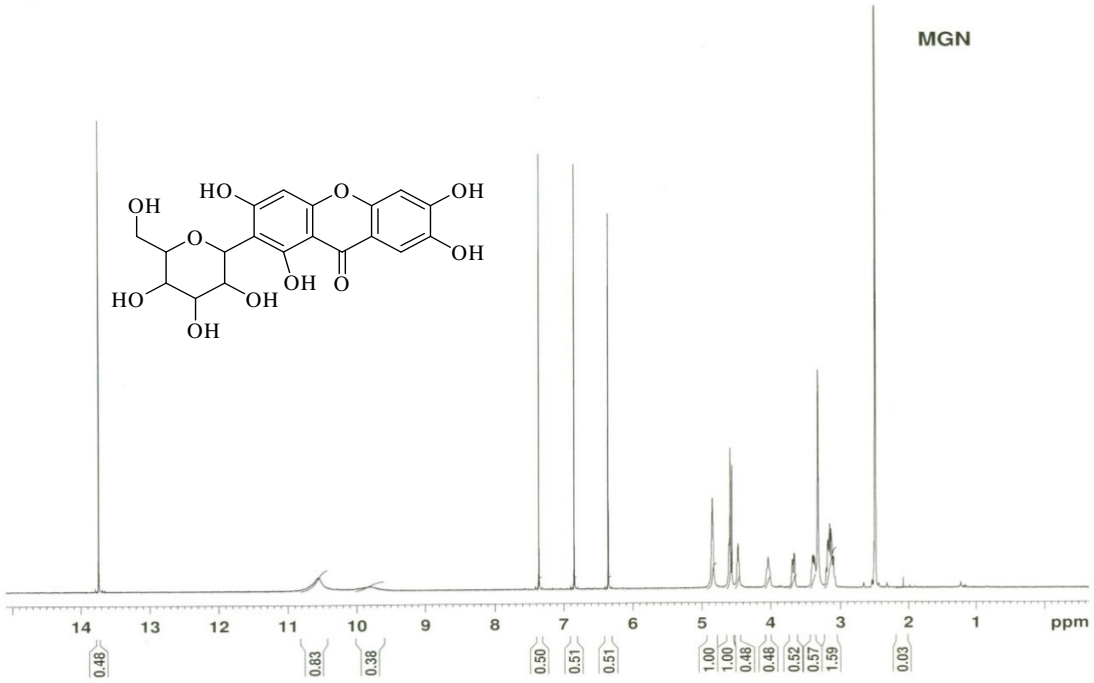


Fig. 114. ^{1}H NMR spectrum of mangiferin (MGN)

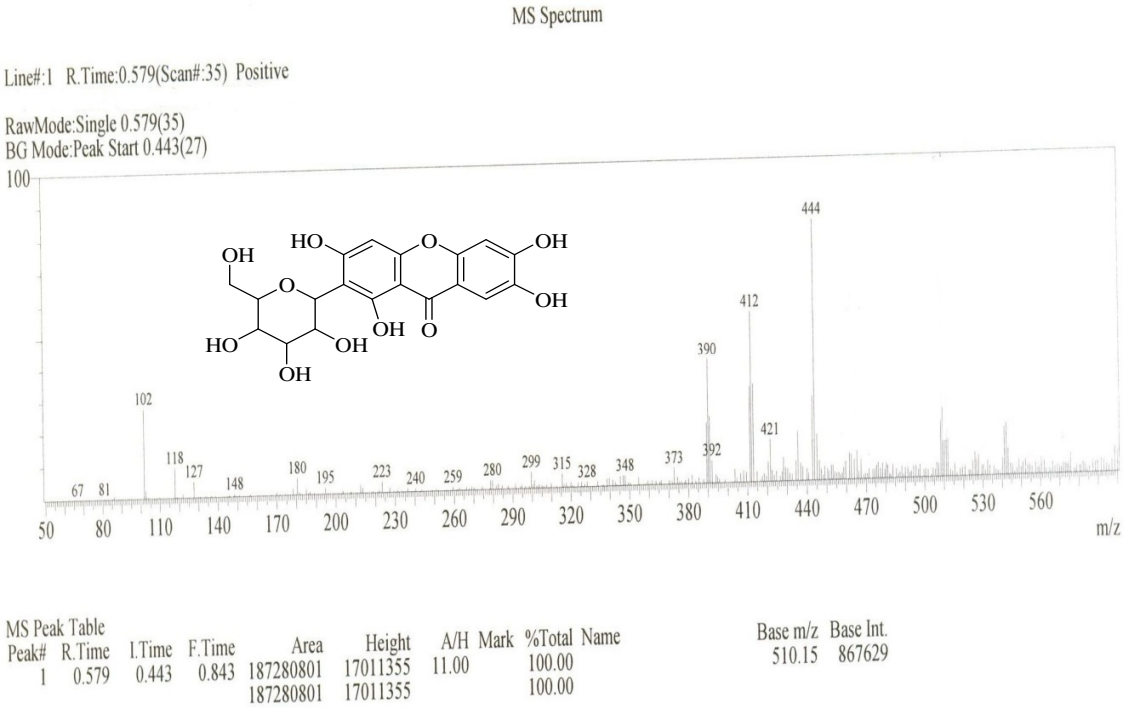


Fig. 115. Mass spectrum of mangiferin (MGN)

5.5.2. 2-b-D-tetrahydroxyglucopyranosyl-3,6,7-tribenzoyl-1-hydroxy-9*H*-xanthen-9-one (MGN 1)

Obtained as yellow crystals, mp 181-183 ºC; yield 0.78 g, 56.07%; IR υ_{max} (KBr) cm^{-1}: 3352 (OH), 1747, 1742 (carbonyl ester), 1618 (C=O), 1600 and 1556 (aromatic C=C), 1259 and 1091 (C–O), 1022, 821, 705, 567 (Fig. 116); ^{1}H NMR (400 MHz, DMSO) δ : see Table 10 and Fig. 117; negative ESI-MS (Fig. 118): m/z calculated for 734.16, Found: 755 for [M +Na – 2H].

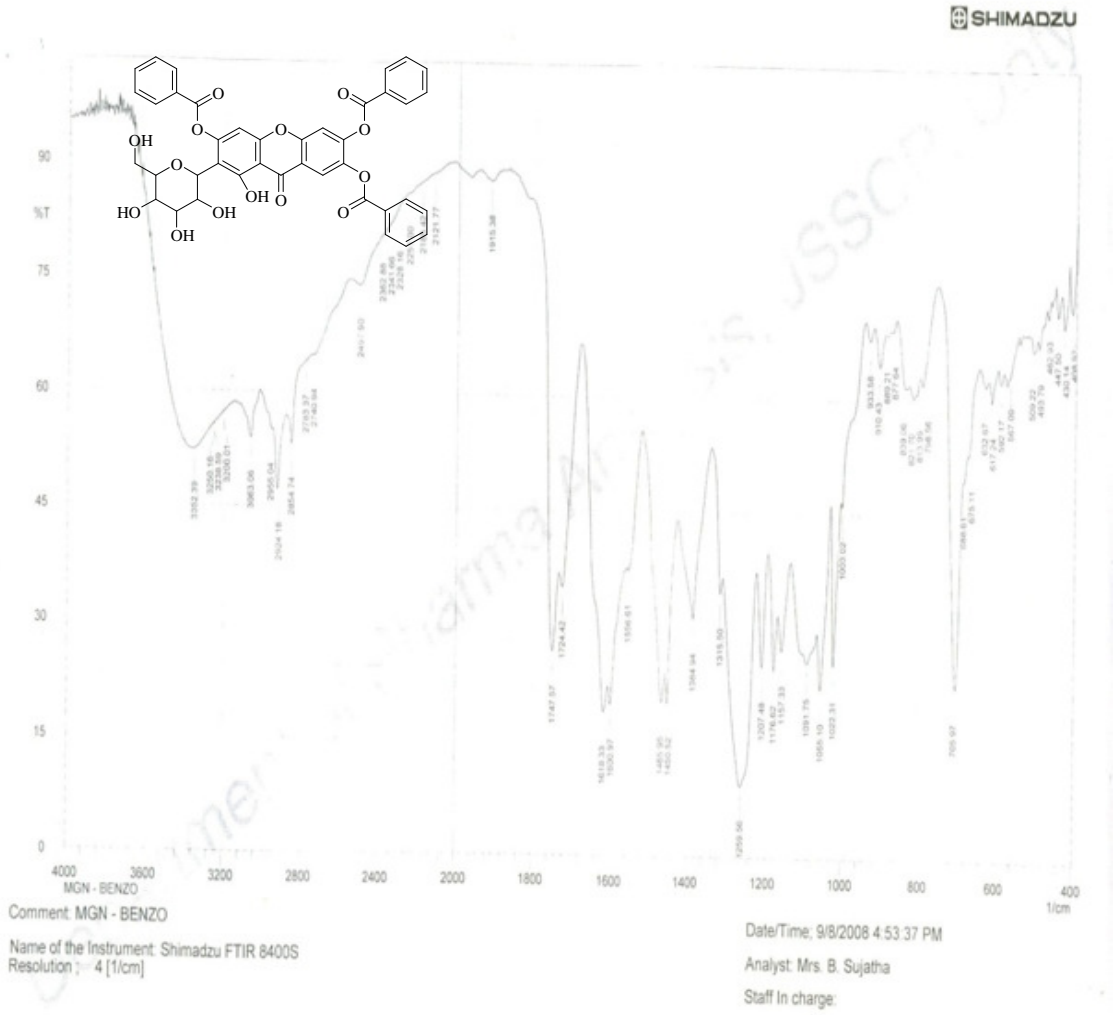


Fig. 116. IR spectrum of benzoyl mangiferin (MGN 1)

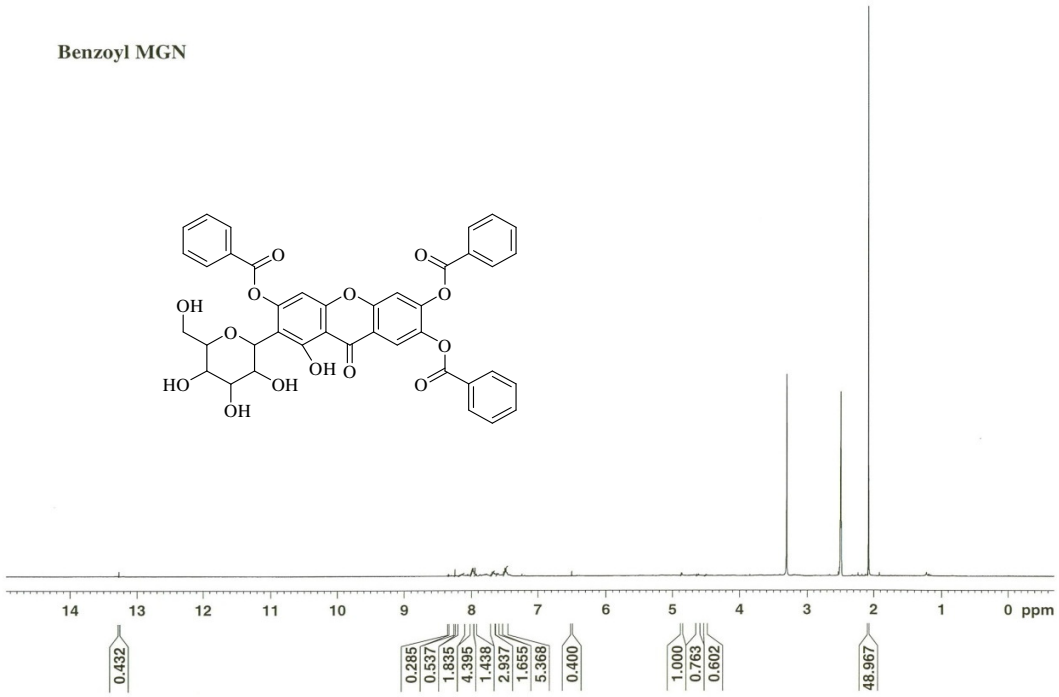


Fig. 117. ^{1}H NMR spectrum of benzoyl mangiferin (MGN 1)

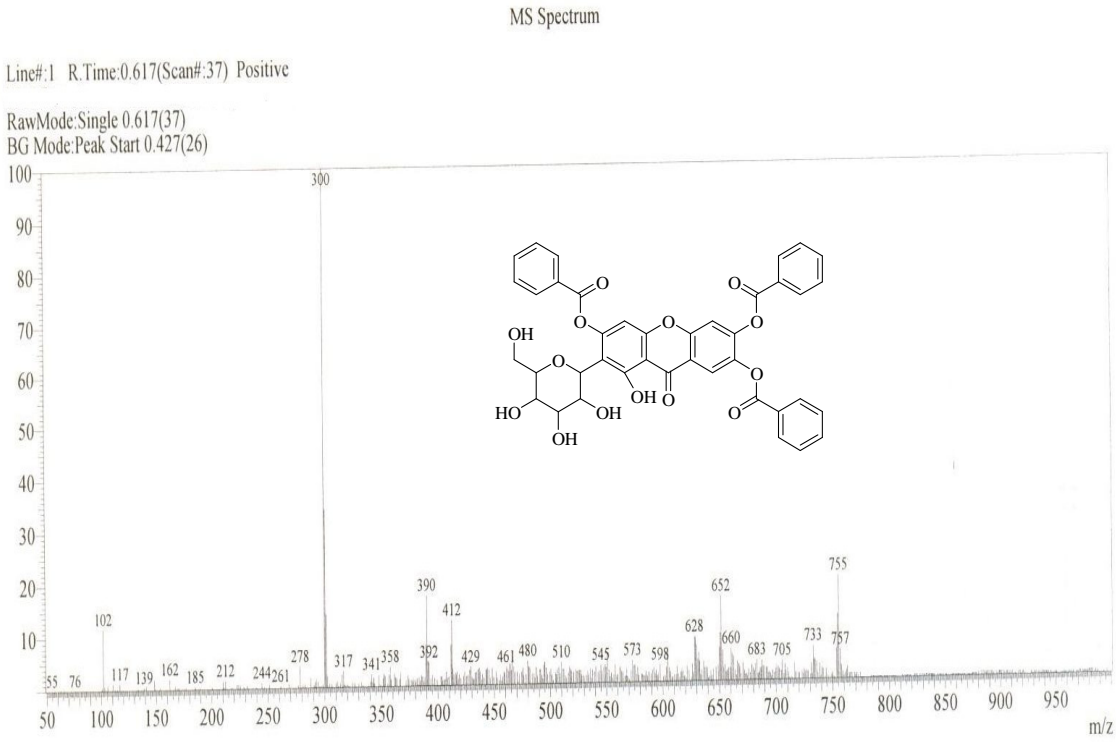


Fig. 118. Mass spectrum of benzoyl mangiferin (MGN 1)

5.5.3. 2-b-D-tetrahydroxyglucopyranosyl-3,6,7-tribenzyl-1-hydroxy-9*H*-xanthen-9-one (MGN 2)

Obtained as yellow powder, mp 137-139 ºC; yield 0.53 g, 82.68%; IR υ_{max} (KBr) cm^{-1}: 3392 (OH), 1647 (C=O), 1608 and 1577 (aromatic C=C), 1190 and 1080 (C–O), 1024, 808, 734, 696 (Fig. 119); ^{1}H NMR (400 MHz, DMSO) δ : see Table 10 and Fig. 120; negative ESI-MS (Fig. 121): m/z calculated for 692.23, Found : 689 for [M – 3H].

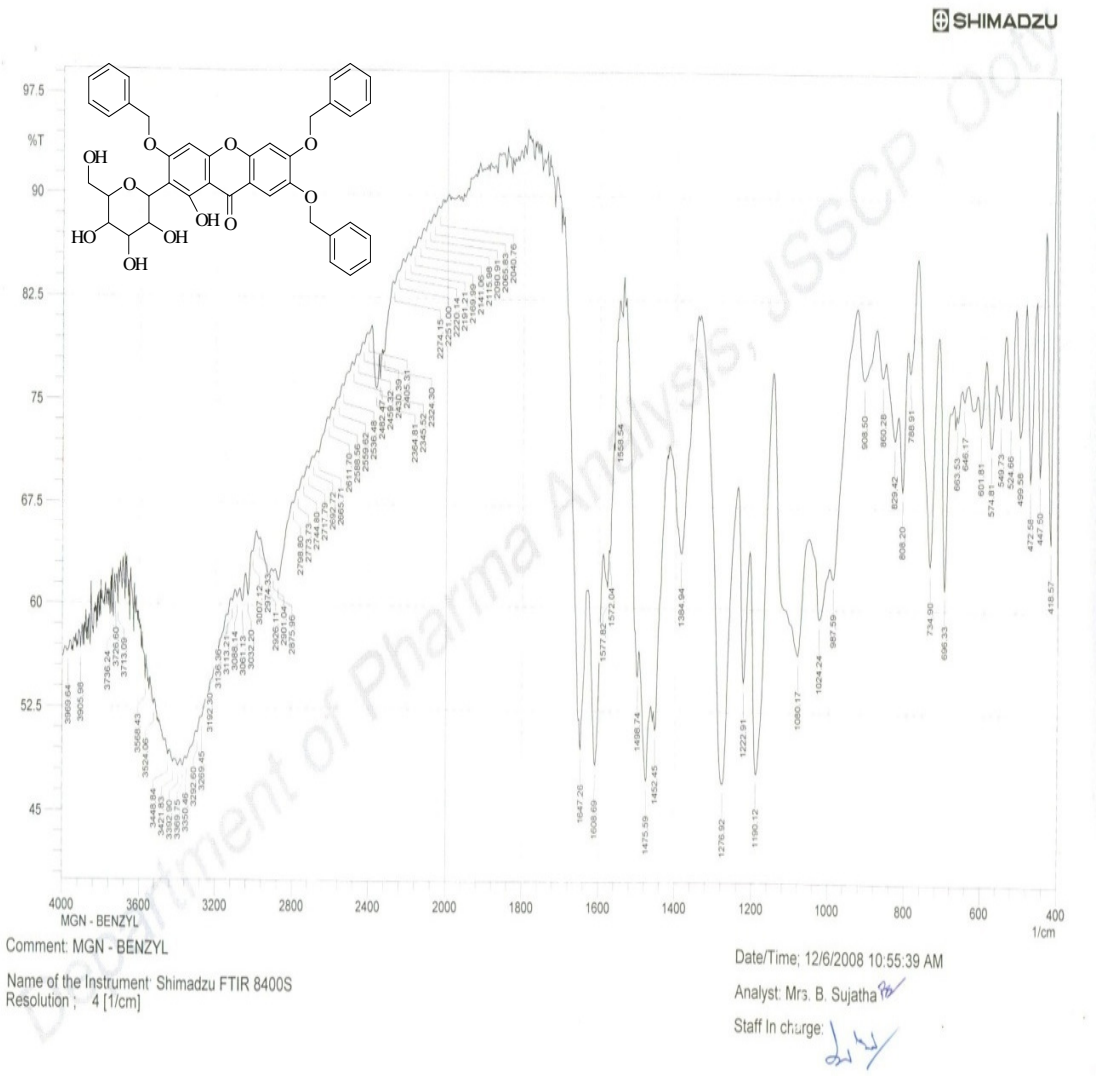


Fig. 119. IR spectrum of benzyl mangiferin (MGN 2)

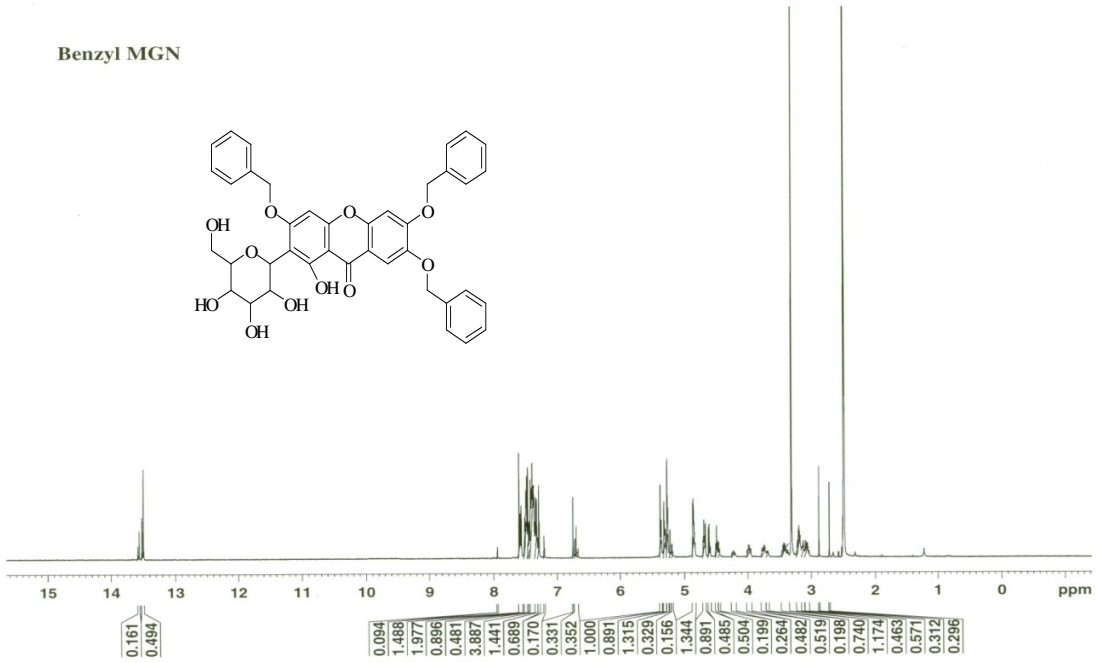


Fig. 120. ^{1}H NMR spectrum of benzyl mangiferin (MGN 2)

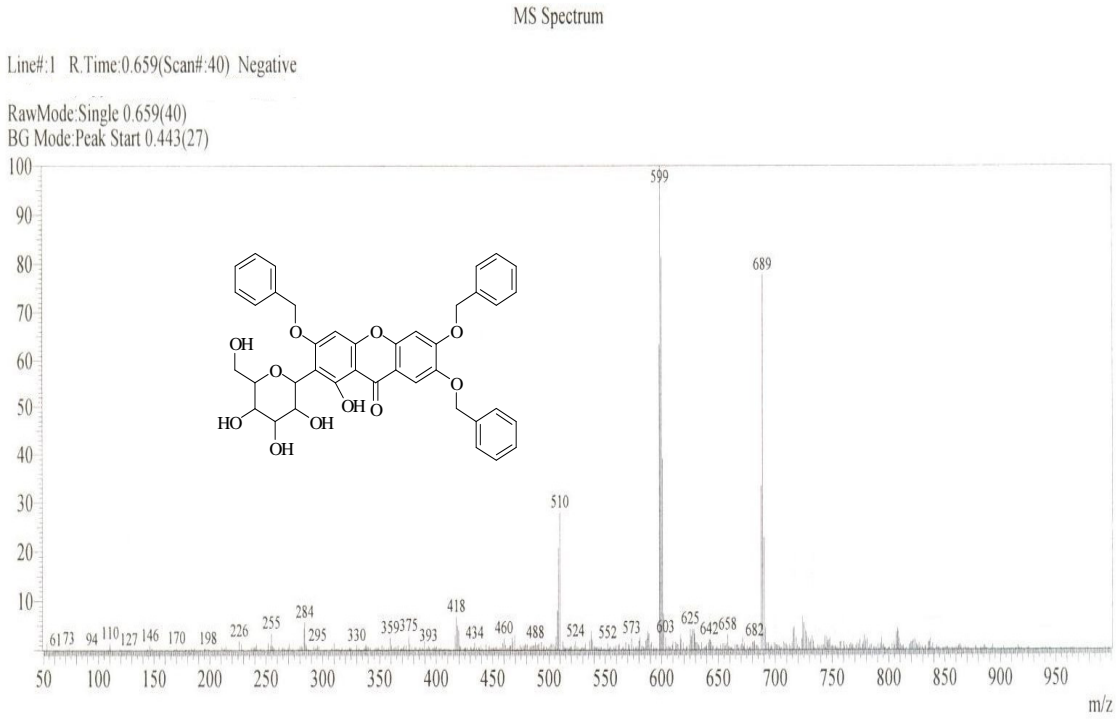


Fig. 121. Mass spectrum of benzyl mangiferin (MGN 2)

5.5.4. 2-b -D-tetraacetoxyglucopyranosyl-1,3,6,7-tetraacetoxy-9*H*xanthen-9-one (MGN 3)

Obtained as white amorphous powder, mp 141-143 ºC; yield 0.60 g, 79.58%; IR υ_{max} (KBr) cm^{-1}: 1751 (Carbonyl ester), 1651 (C=O), 1620 (C=C), 1224 and 1055 (C–O), 1039, 920, 839, 601 (Fig. 122); ^{1}H NMR (400 MHz, DMSO) δ : see Table 10 and Fig. 123; negative ESI-MS (Fig. 124): m/z calculated for 758.17, Found : 755 for [M – 3H].

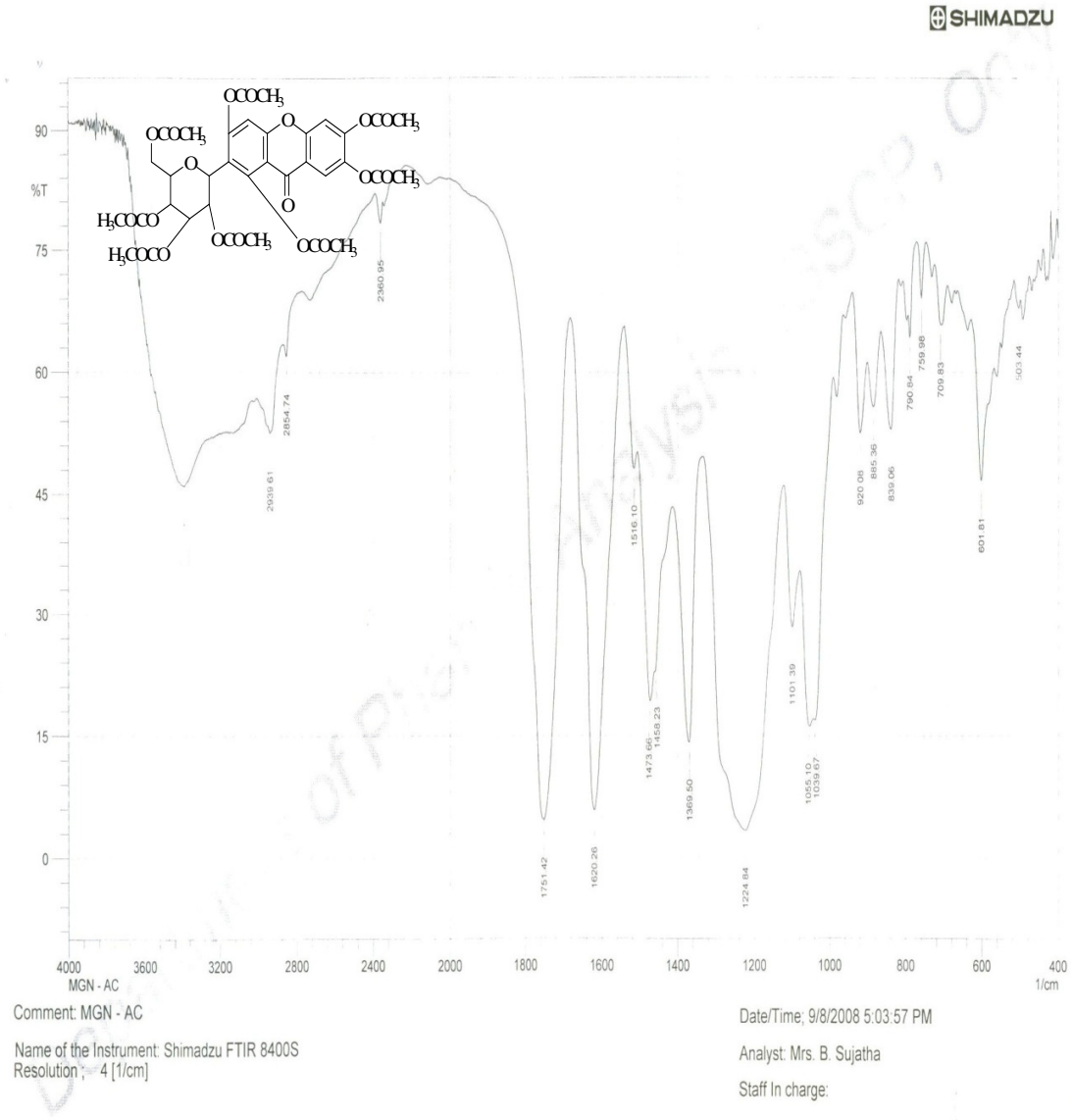


Fig. 122. IR spectrum of acetyl mangiferin (MGN 3)

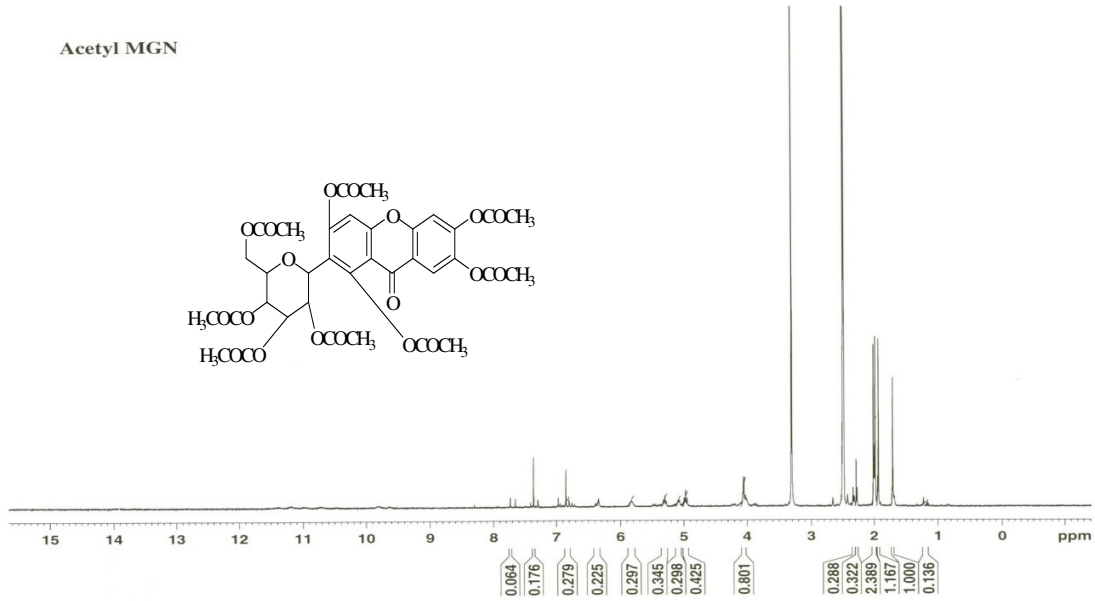


Fig. 123. ^{1}H NMR spectrum of acetyl mangiferin (MGN 3)

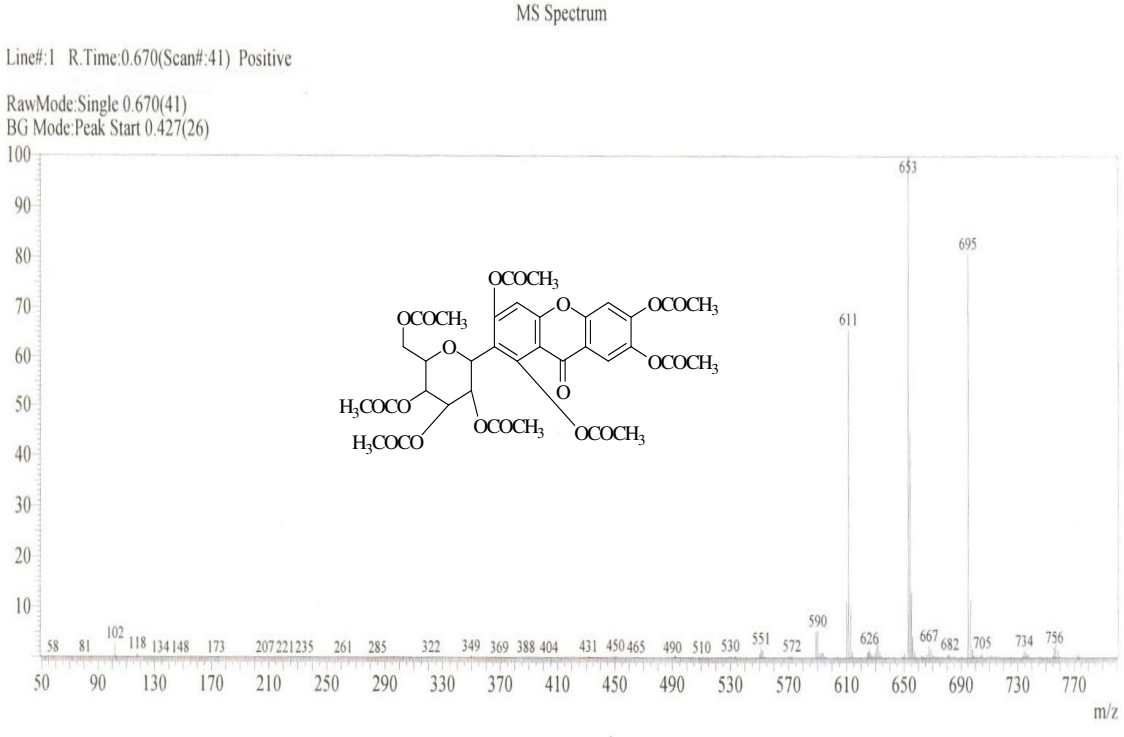


Fig. 124. Mass spectrum of acetyl mangiferin (MGN 3)

5.5.5. 2-b -D-tetrahydroxyglucopyranosyl-3,6,7-trimethoxy-1-hydroxy-9*H*-xanthen-9-one (MGN 4)

Obtained as white amorphous powder, mp 193-195 ºC; yield 0.40 g, 86.58%; IR υ_{max} (KBr) cm^{-1}: 3367 (OH), 1647 (C=O), 1606 (C=C), 1217 and 1064 (C–O), 1022, 819, 759, 619, 576 (Fig. 125); ^{1}H NMR (400 MHz, DMSO) δ : see Table 10 and Fig. 126; negative ESI-MS (Fig. 127): m/z calculated for 464.13, Found : 464.

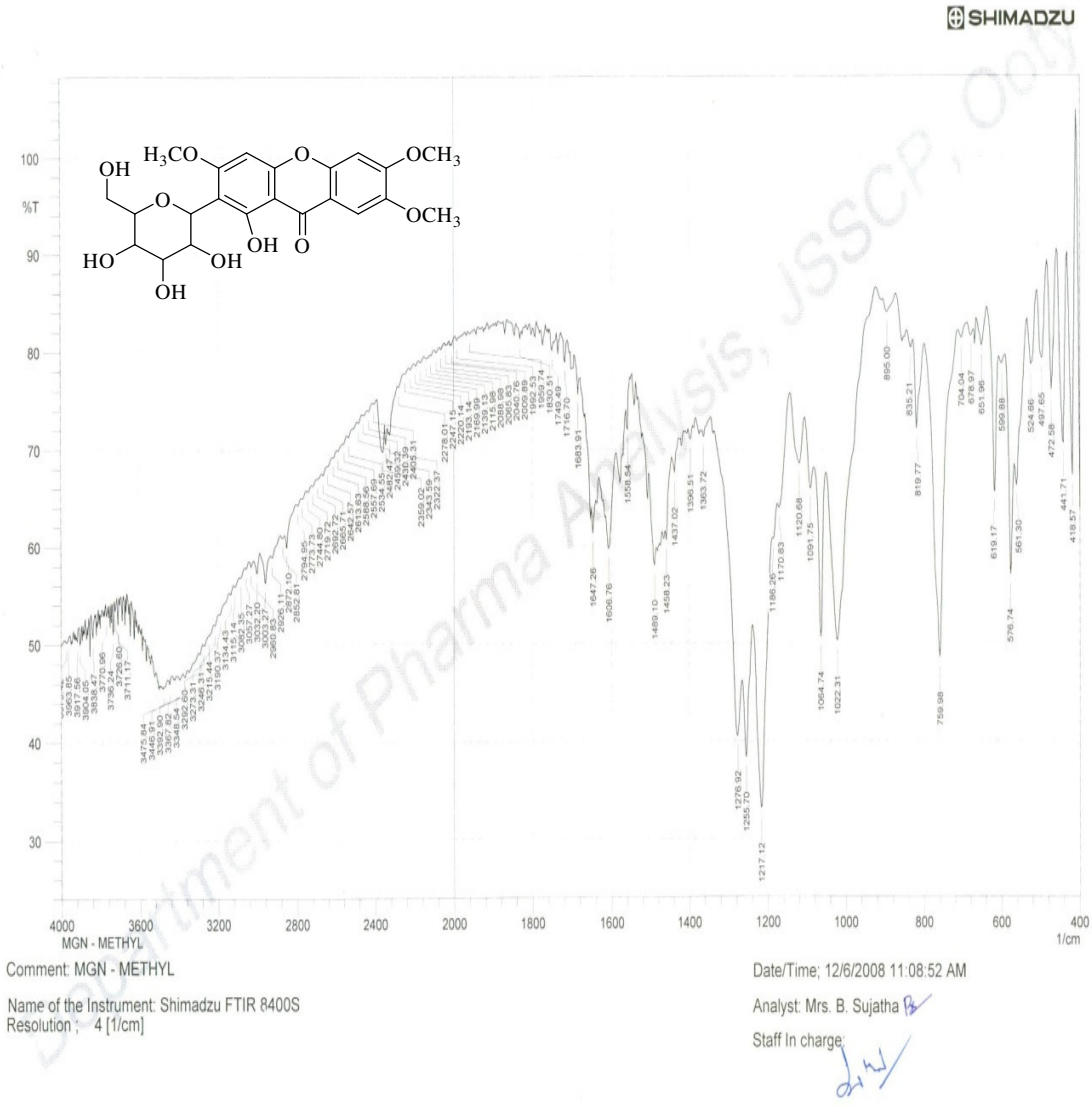


Fig. 125. IR spectrum of methyl mangiferin (MGN 4)

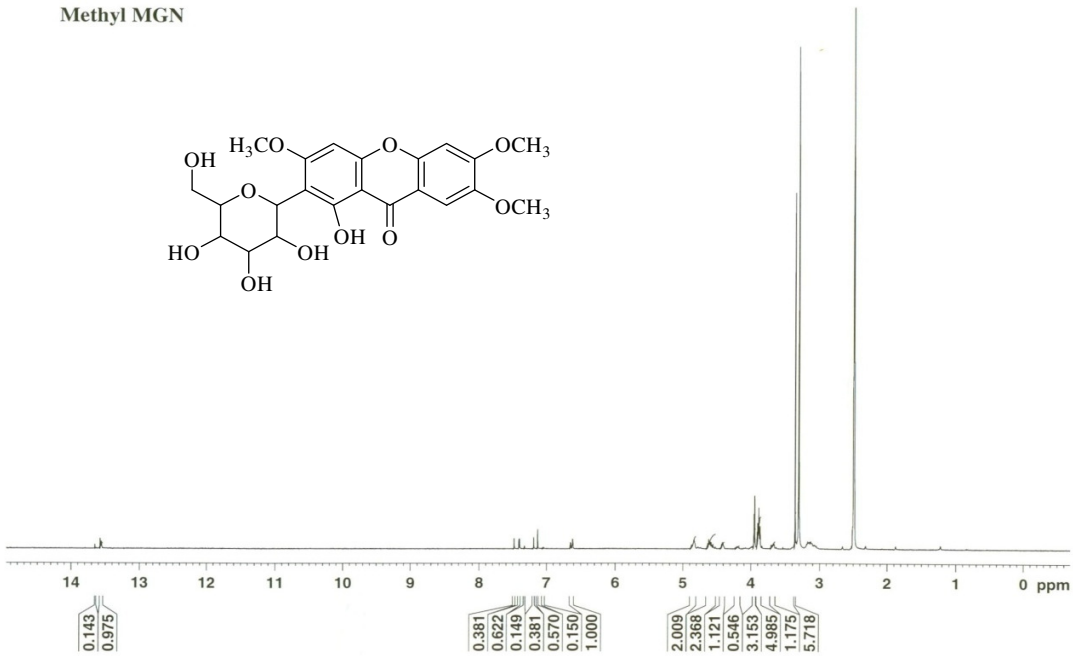


Fig. 126. ^{1}H NMR spectrum of methyl mangiferin (MGN 4)

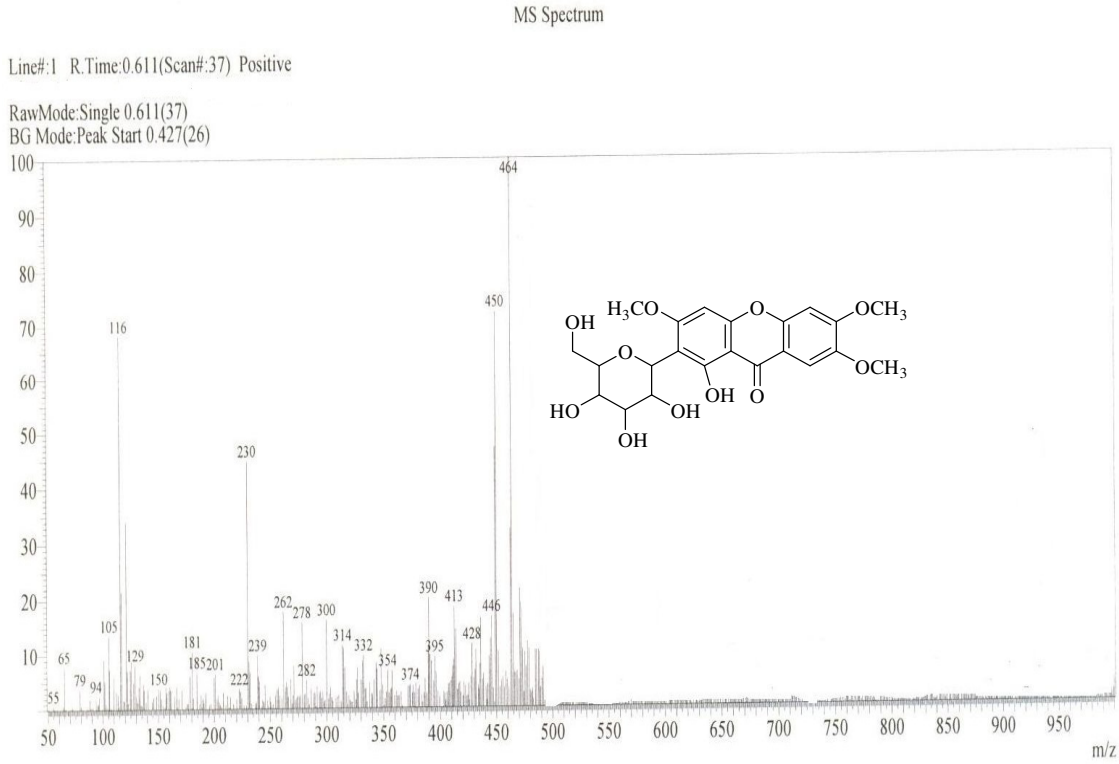


Fig. 127. Mass spectrum of methyl mangiferin (MGN 4)

Table 10. ^{1}H NMR spectrum of mangiferin and its derivatives

Proton	Reported	MGN	MGN 1	MGN 2	MGN 3	MGN 4
1-OH	13.76 (1H)	13.50 (1H)	13.50 (1H)	13.50 (1H)	-	13.50 (1H)
6-OH	10.55 (1H)	10.80 (1H)	-	-	-	-
7-OH	10.55 (1H)	10.80 (1H)	-	-	-	-
3-OH	9.86 (1H)	9.80 (1H)	-	-	-	-
3′,4′-OH	4.86 (2H)	4.90 (2H)	4.95 (2H)	4.90 (2H)	-	4.90 (2H)
6′-OH	4.49 (1H)	4.49 (1H)	4.50 (1H)	4.50 (1H)	-	4.49 (1H)
2′-OH	3.87 (1H)	4.60 (1H)	4.65 (1H)	3.75 (1H)	-	3.75 (1H)
8-H	7.38 (1H)	7.40 (1H)	7.45 (1H)	7.45 (1H)	7.40 (1H)	7.45 (1H)
5-H	6.86 (1H)	6.90 (1H)	7.00 (1H)	7.30 (1H)	6.90 (1H)	6.65 (1H)
4-H	6.37 (1H)	6.40 (1H)	6.50 (1H)	6.75 (1H)	6.40 (1H)	6.65 (1H)
1′-H	4.60 (1H)	4.60 (1H)	4.85 (1H)	4.70 (1H)	4.90 (1H)	4.60 (1H)
2′-H	4.05 (1H)	4.05 (1H)	4.00 (1H)	4.00 (1H)	4.00 (1H)	3.95 (1H)
6′a-H	3.69 (1H)	3.69 (1H)	4.50 (1H)	3.40 (1H)	4.00 (1H)	3.65 (1H)
6′b-H	3.42 (1H)	3.41 (1H)	4.60 (1H)	3.20 (1H)	4.00 (1H)	3.30 (1H)
3′-H	3.18 (1H)	3.18 (1H)	4.60 (1H)	3.40 (1H)	5.80 (1H)	3.30 (1H)
4′-H	3.18 (1H)	3.18 (1H)	4.85 (1H)	3.20 (1H)	5.30 (1H)	3.30 (1H)
5′-H	3.18 (1H)	3.18 (1H)	4.90 (1H)	3.20 (1H)	5.20 (1H)	3.30 (1H)
CH_3CO	-	-	-	-	1.20-2.00 (24 H)	-
Benzene ring	-	-	7.20-8.30 (15H)	7.30 -7.50 (15H)	-	-
$-CH_2$	-	-	-	5.25-5.40 (6H)	-	-
$-OCH_3$	-	-	-	-	-	4.60-4.90 (9H)
Total number of protons	18	18	30	36	34	24

5.6. *In vitro* antioxidant screening of mangiferin and its derivatives

Mangiferin showed potent antioxidant activity with IC_{50} values 1.72 ± 0.07, 0.09 ± 0.01, 8.39 ± 1.09 and 32.58 ± 0.34 µg/ml in DPPH, ABTS, H_2O_2 and nitric oxide methods, respectively. Mangiferin failed to show potent activity in rest of the methods. Benzoyl (MGN 1) and acetyl (MGN 2) substituted mangiferin were showed potent to moderate activity in all the methods. MGN 1 and 3 were showed better activity than mangiferin in deoxyribose, p-NDA and lipid peroxidation methods. In alkaline DMSO method, only MGN 1 exhibited potent activity (Table 11). In p-NDA, lipid peroxidation and alkaline DMSO methods, MGN 1 showed better activity than the standards. Mangiferin substituted with acetyl and benzoyl groups was beneficial for the activity. Based on these results, MGN 1 and 3 along with mangiferin were chosen for comparing their *in vivo* analgesic and anti-inflammatory activities.

Table 11. *In vitro* antioxidant acivity of mangiferin and its derivatives using standard methods

Compound	IC_{50} values ± SEM (µg/ml) by methods*							
	DPPH	**ABTS**	**Deoxyribose**	**p-NDA**	**H_2O_2**	**Nitric oxide**	**Lipid peroxidation**	**Alkaline DMSO**
Mangiferin	1.72 ± 0.07	0.09 ± 0.01	502.20 ± 3.20	106.84 ± 0.07	8.39 ± 1.09	32.58 ± 0.34	433.20 ± 4.08	>1000
MGN 1	23.80 ± 0.89	0.65 ± 0.02	87.58 ± 2.21	24.13 ± 1.05	23.57 ± 0.34	52.67 ± 1.30	8.90 ± 0.98	152.30 ± 3.63
MGN 2	>1000	2.41 ± 0.08	>1000	>1000	215.02 ± 3.11	>1000	>1000	>1000
MGN 3	36.47 ± 0.81	3.71 ± 0.14	406.30 ± 2.85	31.53 ± 3.72	81.23 ± 1.05	>1000	29.80 ± 1.27	>1000
MGN 4	>1000	2.23 ± 0.06	>1000	>1000	>1000	>1000	>1000	>1000
Ascorbic acid	4.92 ± 0.28	11.25 ± 0.49	-	>1000	193.45 ± 2.30	-	-	>1000
Rutin	8.91 ± 0.10	0.52 ± 0.04	-	205.54 ± 3.25	32.35 ± 1.02	65.21 ± 2.97	-	>1000
BHA	-	-	83.46 ± 4.34	-	22.16 ± 0.56	-	110.02 ± 3.41	-

*Average of three determinations

5.7. Analgesic activity of selected mangiferin derivatives

In the hot plate method, a significant increase in the response time was observed for mangiferin and MGN 1 at both the doses after 15, 30 and 45 min, MGN 3 at 20 mg/kg after 30 and 45 min, when compared to control. Similar results were observed for standard pentazocine at 20 mg/kg. MGN 1 showed better results than mangiferin. However, the strandard pentazocine produced better results compared to the other treated groups. The percentage protection after 45 min for all the compounds ranged between 51.59 to 72.45% (Table 12). A significant increase in the tail withdrawal reflex was observed for MGN 1 at both the doses and mangiferin at 20 mg/kg. MGN 1 showed better activity than mangiferin. The percent protection was found to be 9.11 to 55.34%.

Injection of acetic acid into control mice produced 43.83 ± 2.32 writhes. Pretreatment with mangiferin and its derivatives at both the doses significantly reduced the number of writhes (Table 12). Mangiferin at both the doses showed better activity than its derivatives. The percent protection was found to be 37.26 to 63.11%. However, the standard pentazocine showed better and significant results than all other treated groups.

5.8. Anti-inflammatory activity of selected mangiferin derivatives

In carrageenan induced paw edema in rats, mangiferin and MGN 1 produced significant activity at 20 mg/kg dose during 30 to 360 min measurements, compared to the control. Mangiferin, MGN 1 at 10 mg/ kg and MGN 3 at 20 mg/kg showed significantly reduced paw edema after 120, 180 and 360 min (Table 13). However, the standard diclofenac at 20 mg/kg also produced similar and better results than the tested samples. The percent protection was found to be 28.17 to 37.09 after 360 min measurements.

Table 12. Analgesic activity of mangiferin and its derivatives using standard methods

Treatment (dose, mg/kg, i.p.)	Hot plate test			Tail immersion	Acetic acid induced writhing
	Latency period , sec (% Protection)			Latency period, sec	No. of writhing
	15 min	30 min	45 min	(% Protection)	(% Protection)
Control	2.32 ± 0.25	2.27 ± 0.26	2.43 ± 0.27	4.39 ± 0.60	43.83 ± 2.32
Mangiferin (10)	3.21 ± 0.25	4.58 ± 0.47[b]	6.46 ± 0.71[b]	5.70 ± 0.50	23.33 ± 2.99[a]
	(27.73)	(50.44)	(62.38)	(22.98)	(46.77)
Mangiferin (20)	4.97 ± 0.32[a]	5.46 ± 0.54[a]	7.35 ± 0.95[a]	8.69 ± 0.49[a]	16.17 ± 2.33[a]
	(53.32)	(58.42)	(66.94)	(49.48)	(63.11)
MGN 1 (10)	3.90 ± 0.47[c]	4.75 ± 0.45[b]	7.14 ± 0.50[a]	7.64 ± 0.60[c]	24.17 ± 2.93[a]
	(40.51)	(52.21)	(65.97)	(42.54)	(44.85)
MGN 1 (20)	6.16 ± 0.72[a]	6.66 ± 0.27[a]	8.82 ± 0.51[a]	9.83 ± 0.89[a]	17.33 ± 1.69[a]
	(62.34)	(65.92)	(72.45)	(55.34)	(60.46)
MGN 3 (10)	3.08 ± 0.19	3.85 ± 0.46	5.02 ± 0.42	4.83 ± 0.41	27.50 ± 2.32[a]
	(24.68)	(41.04)	(51.59)	(9.11)	(37.26)
MGN 3 (20)	3.61 ± 0.33	4.58 ± 0.50[b]	5.79 ± 0.44[b]	7.04 ± 0.81	23.50 ± 2.69[a]
	(35.73)	(50.44)	(58.03)	(37.64)	(46.38)
Pentazocine (20)	5.11 ± 0.32[a]	6.85 ± 0.50[a]	10.61 ± 1.14[a]	10.21 ± 1.00[a]	7.67 ± 1.45[a]
	(54.60)	(66.86)	(77.10)	(57.00)	(82.50)

Values are given as mean ± S.E.M. for groups of six animals each, Dunnet′s test; values are statistically significant at [a]P<0.001, [b]P<0.01, [c]P<0.05 between control and treated groups.

Table 13. Anti-inflammatory activity of mangiferin and its derivatives against carrageenan induced paw edema in rats

Treatment	Paw volume, ml after min (% Protection)					
(dose, mg/kg, i.p.)	0	30	60	120	180	360
Control	0.84 ± 0.02	1.63 ± 0.04	1.77 ± 0.05	1.90 ± 0.07	1.96 ± 0.07	2.13 ± 0.06
Mangiferin (10)	0.91 ± 0.04	1.51 ± 0.07 (7.36)	1.60 ± 0.06 (9.60)	1.56 ± 0.03[a] (17.89)	1.52 ± 0.08[a] (22.45)	1.48 ± 0.08[a] (30.52)
Mangiferin (20)	0.98 ± 0.08	1.39 ± 0.04[b] (14.72)	1.46 ± 0.04[b] (17.51)	1.43 ± 0.06[a] (24.74)	1.38 ± 0.05[a] (29.59)	1.34 ± 0.04[a] (37.09)
MGN 1 (10)	0.98 ± 0.05	1.55 ± 0.04 (4.90)	1.57 ± 0.04 (11.30)	1.59 ± 0.04[a] (16.32)	1.55 ± 0.09[a] (20.92)	1.52 ± 0.05[a] (28.64)
MGN 1 (20)	1.00 ± 0.06	1.40 ± 0.04[b] (14.10)	1.40 ± 0.06[a] (20.90)	1.41 ± 0.04[a] (25.79)	1.36 ± 0.03[a] (30.61)	1.37 ± 0.05[a] (35.68)
MGN 3 (10)	0.95 ± 0.05	1.58 ± 0.03 (3.07)	1.67 ± 0.06 (5.65)	1.60 ± 0.07[b] (15.79)	1.55 ± 0.07[a] (20.92)	1.53 ± 0.06[a] (28.17)
MGN 3 (20)	1.02 ± 0.03	1.51 ± 0.06 (7.36)	1.55 ± 0.04 (12.43)	1.57 ± 0.04[a] (17.37)	1.45 ± 0.08[a] (26.02)	1.42 ± 0.07[a] (33.33)
Diclofenac (20)	0.89 ± 0.04	1.22 ± 0.05[a] (25.15)	1.25 ± 0.05[a] (29.38)	1.30 ± 0.03[a] (31.58)	1.16 ± 0.02[a] (40.82)	1.13 ± 0.03[a] (46.94)

Values are given as mean ± S.E.M. for groups of six animals each, Dunnet′s test; values are statistically significant at [a]P<0.001, [b]P<0.01, [c]P<0.05 between control and treated groups.

5.9. Anticonvulsant activity of embelin

The duration of HLTE for the control group was 11.80 ± 0.37 sec after an electroshock in MES model. Administration of embelin (2.5 and 5 mg/kg, i.p) showed significant ($P<0.001$) reduction in the duration of HLTE to 5.40 ± 0.24 and 1.50 ± 0.12 sec, respectively compared to the control (Table 14). However, embelin at the dose of 100 mg/kg and phenytoin at 25 mg/kg. dose showed total absence of extension. The onset of stupor for the control animals was 131.40 ± 2.29 sec. Embelin at all the three doses significantly ($P<0.001$) decreased the onset of stupor when compared to the control. At 10 mg/kg. dose it showed lower onset time when compared to standard phenytoin. In the control group 66.66 percent animals survived the electroshock, but embelin at all the three doses and phenytoin treatments exhibited 100 percent protection against mortality.

The onset of clonic and tonic actions induced by PTZ in the control animals was found to be 47.80 ± 1.69 and 72.60 ± 1.78 sec, respectively. Embelin at all the three doses significantly ($P<0.001$) increased both the clonic and tonic onsets (Table 15). At 5 and 10 mg/kg. doses it exhibited 50 and 83.33 percent protection against mortality. However, the standard diazepam 4 mg/kg. showed total absence of convulsions and 100 percent protection against mortality. Embelin at all the three doses and standard diazepam at 1 mg/kg significantly ($P<0.001$) reduced the locomotor activity in mice when compared to the control animals (Table 16). At 10 mg/kg. dose it showed better reduction in locomotor activity (87.78%) when compared to standard diazepam (76.52%) at 1 mg/kg.

Table 14. Effect of embelin on MES – induced seizure in rats

Treatment (Dose, mg/kg, i.p)	Duration of HLTE (sec) Mean ± SEM	Stupor (sec) Mean ± SEM	No. of animals recovered/used	Protection against mortality (%)
MES Control	11.80 ± 0.37	131.40 ± 2.29	4/6	66.66
Embelin (2.5)	5.40 ± 0.24[a]	109.00 ± 1.88[a]	6/6	100.00
Embelin (5)	1.50 ± 0.12[a]	84.00 ± 1.87[a]	6/6	100.00
Embelin (10)	Absence of extension	49.40 ± 0.87[a]	6/6	100.00
Phenytoin (25)	Absence of extension	55.00 ± 3.30[a]	6/6	100.00

[a]P<0.001, when compared to control (n = 6), Tukey-Kramer

Table 15. Effect of embelin on PTZ – induced seizure in mice

Treatment (Dose, mg/kg, i.p)	Onset of clonic action in sec Mean ± SEM	Onset of tonic action in sec Mean ± SEM	No. of animals recovered/used	Time to death in min Mean ± SEM (No. of deaths)	Protection against mortality (%)
PTZ Control (85)	47.80 ± 1.69	72.60 ± 1.78	0/6	11.20 ± 0.58 (6)	0.00
Embelin (2.5)	66.60 ± 1.40[a]	88.60 ± 1.17[a]	0/6	18.60 ± 0.75 (6)	0.00
Embelin (5)	88.20 ± 0.92[a]	143.40 ± 1.40[a]	3/6	43.50 ± 1.50 (3)	50.00
Embelin (10)	230.00 ± 4.44[a]	349.40 ± 3.37[a]	5/6	125. 45 (1)	83.33
Diazepam (4)	Absence of convulsions	Absence of convulsions	6/6	Nil	100.00

[a]P<0.001, when compared to control (n = 6), Tukey-Kramer.

Table 16. Effect of embelin on locomotor activity in actophotometer

Treatment (Dose mg/kg, i.p)	Locomotor activity (score) in 10 min		Reduction in activity (%)
	Before treatment Mean ± SEM	After treatment Mean ± SEM	
Normal Control	568.0 ± 16.89	512.0 ± 13.62	9.86
Embelin (2.5)	551.0 ± 27.41	246.4 ± 16.38[a]	59.42
Embelin (5)	527.0 ± 20.83	145.6 ± 10.22[a]	72.38
Embelin (10)	577.6 ± 22.98	70.6 ± 9.59[a]	87.78
Diazepam (1)	540.8 ± 17.16	127.0 ± 10.36[a]	76.52

[a]P<0.001, when compared to before treatment (n = 6), Tukey-Kramer.

5.10. Antidiabetic activity of embelin

Alloxan induced diabetic control rats showed a significant (P<0.001) reduction in body weight on days 7, 14 and 21 when compared to the normal rats. Embelin treated at 25 and 50 mg/kg doses and standard glibenclamide at 10 mg/kg dose given to alloxan induced diabetic rats caused a significant increase in body weights when compared to diabetic control animals (Fig. 128). Significant increase in the fasting serum blood glucose level was observed in alloxan induced diabetic control rats (P<0.001) when compared to normal. Embelin and glibenclamide treated groups caused a significant reversal of the same on days 7-21 (Fig. 129) measurements. A significant (P<0.001) fall in serum blood glucose level of diabetic rats i.e. 41.15% (from 238.83 ± 2.73 mg/dl to 131.50 ± 3.06 mg/dl), 56.85% (from 240.33 ± 3.65 mg/dl to 92.17 ± 2.20 mg/dl) in group III and IV after 21 days of embelin treatments was observed. However, glibenclamide produced 66.16% (from 229.83 ± 2.33 mg/dl to 77.67 ± 2.36 mg/dl) reduction during the same period.

Treatment with alloxan caused a significant (P<0.001) increase in the levels of TGL, TC, TB, CR, LDH, ALP and VLDL and a significant (P<0.001) decrease in the levels of TP and albumin when compared to normal (Table 17). Treatment with embelin at both the doses and glibenclamide caused a significant reversal of all these changes towards the normal. Among the two doses, embelin at 50 mg/kg dose was found to be more active. However, the standard glibenclamide exhibited better activity than embelin in all the above parameters.

In the histopathological studies, normal animals liver tissue showed sinusoidal cords of hepatocytes with central vein and portal tracts. In the diabetic control, liver section showed distortion in the arrangement of cells around the central vein and periportal fatty infiltration with focal necrosis of hepatocytes. The treatment with embelin at both the doses and glibenclamide treated animals exhibited normal cellular arrangement around the central vein, blood vessels and reduced necrosis (Fig. 130). The kidney sections of diabetic rat showed tubular damage, proteinuria and haemorrhage. Due to glomerular damage, haemorrhage is seen in the Bowman′s space. The sections of animals treated with embelin at 25 mg/kg showed mild tubular epithelial atrophy and congestion of capillaries (Fig. 131). However, embelin at 50 mg/kg and glibenclamide at 10 mg/kg treated diabetic rats kidney showed glomeruli and tubules without proteinuria and haemorrhage. The pancreas of the normal animals showed lobules of exocrine acini and exocrine islets of langerhans which were not observed in alloxan induced diabetic pancreas (Fig. 132). However, the treatment with embelin at both the doses and standard glibenclamide treated pancreas appeared normal, cells gathered together and preserved islets similar to the normal.

Table 17. Effect of embelin on serum profile in alloxan (120 mg/kg) induced diabetic rats after 21 days of treatment

Parameters	**TGL (mg/dl)**	**TC (mg/dl)**	**TB (mg/dl)**	**TP (g/dl)**	**CR (mg/dl)**	**Albumin (g/dl)**	**LDH (IU/l)**	**ALP (IU/l)**	**VLDL (mg/dl)**
Normal control	81.17 ± 0.95	144.01 ± 2.96	0.74 ± 0.02	6.78 ± 0.11	0.31 ± 0.02	3.78 ± 0.13[c]	185.50 ± 1.34	170.17 ± 1.40	16.70 ± 0.60
Diabetic control	199.17 ± 2.41[a]	270.67 ± 3.39[a]	1.07 ± 0.03[a]	3.53 ± 0.12[a]	0.59 ± 0.04[a]	2.25 ± 0.17[a]	380.67 ± 3.91[a]	364.50 ± 2.68[a]	39.83 ± 0.48[a]
Alloxan + embelin (25 mg/kg)	138.67 ± 2.33[d]	186.17 ± 1.96[d]	0.92 ± 0.02[d]	4.78 ± 0.14[d]	0.43 ± 0.02[b]	3.10 ± 0.10[c]	287.50 ± 2.62[d]	244.83 ± 3.28[d]	27.73 ± 0.47[d]
Alloxan + embelin (50 mg/kg)	104.83 ± 3.96[d]	169.50 ± 3.61[d]	0.82 ± 0.01[d]	5.92 ± 0.06[d]	0.37 ± 0.03[d]	3.23 ± 0.15[d]	242.33 ± 3.09[d]	201.83 ± 2.30[d]	20.97 ± 0.79[d]
Alloxan + glibenclamide (10 mg/kg)	99.00 ± 1.71[d]	151.33 ± 2.20[d]	0.77 ± 0.02[d]	6.50 ± 0.14[d]	0.29 ± 0.05[d]	3.61 ± 0.10[d]	244.83 ± 2.93[d]	183.17 ± 2.18[d]	19.80 ± 0.34[d]

Values are given as mean ± S.E.M. for groups of six animals each, Tukey-Kramer; values are statistically significant at [a]$P<0.001$ between normal and diabetic control and [b]$P<0.05$, [c]$P<0.01$, [d]$P<0.001$ between diabetic control and treated groups.

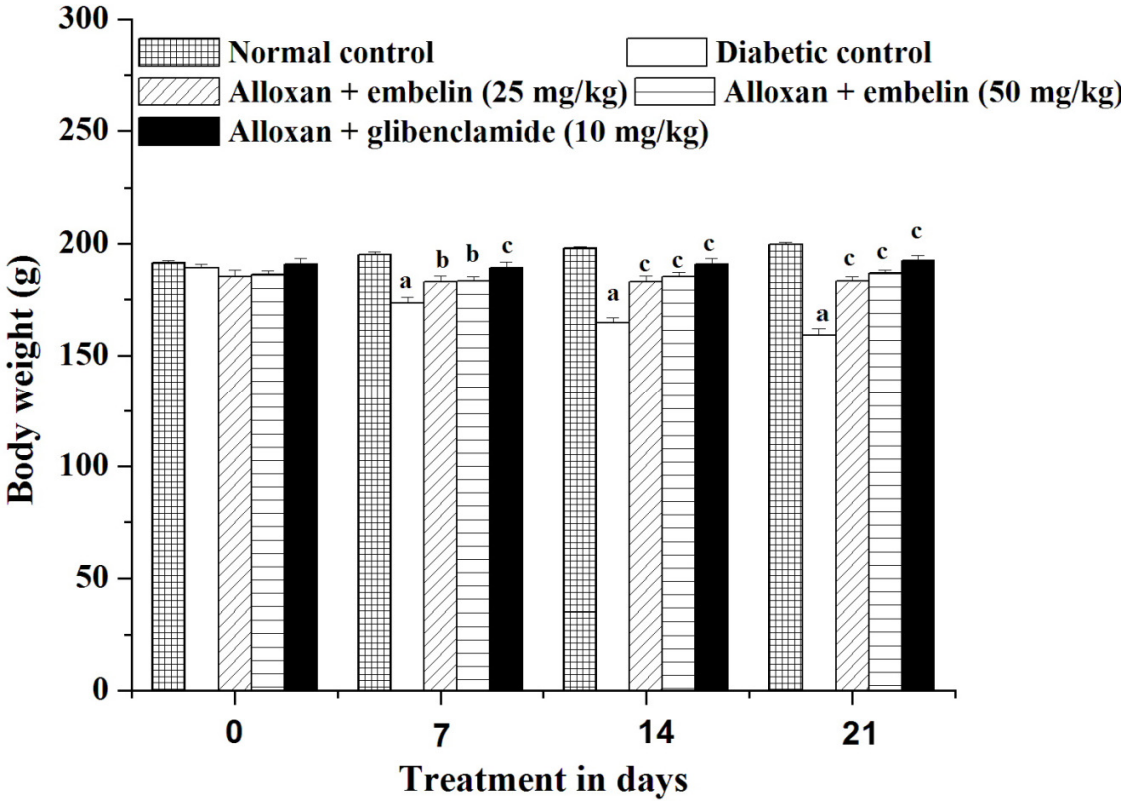


Fig. 128. The effect of 3-week treatment with embelin on body weight (g) after alloxan (120 mg/kg) induced diabetes in rats. Values are given as mean ± S.E.M. for groups of six animals each, Tukey-Kramer; values are statistically significant at $^{a}P<0.001$ between normal and diabetic control and $^{b}P<0.05$, $^{c}P<0.001$ between diabetic control and treated groups.

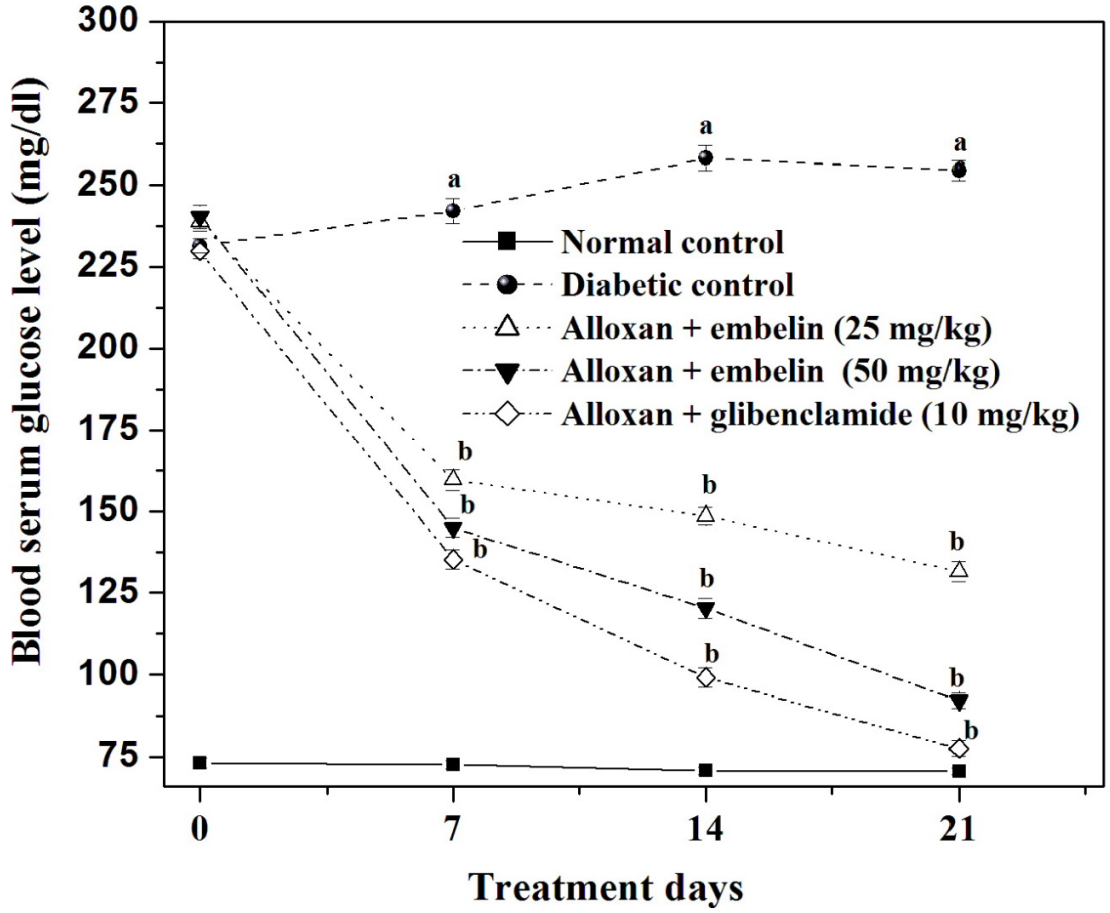


Fig. 129. Comparative effect of embelin on blood serum glucose level in alloxan (120 mg/kg) induced diabetes in rats. Values are given as mean ± S.E.M. for groups of six animals each, Tukey-Kramer; [a]P<0.001 between normal and diabetic control and [b]P<0.001 between diabetic control and treated groups.

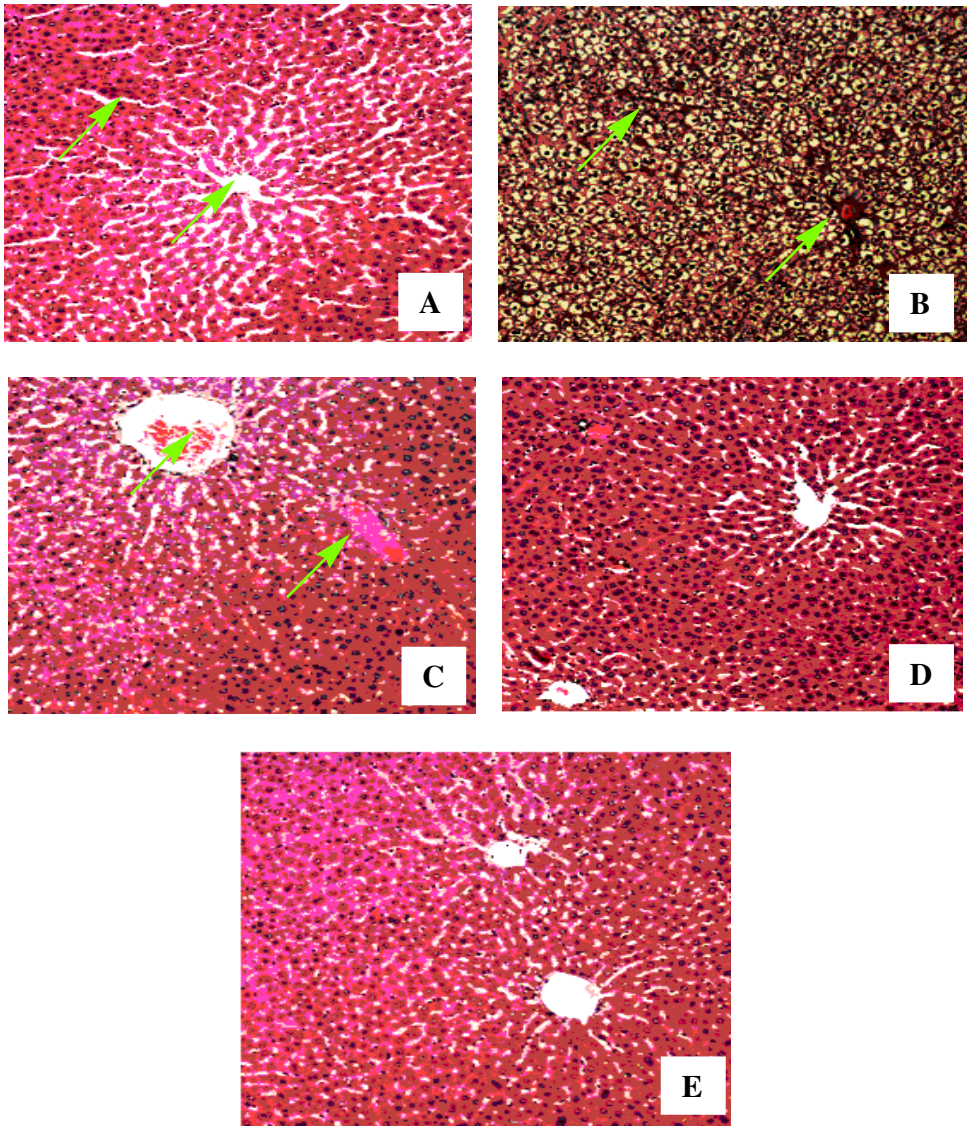


Fig. 130. Histopathological changes in liver of experimental rats. A: Group I – Normal liver showing the central vein with radiating cords of hepatocytes. B: Group II – Diabetic control, alloxan treated liver shows periportal fatty infiltration with focal fat necrosis. C: Group III – Alloxan + embelin (25 mg/kg) shows moderate degeneration changes with bleeding area in hepatic lobes. D: Group IV – Alloxan + embelin (50 mg/kg) shows mild degeneration changes with normal histological structure. E: Group V – Alloxan + glibenclamide 10 mg/kg shows normal hepatic structure.

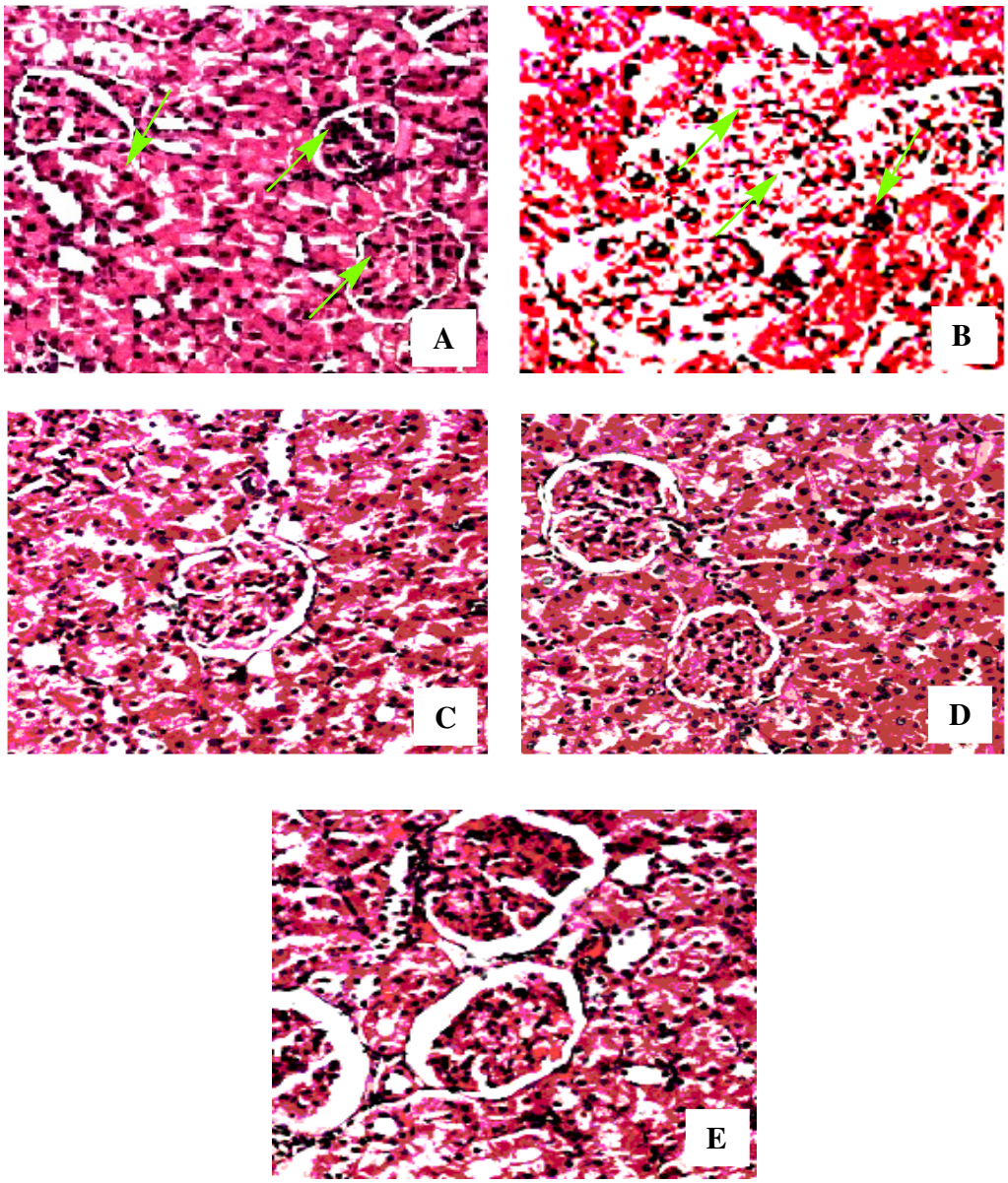


Fig. 131. Histopathological changes in kidney of experimental rats. A: Group I – Normal kidney show glomeruli and proximal convoluted tubules. B: Group II – Diabetic control, alloxan treated kidney shows tubular damage proteinuria and hemorrhage. C: Group III – Alloxan + embelin (25 mg/kg) shows mild tubular epithelial atrophy and congestion of capillaries. D: Group IV – Alloxan + embelin (50 mg/kg) shows normal histological structure. E: Group V – Alloxan + glibenclamide 10 mg/kg shows normal architecture of kidney.

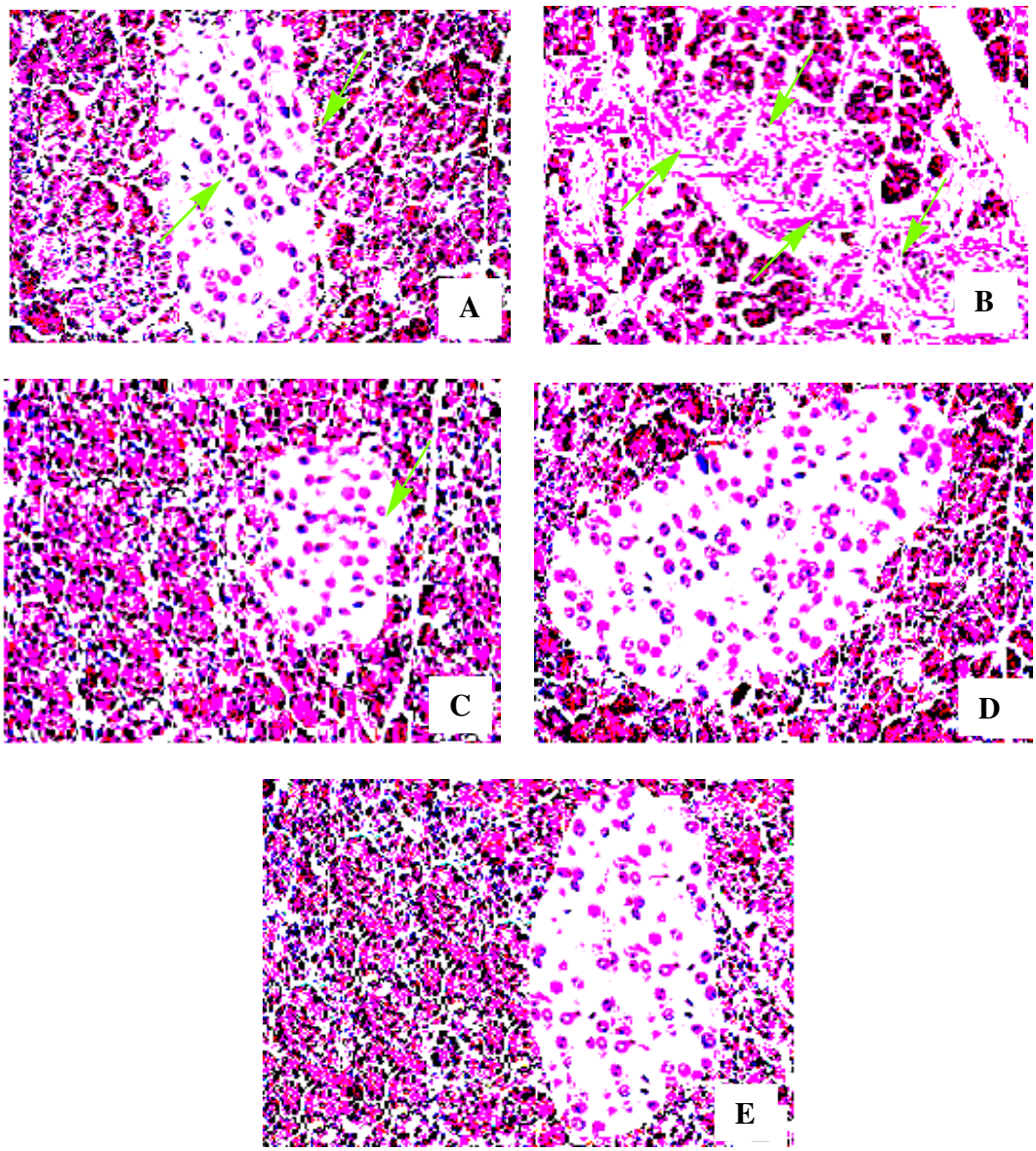


Fig. 132. Histopathological changes in pancreas of experimental rats. A: Group I – Normal pancreas showing normal round exocrine acini and endocrine islets. B: Group II – Diabetic control, alloxan treated pancreas shows depleted islets. C: Group III – Alloxan + embelin (25 mg/kg) shows exocrine acini and islets were shrunken moderately. D: Group IV – Alloxan + embelin (50 mg/kg) islets resemble normal rats islets. E: Group V – Alloxan + glibenclamide 10 mg/kg shows apparently normal architecture of islets.

5.11. Effect of embelin against inflammatory bowel disease

Acetic acid caused severe macroscopic edematous inflammation in the colon. The clinical score, gross lesion score, percent area affected, wet colon weight for colitis control group were found to be 3.35 ± 0.39, 4.80 ± 0.58, 64.80 ± 12.91 and 142.80 ± 3.02, respectively (Table 18). Embelin at both the doses (25 and 50 mg/kg) showed significant decrease in the clinical activity scores (1.98 ± 0.12 and 1.20 ± 0.13, $P<0.001$), gross lesion score (2.40 ± 0.68 and 1.80 ± 0.37, $P<0.01$ and $P<0.001$), percent affected area (16.40 ± 8.80 and 12.00 ± 5.39%, $P<0.001$) and wet colon weight (117.60 ± 3.41 and 116.80 ± 8.38 mg/cm, $P<0.05$ and $P<0.01$), respectively. However, the standard sulfasalazine (100 mg/kg) showed better results in all these parameters, indicating its potent activity at the dose tested. Embelin treated alone at 50 mg/kg caused no significant change in these parameters when compared to normal control.

Intra-rectal administration of acetic acid showed a significant increase in the concentrations of MPO and lipid peroxides (4.27 ± 0.20 U/g and 14.48 ± 0.36 µmol/g of wet tissue, $P<0.001$), decrease the GSH level (821.93 ± 15.89 nmol/g of wet tissue, $P<0.001$) in colonic tissue and increase the LDH in serum (2002.00 ± 96.60 U/l, $P<0.001$) when compared to normal control. The pre-treatment with embelin at 25 and 50 mg/kg significantly ($P<0.001$) reduced the alterations in these biochemical parameters when compared to colitis control and towards the normal level (Fig. 133). Embelin at 50 mg/kg was found to be more potent and the activity was comparable to standard sulfasalazine at 100 mg/kg treatment. When treated alone, embelin at 50 mg/kg caused no significant change in these biochemical parameters when compared to normal indicating its safety.

In the histopathological observations of colon, acetic acid induced colitis showed massive necrotic destruction of epithelium, submucosal edema, areas of haemorrhages and inflammatory cellular infiltration. Emn at low dose level showed minimal damage of the mucosa with slight submucosal edema and mild inflammatory cell infiltration. Embelin at 50 mg/kg and sufasalazine at 100 mg/kg showed remarkable recovery of colonic mucosa from acetic acid induced colitis damage (Fig. 134).

Table 18. Clinical and macroscopic characters of embelin treated acetic acid induced colitis in rats

Treatment	Clinical score (% protection)	Gross lesion score (% protection)	Area affected (%)	Wet colon weight/length (mg/cm)
Normal control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	107.80 ± 4.03
Embelin alone (50 mg/kg, p.o.)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	95.00 ± 5.17
Colitis control (2 ml of 3% acetic acid, intra-rectal)	3.35 ± 0.39[a]	4.80 ± 0.58[a]	64.80 ± 12.91[a]	142.80 ± 3.00[b]
Acetic acid + embelin (25 mg/kg, p.o.)	1.98 ± 0.12[c] (40.90)	2.40 ± 0.68[d] (50.00)	16.40 ± 8.80[c]	117.60 ± 3.41[e]
Acetic acid + embelin (50 mg/kg, p.o.)	1.20 ± 0.13[c] (64.18)	1.80 ± 0.37[c] (62.50)	12.00 ± 5.39[c]	116.80 ± 8.38[d]
Acetic acid + sulfasalazine (100 mg/kg, p.o.)	0.89 ± 0.06[c] (73.43)	0.83 ± 0.16[c] (82.70)	4.00 ± 1.00[c]	112.00 ± 3.80[d]

Values are given as mean ± S.E.M. for groups of six animals each, Tukey-Kramer; values are statistically significant at [a]$P<0.001$, [b]$P<0.01$ between normal and colitis control, [c]$P<0.001$, [d]$P<0.01$, [e]$P<0.05$ between colitis control and treated groups.

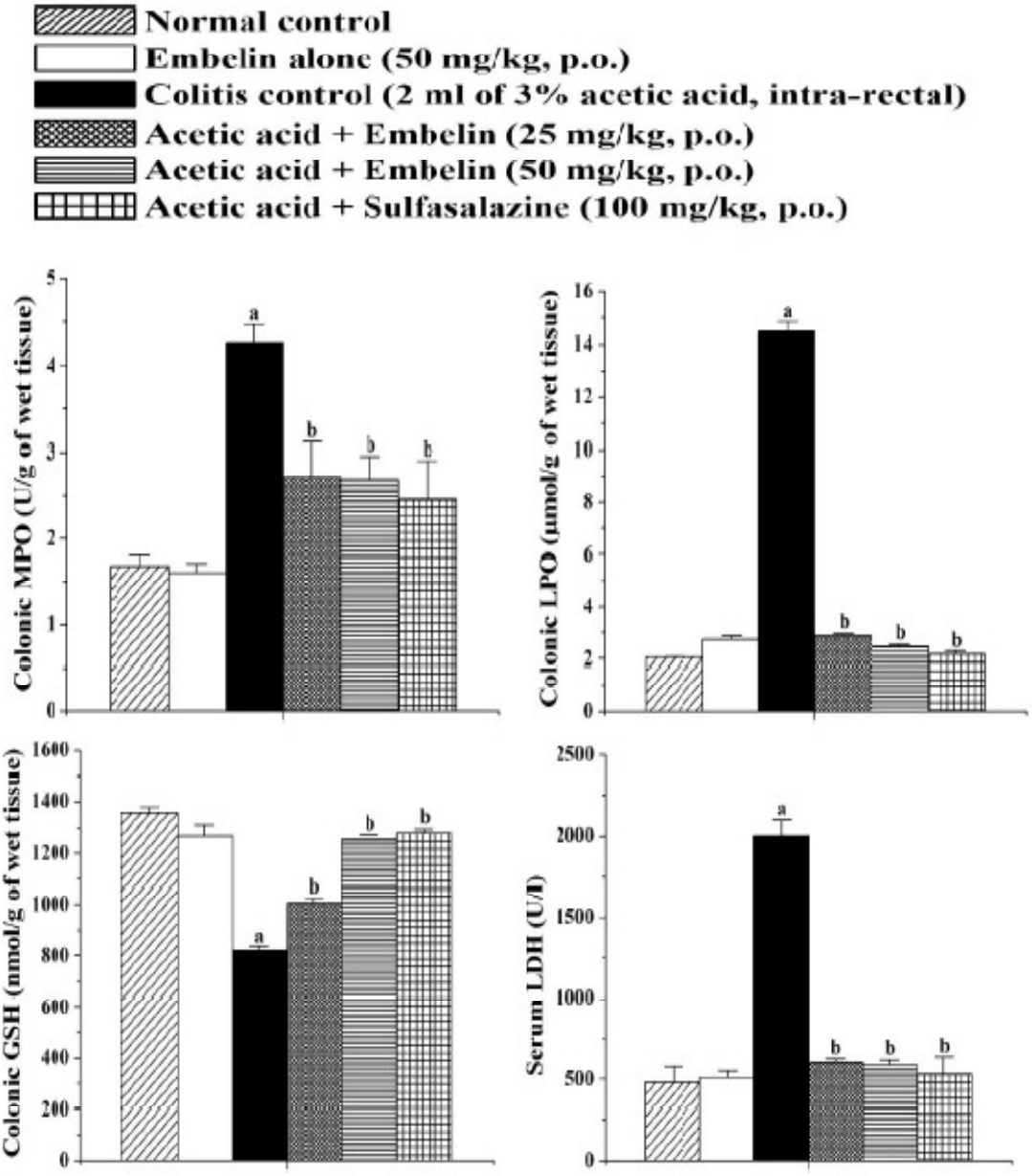


Fig. 133. Effect of embelin on biochemical profile in acetic acid induced colitis in rats. Values are given as mean ± S.E.M. for groups of six animals each, Tukey-Kramer; values are statistically significant at [a]P<0.001 between normal and colitis control and [b]P<0.001 between colitis control and treated groups.

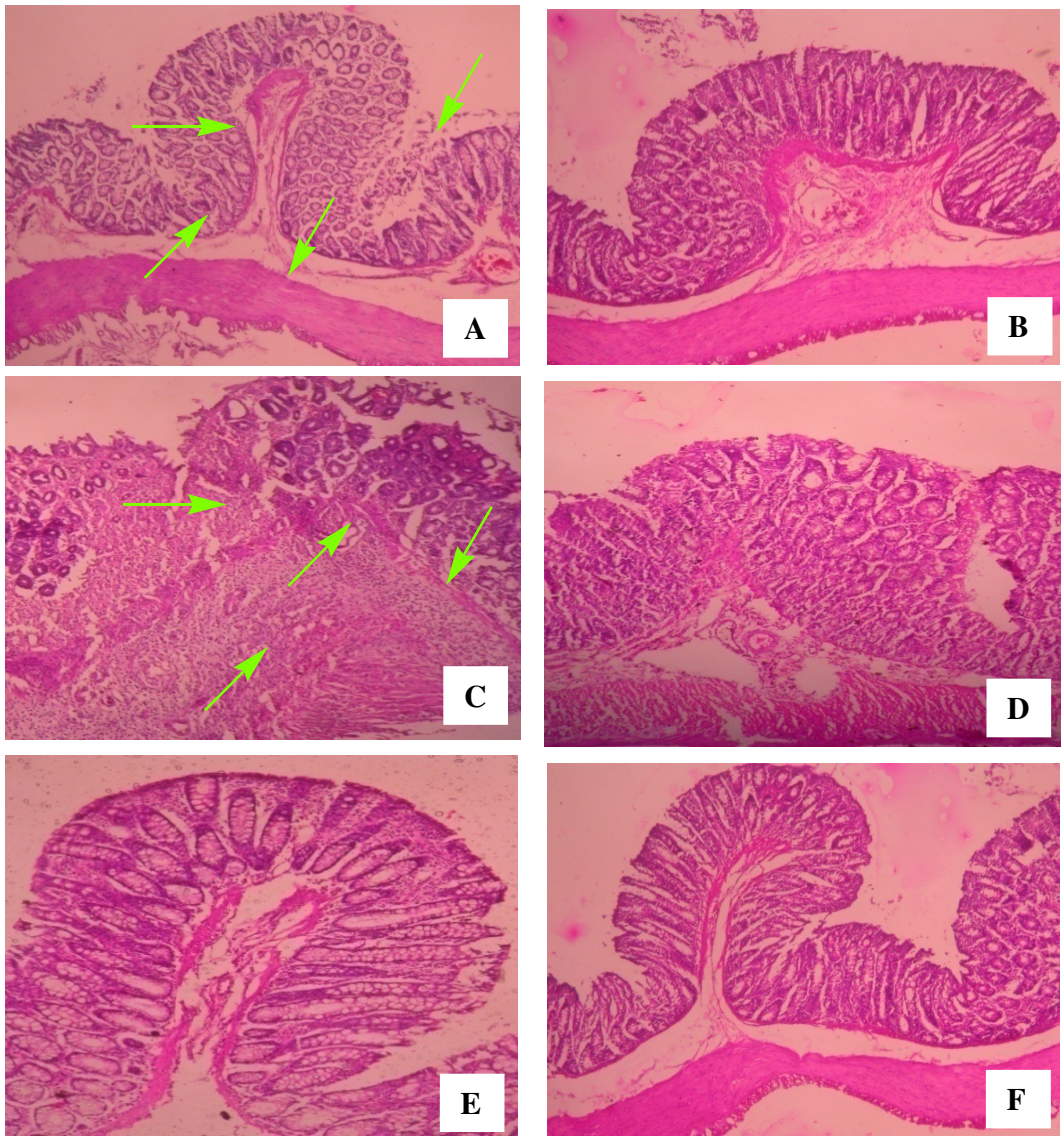


Fig. 134. Histopathological changes in colon of experimental rats. A and B: Group I and II – Normal intact mucosa from normal control and embelin alone treated animals showing intact epithelial surface. C: Group III – Acetic acid induced colitis showing massive necrotic destruction of epithelium, submucosal edema, areas of haemorrhages and inflammatory cellular infiltration. D: Group IV – Acetic acid + embelin 25 mg/kg showing minimal damage of the mucosa with slight submucosal edema and mild inflammatory cell infiltration. E: Group V – Acetic acid + embelin 50 mg/kg showing significant protection of colonic mucosa from acetic acid induced colitis damage. F: Group VI – Acetic acid + sufasalazine 100 mg/kg showing normal colonic structure.

5.12. Neuroprotective effect of embelin against global cerebral ischemia in rats

Global cerebral ischemia for 30 min followed by reperfusion for 24 h significantly ($P<0.01$) decreased the locomotion in ischemic control (48.00 ± 6.97) when compared to sham control (174.80 ± 35.30). A significant increase ($P<0.05$) in the locomotion was observed in both the doses of embelin (25 and 50 mg/kg) treated groups (144.00 ± 20.00 and 172.80 ± 35.62, respectively) compared to ischemic control. In the hanging wire test, ischemic animals exhibited inability to hold the wire and also the time of fall was reduced significantly (16.22 ± 4.95 sec, $P<0.05$) when compared to sham control (65.40 ± 11.63 sec).

Pretreatment with embelin at both the doses showed significant ($P<0.05$) increase (58.50 ± 10.59 and 61.50 ± 4.87 sec) as compared to ischemic control. In the beam walking task, animals in sham group were able to traverse the beam in short time (14.23 ± 5.10 sec), whereas, in ischemic group, the animals were unable to balance on the beam and the time taken to traverse were significantly increased ($P<0.01$) to 41.21 ± 8.21 sec. The pretreatment with embelin at both the doses showed significant reduction ($P<0.01$) in latency at both the doses (14.52 ± 1.23 and 14.25 ± 3.68 sec, respectively) compared to ischemic control (Table 19).

A significant ($P<0.001$) elevation in the levels of MDA was observed in BCCA occluded rats (3.70 ± 0.42) when compared to sham control (1.53 ± 0.07). Pretreatment with embelin at both the doses significantly ($P< 0.01$ and $P< 0.001$) reduced the MDA levels to 1.85 ± 0.44 and 0.75 ± 0.12, respectively as compared to ischemic control in a dose dependent manner (Fig. 135). Ischemia for 30 min, significantly ($P< 0.01$) reduced the

total thiol level to 27.31 ± 1.15 as compared to sham control (45.01 ± 4.95). Pretreatment with embelin at both the doses increased the total thiols (37.54 ± 2.98 and 34.88 ± 3.60, respectively) when compared to ischemic control.

Reduction in GST activity was significant (P<0.001) in ischemic control (17.33 ± 1.65) when compared to sham control (35.39 ± 4.27). A significant increase (P<0.001) in the enzyme level was found in the pretreatment of embelin at both the doses (32.31 ± 2.14 and 32.90 ± 1.96, respectively) when compared to ischemic control. In the TTC staining of brain (Fig. 136), pretreatment of embelin at both the doses markedly attenuated ischemia induced higher cerebral infarct volume dose dependently and significantly. The percentage of infarction of embelin treated groups were reduced to 47.04 ± 2.31% and 21.88 ± 0.36%, respectively when compared to ischemic control (100%).

In the histopathological observation of brain, ischemia showed congested blood vessels, increased lymphocyte infiltration and neuronal necrosis. There was significant protection against brain damage observed in the pretreatment with embelin at both the tested doses. However, pretreatment of embelin at 50 mg/kg did not show any pathological changes, when they were administered with ischemia (Fig. 137).

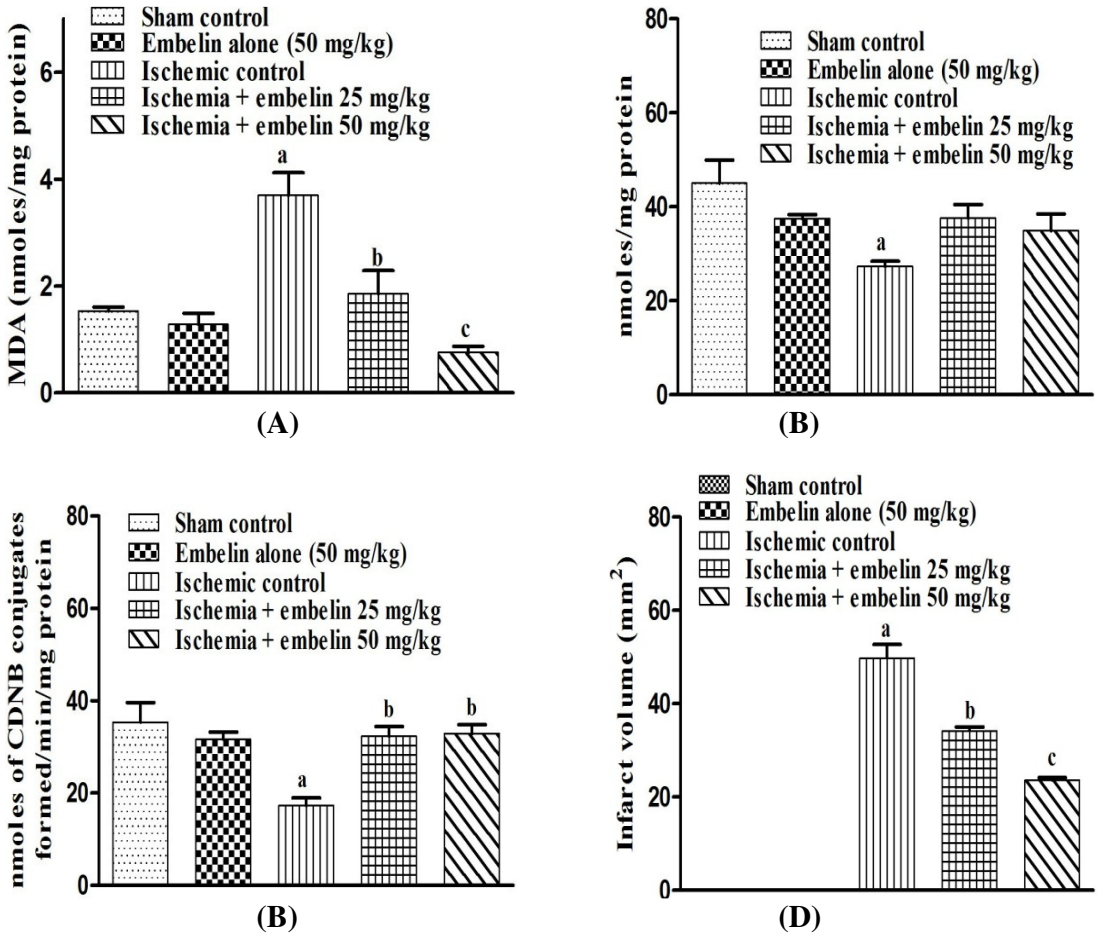


Fig. 135. Effect of embelin on biochemical profile in global cerebral ischemia in rats. Values are given as mean ± S.E.M., Tukey-Kramer; **(A)** Lipid peroxidation, $^{a}P<0.001$ between sham and ischemic control; $^{b}P<0.01$, $^{c}P<0.001$ ischemic control and treated groups. **(B)** Total thiol levels, $^{a}P<0.01$ between sham and ischemic control. **(C)** Glutathione-S-transferase, $^{a}P<0.001$ between sham and ischemic control; $^{b}P<0.001$ ischemic control and treated groups. **(D)** Brain infarction volume, $^{a}P<0.001$ between sham and ischemic control; $^{b}P<0.01$, $^{c}P<0.001$ ischemic control and treated groups (n = 3).

Table 19. Effect of Embelin on behavioural parameters in BCCA occluded rats

Treatment	Locomotor activity Counts/5 min	Hanging latency time (sec)	Beam walking Latency (sec)
Sham control (n = 6)	174.80 ± 35.30	65.40 ± 11.63	14.23 ± 5.10
Embelin alone (50 mg/kg) (n = 7)	157.00 ± 28.78	42.00 ± 10.43	16.09 ± 2.51
Ischemic control (n = 8)	48.00 ± 6.97[b]	16.22 ± 4.95[a]	41.21 ± 8.21[b]
Ischemia + embelin 25 mg/kg (n = 8)	144.00 ± 20.00[c]	58.50 ± 10.59[c]	14.52 ± 1.23[d]
Ischemia + embelin 50 mg/kg (n = 7)	172.80 ± 32.62[c]	61.50 ± 4.87[c]	14.25 ± 3.68[d]

Values are given as mean ± S.E.M, Tukey test. [a]P<0.05, [b]P<0.01 between sham and ischemic control; [c]P<0.05, [d]P<0.01 ischemic control and treated groups.

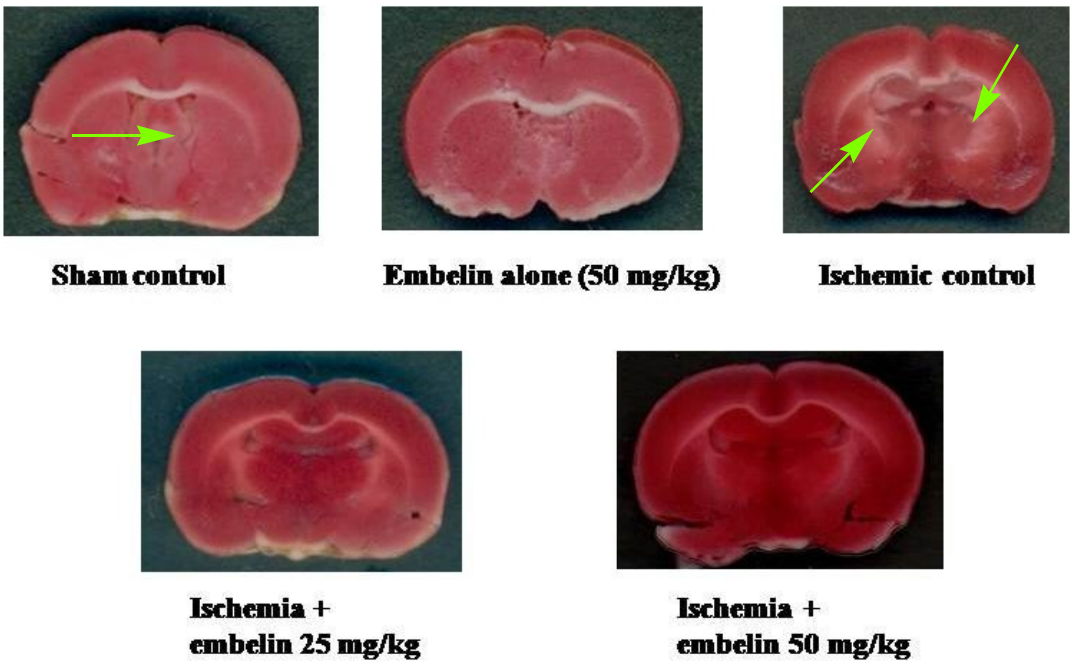


Fig. 136. A representative photograph of rat brain coronal sections after staining with TTC in experimental rats.

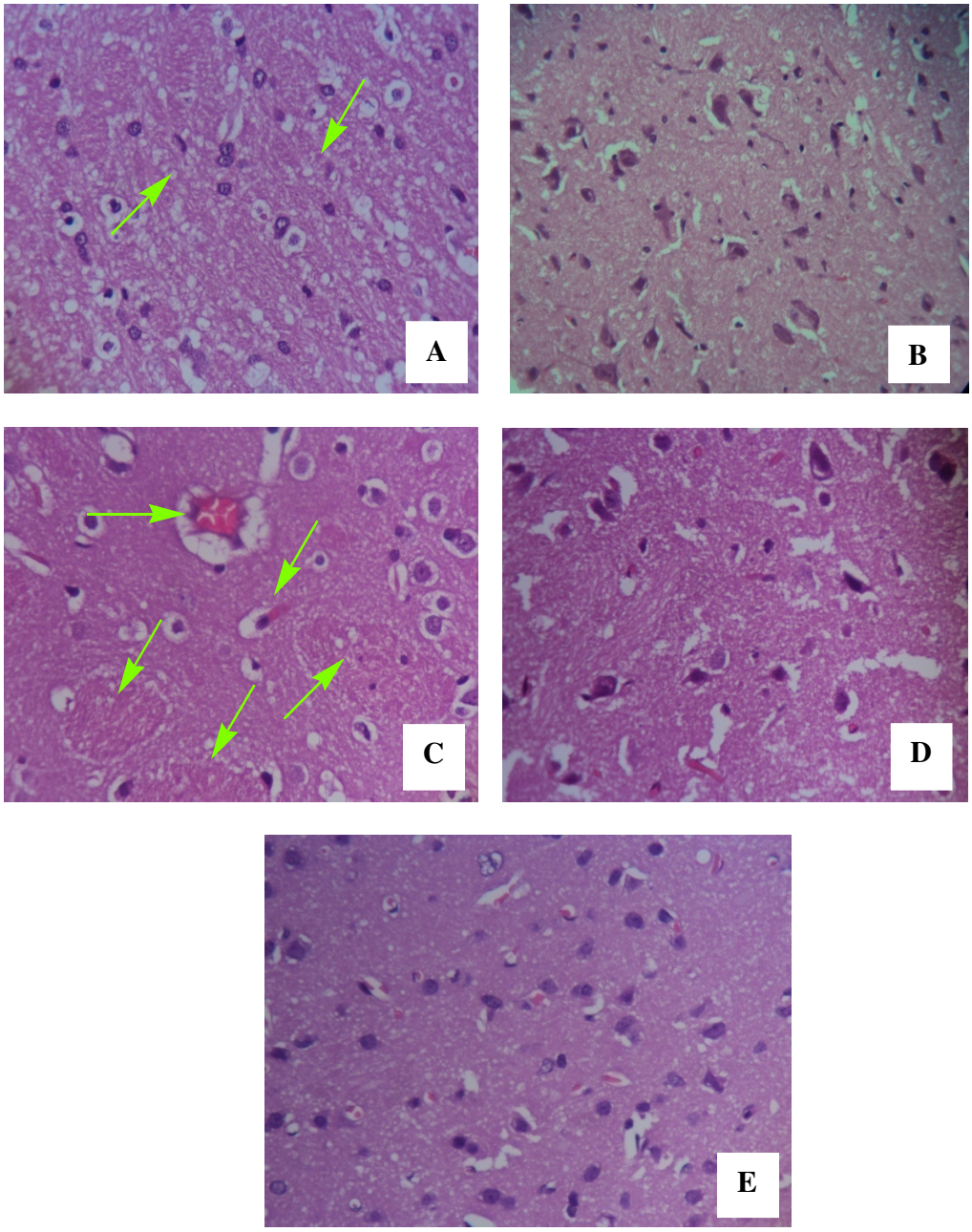


Fig. 137. Histopathological changes in brain of experimental rats. A and B: Sham control and embelin alone, normal brain showing normal blood vessels and brain parenchyma. C: Ischemic control showing congested blood vessels, increased lymphocyte infiltration and neuronal necrosis. D: Ischemia + embelin 25 mg/kg showing reduced congested blood vessels, lymphocytes and absence of necrosis. E: Ischemia + embelin 50 mg/kg showing normal architecture of brain.

Herbal medicine is a major component in all indigenous people's traditional medicine and a common element in Ayurvedic, Homeopathic, Naturopathic, Traditional, Oriental, and Native American Indian medicine. Many drugs commonly used today are of herbal origin. Indeed, about 25 percent of the prescription drugs dispensed in the United States contain at least one active ingredient derived from plant material. Some are made from plant extracts; others are synthesized to mimic a natural plant compound. Major pharmaceutical companies are currently conducting extensive research on plant materials gathered from the rain forests and other places for their potential medicinal values.

Numerous methods have been utilized to acquire compounds for drug discovery, including isolation from plants and other natural sources, synthetic chemistry, combinatorial chemistry and molecular modelling. Despite the recent interest in molecular modelling, 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). According to a recent report [148], 61% of the 877 small-molecule NCEs introduced as drugs worldwide during 1981–2002 were inspired by natural products. These include natural products (6%), natural products derivatives (27%), synthetic compounds with natural products derived pharmacophore (5%) and synthetic compounds designed from natural products (natural products mimic, 23%).

Once a natural product compound has been screened for biological activity, isolated, purified, its structure identified, and the pharmacological profile refined, the journey is not over. The molecule may turn out to be too complex in nature and too expensive to be

synthesized. Indeed, when compared to a purely man-made synthetic alternative, many times the natural product compound is quickly eliminated from further consideration because of considerations of time and potential costs of synthetic production. Any given natural product compound may possess unacceptable physicochemical, pharmacodynamic, pharmacokinetic, bioavailability properties or demonstrate excessive toxicity and will therefore require optimization of its chemical structure. Optimization involves a dissection of the lead molecule and the synthetic addition, removal, replacement, or modification of substituent groups so as to enhance the utility and efficacy of the molecule.

With this background, in the present study an attempt was made to modify the structure of embelin and mangiferin isolated from *Embelia ribes* and *Mangifera indica*, respectively and submit these compounds for *in vitro* antioxidant activities. The potent derivatives were subjected to *in vivo* analgesic and anti-inflammatory activities. The parent compound embelin was screened for anticonvulsant, antidiabetic and the effect of embelin in inflammatory bowel disease along with neuroprotective studies.

Chemical modification of embelin for obtaining compounds with better activity has been tried. Sixteeen new and eight known embelin derivatives were prepared and characterized.

Embelin (**1**) was isolated and characterized by comparison of its physical and spectral data with literature values [107]. The ^{13}C NMR spectrum of embelin did not exhibit all the ring carbon signals, but showed only two peaks at δ 116.99 (C-3) and 102.15 (C-6). Normally, the ^{13}C NMR of 2,5-dihydroxy-3alkyl-1,4-benzoquinones do not show the ring

carbon peaks particularly those attached to oxygen atoms due to fluxional effect caused by intramolecular hydrogen bonding. The result of this is long spin relaxation time which leads to saturation of oxygen-carbon signals [149]. The fluxional effect can be precluded if at least one of the hydroxyl group is removed through structural modification. Such modifications led to observation of all the ring carbons except in compounds **4**, **5**, **9**, **12, 16-19** and **21-23**. The IR, ^{1}H NMR and mass spectral data of these compounds confirmed their structures.

Compound **2** exhibited a pseudomolecular ion at m/z 307 for the $[M-H]^{-1}$, corresponding to the addition of one methyl group at 5th position. The IR spectra of **2** showed absorptions at 3352 cm^{-1} for –OH group, 2918, 2850 cm^{-1} for aliphatic –CH stretching, 1635 cm^{-1} for α, β- unsaturated C=O, 1599 cm^{-1} is due to the presence of C=C, and at 1079 cm^{-1} for C-O groups. ^{1}H NMR spectrum of **2** showed a signal at δ 3.85 (s, 3H) for the methoxy group. The signal at δ 7.80 (1H, br. s) for one phenolic hydroxyl group at C-2 position and at δ 5.80 for H-6 were assigned. The signal at δ 0.87 (t, 3H, H-11′), the broad singlet at δ 1.56 (18H, H-2′ – H-10′) and a signal at δ 2.46 (t, 2H, H-1′) showed the presence of the undecyl chain at C-3 position of embelin. The methoxy carbon signal at δ 56.71 and the two carbonyl carbon signals at δ 182.82 (C-1) and 181.67 (C-4) were also observed in ^{13}C NMR spectra.

Compound **3**, exhibited a molecular ion at m/z 444.68, corresponding to the formation of a di-imine substituted compound. Accordingly, absence of α, β- unsaturated carbonyl absorption at 1630 cm^{-1} and appearance of absorption at 1518 cm^{-1} for C=N in the IR spectra manifested the change from carbonyl to di-imine group. In the ^{1}H NMR

spectrum of **3**, the signals at δ 7.45 (br. s, 2H) was assigned to two phenolic hydroxyl groups at C-2 and C-5 positions, δ 6.07 to H-6 and δ 7.19 – 7.40 (m, 10H) was assigned for ten aromatic protons. The remaining signals in the upfield region due to undecyl chain were similar to the parent compound **1**. The ^{13}C NMR spectrum of **3** showed signals at δ 170.10 assigned for C=N at positions 1 and 4. The signals between δ 123.29 – 137.25 were assigned for aromatic carbons and the remaining carbon signals were similar to the parent compound **1**.

All the other compounds (**4-7, 9-17, 19, 20** and **25**) showed pseudomolecular ion peaks in the mass spectra at m/z 414 (**4**), 414.68 (**5**), 403 (**6**), 403.65 (**7**), 385.68 (**9**, **10**), 383.68 (**11**), 412.82 (**12**), 448.58 (**13**), 306.45 (**14**), 320.65 (**15**), 349.58 (**16**), 411 (**17**), 383.68 (**19**), 413.76 (**20**) and 366.82 (**25**). Compounds **8**, **18**, **22** and **23** showed pseudomolecular ions for the $[M-H]^-$ ion at m/z 398, 498, 382 and 473, respectively. Compounds 21 and 24 showed $(M-COOH)^-$ ion at 395 and 435, respectively.

The IR spectra of compounds **4-24** showed absorptions between 3230 – 3400 cm^{-1} due to –OH and –NH groups and the absorption bands at 2840 to 2950 cm^{-1} are due to the presence of long aliphatic chain. The compounds also exhibited absorptions at 1635 to 1645 cm^{-1} due to the presence of α, β- unsaturated C=O and at 1550 – 1620 cm^{-1} due to the presence of aromatic C=C. Complementing these data, the reaction has taken place in the position C-5 and the -OH group of the embelin was substituted with –NH group. In ^{1}H NMR spectrum of **4-24**, -NH proton was observed at δ 7.25 – 10.84 and H-6 was observed at δ 5.00 to 6.00. The signals between δ 6.00 – 8.50 were assigned for aromatic protons and the rest of the signals between δ 0.80 – 2.50 were due to the presence of

aliphatic chain. In ^{13}C NMR spectrum of **4-24**, aromatic carbons appeared between δ 110.00 – 160.00, C-6 appeared between δ 91.00 – 106.00 and C-5 appeared at δ 138.00 – 155.00.

Further in ^{1}H NMR spectrum of compound **8**, the peak present at δ 3.87 (s, 3H) is assigned for the presence of $–OCH_3$ group and it exhibited a signal at δ 55.55 in ^{13}C NMR. Similarly, due to the presence of $–CH_3$ group in compound **11**, it showed the signal at δ 2.36 in the ^{1}H NMR spectrum and at δ 20.99 in ^{13}C NMR. In compound **12**, signal at δ 10.79 (s, 2H, $-CONH_2$) in ^{1}H NMR and at δ 170.02 for C=O in ^{13}C NMR showed the presence of amide group. In compound **13**, the signal exhibited at δ 6.59 (s, 2H, $-NH_2$) was due to the $–NH_2$ group present in sulfanilamide. The IR and NMR spectral data of compound **14** are almost identical with the parent compound **1.** In addition a singlet signal at δ 2.73 for three protons in ^{1}H NMR and the corresponding signal at 31.27 in ^{13}C NMR for the presence of the N-methyl group. The compound **15** in addition to the embelin moiety signals, showed characteristic signals at δ 3.14 (t, 2H, H-1″), 1.09 (t, 3H, H-2″) showing the presence of N-ethyl group. It was supported by the respective signals at δ 36.77 (C-1″) and 12.95 (C-2″) in the ^{13}C NMR spectrum. In compound **16**, the butyl chain protons resonated at δ 3.33 (t, 3H, H-1″), 3.10 (t, 2H, H-2″), 1.32 (t, 2H, H-3″) and 0.87 (3H, t, H-4″) in the ^{1}H NMR and supported the signals at δ 41.70 (C-1″), 29.42 (C-2″), 19.64 (C-3″) and 13.61 (C-4″) in the ^{13}C NMR spectrum.

In compound **17**, the singlet signal at δ 2.55 for three protons is due to the methyl group attached to the keto carbonyl group. It was supported by its ^{13}C NMR spectral signals by exhibiting a peak at δ 31.76 and 177.44, respectively. The compound **18**, ^{1}H NMR

spectrum in addition to the signals of compound **5** except the methyl signal at δ 2.55, it exhibited a pair of doublet signals at δ 7.71 (H-7‴) and 7.97 (H-8‴) each for one proton with a coupling constant of J=16 Hz indicating the presence of a trans alkene moiety. The signals at δ 8.17-8.19 (2H, m, H-11″, H-15″), 7.45-7.50 (3H, m, H-12″-H-14″) for the aromatic ring of benzaldehyde.

In compound **19**, the ^{1}H NMR spectrum, the methyl group resonated at δ 2.31 as a singlet for three protons, corresponding to the peak at δ 17.31 in the ^{13}C NMR. Compound **20**, exhibited characteristic signal at δ 10.86 assigned for –COOH, corresponding to the peak at δ 197.01 in the ^{13}C NMR. Compound **21**, ^{1}H NMR in addition to the signals of the embelin moiety it showed signals for two benzylic protons at δ 5.36 and one –CH group of phenylalanine peak appeared at δ 2.49, corresponding to the peak at δ 36.19 and 56.12 in ^{13}C NMR, respectively. The peak present at δ 13.05 is due to the presence of –COOH group. The aromatic protons resonated between δ 7.20 to 7.50 and the peak present at δ 192.00 for C=O in ^{13}C NMR spectrum. Compound **22** obtained by reaction between embelin and phenyl hydrazine exhibited two –NH protons in its ^{1}H NMR spectrum resonated at δ 9.22 (s, 2H). Compound **23** in its IR spectrum exhibited characteristic IR bands at 1330 cm^{-1} for NO_2 groups, δ 9.22 (s, 2H) for two –NH groups in the ^{1}H NMR. Compound **24,** the ^{1}H NMR exhibited signals for –COOH at δ 10.58, the signal at δ 3.82 for one proton is due to the –CH group attached to the –NH and –COOH groups of tryptophan. The corresponding carbon signal appeared at δ 63.32 in the ^{13}C NMR. The peak present at δ 2.70 for two protons of –CH_2 group are corresponding to δ 29.99 in ^{13}C NMR.

The absence of carbonyl carbon signals in the ^{13}C NMR at δ 180-184 and in IR spectrum at 1635-1645 cm^{-1} suggested that the carbonyl groups of embelin have reacted with O-phenylenediamine to form compound **25.** The aromatic protons resonated at δ 8.05 (d, 1H, H-5), 7.73 (t, 1H, H-6), 7.80 (t, 1H, H-7) and at 8.13 (d, 1H, H-8). The respective carbon signals appeared at 114.78 to 152.00 (C-1 to C-12) in ^{13}C NMR.

Chemical modification of natural phenolic antioxidant by use of ninhydrin has enormous potential for the development of pharmacologically important chemicals [34]. Hence, in the present study novel embelin-ninhydrin adduct was prepared and characterized. Embelin-ninhydrin adduct, in its hemi-ketal form showed a pseudomolecular ion peak at m/z 453 for $[M-H]^{-1}$ in its mass spectrum. Subsequently, it fragmented by loosing one oxygen atom (m/z 437) followed by elimination of a water molecule (m/z 419). The analysis by ^{1}H NMR of embelin-ninhydrin adduct in DMSO exhibited a spectrum showing disappearance of the 6′-H and 5′-OH signals while other protons assigned to embelin remained, indicating that the addition of ninhydrin proceeded at these positions to afford embelin-ninhydrin adduct. Further, the aromatic protons appeared at δ 7.94-7.99 for four protons and three hydroxyl groups were assigned at δ 7.83, 7.89 and 8.05 for H-2′, H-2 and 3, respectively. In the ^{13}C NMR spectrum, signal at δ 198.53 for C=O group, aromatic carbons assigned in the range of δ 117.83 – 142.20 for C-4 to C-9 and δ 51.29, 104.29 assigned for C-2 and C-3, respectively.

All the synthesised compounds were screened for their *in vitro* antioxidant activity using standard ABTS and DPPH methods. Methylation of embelin (**2**), reduced its activity in both the methods indicating that phenolic –OH group of embelin at C-5 is essential for

the activity. Similarly, when the di-ketone groups at C-1 and C-4 in embelin were substituted with aniline to form a di-imine derivative, the activity was found to be decreased. Hence, the quinone moiety of embelin is again essential for the activity. When the p-hydroxy aniline (**10**), phenyl alanine (**21**), 2,4-dinitro phenyl hydrazine (**23**) and tryptophan (**24**) were substituted at C-5 of embelin, the activity was found to be more than embelin in both the methods. The addition of phenolic group, hydrazines and amino acids was found to be beneficial for the activity. Compound **13** was found to be more active in ABTS and compound **22** in DPPH methods than embelin indicating that the substitution of p-sulfonylamine phenylamino group and phenyl hydrazine substitution helped to improve the activity. Compound **25** was also found to be more active in both the methods indicating phenazine formation is beneficial for the activity. Among all the para substituted phenyl derivatives, compound **10** in both the methods and **13** in ABTS method were found to be more active than embelin (**1**). Further, hydrazines, amino acids substitutions (**21**-**24**) and phenazine nucleus (**25**) also more active than embelin. Based on these results, compounds **10, 13** and **21-25** along with **1** were chosen for comparing their *in vivo* analgesic and anti-inflammatory activities with embelin.

In the present study analgesic activity of embelin and its derivatives (**1**, **10**, **13** and **21-25**) was evaluated by hot plate, tail immersion and acetic acid induced writhing methods. These tests allows to analyze peripheral and centrally mediated antinociceptive responses. Hot plate test and tail withdrawal response has selectivity for opioid derived centrally mediated analgesics [150]. Animals treated with embelin or its derivatives showed significantly longer latency than the control group in both the methods indicating that these compounds cause analgesia by their actions at CNS. Acetic acid causes an

increase in peritoneal fluids of PGE_2 and $PGF_{2\alpha}$, serotonin and histamine involved in part, which is a model commonly used for screening peripheral analgesics [151]. All the compounds abolished the acetic acid induced writhing at both the doses indicating their potent activity by peripheral antinociceptive action. These results indicates that the analgesic effect of compounds **1**, **10**, **13** and **21-25** might be mediated by its peripheral effects by inhibiting the synthesis or action of prostaglandins. The activity was found to be better than standard pentazocine.

Carrageenan induced inflammation is a non-specific inflammation resulting from a complex of diverse mediators [35]. This model is conventional, sensitive, accepted for screening of newer anti-inflammatory agents and reliably predicts the anti-inflammatory efficacy based on inhibition of prostaglandin amplification. In the present study, compounds **13** and **25** exhibited potent effect indicating them to be good candidates for anti-inflammatory activity. The potent activity may be due to the presence of p-sulfonamide nucleus in the molecule. The observed potent analgesic and anti-inflammatory properties of embelin derivatives in the present study may be due to their potent antioxidant nature.

Chemical modification of natural phenolic antioxidant by the use of ninhydrin has enhanced the potential for the development of pharmacologically important chemicals [34]. Hence, in the present study embelin-ninhydrin adduct was prepared and evaluated for its antioxidant, analgesic and anti-inflammatory properties. The adduct produced slightly better results than embelin in all these activities.

Chemical modification of mangiferin for obtaining compounds with better activity has also been tried. One new and three known mangiferin derivatives were prepared and characterized.

Mangiferin on benzoylation, showed an intense ionic peak at m/z 755 for [M+Na-2H) of tribenzoyl derivative in its positive mode ESI-MS spectrum. In its IR spectrum, it exhibited characteristic absorption bands at 3352 (OH), 1747, 1742 (ester carbonyl), 1618 (C=O), 1600 and 1556 (C=C of aromatic ring) and at 1091 cm^{-1} (C-O). The presence of signals at δ 7.20-8.30 (15H) indicated the presence of benzoyl groups in the molecule. Further disappearance of –OH group signals at 3, 6 and 7^{th} position in ^{1}H NMR suggesting the position of the benzoylation in the hydroxyl groups of the basic molecule.

Mangiferin on benzylation, showed intense ionic peaks at m/z 689, 599 and 510 for tribenzyl, dibenzyl and monobenzyl derivatives in their negative mode ESI-MS spectrum. In its IR spectrum it exhibited characteristic absorption bands at 3392 (OH), 1647 (C=O), 1608 and 1577 (C=C of aromatic ring) and at 1080 cm^{-1} (C-O). The presence of three signals at δ 5.40, 5.30 and 5.25 each for two protons accompanied with the aromatic protons at δ 7.30, 7.40 and 7.50 suggests the presence of a tribenzyl derivative of mangiferin.

Mangiferin on acetylation, showed an intense ionic peak at m/z 611, 653, 695 and 755 for [M–3H] ion of tetra acetyl, penta acetyl, hexa acetyl and octa acetyl derivatives, respectively in its positive mode ESI-MS spectrum. In its IR spectrum, it exhibited characteristic absorption bands at 1751 (ester carbonyl), 1651 (C=O), 1620 (C=C of aromatic ring) and at 1055 cm^{-1} (C-O). The ^{1}H NMR values showed signals at δ 1.20-

2.00 (24H) indicating the formation of an octa acetyl derivative. The position of the acetylation in the octa acetyl derivative is at all the –OH groups, which is evident from the downfield shift of the corresponding protons in the ^{1}H NMR spectrum. The hydroxyl group signals disappeared in the acylated spectrum.

Mangiferin on methylation, showed an intense ionic peak at m/z 450, and a pseudo molecular ion peak at m/z 464 for $[M]^{+}$ ion and analyzed for $C_{22}H_{24}O_{11}$ in its positive mode ESI-MS spectrum. In its IR spectrum, it exhibited characteristic absorption bands at 3367 (OH), 1647(C=O), 1606 (C=C of aromatic ring) and at 1064 cm^{-1} (C-O). The presence of signals at δ 4.60-4.90 (9H) suggests a trimethyl derivative of mangiferin. The signals of the remaining protons are same as that of mangiferin. This suggests that methylation occurred in the xanthone nucleus (C-3, 6 and 7).

All the synthesised mangiferin derivatives were screened for their *in vitro* antioxidant activity using various standard methods. Mangiferin showed better activity in DPPH, ABTS, H_2O_2 and nitric oxide methods when compared to all the derivatives. Furthermore, mangiferin was poorly active in lipid peroxidation, p-NDA, deoxyribose and alkaline DMSO methods. A possible reason for these results is that mangiferin has ability to scavenge free radicals formed in the initial step of lipid peroxidation as has been noticed previously in rat liver microsomes [152]. It is more likely that in the case of other methods also similar situation may also be operative. MGN 1 and MGN 3 showed potent activity than mangiferin in lipid peroxidation, p-NDA, deoxyribose and alkaline DMSO methods revealed that acetyl and benzoyl group substitution is beneficial for the activity. However, methyl and benzyl mangiferin abolishes the antioxidant activity in most of the

methods. Based on these results, compounds MGN 1 and MGN 3 along with mangiferin were chosen for comparing their *in vivo* analgesic and anti-inflammatory activities with mangiferin.

In analgesic and anti-inflammatory studies MGN 1 and MGN 3 does not produce significant activity when compared to mangiferin. However, MGN 1 showed slightly better results than mangiferin in analgesic models, but it failed in acetic acid induced writhing and anti-inflammatory studies.

In anticonvulsant activity of embelin, protection against HLTE in MES predicts the ability of a sample to prevent the spread of seizure discharge from the epileptic focus in brain and suppressing generalized tonic-clonic and partial seizures [56]. The dose dependant blockage of HLTE, decrease in the time taken for stupor, a complete abolition of extension and fast onset of stupor observed by embelin indicates its antiepileptic activity. Embelin also exhibited significant and dose dependant delayed onset of clonic-tonic actions and protection from PTZ induced mortality and may be interfering with GABAergic mechanism to exert its anticonvulsant effect [153]. Decrease in locomotion is due to decrease in dopaminergic transmission and thus increase in GABAergic transmission [154]. Locomotor activity is considered as an index of alertness, and a decrease indicates a sedative effect [155]. In the present study, embelin was found to decrease the locomotion dose-dependently, supporting the earlier evidence [120]. In conclusion, Embelin exhibited significant activity in MES and PTZ induced seizure models, it can be an effective compound against both grand mal and petit mal epilepsies

[156]. Further research is warranted to determine the specific mode of its anticonvulsant activity.

Several medicinal plants are widely used for the treatment of diabetes mellitus in the traditional medicine of many countries. *Embelia ribes* is an important ingredient of a number of Ayurvedic formulations in India [157,158]. Studies confirmed its antihyperglycemic potential along with its antioxidant and hepatoprotective effects [11,16,17,64,65,159]. The present work was carried out to know whether embelin, a major phytoconstituent present in it is the active molecule for its antidiabetic effects.

Embelin exhibited restoration of body weight, blood glucose and biochemical parameters towards the normal when compared to alloxan induced hyperglycemic rats. There will be an increase in body weight after attaining normal glycemic levels in the diabetes, particularly after treatment with sulfonyl ureas or insulin or both. Weight gain is not seen normally with the use of acarbose, troglitazone or metformin, in part because these antidiabetic agents do not stimulate the pancreas and do not elevate circulating insulin levels [160]. Treatment with embelin induced an increase in the body weight in the diabetic animals, probably due to the improvement in insulin secretion and glycemic control. The significant reduction in the serum blood glucose levels after the treatment with embelin is consistant with the findings of ethanolic extract of *Embelia ribes* by Bhandari et al. [65].

Embelin at both the doses, significantly reduced TGL, TC and VLDL in serum of alloxan induced diabetic rats. Thus, the normalization of lipids in diabetic rats treated with embelin may be due to its stimulatory effect on insulin secretion from pancreatic β-cells

[161]. The diabetic hyperglycemia induces elevation of creatinine which was considered as a significant marker of renal dysfunction [162]. The decrease in TP and albumin may be due to microproteinurea and albuminurea, which are important clinical markers of diabetic nephropathy, and/or may be due to increased protein catabolism [162]. The results of the present study demonstrated significant decrease in CR and elevation in TP and albumin levels to be comparable with their normal levels after the treatment of the diabetic rats with embelin.

The levels of serum LDH and ALP were significantly increased in alloxan induced diabetes in rats. It is mainly due to the leakage of these enzymes from the liver cytosol into the blood stream, which gives an indication on the hepatotoxic effect of alloxan [163], which was reversed with the treatment of embelin. This could be further confirmed by the normal level of TB observed in the embelin treated groups. From these results, it could be concluded that embelin is capable of ameliorating the impaired diabetic kidney function in addition to its hypoglycemic and hepatoprotective activities.

Histologically, liver section of alloxan induced diabetic rats showed periportal fatty infiltration and necrosis of hepatocytes as a result of absence of insulin [164]. Diuresis is a common feature associated with diabetes which may be the reason for structural changes observed with glomerulus [165]. Histological observations in the present study proved the excellent recovery of liver and kidney functions after embelin treatment. The damage of pancreas in alloxan-treated diabetic control rats was also reversed by embelin indicating its positive effects on the production of insulin.

It is well known that oxygen free radicals are involved in the diabetogenic action of alloxan and antioxidants have been shown to be effective in diabetes [166]. Embelin significantly scavenged free radicals and resulting in hepatic glutathione antioxidant defense, decreases lipid peroxidation and minimizes the histological (liver) alterations induced by N-nitrosodiethylamine [159]. Hence, the antidiabetic activity of embelin may be due to its potent antioxidant properties. Our results also confirm that the potent antidiabetic action of *Embelia ribes* observed earlier by many studies may be due to the presence of embelin in the extract [11,64,65]. In conclusion, the present study demonstrated that the treatment of diabetic rats with embelin has exerted a considerable hypoglycemic effect. In addition, embelin could ameliorate the impaired renal function, inhibition of liver damage and resemble of islets of pancreas associated with alloxan diabetes. Due to its safety and activity, it is an ideal lead molecule for further antidiabetic drug development.

Acetic acid induced colitis is a model wherein inflammatory mediators such as reactive oxygen species, vasoactive amines and eicosanoids play a prominent role [167]. The underlying pathophysiological mechanisms involved include colon structure and mucosa barrier destruction by chemical stimulation, enhanced vessel permeability, increased inflammatory mediators, promotion of fibrin hydrolysis, and disturbance of cruor process.

The wet weight of the inflamed colon tissue is considered a reliable and sensitive indicator of the severity and extent of inflammatory response [168]. In the present study, pre-treatment with embelin in the acetic acid induced colitis significantly reduced the wet

weight of colon, clinical activity, gross lesion score and percentage of affected area compared with colitis control, indicating its protective effect from ulcerative colitis.

Myeloperoxidase is an enzyme present in neutrophils and at a much lower concentration in monocytes and macrophages. The level of MPO activity is directly proportional to the neutrophil concentration in the inflamed tissue. Therefore, a measurement of MPO activity has been considered a quantitative and sensitive assay for acute intestinal inflammation. In addition, increased MPO activity has been reported to be an index of neutrophil infiltration and inflammation [131]. Embelin at both the doses exhibited a significant decrease in the MPO levels when compared to acetic acid induced colitis control.

Increased lipid peroxides that occurs in colonic tissue can initiate a vicious cycle that generate more and more reactive metabolites, which exhausts cellular antioxidants, vitamin C and E and ultimately favors the consequent development of further inflammation and ulceration. Embelin at both the doses significantly inhibited the increase in the lipid peroxides activity in the colonic tissue. It is therefore reasonable to assume that the embelin treatment improves colonic oxidative balance in colitis induced animals, because embelin was able to reduce the level of malondialdehyde, a good indicator of lipid peroxidation [138].

GSH is involved in the synthesis and repair of DNA, assists the recycling of vitamins C and E, blocks free radical damage, enhances the antioxidant activity of vitamin C, facilitates the transport of amino acids and play a critical role in detoxification [169]. Pre-treatment of embelin reversed the depletion of GSH and restored the levels towards the

normal. LDH, a cytosolic enzyme is involved in the biochemical regulation reaction of the body tissues and fluids. An elevation of LDH in serum indicates a shift towards anaerobiosis resulting in the enhanced production of lactic acid [170]. In the present study, the embelin pre-treatment altered the serum LDH level induced by acetic acid towards the normal.

The clinical, macroscopic and biochemical evidences for the protective effect of embelin on acetic acid induced colitis in rats was well correlated by the histopathological studies. The histological science of inflammation such as leukocyte infiltration, edema and tissue injury were found to be low following the pre-treatment with embelin.

The results obtained from embelin treated acetic acid induced colitis in the present study are in well correlation with earlier results of its ability to inhibit carrageenan induced paw edema in rats, inhibition of NF-kappaB activation, which makes it a potentially effective suppressor of tumor cell survival, proliferation, invasion, angiogenesis and inflammation [15,32,171]. In addition, embelin is known to suppress the NF-kappaB activation induced by TNF-α and various other inflammatory and carcinogenic agents [171]. The results from the present study are also in consistent with the earlier study of its ability to scavenge physiologically relevant oxidizing radicals [16]. The present data suggest that the pre-treatment of embelin prevents acetic acid induced colitis in rats and this protective effect may at least in part be due to its antioxidant and anti-inflammatory actions. However, further investigations are necessary to evaluate whether a similar efficacy can be achieved in other models of experimental colitis that simulate human inflammatory bowel disease.

Embelia ribes was reported to possess neuroprotection [76,77] against cerebral ischemia and methionine induced oxidative stress in rat brain. The present work was carried out to know whether embelin, a major phytoconstituent present in it is the active molecule for its neuroprotective effects.

BCCA occlusion for 30 min followed by 24 h reperfusion showed significant impairment of sensorimotor activities (locomotor activity, hanging wire test and beam walking test). These findings were correlated with earlier reports which also documented sensorimotor and neurological deficits in the animals following BCCA occlusion and reperfusion injury [172]. Global cerebral ischemia and reperfusion induced behavioral alterations are similar to clinical symptoms in stroke patients [173]. In the present study, a significant decrease in locomotor activity and an increase in beam walking latency have been observed after ischemia reperfusion which was reversed by embelin pretreatment at both the doses. Similarly, global cerebral ischemia has been reported to cause significant decrease in grip and muscle strength of the limbs [141,174]. Consistent to the above studies, a marked decrease in muscle strength and grip has been observed in the present study, which was significantly reversed by embelin pretreatment at both the doses suggesting the therapeutic potential of embelin against ischemic reperfusion injury.

Oxidative stress has been implicated to play a major role in the ischemic reperfusion injury. Neurons contain polyunsaturated fatty acids, which are vulnerable to free radical attack, making the brain prone to damage by oxidative stress [175]. The ROS generated during ischemia have multiple mechanisms to cause cell death. The endogenous antioxidants play a vital role in detoxifying the free radicals while depleting their own

cellular stores [176]. In the present investigation, BCCA occlusion for 30 min increased the MDA levels and decreased the total thiols and enzymatic antioxidant GST significantly. These results are in harmony with other studies [177,178]. Pretreatment with embelin significantly elevated the levels of total thiols, enzymatic antioxidant GST and markedly decreased the MDA levels. Thus the protection offered by embelin may also be due to its antioxidant potential.

TTC staining is widely used to identify the extent of ischemia in the tissues. The colorless TTC is converted to red formazan product by nicotinamide adenine dinucleotide (NAD) and dehydrogenase enzymes, which are found abundant in mitochondria. Hence, viable cells take deep red stain and the infarcted cells remain unstained [176,179,180]. Pretreatment of embelin reduced the infarct volume, indicates its significant protection against neuronal injury by preventing mitochondrial damage through scavenging ROS.

The sensorimotor activities, infarct volume and biochemical evidences for the protective effect of embelin on global ischemia induced neuronal damage in rats was well correlated by the histopathological studies. The histological science of ischemia such as congested blood vessels, increased lymphocyte infiltration and neuronal necrosis were found to be low following the pretreatment with embelin. The present data suggests that the pretreatment of embelin protected rats from global ischemia induced neuronal damage. The protective effect may at least in part be due to its antioxidant action. However, further investigations are necessary to determine its specific mode of action.

- Natural products provide a starting point for new synthetic compounds with diverse structures and often with multiple stereocenters that can be challenging synthetically. Many structural features common to natural products (e.g., chiral centers, aromatic rings, complex ring systems, degree of molecule saturation, and number and ratio of heteroatoms) have been shown to be highly relevant to drug discovery efforts. Furthermore, since the escalation of interest in combinatorial chemistry and the subsequent realization that these compound libraries may not always be very diverse, many synthetic and medicinal chemists are exploring the creation of natural product and natural-product like libraries that combine the structural features of natural products with the compound-generating potential of combinatorial chemistry. Drugs derived from medicinal plants can serve not only as new drugs themselves but also as drug leads suitable for optimization by medicinal and synthetic chemists. Even when new chemical structures are not found during drug discovery from medicinal plants, known compounds with new biological activity can provide important drug leads.

- Since the sequencing of the human genome, thousands of new molecular targets have been identified as important in various diseases. With the advent of high-throughput screening assays directed towards these targets, known compounds from medicinal plants may show promising and possibly selective activity. Hence, in the present study, embelin and mangiferin were selected as lead compounds for semi synthetic drug development. Embelin showed antifertility, anti-implantation, antitumour, anti-inflammatory, analgesic, antioxidant, hepatoprotective, wound healing and antibacterial activities [13-19]. Except these studies so far no other

biological studies have been carried out. However, embelin has not been tested so far for anticonvulsant, antidiabetic, effect against inflammatory bowel disease and neuroprotection activities, though the plant (*Embelia ribes*) is reported against such a disorders [9,10,32,63,64,76,77]. Hence, in the present study, anticonvulsant, antidiabetic, effect in inflammatory bowel disease and neuroprotective activities of embelin were also carried out.

- The plants selected for the study were berries of *Embelia ribes* and leaves of *Mangifera indica*. The plant materials were collected and authenticated. The dried materials were extracted and isolation of embelin and mangiferin was carried out from the respective plants.

- Structural modification of embelin was carried out for structure activity relationship studies. Embelin was condensed with various aromatic substituted primary amines to yield sixteen new and eight known derivatives along with novel embelin-ninhydrin adduct were prepared. All these compounds were synthesized and purified by standard procedures, identified by using physical and spectral (IR, ^{1}H NMR, ^{13}C NMR and MS) properties.

- During the last two decades, there has been a growing interest in studies that concern 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. Antioxidant and related properties of the plant *Embelia*

ribes and embelin are well known. Hence, all the synthesized compounds along with embelin were tested for *in vitro* antioxidant activity using ABTS and DPPH methods. When p-hydroxy aniline (**10**), sulphanilamide (**13**) phenyl alanine (**21**), phenyl hydrazine (**22**), 2,4-dinitro phenyl hydrazine (**23**) and tryptophan (**24**) were substituted at C-5 of embelin and substitution of o-phenylene diamine (**25**), the activity was found to be more potent than embelin in ABTS and DPPH methods. These compounds along with embelin were studied for analgesic and anti-inflammatory activities at 10 and 20 mg/kg doses by standard methods. Potent analgesic activity higher than the standard pentazocine was observed. Embelin and its derivatives almost completely abolished the acetic acid induced writhing. p-Sulfonylamine, phenyl propionic acid and phenazine derivatives showed better anti-inflammatory activity than embelin.

- Based on a reaction of ninhydrin with phenols, embelin-ninhydrin adduct was prepared and characterized. It exhibited better antioxidant activity in DPPH method. The analgesic and anti-inflammatory activities were also found to be better than the parent moiety, embelin. In the acetic acid induced writhing almost complete abolition of writhing was observed at 10 and 20 mg/kg body weight doses of embelin-ninhydrin adduct and the results were found to be better than the standard pentazocine.

- Structural modification of mangiferin was carried out for structure activity relationship studies. One new compound and three known derivatives were prepared from mangiferin. All these compounds were synthesized and purified by

standard procedures, identified by using physical and spectral (IR, ^{1}H NMR, ^{13}C NMR and MS) properties.

- The synthesized mangiferin derivatives were tested for *in vitro* antioxidant properties. Benzyl and methyl substituted mangiferin showed poor activity than mangiferin. However, mangiferin derivatives substituted with acetyl and benzoyl groups were showed potent activity than mangiferin in lipid peroxidation, p-NDA, deoxyribose and alkaline DMSO methods. But both the compounds failed to show potent analgesic and anti-inflammatory activities. In all these methods, standard drugs showed better activity than mangiferin and its derivatives.

- Anticonvulsant activity of embelin (2.5, 5 and 10 mg/kg, i.p.) was studied. It showed a significant inhibition of the seizures induced by electroshock and pentylenetetrazole in a dose dependent manner and the activity was comparable to phenytoin and diazepam. Significant decrease in locomotion revealing its CNS depressant activity was observed. The findings suggest that embelin possess anticonvulsant activity against both grand mal and petit mal epilepsies.

- In the antidiabetic activity, embelin (25 and 50 mg/kg, p.o.) administered orally to alloxan induced diabetic rats for 21 days caused a significant reduction in fasting serum blood glucose levels and significant improvement in body weights. Significant antidiabetic effects were obtained as evident from the restoration of biochemical parameters altered by alloxan towards the normal. Almost normal histological appearance of liver, kidney and pancreas tissues were observed in treated groups. Among the two doses, 50 mg/kg showed better activity. However,

the activity was found to be less than the standard glibenclamide given at 10 mg/kg dose. The results showed that embelin has potential hypoglycemic effect along with recovery of liver, kidney and pancreas functions.

- In the present study, protective activity of embelin against acetic acid induced colitis was investigated Experimental animals received embelin (25 and 50 mg/kg, p.o.) and sulfasalazine (100 mg/kg, p.o.) for five consecutive days before induction of colitis by intra-rectal acetic acid (3% v/v) administration and the treatment continued upto 7 days. The colonic mucosal injury was assessed by clinical, macroscopic, biochemical and histopathological examination. Embelin treatment significantly decreased clinical activity score, gross lesion score, percent affected area and wet colon weight when compared to acetic acid induced controls. The treatment also reduced significantly the colonic myeloperoxidase activity, lipid peroxides and serum lactate dehydrogenase and significantly increased the reduced glutathione. The histopathological studies also confirmed the above findings. The protective effect may be due to its antioxidant and anti-inflammatory activities.

- In the neuroprotective activity of embelin, global ischemia and reperfusion were induced by occluding bilateral common carotid arteries for 30 min followed by 24 h reperfusion. Neurological functions were measured by using sensorimotor tests. Ischemia reperfusion induced neuronal injury was assessed by cerebral infarct volume, biochemical and histopathological examination. Pretreatment of embelin (25 and 50 mg/kg, p.o.) significantly increased locomotor activity and hanging

latency time and decreased beam walking latency when compared to ischemic control. The treatment also reduced significantly the lipid peroxidation and increased the total thiol and glutathione-S-transferase activity. Cerebral infarction area and histopathological findings also confirmed the above findings. These findings suggested that embelin is a neuroprotective and may prove to be useful adjunct in the treatment of stroke.

- In conclusion, in the present study structural modification of two natural products was carried out for structure activity relationship studies. Including sixteen new compounds twenty four derivatives along with a novel embelin-ninhydrin adduct were prepared from embelin. A new and three known mangiferin derivatives were also prepared. All these compounds were characterized by physical and spectral properties. The derivatives of embelin and mangiferin were tested for *in vitro* antioxidant properties. Some of the derivatives showed potent *in vitro* antioxidant properties compared to the parent moieties. The potent antioxidant compounds also exhibited potent analgesic and anti-inflammatory activities. Hence, the structural modification of natural compounds played an important role to improve the biological activities. Embelin exhibited potent anticonvulsant properties. It exhibited potent antidiabetic properties in alloxan induced diabetes, protective effect against acetic acid induced colitis and neuroprotective effect against global cerebral ischemia in rats. Hence, new biological properties of embelin were established, which supports the traditional claim of the plant. Further research would be of interest to explain the exact mechanism of the parent compounds and their chemical modifications.

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INSTITUTIONAL ANIMAL ETHICS COMMITTEE (IAEC)

CERTIFICATE

Title of the Project: **Structure activity relationship of bioactive compounds from medicinal plants**

Proposal number: JSSCP/IAEC/M.PHARM/PH.D/04/2008-09

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Approval date: 22.10.08.

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Number of animals sanctioned: 42

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Expiry date: TWO MONTHS

Name of IAEC/CPCSEA chairperson: Dr. K. Elango, Professor

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Signature

Dr. K. Elango
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RESEARCH PUBLICATIONS

1. **Mahendran S**, Thippeswamy BS, Veerapur VP, Badami S. Anticonvulsant activity of embelin isolated from *Embelia ribes*. **Phytomedicine** (2010, In Press).

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3. Thippeswamy BS, **Mahendran S,** Biradar MI, Pooja R, Kamya S, Badami S, Veerapur VP. Protective effect of embelin against acetic acid induced colitis in rats. **European Journal of Pharmacology (Accepted, 2010).**

4. **Mahendran S,** Badami S, Ravi S, Thippeswamy BS, Veerapur VP. Antioxidant, analgesic and anti-inflammatory studies of novel ninhydrin adduct of embelin **(**Communicated to **European Journal of Medicinal Chemistry).**

5. **Mahendran** S, Badami S, Ravi S, Thippeswamy BS, Veerapur VP. Synthesis and evaluation of analgesic and anti-inflammatory activities of most active antioxidant derivatives of embelin – A structure activity relationship (Communicated to **Chemical & Pharmaceutical Bulletin**).

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Short communication

Anticonvulsant activity of embelin isolated from *Embelia ribes*

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ABSTRACT

Anticonvulsant activity of embelin (2.5, 5 and 10 mg/kg, i.p.) was studied. It showed a significant inhibition of the seizures induced by electroshock and pentylenetetrazole in a dose dependent manner and the activity was comparable to phenytoin and diazepam. Significant decrease in locomotion revealing its CNS depressant activity was observed. The findings suggest that embelin possess anticonvulsant activity against both grand mal and petit mal epilepsy.

Introduction

Embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone) is a major constituent of *Embelia ribes* Burm. (Family: Myrsinaceae). The whole plant is used as anti-inflammatory to relieve rheumatism and fever (Kapoor et al. 1983). The fruit is good appetizer, cures tumors, ascites, bronchitis, jaundice, brain tonic, mental disorders, dyspnaea, diseases of the heart; urinary discharges, scorpion-sting, snake-bite and tooth ache (Varier 2006). Embelin showed antifertility, antitumor, anti-inflammatory, analgesic, antioxidant, hepatoprotective, wound healing and antibacterial (Chitra et al. 2003a; Dharmendra et al. 2009; Kumara Swamy et al. 2007) activities.

Incidence of epilepsy in developed countries is approximately 50 per 100,000 while that of developing countries is 100 per 100,000. The entire currently available antiepileptic drugs are synthetic molecules associated with side effects and approximately 30% of the patients continue to have seizures with this therapy (Poole et al. 2000). Several natural products including quinone derivatives, which are considered to possess better safety and efficacy profile, are known for their anticonvulsant effects (Hosseinzadeh and Parvardeh 2004). However, embelin, a benzoquinone has not been tested so far for anticonvulsant properties, though the plant is traditionally used against such disorders (Varier 2006). In the present study, we investigated the anticonvulsant activity of embelin by using maximal electroshock (MES), pentylenetetrazole (PTZ) induced seizures along with locomotor activity.

Materials and methods

Extraction and isolation of embelin

The berries of *Embelia ribes* were purchased from Abirami Botanicals, Tuticorin, TN, India and authenticated by Medicinal Plants Survey and Collection Unit, Ootacamund, TN, India. Coarsely powdered berries (1 kg) were extracted for 72 h with *n*-hexane by cold maceration (3 × 2 l). The extract was concentrated to dryness in a rotavapor and chromatographed over silica gel (100–200 mesh). Elution with benzene and re-crystallization with ether afforded orange plates of embelin (yield 3 g, 0.3%, Chitra et al. 2003b). It was found to be 100% pure by HPTLC using the solvent system ethyl acetate: benzene (70:30) and characterized by comparing its physical and spectral values with literature (Feresin et al. 2003). The structure of embelin is depicted in Fig. 1.

Animals

The animals were obtained from the animal house Sree Siddaganga College of Pharmacy, Tumkur, India, maintained under standard conditions (12 h light/dark cycle; 25 ± 3 °C, 45–65% humidity), had free access to standard rat feed and water *ad libitum* and studies were performed as per CPCSEA, India.

Chemicals

Diazepam (Ranbaxy, New Delhi, India), Phenytoin (Sun Pharma, Mumbai, India), and PTZ (Sigma Aldrich, St. Louis, USA) were purchased. All other chemicals used were of analytical grade. PTZ was dissolved in normal saline and embelin was suspended in Tween 80 (1%, v/v) in saline and used.

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Table 1
Effect of embelin on MES-induced seizure in rats.

Treatment (dose, mg/kg, i.p.)	Duration of HLTE (s) Mean ± SEM	Stupor (s) Mean ± SEM	No. of animals recovered/used	Protection against mortality (%)
MES Control	11.80 ± 0.37	131.40 ± 2.29	4/6	66.66
Embelin (2.5)	5.40 ± 0.24[a]	109.00 ± 1.88[a]	6/6	100.00
Embelin (5)	1.50 ± 0.12[a]	84.00 ± 1.87[a]	6/6	100.00
Embelin (10)	Absence of extension	49.40 ± 0.87[a]	6/6	100.00
Phenytoin (25)	Absence of extension	55.00 ± 3.30[a]	6/6	100.00

[a] $P < 0.001$, when compared to control ($n = 6$), Tukey–Kramer.

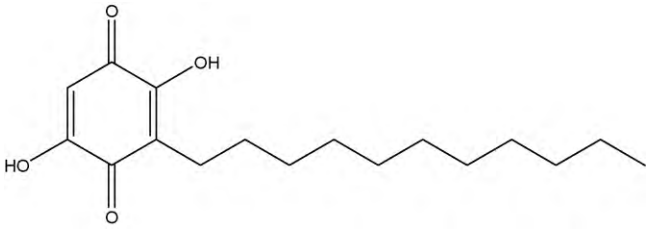

Fig. 1. Structure of embelin.

MES induced seizure model

Swiss albino rats (150–200 g) were divided into five groups with six animals in each group. Group I was served as solvent control and received 0.9% (w/v) of saline (1 ml/100 g). Groups II, III and IV were received embelin 2.5, 5 and 10 mg/kg suspended in 1% Tween 80 (v/v) and Group V treated as positive control was received phenytoin (25 mg/kg). All the treatments were administered intraperitoneally 30 min prior to the electroshock. The electroshock was induced in animals by passing a current of 150 mA for 0.2 s duration through electroconvulsiometer (INCO, Ambala Pvt. Ltd., India) using corneal electrodes. A drop of electrolyte solution (0.9% sodium chloride) was instilled in the eyes of the animals prior to delivery of electroshock for good electrode contact (Swinyard and Kupferberg 1985). Total duration of Hind Limb Tonic Extension (HLTE) was recorded after the delivery of the electroshock for control and experimental groups of animals. The onset of stupor, time taken for death/recovery as well as the percentage of protection against mortality (Hosseinzadeh and Parvardeh 2004) were also recorded.

PTZ induced seizure model

Swiss albino mice (20–25 g) were divided into five groups with six animals in each group. Groups I, II, III and IV were received the treatments as described above. Group V, positive control, was received diazepam (4 mg/kg). All the treatments were administered intraperitoneally 30 min prior to the administration of PTZ (85 mg/kg). Each animal was observed for 1 h by placing in a separate cage. The onset of action showing fore limb and hind limb clonus (clonic and tonic action, Salahdeen and Yemitan 2006), and the time taken for death/recovery as well as the percentage of protection against mortality (Hosseinzadeh and Parvardeh 2004) were recorded.

Locomotor activity

Mice were acclimatized with environment and placed individually in an actophotometer (INCO, Ambala Pvt. Ltd., India) for 10 min and a basal activity score was obtained. Subsequently they were divided into five groups with six animals in each group and all the groups except Group V, were treated as per MES induced seizure model. Group V, positive control was received diazepam (1 mg/kg, i.p.). After 30 min, the activity score was recorded (Kulkarni 1999). The percentage reduction in locomotor activity was calculated.

Statistical analysis

The values were expressed as mean ± SEM. The statistical analysis was carried out by one way analysis of variance (ANOVA) followed by multiple comparison test of Tukey–Kramer. P values < 0.05 were considered as significant.

Results and discussion

The duration of HLTE for the control group was 11.80 ± 0.37 s after an electroshock in MES model. Administration of embelin (2.5 and 5 mg/kg, i.p.) showed significant ($P < 0.001$) reduction in the duration of HLTE to 5.40 ± 0.24 and 1.50 ± 0.12 s, respectively compared to the control (Table 1). However, embelin at the dose of 10 mg/kg and phenytoin at 25 mg/kg dose showed total absence of extension. The onset of stupor for the control animals was 131.40 ± 2.29 s. Embelin at all the three doses significantly ($P < 0.001$) decreased the onset of stupor when compared to the control. At 10 mg/kg dose it showed lower onset time when compared to standard phenytoin. In the control group 66.66% animals survived the electroshock, but embelin at all the three doses and phenytoin treatments exhibited 100% protection against mortality.

The onset of clonic and tonic actions induced by PTZ in the control animals was found to be 47.80 ± 1.69 and 72.60 ± 1.78 s, respectively. Embelin at all the three doses significantly ($P < 0.001$) increased both the clonic and tonic onsets (Table 2). At 5 and 10 mg/kg doses it exhibited 50 and 83.33% protection against mortality. However, the standard diazepam 4 mg/kg showed total absence of convulsions and 100% protection against mortality.

Table 2
Effect of embelin on PTZ-induced seizure in mice.

Treatment (dose, mg/kg, i.p.)	Onset of clonic action in s Mean ± SEM	Onset of tonic action in s Mean ± SEM	No. of animals recovered/used	Time to death in min Mean ± SEM (no. of deaths)	Protection against mortality (%)
PTZ Control (85)	47.80 ± 1.69	72.60 ± 1.78	0/6	11.20 ± 0.58 (6)	0.00
Embelin (2.5)	66.60 ± 1.40[a]	88.60 ± 1.17[a]	0/6	18.60 ± 0.75 (6)	0.00
Embelin (5)	88.20 ± 0.92[a]	143.40 ± 1.40[a]	3/6	43.50 ± 1.50 (3)	50.00
Embelin (10)	230.00 ± 4.44[a]	349.40 ± 3.37[a]	5/6	125. 45 (1)	83.33
Diazepam (4)	Absence of convulsions	Absence of convulsions	6/6	Nil	100.00

[a] $P < 0.001$, when compared to control ($n = 6$), Tukey–Kramer.

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Table 3
Effect of embelin on locomotor activity in actophotometer.

Treatment (dose mg/kg, i.p.)	Locomotor activity (score) in 10 min		Reduction in activity (%)
	Before treatment Mean ± SEM	After treatment Mean ± SEM	
Normal Control	568.0 ± 16.89	512.0 ± 13.62	9.86
Embelin (2.5)	551.0 ± 27.41	246.4 ± 16.38[a]	59.42
Embelin (5)	527.0 ± 20.83	145.6 ± 10.22[a]	72.38
Embelin (10)	577.6 ± 22.98	70.6 ± 9.59[a]	87.78
Diazepam (1)	540.8 ± 17.16	127 ± 10.36[a]	76.52

[a] $P < 0.001$, when compared to before treatment ($n = 6$), Tukey–Kramer.

Embelin at all the three doses and standard diazepam at 1 mg/kg, significantly ($P < 0.001$) reduced the locomotor activity in mice when compared to the control animals (Table 3). At 10 mg/kg dose it showed better reduction in locomotor activity (87.78%) when compared to standard diazepam (76.52%).

Protection against HLTE in MES predicts the ability of a sample to prevent the spread of seizure discharge from the epileptic focus in brain and suppressing generalized tonic-clonic and partial seizures (Hosseinzadeh and Parvardeh 2004). The dose dependant blockage of HLTE, decrease in the time taken for stupor, a complete abolition of extension and fast onset of stupor observed by embelin indicates its antiepileptic activity. Embelin also exhibited significant and dose dependant delayed onset of clonic-tonic actions and protection from PTZ induced mortality and may be interfering with GABAergic mechanism to exert its anticonvulsant effect (Gale 1992). Decrease in locomotion is due to decrease in dopaminergic transmission and thus increase in GABAergic transmission (Sokoloff et al. 1990). Locomotor activity is considered as an index of alertness, and a decrease indicates a sedative effect (Thakur and Mengi 2005). In the present study, embelin was found to decrease the locomotion dose-dependently, supporting the earlier evidence (Atal et al. 1984). In conclusion, embelin exhibited significant activity in MES and PTZ induced seizure models, it can be an effective compound against both grand mal and petit mal epilepsies (Vida 1995). Further research is warranted to determine the specific mode of its anticonvulsant activity.

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Original article

Evaluation of antidiabetic effect of embelin from *Embelia ribes* in alloxan induced diabetes in rats

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ABSTRACT

Embelin, a major constituent of *Embelia ribes* is therapeutically evaluated for antihyperglycemic potential against alloxan induced diabetes in rats. Embelin (25 and 50 mg/kg b.w.) administered orally to alloxan induced diabetic rats for 21 days caused a significant reduction in fasting serum blood glucose levels and significant improvement in body weights. Significant antidiabetic effects were obtained as evident from the restoration of biochemical parameters altered by alloxan towards the normal. Almost normal histological appearance of liver, kidney and pancreas tissues were observed in treated groups. Among the two doses, 50 mg/kg b.w. showed better activity. However, the activity was found to be less than the standard glibenclamide given at 10 mg/kg b.w. dose. The results showed that embelin has potential hypoglycemic effect along with recovery of liver, kidney and pancreas functions.

1. Introduction

Diabetes is a major endocrine disorder causing morbidity and mortality worldwide. The problem of diabetes is particularly relevant to India, as several studies have clearly documented an increased ethnic susceptibility to diabetes in-migrant Asian Indians [1]. Recent epidemiological studies have pointed to the growing epidemic of diabetes in India [2]. Indeed, according to the recent Diabetes Atlas produced by the International Diabetes Federation (IDF), India is home to the largest number of people with diabetes in the world, 40.9 million diabetic subjects in 2007, and these numbers are predicted to increase to 69.9 million by 2025 [3].

Plants have always been an exemplary source of drugs and many of the currently available drugs have been derived directly or indirectly from them. The ethnobotanical information reports about 800 plants that may possess antidiabetic potential [4]. Several herbs have shown antidiabetic activity when assessed using presently available experimental techniques [5]. Wide arrays of plant derived active principles representing numerous chemical compounds have demonstrated activity consistent with their possible use in the treatment of non-insulin-dependent diabetes mellitus [6].

Embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone) is a naturally occurring alkyl substituted hydroxy benzoquinone and a major constituent from all the parts of *Embelia ribes* Burm. (Family: Myrsinaceae). The plant is indicated in traditional medicine for the treatment of various diseases. The whole plant is used in the treatment of anti-inflammatory to relive rheumatism and fever [7]. The fruit is bitter in taste, good appetizer, cures tumors, ascites, bronchitis, jaundice, brain tonic, mental disorders, dyspnoea, diseases of the heart, urinary discharges, scorpion-sting, snake-bite and tooth ache [8,9]. Embelin showed antifertility [10], anti-implantation [11], antitumour, anti-inflammatory, analgesic [12], antioxidant [13], hepatoprotective [14], wound healing [15], antibacterial [16] and anticonvulsant [17] activities.

In a preliminary study, Tripathi [18], reported the antihyperglycaemic activity of the decoction of *Embelia ribes* in glucose-fed albino rabbits. Further, Bhandari et al. [19] reported the ethanolic extract of the plant in diabetic dyslipidemia and protection from lipid peroxidation in tissues in streptozotocin-induced diabetes in rats. The effect of chronic oral treatment with ethanolic extract of the plant on the basal level of some key serum, tissue antioxidants, blood pressure and glycosylated haemoglobin was investigated in streptozotocin-induced oxidative damage in rats [20,21].

However, embelin has not been tested so far for its antidiabetic properties. The LD_{50} of embelin is as high as 2000 mg/kg b.w. in rats and mice with no mortality or adverse effects indicating its safety [22]. Hence, in the present study embelin isolated from *Embelia ribes* was evaluated for antidiabetic effect against alloxan induced diabetes in rats.

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2. Materials and methods

2.1. Chemicals

Alloxan monohydrate was purchased from Loba Chemie, Bombay. Assay kits for serum triglycerides (TGL), total cholesterol (TC), total bilirubin (TB), total protein (TP), creatinine (CR), albumin, lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) were obtained from Merck Ltd., Ambemath, India. All other chemicals used were of analytical grade.

2.2. Collection and authentication

The berries of *Embelia ribes* were purchased from Abirami Botanicals, Tuticorin, Tamilnadu, India and authenticated by Medicinal Plants Survey and Collection Unit, Ootacamund, Tamil Nadu, India, where a voucher specimen is preserved for further reference.

2.3. Isolation of embelin

Coarsely powdered berries of *Embelia ribes* (1 kg) were exhaustively extracted with *n*-hexane by cold extraction method (3 × 2 L). After 72 h, the extracts were concentrated to dryness in a rotavapor under reduced pressure and controlled temperature (40–50 °C). The extract so obtained was subjected to column chromatography over silica gel (100–200 mesh) and elution with benzene yielded an orange coloured powder [23] which on crystallization with ether afforded orange plates of embelin (yield 3 g, 0.3%). It was found to be 100% pure by HPTLC (solvent system, ethyl acetate 70: benzene 30). It was characterized by comparison of melting point, IR, NMR and mass spectra with literature values [24] and with authentic sample. The structure of embelin is depicted in Fig. 1.

2.4. Animals

Male Wistar rats (150–200 g) were selected for the study and maintained at a controlled temperature of 19–25 °C with 12 h light/dark cycle and fed a standard diet and water *ad libitum*. The experiments were conducted according to the Institutional Animal Ethics Committee regulations approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals.

2.5. Antidiabetic activity

Animals were divided into five groups of six animals each. Group I was served as normal control and received 1% w/v of Tween 80 (1 ml/100 g) in saline. Groups II – V animals were fasted overnight and alloxan monohydrate at 120 mg/kg b.w. was administered by intraperitoneal injection to induce diabetes [25]. The fasting blood glucose level was determined after 72 h of alloxan injection. The rats having serum blood glucose levels above 200 mg/dl were used for the study. This is considered as day

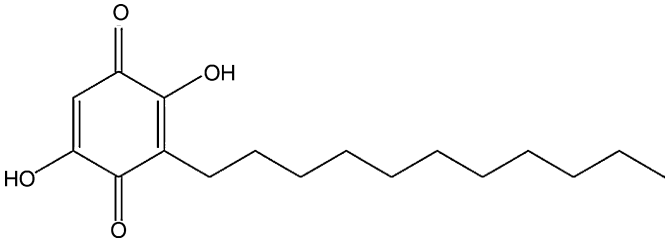

Fig. 1. Structure of embelin.

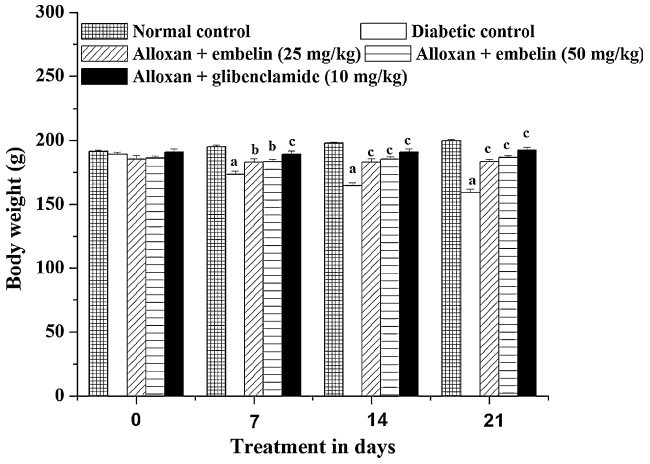

Fig. 2. The effect of 3-week treatment with embelin on body weight (g) after alloxan (120 mg/kg b.w.) induced diabetes in rats. Values are given as mean ± S.E.M. for groups of six animals each, Tukey-Kramer; values are statistically significant at (a) $P < 0.001$ between normal and diabetic control and (b) $P < 0.05$, (c) $P < 0.001$ between diabetic control and treated groups.

zero of the study. Group II was served as diabetic control and received 1% Tween 80 in saline. Groups III and IV were received embelin 25 and 50 mg/kg b.w. suspended in 1% Tween 80 (v/v) and Group V treated as positive control was received glibenclamide (10 mg/kg b.w.) dissolved in saline. All these treatments were given orally for 21 days. The animals were fasted on days 6, 13 and 20, blood samples were collected on 7, 14 and 21 days by retro-orbital plexus puncture method and fasting serum blood glucose and body weight were measured.

On day 21, blood was collected by cardiac puncture under mild ether anesthesia from overnight fasted rats and serum was separated and analyzed for TGL, TC, TB, TP, CR, albumin, LDH and ALP by autoanalyzer using Ecoline Kits. Very low density lipoprotein (VLDL) was calculated as per Friedevald's equation, VLDL Cholesterol = TGL/5.

After the collection of blood samples, the liver, kidney and pancreas were excised and rinsed in ice-cold normal saline. A portion of the tissues were fixed in 10% formalin, cut into 5 μm thick sections and stained using heamatoxylin-eosin and histo-pathological observations were made.

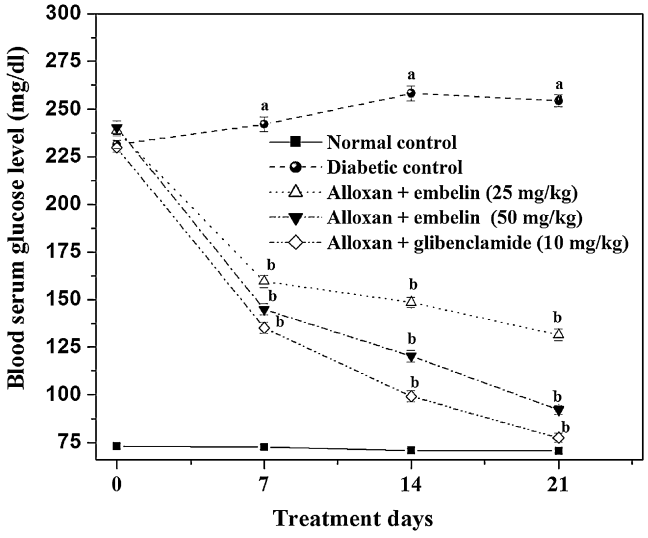

Fig. 3. Comparative effect of embelin on blood serum glucose level in alloxan (120 mg/kg b.w.) induced diabetes in rats. Values are given as mean ± S.E.M. for groups of six animals each, Tukey-Kramer; (a) $P < 0.001$ between normal and diabetic control and (b) $P < 0.001$ between diabetic control and treated groups.

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2.6. Statistical analysis

The values were expressed as mean ± SEM. The statistical analysis was carried out by one way analysis of variance (ANOVA) followed by multiple comparison test of Tukey-Kramer. P values < 0.05 were considered as significant.

3. Results

Alloxan induced diabetic control rats showed a significant ($P < 0.001$) reduction in body weight on days 7, 14 and 21 when compared to the normal rats. Embelin treated at 25 and 50 mg/kg b.w. doses and standard glibenclamide at 10 mg/kg b.w. dose given to alloxan induced diabetic rats caused significant increase in body weights when compared to diabetic control animals (Fig. 2). Significant increase in the fasting serum blood glucose level was observed in alloxan induced diabetic control rats ($P < 0.001$) when compared to normal. Embelin and glibenclamide treated groups caused a significant reversal of the same on days 7–21 (Fig. 3) measurements. A significant ($P < 0.001$) fall in serum blood glucose level of diabetic rats i.e. 41.15% (from 238.83 ± 2.73 mg/dl to 131.50 ± 3.06 mg/dl), 56.85% (from 240.33 ± 3.65 mg/dl to 92.17 ± 2.20 mg/dl) in group III and IV after 21 days of embelin treatments was observed. However, glibenclamide produced 66.16% (from 229.83 ± 2.33 mg/dl to 77.67 ± 2.36 mg/dl) reduction during the same period.

Treatment with alloxan caused a significant ($P < 0.001$) increase in the levels of TGL, TC, TB, CR, LDH, ALP and VLDL and a significant ($P < 0.001$) decrease in the levels of TP and albumin when compared to normal (Table 1). Treatment with embelin at both the doses and glibenclamide caused a significant reversal of all these changes towards the normal. Among the two doses, embelin at 50 mg/kg b.w. dose was found to be more active. However, the standard glibenclamide exhibited better activity than embelin in all the above parameters.

In the histopathological studies, normal animals liver tissue showed sinusoidal cards of hepatocytes with central vein and portal tracts. In the diabetic control liver section showed distortion in the arrangement of cells around the central vein and periportal fatty infiltration with focal necrosis of hepatocytes. The treatment with embelin at both the doses and glibenclamide treated animals exhibited normal cellular arrangement around the central vein,

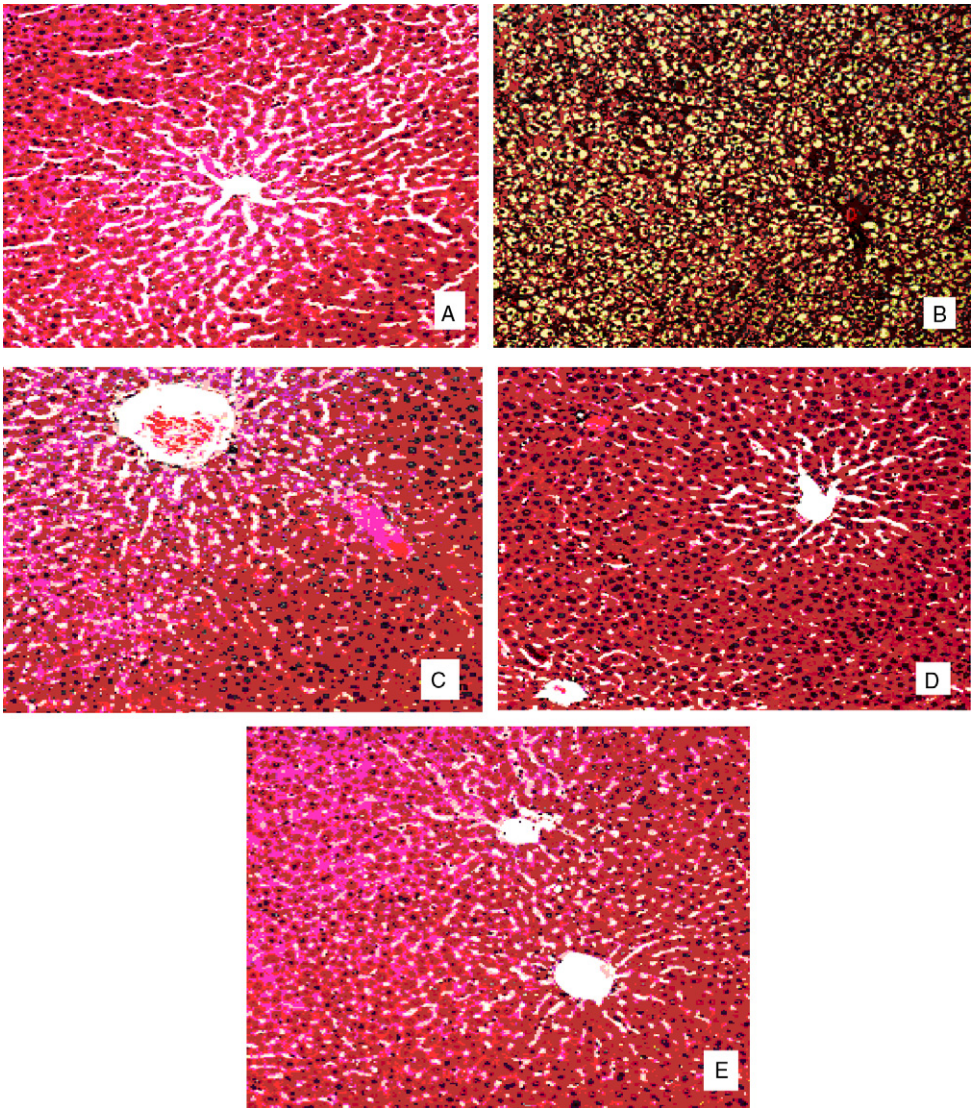

Fig. 4. Histopathological changes in liver of experimental rats. Group I (A): normal liver showing the central vein with radiating cords of hepatocytes; Group II (B): diabetic control, alloxan treated liver shows periportal fatty infiltration with focal fat necrosis; Group III (C): alloxan + embelin (25 mg/kg b.w.) shows moderate degeneration changes with bleeding area in hepatic lobes; Group IV (D): alloxan + embelin (50 mg/kg b.w.) shows mild degeneration changes with normal histological structure; Group V (E): alloxan + glibenclamide 10 mg/kg b.w. shows normal hepatic structure.

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Table 1
Effect of embelin on serum profile in alloxan (120 mg/kg b.w.) induced diabetic rats after 21 days of treatment.

Parameters	TGL (mg/dl)	TC (mg/dl)	TB (mg/dl)	TP (g/dl)	CR (mg/dl)	Albumi (g/dl)	LDH (IU/l)	ALP (IU/l)	VLDL (mg/dl)
Normal control	81.17 ± 0.95	144.01 ± 2.96	0.74 ± 0.02	6.78 ± 0.11	0.31 ± 0.02	3.78 ± 0.13[c]	185.50 ± 1.34	170.17 ± 1.40	16.70 ± 0.60
Diabetic control	199.17 ± 2.41[a]	270.67 ± 3.39[a]	1.07 ± 0.03[a]	3.53 ± 0.12[a]	0.59 ± 0.04[a]	2.25 ± 0.17[a]	380.67 ± 3.91[a]	364.50 ± 2.68[a]	39.83 ± 0.48[a]
Alloxan + embelin (25 mg/kg)	138.67 ± 2.33[d]	186.17 ± 1.96[d]	0.92 ± 0.02[d]	4.78 ± 0.14[d]	0.43 ± 0.02[b]	3.10 ± 0.10[c]	287.50 ± 2.62[d]	244.83 ± 3.28[d]	27.73 ± 0.47[d]
Alloxan + embelin (50 mg/kg)	104.83 ± 3.96[d]	169.50 ± 3.61[d]	0.82 ± 0.01[d]	5.92 ± 0.06[d]	0.37 ± 0.03[d]	3.23 ± 0.15[d]	242.33 ± 3.09[d]	201.83 ± 2.30[d]	20.97 ± 0.79[d]
Alloxan + glibenclamide (10 mg/kg)	99.00 ± 1.71[d]	151.33 ± 2.20[d]	0.77 ± 0.02[d]	6.50 ± 0.14[d]	0.29 ± 0.05[d]	3.61 ± 0.10[d]	244.83 ± 2.93[d]	183.17 ± 2.18[d]	19.80 ± 0.34[d]

Values are given as mean ± S.E.M. for groups of six animals each, Tukey-Kramer; values are statistically significant at (a) $P < 0.001$ between normal and diabetic control and (b) $P < 0.05$, (c) $P < 0.01$, (d) $P < 0.001$ between diabetic control and treated groups.

blood vessels and reduced necrosis (Fig. 4). The kidney sections of diabetic rat showed tubular damage, proteinuria and haemorrhage. Due to glomerular damage, haemorrhage is seen in the Bowman's space. The sections of animals treated with embelin at 25 mg/kg b.w. showed mild tubular epithelial atrophy and congestion of capillaries (Fig. 5). However, embelin at 50 mg/kg b.w. and glibenclamide at 10 mg/kg b.w. treated diabetic rats kidney showed glomeruli and tubules without proteinuria and haemorrhage.

The pancreas of the normal animals showed lobules of exocrine acini and exocrine islets of langerhans, which were not observed in

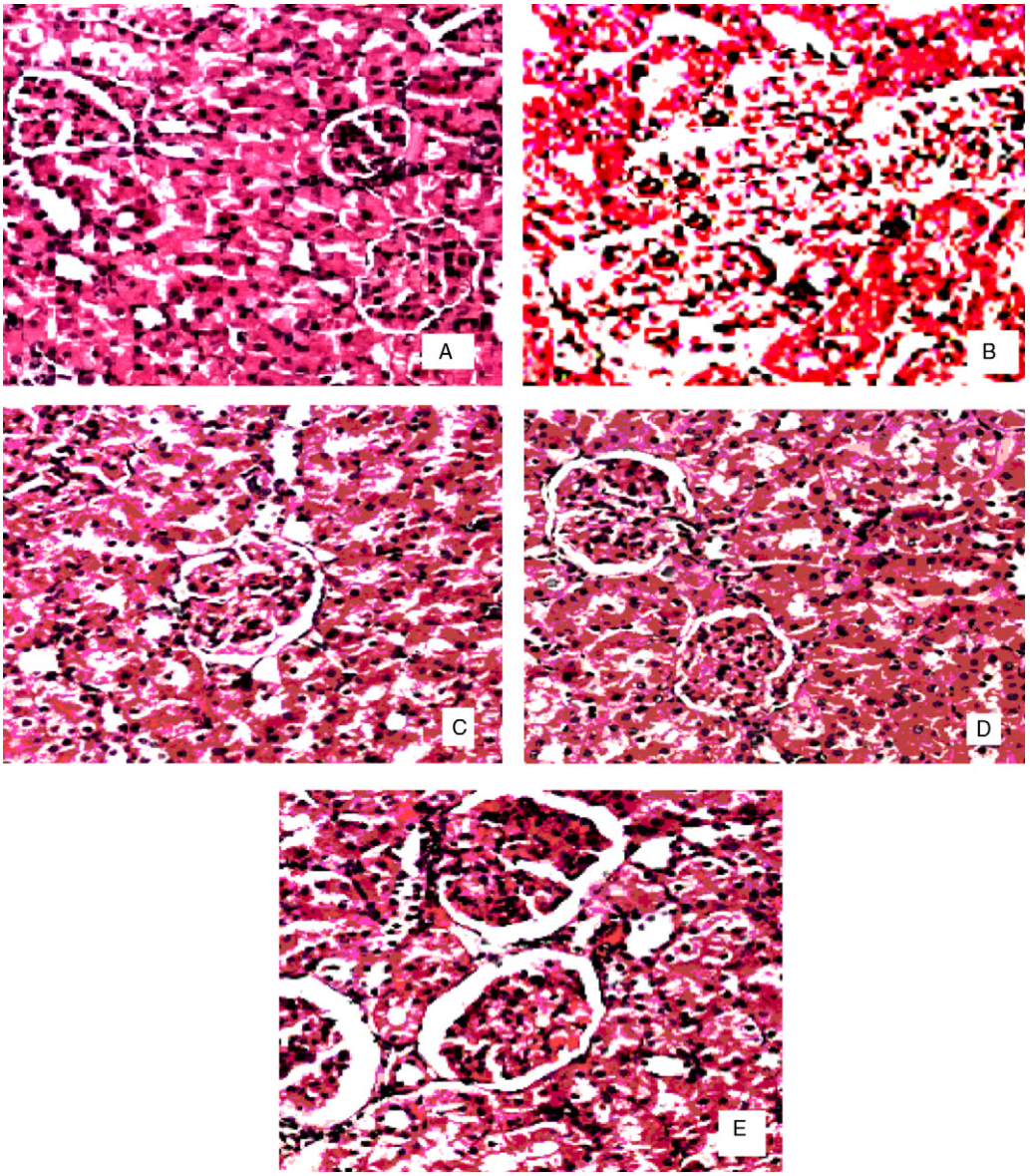

Fig. 5. Histopathological changes in kidney of experimental rats. Group I (A): normal kidney show glomeruli and proximal convoluted tubules; Group II (B): diabetic control, alloxan treated kidney shows tubular damage proteinuria and hemorrhage; Group III (C): alloxan + embelin (25 mg/kg b.w.) shows mild tubular epithelial atrophy and congestion of capillaries; Group IV (D): alloxan + embelin (50 mg/kg b.w.) shows normal histological structure; Group V (E): alloxan + glibenclamide 10 mg/kg b.w. shows normal architecture of kidney.

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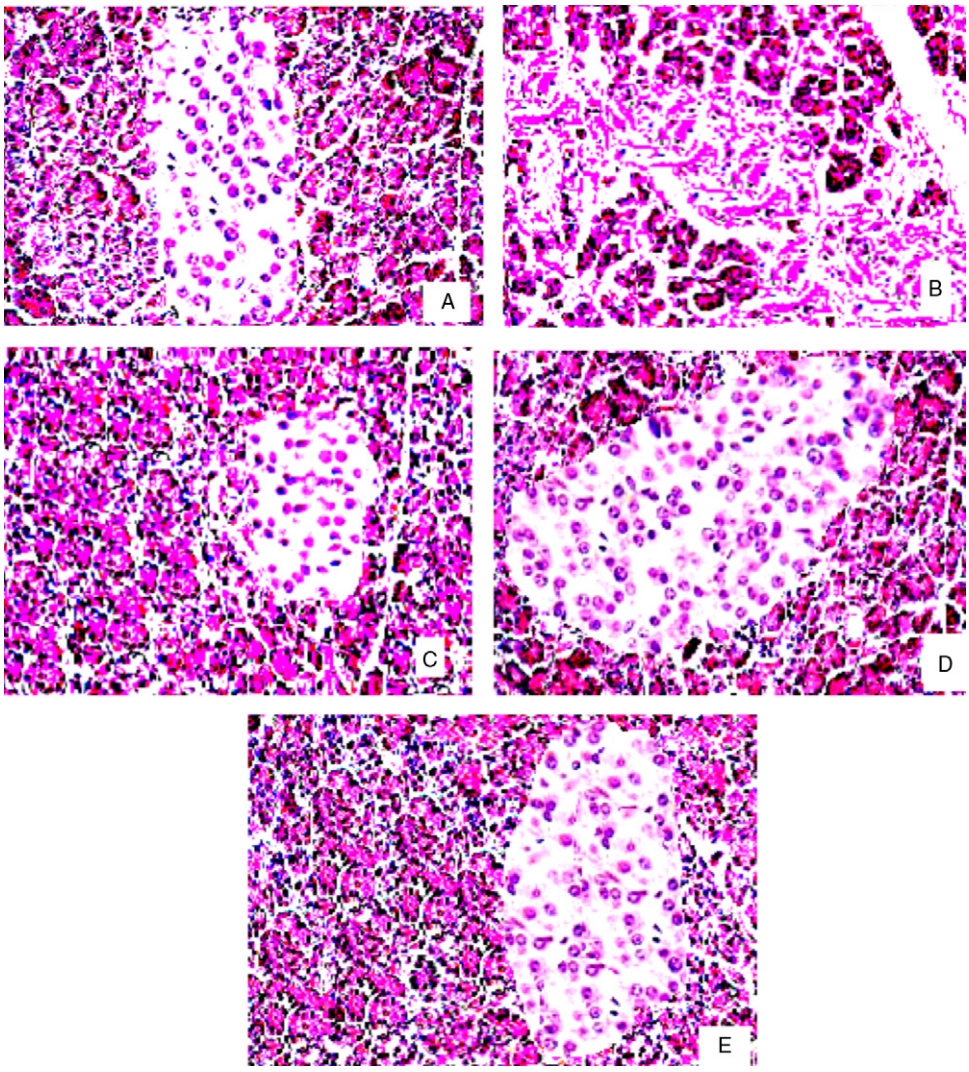

Fig. 6. Histopathological changes in pancreas of experimental rats. Group I (A): normal pancreas showing normal round exocrine acini and endocrine islets; Group II (B): diabetic control, alloxan treated pancreas shows depleted islets; Group III (C): alloxan + embelin (25 mg/kg b.w.) shows exocrine acini and islets were shrunken moderately; Group IV (D): alloxan + embelin (50 mg/kg b.w.) islets resemble normal rats islets; Group V (E) – alloxan + glibenclamide 10 mg/kg b.w. shows apparently normal architecture of islets.

alloxan induced diabetic pancreas (Fig. 6). However, the treatment with embelin at both the doses and standard glibenclamide treated pancreas appeared normal, cells gathered together and preserved islets similar to the normal.

4. Discussion

Several medicinal plants are widely used for the treatment of diabetes mellitus in the traditional medicine of many countries. *Embelia ribes* is an important ingredient of a number of Ayurvedic formulations in India [26,27]. Studies confirmed its antihyperglycemic potential along with its antioxidant and hepatoprotective effects [13,14,19–21,28]. The present work was carried out to know whether embelin, a major phytoconstituent present in it is the active molecule for its antidiabetic effects.

Embelin exhibited restoration of body weight, blood glucose and biochemical parameters towards the normal when compared to alloxan induced hyperglycemic rats. There will be an increase in body weight after attaining normal glycemic levels in the diabetes, particularly after treatment with sulfonyl ureas or insulin or both. Weight gain is not seen normally with the use of acarbose, troglitazone or metformin, in part because these antidiabetic agents do not stimulate the pancreas and do not elevate circulating insulin levels [29]. Treatment with embelin induced an increase in the body weight in the diabetic animals, probably due to the improvement in insulin secretion and glycemic control. The significant reduction in the serum blood glucose levels after the treatment with embelin is consistant with the findings of ethanolic extract of *Embelia ribes* by Bhandari et al. [21].

Embelin at both the doses, significantly reduced TGL, TC and VLDL in serum of alloxan induced diabetic rats. Thus, the normalization of lipids in diabetic rats treated with embelin may be due to its stimulatory effect on insulin secretion from pancreatic β-cells [30]. The diabetic hyperglycemia induces elevation of creatinine, which was considered as a significant marker of renal dysfunction [31]. The decrease in TP and albumin may be due to microproteinurea and albuminurea, which are important clinical markers of diabetic nephropathy, and/or may be due to increased protein catabolism [31]. The results of the present study demonstrated significant decrease in CR and elevation in TP and albumin levels to be comparable with their normal levels after the treatment of the diabetic rats with embelin.

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The levels of serum LDH and ALP were significantly increased in alloxan induced diabetes in rats. It is mainly due to the leakage of these enzymes from the liver cytosol into the blood stream, which gives an indication on the hepatotoxic effect of alloxan [32], which was reversed with the treatment of embelin. This could be further confirmed by the normal level of TB observed in the embelin treated groups. From these results, it could be concluded that embelin is capable of ameliorating the impaired diabetic kidney function in addition to its hypoglycemic and hepatoprotective activities.

Histologically, liver section of alloxan induced diabetic rats showed periportal fatty infiltration and necrosis of hepatocytes as a result of absence of insulin [33]. Diuresis is a common feature associated with diabetes, which may be the reason for structural changes observed with glomerulus [34]. Histological observations in the present study proved the excellent recovery of liver and kidney functions after embelin treatment. The damage of pancreas in alloxan-treated diabetic control rats was also reversed by embelin indicating its positive effects on the production of insulin.

It is well-known that oxygen free radicals are involved in the diabetogenic action of alloxan and antioxidants have been shown to be effective in diabetes [35]. Embelin significantly scavenged free radicals and resulting in hepatic glutathione antioxidant defense, decreases lipid peroxidation and minimizes the histological (liver) alterations induced by N-nitrosodiethylamine [28]. Hence, the antidiabetic activity of embelin may be due to its potent antioxidant properties. Our results also confirms that the potent antidiabetic action of *Embelia ribes* observed earlier by many studies may be due to the presence of embelin in the extract [19–21].

5. Conclusion

In conclusion, the present study demonstrated that the treatment of diabetic rats with embelin have exerted a considerable hypoglycemic effect. In addition, embelin could ameliorate the impaired renal function, inhibition of liver damage and resemble of islets of pancreas associated with alloxan diabetes. Due to its safety and activity, it is an ideal lead molecule for further antidiabetic drug development.

Conflict of interest statement

There are no conflicts of interest.

Acknowledgement

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