KUVEMPU UNIVERSITY

"Extraction, Evaluation and Characterization of *Myristica dactyloides*, *Hopea ponga* medicinal plants for their biological activities"

Thesis Submitted to the Faculty of Science, Kuvempu University
for the award of the Degree of

DOCTOR OF PHILOSOPHY

in

CHEMISTRY

Submitted By

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Research Guide

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February-2019

DECLARATION

I hereby declare that the research work presented in this thesis entitled **"Extraction, Evaluation and Characterization of *Myristica dactyloides, Hopea ponga* medicinal plants for their biological activities"** is entirely original and was carried out by me in the Department of Chemistry under the supervision of **Dr. H.M. Vagdevi,** Principal, Sahyadri Commerce & Management College, Shivamogga. I further declare that the results presented in the thesis or any part thereof has not been submitted elsewhere for any other degree, diploma of similar title in any other Universities.

Date:

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Certificate

This is to certify that the work reported in this thesis entitled "Extraction, Evaluation and Characterization of *Myristica dactyloides, Hopea ponga* medicinal plants for their biological activities" submitted by **Mr. Ajish A.D** to the Faculty of Science, Kuvempu University, for the award of **Doctor of Philosophy in Chemistry** is a record of the bonafide and original research work carried out by her under my guidance and direct supervision. The work reported in this thesis has not formed the basis for the award of any degree or diploma or any other similar title in any other Universities.

Date: 28/2/19

Place: Shivamogga

Dr. H.M. Vagdevi

(Guide)

DEDICATED TO

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Instrumentation Details

Melting points

Melting points were recorded on electro-thermal melting point apparatus and are uncorrected.

IR spectroscopy

The FT-IR spectra of the compounds were taken in KBr pellet (100 mg) using Shimadzu Fourier Transformed Infrared (FT-IR) Spectrophotometer.

NMR spectroscopy

^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker 400 MHz spectrometer in IISc, Bangalore, Karnataka, IIIT Chennai and MIT Manipal, India. The chemical shifts are shown in δ values (ppm) with tetramethylsilane (TMS) as an internal standard.

Mass spectroscopy

LC-MS were obtained using C 18 column on Shimadzu, LCMS 2010A, Japan. The column chromatography was performed using silica gel (230-400 mesh).

Thin Layer Chromatography (TLC)

Silica gel GF254 plates from Merck were used for TLC and spots located and identified by UV Chamber. The chemicals were purchased from Sigma-Aldrich Co and from SD Fine chemicals. The solvents for column chromatography were of reagent grade and were purchased from commercial source.

CHAPTER-I

Introduction to Medicinal Plants

1.1 Introduction to Medicinal Plants

Human beings are dependent on nature for their simple requirements as being the sources for medicines, shelters, food stuffs, fragrances, clothing, flavors, fertilizers and means of transportation throughout the ages. For the large proportions of world's population medicinal plants continue to play a dominant role in the healthcare system and this is mainly true in developing countries, where herbal medicine has continuous history of long use. The development and recognition of medicinal and financial aids of these plants are on rise in both industrialized and developing nations[1].

The foundations of typical traditional systems of medicine for thousands of years, that have been in existence have formed from plants. The plants remain to offer mankind with new medicines. Some of the beneficial properties ascribed to plants have recognized to be erroneous and medicinal plant treatment is based on the experimental findings of hundreds to thousands of years. The earliest reports carved on clay tablets in cuneiform date from about 2600 BC are from Mesopotamia, among the materials that were used were oils of *Commiphora* species (Myrrh), *Cedrus* species (Cedar), *Glycyrrhiza glabra* (Licorice), *Papaver somniferum* (Poppy juice) and *Cupressus sempervirens* (Cypress) are still used today for the cure of diseases extending from cold and cough to inflammation and parasitic infections[2].

The traditional medicine practice is widespread in India, Japan, Sri Lanka, Thailand, China and Pakistan. About 40% of the total medicinal consumption is attributed to traditional tribal medicines alone by China. In Thailand, herbal medicines make use of legumes encountered in the *Caesalpiniaceae, Fabaceae* and *Mimosaceae*. It is estimated that in mid-90s, more than US$2.5 billion have resulted from the sales of herbal medicines. The herbal medicinal preparations are more in demand than mainstream pharmaceutical products in Japan.

In diversified industries the contribution of plants is remarkable such as fine chemicals, cosmetics, pharmaceuticals drugs and industrial raw materials etc. For the development of new drug discovery medicinal plants perform a dynamic part. Medicinal plants have proved their sole role in coping with a number of deadly diseases including cancer and the diseases associated with viral incursion viz. Hepatitis, AIDS etc. In USA drug market approximately 100 plant made new drugs were presented during 1950-1970 which includes vincristine, reseinnamine, vinblastin, deseridine and reserpine which are from different plants. During 1971-1990 fresh medicines i.e., Artmisinin, Zguggulsterone,

Ginkgolides, Lectinam, E-Guggulsterone, Teniposide, Ectoposide, Plaunotol and Nabilone appeared all around the world. The 2% medicines which were presented during 1991-1995 include Irinotecan, Toptecan, Paclitaxel and Gomishin etc. The isolation of Serpentine in 1953 from the Indian plant *Rauwolfia serpentine* root was an innovatory episode in treatment of hypertension and lowering of blood pressure. The Vinblastine used for the treatment of leukemia in children, Hodgkins choriocarinoma, non-Hodgkins lymphomas, testicular and neck cancer was isolated from the *Catharanthus rosesus*[3-4]. Indian indigenous tree of *Nothapodytes nimmoniana* (Mappia foetida) are frequently used in Japan for the cure of cervical cancer.

Even today, plants are not only indispensable in health care, but form the best hope of source for safe future medicines[5]. In spite of the fact that now we have at our command a number of modern drugs, it is still genuinely urgent to discover and develop new therapeutic agents. It has been estimated that the acceptable therapy is available only for one third of the known human ailments. Therefore, the fight against diseases must be carried on relentlessly. Traditional plant medicines still enjoy significant position in the modern-day drug industries due to the minor side effects as well as the synergistic action of the combination of compounds.

Most of the important drugs of the past 50 years, which have revolutionized modern medicinal practice, have been isolated/derivatized from plants. These chemical ingredients exhibit therapeutic properties of plant and animal drugs. The WHO endorses and promotes the addition of herbal drugs in national health care programs because they are easily accessible at a price within the reach of a common man and are time tested and thus considered to be much safer than the modern synthetic drugs[6]. Thus, the research of pharmacologically/biologically active agents obtained by screening natural sources such as plant extracts had led to the detection of many pharmaceutically valuable drugs, that play a key role in the treatment of human diseases[7]. The phytochemical-pharmacological research work has recently yielded effective solutions to certain diseases which synthetic drug industry has failed to afford. The most important among them are the research work on *Artimisia annua, Cathranthus roseus, Taxus spp, Lantana camara* and *Baccopa spp,* etc. Such plants were earlier considered as poisonous or useless, but now have been found to contain molecules of high drug values and are considered as medicinal herbs of great significance.

Modern searches for bioactive molecules typically make use of sophisticated bioassays and bioassay-guided fractionation of medicinal plants used by traditional healers. This has led to the isolation of several new therapeutically important compounds. A good number of potent drugs and a large number of therapeutic leads and many new pharmacologically active constituents have been developed from herbal drugs due to the dedicated efforts of researchers[8]. The manufacture of morphine on industrial scale by E. Merck, Germany in 1826 marks the beginning of commercialization of plant-derived drugs[9]. Nearly half of the top selling pharmaceuticals in 1991 were either natural products or their derivatives[10].

Medicinal plants, as a group, comprise approximately 8000 species and account for about 50% of all the higher flowering plant species in India. A large number of the country's rural population depends on medicinal plants for treating various illnesses as well as a source of livelihood. About 1.5 million practitioners of the Indian Systems of Medicine and Homeopathy (ISM & H) use medicinal plants for preventive and curative applications. Furthermore, there are 7843 registered ISM pharmacies, 851 homoeopathy units as well as a number of unlicensed small scale enterprises. Besides meeting national demands, India caters to 12% of the global herbal trade. In recent years, trade in herbal-based products has quantum leaped, particularly in the volume of plant materials traded locally and internationally. Estimates by the EXIM Bank put medicinal plants-related international trade at US$ 60 billion per year and still growing at a rate of 7% annually. India is blessed with two mega centres of biodiversity: the Hindustan Centre of Origin and the Central Asia Centre of Origin. This biodiversity is mainly distributed in Western Ghat, North Eastern India and the Himalayan Region. Floristically rich, India has about 141 endemic genera of 5150 species belonging to 47 families of higher plants. Among the different endemic species, 2532 species are distributed in Himalayas, 1788 species in the peninsular region and 185 species in the Andaman and Nicobar Islands. About 43000 plant species are said to exist in India, of which 7500 plant species are referred to in Indian folklore but only about 1700 plant species have actually been documented in old literature. The vast degree of diversity present in this country is directly related to the highly divergent ecosystem and altitudinal variations. The agro-biodiversity in India is distributed in eight very diverse phyto geographical and 15 agro ecological regions. The range of distribution of these plants varies from the wet evergreen forests in the Western Ghats to the Alpine scrubs of the Himalayas, from the arid deserts of Rajastan to the mangroves along the east coast, from the vast deciduous forests of the Decan to the

Shoals of the high ranges, from the swamps of the Ganghes to the moss laden tree trunks of the Silent Valley. The indigenous diversity of plant species of medicinal and aromatic value in the region is also unique. This is reflected from the Arogyapacha (*Trichopus zeylanicus*) of the Agastiar Hills to the Saalam Panja of the Himalayas from the tiny Drosera of the Sholas to the huge Dipterocarps of the Western Ghats, from the xerophytic Aloes to the marshy land Brahmis, from the wild turmeric to the cultivated peppers.

Over 7000 species belonging mainly to the families *Fabaceae, Euphorbiaceae, Asteraceae, Poaceae, Rubiaceae, Cucurbitaceae, Apiaceae, Convolvulaceae, Malvaceae* and *Solanaceae,* have been used from ancient times by various indigenous peoples in the country. This number corresponds to more than 25% of the world's known medicinal plants, estimated to be at around 30,000 species. Analyses of these plants show that they include all the major forms (i.e., trees, shrubs, climbers and herbs), with the proportion of ferns and lichens being much smaller compared to flowering plants. Although India has rich biodiversity and is one of the 12 mega diversity centres, the growing demand for medicinal plants is putting a heavy strain on the existing resources, causing a number of species to be either threatened or endangered. The 2000 report of the International Union for Conservation of Nature and Natural Resources (IUCN) revealed that India ranked fifth highest in the number of threatened plant species and birds globally. Recently, some rapid assessment of the threat status of medicinal plants using the IUCN-designed CAMP methodology revealed that about 112 species in Southern India, 74 species in Northern and Central India and 42 species in the high altitudes of the Himalayas are threatened in the wild.

1.2 Distribution of Medicinal Plants

Macro analysis of the distribution of medicinal plants showed that they are distributed across diverse habitats and landscapes. Around 70% of India's medicinal plants are found in tropical areas mostly in the various forest types spread across the Western and Eastern ghats, the Vindhyas, Chotta Nagpur plateu, Aravalis and Himalayas. Although less than 30% of the medicinal plants are found in the temperate and alpine areas and higher altitudes, they include species of high medicinal value. The studies also showed that a larger percentage of the known medicinal plants could be found in the dry and moist deciduous vegetation as compared to the evergreen or temperate habitats.

Analysis of medicinal plant types indicated that about 34% are trees, another 34% are shrubs and the remaining 32% are composed of herbs, grasses and climbers. A very small portion of medicinal plants belong to lower plants like lichens, ferns algae, etc. while majority are classified as higher flowering plants. Of the 386 families and 2200 genera of medicinal plants recorded in India, the families *Asteraceae, Euphorbiacae, Laminaceae, Fabaceae, Rubiaceae, Poaceae, Acanthaceae, Rosaceae* and *Apiaceae* comprise the largest proportion of medicinal plant species, with the highest number of species (419) falling under *Asteraceae*. About 90% of medicinal plants used by related industries are collected from the wild. While over 800 species are used in industries, less than 20 species of plants are under commercial cultivation. Over 70% of the plant collections involve destructive harvesting practices as virtually all parts of the plants like the roots, bark, wood, stem and the whole plant (in the case of herbs) have known uses. This poses a definite threat to the genetic stocks and to the diversity of medicinal plants if they are not sustainably harvested and used.

Medicinal plants are living resources, exhaustible if overused but sustainable if used with care and wisdom. At present, 95% of medicinal plants collected are from the wild. Current practices of harvesting are unsustainable and many studies have highlighted depletion of resource base. Many medicinal plants-based industries are still being managed using traditional methods and practices. A number of studies have confirmed that pharmaceuticals companies are also responsible for inefficient and opportunistic marketing of medicinal plants. As a result, the raw-material supply situation is shaky, unsustainable and exploitative. There is a vast, secretive and largely unregulated trade in mainly wild medicinal plants which continues to grow dramatically in the absence of a national policy addressing environmental planning. Confusion also exists in the identification of plant materials where the origin of a particular drug is assigned to more than one plant, sometimes having vastly different morphological and taxonomic characters. There are few others where the identity of plant sources is doubtful or still unknown, therefore, in such cases, adulteration is very common.

The medicinal plants have been used by humans from the pre-historical times. Studies have pointed out that many drugs that are used in commerce have come from folk-use and use of plants by indigenous cultures[11]. About 50 drugs have been discovered from ethnobotanical leads by translating folk knowledge into new pharmaceuticals. Some examples of medicinal plant from the Asia-Pacific region are of species such as

Rauvolfia, *Hyoscyamus*, *Cassia*, *Atropa*, *Podophyllum*, *Psoralea*, *Catharanthus*, and Some 75% of the world's population rely for health care on traditional medicines, which are derived directly from natural sources (UNDP, UNEP, World Bank and WRI 2000). Medicinal plants contribute substantially to health, cultural integrity and local economies, particularly among the poor, and particularly for women, children and the elderly[12].

1.3 Traditional practices and importance of medicinal plants

People who live in rural areas of the Asia-Pacific are familiar with the medicinal properties of plants, growing close to their homes, in the open fields, water margins, waste lands, both inside and outside the nearby forest areas and under different growth conditions. Most of the plant materials collected is used fresh either to obtain the extract from the whole plant or parts thereof, whether they be leaves, roots, flowers or fruits. In case of woody forms, mostly the bark, roots and other parts are used. Carminatives like ginger, cloves and coriander are also usually added as fresh or dried materials. Though dried plant parts are frequently used, often the easy availability of fresh material is a critical point and the herbal doctor in the village is well familiar with various plants he/she needs, their growth patterns, seasonality, habitat and other details. Such details were usually passed on in the past from parent to offspring in the family and uses of plants and the various combinations or mixes made were kept as a family secret. Along with the development of knowledge at family level, tremendous progress has been made at using the plant products at professional level in different societies, which have grown into branches of science in their own right. Most of the methods and uses were taught orally and through demonstration and very few records or writings were maintained. Such professional practices are continuing even today. As villagers migrated to city, losing touch with past practices or when there was no heir apparent to the village doctor, the precious knowledge was usually lost, although there are a number of treatises that exist in different countries[13]. Refinement of such practices lead to the well established Asian systems of medicines including Ayurveda and Siddha of India, Unani system of middle and Far East Asia, Ying and Yan principles of Chinese herbal medicines, Jamu of Indonesia and others[14-15]. About 400 plant species are used in regular production of Ayurvedic, Unani, Sidhha and tribal medicine[16]. Recently, a regional inventory of medicinal and aromatic plants and polyherbal formulations dealing with 65 Indian medicinal plants, 10 important Indonesian and 25 medicinal plants of Malaysia, along with important traditional and polyherbal formulations used in these countries has been

brought out by CIMAP and supported by the Department of Biotechnology, Government of India[17]. It is only in the last 40 to 50 years that many of the medicines were produced industrially and sold in shops and markets with trade names.

The practice of various indigenous medicinal systems is flourishing in different countries even today, with nearly 80% of the rural population still dependent on plant-based medicines for primary health care[18]. It is said that US$ 1 per day is enough to provide the basic nutritional needs of an individual. About 1.3 billion people in the world earn less than this and it would not require much imagination to realize that such people can hardly afford to spend any money on modern medicines or avail of modern medical services. This stresses the importance of turning to local plants which are useful medicinally and obtained almost free of cost. Thus, the utilization of medicinal plants in traditional remedies is very important to the people in developing countries particularly the rural population. Medicinal plant resources in nature though much information exists on the species diversity in medicinal plants in the Asia-Pacific region, relatively very little is known about the distribution, abundance, ecology and genetic diversity of the great majority of medicinal and aromatic plants, although some efforts have started in recent years[19-22], including the use of molecular markers[20]. Out of the 3,50,000 plant species identified so far, about 35,000 (some estimate up to 70,000) are used worldwide for medicinal purposes and less than about 0.5% of these have been chemically investigated. The figures published vary in different reports. About 100 plant species are involved in 25% of all drugs prescribed in advanced countries[20] (Comer and Debus 1996) and the annual market value of herbal drugs used worldwide was estimated to be US$ 45 billion in 1996 and it must be much more by now. The global market for the medicinal plants and herbal medicine is estimated to be worth US$800 billion a year[22]. More than 8,000 plant species are known for their medicinal properties in the Asia-Pacific and about 10% of them are used regularly, mostly collected from wild. For example, it has been estimated that not less than 7,500 species of medicinal plants exist in the Indonesian archipelago, of which only about 187 species are used as basic materials in traditional medicines industries[23]. In China, over 4,000 species of medicinal plants have been reported[24].

In India, about 2,500 species are used for medicinal purposes, and about 90% of the medicinal plants provide raw materials for the herbal pharmaceuticals, which are collected from the wild habitats[25]. About 2,000 medicinal plants species are reported from

Malaysia[25], while in another account 1,200 species have been reported to have potential pharmaceutical value, some of which are being used as herbal medicines[26]. For the Indian Himalayan Region, a total of 1,748 species of medicinal plants-1,020 herbs, 338 shrubs, 339 trees, apart from 51 pteridophytes have been listed[27]. These include several of the endangered medicinal plant species, using current IUCN, Red Data criteria under the Biodiversity Conservation Prioritization Project (BCPP), by Conservation Assessment and Management Plan (CAMP) workshop organized by WWF at Lucknow from 21-25 January. Some examples of the endangered Himalayan medicinal plant species include: *Aconitum balfourii, A. deinorrhizum, Acorus calamus, Angelica glauca, Atropa belladonna, Berberis kashmiriana, Coptis teeta, Dioscorea deltoidea, Gentiana kurrooa, Nardostachys grandiflora, Picrorhiza kurrooa, Podophyllum hexandrum, Saussurea costus, Sweria chirayita, Taxus baccata* and the sub-tropical/sub-temperate species *Aquilaria malaccensis*. The availability of fresh or dried plant materials required to prepare various medicines was not a major problem when human population was small, and plant material collected were within limits, allowing enough number of plants to regenerate or re-grow in the following years. However, the current industrial practice of manufacturing herbal products requires large quantities of plant materials resulting in over collecting leading to scarcity of materials, especially the well known and slow growing species that are in great demand, for example *Rauvolfia, Ginseng* and different gingers. The depletion of resources is less in tree or woody forms when compared to the herbaceous species. Cultivation was encouraged, which also became profitable. But very soon it was discovered that in many cases the ingredients obtained from natural habitats were usually superior to the cultivated ones and the quality of products did not match in many cases.

In almost every Asian country, there is a vast indigenous knowledge on the use of medicinal plants. Although traditional and local identification systems existed for long, actual and formal scientific identification of these plants only started in the 1900's[28-31]. However, as the availability of plant materials was not a problem, very little or no attention was paid by the earlier authors to the occurrence, growth habit, distribution and other ecological details of the plants. Only recently, publications regarding the resources of medicinal plants in Asia are becoming available, information on their relative abundance or scarcity, ecological conditions of growth, distribution patterns, etc., are being recorded[31]. More recently, the publication of red data book by IUCN as well as

proceedings of a few regional meetings on this topic have helped to understand the relative abundance or scarcity of various medicinal plant species including the rare, threatened, endangered, or species about to become extinct[33-34]. In India, the Foundation for Rehabilitation of Local Health Traditions (FRLHT), a nongovernment voluntary organization based at Bengaluru, has compiled a list of 352 medicinal plant species of South India which require urgent conservation measures and of these, 226 are collected from the forest for their use by the pharmaceutical industries. The CAMP workshops identified 112 threatened medicinal plants in South India. These include critically endangered species such as *Coscinium fenestratum, Kaempferia galanga, Piper barberi, Trichopus zeylanicus, Valeriana leschenaultii* and *Vateria macrocarpa; endangered species such as Rauvolfia serpentina, Pterocarpus santalinus, Santlum album, Swertii lawii,* and *Gymnema sylvestris*. Few species were designated extinct namely: *Aerva wightii, Asparagus rottlerii, Madhuca insignis* and *Plectranthus vettivariodes*. Recent compilations by IUCN/SSC in producing medicinal plant conservation bibliography[34] have provided more information on this aspect, just like some of the international conferences on medicinal plants, such as the conservation, utilization, trade and culture held at Bengaluru, India in January 1996.

Many drugs listed as conventional medications were originally derived from plants. Salicylic acid, a precursor of aspirin, was originally derived from white willow bark and the meadowsweet plant. Cinchona bark is the source of malaria-fighting quinine. The opium poppy yields morphine, codeine and paregoric, a remedy for diarrhoea. Laudanum, a tincture of the opium poppy, was the favoured tranquilizer in Victorian times. Even today, morphine the most important alkaloid of the opium poppy remains the standard against which new synthetic pain relievers is measured[35]. Similarly, tetrahydrocannabinol (THC), the component of *Cannibas sativa* responsible for the CNS effect, has also been found to reduce nausea associated with cancer chemotherapy. Another therapeutic area where natural products have had a major impact on longevity and quality of life is in the treatment of cancer. In fact, most of the major anticancer drugs are natural products either from plants or micro-organisms. Examples include important anticancer drugs such as Bleomycin, Doxorubicin, Vincristine, Vinblastine, and now the recent addition of Paclitaxel (Taxol), Ironotecan (a camptothecin derivative) and Etoposide and Tenoposide (Podophyllotoxin derivatives). Some of the most exciting natural products discovered in the recent years are the cholesterol-lowering agents derived from fungi.

Medicinal plants play a central role not only as traditional medicines but also as trade commodities, meeting the demand of distant markets. Ironically, India has a very small share (1.6%) of this ever growing global market. To compete with the growing market, there is urgency to expeditiously utilize and scientifically validate more medicinally useful plants while conserving these species, which seems a difficult task ahead.

1.4 Medicinal and Aromatic Plants

India has 2.4% of worlds area with 8% of global bio-diversity. It is one of the 12 mega-diversity hot-spot regions of the world. Other countries being Brazil, Colombia, China, South Africa, Mexico, Venezuela, Indonesia, Ecuador, Peru, USA and Bolivia. Across the country, the forests of India are estimated to harbour 90% of Indias medicinal plants diversity. Only about 10% of the known medicinal plants of India are restricted to non-forest habitats. The estimated numbers of plant species and those used for medicinal purpose vary. The world average stands at 12.5% while India has 20% plant species of medicinal value[36]. But according to Hamilton (2003) [37], India has about 44% of flora, which is used medicinally. Although it is difficult to estimate the number of medicinal and aromatic plants present worldwide, the fact remains true that India with its rich biodiversity ranks first in its per cent flora, which contain active medicinal ingredient. The existence of traditional medicine, depends on plant species diversity and the related knowledge of their use as herbal medicine. Both plant species and traditional knowledge are important to the herbal medicine trade and the pharmaceutical industry where plants provide raw materials and the traditional prerequisite information[38].

India has one of the richest plant medical traditions in the world. It is the tradition that is of remarkable contemporary relevance for ensuring health security to the teeming millions. There are estimated to be around 25,000 effective plant-based formulations, are used in folk medicine and are known to rural communities in India. There are over 1.5 million practitioners of traditional medicinal system using medicinal plants in preventive, promotional and curative applications. It is estimated that there are over 7,800 medicinal drug manufacturing units in India, which consume about 2,000 tonnes of herbs annually[39]. Two of the largest users of medicinal plants are China and India[40-42].

1.5 Herbal Medicine:

Herb has various meanings, but in simplest form, it refers to “crude drugs of vegetable origin utilized for the treatment of diseases, often of a chronic nature, or to attain or

maintain a condition of improved health". Herbal medicine, sometimes referred to as Herbalism or Botonical Medicine, is the use of herbs for their therapeutic or medicinal value. A herb is a plant or plant part valued for its medicinal, aromatic or savory qualities. Herbs produce and contain a variety of chemical substances that act upon the body. Herbal preparations called "phytopharmaceuticals", "phytomedicinal" or "phytomedicine", are the preparations made from different parts of herbs. They come in different formulations and dosage forms including tablets, capsules, elixir, powder, extract, tincture, cream and parenteral preparations. A single isolate or active principle derived from plants such as digoxin or reserpine tablet is not considered as herbal medicine[43-44]. The effectiveness of herbal remedies, their easy availability, low cost and comparatively being devoid of serum toxic effects popularized in society.

1.6 Popularity of Herbal Medicine:

The herbal medicines are largely gaining popularity over allopathic medicine because of the following reasons,

- Rising costs of medicinal care.
- As these are from natural origin, so free from side effects in several cases.
- Goes to the root cause and removes it, so that the disease does not occur again.
- Freedom from approaching various specialists.
- Cure for many obstinate diseases.
- Easy availability of drugs from natural sources.

1.7 Need and Scope of Herbal Therapy:

The treatment of diseases with pure pharmaceutical agents is a relatively modern phenomenon. However, as European explorers and merchants spread out to the Western and Eastern parts of the world, some of the benefits they would bring back were newly discovered pharmaceutical preparation of natural origin. One of the earliest success stories in developing a drug from a natural product was aspirin. Today we are more concerned with the life-style disease like depression, cancer and heart troubles caused by faulty nutrition and stress. The need of alternative therapy is to cover good health for all. Herbal therapy is one of the best practices to overcome illness. Traditional Indian practice held that certain drugs should be formulated through the addition of chosen substance that enhances bioavailability of the drug. Pepper has confirmed bioavailability enhancer property and point to the active component as the molecule piperine. An anti-TB drug,

Rifampicin has to be given at a higher dosage than required in order to compensate for losses on the way to the target site. Formulation of Piperine with Rifampicin will have counter effects[45-46]. Hence one needs to be cautious while administrating herbal medicine with any other formulations.

1.8 Future Prospects of Medicinal Plants

There is a promising future of medicinal plants as there are about half million plants around the world and most of them are not investigated yet for their medical activities and their hidden potential of medical activities could be decisive in the treatment of present and future studies[47].

In the development of human culture medicinal plants have played an essential role, for example religions and different ceremonies[48]. Among the variety of modern medicines, many of them are produced indirectly from medicinal plants, for example aspirin. Many food crops have medicinal effects, for example garlic. Studying medicinal plants helps to understand plant toxicity and protect human and animals from natural poisons. The medicinal effects of plants are due to secondary metabolite production of the plants. Keeping this in consideration there have been increased waves of interest in the field of research in natural product chemistry. This interest can be due to several factors, including therapeutic needs, the remarkable diversity of both chemical structure and biological activities of naturally occurring secondary metabolites, the utility of novel bioactive natural compounds as biochemical probes, the development of novel and sensitive techniques to detect biologically active natural products, improved techniques to isolate, purify, and structurally characterize these active constituents and advances in solving the demand for supply of complex natural products[49]. The importance of traditional medicine has also recognized by World Health Organization (WHO) and has created strategies, guidelines and standards for botanical medicines. For the cultivation, processing of medicinal plants and the manufacture of herbal medicines agro-industrial technologies need to be applied[50]. Medicinal plants are resources of new drugs and many of the modern medicines are produced indirectly from plants.

1.9 Alternative Medicine

These days the term “Alternative Medicine” became very common in western culture, it focus on the idea of using the plants for medicinal purpose. But the current belief that medicines, which come in capsules or pills are the only medicines that we can trust and use. Nevertheless, most of these pills and capsules we take and use during our daily life

came from plants. Medicinal plants frequently used as raw materials for extraction of active ingredients which used in the synthesis of different drugs. Like in case of laxatives, blood thinners, antibiotics and antimalaria medications contain ingredients from plants. Moreover the active ingredients of Taxol, vincristine, and morphine isolated from foxglove, periwinkle, yew and opium poppy, respectively.

Literature survey revealed that the extracts of seeds of *Psoralea corylifolia* showed considerable antifungal activity against *T. rubrum, T. mentagrophytes, E. floccosum* and *M. gypseum*. The methanol extracts of the seeds were found to be most effective. An active compound 4-methoxy flavone was isolated from the active fraction[51].

Selected medicinal plants viz., *Ocimum sanctum (Tulsi), Origanum majorana (Ram Tulsi), Cinnamomum zeylanicum (Dalchini),* and *Xanthoxylum armatum (Timur),* were screened for antibacterial activity[52] against 10 medically important bacterial strains by agar well diffusion method. The plant extracts were more active against Gram-positive bacteria than against Gram-negative bacteria. The most susceptible bacteria were *B. subtilis*, followed by *S. aureus*, while the most resistant bacteria were *E.coli,* followed by *Shigella dysenteriae*, *Klebsiella pneumoniae* and *Salmonella typhi*. From the screening experiment, the largest zone of inhibition was obtained with *Xanthoxylum armatum* against *Bacillus subtilis* (23mm) and the minimum bactericidal concentration (MBC) value of 2.5 mg/ml was obtained. This antibacterial study of the plant extracts demonstrated that, folk medicine can be as effective as modern medicine to combat pathogenic microorganisms.

Girish and Satish[53] investigated the leaves of five different plants, belonging to different family for antibacterial against human pathogenic bacteria. It has been showed that the methanol extracts had wider range of activity on these organisms than the aqueous extracts, which indicated that the methanol extracts of all selected plants may contain the active components. This study supports the traditional medicines (herbal extracts) to cure many diseases like diarrhoea, intestinal tract, throat, ear infections, fever and skin diseases**.**

The antibacterial activity of some medicinal plants of western region of India was carried out by Rathish Nair and Sumitra chanda[54]. According to them the ethanol extract was more potent than aqueous extract of all the plants studied. *P. aeruginosa* and *S.typhimurium* were the most resistant strains while the most susceptible bacterial strains were *Bacillus cereus* and *Proteus mirabilis*. *Emblica officinalis* showed remarkable activity against all the tested bacterial strains. Hence, this plant can be used to discover

bioactive natural products that may serve as leads in the development of new pharmaceutics that address unmet therapeutic needs.

All the above reports on various medicinal plants and their antimicrobial and other pharmacological activities prompted us to take up the present research work. The main objective of the work directed towards the identification of various medicinal plants in Muthinakoppa, N.R. Pura and Agumbe region, extraction, evaluation of phytochemical constituents and screening of crude extracts for possible biological activities. The active fraction was purified, three bioactive molecule has been isolated by column chromatography and characterized by spectral data.

References

1. WHO, Regulatory situation of herbal medicines. A worldwide review. Geneva, Switzerland, 1, **1998**.
2. A.G. Fakim, Medicinal plants: Traditions of yesterday and drugs of tomorrow. *Molecular aspects of medicine.*, 27, 1, **2006**.
3. P. Harrison, Herbal medicine takes roots in Germany. *Canadian Medical Association J.*, 10, 637, **1998**.
4. W.B. Jones, Alternative medicine-learning from the past examining the present advancing to the future. *J of American Medical Association.*, 280, 1616, **1998**.
5. M. Hamburger and K. Hostettmann, Bioactivity in plants: The link between phytochemistry and medicine. *Phytochemistry.*, 30, 3864, **1991**.
6. P. Singh, and C.L. Singh, Chemical investigations of Clerodendraon fragrans. J of Indian Chemical Society., 58, 626, **1981**.
7. P.R. Rastogi and B.N. Meharotra, In Compendium of Indian Medicinal Plants., 1, 339; a) III: 194. PID, CSIR, New Delhi, India, **1990**.
8. M.N. Philipson, A symptomless endophyte of ryegrass (*Lolium perenne*) that spores on its host a light microscope study. *New Zealand J of Botany.*, 27, 513, **1990**.
9. S. Galbley and R. Thiericke, Drug Discovery from Nature, Series: Springer Desktop Editions in Chemistry, Springer, Berlin., **1999**.
10. G.M. Cragg, D.J. Newman and K.M. Snader., Natural products in drug discovery and development. J of Natural Products., 60, 52, **1997**.
11. Anonymous. Ethnobotany in the search for new drugs. Ciba Foundation Symposium 188, John Wiley and Sons, New York, **1994.**
12. D. Leaman, H. Fassil and I. Thormann. Conserving medicinal and aromatic plant species: Identifying the contribution of the International Plant Genetic Resources Institute (IPGRI). A consultancy report. IPGRI, Rome, **1999**.
13. A.N. Rao and V. Ramanatha Rao. Strategies for conservation of medicinal plants. Paper presented at a symposium on Medicinal Plants: CURE for the 21st Century., 15, **1998**.
14. S.K. Sharma, K.C. Chunrkar and C.L Yadav (eds). Medicinal plants used in Ayurveda. National Academy for Ayurveda, New Delhi, **1998**.
15. S. Natesh, Biotechnology in the conservation of medicinal and aromatic plants. in Biotechnology in Horticulture and Plantation Crops., 548, **2000.**

16. P.E. Rajasekharan, and S. Ganeshan. Conservation of medicinal plant biodiversity in Indian perspective. *J of Medicinal and Aromatic Plant Sciences* 24, 132, **2002.**
17. Anonymous. Asian Regional Inventory of Medicinal and Aromatic Plants and Polyherbals Formulations. Central Institute of Medicinal and Aromatic Plants, **1999.**
18. A. Sasson, Biotechnologies and use of plant genetic resources for industrial purposes: Benefits and constraints for developing countries, 469, **1996**.
19. K.L. Chadha and R. Gupta (eds). Advances in Horticulture: Medicinal and Aromatic Plants., 11, **1995.**
20. KPS Chandel, G. Shukla and N. Sharma. Biodiversity in Medicinal and Aromatic Plants in India, **1996.**
21. S. Kumar, S.A Hassan, S. Dwivedi, A.K. Kukreja, A. Sharma, A.K Singh, S. Sharma and R. Tewari (eds).. *J Medicinal and Aromatic Plant Sciences*., 22/4A-23/1A, **2000**.
22. Y. Paisooksantivatana, S. Kako and H. Seko. Isozyme polymorphism in Curcuma alismatifolia Gagnep. (*Zingiberaceae*) populations from Thailand. Scientia Horticulturae., 88, 299, **2001**.
23. A. Hamid and D. Sitepu. An understanding of native herbal medicine in Indonesia. Industrial Crops Research *J.*, 3, 11, **1990.**
24. E.S. Ayensu, World Medicinal Plant Resources. Conservation for Productive Agriculture., 11, **1996.**
25. A. Latif, G. Ismail, M. Omar, M.I. Said and A. Kadri. A multivariate approach to the study of medicinal plants in Malaysia., 13, 101, **1984**.
26. A.A. Kadir, Conservation and economic potential of plant genetic resources in Malaysia. Medicinal and Aromatic Plants. Strategies and Technologies for Conservation., **1997**.
27. S.S. Samant, U. Dhar and LMS Palni. Medicinal Plants of Indian Himalayas: Diversity, Distribution, Potential values. Himavikas Publication No. 13, **1998**.
28. W. Dymock, C.J.H Warden and D Hooper. *Pharmacographica Indica.* **1890-93**.
29. G. Dragendorff, Wie Heilpflanzen der Verschidenen, Vlkerund Zeitan, Ferdinand ENKE, Stuhgart, **1898.**
30. W.G. Boorsma, Notes about eastern medicine in Java, Botanical Garden, Bogor, Indonesia, **1926**.

31. I.H. Burkill, A Dictionary of the Economic Products of Malay Peninsula. Ministry of Agriculture, Kuala Lumpur, Malaysia, **1935**.
32. M.K. Salleh, S. Natesh, A. Osman and A.A. Kadir (eds). Medicinal and aromatic plants: Strategies and technologies for conservation, **1997**.
33. P.L. Gautam, R. Raina, U. Srivastava, S.P. Raychaudhari and B.B. Singh (eds). Prospects of medicinal plants, **1998**.
34. V. Tandon, N.K. Bhattarai and M. Karki (eds). Conservation assessment and management plan workshop report on selected medicinal plant species of Nepal, **2001**.
35. Manuchair Ebadi, Pharm. dynamic basis of herbal medicine, CRC Press, **2002**.
36. U. Schippmann, D.J. Leaman and A.B. Cunningham. Impact of Cultivation and Gathering of medicinal plants on Biodiversity: Global Trends and Issues. 1-21, **2002**.
37. A. Hamilton, Medicinal plants and conservation: issues and approaches. UK, WWF, **2003**.
38. J.R.S Tabuti, K.A. Lye and S.S. Dhillon, Traditional herbal drugs of Bulamogi, Uganda: plants, use and administration. J of Ethnopharmacology., 88, 19, **2003**.
39. K. Ramakrishnappa, Impact of Cultivation and Gathering of Medicinal Plants on Biodiversity: Case studies from India. **2002**.
40. E. Masood, Medicinal plants threatened by over-use. *Nature*, 570, **1997**.
41. Rawat, R.B.S. Medicinal Plants Sector in India with reference to Traditional Knowledge and IPR issues. **2002**.
42. Wang, Zhen-Gang and J. Ren. Current status and future direction of Chinese herbal medicine. *Trends in Pharmacological Sciences*. 23, 347, **2002**.
43. Thaibinh Ton Thot. Herbal Medicine. *Ind. J. Pharma. Edu.*, 32, 104, **1998**.
44. www.dhyansanjivani.org/herbal-therapy.asp
45. A.K. Gupta, H.R. Chitme. Herbal Medicine for Health. Eastern Pharmacist. 41, 41, **2000**.
46. Amit Singh, Smita Verma, Nouh M H Al Jarari , Ajay Pal Singh , Neeraj Kumar Fuloria, Shivkanya Fuloria, Pradeep Kumar Sharma, Chhotelal, Effect of Piperine on Pharmacokinetics of Rifampicin and Isoniazid: Development and Validation of High Performance Liquid Chromatography Method, Journal of Applied Pharmaceutical Science., 8, 72, **2018.**

47. R. Singh. Medicinal Plants: A Review. *J of Plant Sciences*. Special Issue: Medicinal Plants. 50, **2015**.

48. S. Hosseinzadeh, A. Jafarikukhdan, A Hosseini and R. Armand. The application of Medicinal Plants in Traditional and Modern Medicine: A Review of Thymus vulgaris. *International J of Clinical Medicine*, 6, 635, **2015**.

49. A.M. Clark. Natural Products as a Source for New Drugs. *Pharmaceutical Research*, 13, 1133, **1996**.

50. WHO, Research Guidelines for Evaluating the Safety and Efficacy of Herbal Medicines. Manila, **1996**.

51. R.N. Prasad, C. Anandi, S. Bala Subramanian and K.V. Pugalendi, *J. Enthnopharmacol*., 91, 21, **2004**.

52. Bishnu joshi, Sunil Lekhak, Anuja Sharma, *J. Sci. Eng. and Technol.,* No.I, January, 5, 143, **2009**.

53. H.V. Girish and S. Satish, *World App. Sci. J.,* 5, 267, **2008**.

54. Rathish Nair and V. Sumitra chanda, *Turk. J. Biol.,* 31, 231, **2007**.

CHAPTER-II

Literature Survey of Medicinal plants

2.1 Literature Survey

India is one of the major hotspots, which inhabits a wide variety of plants and has been identified as one of the mega diversity centers of the world. Although it's total land area comprises of 2.4% of the total geographical area of the world. The country accounts for an average of 8% of the total global biodiversity with approximately 49,000 species of plants of which 4,900 are endemic[1]. The southern peninsular India and Western Ghat ecosystem contains more than 6000 species of higher plants including an estimated 2,000 endemic species. Out of these 2,500 species representing over 1,000 genera and 250 families have been used in traditional systems of medicine[2]. The special importance has to be given for traditional medicine usage practices, although a good proportion of medicinal plant species exists throughout the country and highly significant diverse species is found in the peninsular Indian forests and Western Ghats[3].

The entire Western Ghat is known for its biodiversity, richness and endemism of different species. Western Ghats of Karnataka is one of the 18 biodiversity hot spots of the world. As early as 1904, Hooker had drawn attention to the distinct flora of the Western Ghats, which he called the Malabar floristic region. It attracts attention from people mainly due to its pleasing climate and wealth of herbal medicines present in the bosom of its forest. Chickmagalur and Shivamogga district form a major part of the Western Ghats that passes through Karnataka. N.R. Pura region of Chickmagalur and Agumbe region of Shivamogga district is a part of the Western Ghats, which run through Karnataka.

The of Western Ghats is covering three district regions viz., i) Malnad region (Chickmagalur dist.), ii) Semi–Malnad region (Shivamogga dist.) and iii) Maidan region (Davangere and Chitradurga dist.). It has enormous wealth of flora and more than 50% of them are useful to mankind in the treatment of several diseases. Hence, the flora around this area has attracted researchers into action for finding out new medicinal plants for the cure of various ailments.

In order to preserve endangered species of flora, many government and non-government organizations have set up medicinal gardens in this area. In these gardens, different types of medicinal plants have been grown and maintained. The important medicinal plant gardens are listed as below:

a) "Ashwini Aushadha Vana"- At Gajanur near Shivamogga. Maintained by Department of Forest, Government of Karnataka.

b) "Medicinal Plant Garden"- At Malladihalli (Chitradurga district). Maintained by private Ayurvedic Hospital.

c) "Garden of Medicinal Plants"- At Agumbe (Shivamogga district). Maintained by Foundation for Revitalisation of Local Health Traditions, Government of Karnataka.

d) "Garden of Aushadi Sasyagalu"- At Kopppa (Chickmagalur district). Maintained by private Ayurvedic College.

e) "Garden of Medicinal Plants"- At Sirigere – Chitradurga District.

The study area, N.R. Pura and Agumbe region located in the midst of the Western Ghats region harbor diverse types of vegetation-evergreen, semi-evergreen, moist and dry deciduous forest and is rich in diversity of plants with medicinal values.

However, literature survey revealed that, not much work has been done regarding phytochemicals, biological and pharmacological investigations of many medicinal plants, which are available in N.R. Pura region of Chickmagalur district and Agumbe region of Shivamogga district. Hence, an organized study on medicinal plant in this area provides plenty of scope for systematic research work.

In this region, many Ayurvedic pandits and local herbal healers use plants for the treatment of skin diseases, asthma, fever, bone fracture, cough, wound healing, stomachaches, heart diseases etc. Hence it was thought to identify various medicinal plants available in this region and to extract two of the medicinal plant and investigate for its biological and pharmacological activities.

Based on the literature survey and discussion with local healers, a list of plants was prepared. While selecting the plants, due care was taken to select only those plants on which there were no reports or very less report on antimicrobial and other pharmacological studies. It was envisaged that a lot of scope still remained in the field of antimicrobial studies. Some of the plants have been shortlisted such as *Ficus Hispida, Kydia calycina Roxb, Helicteres isora L., Acacia pennata, Anona muricata, Terminalia tomentosa, Myristica dactyloides Gaertn, Hopea ponga (Dennst.).*

Botanical name : ***Ficus Hispida***[4-5]

Family : *Moracea*

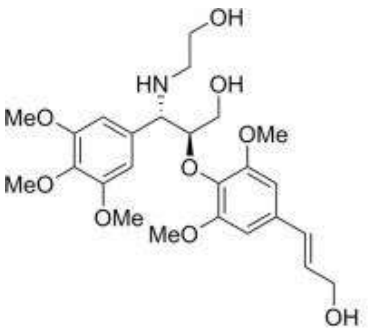
Common name : Kannada: Kaduatthi

Sanskrith: Kakodumbarika

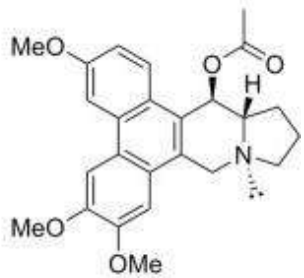
Habitate : It grows along rivers and forest edges.

Uses : Different parts of the plant have been used in the treatment of ulcers, psoriasis, anemia, piles jaundice, vitiligo, hemorrhage, diabetes, convulsion, hepatitis, dysentery, biliousness, and as lactagogue and purgative

Some isolated compounds:



Hispidacine



Hispiloscine

Botanical name : ***Helicteres isora* L.**[6-7]

Family : Malvaceae

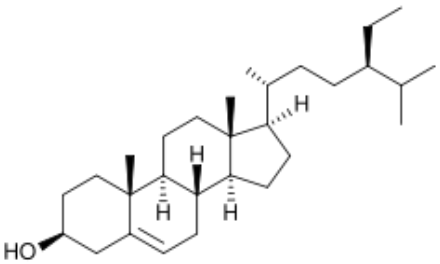
Common name : Kannada: Yedmuri

Sanskrith: Avartani; avartphala

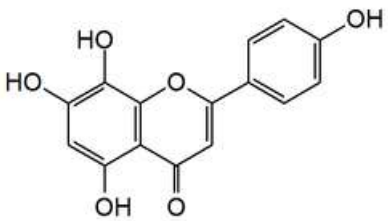
Habitate : It is a tropical Asian shrub or medium-sized tree. The shrub/plant is found all throughout India from Punjab to Bengal; Jammu to South India. Pakistan, Nepal, Myanmar, Thailand, and Sri Lanka.

Uses : The roots and the bark are used as an expectorant, gastropathy, diabetes, diarrhoea and dysentery. The fruits are used as astringents, refrigerant, stomachachic, antispasmodic, haemostatic and vermifuge. They are useful in griping of bowels, flatulence, colic, diabetes, diarrhea and dysentery. The root juice and fruits are topically applied to cure snake bite

Some isolated compounds:



β-Sitosterol



Isoscutellarein

Botanical name : ***Acacia pennata***[8]

Family : *Mimosaceae*

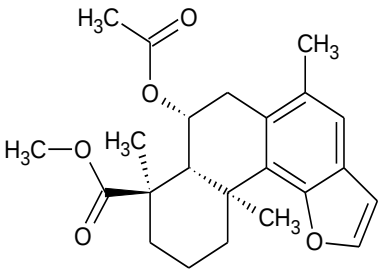
Common name : Kannada: Kaadu seege

Sanskrith: Khadiravallari

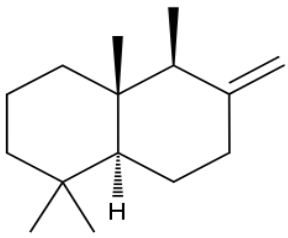
Habitate : It grows in lowland and medium altitude deciduous bushlands, dry rocky plains

Uses : *A. pennata* has been used in Laos against anaemia. In India, a decoction of young leaves is taken to treat body pain, headache and fever, and a decoction of the roots is applied against rheumatism and cough.

Some isolated compounds:



Taepeenin-D



Drim-8(12)-ene

Botanical name : ***Terminalia tomentosa***[9-10]

Family : *Combretaceae.*

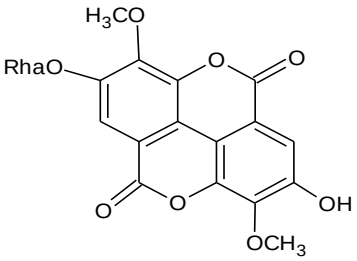
Common name : Kannada: Hole matthi

Sanskrith: Raktarjun

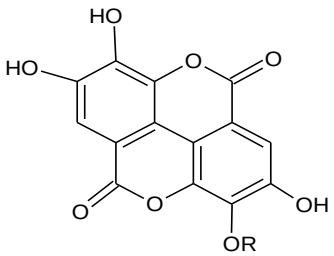
Habitate : It is found growing on river banks or near dry river beds.

Uses : The bark is astringent. It is used in the treatment of diarrhea The juice of the bark is applied externally to cuts and wounds It can also be boiled then rubbed onto the head to remove dandruff A paste of the gum is applied externally to burns and is also used to treat swellings caused by inflammation

Some isolated compounds:



3,3'-di-O-methylellagic acid-4'-O-rhamnopyranoside



ellagic acid: R=H

3-O-methyl ellagic acid: R=CH3

Botanical name : ***Kydia calycina* Roxb.**[11-12]

Family : *Malvaceae*

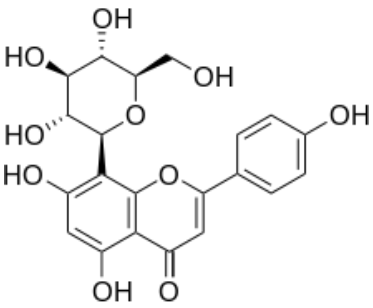
Common name : Kannada: Kaadu Bende

Sanskrith: Pula

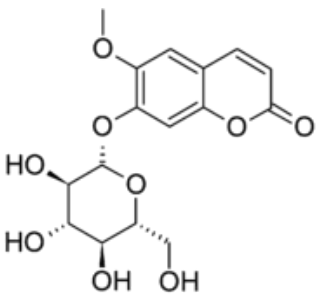
Habitate : Sparse mixed forests in valleys at elevations of 500 - 1,600 metres in southern China

Uses : Leaves for skin diseases, abscess, wounds, cuts, boils, leaf paste applied to the body to get relief from pain; leav es chewed to overcome the deficiency of saliva and for stomatitis; leaf decoction eaten for reducing the temperature of the body. Root febrifuge, and for rheumatism.

Some isolated compounds:



Vitexin



Scopolin

Botanical name : ***Myristica dactyloides Gaertn***[13]

Family : *Myristicaceae*

Common name : Kannada: Kadu jayikai

Sanskrith: Jatisasga

Habitate : It is mainly found in the rainforests of Malaysia, Borneo, Polynesia, India, Madagascar, Africa, Amazon basin and Sri Lanka.

Uses : Medicinally used for diarrhea, nausea, vomiting, chronic bowel complaints, spermatorrhoea, impotency, amenorrhoea, ulcers, splenic disorders, rheumatism, asthma. It is useful as tonic for the heart and brain and also in general debility.

Botanical name : ***Hopea ponga (Dennst.)***[14]

Family : *Dipterocarpaceae*

Common name : Kannada: Doddele bogi, Haiga

Sanskrith: Kalhoni

Habitate : In evergreen and semi-evergreen forests of Western Ghats. Maharashtra, Karnataka, Tamil Nadu and Kerala.

Uses : Plants belonging to the Dipterocarpaceae family have been disclosed to be a rich source of stilbene oligomers. This genus has been well documented to be a good source of biologically active stilbenoids. Stilbenoids have anti-human immune deficiency virus (HIV), cytotoxic, and acetylcholin- esterase inhibitory activities.

Among the above short listed plants, bark part of ***Myristica dactyloides Gaertn*** and ***Hopea ponga (Dennst.)*** has been selected to carry out the research work. Because, local healers as well as ayurvedic vaidyas in Agumbe region prescribe the bark part of ***Myristica dactyloides Gaertn*** and ***Hopea ponga (Dennst.)*** as tonic for the heart and brain and also in general debility, boils, wound healing, microbial infections and etc. Also the literature survey emphasised that, there were no systematic and scientific reports on biological activities of the bark part of these plants.

Hence, the bark of ***Myristica dactyloides Gaertn*** and ***Hopea ponga (Dennst.)*** plant was selected for extraction, phytochemical investigation, antimicrobial and pharmacological studies.

References

1. V. Kumar and Asija, Agrobiosis Biodiversity Conservation in Biodiversity-Principles and Conservation, India, Jodhpur, **2000**.
2. S.K. Jain, Dictionary of Indian Folk Medicine and Ethnobotany. Deep Publication, New Delhi, **1991**.
3. J.A. Parrota, Healing Plants of Peninsular India. CABI, New York, **2001**.
4. Dewan Md. Sumsuzzman, Turabe Farah Chowdhury Dewan Md. Sumsuzzman, Turabe Farah Chowdhury. Antibacterial, antioxidant and cytotoxic activities of *Ficus hispida* leaves and fruits. *Indo American J of Pharmaceutical Research.*, 3, 841, **2016**.
5. Ramandeep Singh, Preeti Thakur, Alok Semwal and Satinder Kakar. Wound healing activity of leaf methanolic extract of *Ficus hispida Linn African J of Pharmacy and Pharmacology.*, 8, 21, **2014**.
6. M. Suthar, G. S. Rathore and A. Pareek. Antioxidant and Antidiabetic Activity of *Helicteres isora (L.)* Fruits. *Indian J of Pharmaceutical Sciences.*, 695, **2009**.
7. Renu Dayal, Amrita Singh, Rudra P. Ojha, K. P. Mishra, Possible therapeutic potential of *Helicteres isora (L.)* and it's mechanism of action in diseases, *J of Medicinal Plants Studies.*, 3, 95, **2015**.
8. Reena Terangpi, Ratan Basumatary, A.K. Tamuli, R. Teron. Pharmacognostic and Physicochemical evaluation of stem bark of *Acacia pennata (L.)* Willd, a folk plant of the Dimasa tribe of *Assam. J of Pharmacognosy and Phytochemistry*, 2, 134, **2013**.
9. Arun Bhimarao Joshi, M. Aswathi, Maya Bhobe. *Terminalia Tomentosa roxb* (ex DC) wight & arn: phytochemical investigation. *American J of Advanced Drug Delivery.*, 224, **2013**.
10. Balaji Meriga, Parim Brahma Naidu, Ganjayi Muniswamy, GEN Hanuma Kumar, Ramavat Ravindar Naik, Suresh Pothani. Ethanolic Fraction of *Terminalia tomentosa* Attenuates Biochemical and Physiological Derangements in Diet Induced Obese Rat Model by Regulating Key Lipid Metabolizing Enzymes and Adipokines, *Pharmacognosy Magazine.*, 385, **2017**.
11. Baburao Bhuky, N Rama, Analgesic and anti-inflammatory activities of leaf extract of *Kydia calycina Roxb*., *Bangladesh J Pharmacol.*, 4, 101, **2009**.

12. Baburao Bhukya, Harikiran Lingabathula, Narsimha Reddy Yellu. Cytotoxic effects of *Kydia calycina* leaf fractions on different human cancer cell cultures. *Int J of Green Pharmacy.*, 11, 708, **2017**.

13. S. Subha, B. Vijayakumar, K. Prudhviraj, M. Vijey Aanandhi, M. Shankar, M. Nishanthi, Pharmacognostic and priliminery phytochemical investigation on leaf extracts of *Myristica dactyloides Gaertn*. *Int J of Phytopharmacology*. 4, 8, **2013**.

14. T. Ito, T. Tanaka, M. Iinuma, I. Iliya, K. Nakaya, Z. Ali, Y. Takahashi, R. Sawa, Y. Shirataki, J. Murata, D. Darnaedi, Tetrahedron, 59, 5347, **2003**.

CHAPTER-III

Description of *Myristica dactyloides Gaertn* and *Hopea ponga (Dennst.)* Plants

3.1 Description of *Myristica dactyloides Gaertn* and *Hopea ponga (Dennst.)* Plants

Literature survey emphasized that, ***Myristica dactyloides Gaertn*** and ***Hopea ponga (Dennst.)*** plants have been reported for a wide variety of uses in traditional medicine. Therefore, these two plants have been selected to carry out the research work and their detailed description is given below.

3.2 *Myristica dactyloides Gaertn*

3.2.1 *Family*: *Myristicaceae*

The ***Myristicaceae*** are a family of blooming plants native to Asia, Pacific islands, Africa, and America has been recognized by most taxonomists[1]. It is sometimes called the "nutmeg family", after its most famous member, *Myristica fragrans*, the source of the spices nutmeg and mace. The best known genera are *Myristica* in Asia and *Virola* in the Neotropics. *Myristicaceae* are found in humid lowland forests, swamp forests, submontane forests and rain forests at elevations up to 2100 m. Some of the anatomical characters presented by this family suggest that in the past they could live in xeric (dry) environments, but now their species are linked to tropical rainforests. The species present anthesis at night and pollination is usually carried out by small beetles from the Anthicidae family that resemble ants and consume pollen (e.g., *Myristica fragrans* is probably pollinated by the beetle Formicomus braminus)[2]. The strong floral scent that attracts beetles emerges from the ends of the connectives of the stamens. However, *Myristica* is probably pollinated by true ants, a case of myrmecophily.

3.2.2 *Genus Myristica*

Myristica is a genus of trees in the family *Myristicaceae*. The family consists of about 21 genera with about 520 species[3] of trees, shrubs and rarely lianas (*Pycnanthus*) found in tropical forests across the world. They are typically trees with reddish sap and distinctive pagoda-like growth (known as myristicaceous branching) in which horizontal branching only occurs at certain nodes along the main axis of the trunk, each node separated by a large gap where no branching occurs. All genera are dioecious, except *Endocomia* and some *Iryanthera*. The inner bark is usually pink to reddish or light colored then oxidizing as such. When cut, the tree trunk exudes a red or orange resin, stems and young twigs often will exude clear sap (not colored) that may smell spicy. The foliage is generally spicy-aromatic and the leaves are glossy, dark green, simple, entire, 2-ranked, undersides often whitish or tomentose, with dark brown punctations or not, usually with complex caducous hairs colored golden yellow to red. The flowers are usually small, highly

reduced, fragrant, with 3-5 petals, inner perianth whitish-green, yellow, orange, reddish-pink to rusty-brown, arranged in axillary paniculate inflorescences or unbranched wart-like structures (like *Knema*). The female flowers are without staminodes, with stigmas often lobed. The male flowers with fused stamens arranged in a synandrium. Pollen is monosulcate, often boat-shaped.

The fruit is a leathery dehiscent capsule, with rusty indument or not[4] containing a single seed that is arillate or not; when present, the aril variously lacinate or entire. In most genera, the aril is colored red but also can be orange or white and translucent. The single seed has ruminated endosperm and is uniform in color or rarely with black blotches (*Compsoneura*). Many species within the family exhibit highly complex phytochemistry with numerous compounds having been described from the leaves, bark, fruits, arils, and seeds of many species.

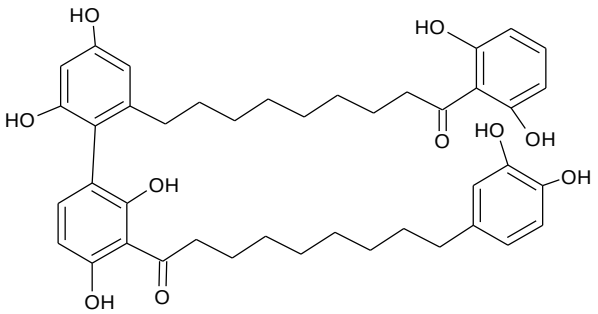
3.2.3 Ethnomedical Information

Essential oils of *Myristicaceae* have antifungal action and antimicrobial activity against *Streptococcus mutans*. The dark-red resin of the tree bark in some genera, such as *Virola*, contains several hallucinogenic alkaloids. Myristicin poisoning can induce convulsions, palpitations, nausea, eventual dehydration and generalized body pain. It is also reputed to be a strong deliriant[5] and some fatal myristicin poisonings in humans have occurred[6].

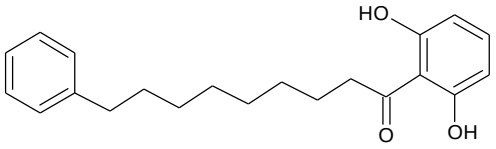
3.2.4 Compounds reported from the genus *Myristica*

The genus *Myristica* was found to contain various phytoconstituents such as Isoflavones, Diarylnonanoids which consists of Malabaricones A-D, Tannins, Isoflavones, and several other phytochemicals are also considered to be present in the plant. Some compounds reported from different species of genus *Myristica* are listed below.

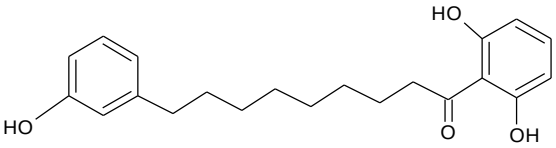
1. *Myristica maxima warb*



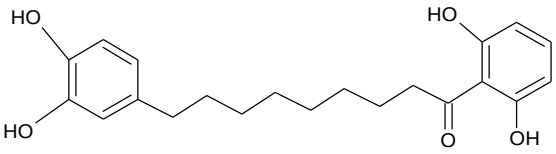
i. Giganteone E



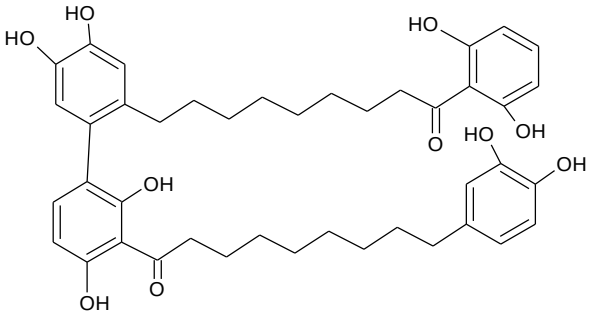
ii. Malabaricone A



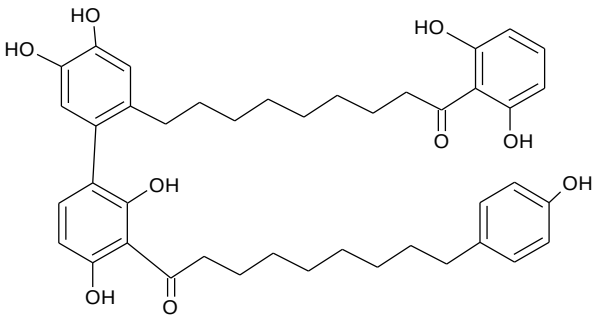
iii. Malabaricone B



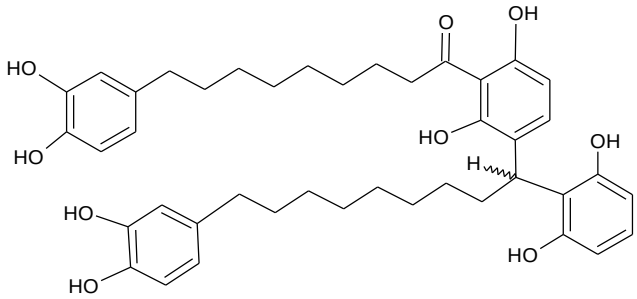
iv. Malabaricone C



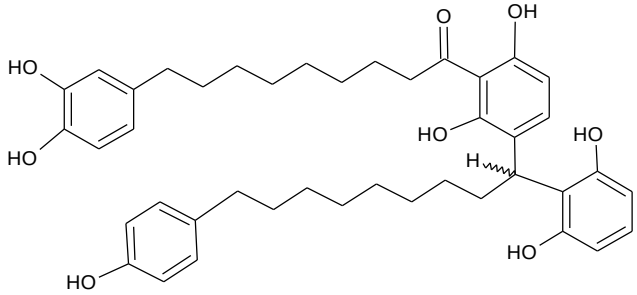
v. Giganteone A



vi. Giganteone C

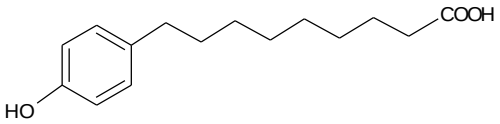


vii. Maingayones A

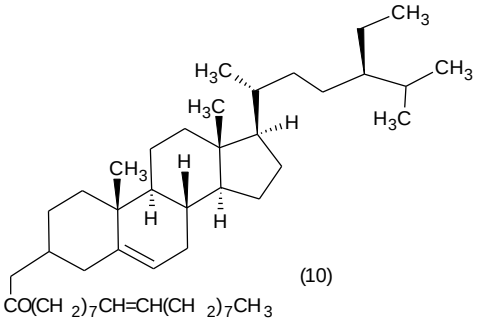


(8)

viii. Maingayones B



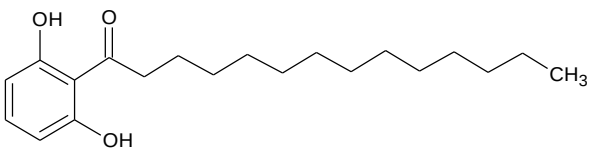
ix. Maingayic acid



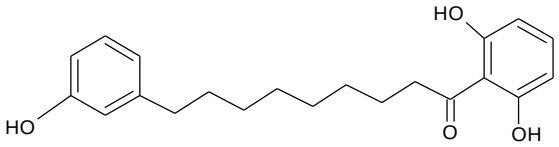
(10)

x. β-Sitosteryloleate

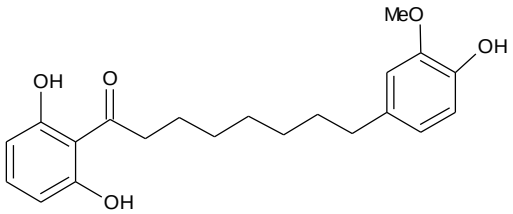
2. *Myristica fragrans*



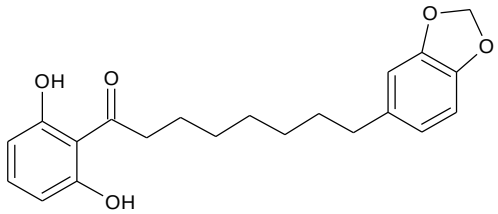
i. 1-(2,6-dihydroxyphenyl)tetradecane-1-one



ii. Malabaricone B



iii. 1-(2,6-dihydroxyphenyl)-9-(4-hydroxy-3-methoxyphenyl)nonane-1-one



iv. Malabaricone D

3.2.5 Myristica *Dactyloides gaertn*

Myristica dactyloides gaertn is a perennial tree. It is endemic to India and found widely distributed in Western Ghats forest region.

Plant Taxonomy

Kingdom : Plantae

Super division : Angiosperms

Phylum : Tracheophyta

Class : Magnoliopsida

Order : Mangoliales

Family : *Myristicaceae*

Genus : Myristica

Species : *Myristica dactyloides Gaertn*

Habit and Habitat: It is a swamp and lowland forest habitat tree. Trees up to 20 m tall, often with still roots.

Pharmacognostical Review Morphology

Trunk & Bark: Bark greenish-black, smooth, blaze reddish.

Branches and branchlets: Branches- horizontal, Branchlets- subtree, glabrous.

Exaduates: Sap red from cut end of bark, profuse.

Leaves: Leaves simple, alternate, distichous; Petiole 1-1.5cm long, caniculate above glabrous, lamina 9.5-22*3.7-10 cm, elliptic or elliptic oblong, apex acute or sub-acute, base acuteor attenuate, margin entire, glossy above, glabrous and glaucous beneath, coriaceous; mid rib raised above.

Inflorescence/Flower: Flowers unisexual, uroceolate, white; male flower numerous in number and smaller than female flowers, in auxillary cymes; female flowers in umbels, 5-6 flowered.

Fruit and seed: Capsule 5-7.5*1.8-3.5, oblong, pubescent; seed one, Aril covering the seed yellow and laciniate.

Fig: 3.1- *Myristica dactyloides Gaertn fruit*

Fig: 3.2- *Myristica dactyloides Gaertn bark*

3.2.6 Uses in Traditional Medicine

The plant *Myristica dactyloides gaertn* is traditionally used as medicine. The seed and seed aril is used as spice in Indian foods. They enhance the taste and aromatic flavor of the food. Recent scientific studies proved their biological activity according to their traditional claims. They are now known to possess Gastroprotective, Antipromastigote, Antioxidant, Antifungal, Nematicidal, Antiproliferative, anti-inflammatory, Analgesic, anti-ulcer, sedative, hypnotic Leukemic and Solid tumor. The aril, combined with dried ginger, is used to check diarrhoea. The aril is also used in treating coughs, bronchitis, fever, burning sensations, inflammation of joints, skin disorders, wounds, sleeplessness, indigestion, liver disorders and worms. The fruits are used in Ayurveda and Sidha

systems of medicine. The fruits are traded in the name of "Jaiphal". The bark and the leaves are boiled and the liquid used as a gargle in the treatment of throat infections.

3.2.7 Biological activities reported in the literature:

Plants of the genus of *Myristica* have been extensively used in folk medicine in Asia, including India. Acylphenols, including malabaricones B and C, extracted from Myristica spp. have been reported to exhibit antimicrobial, cytotoxic nematocidal activities and healing properties for gastric ulcers[7-11].

Myristica fragrans Houtt (Myristicaceae) is a traditional Chinese medicinal plant and its fruits, nutmeg, are used as an aromatic stomachic, analgesic and anti-inflammatory agent[12]. Up to now, more than 70 compounds have been identified from this plant, which showed various bioactivities, such as antioxidative[13], antitumor[14], antibacterial[15] and hepatoprotective effects[16].

3.3 *Hopea ponga*

3.3.1 Family: *Dipterocarpaceae*

Dipterocarpaceae are a family of 16 genera and approximately 695 known species[17] of mainly tropicaly lowland rainforest trees. The family name, from the type genus *Dipterocarpus*, is derived from Greek (*di*=two, *pteron*=wing and *karpos*=fruit) and refers to the two-winged fruit. The largest genera are *Shorea* (196 species), *Hopea* (104 species), *Dipterocarpus* (70species) and *Vatica* (65species). Many are large forest emergent species, typically reaching heights of 40–70m, some even over 80 m (in the genera *Dryobalanops*[18], *Hopea* and *Shorea*)[19], with the tallest known living specimen (*Shorea faguetiana*) 93.0 m tall[20]. The species of this family are of major importance in the timber trade. Their distribution is pantropical, from northern South America to Africa, the Seychelles, India, Indochina, Indonesia and Malaysia. The greatest diversity of *Dipterocarpaceae* occurs in Borneo. In south Asia, the dipterocarps are mainly distributed in tropical peninsula from Karnataka coast to the tip of southern India and northeast India.

Hopea is the largest genus among the family *Dipterocarpaceae* and well known for its timber value among tree species. The genus *Hopea* has reported with 104 species throughout the world out of which, 11 species reported from India. They are typically tropical trees, adapted to warm and uniformly moist conditions and it was not found on mountain slopes above altitudes of 900 m asl. It has a wide range of distribution in the

Western Ghats from lowland evergreen forest in the coastal plains to the tropical evergreen forest (1200 m asl). Currently, they predominate in the international timber market and therefore, play an important role in the economy of many of the Southeast Asian countries. They constitute important timber for domestic needs and a source of a variety of minor products on which many forest dwellers are directly dependent for their survival. Since being an economically important genus, potential benefits of this genus have not been recognized properly until, and the present situation leads to the loss of many valuable timber species. As per the current literature, relatively little research work has been conducted on the genus *Hopea* when compared to other genera of *Dipterocarpaceae*. Most of the species of *Hopea* reported in India were found to be endemic. Of the 11 species reported from India, 8 species are recorded as endemic to the Western Ghats particularly to the states of Karnataka, Kerala and Tamil Nadu. In particular, the lower altitude, warm and humid part of Karnataka is most suitable for the species. All the 11 species of *Hopea* reported from India are under severe threat and included in the IUCN red list category. Three species viz., *Hopea erosa, H. helferi and H. jacobi* were included under critically endangered, 5 species (*H.glabra, H. parviflora, H. ponga, H. racophloea and H. utilis*) included under endangered. Unfortunately, two species *Hopea jacobi* (IUCN, 2015) and *H. canarensis* were thought to be extinct and there was no recent record on distribution. This indicates that the entire genus of *Hopea* in India is under severe threat due to various anthropogenic activities and illegal timber trade.

Hopea ponga is a common sub canopy tree of wet evergreen forests that grows up to 900m asl and the species is endemic to the Western Ghats. It is distributed in isolated patches and along the rivers of the Western Ghats of Tamil Nadu, Kerala, Karnataka and Maharashtra. It has been also recorded from all parts of Western Ghats from Agasthyamalai in the south to some parts of Goa in North. It looks very beautiful when it gets covered with the reddish fruits towards the end of July. The trees have a smooth and straight trunk that grows up to a larger height and the base of the tree may be often buttressed. *Hopea ponga* is an economically important timber species and the bark used as a good tanning material and astringent with the slow speed of diffusion. Heartwood obtained from the tree was used for building construction, for posts and for making cartwheels.

Due to its greater importance in the timber market, it has been largely cut down in the past few decades which caused a severe reduction in the population that leads the species to include under endangered category in the ICUN red list. In this present situation, it is very much important to understand the reproductive constraints of the *Hopea ponga* for the conservation this valuable timber species from extinction in the near future.

3.3.2 Genus: Hopea[21-27]

The genus Hopea is distributed from Sri Lanka, to Bangladesh, India, Myanmar, South China, Hainan, Indochina and Malaysia. There are 14-15 species in Thailand. The genus *Hopea* is having big or vast trees resinous, with transparent resin. Stipule oblong to linear-lanceolate, small, caducous. Leaves not plicate, symmetrical or asymmetrical at base, chartaceous or coriaceous secondary nerves pinnate or dryobalanoid, arched near to margin intermediate nerves present in dryobalanoid type tertiary nerves scalariform or reticulate. Domatia frequently present. Flowers rather small in panicled unilateral racemes. Sepal 2 external, 3 internal, imbricate. Petal silky or tomentose on the part exposed in bud only, contort, narrow, yellow, red or white. Stamen usually 15, rarely 10. Anther ovate with 4 pollen sac. Ovary 3 celled, 2-ovuled each. Fruit surrounded by 5 enlarged sepal, free to base, 2 outer wings long linear, 3 inner ones not longer than fruit, connate in a cup at base, closely appressed to the small nut. Cotyledon fleshy, bilobed, unequal. Bark usually smooth, paper flaky, or distantly fissured. Timber light red and rather soft.

3.3.3 Ethanomedical Information

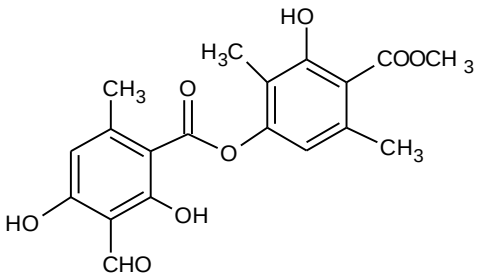
Plants of the family *Dipterocapaceae* have been shown to be a rich source of resveratrol (3,5,4′-trihydroxystibene) oligomers[28-31], which are receiving considerable chemical and biological attention owing to their structural complexity as well as their array of bioactivities exhibited such as antifungal[32-33], anti-HIV[34], cytotoxic[35-36], anti-inflammatory[37], and antibacterial effects[38]. In particular, species in the genus *Hopea* seem to be abundant in oligostilbenoids[39]. Research on the metabolites from the *Hopea* genus of the *Dipterocarpaceae* family contain several structurally novel oligostilbenoids have been isolated from the bark, which exhibited significant cytotoxic activity or potent inhibitory activity against acetylcholinesterase[40-42]. In Vietnam, the extract of the *H. odorata* bark has been used as a remedy for gingivitis and for diarrhea treatment. The resin of the trunk of *H. odorata* can be applied to sores and wounds for the relief of

inflammation and to stop bleeding. A recent study showed that the methanol extract of *H. odorata* strongly inhibited gene expression of proinflammatory cytokines and chemokines, such as interferon-beta, interleukin-12, and monocyte chemotactic protein-1[43]. To our knowledge, there is no information of *H. odorata* as a cancer treatment, from indigenous medicine until now.

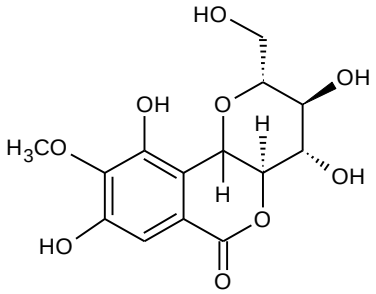
3.3.4 Compounds reported from the genus *Hopea*

The plant ***Hopea ponga (Dennst.)*** was found to contain various phytoconstituents such as Phenols, Saponins, Flavonoids, Tannins, Terpenes, Alkaloids, Glycosides and several other phytochemicals are also considered to be present in the plant. Some compounds reported from different species of genus *Hopea* are listed below.

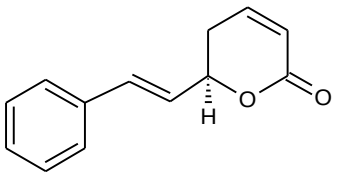
1. ***Hopea sangal***



i. Atranorin

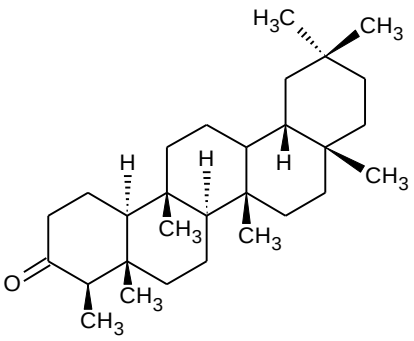


ii. Bergenin

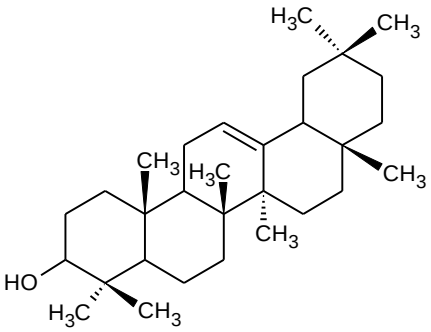


iii. Goniothalamine

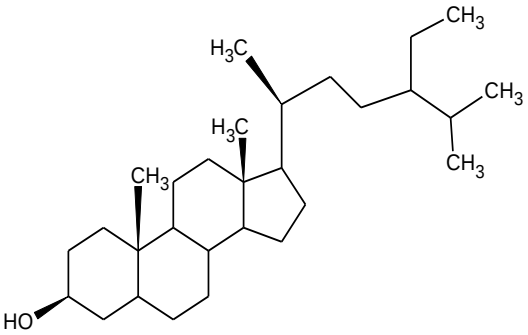
3. ***Hopea odorata***



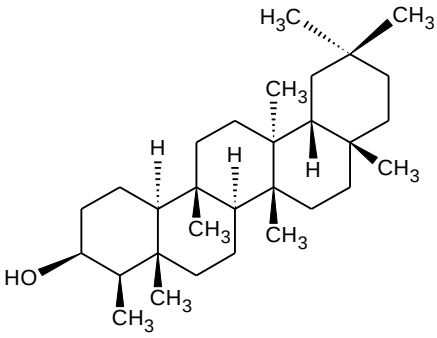
i. Friedelin



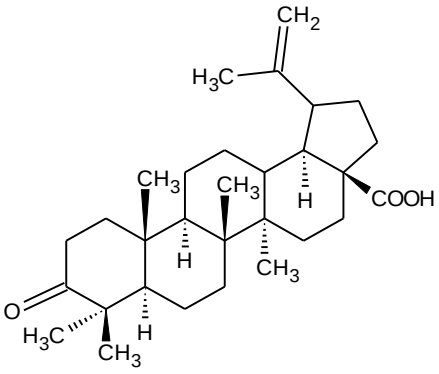
ii. β-Amyrin



iii. β-Sitosterol

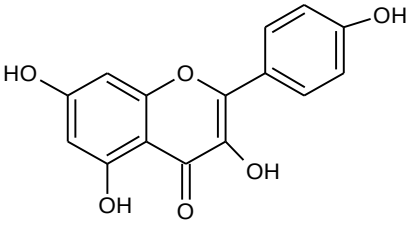


iv. Epifriedelanol

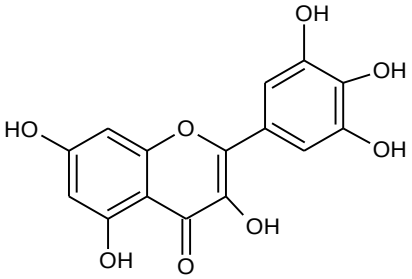


v. Betulonic acid

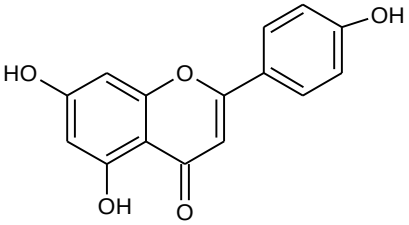
4. *Compounds* isolated from the family diptercarpaceae



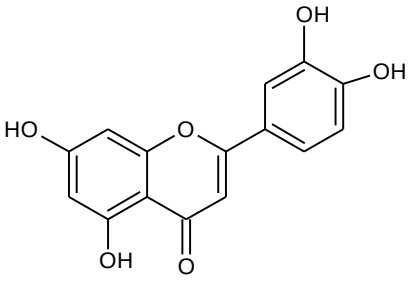
i. Kaempferol



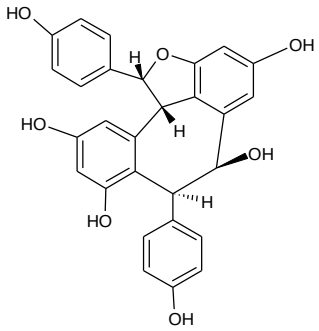
ii. Myricetin



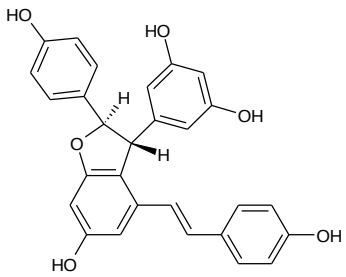
iii. Apigenin



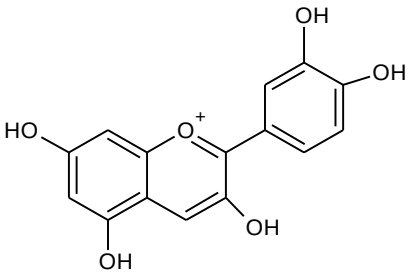
iv. Luteolin



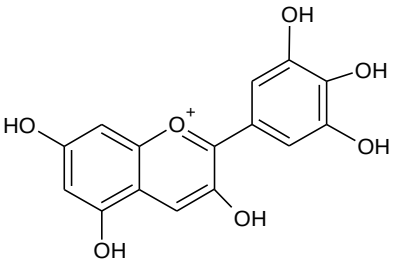
v. Balanocarpol



vi. Viniferin



vii. Cyanidin



viii. Delphinidin

3.3.5 *Hopea ponga (Dennst.)*

Hopea ponga (Dennst.) is a species of plant in the family *Dipterocarpaceae.* It is endemic to India.

Plant Taxonomy

Kingdom **:** Plantae

Super division : Angiosperms

Phylum **:** Tracheophyta

Class **:** Magnoliopsida

Order **:** Malvales

Family **:** *Diptercarpaeceae*

Genus **:** Hopea Roxb.

Species **:** *Hopea ponga (Dennst.)*

Habit and Habitat: Common subcanopy trees in wet evergreen forests, up to 900 m. Trees up to 18 m tall.

Pharmacognostical Review Morphology

Trunk & Bark: Bark thin smooth, flaky.

Branches and branchlets: Branches- horizontal, Branchlets- subtree, glabrou Branchlets usually drooping, terete, tomentose.

Leaves: Leaves simple, alternate, spiral; stipules caducous; petiole stout, terete, whitish tomentose, 1.3 cm long; lamina 11-31 x 2.5-7.5 cm, narrow oblong to oblong, apex bluntly acute or acuminate, often rounded, base rounded or subcordate, chartaceous or subcoriaceous; secondary nerves 7-12 pairs, gradually curved; tertiary nerves reticulo-percurrent.

Inflorescence/Flower: Inflorescence panicled racemes, glabrous; flowers white.

Fruit and seed: Nut with 3 shorter and 2 longer accrescent calyx lobes; seeds 1.

Fig: 3.1- *Hopea ponga (Dennst.)* leaves

Fig: 3.2- *Myristica dactyloides Gaertn* bark

3.3.6 Uses in Traditional Medicine

The methanolic extract of leaf of *Hopea ponga (Dennst.)* exhibits high scavenging ability against different free radicals. It was also found to contain active compounds which have effective antioxidant and antimicrobial activities. The crude extract of seed wings of *Hopea ponga (Dennst.)* exhibits a high degree of antimicrobial activity against several pathogenic bacteria. This family of plant is known to produce a variety of resveratrol oligomers. Resveratrol, a stilbene-based phytochemical, has been found to be substantially helpful for human health owing to its significant anticancer[44-48], antioxidative[49-51], anti-HIV[48], antimicrobial[48] and lung-microcirculation-improving actions[52].

3.3.7 Biological activities reported in the literature

Its oligomers have also received intense medicinal attention for their promising anticancer[53-55], antioxidant[56] and antifungal potentials[57]. Plants belonging to the *Dipterocarpaceae* family have been disclosed to be a rich source of stilbene oligomers[58-

[60]. Thus *Dipterocarpaceae* plants are very promising for chemical research in natural product and pharmaceutical industry.

The oligoresveratrol isolated from the stem bark of *Hopea odorata* consist of dimer and tetramer resveratrol. Some compounds have biological activity as antioxidant and cytotoxic effect against Raji and HeLa-S3 lines cell. Hopeaphenol showed the highest activity as antioxidant, whereas ampelopsin gave the highest cytotoxic effect against HeLa-S3 and Raji cell[61].

Aqueous and ethanolic crude extracts of *Hopea utilis* screened for antibacterial and cytotoxic activities were studied. Antibacterial activity of ethanolic extracts of *H. utilis* was more successful with the pathogens *Salmonella typhi* and *Streptococcus aureus* respectively. Oligostilbenoids from *Hopea hainanansis*, hopeahainol A, a dimer, and neohopeaphenol A, a tetramer, were found to be acetylcholine esterase inhibitors. Hopeanolin, an unusual resveratrol trimer with an ortho-quinone nucleus, was isolated and characterized from the stem bark of *Hopea exalata*. Also obtained were six known stibenoids, shoreaphenol, Vaticanol G, Viniferin, Pauciflorol A, Vaticanol A and trans-3,5,4'-trihydroxystilbene-2-C-glucoside.

Timber/bark of *Hopea cordifolia* and *Hopea jucunda*[62] contained sitosterol, lupeol, ursolic acid, β –amyrin, betulinic acid, dipterocarpol (in the order of quantity from high to low). On the other hand, it showed that there was more lupane triterpene in genus *Hopea* than dammarane triterpene.

The isolation of the extract from the stem bark of *Hopea odorata*, *H. mengarawan* and *H. nigra*, found seven known resveratrol derivatives, named balanocarpol, heimiol A, vaticanol G, vaticanol B, hopeaphenol, ampelopsin H and hemlesyanol C. Hopeaphenol was more active in antioxidant than ascorbic acid whereas vaticanol B and ampelopsin H were very active against HeLa-S3 (human epithelial carcinoma cell line) and Raji cell (human Burkitt's lymphoma cell line)[63]. Two oligostilbene, hopeaphenol and malibatol A, from the methanolic extract of the stem bark of *Hopea odorata Roxb*[64]. Malibatol-A had been reported to have significant effect against cancer cell line CEM-SS (human T lymphoblastoid cell line). Quercetin, kaempferol, apigenin and quercetin-3-glucoside were isolated in the study of flavanoid pattern of *H. odorata* leaves[65].

References

1. Bingtao Li and K. Thomas. Wilson, "Myristicaceae", *Flora of China online.*, **7, 2008**.
2. D. Poore. *No timber without trees. Sustainability in the tropical forest*. Earthscan Publications Ltd, London., 6, 341, **1990.**
3. P.S. Ashton, *Dipterocarpaceae.* In *Tree Flora of Sabah and Sarawak.* E. Soepadmo, L.G. Saw, and R.C.K. Chung, eds. Government of Malaysia, Kuala Lumpur, Malaysia., **2004**.
4. *Borneo". Eastern Native Tree Society. Retrieved.,* **2009**.
5. K.S. Murali and R. Sukumar Reproductive Penology of a Tropical Dry Forest in Mudumalai, *Southern India* J of Ecology*.,* 82, 759, **1994.**
6. K. Pradeep, D. Divakar Steven. M. Leavitt. Carmen Molina Ruth Del-Prado H. Thorsten Lumbsch Ana Crespo A DNA barcoding approach for identification of hidden diversity in *Parmeliaceae (Ascomycota): Parmelia sensu stricto* as a case study, Wiley online library., **2015.**
7. A. Maia, I. Schmitz-Afonso, M.T. Martin, K. Awang, O. Laprevote, F. Gueritte, M. Litaudon. *Planta Med*., 74, 1457, **2008**,
8. K.Y. Orabi, J.S. Mossa, El-Feraly, *F. S. J. Nat. Prod*., 54, 856, **1991**.
9. P. Van Cuong, A. Jossang, T. Sevenet, B. Bodo. *Tetrahedron.*, 58, 5709, **2002**.
10. S. Hosoi, F. Kiuchi, N. Nakamura, M. Imasho, M. Ahad Ali, Y. Sakaki. *Chem. Pharm. Bull.,* 47, 37, **1999**.
11. B. Debashish, B.K. Ajay, G.K. Ranjit, B.K. Sandip, C. Subrata. *Eur. J. Pharmacol.,* 578, 300, **2008.**
12. Jiangshu New College of Medicine. Zhongyao dacidian (a dictionary of traditional Chinese medicine). 894, **1977.**
13. G.F. He. Natural antioxidants from *Myristica fragrans Houtt*. *Food Sci.*, 12, 43, **1986.**
14. N. Nakamura, F. Kiuchi, Y. Tsuda, K. Kondo. Studies on crude drugs effective on visceral larva migrans. V: the larvicidal principle in mace (aril of *Myristica fragrans*). *Chem Pharm Bull.*, 36, 2685, **1988.**
15. M. Hattori, S. Hada, A. Watahiki, H. Ihara, Y.Z. Shu, N. Kakiuchi, T. Mizuno, T. Namba. Studies on dental caries prevention by traditional medicines. X: antibacterial action of phenolic components from mace against Streptococcus mutans. *Chem Pharm Bull.*, 34, 3885, **1986**.

16. T. Morita, K. Jinno, H. Kawagishi, Y. Arimoto, H. Suganuma, T. Inakuma, K. Sugiyama. Hepatoprotective effect of myristicin from nutmeg (*Myristica fragrans*) on lipopolysaccharide/D-galactosamine-induced liver injury. *J Agric Food Chem.,* 51, 1560, **2003**.

17. *M.J.M Christenhusz, & J.W. Byng,*. "The number of known plants species in the world and its annual increase". *Phytotaxa., 261, 201, **2016**.*

18. P.S. Ashton, *Dipterocarpaceae.* In Tree Flora of Sabah and Sarawak, E. Soepadmo, L.G. Saw, and R.C.K. Chung, eds. Government of Malaysia, Kuala Lumpur, Malaysia. 5, **2004**.

19. "Borneo". Eastern Native Tree Society. Retrieved., 04, **2009**.

20. Simon Gardner, Pindar Sidisunthorn and Lai Ee May, *Heritage Trees of Penang*. Penang: Areca Books, **2011**.

21. R. Pooma, *"Dipterocarpaceae* in Thailand: Taxonomic and Biogeographical Analysis", Department of Botany, Kasetsart University, Bangkok, 285, **2003.**

22. H. N. Ridley, The Flora of the Malay Peninsula Vol. I. London: L.Reeve & Co., LTD, **1967**.

23. S. Gardner, P. Sidisunthorn, and V. Anusarnsunthorn. A Field Guide to Forest Trees of Northern Thailand. Bangkok: Kobfai Publishing Project. 560, **2000.**

24. P.S. Ashton, "Dipterocarpaceae." in Flora Malesiana, Kuala Lumpur: Goverment of Malaysia., 237, **1982**.

25. Y. Mitinand et al. "The manual of Dipterocarpaceae of mainland SouthEast Asia." Thai Forest Bulletin (Botany) 12, 1, **1980**.

26. T. Yuwa Amornpitak, T. Vichitsoonthonkul, and M. Tanticharoen. "Molecular phylogeny of *diptercarpaceae* in Thailand using trnL-trnF and atpBrbcL intergenicspacer region in chloroplast DNA." *Pakistan J of Biological Sciences* 9, 649, **2006**.

27. R. Pooma, and M. Newman. "Checklist of *Dipterocarpaceae* in Thailand." Thai Forest Bulletin (Botany) 29, 110, **2017**.

28. E.K. Seo, H. Chai, H.L. Constant, T. Santisuk, V. Reutrakul, C.W.W. Beecher, N.R. Farnsworth, G.A. Cordell, J.M. Pezzuto, A.D. Kinghorn., *J. Org. Chem.*, 64, 6976, **1999**.

29. T. Ito T. Tanaka, M. Iinuma, K. Nakaya, Y. Takahashi, R. Sawa, J. Murata, D. Darnaedi, D. *J. Nat. Prod*., 67, 932, **2004**.

30. J.Y. Liu, Y.H. Ye, L. Wang, D.H. Shi, R.X. Tan., **HelV. Chim. Acta**, 88, 2910, **2005**.

31. J. R. Zgoda-Pols, A.J. Freyer, L.B. Killmer, J.R. Porter., *J. Nat. Prod.*, 65, 1554, **2002**.

32. M. Bokel, C. Diyasena, A.A.L. Gunatilaka, W. Kraus, S. Sotheeswaran, **Phytochemistry**, 27, 377, **1988**.

33. S. Sotheeswaran, M.U.S Sultanbawa, S, Surendrakumar, S. Balasubramaniam, P.J. Bladon., *J. Chem. Soc., Perkin Trans*., 1, 159, **1985**.

34. J. R. Dai, Y.F. Hallock, J. H. Cardellina, *M. R. J. Nat. Prod*., 61, 351, **1998**.

35. M. Ohyama, T. Tanaka, T. Ito, M. Iinuma, K.F. Bastow, K.H. Lee., *Bioorg. Med. Chem. Lett*., 9, 3057, **1999**.

36. T. Ito, Y. Akao, T. Tanaka, M. Iinuma, Y. Nozawa., *Biol. Pharm. Bull*., 25, 147, **2002**.

37. K. S. Huang, M.Lin, L.N. Yu, M. Kong., *Tetrahedron.*, 56, 1321, **2000**.

38. Y.A.G.P. Gunawardana, S. Sotheeswaran, M.U.S. Sultanbawa, S. Surendrakumar, P. Bladon, *Phytochemistry*., 25, 1498, **1986**.

39. T. Tanaka, T. Ito, Y. Ido, T.K. Son, K. Nakaya, M. Iinuma, M. Ohyama, V. Chelladurai, *Phytochemistry.*, 53, 1015, **2000.**

40. J.Y. Liu, Y.H. Ye, L. Wang, D.H. Shi, R.X. Tan, New resveratrol oligomers from the stem bark of *Hopea hainanensis*. *Helv. Chim. Acta.*, 88, 2910, **2005**.

41. H.M. Ge, C. Xu, X.T. Wang, B. Huang, R.X. Tan, Hopeanol: a potent cytotoxin with a novel skeleton from *Hopea exalata*. *Eur. J. Org. Chem.*, 5551, **2006**.

42. H.M. Ge, C.H. Zhu, D.H. Shi, L.D. Zhang, D.Q. Xie, J. Yang, S.W. Ng, R.X. Tan, Hopeahainol A: an acetylcholinesterase inhibitor from *Hopea hainanensis*. Chem., *Eur. J*., 14, 376, **2008.**

43. Y. Yang, T. Yu, Y.G. Lee, et al. Methanol extract of *Hopea odorata* suppresses inflammatory responses via the direct inhibition of multiple kinases. *J Ethnopharmacol*. 145, 598, **2013.**

44. M. Jang, L. Cai, G. O. Udeani, K. V. Slowing, C.F. Thomas, C. W. W. Beecher, H. H. S. Fong, N. R. Farnsworth, A. D. Kinghorn, R. G. Mehta, C. M. Richard, J. M. Pezzuto, Cancer Chemopreventive Activity of Resveratrol, a Natural Product Derived from Grapes., *Science.*, 275, 218, **1997**.

45. E.J. Park, J. M. Pezzuto, *Cancer Metast. Rev*., 21, 231, **2002**.

46. A.D. Kinghorn, B.N. Su, D. S. Jang, L.C. Chang, D. Lee, J.Q. Gu, E. J. Carcache-Blanco, A.D. Pawlus, S.K. Lee, E. J. Park, M. Cuendet, J. J. Gills, K. Bhat, H.S. Park, E. Mata-Greenwood, L. L. Song, M. Jang, J.M. Pezzuto, *Planta Med*., 70, 691, **2004**.

47. J. R. Stewart, M. C. Artime, C. A. O'Brian, *J. Nutr*., 133, 2440, **2003**.

48. Y. Shirataki, N. Motohashi, S. Tani, H. Sakagami, K. Satoh, H. Nakashima, S. K. Mahapatra, K. Ganguly, S.G. Dastidar, A.N. Chakrabarty, *Anticancer Res.*, 21, 275, **2001**.

49. B. Olas, P. Nowak, B. Wachowicz, *Cell. Mol. Biol. Lett*., 9, 577, **2004.**

50. B. Olas, B. Wachowicz, *Gen. Physiol. Biophys*., 23, 315, **2004**.

51. Y. J. Cai, Q. Y. Wei, J. G. Fang, L. Yang, Z. L. Liu, J. H. Wyche, Z. Han, *Anticancer Res.*, 24, 999, **2004**.

52. Y. Meng, M. Zhang, J. Xu, X. M. Liu, Q. Y. Ma, *World J. Gastroenterol*., 21, 433, **2005**.

53. R. H. Cichewicz, S. A. Kouzi, *Stud. Nat. Prod. Chem. (Bioact. Nat. Prod. (Part G))*., 26, 507, **2002**.

54. T. Ito, Y. Akao, H. Yi, K. Ohguchi, K. Matsumoto, T. Tanaka,M. Iinuma, Y. Nozawa, *Carcinogenesis.*, 24, 1489, **2003**.

55. M. Ohyama, T. Tanaka, T. Ito, M. Iinuma, K.F. Bastow, K.H. Lee, *Bioorg.Med. Chem.Lett.*, 9, 3057, **1999**.

56. J. H. Kang, Y. H. Park, S. W. Choi, E. K. Yang, W. J. Lee, *Exp. Mol. Med.*, 35, 467, **2003.**

57. A. E. Bala, A. Kollmann, P.-H. Ducrot, A. Majira, L. Kerhoas, R. Delorme, *J. Einhorn, Pest. Sci.*, 55, 206, **1999**.

58. T. Ito, T. Tanaka, M. Iinuma, I. Iliya, K. Nakaya, Z. Ali, Y. Takahashi, R. Sawa, Y. Shirataki, J. Murata, D. Darnaedi, *Tetrahedron.*, 59, 5347. **2003**.

59. J. R. Zgoda-Pols, A. J. Freyer, L. B. Killmer, J. R. Porter, *J. Nat. Prod*., 65, 1554, **2002**.

60. T. Ito, T. Tanaka, M. Iinuma, K. Nakaya, Y. Takahashi, R. Sawa, H. Naganawac, V. Chelladurai, *Tetrahedron*, 59, 1255, **2003**.

61. Sri Atun, Nurfina Aznam and Retno Arianingrum, Oligoresveratrol Isolated from Stem Bark of *Hopea Odorata* as Antioxidant and Cytotoxicity againt Human

Cancer Cell Line. 3rd International Conference on Chemistry and Chemical Engineering, **2012.**

62. Y.A. Geevananda, et al. "Distribution of some triterpenes and phenolic compounds in the extractives of endemic *Dipterocarpaceae* species of Sri Lanka." *Phytochemistry.*, 19, 1099, **1980**.
63. S. Atun et al. "Resveratrol derivatives from stem bark of *Hopea* and their biological activity test." *Journal of Physical Science.*, 19, 7, **2008**.
64. Phytochemical screening of two thai tropical rain forest Dipterocorps: *Hopea Odorata Roxb and Dipterocarpus costatus Gaertn.f. Tap Chi Duoc Hoc.*, 47, 4, 37.
65. K. Joshi, "Leaf flavonoid patterns in *Dipterocarpus* and *Hopea (Dipterocarpaceae)*." *Botanical J of the Linnean Society.*, 143, 43, **2003**.

CHAPTER-IV

Extraction, Phytochemistry and Soil analysis

4.1 Soxhlet extraction of *Myristica dactyloides Gaertn* and *Hopea ponga (Dennst.)* bark

To achieve preliminary separation depending upon polarity of biomolecules present in plants, successive hot extraction using Soxhlet extractor was carried out with solvents of increasing polarity such as petroleum ether (60-80°C), ethyl acetate and methanol.

The bark part of the plant ***Myristica dactyloides Gaertn*** and ***Hopea ponga (Dennst.)*** was collected in Agumbe region of Shivamogga district and N.R.Pura region of Chickmagalur and was authenticated by expert taxonomist Rtd. Prof. M.S. Pushpalatha,, Department of Botany, Sahyadri Science College, Shimoga. The freshly collected plant material was sprayed with ethanol. Later it was chopped into pieces, the chopped plant material was then shade dried and coarsely powdered in a mixer.

Weighed amount of coarsely powdered material was successively extracted with petroleum ether (60-80°C), ethyl acetate and methanol. Each extraction was carried out for nearly 18 h (approx. 45 cycles). After each extraction, the plant material was removed from extractor, dried and reloaded in the extractor for subsequent extraction. The extracts were concentrated by evaporating solvent using Buchi Type Rotary Evaporator under reduced pressure and at controlled temperature. The extracts obtained was dried under vaccum, packed and stored in a refrigerator. The details of the extraction are presented in the table **4.1** and **4.2.**

Table 4.1 Details of the extraction of *Myristica dactyloides Gaertn* bark

Weight of plant material used for extraction (3000g)	**Solvent used**	**Colour & Consistency**	**Yield (g)**
	Pet. ether	Yellowish semisolid	17.53
	Ethyl acetate	Dark brown paste	31.80
	Methanol	Reddish brown solid	72.00

Table 4.2 Details of the extraction of *Myristica dactyloides Gaertn* bark

Weight of plant material used for extraction (3000g)	Solvent used	Colour & Consistency	Yield (g)
	Pet. ether	Yellowish semisolid	13.24
	Ethyl acetate	Dark brown paste	25.60
	Methanol	Reddish brown solid	51.00

4.2 Phytochemistry

Most of the herbal medicines and their derivative products were often prepared from the plant extracts, which comprise a complex mixture of different phytochemical constituents (plant secondary metabolites). There may be hundreds of active components in these herbs. The chemical features of these constituents differ considerably among the different species. Even the same herbal extracts may vary depending upon the harvest season, plant origin, drying process and other factors. Therefore, the quality control of the herbal medicines and their derived products is difficult.

Freshly prepared extracts were subjected to standard phytochemical analysis to ensure the presence of phytoconstituents namely tannins, saponins, alkaloids, flavonoids, sesquiterpenes, etc. The phytochemical investigations were carried out using standard procedure[1-2] and the results are tabulated in the tables **4.3** and **4.4**.

Qualitative Tests

4.2.1 Test for Alkaloids

A small quantity of each extract was added to the ammonia solution in a test tube shaken well and then extracted with chloroform. The chloroform solution was then extracted with dilute hydrochloric acid. The acid layer was used for the following chemical test for alkaloids.

a) Mayer's test

The acid layer was mixed with few drops of Mayer's reagent and shaken well. A creamy precipitate indicates the presence of alkaloids in the extract.

b) Wagner's test

The acid layer when mixed with few drops of Wagner's reagent gives reddish brown precipitate for alkaloids in the extract.

c) Dragendroff's test

The acid layer when mixed with few drops of Dragendroff's reagent gives reddish brown precipitate for alkaloids.

4.2.2 Test for Steroids

a) Salkowski's test

A little of each extract was shaken with chloroform. The chloroform layer of the sample was treated with a few drops of concentrated sulphuric acid and shaken well. On standing it gives red colour for steroids in extracts.

b) Liebermann-Borchard's test

Chloroform solution of the sample was taken in a test tube. To this a few drops of acetic anhydride was added carefully and 1ml of concentrated sulphuric acid was added along the sides of the test tube. A brown ring formed at the junction of two liquid layers indicates the presence of steroids in the extract under investigation.

4.2.3 Tests for carbohydrates

a) Molisch test

Few drops of Molisch reagent was added to 2ml of test solution of the extract and mixed well. About 1ml of concentrated sulphuric acid was added along the sides of the test tube and the brown colour was observed at the junction of two liquid layers indicates the presence of carbohydrates in the extract taken for the test.

4.2.4 Test for Flavonoids

A small quantity of each extract was taken in a test tube, Molisch reagent was added to it and shaken well. Then 2 ml of concentrated sulphuric acid was added along the sides of the test tube and allowed it to stand for 2 min. Reddish-violet ring is formed at the junction of two liquid layers for flavonoids in the extract.

4.2.5 Tests for Phenolics/Tannins

a) Ferric Chloride test

A small amount of each extract was taken in a test tube, ferric chloride was added to it and shaken well. The precipitate or violet colour is observed in the extract.

b) Sodium Chloride test

A small amount of each extract was taken in chloride was added to it and shaken well. It gives phenolics/tannins. a test tube, 10% of sodium the precipitate confirms the presence of phenolics/tannins.

4.2.6 Test for Saponins

a) Ferric chloride test

Alcoholic solution of each extract was treated with few drops of neutral ferric chloride solution. Formation of green colour indicates for saponins in the extract.

b) Zinc hydrochloride acid reduction test

Few drops of concentrated hydrochloric acid when mixed with a mixture of alcoholic solution of the extract and a pinch of Zinc dust give Magenta colour for saponins.

A small amount of each extract is taken in a test tube. To this 5 ml of aqueous sodium hydroxide was added and shaken well. Yellow colour solution indicates the presence of saponins in the sample.

c) Lead acetate test

Alcoholic solution of each extract when treated with few drops of 10% lead acetate solution gives yellow precipitate for saponins.

4.2.7 Test for glycosides

a) Foam test

A small quantity of each extract was shaken with 5 ml of water in a test tube. Formation of foam suggests glycosides in the extract.

4.2.8 Test for aminoacids

a) Ninhydrin test

A small quantity of extract was taken in a test tube, to this few drops of ninhydrin reagent was added. If the blue colour appears then it confirms the aminoacids.

4.2.9 Millon's test

A small amount of each extract was taken in a test tube, 2 ml of the Millon's reagent added to it. If white precipitate formed, then it turns red on heating indicating the presence of aminoacids.

Table 4.3 Summary of Phytochemical investigation of *Myristica dactyloides Gaertn*

Sl. No.	Extract	Constituents
1	Pet. ether	Alkaloides, Steroides, Carbohydrates, FlavanoidesGlycosides and Amino acids
2	Ethyl acetate	Alkaloides, Steroids, Carbohydrates, Saponins, Glycosides and Amino acids
3	Methanol	Steroides, Carbohydrates, Glycosides

Table 4.4 Summary of Phytochemical investigation of *Hopea ponga*

Sl. No.	Extract	Constituents
1	Pet. ether	Alkaloides, Steroides, Carbohydrates, Glycosides and Amino acids
2	Ethyl acetate	Alkaloides, Steroids, Carbohydrates, Saponins, Glycosides and Phenolics
3	Methanol	Steroides, Carbohydrates, Glycosides, Flavanoides

The phytochemical investigation of ***Myristica dactyloides Gaertn*** and ***Hopea Ponga*** bark revealed that, petroleum ether extract contains alkaloids, steroids, carbohydrates, flavonoids, glycosides and aminoacids while alkaloids, steroids, carbohydrates, saponins, glycosides and aminoacids are present in chloroform extract and methanol extract contains steroids, carbohydrates and glycosides. Isolation and identification of bioactive molecules from the bark of ***Myristica dactyloides Gaertn*** and ***Hopea Ponga*** is the aim of research, hence the detailed study of this plant is carried out in the following chapters.

For convenient presentation, this chapter is divided into two sections,

4.3 Section A: Soil analysis

4.4 Section B: Pharmacological investigation of crude extracts

4.3 Section A: Soil analysis

It is well known fact that chemical constituents of the given plant, vary with climatic conditions and nature of the soil. The composition of the soil plays vital role in the biosynthesis of various chemical constituents of plants. Hence, number of components and their relative percentage present in a plant changes from place to place, season to season and age of the plant. Therefore, it was contemplated to carry out soil analysis of the area where the plant material i.e ***Myristica dactyloides Gaertn*** and ***Hopea Ponga*** bark was collected.

The soil analysis was carried out in the following steps:

4.3.1 Sample Collection

Soil samples were collected from different sites, from the place of collection of plant material i.e. at Agumbe region of Shimoga District and N.R.Pura region of Chickmagalur District respectively. A Total of three samples were collected as described below:

Sample -1: Collected near the plant of ***Myristica dactyloides Gaertn*** and ***Hopea Ponga*** at 1 foot depth surrounding the roots

Sample -2: Collected 3 meters away from the plant

Sample -3: Collected 15 meters away from the plant

All the samples were sun dried, powdered and mixed in equal proportions to prepare the sample for testing. The mixed sample was divided into four quarters. Opposite quarters were discarded and the remaining quarters were once again mixed and divided into four quarters. This process was repeated so as to get around 500 gm of the sample for soil analysis.

4.3.2 Soil Testing

The collected soil sample was investigated for the following parameters using standard procedures available in the literature[3-7].

- Determination of pH
- Determination of available carbon
- Determination of available phosphorous

- Determination of potassium content and
- Determination of micronutrients

4.3.3 Determination of pH of the Soil

The pH of the soil is a measure of chemical and biochemical properties of the soil under investigation. It includes ion exchange, retention of ions by colloids, biological activity and nutrient availability in soil. Thus the soil pH assists in understanding the fertility status of the soil and also it is a parameter used in the classification of the soil into acidic and alkaline.

The pH of the soil was measured by Potentiometric method.

4.3.4 Potentiometric method

Soil sample (10 g, 2 mm sieved) was mixed with distilled water (20 ml) and the mixture was stirred for half an hour. The glass electrode, serves as indicator electrode and calomel electrode, as reference electrode were immersed in the suspension of the soil and e.m.f of the cell, was recorded. The pH of the solution was calculated by using the following equation,

$$\text{pH} = \frac{\text{e.m.f of the cell (E)}}{2.303\frac{RT}{nF}} \rightarrow (1)$$

Where, R= Gas constant

T= Temperature (Absolute)

n= Number of electrons

F= Faraday constant

E= Electrical potential of the cell

At 25^0C, the equation (1) becomes

$$\text{pH} = \frac{\text{e. m. f of the cell}}{0.0591}$$

The e.m.f of the cell was found to be 0.36642, substituting this value in equation (2), we get,

$$pH = \frac{0.3246}{0.0591}$$

$$pH = 5.62$$

Therefore, the pH of the soil under investigation was found to be 5.62. This value indicates that the soil sample was slightly acidic in nature.

4.3.5 Determination of organic carbon in soil

Nitrogen is an essential constituent required for the plant, which they get from soil. Thus percentage of nitrogen in the soil, plays a significant role as for as fertility is concerned. Generally, carbon and nitrogen exist in a definite ratio of 10:1 in almost all soils. Hence, the determination of organic carbon in the soil, leads to the percentage of nitrogen in it. Generally, colorimetric method was adopted for the determination of organic carbon.

Principle

Organic matter of the soil was oxidized by potassium dichromate solution in presence of dilute sulphuric acid. Potassium dichromate, being an oxidizing agent, oxidizes the carbon of the soil into carbon dioxide. The corresponding amount of potassium dichromate was reduced to green coloured chromium sulphate. The intensity of this colour so produced was dependent on the amount of organic carbon present in the soil. Thus, by measuring the amount of chromium sulphate formed, by colorimetric method, the amount of carbon and hence nitrogen can be determined.

$K_2Cr_2O_7 + 4H_2SO_4 \longrightarrow K_2SO_4 + Cr_2(SO_4)_3 + 4H_2O + 3[O]$

$3C + 6[O] \longrightarrow 3CO_2$

4.3.6 Colorimetric method

The procedure for the determination of carbon content by colorimetric method involves the following steps:

Preparation of reagents

Potassium dichromate solution (1N) was prepared by dissolving potassium dichromate (29.04 g) in water (1000 ml) Standard sucrose solution was prepared by dissolving sucrose (0.5 g) in distilled water (1000 ml)

Standard curve

Standard curve was obtained by oxidizing the carbon content in sucrose. Accurate volumes of standard sucrose solution, i.e. 0, 1, 2, 3, 4, 5 and 6 ml were pipetted out into different conical flasks and the volume was made up to 10 ml by adding required volume of potassium dichromate solution. Then, to each conical flask, concentrated sulphuric acid (20 ml) was added slowly. The resulting solution was allowed to stand for half an hour and distilled water (10 ml) was added and left overnight. The intensities of the colours developed in different conical flasks were measured next day with the help of colorimeter by selecting filter at 660 nm. The graph of concentration against absorption was plotted to get the standard curve.

Soil sample (15 g) was ground and sieved through 100 mesh sieve. Then, 1g of this sample was transferred to a conical flask (100 ml). To this, standard potassium dichromate solution (10 ml) and concentrated sulphuric acid (20 ml) were added slowly. After half an hour, this solution was diluted by adding distilled water (10 ml). It was left overnight and the intensity of the colour was measured by using colorimeter.

With the help of this reading, using standard curve, the percentage of organic Carbon present in the soil was calculated using the equation,

$$Percentage\ of\ organic\ carbon = Graph\ reading \times 0.042$$

$$= 15.8 \times 0.042$$

$$= 0.66 \longrightarrow$$

The percentage of carbon in soil sample was found to be 0.66% and corresponding nitrogen content was 0.066%. This result indicates that the soil was less fertile with respect to organic carbon and nitrogen.

4.3.7 Determination of available phosphorus (Kg/acre)

Molybdenum blue method was used for accurate determination of available phosphorus.

Generally, available phosphorus in the soil sample is extracted in the solution form. For this purpose mainly two extractants are commonly used.

- ✓ Olsen extractant.
- ✓ Bray‘s extractant.

The proper extract was selected on the basis of the nature of the soil. Olsen extractant is used for neutral and alkaline soils, whereas Bray‘s extractant is used for acidic soils. pH

value of the soil suggests Bray‘s extractant to be used to determine the available phosphorus in the solution form.

It was estimated by converting the dissolved phosphorus into phosphomolybdate complex. This complex was reduced by using standard solution of ascorbic acid. The reduced compound has blue colour, the intensity of which depends upon the concentration of phosphorus present in the solution. The intensity of the colour was measured colorimetrically.

$$H_3PO_4 + 12\ H_3MoO_4 \longrightarrow H_3P\ [Mo_3O_{10}]_4 + 12\ H_2O$$

Colorimetric method

The determination of available phosphorus by colorimetric method, involved the following steps:

Preparation of Bray’s extractant

Reagent A

Ammonium molybdate (12 g) was dissolved in distilled water (200 ml). Similarly potassium antimony tartarate (0.2908 g) was dissolved in distilled water (200 ml). These two solutions were mixed and to the mixture, sulphuric acid (5 N, 1000 ml) was added and the solution was made upto 2 litres by using distilled water. The solution was stored in cool and dark place.

Reagent B

It was prepared by dissolving ascorbic acid (1.056 g) in reagent A (200 ml).

This solution was prepared just before the experiment.

Stock solution of phosphorus pentoxide

Ammonium dihydrogen orthophosphate (0.479 g) was dissolved in distilled water and the volume was made upto 1000 ml with distilled water. This solution had 250 ppm concentration of P_2O_5.

Working standard

The stock solution (20 ml) of P_2O_5 was diluted to 1000 ml by using distilled water, to reduce the concentration of P_2O_5 to 5.0 ppm.

Standard curve

The working standard solution was accurately pipetted out 0, 2, 4, 6 and 10 ml to beakers (50 ml). To this solution, Bray's extractant (5 ml) was added and the volume was made up to 20 ml by adding appropriate volume of distilled water. To the resulting solution, reagent B (4 ml) was added and kept aside for 15-20 minutes. The intensity of the colour was measured using colorimeter. The graph of concentration against absorption was plotted to obtain standard curve.

Soil sample (2.5 g) was taken and Bray's extractant (50 ml) was added, shaken thoroughly and filtered. The filtrate (5 ml) was taken in a separate conical flask and to this, sulphuric acid (5N, 0.5 ml) and distilled water (16 ml) were added. To this solution reagent B (4 ml) was added, stirred well and allowed to stand for 15-20 minutes. The intensity of the colour developed was measured colorimetrically. The concentration of follows

Average P_2O_5 Kg/acre = A x 3.6 ⟶ **(4)**

Where _A' is graph reading = 3.19

Therefore, Average P_2O_5 Kg/acre = 3.19 x 3.6

Average P_2O_5 Kg/acre = 11.48

Thus, the amount of phosphorus in soil sample under examination was found to be 11.48. This value indicates that the soil is less fertile with respect to available phosphorus.

4.3.8 Determination of potash content (Kg/acre) in the soil sample

Mineral soils generally contain potassium in larger quantities than nitrogen and phosphorus. The technique of Flame Emission Spectroscopy (FES), formerly called flame photometry, was employed for the determination of potassium present in the soil in terms of available potassium oxide (in kg/acre).

Principle of FES

In this technique, a solution containing a metallic salt is aspirated into the flame. The vapours now contain atoms of the metal. Some of these gaseous metal atoms absorb energy and raised to higher energy level to permit emission of radiation characteristic of the metal. The intensity of the radiation can be measured by using photoelectric detectors. The experiment is carried out in the following steps.

Preparation of standard solution

The standard solution of 1000 ppm potassium oxide was prepared by dissolving potassium chloride (1.583 g) in distilled water (1000 ml). From this stock solution, 10, 20, 30 and 40 ppm K_2O solutions were prepared by diluting 10, 20, 30 and 40 ml of stock solution with neutral ammonium acetate solution (1N, 1000 ml). The neutral ammonium acetate solution helps to extract potassium present in the soil sample in solution form.

Procedure

The standard solutions having different concentration (1.0 ml each) was aspirated into the flame and intensities of the radiation was measured by using flame emission spectrometer. The standard curve was obtained by drawing a graph of concentration (known solution) against intensity of radiation and the similar procedure was employed for soil sample.

Soil sample (10g) was ground and neutral ammonium acetate solution (1 N, 25 ml) was added and the mixture was shaken thoroughly to get phosphorus present in the soil sample to solution form. Then, the solution was filtered and 0.5 ml of this solution was aspirated into the flame in the instrument. The intensity of radiation was recorded and compared with standard curve. The amount of potassium present in the soil was determined by using the following Equation,

$$\text{Available } K_2O \text{ (Kg/acre)} = \frac{A}{10^6} \times \frac{\text{Volume of the extractant}}{\text{Weight of the sample}} \times \frac{2 \times 10^6}{2.2}$$

$$\text{Available } K_2O \text{ (Kg/acre)} = \frac{A}{10^6} \times \frac{25}{10} \times \frac{2 \times 10^6}{2.2}$$

$$= A \times 2.27$$

Where A is concentration of K_2O in ppm

Therefore available K_2O (Kg/acre) $= A \times 2.27$

$= 35.78 \times 2.27$

$= 81.22$

Therefore, available K_2O (Kg/acre) $= 81.22$

Therefore, from this value, it can be concluded that the soil under examination was found to be moderately fertile in nature with respect to potash.

4.4 Determination of Micronutrients

Sixteen elements are essential for plants for their normal growth and development. Out of these, seven are classified as micro nutrients since their requirement is in small amounts. They are Zn, Cu, Mo, Cl, Mn, B and Fe. These nutrients are present in the soil in two forms. i.e., in available and unavailable forms.

The concentration of micro nutrients like Fe, Cu, Zn and Mn was determined by using Atomic Absorption Spectrophotometer (AAS).

Procedure

Soil sample (10 gm) was mixed with DTPA (diethylene triamine penta acetic acid) solution (20 ml). The mixture was stirred mechanically for 2 hrs. The solution was filtered and the filtrate (0.5 ml) was aspirated into the flame of AAS and the intensity of radiation was recorded for Zn, Cu, Mn and Fe using appropriate filters. By using this intensity of radiation, the concentration of these metals was determined with the help of standard curves. The exact amount of these micro nutrients was calculated by using the following equation,

$$\text{Available micronutrients} = \frac{\text{X ppm}}{10^6} \times \frac{\text{Volume of the extractant}}{\text{Weight of the sample}} \times 10^6$$

$$\text{Available micronutrients} = \frac{\text{X ppm}}{10^6} \times \frac{20}{10} \times 10^6$$

$$= X \times 2$$

Thus,

(i) $\text{Available Zn in the soil} = 0.46 \times 2 = 0.92\text{ppm}$

(ii) $\text{Available Cu in the soil} = 1.24 \times 2 = 2.48\text{ppm}$

(iii) $\text{Available Mn in the soil} = 6.29 \times 2 = 12.58\text{ppm}$

(iv) $\text{Available Fe in the soil} = 17.43 \times 2 = 34.86\text{ppm}$

Therefore, the values obtained above indicate that the soil sample under examination was moderately fertile with respect to available Zn, Cu, Mn and Fe. The results are tabulated in table **4.5** and **4.6**.

Table 4.5 Summary of the Soil Analysis (Agumbe Region)

Sl. No	Examined Parameters	Unit	Report	Nature of the Soil
1	pH	-	5.62	Less acidic
2	Organic Carbon	%	0.66	Less fertile
3	Organic Nitrogen	%	0.066	Less fertile
4	Available Phosphorus	Kg/acre	11.48	Less fertile
5	Available Potash	Kg/acre	81.22	Moderately fertile
6	Available Zinc	Ppm	0.92	Moderately fertile
7	Available Cu	Ppm	2.48	Moderately fertile
8	Available Mn	Ppm	12.58	Moderately fertile
9	Available Fe	Ppm	34.86	Moderately fertile

Data of soil analysis suggests that the soil, where the plant was collected is slightly acidic, less fertile with respect to organic carbon and nitrogen, moderately fertile with respect to P, K, Zn, Cu, Mn and Fe.

Table 4.6 Summary of the Soil Analysis (N.R. Pura Region)

Sl. No	Examined Parameters	Unit	Report	Nature of the Soil
1	pH	-	5.92	Less acidic
2	Organic Carbon	%	0.66	Less fertile
3	Organic Nitrogen	%	0.066	Less fertile
4	Available Phosphorus	Kg/acre	11.48	Less fertile
5	Available Potash	Kg/acre	71.22	Moderately fertile
6	Available Zinc	Ppm	0.72	Moderately fertile
7	Available Cu	Ppm	3.48	Moderately fertile
8	Available Mn	Ppm	19.58	Moderately fertile
9	Available Fe	Ppm	43.86	Moderately fertile

Data of soil analysis suggests that the soil, where the plant was collected is slightly acidic, less fertile with respect to organic carbon and nitrogen, moderately fertile with respect to P, K, Zn, Cu, Mn and Fe.

References

1. P. Aida, V. Rosa, F. Blamea, A. Tomas and Salvador, *J. Ethnopharmacol*., 16, 93, **2001**.

2. I.I. Koleva, J.P.H. Linssen, A. De Groot and L.N. Evstatieva, *Phytochemical Analysis.*,13, 17, **2002**.

3. M.L. Jackson, Soil Chemical Analysis, *Prentice Hall Publication*., 128, **1973**.

4. M.D. Kerwin, K. Pooch, Exchangeable Soil Science, *Soc. Anor. Proc.*, 125, **1951**.

5. B.V. Subbaiah and G.L. Asija, *Curr. Sci.*, 259, **1956**.

6. A.I. Vogel, *A Text Book of Quantitative Chemical Analysis*., **1989**.

7. B.R. Puri, L.R. Sharma and S. Pathania, *Principles of Physical Chemistry*., **1994**.

CHAPTER-V

Biological Activities of bark extracts of *Myristica dactyloides Gaertn* and *Hopea ponga (Dennst.)* Plants

5.1 Screening for Biological Activities of Crude Extracts

The use of medicinal plants as a source for relief from illness can be traced back over five millennia to written documents of the early civilization in India, China and the Near east, but it is doubtless an art as old as mankind. Neanderthals living 60,000 years ago in present day Iraq used plants such as hollyhock, these plants are still widely used in ethnomedicine around the world[1-2].

The potential of higher plants as source for new drugs is still largely unexplored. Among the estimated 250,000-500,000 plant species, only a small percentage has been investigated phytochemically and the fraction submitted to biological or pharmacological screening is even smaller. Thus, any phytochemical investigation of a given plant will reveal only a very narrow spectrum of its constituents. Historically pharmacological screening of compounds of natural or synthetic origin has been the source of innumerable therapeutic agents. Random screening as tool in discovering new biologically active molecules has been most productive in the area of antibiotics[3-4]. Even now, contrary to common belief, drugs from higher plants continue to occupy an important niche in modern medicine. On a global basis, atleast 130 drugs, all single chemical entities extracted from higher plants, or modified further synthetically, are currently in use, though some of them are now being made synthetically for economic reasons[5].

In the recent times, the rapid development of multi-resistant bacterial and fungal strains of clinically important pathogens fetches the interest of scientist to develop newer broad spectrum antimicrobial agents[6]. A number of herbs with significant antimicrobial activity have been reported in literatures[7-9]. Traditionally, the plants ***Myristica dactyloides Gaertn*** and ***Hopea ponga (Dennst.)*** are used in the treatment of various diseases. The preliminary phytochemical examination revealed the presence of phytoconstituents like alkaloids, flavonoids, steroids, glycosides, carbohydrates, saponins and tannins in the crude extracts of ***Myristica dactyloides Gaertn*** and ***Hopea ponga (Dennst.)*** bark. These phytoconstituents exhibit a wide range of pharmacological activities. Hence, it was contemplated to screen the crude extracts for the following activities.

- Antimicrobial Activity
- Anthelmintic Activity
- Antioxidant Activity
- Anticancer activity

- Antitubercular Activity
- DNA cleavage studies

5.2 Antimicrobial Activity

Bacteria are microscopic, single-celled organisms that are the most numerous organisms on earth. Bacteria are the oldest, structurally simplest, and the most abundant forms of life on earth. They are also the only organisms with prokaryotic cellular organization. Represented in the oldest rocks from which fossils have been obtained, 3.5 to 3.8 billion years old, bacteria were abundant for over 2 billion years before eukaryotes appeared in the world Bacteria are mostly simple in form and exhibit one of three basic structures: bacillus (plural, bacilli) straight and rod shaped, coccus (plural, cocci) sphericalshaped, and spirillus (plural, spirilla) long and helical-shaped, also called spirochetes. Spirilla bacteria generally do not form associations with other cells and swim singly through their environments. They have a complex structure within their cell membranes that allow them to spin their corkscrew-shaped bodies which propel them along. Some rod-shaped and spherical bacteria form colonies, adhering end-to-end after they have divided, forming chains.

Human Bacterial Diseases Bacteria cause many diseases in humans, including cholera, leprosy, tetanus, bacterial pneumonia, whooping cough, diphtheria and lyme disease. Members of the genus *Streptococcus* are associated with scarlet fever, rheumatic fever, pneumonia, and other infections. Tuberculosis (TB), another bacterial disease, is still a leading cause of death in humans. Some of these 180 diseases like TB are mostly spread through the air in water vapour. Other bacterial diseases are dispersed in food or water, including typhoid fever, paratyphoid fever, and bacillary dysentery. Typhus is spread among rodents and humans by insect vectors. In light of the recent emergence of bacteria, which are resistant to multiple antimicrobial drugs posing a challenge for the treatment of infections, the need to discover new antimicrobial substances to combating such micro-organisms represents a challenge in the treatment of infections.

Medicinal plants represent a rich source of antimicrobial agents. Plants are used medicinally in different countries and are a source of many potent and powerful drugs[10]. A wide range of medicinal plant parts is used for extract as raw drugs and they possess varied medicinal properties. The different parts used include root, stem, flower, fruit, twigs exudates and modified plant organs. While some of these raw drugs are collected in

smaller quantities by the local communities and folk healers for local used, many other raw drugs are collected in larger quantities and traded in the market as the raw material for many herbal industries[11]. Although hundreds of plant species have been tested for antimicrobial properties, the vast majority of have not been adequately evaluated[12].

Clinical microbiologists have two reasons to be interested in the topic of antimicrobial plant extracts. First, it is very likely that these phytochemicals will find their way into the arsenal of antimicrobial drugs prescribed by physicians, several are already being tested in humans. It is reported that, on average, two or three antibiotics derived from microorganisms are launched each year[13]. After a downturn in that pace in recent decades, the pace is again quickening as scientists realize that the effective life span of any antibiotic is limited. Worldwide spending on finding new anti-infective agents (including vaccines) is expected to increase 60% from the spending levels in 1993. New sources, especially plant sources, are also being investigated. Second, the public is becoming increasingly aware of problems with the over prescription and misuse of traditional antibiotics. In addition, many people are interested in having more autonomy over their medical care. A multitude of plant compounds (often of unreliable purity) is readily available over-the- counter from herbal suppliers and natural-food stores, and self-medication with these substances is commonplace. The use of plant extracts, as well as other alternative forms of medical treatments, is enjoying great popularity in the late 1990s. Earlier in this decade, approximately one-third of people surveyed in the United States used at least one "unconventional" therapy during the previous year. It was reported that in 1996, sales of botanical medicines increased 37% over 1995. It is speculated that the American public may be reacting to over prescription of sometimes toxic drugs, just as their predecessors of the 19th century reacted to the overuse of bleeding, purging, and calomel[14].

Historically, plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and well- being. Their role is twofold in the development of new drugs: first: they may become the base for the development of a medicine, a natural blueprint for the development of new drugs, or second: a phytomedicine to be used for the treatment of disease. There are numerous illustrations of plant derived drugs. Some selected examples, including those classified as anti-infective, are presented below. The isoquinoline alkaloid emetine obtained from the underground part of *Cephaelis ipecacuanha*, and related species, has been used for many

years as and amoebicidal drug as well as for the treatment of abscesses due to the spread of *Escherichia histolytica* infections. Another important drug of plant origin with a long history of use, is quinine. This alkaloid occurs naturally in the bark of *Cinchona* tree. Apart from its continued usefulness in the treatment of malaria, it can be also used to relieve nocturnal leg cramps. Currently, the widely prescribed drugs are analogs of quinine such as chloroquine. Some strains of malarial parasites have become resistant to the quinines, therefore antimalarial drugs with novel mode of action are required. Similarly, higher plants have made important contributions in the areas beyond antiinfectives, such as cancer therapies. Early examples include the antileukaemic alkaloids, vinblatine and vincristine, which were both obtained from the Madagascan periwinkle (*Catharanthus roseus* syn. *Vinca roseus*)[14].

It is estimated that today, plant materials are present in, or have provided the models for 50% Western drugs[15]. Many commercially proven drugs used in modern medicine were initially used in crude form in traditional or folk healing practices, or for other purposes that suggested potentially useful biological activity. The primary benefits of using plant derived medicines are that they are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatment.

Much of the exploration and utilization of natural products as antimicrobials arise from microbial sources. It was the discovery of penicillin that led to later discoveries of antibiotics such as streptomycin, aureomycin and chloromycetin. Though most of the clinically used antibiotics are produced by soil microorganisms or fungi, higher plants have also been a source of antibiotics[16]. Examples of these are the bacteriostatic and antifugicidal properties of *Lichens,* the antibiotic action of allinine in *Allium sativum* (garlic), or the antimicrobial action berberines in goldenseal (*Hydrastis canadensis*)[16].

5.2.1 Major groups of antimicrobial compounds from plants

Over the centuries man made use of medicinal plants, even though he was unable to find a rational explanation for their effects. It was not until the 19th century and the rapid development of organic chemistry and pharmacology, that man determined which active principles of group of principles are responsible for a given therapeutic effect[17].

All plants containing active compounds are important. The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant. In plants, these compounds are mostly secondary metabolites such as alkaloids,

steroids, tannins, and phenol compounds, which are synthesized and deposited in specific parts or in all parts of the plant. These compounds are more complex and specific and are found in certain taxa such as family, genus and species, but heterogeneity of secondary compounds is found in wild species. The medicinal actions of plants are unique to a particular plant species or group, consistent with the concept that the combination of secondary products in a particular plant is taxonomically distinct. The plants secondary products may exert their action by resembling endogenous metabolites, ligands, hormones, signal transduction molecules or neurotransmitters and thus have beneficial medicinal effects on humans due to similarities in their potential target sites. Therefore, random screening of plants for active chemicals is as important as the screening of ethnobotanically targeted species.

5.2.1.1 Phenolics, polyphenols, Simple phenols and phenolic acids

Some of the simplest bioactive phytochemicals consist of a single substituted phenolic ring. Cinnamic and caffeic acids are common representatives of a wide group of phenyl propane-derived compounds which are in the highest oxidation state.

The common herbs tarragon and thyme both contain caffeic acid, which is effective against viruses, bacteria, and fungi. Catechol and pyrogallol both are hydroxylated phenols, shown to be toxic to microorganisms. Catechol has two 2OH groups, and pyrogallol has three. The site(s) and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity. In addition, some authors have found that more highly oxidized phenols are inhibitorier. The mechanisms thought to be responsible for phenolic toxicity to microorganism.

5.2.1.2 Quinones

Quinones are aromatic rings with two ketone substitutions. They are ubiquitous in nature and are characteristically highly reactive. These compounds, being colored, are responsible for the browning reaction in cut or injured fruits and vegetables and are an intermediate in the melanin synthesis pathway in human skin.

Vitamin K is a complex naphthoquinone. Its antihemorrhagic activity may be related to its ease of oxidation in body tissues. In addition to providing a source of stable free radicals, quinones are known to complex irreversibly with nucleophilic amino acids in proteins , often leading to inactivation of the protein and loss of function. For that reason,

the potential range of quinone antimicrobial effects is great. Probable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides and membrane-bound enzymes. Quinones may also render substrates unavailable to the microorganism. As with all plant-derived antimicrobials, the possible toxic effects of quinones must be thoroughly examined. Kazmi et al[18] described an anthraquinone from *Cassia italica*, a Pakistani tree, which was bacteriostatic for *Bacillus anthracis*, *Corynebacterium pseudodiphthericum,* and *Pseudomonas aeruginosa* and bactericidal for *Pseudomonas pseudomalliae.* Hypericin, an anthraquinone from St. John's wort (*Hypericum perforatum*), has received much attention in the popular press lately as an antidepressant, and Duke reported in 1985 that it had general antimicrobial properties.

5.2.1.3 Flavones, flavonoids and flavonols

Flavones are phenolic structures containing one carbonyl group (as opposed to the two carbonyls in quinones). Flavonoids are also hydroxylated phenolic substances but occur as a C6-C3 unit linked to an aromatic ring. Since they are known to be synthesized by plants in response to microbial infection, it should not be surprising that they have been found in vitro to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls, as described above for quinones. More lipophilic flavonoids may also disrupt microbial membranes.

Catechins, the most reduced form of the C3 unit in flavonoid compounds, deserve special mention. These flavonoids have been extensively researched due to their occurrence in oolong green teas. It was noticed some time ago that teas exerted antimicrobial activity and that they contain a mixture of catechin compounds. These compounds inhibited in vitro *Vibrio cholerae*, *Streptococcus mutans,*, *Shigella*, and other bacteria and microorganisms.

Flavonoid compounds exhibit inhibitory effects against multiple viruses. Numerous studies have documented the effectiveness of flavonoids such as swertifrancheside, glycyrrhizin (from licorice), and chrysin[19] against HIV.

5.2.1.4 Tannins

"Tannin" is a general descriptive name for a group of polymeric phenolic substances capable of tanning leather or precipitating gelatin from solution, a property known as astringency. Their molecular weights range from 500 to 3,000, and they are found in

almost every plant part: bark, wood, leaves, fruits, and roots. They are divided into two groups, hydrolyzable and condensed tannins. Hydrolyzable tannins are based on gallic acid, usually as multiple esters with D-glucose, while the more numerous condensed tannins (often called proanthocyanidins) are derived from flavonoid monomers. Tannins may be formed by condensations of flavan derivatives which have been transported to woody tissues of plants. Alternatively, tannins may be formed by polymerization of quinone units[20]. This group of compounds has received a great deal of attention in recent years, since it was suggested that the many human physiological activities, such as stimulation of phagocytic cells, host-mediated tumor activity, and a wide range of anti - infective actions, have been assigned to tannins . Thus, their mode of antimicrobial action, as described in the section on quinones may be related to their ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins, etc.

The antimicrobial significance of this particular activity has not been explored. Scalbert reviewed the antimicrobial properties of tannins in 1991. He listed 33 studies which had documented the inhibitory activities of tannins up to that point. According to these studies, tannins can be toxic to filamentous fungi, yeasts, and bacteria. Condensed tannins have been determined to bind cell walls of ruminal bacteria, preventing growth and protease activity[21].

5.2.1.5 Coumarins

Coumarins are phenolic substances made of fused benzene and a-pyrone rings. Coumarins are found to posses antithrombotic, anti-inflammatory, and vasodilatory[22] activities. Several other coumarins have antimicrobial properties. R. D. Thornes, working at the Boston Lying-In Hospital in 1954, sought an agent to treat vaginal candidiasis in his pregnant patients. Coumarin was found in vitro to inhibit *Candida albicans*. Its estrogenic effects were later described. Also, phytoalexins, which are hydroxylated derivatives of coumarins, are produced in carrots in response to fungal infection and can be presumed to have antifungal activity. General antimicrobial activity was documented in woodruff (*Galium odoratum*) extracts[23]. All in all, data about specific antibiotic properties of coumarins are scarce, although many reports give reason to believe that some utility may reside in these phytochemicals.

5.2.1.6 Terpenoids and essential oils

The fragrance of plants is carried in the so called quinta essentia, or essential oil fraction. These oils are secondary metabolites that are highly enriched in compounds based on an isoprene structure. They are called terpenes, their general chemical structure is $C_{10}H_{16}$, and they occur as diterpenes, triterpenes, and tetraterpenes (C20, C30, and C40), as well as hemiterpenes (C5) and sesquiterpenes (C15). When the compounds contain additional elements, usually oxygen, they are termed terpenoids.

Terpenoids are synthesized from acetate units, and as such they share their origins with fatty acids. They differ from fatty acids in that they contain extensive branching and are cyclized. Examples of common terpenoids are methanol and camphor (monoterpenes) and farnesol and artemisin (sesquiterpenoids). Terpenenes or terpenoids are active against bacteria, fungi, viruses, and protozoa. In 1977, it was reported that 60% of essential oil derivatives examined to date were inhibitory to fungi while 30% inhibited bacteria . The ethanol-soluble fraction of purple prairie clover yields a terpenoid called petalostemumol, which showed excellent activity against *Bacillus subtilis* and *Staphylococcus aureus* and lesser activity against gram-negative bacteria as well as *Candida albicans*. The diterpenes isolated by Batista et al. were found to be more democratic; they worked well against *Staphylococcus aureus*, *V. cholerae*, *P. aeruginosa*, and *Candida* spp.

5.2.1.7 Lectins and polypeptides

Peptides which are inhibitory to microorganisms were first reported in 1942. Recent interest has been focused mostly on studying anti-HIV peptides and lectins, but the inhibition of bacteria and fungi by these macromolecules, such as that from the herbaceous *Amaranthus*, has long been known. Thionins are peptides commonly found in barley and wheat and consist of 47 amino acid residues. They are toxic to yeasts and gram-negative and gram-positive bacteria[24]. Fabatin, a newly identified 47-residue peptide from fava beans, appears to be structurally related to g-thionins from grains and inhibits *E.coli*, *P.aeruginosa*, and *Enterococcus hirae* but not *Candida* or *Saccharomyces.*

5.2.1.8 Alkaloids

Alkaloids rank among the most efficient and therapeutically significant plant substances. They are chemically very diverse group of organic nitrogen compounds. Generally they are extremely toxic though they do have a marked therapeutic effect in minute quantities.

For this reason plants containing alkaloids were not often used in folk medicine and then for external application only. Pure, isolated plant alkaloids and their synthetic derivates are used as basic medicinal agents all over the world for their analgesic, antispasmodic, and bactericidal effects[25].

It is a well known fact that the interactions between microorganisms, plants and animals are natural and constant. The ecological role of microorganisms and their importance in the bio-geochemical cycles in nature is well documented. The human food supply consists basically of plants and animals or products derived from them. Microorganisms are ubiquitous and are so small that they can only be visualized with the aid of high-resolution microscopes.

Microbes are heterogeneous group of several classes of living things. These were originally classified under the plant and animal kingdom. As this proved unsatisfactory, a five-kingdom system was introduced namely,

a. Monera (Unicellular prokaryotes)
b. Protista (Unicellular Eukaryotes)
c. Fungi
d. Plantae
e. Animalia

Protozoa are unicellular, non-photosynthetic eukaryotic microorganisms. Various groups of protozoa exhibit different strategies of locomotion, for example *Ameoba* can change its cell shape by extending the cell wall and thus migrates along, whereas *Paramaecium* is propelled by numerous beating structures called cilia.

As a group, fungi are microorganisms that obtain their nutrition from organic compounds. Their cells are usually surrounded by protective cell wall composed of chitin or other polysaccharides. Fungi produce spores, which are specialized cells, involved in reproduction, dissemination and survival. Depending on the cell morphology, fungi can be divided into four classes such as, yeast, yeast like fungi, moulds and dimorphic fungi.

Yeasts are unicellular and moulds are filamentous. Dimorphic fungi may grow as mycelial or yeast-form depending upon prevalent growth conditions. Fungi form multicellular filaments called hyphae. The only pathogenic yeast is *Cryptococcus neoformans*. Fungi have been recognized as causative agents of human disease earlier

than bacteria. Fungal infections are extremely common and some of them are serious and even fatal.

Antimicrobial activity refers to the activity of a compound to kill or inhibit the growth of micro-organisms. Micro-organism is any living thing of microscopic size, including bacteria, viruses, yeast, fungi and protozoa. Antimicrobial drugs are the greatest contribution of the 20^{th} century to therapeutics. Drugs of this class differ from all others in that they are designed to inhibit or kill the infecting organism and to have no or minimal effects on the recipient. Thus, they play an important role in chemotherapy. Therefore, it was aimed to explore antimicrobial activity of crude extracts of ***Myristica dactyloides Gaertn*** and ***Hopea ponga (Dennst.)***

5.2.2 Antibacterial activity

The antibacterial activity of different extracts of ***Myristica dactyloides Gaertn*** and ***Hopea ponga (Dennst.)*** was studied comparatively with that of standard Tetracycline by Agar well diffusion method[26].

Materials and Method

i. Nutrient agar
ii. Sterilized Petri dishes, pipettes, boiling tubes and beakers
iii. Sterile test-tubes
iv. Sterile 6 mm borer
v. Sterile inoculation loops
vi. Sterile fine pointed forceps
vii. Standard drug : Tetracycline
viii. DMSO : Dimethyl sulphoxide as a negative control

The different organisms used in the studies were *Staphylococcus aureus , Staphylococcus faecalis, Streptococcus pyrogenus, Shigella flexneri, Enterobacter aerogenes, Pseudomonas fluorescens. Basilis subtilis, Bacillus cereus, Enterobactere aerogenes, Proteus vulgaris* and *Klebsiella pneumonia*. The bacterial cultures were maintained on nutrient agar (NA), while fungal cultures on Sabouraud dextrose agar (SDA).

Preparation of Media

Nutrient agar was prepared by dissolving the nutrient agar (14 g) in distilled water (500 ml). The pH of the solution was adjusted to 7.4 and then sterilized for 20 min at 15 lb pressure at 121ºC in an autoclave.

Sterilization of media and glassware

The media used in the present study, nutrient agar and nutrient broth, were sterilized in conical flasks of suitable capacity by autoclaving at 15 lb pressure for 20 min. The cork borer, Petri dishes, test tubes and pipettes were sterilized in hot air oven at 160° C for an hour.

Preparation of sub-cultures

One day prior to the experiment, the micro organisms were inoculated into sterilized nutrient broth tubes and incubated at 37°C for 24 h. These organisms were procured from the National Collection of Industrial Microorganism (NCIM), Pune, India. The organisms were sub-cultured into sterile nutrient broth. After incubating the same for 3 h, the growth thus obtained was used as inoculums for the test.

Preparation of test solutions of the extracts

Accurately weighed 1000 mg of each extract was dissolved in dimethyl sulphoxide (DMSO) (10 ml). From this stock solution, different concentrations (25, 50, 100 mg/ml) were prepared by diluting suitably with DMSO and preserved in sterile test tubes separately.

Agar well diffusion method

Agar well radial diffusion technique was used for the assessment of antibacterial activity of the test samples. Sterilized nutrient agar medium was poured into sterilized petridishes. Nutrient broth containing 100 µl of 24 h old cultures of respective bacterial strains was spread separately on the agar medium. Wells were made using a stainless steel sterilized cork borer under aseptic conditions. 25, 50, 100 µg/ml of pet ether, ethyl acetate and methanol crude extracts were loaded into corresponding wells. The antibiotic Tetracycline was used as standard (1µg/ml of sterile water). The plates were incubated for 24 h at 37°C and the diameter of the zone of bacterial growth inhibition was measured and readings were recorded in millimeter. The tests were carried out in triplicates and

results were recorded as mean ± SEM (Standard Error Mean). The results are tabulated in table **5.1**and **5.2.**

Table 5.1 Antibacterial activity of bark extracts of *Myristica dactyloides Gaertn*

Results are expressed as Mean±SEM of triplicates

Organisms	Control	Std	Pet ether			Ethyl acetate			Methanol		
			25	50	100	25	50	100	25	50	100
S. aureus	3.00 ±0.23	22.33 ±0.46	19.00 ±0.06	21.03 ±0.09	21.93 ±0.12	11.87 ±0.09	13.03 ±0.03	14.10 ±0.15	20.23 ±0.12	21.03 ±0.03	23.10 ±0.15
S. faecalis	2.30 ±0.43	21.93 ±0.76	16.10 ±0.06	18.00 ±0.06	20.07 ±0.12	10.07 ±0.12	10.83 ±0.09	11.00 ±0.00	17.97 ±0.09	20.00 ±0.17	21.00 ±0.06
S. pyrogenus	2.67 ±0.33	18.95 ±0.51	19.10 ±0.06	20.07 ±0.12	20.10 ±0.06	10.23 ±0.12	10.03 ±0.15	11.97 ±0.09	18.10 ±0.06	16.97 ±0.09	19.00 ±0.00
S. flexneri	3.46 ±0.27	22.79 ±0.51	12.97 ±0.09	14.87 ±0.09	16.97 ±0.15	07.10 ±0.15	08.97 ±0.03	09.10 ±0.06	15.03 ±0.15	17.07 ±0.12	17.97 ±0.09
E. aerogenes	3.08 ±0.42	20.73 ±0.41	15.93 ±0.12	16.97 ±0.03	18.00 ±0.12	07.97 ±0.09	08.13 ±0.13	09.07 ±0.12	16.97 ±0.09	19.03 ±0.03	19.07 ±0.12
P. fluorescens	2.86 ±0.37	19.67 ±0.66	16.97 ±0.15	17.93 ±0.12	19.17 ±0.12	10.00 ±0.17	11.13 ±0.09	13.07 ±0.09	17.97 ±0.09	19.83 ±0.12	21.03 ±0.03

Results and Discussion

The results revealed that, all the extracts of ***Myristica dactyloides Gaertn*** bark exhibited potent antibacterial activity against all the tested organisms when compared with the standard drug. Among the tested extracts, methanol showed a potent activity against all the organisms, it exhibited good zone of inhibition rather than pet ether and ethyl acetate extracts when compared to standard. Ethyl acetate and pet ether extract exhibited considerable zone of inhibition against the tested organisms.

Table 5.2 Antibacterial activity of bark extracts of *Hopea ponga (Dennst.)*

Organisms	Control	Std	Pet ether			Ethyl acetate			Methanol		
			25	50	100	25	50	100	25	50	100
S. aureus	3.00 ±0.23	22.33 ±0.46	-	10.93 ±0.11	11.23 ±0.01	28.00 ±0.15	31.23 ±0.19	32.93 ±0.13	21.00 ±0.15	22.93 ±0.12	24.93 ±0.01
B. subtilis	2.30 ±0.43	21.93 ±0.76	-	11.07 ±0.12	13.07 ±0.13	21.10 ±0.14	27.07 ±0.12	31.07 ±0.12	27.10 ±0.18	26.97 ±0.03	26.07 ±0.12
B. cereus	2.67 ±0.33	18.95 ±0.51	10.07 ±0.12	16.97 ±0.03	18.00 ±0.10	20.07 ±0.12	26.97 ±0.03	28.00 ±0.02	20.07 ±0.12	27.07 ±0.12	28.00 ±0.09
E. aerogenes	3.46 ±0.27	22.79 ±0.51	-	10.10 ±0.06	11.07 ±0.12	23.10 ±0.15	29.07 ±0.12	30.07 ±0.12	20.10 ±0.17	21.07 ±0.12	22.07 ±0.13
P. vulgaris	3.08 ±0.42	20.73 ±0.41	09.00 ±0.12	14.87 ±0.09	15.97 ±0.15	11.07 ±0.07	14.87 ±0.09	15.97 ±0.05	16.07 ±0.07	19.87 ±0.09	25.97 ±0.15
K. pneumonia	2.86 ±0.37	19.67 ±0.66	-	17.93 ±0.12	19.17 ±0.07	28.07 ±0.18	33.93 ±0.11	35.17 ±0.13	20.07 ±0.18	20.83 ±0.12	21.17 ±0.12

Results and Discussion

The results revealed that, all the extracts of bark of ***Hopea ponga (Dennst.),*** exhibited potent antibacterial activity against all the tested organisms when compared with standard drug. Among the tested extracts, ethyl acetate extract showed a potent activity against all the tested organisms, i.e., it exhibited good zone of inhibition rather than pet

ether and methanol extracts when compared to standard. Pet ether and methanol extract exhibited considerable zone of inhibition against the tested organisms.

5.2.2.1 Minimum Inhibitory Concentration

Minimum Inhibitory Concentration is defined as the lowest concentration of test samples that result in a complete inhibition of visible growth. MIC of all the extracts was determined by micro dilution method[27-29]. The respective clinical strain was spread separately on the medium. The wells were created using a stainless steel sterilized cork borer under aseptic conditions. The extracts at different concentrations was made using DMSO and later loaded into corresponding wells. The standard drug Ciprofloxacin and Fluconazole were used for the comparison of antibacterial and antifungal activities respectively. The zone of inhibition was compared with the standard drug after 24 h of incubation at 37°C for antibacterial activity and 72 h at 25°C for antifungal activity. The results are recorded in table **5.3** and **5.4.**

Table 5.3 Minimum Inhibitory Concentration of bark extracts of *Myristica dactyloides Gaertn*

Sl. No	Bacterial Strains	Minimum Inhibitory Concentration (µg/ml)		
		Petroleum ether	Ethyl acetate	Methanol
1	*S. aureus*	25	100	50
2	*S. faecalis*	125	250	250
3	*S. pyrogenus*	100	250	125
4	*S. flexneri*	250	500	500
5	*E. aerogenes*	100	125	250
6	*P. fluorescens*	50	100	250

Results and Discussion

The MIC values of the plant extract against the tested bacterial isolates ranged from 25 to 500 µg/ml. The pet ether extract at the least concentration of 25 µg/ml and 50 µg/ml inhibits the growth of *Streptococcus aureus* and *P.fluorescens* respectively.

Table 5.4 Minimum Inhibitory Concentration of bark extracts of *Hopea ponga (Dennst.)*

Sl. No	Bacterial Strains	Minimum Inhibitory Concentration (µg/ml)		
		Petroleum ether	Ethyl acetate	Methanol
1	*S. aureus*	250	100	250
2	*B. subtilis*	500	125	250
3	*B. cereus*	250	100	125
4	*E. aerogenes*	500	250	500
5	*P. vulgaris*	250	125	250
6	*K. pneumonia*	100	500	250

Results and Discussion

The MIC values of the plant extract against the tested bacterial isolates ranged from 100 to 500 μg/ml. The ethyl acetate extract at the least concentration of 100 μg/ml inhibits the growth of *Streptococcus aureus and Bacillus cereus*.

5.2.3 Antifungal activity

The prevalence of resistance to antifungal agents significantly increased in the past decade. Resistance to antifungal agents has important implications for morbidity, mortality, and healthcare in the community. Humans and fungi share some of the same molecular processes; therefore, there is always the risk that what is toxic to the fungal cells will be toxic to the host cells. Patients with AIDS, organ transplant patients, patients receiving chemotherapy, and diabetes patients represent current medical challenges[30]. The drugs currently available to treat fungal infections have serious drawbacks such as the development of fungal resistance and toxic side effects. The broad-spectrum drug amphotericin B was the sole drug for nearly 30 years, and it is one of the few drugs that actually kill fungal cells, but can cause significant nephrotoxicity in the patients. The imidazoles and the triazoles in late 1980s and early 1990s, respectively, were major advances which act by inhibiting processes of the fungal cell, but they have been found to result in recurrence of the infection and the development of resistance to the drug[31]. Therefore, there is need to search new, safer, and more potent agents to combat serious fungal infections. There is already a rich history of research that has been carried out to verify folk medicine practices.

Medicinal plants have been a source of wide variety of biologically active compounds for many centuries and used extensively as crude material or as pure compounds for treating various disease conditions. Relatively 1-10% of plants are used by humans out of the estimated 250,000–500,000 species of plants on earth[32]. The chemical diversity of natural products is complementary to the diversity found in synthetic libraries. However, natural products are stearically more complex and have greater ring system diversity because of the long evolutionary selection process. Therefore, strategies to exploit the natural sources and to develop methodologies for the preparation of natural product like libraries through the diversification of natural product mixtures by combinatorial biosynthesis and related techniques are possible. Mainstream medicine is increasingly receptive to the use of antimicrobial and other drugs derived from plants, as traditional antibiotics become

ineffective[34]. A number of compounds isolated from plants such as phenolic compounds, tannins, flavonoids, azulenes, sesquiterpenes, essential oils, coumarins, quinones from natural sources showed antifungal activity.

The antifungal activity of bark extracts of ***Myristica dactyloides Gaertn*** **and** ***Hopea ponga (Dennst.)*** was studied in comparison with that of standard Fluconazole, by cup plate method[33-34] against *Candida tropicalis, Trichophyton mentagrophytes* and *Trichoderma viride.*

Materials and Method

i. Potato dextrose agar
ii. Micropipette
iii. Sterile petridishes
iv. Potato dextrose broth (48 h old)
v. Sterile test tubes for preparation of solutions of the test compounds

Sterilization of media and glassware

The media used in the present study were nutrient agar and nutrient broth, were sterilized in conical flasks of suitable capacity by autoclave at 15 lbs pressure for about 20 min. The cork borer, petridishes, test tubes and pipettes were sterilized in hot air oven at 160ºC for an hour.

Preparation of test solutions of the extracts

The suspension of each extract (1000 mg in 10 ml DMF) was prepared and kept in a suitably labeled sterile test tube separately, to get the solution of the extract of 100 mg/ml concentration. It was diluted by DMF.

Preparation of media (PDA)

Potato dextrose agar was prepared by dissolving potato dextrose (20 g) agar in distilled water (500 ml) and then the pH of the solution was adjusted to 5.6 and sterilized for 20 min at 120ºC at 15 lb pressure in an autoclave. (Bacteriological Peptone (1 g) and glucose (4 g) were dissolved in distilled water (100 ml) and filtered; agar powder (2 g) was added and sterilized for 30 min at 15 lb pressure.

Preparation of sub-culture

Two days prior to the experiment, the microorganisms were made in sabourauds broth and incubated at 37ºC for 48 h.

Method of testing-Cup-plate method

This method depends on the diffusion of an antifungal agent from a cavity through the solidified agar layer in a petridish to an extent such that the growth of the added micro organisms is prevented entirely in a circular area or zone around the cavity containing a solution of antifungal agent.

Previously liquified medium was inoculated appropriate to the assay with the requisite quantity of suspension of the micro organisms between 40-50°C and the inoculated medium was poured into petridishes to give a depth of 3 to 4mm, ensuring that the layers of medium were uniform in thickness by placing the dishes on a leveled surface.

The dishes thus prepared were stored in a manner so as to ensure that no significant growth of the test organism occurs before the dishes were used and the surface of the agar layer was dry at the time of use, with the help of a sterile cork borer, three cups of each 6 mm diameter were punched and scooped out. To the set agar in each petridish standard sample of extract and control of known concentration were fed into the bored cups.

The dishes were left standing for 2 h at room temperature as a period of pre-incubation diffusion to minimize the effects of variation in time among the applications of different solutions. These were then incubated for 48 h at 25ºC. The zone of inhibition developed, if any, was then accurately measured. The results of antifungal activity of ***Myristica dactyloides Gaertn*** and ***Hopea Ponga (Dennst.)*** bark extracts are tabulated in table **5.5** and **5.6**

Table 5.5 Antifungal activity of bark extracts of *Myristica dactyloides Gaertn*

Organisms	Control	Std	Pet ether			Ethyl acetate			Methanol		
			25	50	100	25	50	100	25	50	100
C. tropicalis	1.48 ±0.34	15.67 ±0.69	17.17 ±0.09	18.93 ±0.12	22.97 ±0.15	11.93 ±0.12	13.83 ±0.09	13.87 ±0.09	07.01 ±0.03	09.37 ±0.03	10.93 ±0.12
T. mentagrophytes	1.75 ±0.44	17.54 ±0.56	17.90 ±0.06	19.00 ±0.06	22.03 ±0.03	06.97 ±0.15	07.97 ±0.15	10.93 ±0.12	08.97 ±0.15	08.97 ±0.15	09.90 ±0.06
T. viride	1.65 ±0.38	16.39 ±0.50	22.87 ±0.09	23.97 ±0.03	25.90 ±0.06	13.17 ±0.09	14.00 ±0.06	14.13 ±0.03	10.93 ±0.12	13.17 ±0.09	12.97 ±0.15

Results and Discussion

From the results, it revealed that pet ether extract showed a promising activity against all the organisms when compared to standard drug Fluconazole, while ethyl acetate and methanol extracts showed a considerable antifungal activity.

Table 5.6 Antifungal activity of bark extracts of *Hopea ponga (Dennst.)*

Organisms	Control	Std	Pet ether			Ethyl acetate			Methanol		
			25	50	100	25	50	100	25	50	100
C. tropicalis	1.48 ±0.34	15.67 ±0.69	07.35 ±0.18	11.83 ±0.12	12.57 ±0.25	11.93 ±0.22	14.71 ±0.19	15.88 ±0.24	08.01 ±0.13	09.37 ±0.33	10.93 ±0.12
T. mentagrophytes	1.75 ±0.44	17.54 ±0.56	09.63 ±0.27	12.72 ±0.36	15.62 ±0.33	06.97 ±0.15	07.97 ±0.25	10.93 ±0.17	08.07 ±0.25	08.97 ±0.15	09.37 ±0.06
T. viride	1.65 ±0.38	16.39 ±0.50	08.21 ±0.19	13.10 ±0.73	11.49 ±0.40	13.17 ±0.19	14.00 ±0.26	14.13 ±0.33	10.93 ±0.12	13.17 ±0.09	12.97 ±0.15

Results and Discussion

From the results, it revealed that ethyl acetate extract showed a promising activity against all the organisms when compared to standard drug Fluconazole, while pet ether and methanol extracts showed a considerable antifungal activity.

5.3 Anthelmintic activity

Helminthes infections are the most common infections in man which affects the large proportions of the world's population. In the treatment of parasitic diseases, the anthelmintics drugs are used indiscriminately. Recently the use of anthelmintics produces toxicity in human beings. Hence the development and discovery of new substances acting as anthelmintics are being derived through plants which are considered to be the best source of bioactive substances. Various plants were used in veneral diseases, to promote healing of wounds, swellings, abscesses, rheumatism and treating pain in lower extremities, skin diseases, leucorrhoea, dysentery, dysuria and fever[35-36].

Anthelmintics are the drugs that either kill (vermicide) or expel (vermifuge) infesting helminths. Thus they are used in the treatment of helminthiasis, which is a disease caused by infestation of parasitic worms living in the alimentary canal or in the other tissues of the host. Helminthiasis is prevalent globally, in fact one –third of the world population

harbours them but it is more common in the developing countries with poorer personal and environmental hygiene[37].

The drugs possessing anthelmintic property will paralyze the worms by causing damage to the mucopolysacharides layer. An anthelmintic drug may act by causing narcosis or paralysis of worms by either penetrating the cuticle or by the worm ingesting. It may damage the cuticle of the worm or partially paralyze it[38].

Natural anthelmintic includes the following list of components: Tobbacco, Walnut , Wormwood, Clove, Kalonji seeds, Garlic, Malefern, Pineapple, Diatomaceous earth, Soya, legumes, Honey, water and vinegar are mixed with warm water act as vermifuges. In other words, anthelmintics are drugs that are used for the treatment of infections caused by the worms, flukes, nematodes, round worms, tapeworms etc.

Anthelmintics are the tropical and veterinary types of medicines which are of huge importance. Parasitic worms also infect the livestock and crops thus affecting the food production with a resultant economic impact. It comes as no surprise, that the drugs available for human treatment were first developed as veterinary medicines.

In some cases, this situation has been exacerbated by the remarkable success of ivermectin over the last twenty years[39], which has decreased motivation for anthelmintic drug discovery programmes[40]. Broad spectrum anthelmintics are effective against parasitic flat worms and nematodes. However, the majority of drugs are more limited in their action, e.g., praziquantel a drug used in the treatment of schistosomiasis and act by disrupting the calcium homeostasis[41] and has no activity against nematodes. Pharmacology of anthelmintics.

Throughout the world, the parasitic helmintic infection increases the mortality and morbidity day by day. This includes the intestinal nematodes (roundworms), trematodes (flukes) and cestodes (tapeworms). It is unevenly distributed disease in low income countries which affected worstly and highest risk of morbidity because it is the major source of environmental contamination and transmission. Albendazole, mebendazole and praziquantel are the commonly used drugs acting as anthelmintics having broad spectrum activity and high cure rates due to the sustainability of the periodic emergence of resistance.

Roundworms

The migration of the larval forms and eggs transmission through skin contact in moist soil and in tropical areas causes migraine, eosinophilic and pulmonary related problems. The common infections occurring with intestinal worms include Ascaris lumbricoides, Trichuris trichiura, Necator americanus and Ancylostoma duodenal with the household aggregation of infection. The eggs are deposited on perianal area that is due to self infection. These infections also occur due to the contaminated surfaces like carpets, curtains etc. The airborne and inhalation of the small number of eggs are transmitted through ingestion of the infected food because the humans are the accidental hosts. After the ingestion of the infected products the immunological lungs, liver and central nervous system damages occur.

Flukes

Flukes are the parasitic trematodes of Schistosoma species which are transmitted through direct contact with fresh water. They penetrate into the intact human skin and enter the capillaries and then migrate to the central and portal system where they mature. Acute schistosomiasis also known as Katayama fever, which is a form of visceral larval migraines. The adult male and female pairs ultimately migrate to the superior mesenteric veins and ureteric vesicles. The eggs are then shed in the faeces and urine.

Tapeworms

Humans are the intermediate host for the Taeniasolium with the development of the tissue cysts. After the ingestion of the ncooked beef (*T.saginata*) or pork it develops the cysts and it causes the mild abdominal symptoms. The infestations of the central nervous systems cause due to the pork tapeworm or flukes are known as neurocysticercosis which is treated through albendazole and praziquantel. Classes of anthelmintic drugs Anthelmintics are the broad and wide range of drugs and are separated into classes on the basis of similar chemical structure and mode of action. The physiological and pharmacological actions of anthelmintics have been obtained from studies on the large parasitic nematodes *A.suum, C.elegans*, has been used in defining molecular targets.

Several herbal products are available all over the world with an acclaimed anthelminthic and antibacterial activity, which are considered to be less toxic and free from side effects[42-43]. Recently some medicinal plants like *Calotropis procera*[44] *Ocimum basilicum* var. Album[45] have been reported for anthelmintic activity. Encouraged by these

observations, it was contemplated to evaluate various solvent extracts of bark of ***Myristica dactyloides Gaertn*** and ***Hopea ponga (Dennst.),*** for anthelmintic activity.

Evaluation of Activity

Earthworms have similar life cycle with that of round worms and tape worms. Round worms need live tissue for experimentation and very difficult to procure, where as earthworms are easily available. Therefore earthworms were chosen for this experiment. They were procured from Dumalli (Shivamogga District, Karnataka state) at the time of carrying out this work

Materials

i. Earthworms (*Pheritima posthuma)*
ii. Petridishes
iii. Beakers and measuring cylinders
iv. Standard drug (Albendazole)
v. Glass rod
vi. Stop watch

Method

The anthelmintic activity was evaluated on Indian adult earthworms *Pheritima posthuma* due to its anatomical and physiological resemblances with the intestinal round worm parasites of the human beings[46-48]. The activity was assessed using earthworms by the reported methods with slight modification[49]. The worms were washed to remove adhering materials and were sorted out for uniform size and length. The worms were kept in 6% dextrose solution for acclimatization. The worms with normal motility and of length having 3-5 cm and 0.1-0.2 mm in width were used for the experiment. All the worms of equal size were divided into 11 groups and each group contains 3 worms. I group was treated with vehicle (1% Tween-80 in normal saline) served as control, II group is treated with albendazole (Standard) 10 mg/ml and III-XI groups were treated with different concentrations (25, 50, and 100 mg/ml in normal saline containing 1% Tween-80) of all the three extracts. Observations were made for the time taken to paralysis and death of individual worm. Paralysis was said to occur when the normal group did not survive in the saline. Death was concluded when the worm lost their motility followed with fading

of their body colour. The experiment was carried out in triplicate for each groups and data was statistically analyzed. Results are tabulated table **5**.7 and **5.8.**

Table 5.7 Anthelmintic activity of bark extracts of *Myristica dactyloides Gaertn*

Treatment groups	Concentration (mg/ml)	Mean paralysis time (min)±SEM	Mean death time (min)±SEM
Control/ Vehicle		------	------
Albendazole	25	31.00±0.0	36.00±0.0
Pet ether	25	13.07±0.6	16.01±0.29
	50	22.83±0.09	25.10±0.21
	100	10.97±0.15	13.97±0.20
Ethyl acetate	25	17±0.44	21.00±0.36
	50	29.33±0.20	36.07±0.23
	100	34.10±0.26	41.43±0.26
Methanol	25	13.02±0.6	19.02±0.29
	50	29.77±0.19	36.97±0.32
	100	42.50±0.29	50.87±0.41

Results and Discussion

All the three extracts showed the potent anthelmintic activity when compared with standard Albendazole. The mean paralyzing time of *Pheretima posthuma* with the dose of 50 mg/ml for pet ether extract were found to be 22.83±0.09 minutes. Albendazole in the concentration of 25 mg/ml has taken 31.00± 0.0 minutes for getting paralysis. The mean death time of *Pheretima posthuma* with the dose of 50 mg/ml for pet ether extract were found to be 32.97±0.32 minutes. By observing the results of each concentration of all the extracts, it was seen that the pet ether extract of *Myristica dactyloides Gaetrn* possess dose dependent anthelmintic activity as compared to the standard drug Albendazole, whereas ethyl acetate and methanol extract has showed moderate activity.

Table 5.8 Anthelmintic activity of different extracts of *Hopea ponga (Dennst.),* bark

Treatment groups	Concentration (mg/ml)	Mean paralysis time (min)±SEM	Mean death time (min)±SEM
Control/ Vehicle		------	------
Albendazole	25	31.00±0.0	36.00±0.0
Pet ether	25	23.01±0.16	56.02±0.27
	50	50.83±0.09	82.10±0.21
	100	62.97±0.15	92.97±0.20
Ethyl acetate	25	33.06±0.57	49.08±0.27
	50	76.33±0.20	90.07±0.23
	100	84.10±0.26	104.43±0.26
Methanol	25	13.01±0.64	16.20±0.29
	50	18.77±0.19	32.97±0.32
	100	36.50±0.29	52.87±0.41

Result and Discussion:

It was seen that the methanol extract of *Hopea ponga (Dennst.)*, possesses dose dependent anthelmintic activity as compared to the standard drug Albendazole, petroleum ether extract exhibited moderate activity, whereas ethyl acetate has showed less activity. The mean paralyzing time of *Pheretima posthuma* with the dose of 50 mg/ml for methanol extract were found to be 18.77±0.19 minutes respectively. Albendazole in the concentration of 25 mg/ml has taken 31.00± 0.0 minutes for getting paralysis. The mean death time of *Pheretima posthuma* with the dose of 50 mg/ml for methanol extract were found to be 32.97±0.32 minutes. In the case of Albendazole at a dose of 25 mg/ml cause paralysis only, no death was observed during the experimental period of 4 hours.

5.4 Antioxidant activity

Antioxidant can be defined as any substance, present at low concentrations compared to those of an oxidiazable subsrate, singnificantly delay or prevents oxidation of the substrate. Antioxidants are the first line of defense against free radical damage and are critical for maintaining optimal health. The need for antioxidants becomes even more critical with increased exposure to free radicals. As part of a healthy lifestyle and a well-balanced wholesome diet, antioxidant supplementation is now being recognized as an important means of improving free radical protection.

Antioxidant properties of a number of external agents are of current interest in the treatment of diseases from common cold to cancer. Global awareness on this subject created urgency to find such substances as evidenced by filing of patents on natural products such as curcuminoids.

Oxidative stress is characterized as an imbalance between the production of reactive species and antioxidant defense activity, and its enhanced state has been associated with many of the chronic diseases such as cancer, diabetes, neurodegenerative and cardiovascular diseases[50]. Based on that, many research groups have driven efforts to assess the antioxidant properties of natural products. These properties have been investigated through either chemical (in vitro) or biological (in vivo) methods, or both[51]. The results of these researches have led some to suggest that the long-term consumption of food rich in antioxidants can retard or avoid the occurrence of such diseases[52-53].

According to Brewer[54], the effectiveness of a large number of antioxidant agents is generally proportional to the number of hydroxyl (OH) groups present in their aromatic

ring(s). Based on that, the natural compounds would seem to have better antioxidant activity than the currently used synthetic antioxidants, making them a particularly attractive ingredient for commercial foods. Despite the large number of natural products that are currently consumed as antioxidant agents, the search for new chemical entities with antioxidant activity still remains a burgeoning field. In this context, the lichens have played an important role as a source for new antioxidant agents.

DPPH (2,2-diphenyl-picryl-hydrazyl radical) scavenging assay

The model assay of scavenging the DPPH radical is a widely used method to evaluate antioxidant activity and it is relatively rapid compared to other antioxidant methods. The DPPH test provided information on the reactivity of test compounds with a stable free radical. Because of its odd electron, 2, 2-diphenyl-picryl-hydrazyl radical (DPPH) gives a strong absorption band at 517nm in visible spectroscopy (deep violet colour). This commercially available radical serves as the oxidizing radical to be reduced by the antioxidant (RH) and as the indicator for the reaction.

$$DPPH + RH \rightarrow DPPH\text{-}H + R^{\cdot}$$

As the electron becomes paired off in the presence of a free radical scavenger, the absorption varnishes, thus the resulting decolorization is stoichiometric with respect to the number of electrons taken up. The scavenging properties of antioxidants are often associated with their ability to form stable radicals. DPPH has long been recognized as a convenient reagent to quantify antioxidants in complex biological systems and has been widely used for this purpose. The DPPH technique is a convenient screening tool for quickly determining anti-oxidant content differences may be of value in helping interpret efficacy results for products tested in biological models for chronic diseases.

Principle

The scavenging reaction between (DPPH) and an antioxidant (RH) can be written as

$$(DPPH) + RH \rightarrow DPPH\text{-}H + R^{\cdot}$$

(Purple) (Yellow)

Antioxidants react with DPPH, which is a stable free radical and is reduced to the DPPH-H and as consequence the absorbance decreased from the DPPH radical to the DPPH-H form. The degree of discoloration indicates the scavenging potential of the antioxidant

complexes in terms of hydrogen donating ability. Hence, it was contemplated to subject bark extracts of *Myristica dactyloides Gaertn* and *Hopea ponga (Dennst.),* during the present work for investigation of their antioxidant activity by DPPH method.

Materials and method

Methanolic solution of DPPH (0.1 mM): 39.4 mg of DPPH was dissolved in one liter of analytical grade methanol.

Method

Different samples and standard solutions were taken in different test tubes. To these 3 ml methanolic solution of DPPH was added, shaken well and mixture was allowed to stand to room temperature for 30 min. The blank was prepared as above without test complexes. Readings were noted at 517nm using spectrophotometer. Each experiment was carried out in triplicates and deviation of absorbance value was less than 10%. The absorbance of blank was first noted at 517nm change of absorbance of samples were measured and scavenging activity was expressed as DPPH Scavenged (%) = $[A_c - A_t/A_c]$ X 100 Where, A_c is the absorbance of the control reaction A_t is the absorbance in the presence of sample. The results of antioxidant DPPH scavenging activity was summarized in table **5.9** and **5.10**. The fig: **5.1** and **5.2** represents the DPPH scavenging activity of *Myristica dactyloides Gaetrn* and *Hopea ponga (Dennst.)* respectively.

Table 5.9 Antioxidant activity of bark extracts of *Myristica dactyloides Gaertn*

Extracts	Concentration µg/ml	DPPH % inhibition
Pet. ether	20	57.69
	40	73.69
	60	85.33
	80	90.59
	100	94.32
Ethyl acetate	20	34.78
	40	50.32
	60	63.45
	80	75.74
	100	86.87
Methanol	20	19.17
	40	33.12
	60	44.23
	80	57.45
	100	70.65

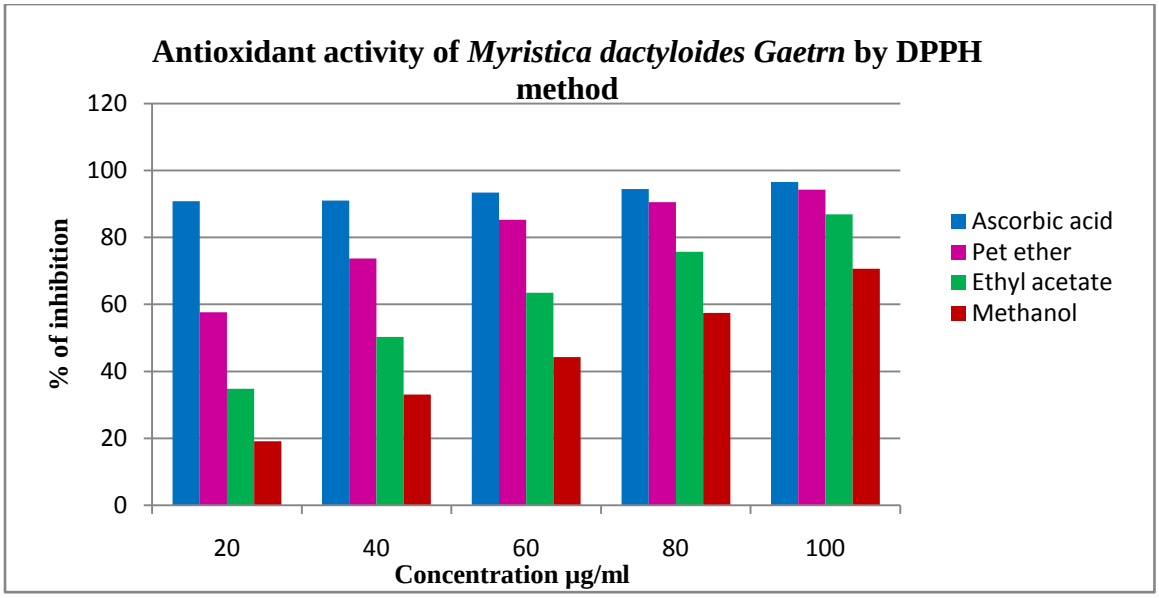


Fig: 5.1 DPPH radical scavenging activity of *Myristica dactyloides Gaertn* extracts.

Result and Discussion

Among the extracts Petroleum ether extract has showed a potent antioxidant activity at the concentration of 10µg/ml showing an IC_{50} value of 10.97±0.07µg/ml, while ethyl acetate and methanol extracts showed IC_{50} value of 21.41±0.13µg/ml and 34.34±0.18 µg/ml respectively. The similar activity was 5.54±0.03µg/ml for standard ascorbic acid. The results revealed that, dose dependent radical scavenging activity in terms of IC_{50} values.

Table 5.10 Antioxidant activity of bark extracts of *Hopea Ponga (Dennst.)*

Extracts	Concentration µg/ml	DPPH % inhibition
Pet. ether	20	49.64
	40	52.86
	60	54.17
	80	79.46
	100	49.64
Ethyl acetate	20	42.27
	40	66.68
	60	68.95
	80	74.21
	100	42.27
Methanol	20	51.32
	40	63.95
	60	71.65
	80	85.51
	100	88.01

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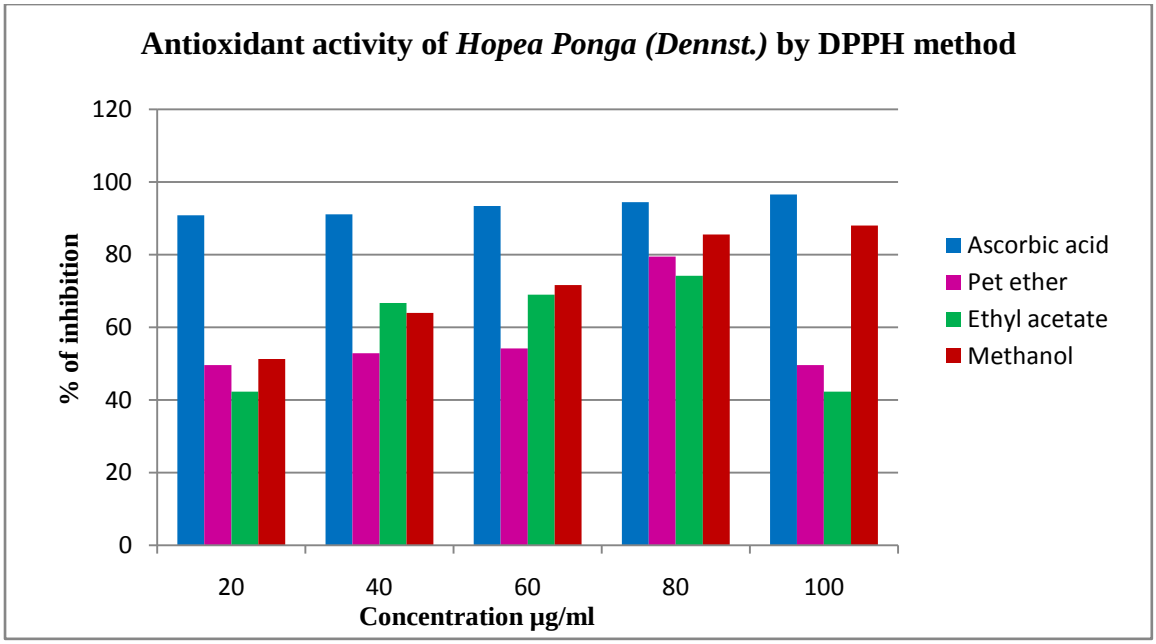


Fig: 5.2 DPPH radical scavenging activity of *Hopea ponga (Dennst.)*, extracts.

Results and Discussion

Among the extracts methanol extract has shown a potent antioxidant activity at the concentration of 20µg/ml, the extract exerted an inhibition of 51.32% and that of Ascorbic acid was 91.06% and the IC_{50} of the extract was 19.48 µg/ml, while that of Ascorbic acid was 11.00 µg/ml. The results revealed that, dose dependent radical scavenging activity in terms of IC_{50} values. This shows that *Hopea ponga* (*Dennst.*) extract presents a good ability to scavenge the DPPH radical.

5.5 Antitubercular activity

Tuberculosis (TB) (caused by Mycobacterium tuberculosis) remains a foremost global health problem and the second leading cause of death worldwide. In 2012 the incidence of the disease was estimated to be about 8.6 million and strikingly around 1.1 million (13%) patients were HIV-positive. India and China alone accounted for 24 and 11% of total cases, respectively[55]. Although India is the second most populous country in the world, it has more new emerging TB cases annually than any other country[56]. The current therapeutic regimens are six month regimen in which initial two months treatment is given with streptomycin, isoniazid, rifampicin and pyrazinamide or ethambutol replacing streptomycin and then followed by treatment with isoniazid and rifampicin for next four months and eight month regimen starting with the same four drugs in the initial phase but continuing with six months of thioacetazone and isoniazid. With time, mycobacterium

has exacerbated the problem in humans by acquiring various types of resistances [multi-drug resistance (MDR), single-drug resistance (SDR), and extensive drug resistance (XDR)] against antimycobacterial drugs[57]. The evaluation of the effectiveness of newly developed anti-tubercular agents against isolates of M. tuberculosis has become more important in the past few years, predominantly due to the appearance of multi-drug resistant tuberculosis[58]. There is an urgent need for new and effective anti-TB agents as an alternative to those drugs currently in use.

Plants with potential therapeutic values have been used from time immemorial to cure various ailments and infectious diseases. Of late, scientific evidences have been provided on the potential therapeutic agent exhibited by certain traditionally used vegetable extracts. The importance of edible plants may be traced to antiquity but systemic studies have not been attempted so far. Recently the exploration and exploitation of the disease fighting properties of a multitude of phytochemicals found in both food and nonfood plants have created arenaissance in human health and nutrition research. At the same time, many opportunities for the development of novel dietary products have been created. With all new fields of study come new term knew as "Nutraceuticals"[59], a term combining the words “nutrition” (a nourishing food or food component) and “pharmaceutical” (a medical drug), is a food or food product that provides health and medical benefits, including the prevention and treatment of disease. Such products may range from isolated nutrients, dietary supplements and specific diets to genetically engineered foods, herbal products and processed foods such as cereals, soups and beverages[60].

With recent advances in medical and nutrition sciences, natural products and health promoting foods have received extensive attention from both health professionals and the public. New concepts have appeared with this trend, such as nutraceuticals, nutritional therapy, phytonutrients, and phytotherapy[61-63]. These functional or medicinal foods and phytonutrients or phytomedicines play positive roles in enhancing health, and improving immune function to prevent specific diseases and also hold great promise to reduce side effects and health care cost[64]. A good number of extracts and pure compounds obtained from plants have exhibited considerable inhibitory activity against Mycobacterium. It is seen that several phytomolecules have shown anti-tubercular potency close to currently used drugs or even better than those. These phytomolecules and their semi-synthesized derivatives are under investigation for anti-tubercular activity by several research groups

globally. New evidence suggests that in order to understand the health benefits of plant based supplements and foods, we will need to take into account the fact that complex mixtures of phytochemicals found in food and other botanicals may act synergistically.

Tuberculosis is a highly infectious disease with about one third of the world's population including 40 per cent from India estimated to be infected it[65]. However, this problem has become serious as *Mycobacterium tuberculosis* developed resistance against both the first line as also the second line drugs. Due to this, there is emergence of multi-drug resistant (MDR) and extensively-drug resistant (XDR) strains of *M. tuberculosis* all over the world[66].

India is one of the few countries in the world which has unique wealth of medicinal plants and vast traditional knowledge of use of herbal medicine for cure of various diseases[67-68]. So far, few plants have been tested against mycobacteria and a few plants which showed anti-TB activity were *Salvia hypargeia, Euclea natalensis*, etc[69-72]. The increasing incidence of MDR-TB and XDR-TB worldwide highlight the urgent need to search for newer antitubercular compounds or drugs. Therefore, the present study was carried out for screening the antitubercular activity of bark extracts ***Myristica dactyloides Gaertn*** and ***Hopea ponga (Dennst.)***.

Materials

i. Sterile wells plate

ii. Micropipette

iii. Middlebrook 7H9 broth

iv. Sterile deionized water

v. Alamar Blue reagent

vi. Tween 80

vii. *M. tuberculosis* (H37 RV strain)

Method

Microplate Alamar Blue Assay (MABA)

The anti-mycobacterial activity of compounds were assessed against *M. tuberculosis* using microplate Alamar Blue assay (MABA)[73-74].This methodology is non-toxic, uses a thermally stable reagent and shows good correlation with proportional and BACTEC

radiometric method. Standard pyrazinamide and streptomycin were tested at 0.8, 1.6, 6.25, 12.5, 25, 50 and 100 µg/ml. The controls maintained for all the tested strains included: medium, DMSO, 1:100 MTB and 2 µg/ml Rifampicin (RIF). In case of the susceptible strains an additional suboptimal RIF (a concentration that allowed a percent reduction of Alamar Blue similar to that of the MTB) control was also maintained. Briefly, 200µl of sterile deionized water was added to all outer perimeter wells of sterile 96 wells plate to minimized evaporation of medium in the test wells during incubation. The 96 wells plate received 100 µl of the Middlebrook 7H9 broth and serial dilution of compounds were made directly on plate. The final drug concentrations tested were 100 to 0.2 µg/ml. Plates were covered and sealed with parafilm and incubated at 37ºC for five days. After this time, 25µl of freshly prepared 1:1 mixture of Alamar Blue reagent and 10% Tween 80 was added to the plate and incubated for 24 h. A blue colour in the well was interpreted as no bacterial growth and pink colour was scored as growth. The MIC was defined as lowest drug concentration which prevented the colour change from blue to pink. The results are tabulated in table **5.11** and **5.12** below.

Table 5.11 Antitubercular activity of bark extracts of *Myristica dactyloides Gaertn* by Microplate Alamar Blue Assay (MABA)

Sl. No	Samples	100	50	25	12.5	6.25	3.12	1.6	0.8
		Concentration in µg/ml							
1	**Pet. ether**	S	S	R	R	R	R	R	R
2	**Ethyl acetate**	S	R	R	R	R	R	R	R
3	**Methanol**	R	R	R	R	R	R	R	R
4	**Streptomycine**	S	S	S	S	S	R	R	R
5	**Pyrazinamide**	S	S	S	S	S	S	R	R

S – Sensitive, **R** - Resistant

Strain used: *M.tuberculosis* (H37 RV strain)

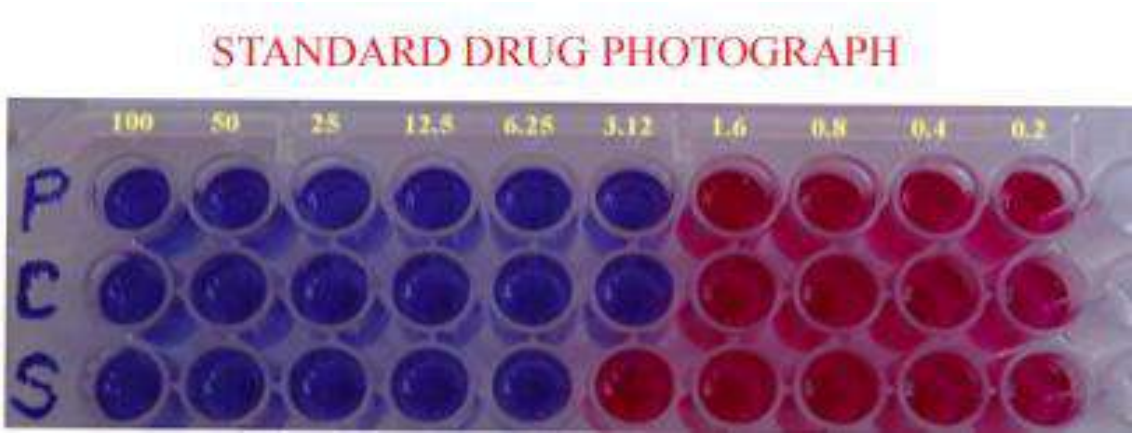


Fig: 5.3 Standard

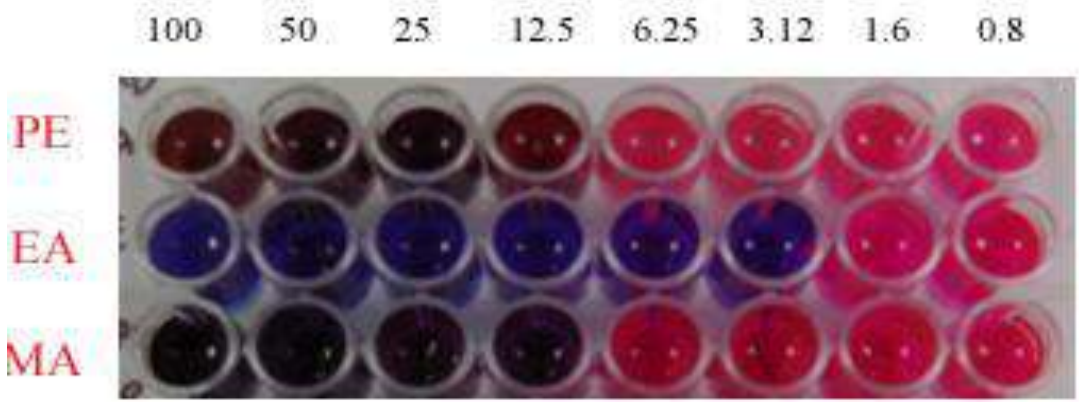


Fig: 5.4 Different solvent extracts of *Myristica dactyloides gaetrn*

Results and Discussion

All the extracts were tested for their *in-vitro* antitubercular activity against *M. tuberculosis* by MABA with the use of middle brook 7H9 broth (fig: 5.3, 5.4). By observing the results, pet ether extract of the plant showed sensitive against *M. Tuberculosis* at the concentration 50 µg/ml, while ethyl acetate and methanol extracts were resistant against *M. Tuberculosis.* The resistance shown by the organism on the extracts means that the extracts were not able to inhibit the *M. tuberculosis* strains. It confirmed that the pet ether extract is having a promising anti-tuberculosis activity rather than pet-ether and chloroform extracts when compared with standard streptomycin and pyarzinamide were sensitive against 6.25 µg/ml and 3.12 µg/ml respectively.

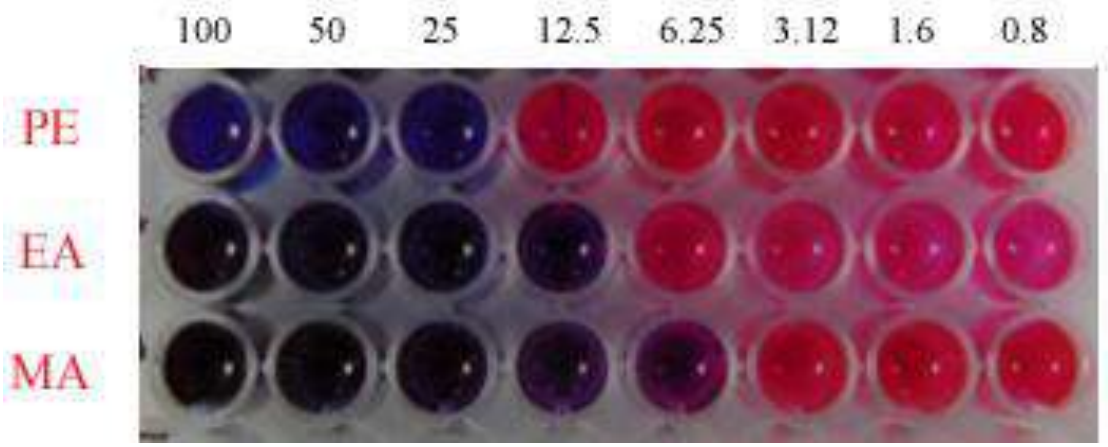


Fig: 5.5 Different solvent extracts of *Hopea ponga (Dennst.)*

Table 5.12 Antitubercular activity of bark extracts of *Hopea Ponga (Dennst.)* by Microplate Alamar Blue Assay (MABA)

Sl. No	Samples	100 µg/ml	50 µg/ml	25 µg/ml	12.5 µg/ml	6.25 µg/ml	3.12 µg/ml	1.6 µg/ml	0.8 µg/ml
1	Pet. ether	S	R	R	R	R	R	R	R
2	Ethyl acetate	S	S	R	R	R	R	R	R
3	Methanol	R	R	R	R	R	R	R	R
4	Streptomycine	S	S	S	S	S	R	R	R
5	Pyrazinamide	S	S	S	S	S	S	R	R

S – Sensitive, **R** - Resistant

Strain used: *M.tuberculosis* (H37 RV strain)

Results and Discussion

By observing the results, ethyl acetate extract of the plant was sensitive to *M. Tuberculosis* at the concentration 50 µg/ml, while pet ether and methanol extracts were resistant against *M. Tuberculosis.* The resistance shown by the organism on the extracts means that the extracts were not able to inhibit the *M. tuberculosis* strains. It confirmed that the pet ether extract is having a promising anti-tuberculosis activity rather than ethyl acetate and methanol extracts, when compared with standard streptomycin and pyarzinamide which were sensitive at 6.25 µg/ml and 3.12 µg/ml respectively(fig: 5.5).

5.6 *In vitro* Cytotoxic Activity

Natural Products, especially plants, have been used for the treatment of various diseases for Thousands of years. Terrestrial plants have been used as medicines in Egypt, China, India and Greece from ancient time and an impressive number of modern drugs have been developed from them. The first written records on the medicinal uses of plants appeared in about 2,600 BC from the Sumerians and Akkaidians[75]. According to World Health Organization, 80 % of the people living in rural areas depend on medicinal herbs as primary healthcare system. A great deal of pharmaceutical research done in technologically advanced countries like USA, Germany, France, Japan and China has considerably improved quality of the herbal medicines used in the treatment of cancer. Some herbs protect the body from cancer by enhancing detoxification functions of the body. Some herbs reduce the toxic side effects of chemotherapy and radiotherapy. Scientists all over the world are concentrating on the herbal medicines to boost immune cells of the body against cancer. By understanding the complex synergistic interaction of various constituents of anticancer herbs, the herbal formulations can be designed to attack the cancerous cells without harming normal cells of the body[76-77]. Cancer is a major

public health burden in both developed and developing countries. It was estimated that there were 10.9 million new cases, 6.7 million deaths, and 24.6 million per-sons living with cancer around the world in 2002. Cancer, after cardiovascular disease, is the second leading cause of death[78-79]. In the United States where one in four deaths is due to cancer. The National Cancer Institute collected about 35,000 plant samples from 20 countries and has screened around 114,000 extracts for anticancer activity. Of the 92 anticancer drugs commercially available prior to 1983 in the US and among worldwide approved anticancer drugs between 1983 and 1994, 60% are of natural origin. Documentation of the Ayurvedic system recorded in Sushruta and Charaka dates from about 1000 BC[80]. The Greeks also contributed substantially to the rational development of the herbal drugs. Dioscorides, the Greek physician (100 A.D.), described in his work "*De Materia Medica*" more than 600 medicinal plants. Phytochemicals have been proposed to offer protection against a variety of chronic ailments including cardiovascular diseases, obesity, diabetes, and cancer. As for cancer protection, it has been estimated that diets rich in phytochemicals can reduce cancer. The old saying "Prevention is always better than cure" is particularly true in the case of cancer where a cure, if at all possible, is associated with high cytotoxic loads and/or invasive procedures. With our growing understanding of the molecular etiology of cancer, it has become apparent that strategies which limit DNA damage and/or increase the probability of DNA repair by inhibiting aberrant proliferation will decrease cancer incidence[81].

Cancer and its Classification

Cancer is a general term applied of series of malignant diseases that may affect different parts of body. These diseases are characterized by a rapid and uncontrolled formation of abnormal cells, which may mass together to form a growth or tumor, or proliferate throughout the body, initiating abnormal growth at other sites. If the process is not arrested, it may progress until it causes the death of the organism. The main forms of treatment for cancer in humans are surgery, radiation and drugs (cancer chemotherapeutic agents). Cancer chemotherapeutic agents can often provide temporary relief of symptoms, prolongation of life, and occasionally cures. In recent years, a lot of effort has been applied to the synthesis of potential anticancer drugs. Many hundreds of chemical variants of known class of cancer chemotherapeutic agents have been synthesized but have a more side effects. A successful anticancer drug should kill or incapacitate cancer cells without causing excessive damage to normal cells. This ideal is

difficult, or perhaps impossible, to attain and is why cancer patients frequently suffer unpleasant side effects when under-going treatment. However, a waste amount of synthetic work has given relatively small improvements over the prototype drugs. There is a continued need for new prototype-new templates to use in the design of potential chemotherapeutic agents: natural products are providing such templates. Recent studies of tumor-inhibiting compound of plant origin have yielded an impressive array of novel structures[82].

Types of Cancers

1) Cancers of Blood and Lymphatic Systems

   a) Hodgkin'sdisease, b) Leukemias, c)Lymphomas, d) Multiple myeloma, e) Waldenstrom's disease

2) Skin Cancers

   a) Malignant Melanoma

3) Cancers of Digestive Systems

   a) esophageal cancer, b) Stomach cancer, c) Cancer of pancreas, d) Liver cancer, e) Colon and Rectal cancer, f) Anal cancer

4) Cancers of Urinary system

   a) Kidney cancer, b) Bladder cancer, c) Testis cancer, d) Prostate cancer

5) Cancers in women a) Breast cancer, b) Ovarian cancer, c) Gynecological cancer, d) Choriocarcinoma

6) Miscellaneous cancers

   a) Brain cancer, b) Bone cancer, c) Carcinoid cancer, d) Nasopharyngeal cancer, e) Retroperitoneal sarcomas f) Soft tissue cancer, g) Thyroid cancer

Breast cancer is the most common form cancer in worldwide[83-84]. Amongst South African women, breast cancer is likely to develop in one out of every 31 women in the country. Breast cancer in India is the second most common cancer in women after the cancer of uterine cervix. The BRCA2 gene is implicated in approximately30-45%familial breast cancer. The absence of any significant BRCA2 mutation in the level expression of the gene indicates that the BRCA2 gene may not be playing an important role in the sporadic breast as well as familial breast carcinogenesis in Indian women[85]. Human papilloma

virus is sexually transmitted and casually related to cervical cancer. Almost of all cervical cancer and high grade cancer precursor caused by specific high risk types of human papilloma virus[86].

Causes of Cancer

The main cause of cancer is mutation; changes in DNA that reduce or eliminate the normal controls over cellular growth, maturation, and programmed cell death. These changes are more likely to occur in people with certain genetic backgrounds (as illustrated by the finding of genes associated with some cases of cancer and familial prevalence of certain cancers) and in persons infected by chronic viruses (e.g., viral hepatitis may lead to liver cancer, HIV may lead to lymphoma). The ultimate cause, regardless of genetic propensity or viruses that may influence the risk of the cancer, is often exposure to carcinogenic chemicals (including those found in nature) and/or to radiation (including natural cosmic and earthly radiation), coupled with a failure of the immune system to eliminate the cancer cells at an early stage in their multiplication. The immunological weakness might arise years after the exposure to chemicals or radiation. Other factors such as tobacco smoking, alcohol consumption, excess use of caffeine and other drugs, sunshine, infections from such oncogenic virus like cervical papillomaviruses, adenoviruses Karposis sarcoma (HSV) or exposure to asbestos A Cancer cell also has the character of immortality even in vitro whereas normal cells stop dividing after 50-70 generations and undergoes a programmed cell death (Apoptosis). Cancer cells continue to grow invading nearby tissues and metastasizing to distant parts of the body. Metastasis is the most lethal aspect of carcinogenesis[87].

Plants Used as Anticancer Agents

The first agents to advance into clinical use were the isolation of the vinca alkaloids, vinblastine and vincristine from the Madagascar periwinkle, *Catharanthus roseus* (Apocynaceae) introduced a new era of the use of plant material as anticancer agents. They were the first agents to advance into clinical use for the treatment of cancer. Vinblastine and vincristine are primarily used in combination with other cancer chemotherapeutic drugs for the treatment of a variety of cancers, including leukemias, lymphomas, advanced testicular cancer, breast and lung cancers, and Kaposi's sarcoma. The discovery of paclitaxel from the bark of the Pacific Yew, *Taxus brevifolia* Nutt. (Taxaceae), is another evidence of the success in natural product drug discovery. Various parts of *Taxus*

brevifolia and other *Taxus* species (e.g., *Taxus Canadensis*, *Taxus baccata*) have been used by several Native American Tribes for the treatment of some noncancerous cases[88]. *Taxus baccata* was reported to use in the Indian Ayurvedic medicine for the treatment of cancer. Paclitaxel is significantly active against ovarian cancer, advanced breast cancer, small and non-small cell lung cancer. Camptothecin, isolated from the Chinese ornamental tree *Camptotheca acuminate* (Nyssaceae), was advanced to clinical trials by NCI in the 1970s but was dropped because of severe bladder toxicity. Topotecan and irinotecan are semi-synthetic derivatives of camptothecin and are used for the treatment of ovarian and small cell lung cancers, and colorectal cancers, respectively[89].

Epipodophyllotoxin is an isomer of podophyllotoxin which was isolated as the active antitumor agent from the roots of *Podophyllum* species, *Podophyllum peltatum* and *Podophyllum emodi* (Berberidaceae). Etoposide and teniposide are two semi-synthetic derivatives of epipodophyllotoxin and are used in the treatment of lymphomas and bronchial and testicular cancers[90]. Homoharringtonine isolated from the Chinese tree *Cephalotaxus harringtonia* (Cephalotaxaceae), is another plantderived agent in clinical use[91]. Combretastatins were isolated from the bark of the South African tree *Combretum caffrum* (Combretaceae). Combretastatin is active against colon, lung and leukemia cancers and it is expected that this molecule is the most cytotoxic phytomolecule isolated so far[92-93].

Herbs with Anticancer Activity

Allium sativum contains more than 100 biologically useful secondary metabolites, which include alliin, alliinase, allicin, Sallyl-cysteine(SAC), diallyldisulphide(DADS), diallyltrisulphide (DATS) and methylallyltrisuphide[94]. *Aloe vera* contains aloe-emodin, which activates the macrophages to fight cancer. *Aloe vera* also contains acemannan, which enhances activity of the immune cells against cancer. *Aloe vera* is found to inhibit metastases[95]. *Annona species* contain acetogenins, which possess significant cytotoxic activity against leukemia and sarcoma. Acetogenins are found to be effective in the treatment of nasopharyngeal carcinoma[96]. *Arctium lappa* contains potent anticancer factors that prevent mutations in the oncogenes. It has been used in the treatment of malignant melanoma, lymphoma and cancers of the pancreas, breast, ovary, oesophagus, bladder, bile duct and the bone. A study revealed that it reduces the size of tumour, relieves the pain and prolongs the survival period[97]. *Betula utilis* contains betulin that can be easily converted into betulinic acid. Studies have revealed that betulinic acid inhibits

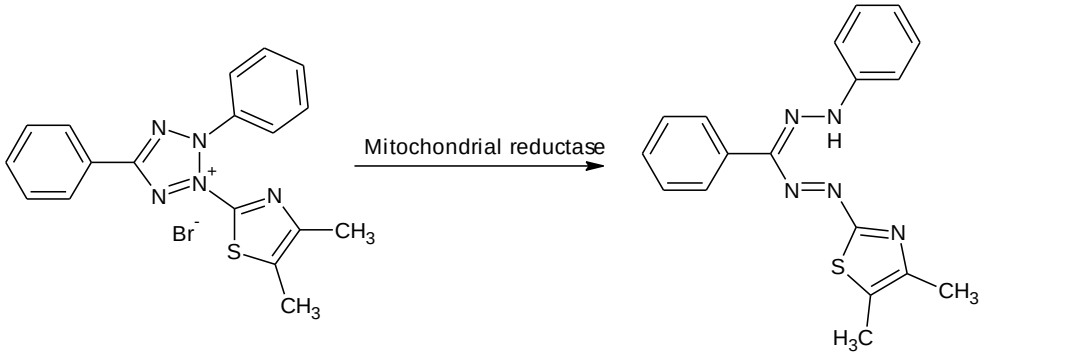
growth of malignant melanoma and cancers of the liver and the lung[98]. Gossypium *barbadense* contains gossypol. Recent studies have revealed that gossypol possesses selective toxicity towards cancerous cells[99]. *Gyrophora esculenta* is a mushroom that inhibits growth of cancer by enhancing activity of the natural killer cells. A study revealed that it inhibits carcinogenesis and metastases. The presence of diverse range of phytoconstituents in ***Myristica dactyloides Gaertn*** and ***Hopea ponga (Dennst.)*** extracts encouraged us to conduct cytotoxic activity on cancer cell lines. Therefore, the crude extracts were screened for cytotoxic activity towards cancer cells lines HeLa, MDA-MB, SiHa and Vero using MTT assay.

MTT Assay

The MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is a fast and reliable method for colorimetric determination of cell viability. This assay measures the cell proliferation rate and conversely the reduction in cell viability due to metabolic events.

Principle

This is a colorimetric assay that measures the reduction of yellow water soluble 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to a water insoluble, dark purplecolouredformazan product. Thecells are then solubilized with an organic solvent like DMSO or isopropanol and the released, solubilized formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells(Fig: 5.6).



MTT
3- (4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
(Yellow)

FORMAZAN
1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformaz
(Dark Purple)

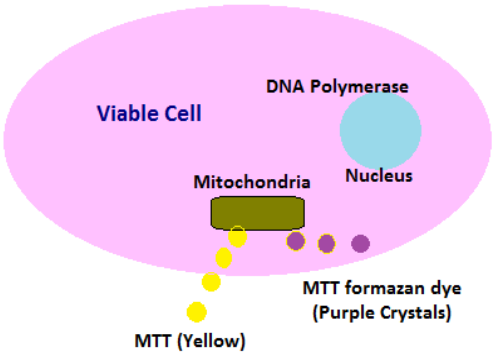


Fig: 5.6 Interaction of MTT with viable cell

Materials

i. Sterile wells plate
ii. Micropipette
iii. DMEM, Fetal bovine serum (FBS)
iv. Sterile deionized water
v. Trypsin, MTT
vi. Vero, HeLa, SiHa and MDA-MB cancer cell lines

Method

The cytotoxicity of ***Myristica dactyloides Gaertn*** and *Hopea ponga (Dennst.)* stem bark was evaluated by using MTT (colorimetric) method as follows[100]. The selected cell lines were seeded in 96-well plates at density of 2.0 x 10^4 cells per well. After incubating for 24 h, samples were added to the cells in phosphate buffer saline (PBS) at different concentrations (0-200µg/ml) and kept for incubation at 37^{o}C in humidified 5% CO_2 atmosphere for 24 h. At the termination of culture, cells were rinsed with PBS solution and 20 µL of MTT solution (5 mg/ml) was added to each well. The cells were cultured for another 4 h. Then, 100 µL of DMSO was added to each well to dissolve formazan crystals and the absorbance of solutions was monitored at 570 nm, with a reference at 630 nm on a microplate reader (Infinite M200 Nanoquant, Tecan). Untreated cells were used as controls and the experiments were tested in triplicate. The percentage of cell viability was calculated by the following equation:

$$\text{Cell viability (\%)} = [\ (A_{\text{Sample}} - A_{\text{Blank}}) / (A_{\text{Untreated}} - A_{\text{Blank}})\ ] \times 100$$

Measurements were performed and the concentration required for a 50% inhibition of viability was determined graphically. The effect of the samples on the proliferation of vero cells was expressed as the % cell viability. The mean of the cell viability values was

compared with the control to determine the effect of the extract. The maximum concentration of the plant extract that was non toxic to Vero cells but toxic to HeLa, SiHa and MDA-MB cell lines was recorded as the effective drug concentration. The results of the cytotoxic activity is shown in table **5.13** and **5.14.**

Table: 5.13 Cytotoxic activity of bark extracts of *Myristica dactyloides Gaetrn*

Extracts	Conc. in µg/ml	Hela		MDA-MB		SiHa		Vero	
		% Inhibition	IC_{50} in µg/ml	% Inhibition	IC_{50} in µg/ml	% Inhibition	IC_{50} in µg/ml	% Inhibition	IC_{50} in µg/ml
Pet-ether	25	70.63	>130 µg/ ml (50.15 %)	71.33	>130 µg/ ml (50.32 %)	69.88	>130 µg/ ml (50.03 %)	91.33	>200 µg/ ml (53.12 %)
	50	70.64		70.53		68.96		90.53	
	100	66.00		66.65		64.68		89.65	
	200	58.57		59.75		56.63		89.75	
Ethyl acetate	25	70.10	100 µg/ ml (50.40 %)	67.83	110 µg/ ml (50.18 %)	66.87	100 µg/ ml (51.30 %)	87.83	>200 µg/ ml (52.30 %)
	50	65.12		64.75		65.96		84.75	
	100	59.46		62.44		64.04		82.44	
	200	51.40		56.85		55.16		76.85	
Methanol	25	73.71	110 µg/ ml (51.10 %)	75.80	120 µg/ ml (50.50 %)	87.11	110 µg/ml (52.23 %)	85.80	>200 µg/ ml (51.82 %)
	50	76.74		74.67		73.48		84.67	
	100	73.90		74.26		69.82		84.26	
	200	72.84		71.37		68.84		81.37	

Results and Discussion

The different extracts of stem bark of ***Myristica dactyloides Gaertn*** were tested for their cytotoxic potential on different cancer cell lines. The extract showed significant inhibition in the cell viability in a dose dependent manner. At the concentration of 25 µg/ml among the extracts pet ether exerted a cell viability of 70.63% with an IC_{50} of > 130 µg/ ml (50.15%) against HeLa, ethyl acetate extract exerted a cell viability of 67.83% and 66.87 % with an IC_{50} of > 110 µg/ ml (50.18%) and > 100 µg/ ml (51.30%) against MDA-MB and SiHa cell lines respectively. Methanol extract exerted a cell viability of 85.80% with an IC_{50} of > 200 µg/ ml (51.82%) against Vero normal cell line.

Table 5.14 Cytotoxic activity of bark extracts of *Hopea ponga (Dennst.)*

Extracts	Conc. in µg/ml	Hela		MDA-MB		SiHa		Vero	
		% Inhibition	IC_{50} in µg/ml	% Inhibition	IC_{50} in µg/ml	% Inhibition	IC_{50} in µg/ml	% Inhibition	IC_{50} in µg/ml
Pet-ether	25	72.04	100 µg/ ml (50.10 %)	73.84	110 µg/ ml (51.14 %)	73.38	100 µg/ ml (5136 %)	93.84	>200 µg/ ml (52.00%)
	50	70.42		72.54		69.14		92.54	
	100	67.30		67.86		68.16		97.86	
	200	60.93		61.06		60.31			
Ethyl acetate	25	65.55	90 µg/ ml (52.30 %)	75.73	80 µg/ ml (51.28 %)	70.08	90 µg/ ml (52.28 %)	85.73	>200 µg/ ml (52.13%)
	50	64.38		73.09		67.33		83.09	
	100	65.43		70.81		65.29		80.81	
	200	62.30		67.89		61.49		77.89	
Methanol	25	77.90	90 µg/ ml (50.90 %)	69.19	80 µg/ ml (50.18 %)	66.64	90 µg/ ml (50.18 %)	89.19	>200 µg/ ml (51.13%)
	50	72.60		68.26		63.65		88.26	
	100	74.62		66.15		61.54		86.15	
	200	69.90		59.89		55.19		79.89	

Results and Discussion

The different extracts of stem bark of *Hopea ponga (Dennst.)* were tested for their cytotoxic potential on different cancer cell lines. The extract showed significant inhibition in the cell viability in a dose dependent manner. At the concentration of 25 µg/ml among the extracts ethyl acetate exerted a cell viability of 65.55%, with an IC_{50} of 90µg/ ml (52.30%) against HeLa, methanol extract exerted a cell viability of 69.19% and 66.64 % with an IC_{50} of 80 µg/ ml (50.18%) against MDA-MB and SiHa cell lines respectively. Methanol extract exerted a cell viability of 89.19% with an IC_{50} of > 200 µg/ ml (51.13%) against Vero normal cell line.

The results revealed that, dose cytotoxicity in terms of IC_{50} values. Different extracts of the plant exhibited different activity on different cell lines. This selectivity could be due to the sensitivity of the cell line to the active compounds in the extract or to tissue specific response. Plant extracts shows more significant activity as compared to the positive control. Among all the extracts tested the methanolic and ethyl acetate extract were found to exert maximum cytotoxicity on cancer cells.

5.6 DNA cleavage studies

Oxygen and its reactive species are very important in oxidative metabolism. They are produced in living cells by normal metabolism and by exogenous sources such as carcinogenic compounds and ionizing radiations[101]. The study of these reactive species and its effects on the organism have been of primary interest, since they induce some

damage to cells by reacting with biomolecules such as proteins, lipids and cause serious lesions to DNA.

Reactive oxygen species (ROS)-induced oxidative DNA damage producing a variety of modifications at DNA level including base and sugar lesions, strand breaks, DNA-protein cross-link and base-free sites. However, DNA of all mammalian cells contains trace amounts of modified bases that are indicative of attack by oxidising species and they are removed by excision repairing enzymes, they are known to accumulate with age being associated with disease processes[102].

Designing drug for cleaving DNA is currently an area of considerable interest from chemical as well as biological stand points and offers potential applications in the field of medicine in the post-genomic era. DNA cleavage is the process, which involved in various biological stages such as inflammation, mutagenesis, carcinogenesis and aging. As a consequence of clinical utility of DNA cleavage agents such as bleomycin, considerable effort has been made to identify and characterize naturally occurring molecules capable of cleaving the DNA[103], as such species may serve as lead structures for the development of novel anti-cancer drugs[104].

Materials

i. Concentrations screened: 100µg
ii. Stock Sample Concentration: 10mg/ml
iii. Agarose gel concentration: 1.2%
iv. DNA used: Calf-thymus DNA (50 µg/test) (Bangalore Genei, Bengaluru Cat.No105850)

Method

DNA cleavage activity by agarose gel electrophoresis

The extract was added separately to the DNA sample. The sample mixtures were incubated at 37°C for 2 hrs. The treatment of DNA samples using electrophoresis was done according to the method adopted by Sambrook et al[105]. Weigh 200 mg of agarose and dissolve in 25 ml of triacetate ethylenediamine (TAE) buffer (4.84 g Tris base, pH 8.0, 0.5 M EDTA/1: l) by gentle heating. As the gel attains ~55°C, pour it into the gel cassette fitted with comb and allow solidifying. Carefully remove the comb, place the gel in the electrophoresis chamber flooded with TAE buffer. Load 20 µl of DNA sample

(mixed with bromophenol blue dye at 1:1 ratio) into the wells, along with DNA marker and pass the constant 50 V of electricity for around 45 minutes. After 45 minutes remove the gel and stain with ETBr solution (10 µg/ml) for 10-15 minutes and observe the bands under UV transilluminator.

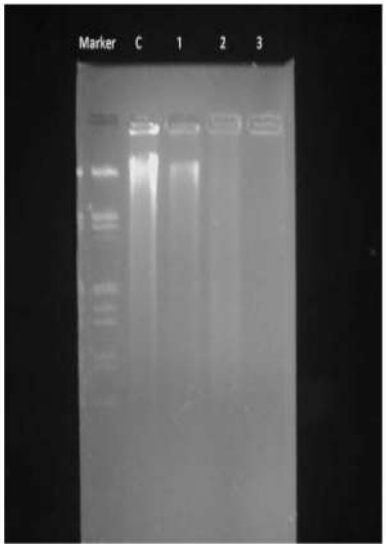


Fig: 5.7 DNA cleavage activity by agarose gel electrophoresis of bark extracts of *Myristica dactyloides gaetrn*

Result and Discussion

The result for DNA cleavage activity by various solvent extracts of *Myristica dactyloides* demonstrates that ethyl acetate and methanol extract cleaved the DNA at the concentrations tested, the pet ether extract has shown partial cleavage (fig: 5.7).

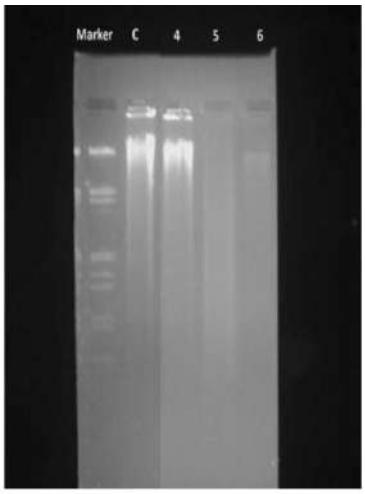


Fig: 5.8 DNA cleavage activity by agarose gel electrophoresis of bark extracts of *Hopea ponga (Dennst.)*

Result and Discussion

The result for DNA cleavage activity by various solvent extracts of *Hopea ponga* demonstrates that pet ether and ethyl acetate cleaved the DNA at the concentrations tested, the methanol extract has shown partial cleavage (5.8).

References

1. C. Stockwell,. Nature's pharmacy. London, United Kingdom. Century Hutchinson Ltd, **1988.**
2. W. Gerhartz, Y.S. Yamamota, F.T. Campbell, Pfefferkorn and J.F. Rounsaville, 1985. Ullmann's Encyclopedia of Industrial, **1985**.
3. J.I. Kroschwitz, and M. Howe-Grant, 1992. Kirk-Othmer encyclopedia of chemical Technology, 2, 893, **1992**.
4. D.J. Newman, G.M. Cragg and K.M. Snader, 2000. The influence of natural products upon drug discovery. Nat. Prod. Res., 17, 215, **2000**.
5. W.A.R. Thomson,. Medicines from the Earth. Maidenhead, United Kingdom. McGraw-Hill Book Co, **1978**.
6. J. Srivastava, J. Lambert and N. Vietmeyer,. Medicinal plants: An expanding role in development. World Bank Technical Paper. No. 320, **1996**.
7. S.K.Uniyal, K.N. Singh, P. Jamwal and B. Lal, Traditional use of medicinal plants among the tribal communities of Chhota Bhangal, Western Himalayan. J Ethnobiol. Ethnomed., 2, 1, **2006**.
8. M.F. Balandrin,, J.A. Klocke, E.S. Wurtele and W.H. Bollinger, Natural plant chemicals: Sources of Industrial and Medicinal materials. Science, 228, 1154, **1985.**
9. A.M. Clark, *Pharm. Res*., 13, **1996**.
10. A. Yankauer,. *Perspect. Biol. Med*., 41,132, **1997**.
11. R. Nelson, *Med. Pediatr. Oncol*., 10,115, **1982**.
12. J. Robbers, M. Speedie, and V. Tyler. *Pharmacognosy and pharmacobiotechnology*. Williams and Wilkins, Baltimore., **1996**.
13. G. Trease, and W. Evans, *Pharmacognosy*, Univ. Press, Aberdeen, Great Britain, **1972**.
14. F. Stary, *The natural guide to medicinal herbs and plants*, Barnes&Noble Inc., **1996.**

15. M.H. Kazmi, A. Malik, S. Hameed, N. Akhtar, and S. Noor Ali. *Phytochemistry.*, 36, 761, **1994**.

16. Critchfield, J. W., S. T. Butera, and T. M. Folks. *AIDS Res. Hum. Retroviruses.*, 12, 39, **1996**.

17. T.A. Geissman, *Flavonoid compounds, tannins, lignins and related compounds*, Elsevier, New York, **1963**.

18. G.A. Jones, T. A. McAllister, A. D. Muir, and K. J. Cheng. 1994. *Appl. Environ. Microbiol*., 60, 1374, **1994**.

19. T. Namba, O. Morita, S.-L. Huang, K. Goshima, M. Hattori, and N. Kakiuchi. *Planta Med*., 54, 277, **1998**.

20. W.A.R. Thomson, (ed.). *Medicines from the Earth*. McGraw-Hill Book Co., Maidenhead, United Kingdom, **1978**.

21. R. Fernandes de Caleya, B. Gonzalez-Pascual, F. Garcia-Olmedo, and P. Carbonero.. *Appl. Microbiol*., 23, 99, 1972.

22. P. Agrawal, V. Rai, R.B. Singh and Randomized, *J. Clinical Pharmacol. and therapeutic.*, 34, 406, **1996.**

23. N.R. Pokhrel, A Dissertation Submitted to Central Department of Microbiology., Tribhuwan University, Kathmandu, **2000.**

24. M.F. Balandrin, J.A. Klocke, E.S. Wutule and W.H. Bollinger, *Sources of industrial and med. materials Sci.*, 228, 1154, **1985.**

25. F.A. Jones, Eur. J. Gastroenterology Hepato., 8, 1227, **1996.**

26. B. Thippeswamy, K.J. Naveenkumar, J. Guruprasad Bodharthi, and S. Shivaprasad, Antimicrobial activity of ethanolic extract of *Usnea longissima*. J of Experimental Sciences, 2, 01, **2011**.

27. R. Sharath, V. Krishna, B.N. Sathyanarayana and B. Harish, *Pharmacol. Online.*, 2, 517, **2008.**

28. H.M. Vagdevi, K.P. Latha, V.P. Vaidya, M.L. Vijaya kumar and K.S.R. Pai, *Indian J. Pharma. Sci.*, 63, 286, **2001.**

29. B.G. Harish, V. Krishna, R. Sharath, S. Kumara, N. Raja and K.M. Mahadevan, *Int. J. Biomed.* and *Pharma. Sci.*, 1, 65, **2007**.

30. C. Beck-Sague, S. Banerjee, and W.R. Jarvis, *J. Public Health*., 83, 1739, **1993**.

31. J.H. Rex, M.G. Rinaldi, and M.A. Pfaller, Antimicrob. *Agents Chemother*, 39, 1 **1995**.

32. R.P. Borris, *J. Ethnopharmacol*., 51, 29, **1996**.

33. W.H. Lewis and M.P. Elvin-Lewis, *Ann. Mo. Bot. Gard*., 82, 16, **1995**.

34. T.G.R. Kumari, P.T. Paul, S. Ayyub, M.A. Iyenger and P.S. Rao, *Indian Drugs.*, 41, 622, **2004**.

35. N. Mimica-Duki, B. Bozin, M. Skovi, B. Mihajlovi and M. Matavuli, *Planta Med.*, 69, 413, **2003**.

36. M. Anisuzzaman, AHMM Rahman, M. Harun-or-Rashid, ATM. Naderuzzaman, AKMR Islam an Ethnobotanical Study of Mdahupur, *Tangail. J App Sci Research*, 3, 519, **2007**.

37. A. Vijayan, V.B. Liju, J.V. Reena John, B. Parthipan, Renekac. Traditional remedies of kani tribes of kotor reserve forest, Agasthyavanam, Thiruvanathapuram kerala. *Ind. J Trad Know*, 6, 589, **2007**.

38. K.D. Tripathi, *Essentials of Med. Pharmacol.*, V Edn, Japee Brothers, New Delhi, 759, **2003**.

39. O.D. Standen, R.J. Schnitzer and Hawking, *Academic Press Inc.*, New York, 3, 701, **1963**.

40. T.G. Geary. Ivermectin 20 years on maturation of a wonder drug. Trends Parasitol, 21, 530, **2005**.

41. R.M. Greenberg. Ca^{2+} signaling, voltage-gated Ca^{2+} channels and praziquantel in flatworm neuromusculature. *Parasitol.*, 131, S97, **2005**.

42. M.S. Akhtar, I. Zafar Iqba, M.N. Khan and Muhammad Lateef, *Small Ruminants Res.*, 38, **2000.**

43. S. Geert and P. Bulletin-des-Seances, *Academic- Royale-des-Sciencesd.*, 3, 401,**1995.**

44. Y.M. Shivkar and V.L. Kumar, *Pharma. Biol.*, 41, 263, **2003**.

45. M.L. Jain and S.R. Jain, *Planta Med*., 22, 66, **1972**.

46. R.D. Vidyarthi, *Text book of Zoology.*, 14th Edn, Chand and Company, New Delhi, 329, **1977.**

47. G.W. Thorn, R.D. Adams, E. Brauwald, K.J. Isselbacher and R.G. Petersdoft, *Harrison's principles of Internal medicine.,* McGraw Hill and Company, New York, 1088, **1977**.

48. Z. Vigar, *Atlas of Medical Parasitology.,* 2nd Edn, P.G. Publishing House, Singapore, 216, **1984**.

49. G. Toker, E. Kupeli, M. Memizolu and E. Yesilada, *J. Ethnopharmacol*., 95, 393, **2004**.

50. McCord, J.M. The evolution of free radicals and oxidative stress. *Am. J. Med*., 108, 652–659, **2000**.

51. M.N. Alam, N.J. Bristi, M. Rafiquzzaman, Review on in vivo and in vitro methods evaluation of antioxidant activity. *Saudi Pharm. J*., 21, 143, **2013**.

52. S.B. Lotito, B. Frei, Consumption of flavonoid-rich foods and increased plasma antioxidant capacity in humans: Cause, consequence, or epiphenomenon*? Free Radic. Biol. Med*., 41, 1727, **2006**.

53. V. Lobo, A. Patil, A. Phatak, N. Chandra, Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacogn. Rev***.,** 4, 118, **2010**.

54. M.S. Brewer, Natural Antioxidants: Sources, Compounds, Mechanisms of Action, and Potential Applications. *Compr. Rev. Food Sci. Food Saf*., 10, 221, **2011**.

55. Global tuberculosis report WHO, **2014**.

56. TB India revised national TB control programme, Annual status report, Government of India, **2013**.

57. E. Stokstad. Drug-resistant TB on the rise. *Science.*, 287, 2391, **2000.**

58. J.S. Bergmann, G.L. Woods. In vitro activity of antimicrobial combinations against clinical isolates of susceptible and resistant Mycobacterium tuberculosis. *Int. J. Tuberc. Lung Dis*., 2, 621, **1998**.

59. B.N. Ames, K.S. Mark, M.H. Tory. Oxidants, Antioxidants and the Degenerative Disease of Aging. *Proc. Natl. Acad. Sci.* USA. 90, 7915, **1993**.

60. H.K. Biesalski, Nutraceuticals: the link between nutrition and medicine. In: K. Kramer, P.P. Hoppe, L. Packer, editors. Neutraceuticals in health and disease prevention. New York: Marcel Dekker Inc, 1, **2001**.

61. J.S. Bland. Phytonutrition, phytotherapy and phytopharmacology. *Altern Ther Health Med.*, 2, 73, **1996**.

62. M.M. Berger, A. Shenkin, Vitamins and trace elements: Practical aspects of supplementation. *Nutrition.*, 22, 952, **2006**.

63. D. Bagchi. Nutraceuticals and functional foods regulations in the United States and around the world. *Toxicol.*, 221, 1, **2006**.

64. C.S. Ramaa, A.R. Shirode, A.S. Mundada, V.J. Kadam. Nutraceuticals-an emerging era in the treatment and prevention of cardiovascular diseases. *Curr Pharm Biotech*., 7, 15, **2006.**

65. S.P. Agarwal, Inter-sectoral cooperation for success of the RNTCP, *Indian J. Tuberc*., 51, 59, **2004**.

66. M.M. Singh, XDR-TB-Danger ahead. 2, *Indian J. Tuberc.*, 54, 1, **2007**.

67. A.K. Gupta and N. Tandon, *Reviews of Indian Med. plants*. New Delhi, India: Indian Council of Medical Research, **2004**.

68. S.K. Sharma, *Medicinal plants used in Ayurveda*, New Delhi, India: National Academy of Ayurveda, Ministry of Health and Family Welfare, Government of India, **1998**.

69. R. Gautam, A. Saklani and S.M. Jachak, *J. Ethnopharmacol*., 110, 200, **2007**.

70. S.M. Newton, C. Lau and C.W. Wright, *Phytother. Res*., 14, 303, **2000**.

71. S.M. Newton, C. Lau, S.S. Gurcha, G.S. Besra and C.W. Wright, *J. Ethnopharmacol*., 79, 57, **2002**.

72. Ulubelen, N. Euren, E. Tuzlaci and C. Johanson, *J. Nat. Prod.*, 812; *Indian J. Med. Res.*, 51, 1178, **2010.**

73. N. Lall and J.J. Meyer, *J. Ethnopharmacol*., 78, 213, **2001**.

74. C.S. Maria Lourenco, V.N. Marcus deSouza, C. Alessandra Pinheiro, L. Marcelle de Ferreira, B. Rasnisb, Goncalves, M. Thais Cristina Nogneira and A. Monica Peralta, *Arkivoc.*, xv, 181, **2007**.

75. G. Samuelson, "*Drugs of Natural Origin: A Textbook of Pharmacognosy"*, 4th Ed., Stockholm, Swedish Pharmaceutical Press, **1999**.

76. T. Larkin, "Herbs Are Often More Toxic Than Magical", *FDA Consum*, 17, 4, **1983**.

77. T.G. Saxe, "Toxicity of Medicinal Herbal Preparations", Am Fam Physician, 35,135, **1987**.

78. T Kutluk, A. Kars, "*General Knowledge About Cancer*", Ankara, Turkey cancer investigation and fight society publications, 7, **1998**.

79. S. Turgay, D. Sar, Turkistan, *Premethod.*, 436, **2005**.

80. L.D. Kappor, "*CRC Handbook of Ayurvedic Medicinal Plants*,Boca Raton, Florida, CRC Press., 416, **1990**.

81. J.S. Bertan, "*Molecular Aspects Medicine"*, 21, 167, **2001.**

82. V. Tyler, "*Herbs of Choice: The Therapeutic Use Of Phytomedicinals*", New York, Haworth Press., 24, **1994**.

83. Cancer index web site. Available at: http/www.cancerindex.org

84. Estrogen & Cancer Website, www.womenshealth.com, www.amazon.com.

85. S. Koduru, D.S. Grierson, and A.J. Afolayan, "Ethno botanical information of medicinal plants used for treatment of cancer in the Eastern Cape Province", *South Africa.Curr.sci.*, 92, 906, **2007**.

86. Neema Goel, Neeraj jain and Suresh Hedau, "Mutation and Expression Analysis of BRCA2 Gene in Breast cancer patients from india", *Institute of Cytology and Preventive oncology* (ICMR), Noida., 1, **2005**.

87. Jayanti, Maria-Pramanik and Shilpa C Kerkar, "Human papilloma virus infection in women with different clinical manifestations", G. S. Medical College and KEM Hospital, Parel, Mumbai, **1995**.

88. K. McNutt, "Medicinals in foods", *Nature Today*; 30,218, **1997**.

89. Cancer web site. Available at: http/www.cancer.gov.

90. G.M. Crag and D.J. Newmann, *Journal of Ethnopharmacology.*, 100, 72, **2005**.

91. G.J. Creemer G. Bolis, G. Scarfome and I. Hudson, *Journal of Clinical oncology.*, 14, 3056-3061, **1996**.

92. J.R. Bertino, *Oncology.*, 24, S18, **1997.**

93. A.L. Harvey, "*Trends Pharmaceutical Sciences*", 20, 196-98, **1999**.

94. H. Itokaw and X. Wang, "*Homoharringtonine and related compounds*", In: Cragg G.M, Kingston DGI. Newman D (eds) "*Anticancer agents from natural products*. Boca Raton, Florida, Brunner- Routledge Psychology Press, Taylor and Francis Group., 47, **2005**.

95. K. Ohsumi, R. Nakagawa, Y. Fukuda and T. Hatanaka, "*Journal of Medicinal Chemistry*"., 41, 705, **1998**.

96. G.R. Petit, S.B. Singh, M.L. Niven and E. Hamel, "*Journal of Natural Products*"., 50, 119, **1987**.

97. R.H. Cichewitz and S.A. Kouzi, "Chemistry, biological activity, and chemotherapeutic potential of betulinic acid for the prevention and treatment of cancer and HIV infection", *Med Res Rev.*, 24, 90, **2004**.

98. J.L. Hartwell, "*Plants used against cancer: a survey*", Lawrence, MA. Quarterman Publications, 438-39. K. Charfenberg, R. Wagner, and K.G. Wagner, "The cytotoxic effect of ajoene, a natural product from garlic, investigated with different cell lines", GBF, Braunschweig, F.R.G. *Cancer Lett*, 53, 103, **1990.**

99. T. Pecere, M.V. Gazzola, and C. Micignat, *et al*, "Aloe-emodin is a new type of anticancer agent with selective activity against neuro-ectodermal tumors", *Cancer Res.*, 60, 280, **2000.**

100. S.P. Ambasta, *et al.*, "*The Useful Plant Of India*", Fourth Edition, National Institution of Sci. Communication., Delhi, 243, **2000**.

101. D. Webster, T.D. Lee, J. Moore, T. Manning, D. Kunimoto, D. LeBlanc, J.A. Johnson and C.A. Gray, *Can. J. Microbiol.*, 56, 487, **2010**.

102. International Agency for Research on Cancer (IARC), World cancer factsheet. January, **2014.**

103. A. Jemal, F. Bray, M. Melissa, J. Ferlay, Elizabeth Ward & Forman D, Global cancer statistics. *CA Cancer J Clin.*, 61, 69, **2011**.

104. B.S. Jayashree, S. Nigam, A. Pai, H.K. Patel, N.D. Reddy, N. Kumar & C.M. Rao, Targets in anticancer research-A review. *Indian J Exp Biol.*, 53, 489, **2015**.

105. J. Sambrook, E.F. Fritsch, T. Maniatis. Molecular Cloning: A Laboratory Manual. 2nd ed. New York: Cold Spring Harbor Laboratory., **1989**.

CHAPTER-VI

Isolation and characterization of bioactive molecules

6.1 Isolation and Characterization of Active Components from *Myristica dactyloides Gaertn*

The pet ether extract of bark of *Myristica dactyloides Gaertn* exhibited considerable antioxidant, antimicrobial and cytotoxic activities. These observations encouraged us to isolate and identify bioactive molecules present in this extract.

Many separation techniques are available to accomplish purification and isolation of active components from extracts of plants. In the present investigation, the following techniques were adopted to achieve the isolation of pure components from pet ether of *Myristica dactyloides Gaertn*.

- Thin Layer Chromatography [TLC]
- Conventional Column Chromatography

6.2 Thin Layer Chromatography [TLC]

Thin Layer Chromatography (TLC) is a technique used for separation of components. It is colour fingerprint method, which is most widely used for quick and easy determination of quality and authenticity of the crude drugs. The separation is based on the differences in adsorption co-efficient of the individual components of a mixture. Components which are strongly adsorbed in the stationary phase move up less readily than those which are adsorbed to a lesser extent, leading to the separation of the compound.

TLC is a mode of chromatography in which sample is applied as a small spot on to the origin of a thin sorbent layer (Silica gel) supported on a glass or metal plate. The mobile phase moves through stationary phase by capillary action, sometimes assisted by gravity or pressure. Mobile phase consists of a single solvent or mixture of solvents.

Thin layer chromatography of pet ether extract of bark of *Myristica dactyloides Gaertn* was carried out to select appropriate mobile phase to achieve reasonably good separation in column chromatography.

For TLC, glass sides (Size 7.5 cm in length and 2.5 cm in width) were cleaned to remove greasy material and dried in oven for an hour at 70-80 $^{\circ}$C. The slurry of silica gel G, which was used as adsorbent, was prepared by dissolving silica gel G in freshly distilled chloroform. The glass slides were coated with the slurry and excess of chloroform was allowed to evaporate. TLC was performed by using various solvents or mixture of solvents of varying polarity on these glass plates. It was observed that, mixture of chloroform and methanol in varying proportion gave good separation.

In the present study an attempt was made to develop a TLC pattern for the extract to identify different the compounds.

6.2.1 Optimization of TLC Solvent system

Different solvent systems were tried for developing TLC for the identification of constituents present in the methanol extract. One showing maximum and clear separation was selected as mobile phase for the study. The different solvent systems used were-

- Pet-ether **:** Ethyl acetate

The detecting agents used were-

- UV at 254 nm and 366 nm
- Iodine vapors

6.2.2 Developing Thin Layer Chromatogram

The sample was dissolved in methanol and spotted on the pre-coated TLC plate. The chromatogram was developed using the above solvent systems and the spots were visualized under UV at 254 nm and iodine vapors. After a number of trial and error, it was observed that, mixture of pet ether and ethyl acetate in the ratio 95:5 by volume showed clear separation of components.

6.3 Column Chromatography

The isolation of pure components involved the following steps

Chromatographic separation using silica gel (100-200 mesh)

Chromatographic separation using silica gel (60-120 mesh)

Column chromatography is one of the most useful methods for separation and purification of both solids and liquids in which it is possible to isolate desired compounds from a mixture. Column chromatography is a solid-liquid separation technique in which the two phases are a solid (stationary phase) and a liquid (moving phase). The sample is dissolved in a small quantity of solvent and placed at the top of the column. There exists an equilibrium between the solute adsorbed on adsorbent and the eluting solvent flowing down through the column. The most common adsorbents are silica gel and alumina.

The pet ether extract of *Myristica dactyloides Gaertn* was subjected to column chromatography for isolation of active compounds using pet ether and ethyl acetate solvent system. The isolation of pure components was done by column chromatography using silica gel 60-120 mesh.

6.3.1 Chromatographic separation using silica gel (60-120 mesh)

The pet ether extract of bark of (5g) of *Myristica dactyloides Gaertn* was subjected to column chromatographic separation by using pet ether and ethyl acetate mixture of various proportions as mobile phase or the chromatographic separation, a column of length 55 cm and diameter of 6 cm was cleaned and dried. Stationary phase was prepared from Silica gel (60-120 mesh) in freshly distilled chloroform and loaded in the column. The column was gently tapped to ensure uniform packing of the column. A small quantity of solvent was allowed to remain at the top of the column to avoid the drying or cracking of the column. The column was left for overnight before charging the extract for complete saturation and removal of air bubbles to make the bed static.

The pet ether extract (5g) was dissolved in minimum amount of distilled pet ether and loaded over the stationary phase in the column. The column was eluted with pet ether and ethyl acetate mixture of various proportions as mobile phase. During elution the polarity of mobile phase was increased gradually by varying the proportions of pet ether and ethyl acetate. The elution was monitored by TLC and the eluted fractions were collected in 100 ml portions. The fractions with similar spots were pooled together. From the collected fractions one pure fraction was identified and separated. The details of column chromatography are given in the below table 6.1.

Table 6.1 The Chromatographic details of pet ether extract of bark of *Myristica dactyloides Gaertn*

Fractions	Eluent (Pet ether- Ethyl acetate)	Colour and consistency of Compounds isolated	Yield in mg
1	100:0	Light brown solid	80
2	95:05	Yellow pasty solid	50
3	90:10	No residue	Negligible
4	85:15	White amorphous solid	970
5	80:20	Light yellow solid	1150
6	70:30	Brown pasty solid	20
7	60:40	Yellow oily liquid	35
8	50:50	No Residue	----
9	40:60	No Residue	----
10	30:70	No Residue	----
11	20:80	No Residue	----
12	10:90	Brown pasty solid	90

Among the obtained fractions, fraction 4 and 5 have got high yield than the other fractions. Hence the fourth and fifth fractions have been taken for their characterization. The fraction **4** and **5** is labeled as compound **I** and **II** respectively.

6.4 Characterization of isolated compound I from the bark extracts of *Myristica dactyloides Gaertn*

The isolated **compound I** and **II** was white amorphous and light yellow solid respectively, the isolated compounds were characterized by physical parameters such as melting point, elemental analysis and spectral studies.

6.4.1 Characterization of compound I

- **Melting point**

Melting point was recorded in an open capillary tube. The Melting point of compound I was found to be 119-121°C.

- **Elemental analysis**

The elemental analysis of the isolated compound I was carried to know the percentage composition of the elements present in the compound. The data of analysis is recorded in the table 6.2.

Table: 6.2-Summary of elemental analysis

Compound	Molecular formula	Molecular Weight in g/mol	Melting Point in ^{0}C	Elemental Analysis (Calculated %)		
				C	H	O
Compound-I	$C_{21}H_{26}O_4$	342.43	119-121	73.64 (73.66%)	7.66 (7.65%)	18.70 (18.69%)

The elemental analysis of the compound indicated the following elemental composition. Carbon= 73.66%, Hydrogen= 7.65%, Oxygen =18.69%. The mass was found to be =342.43. Based on the data, the following formula has been assigned to **compound I**.

Molecular formula = $C_{21}H_{26}O_4$

Molecular weight = 342.43

6.4.2 Spectroscopic Study

6.4.2.1 Infrared spectrum

The infrared spectrum of the compound was recorded in KBr in the range of 4000-400 cm^{-1} on Unicam FTIR (Research Spectrometer Series). In the IR spectrum, the peak observed at the region of 1085 cm^{-1} is due to the presence of (-C-O-C-) ether functional group. Strong absorbance frequency at 1713 cm^{-1} is observed for the presence of carbonyl group (-C=O). The IR-spectrum exhibited broad absorption band at 3310cm^{-1} indicating the presence of phenolic (–OH) group.

6.4.2.2 Proton Magnetic Resonance Spectrum (^{1}H NMR)

The magnetic resonance spectra of the compounds were recorded on AV 400 FT NMR Spectrophotometer (400 MHz) using TMS as an internal standard. The samples were dissolved in $CDCl_{3..}$ The ^{1}H NMR spectrum, fig: (6.2) the appearance of peak at δ 1.20-3.11 values confirmed the presence of $–CH_2$ protons. The peak appeared at δ 5.54-5.87 confirmed the presence of phenolic –OH protons. The peak appeared at δ 6.33-7.26 for aromatic protons.

6.4.2.3 ^{13}C Nuclear Magnetic Resonance Spectrum (^{13}C NMR)

In the ^{13}CNMR spectrum (Fig: 6.3), In the ^{13}C NMR spectrum, The aliphatic carbons appeared at δ 25.88-45.97 The peaks appeared at δ 108.55-163.35 confirms the presence of aromatic carbons. The peak appeared at δ 189.06 confirms the presence of carbonyl carbon (–C=O).

6.4.2.4 Mass Spectrum

The mass spectrum was recorded in Auto Spec E_l mass Spectrometer at 70eV ionisation energy with direct inlet system. The mass spectrum (Fig: 6.4) confirms the molecular weight of compound I is m/z 342.

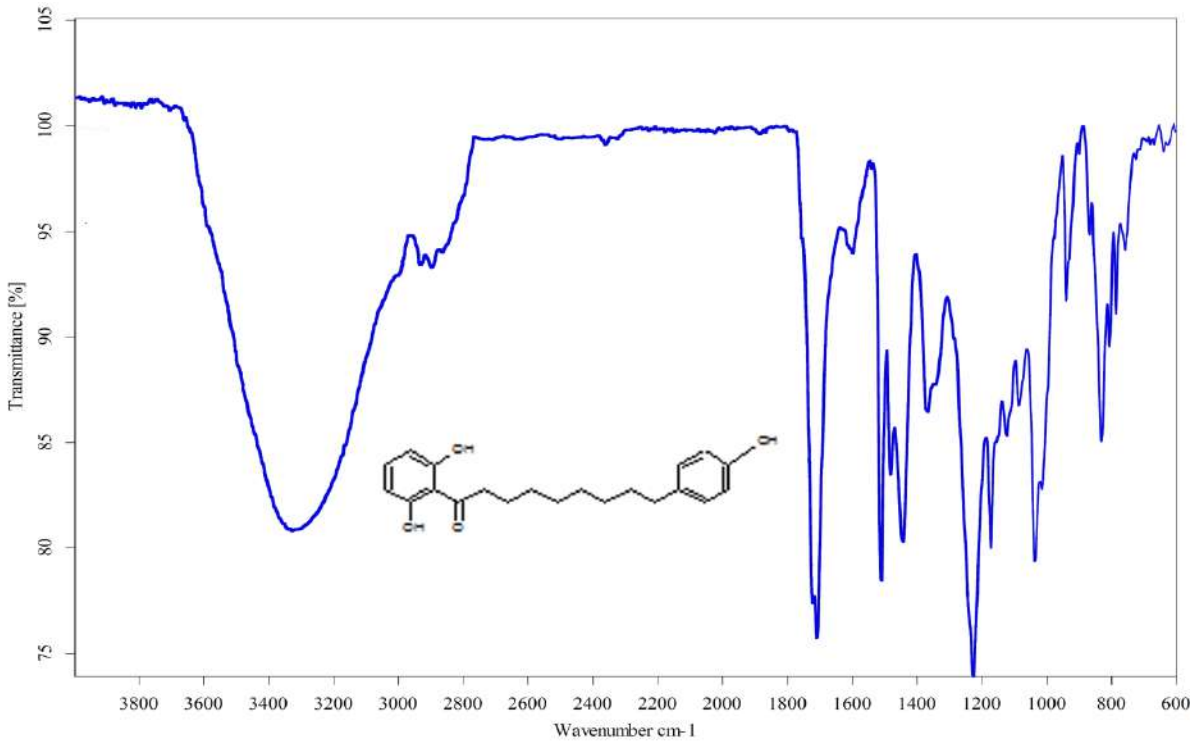


Fig: 6.1 IR Spectrum of Compound I

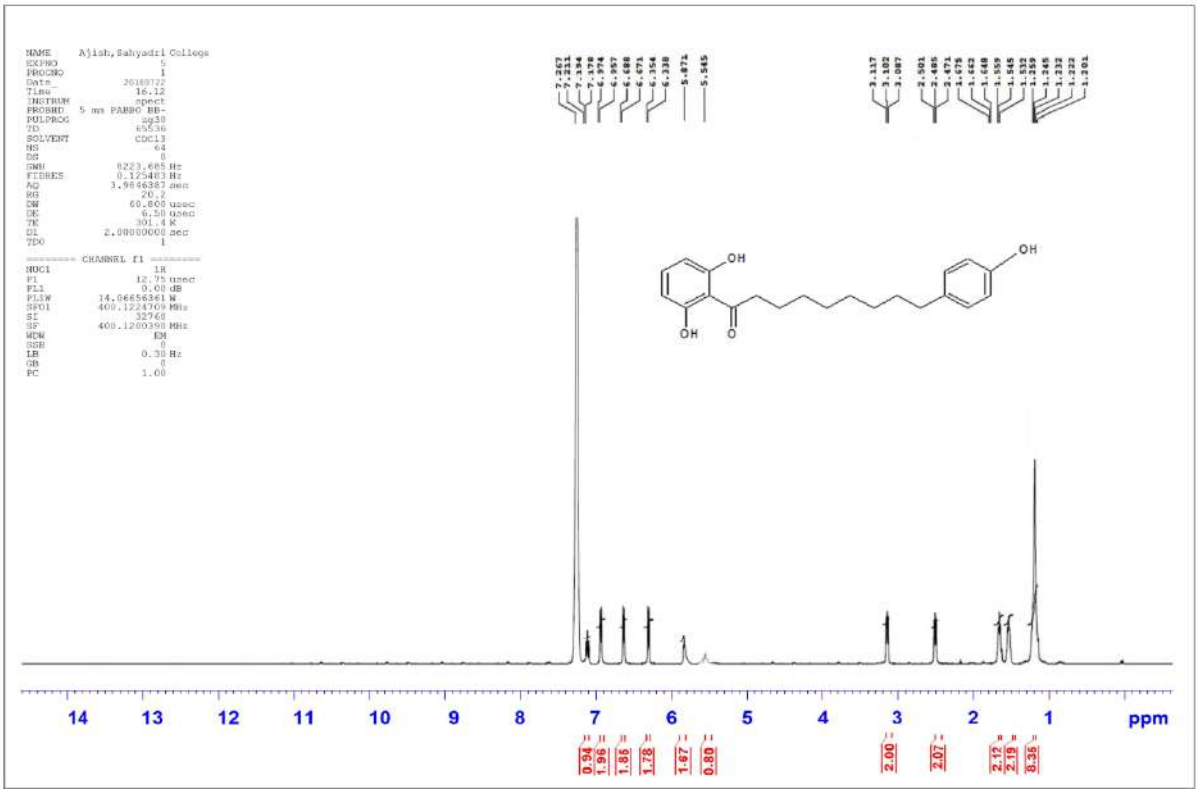


Fig: 6.2 ^{1}H NMR Spectrum of Compound I

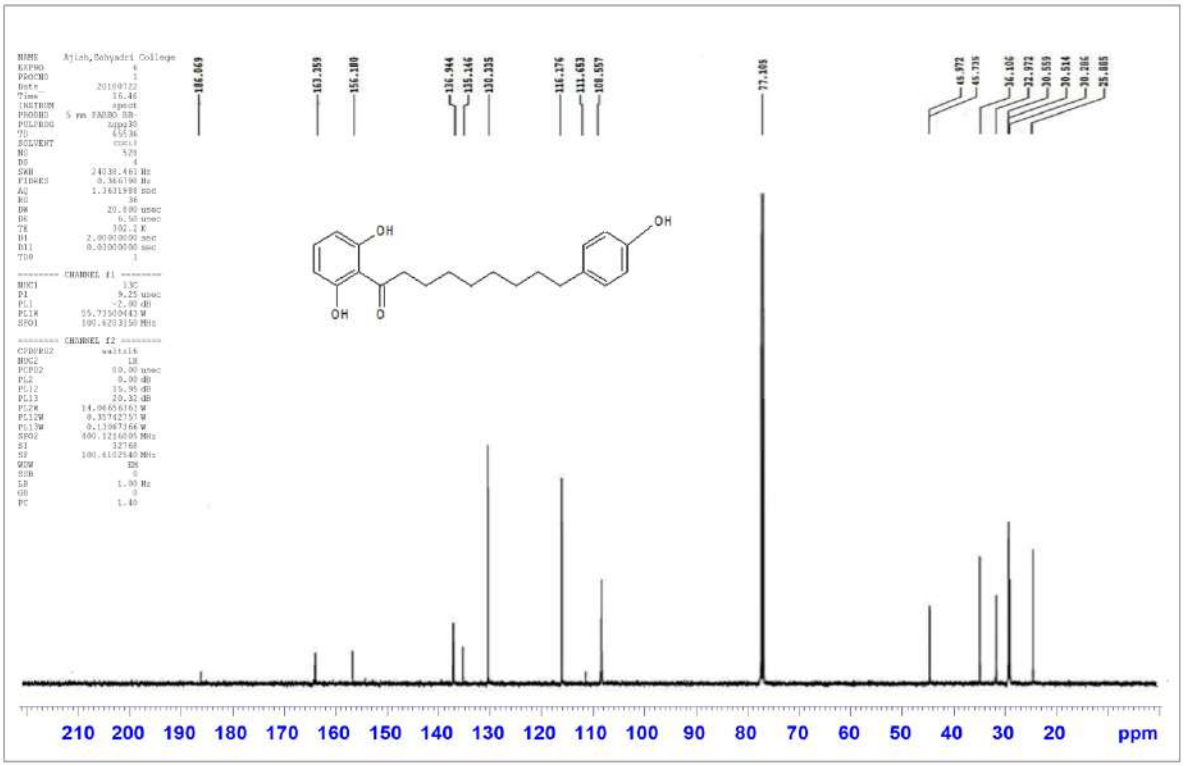


Fig: 6.3 ^{13}C NMR Spectrum of Compound I

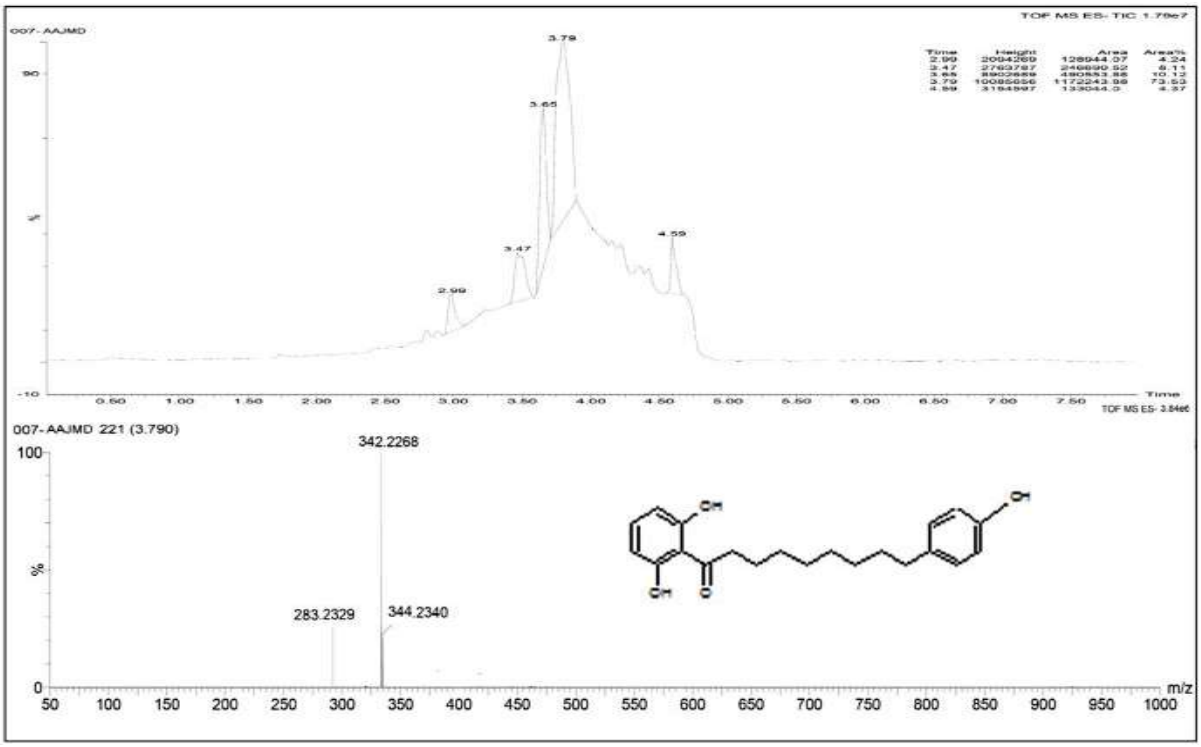
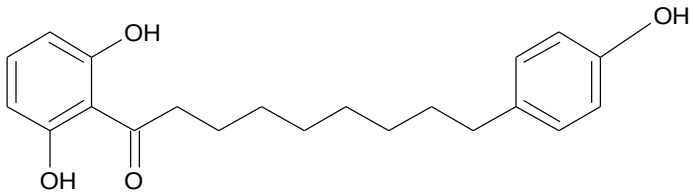


Fig: 6.4 LC-MS Spectrum of Compound I

By considering all the above data such as melting point, elemental analysis and spectral studies the following structure has been assigned to compound I.



IUPAC Name: 1-(2,6-Dihydroxyphenyl)-9-(4-hydroxyphenyl)-1-nonanone;

Common Name: Malabaricone B

6.4.3 Biological Importance of Malabaricone B

Malabaricone B is an antimicrobial resorcinol. This compound exhibits antibacterial, antifungal, radical scavenging activity[1] and anti-quorum sensing activity[2].

6.4.4 Charecterization of Compound II

- **Melting point**

  Melting point was recorded in an open capillary tube. The Melting point of compound II was found to be 126-129°C.

- **Elemental analysis**

  The elemental analysis of the isolated compound-I was carried to know the percentage composition of the elements present in the compound. The data of analysis is recorded in the table 6.3.

Table: 6.3-Summary of elemental analysis

Compound	Molecular formula	Molecular Weight in g/mol	Melting Point in °C	Elemental Analysis (Calculated %)		
				C	H	O
Compound-II	$C_{21}H_{26}O_5$	358.43	126-129°C	70.36 (70.37%)	7.32 (7.31%)	22.33 (22.32%)

The elemental analysis of the compound indicated the following elemental composition. Carbon = 70.37%, Hydrogen = 7.31%, Oxygen = 22.32%. The mass was found to be = 358.43. Based on the data, the following formula has been assigned to compound-II.

Molecular formula = $C_{21}H_{26}O_5$

Molecular weight = 358.43

6.4.5 Spectroscopic Study

6.4.5.1 Infrared spectrum

The infrared spectrum of the compound was recorded in KBr in the range of 4000-400 cm^{-1} on Unicam FTIR (Research Spectrometer Series). The IR-spectrum (fig: 6.5) the peak observed at the region of 1070 cm^{-1} is due to the presence of (-C-O-C-) ether functional group. A strong absorbance frequency at 1700 cm^{-1} is observed for the presence of carbonyl group (-C=O). The broad absorption band at 3380cm^{-1} indicating the presence of phenolic (–OH) group.

6.4.5.2 Proton Magnetic Resonance Spectrum (^{1}H NMR)

The magnetic resonance spectrum of the compound was recorded on AV 400 FT NMR Spectrophotometer (400 MHz) using TMS as an internal standard. Samples were dissolved in $CDCl_3$. The ^{1}H NMR spectrum (fig: 6.6) appearance of peak at δ 1.21-3.11 values confirmed the presence of $–CH_2$ protons. The peak appeared at δ 5.26-5.75 confirmed the presence of phenolic (–OH) protons. The peak at δ 6.32-7.26 for aromatic protons.

6.4.5.3 ^{13}C Nuclear Magnetic Resonance Spectrum (^{13}C NMR)

In the ^{13}CNMR spectrum (Fig: 6.7) The aliphatic carbons appeared at δ 25.88-36.10. The peaks appeared at δ 108.57-163.39 confirms the presence of aromatic carbons. The peak appeared at δ 186.06 confirms the presence of carbonyl carbon (–C=O).

6.4.5.4 Mass Spectrum

The mass spectrum (Fig: 6.8) was recorded in Auto Spec E_l mass Spectrometer at 70eV ionisation energy with direct inlet system. The mass spectrum (Fig: 6.4) confirms the molecular weight of compound II is m/z 358.

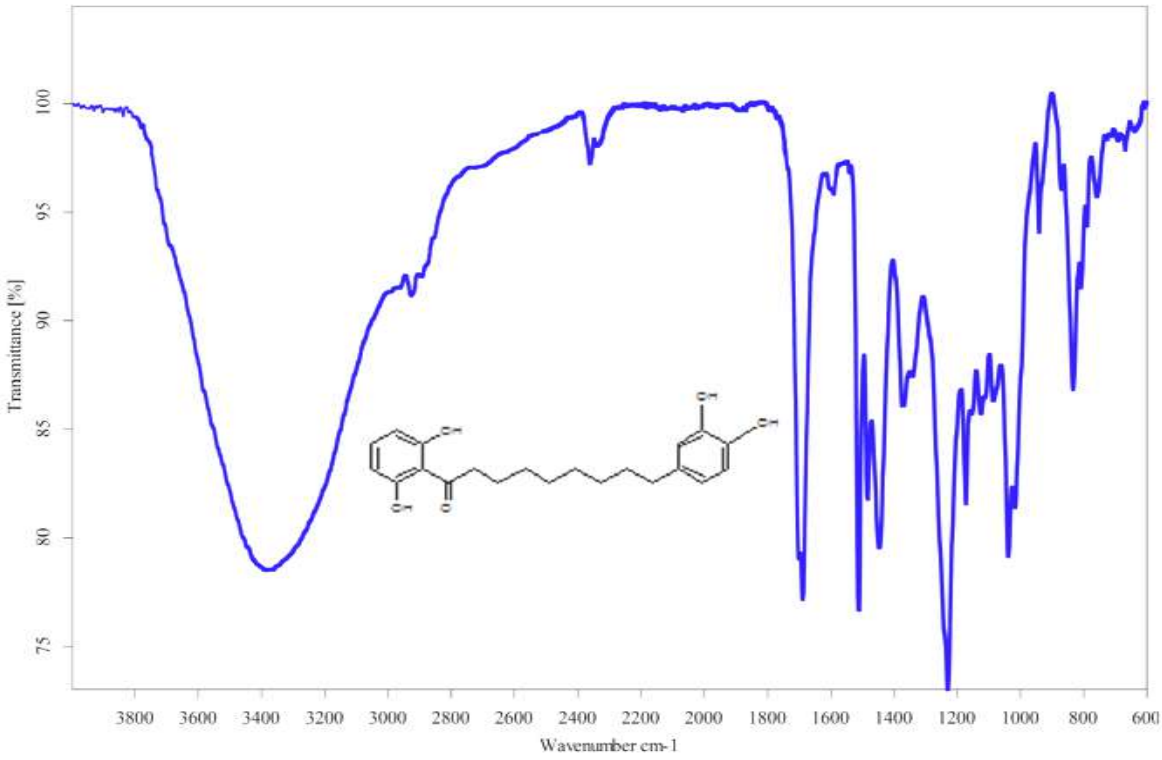


Fig: 6.5 IR Spectrum of Compound II

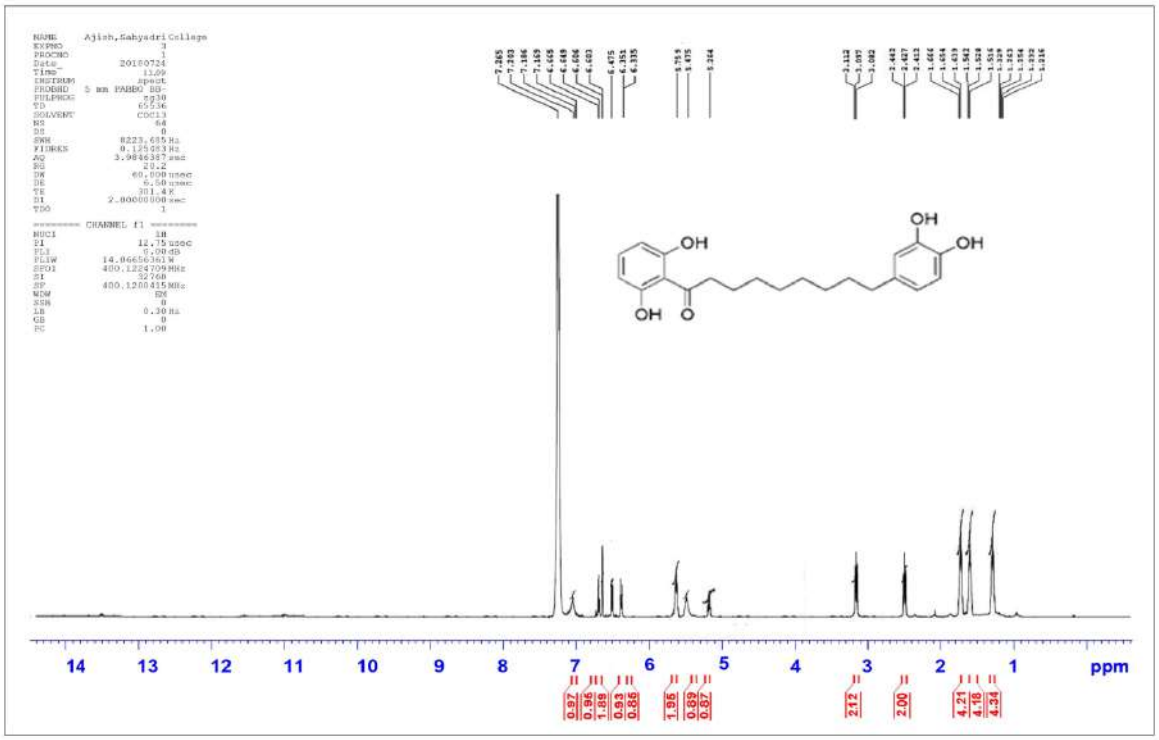


Fig: 6.6 ^{1}H NMR Spectrum of Compoun II

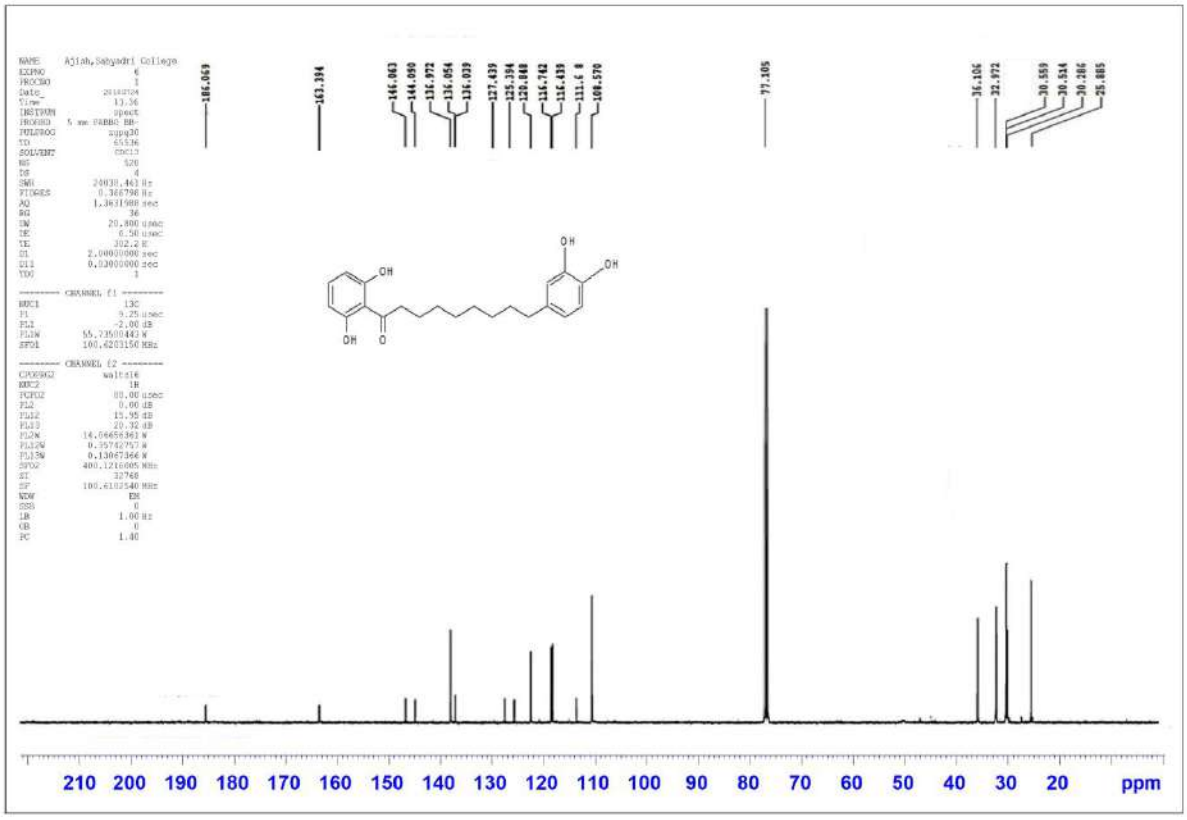


Fig: 6.7 ^{13}C NMR Spectrum of Compound II

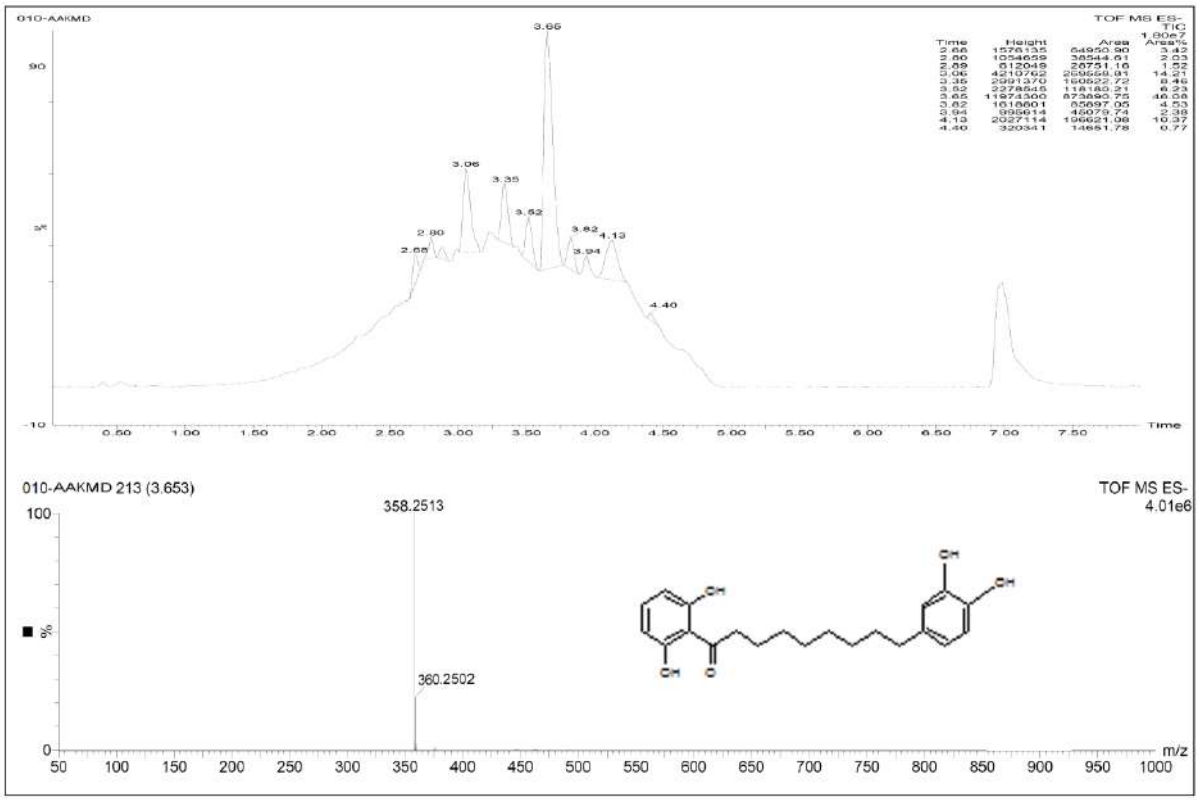
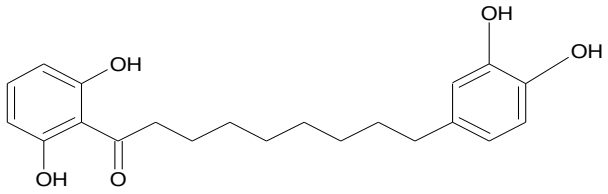


Fig: 6.8 LC-MS Spectrum of Compound II

By considering all the above data such as melting point, elemental analysis and spectral studies the following structure has been assigned to compound II.

Structure of compound II is,



IUPAC Name: 1-(2,6-dihydroxyphenyl)-9-(3,4-dihydroxyphenyl)nonan-1-one

Common Name: Malabaricone C

6.5 Biological Importance of Malabaricone C

Malabaricone A-D was first reported as constituents of M. malabarica[3]. Another study reported the healing power of malabaricone C isolated from M. malabarica against indomethacin-induced gastric ulceration in mice. Malabaricone C was effective in controlling mucin secretion, PGE(2) synthesis and expression of EGF receptor and COX isoforms[4]. The relative healing capacities of malabaricone B and malabaricone C of indomethacin-induced gastric ulceration correlated well with their respective abilities to modulate the angiogenic factors[5]. Malabaricone C from M. malabarica showed maximum DPPH scavenging activity and it could prevent both Fe(II)- and 2,2'-azobis(2amidinopropane) dihydrochloride-induced lipid peroxidation (LPO) of rat liver mitochondria[6]. Another study reported that malabaricone C exhibited stronger antioxidant activity than the commonly used synthetic antioxidant BHT[7]. Furthermore, malabaricones A and B isolated from M. malabarica exhibited antileishmanial activity[8]. An earlier study reported the isolation of two resorcinols, malabaricones B and C from the dried seed covers of M. fragrans which exhibited strong antifungal and antibacterial activities[9]. A recent study reported that malabaricone C from M. fragrans exhibited an anti-inflammatory effect through the inhibition of NF-κB activation by inhibiting interconnected ROS/Akt/IKK/NF-κB signaling pathways[10]. The molecular mechanism of the antiinflammatory activity of a natural diarylnonanoid, malabaricone-C has been reported[11]. Furthermore, malabaricone-C from nutmeg (*M. fragrans*) inhibits PDGF-induced proliferation and migration of aortic smooth muscle cells through induction of heme oxygenase-1[12]. *M. fragrans* was also reported as an aphrodisiac agent[13] and an antidepressant[14]. Malabaricone-C exhibited cytotoxicity (IC50=5.26±1.20 μM) against the MCF-7 human breast cancer cell line. The malabaricone-C induced killing of the

MCF-7 cells followed an apoptotic pathway involving oxidative damage to the cellular DNA[15]. Malabaricone-C isolated from *Myristica cinnamomea* inhibited violacein production by Chromobacterium violaceum CV026 and it also inhibited the quorum sensing regulated pyocyanin production and biofilm formation in Pseudomonas aeruginosa PAO[16]. Malabaricones B and C isolated from *Myristica crassa* were reported to possess significant inhibitory activity on acetylcholine esterase[17]. Malabaricone-C exhibited high nematocidal activity against Toxocara canis with MLC of 6-10µM[18]. Myristica maingayi afforded malabaricones B and C with IC50values of 3 and 4mg/ml, respectively[19]. *Myristica gigantea*[20] and *Myristica dactyloides*[21] also afforded malabaricones B and C which have high cytotoxicity activity against breast carcinoma (MCF-7) cells line[22].

6.6 Isolation and Characterization of active Components from *Hopea ponga (Dennst.)* The ethyl acetate extract of bark of *Hopea ponga (Dennst.)* exhibited considerable antioxidant, antifungal and cytotoxic activities. These observations encouraged us to isolate and identify bioactive molecules present in this extract.

Many separation techniques are available to accomplish purification and isolation of active components from extracts of plants. In the present investigation, the following techniques were adopted to achieve the isolation of pure components from the ethyl acetate extracts of *Hopea ponga (Dennst.)*.

- Thin Layer Chromatography [TLC]
- Conventional Column Chromatography

6.7 Thin Layer Chromatography [TLC]

Thin Layer Chromatography (TLC) is a technique used for separation of components. It is colour fingerprint method, which is most widely used for quick and easy determination of quality and authenticity of the crude drugs. The separation is based on the differences in adsorption co-efficient of the individual components of a mixture. Components which are strongly adsorbed in the stationary phase move up less readily than those which are adsorbed to a lesser extent, leading to the separation of the compound.

TLC is a mode of chromatography in which sample is applied as a small spot on to the origin of a thin sorbent layer (Silica gel) supported on a glass or metal plate. The mobile phase moves through stationary phase by capillary action, sometimes assisted by gravity or pressure. Mobile phase consists of a single solvent or mixture of solvents.

Thin layer chromatography of ethyl acetet extract of bark of *Hopea ponga (Dennst.) w*as carried out to select appropriate mobile phase to achieve reasonably good separation in column chromatography.

For TLC, glass sides (Size 7.5 cm in length and 2.5 cm in width) were cleaned to remove greasy material and dried in oven for an hour at 70-80 ˚C. The slurry of silica gel G, which was used as adsorbent, was prepared by dissolving silica gel G in freshly distilled chloroform. The glass slides were coated with the slurry and excess of chloroform was allowed to evaporate. TLC was performed by using various solvents or mixture of solvents of varying polarity on these glass plates. It was observed that, mixture of chloroform and methanol in varying proportion gave good separation.

6.7.1 Optimization of TLC Solvent system

Different solvent systems were tried for developing TLC for the identification of constituents present in the methanol extract. One showing maximum and clear separation was selected as mobile phase for the study. The solvent systems used was

- Chloroform : Methanol

The detecting agents used were-

- UV at 254 nm and 366 nm
- Iodine vapors

6.7.2 Developing Thin Layer Chromatogram

The sample was dissolved in methanol and spotted on the pre-coated TLC plate. The chromatogram was developed using the above solvent systems and the spots were visualized under UV at 254 nm and iodine vapors. After a number of trial and error, it was observed that, mixture of pet ether and ethyl acetate in the ratio 95:5 by volume showed clear separation of components.

6.8 Column Chromatography

The isolation of pure components involved the following steps

- Chromatographic separation using silica gel (100-200 mesh)
- Chromatographic separation using silica gel (60-120 mesh)

Column chromatography is one of the most useful methods for the separation and purification of both solids and liquids in which it is possible to isolate desired compounds from a mixture. Column chromatography is a solid-liquid separation technique in which the two phases are a solid (stationary phase) and a liquid (moving phase). The sample is

dissolved in a small quantity of solvent and placed at the top of the column. There exists an equilibrium between the solute adsorbed on adsorbent and the eluting solvent flowing down through the column. The most common adsorbents are silica gel and alumina.

The ethyl acetate extract of *Hopea ponga (Dennst.)* was subjected to column chromatography for isolation of active compounds using pet ether-ethyl acetate solvent system. The isolation of pure components was done by column chromatography using silica gel 60-120 mesh.

6.8.1 Chromatographic separation using silica gel (60 -120 mesh)

The ethyl acetate extract of bark of (10 g) of *Hopea ponga (Dennst.)*was subjected to column chromatographic separation by using chloroform and methanol mixture of various proportions as mobile phase or the chromatographic separation, a column of length 55 cm and diameter of 6 cm was cleaned and dried. Stationary phase was prepared from Silica gel (60-120 mesh) in freshly distilled chloroform and loaded in the column. The column was gently tapped to ensure uniform packing of the column. A small quantity of solvent was allowed to remain at the top of the column to avoid the drying or cracking of the column. The column was left for overnight before charging the extract for complete saturation and removal of air bubbles to make the bed static.

The ethyl acetate extract (5g) was dissolved in minimum amount of distilled ethyl acetate and loaded over the stationary phase in the column. The column was eluted with chloroform and methanol mixture of various proportions as mobile phase. During elution the polarity of mobile phase was increased gradually by varying the proportions of pet ether and ethyl acetate. The elution was monitored by TLC and the eluted fractions were collected in 100 ml portions. The fractions with similar spots were pooled together. From the collected fractions one pure fraction was identified and separated. The details of column chromatography are given in the below table 6.4.

Table 6.4 The chromatographic details of chloroform extract of bark of *Hopea ponga (Dennst.)* (100-200 mesh)

Fractions	Eluent (Pet ether- Ethyl acetate)	Colour and consistency of Compounds isolated	Yield in mg
1	100:0	Light yellow oily liquid	30
2	95:05	Yellow pasty solid	60
3	90:10	Light brown pasty solid	150
4	85:15	Light yellow solid	negligible
5	80:20	Yellow-Brown pasty solid	20
6	70:30	Brown pasty solid	1050
7	60:40	Yellow pasty solid	35
8	50:50	No Residue	----
9	40:60	Dark brown pasty solid	40
10	30:70	No Residue	----
11	20:80	Brown oily liquid	10
12	10:90	No Residue	----

Among the obtained fractions, fraction 6 have got high yield than the other fractions. Hence the sixth fraction has been taken for the characterization. The fraction **6** is labelled as compound **III**.

6.9 Characterization of isolated compound from the bark extracts of *Hopea ponga (Dennst.)*

The isolated compound III, which is a brown pasty solid, was characterized by physical parameters such as melting point, elemental analysis and spectral studies.

6.9.1 Characterization of Compound III

- **Melting point**

Melting point was recorded in an open capillary tube. The Melting point of the compound III was found to be 259-262°C.

- **Elemental analysis**

The elemental analysis of the isolated compound III was carried to know the percentage composition of the elements present in the compound. The data of analysis is recorded in the table 6.5.

Table: 6.5 Summary of elemental analysis

Compound	Molecular formula	Molecular Weight in g/mol	Melting Point in °C	Elemental Analysis (Calculated %)		
				C	H	O
Compound-I	$C_{28}H_{22}O_6$	454.47	259-262°C	73.99 (74.00%)	4.88 (4.88%)	21.13 (21.12%)

The elemental analysis of the compound indicated the following elemental composition. Carbon = 74.00%, Hydrogen = 4.88%, Oxygen = 21.12%. The mass was found to be = 342.43. Based on the data, the following formula has been assigned to compound I.

Molecular formula = $C_{28}H_{22}O_6$

Molecular weight = 454.47

6.9.2 Spectroscopic Study

6.9.2.1 Infrared spectrum

The infrared spectrum of the compound was recorded in KBr in the range of 4000-400 cm^{-1} on Unicam FTIR (Research Spectrometer Series). In the IR-spectrum (fig: 6.9) The peak observed at the region of 1010 cm^{-1} is due to the presence of (-C-O-C-) ether functional group. The strong absorbance frequency at 1600 cm^{-1} is observed for the presence of alkene group (-C=C-). A broad absorption band at 3250 cm^{-1} indicates the presence of phenolic (–OH) group.

6.9.2.2 Proton Magnetic Resonance Spectrum (^{1}H NMR)

The magnetic resonance spectrum of the compound was recorded on AV 400 FT NMR Spectrophotometer (400 MHz) using TMS as an internal standard. Samples were dissolved in $CDCl_3$. In the ^{1}H NMR spectrum (fig: 6.10) the appearance of peak at δ 3.71-4.01 confirmed the presence of alkene -C=C- protons. The peak appeared at δ 5.38-5.65 confirmed the presence of phenolic (–OH) protons. The aromatic protons appeared at δ 6.10-7.27.

6.9.2.3 ^{13}C Nuclear Magnetic Resonance Spectrum

In the ^{13}CNMR spectrum (fig: 6.11) the peaks appeared at δ 58.85 confirms the presence of aliphatic carbons. The peak appeared at δ 102.51-161.06 indicate the presence of alkene carbons.

6.9.2.4 Mass Spectrum

The mass spectrum was recorded in Auto Spec E_l mass Spectrometer at 70eV ionisation energy with direct inlet system. The mass spectrum (Fig: 6.12) confirms the molecular weight of compound to be m/z 454.47.

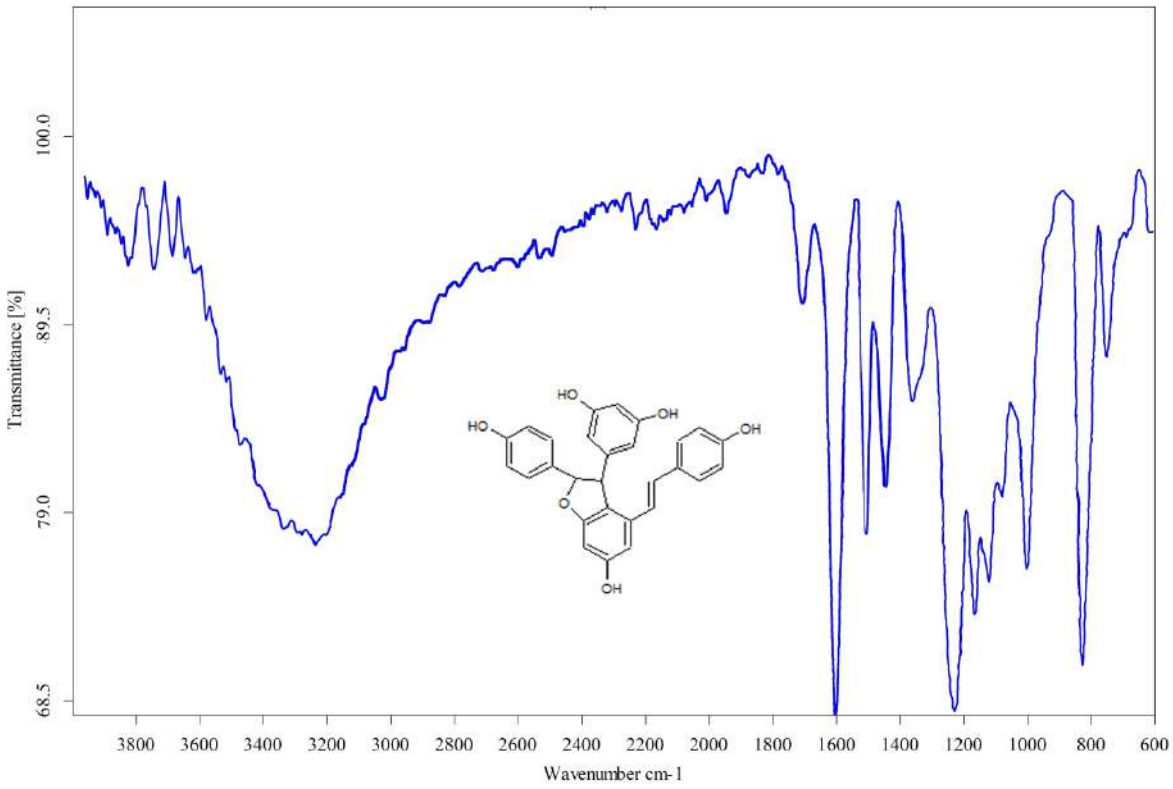


Fig: 6.9 IR Spectrum of Compound III

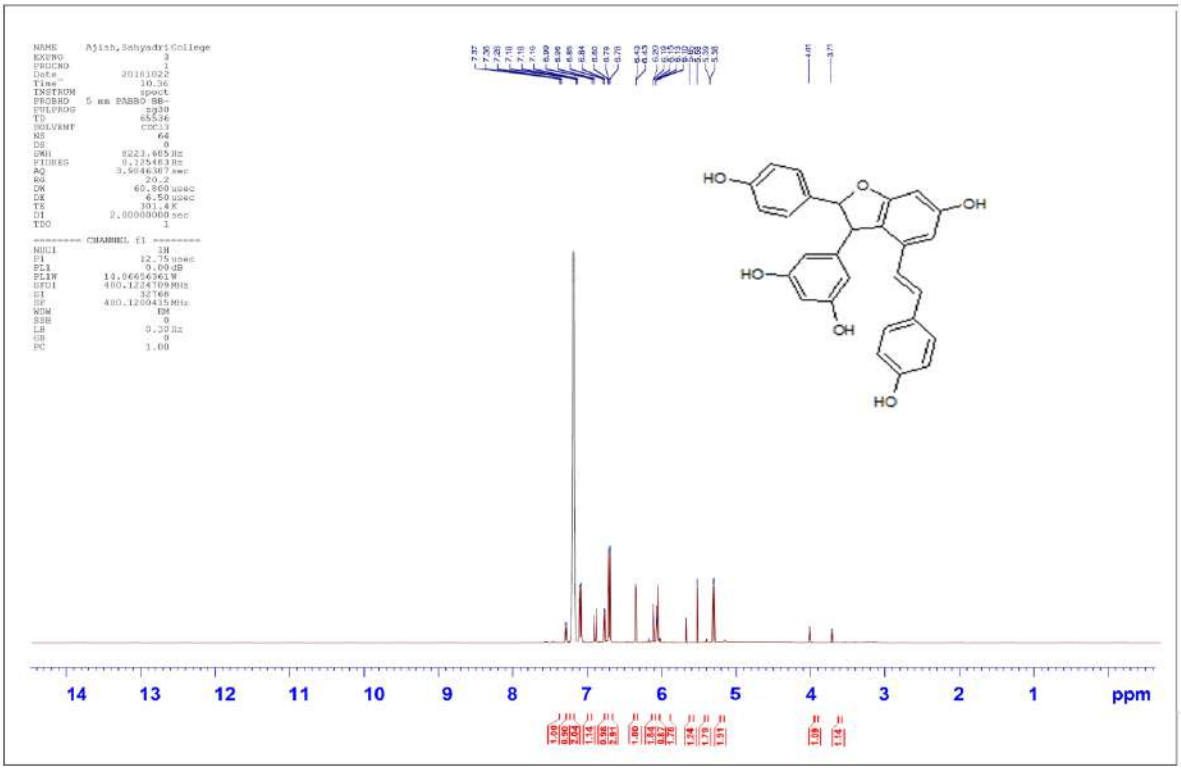


Fig: 6.10 ^{1}H NMR Spectrum of Compound III

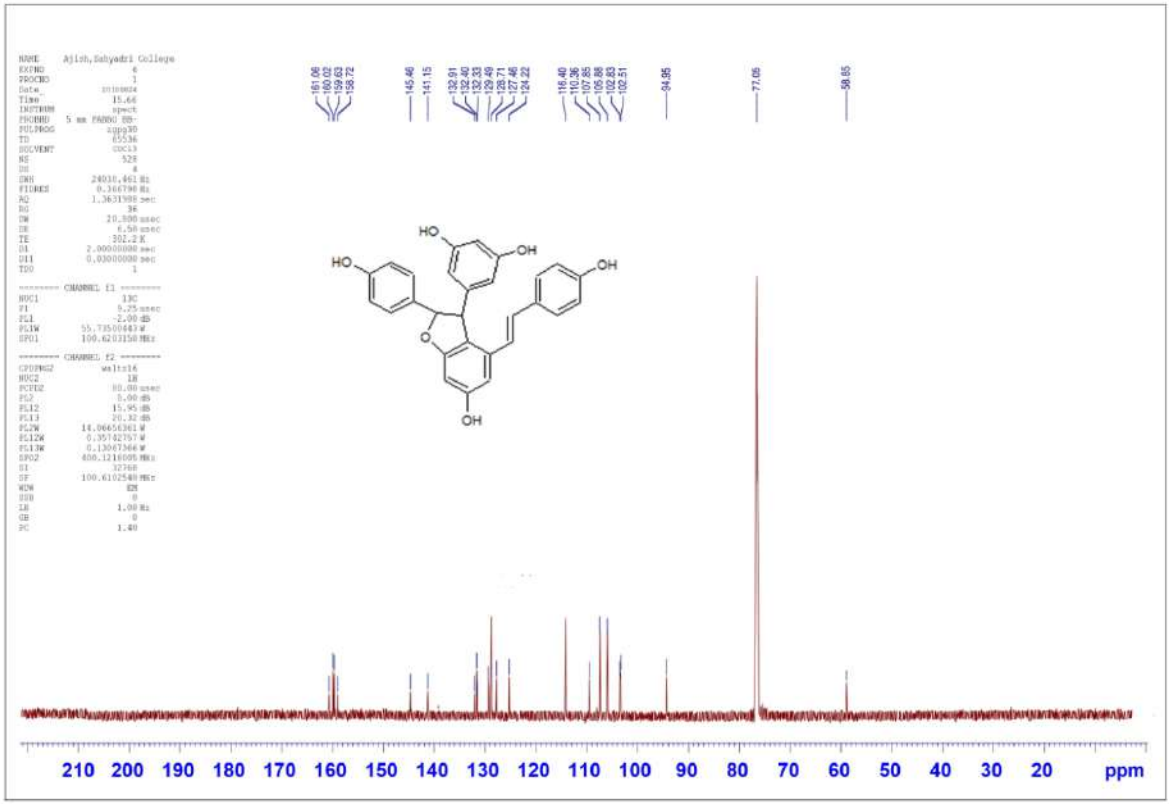


Fig: 6.11 ^{13}C NMR Spectrum of Compound III

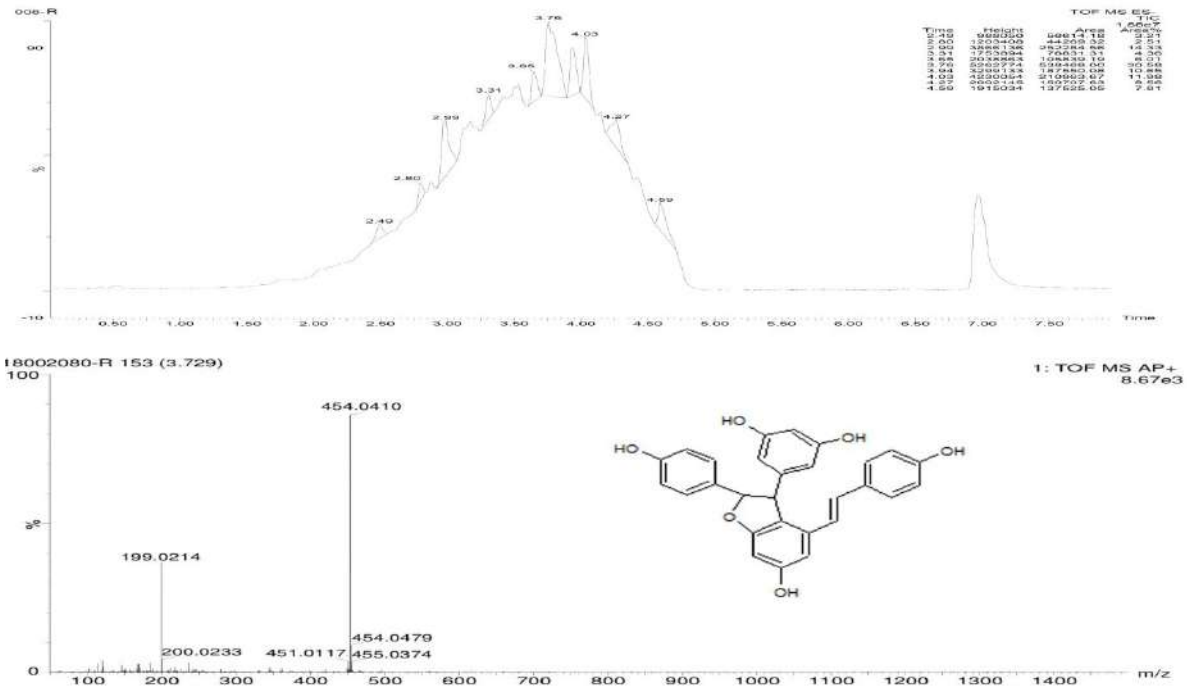
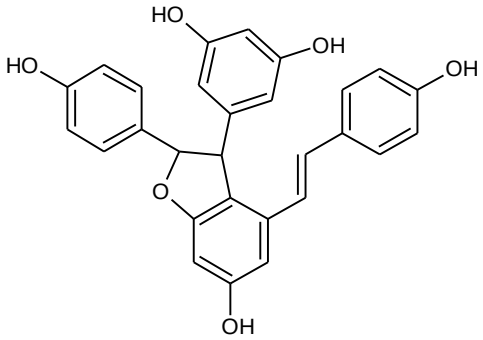


Fig: 6.12 LC-MS Spectrum of Compound III

By considering all the above data such as melting point, elemental analysis and spectral studies the following structure has been assigned to the compound III.



IUPAC Name:5-[(2*R*,3*R*)-6-Hydroxy-2-(4-hydroxyphenyl)-4-[(*E*)-2-(4-hydroxyphenyl)ethenyl]-2,3-dihydro-1-benzofuran-3-yl]benzene-1,3-diol

Common Name: Viniferin

6.10 Biological Importance of viniferin.

Viniferin is a resveratrol dimer. It is well known for its extensive bioactivities, such as antioxidation, anti-inflammatory and anticancer[23].

References

1. B.S. Patro, A.K. Bauri, S. Mishra, S.J. Chattopadhyay, Antioxidant activity of *Myristica malabarica* extracts and their constituents, **2005.**
2. Y.M. Chong, W.F. Yin, C.Y, Ho M.R. Malabaricone C from *Myristica cinnamomea* exhibits anti-quorum sensing activity. *Journal of Natural Product,* **2011.**
3. K.K. Purushothaman, A. Sarada, J.D. Connolly, *J. Chem. Soc., Perkin Trans.*, 1, 587, **1977**.
4. D Banerjee, A.K Bauri, R.K Guha, S.K Bandyopadhyay, S. Chattopadhyay, Eur. J. Pharmacol., 578, 300, **2008**.
5. D. Banerjee, B. Maity, A.H. Bandivdeker, S.K. Bandyopadhyay, S. Chattopadhyay, *Pharm. Res.,* 25, 1601, **2008.**
6. B.S. Patro, M. Tyagi, J. Saha, *Bioorg. Med. Chem.,* 18, 7043, **2010**.
7. J.P. Hou, W. Hou, Y. Wang, X.C. Weng, *Czech J. Food Sci.,* 30, 164, **2012**.
8. R. Sen, A.K. Bauri, S. Chattopadhyay, M. Chatterjee, *Phytother. Res.,* 21, 592, **2007**.
9. K.Y. Orabi, J.S .Mossa, F.S. el-Feraly, *J. Nat. Prod.,* 54, 856, **1991**.
10. J. Kang, N. Tae, B.S. Min, *Int. Immunopharmacol.,* 14, 302, **2012**.
11. B. Maity, S.K. Yadav, B.S. Patro, M. Tyagi, S.K. Bandyopadyay, S Chattopadhyay, *Free Radical Biol. Med.,* 52, 1680, **2012**.
12. S. Lee, J. Seo, S. Ryoo, T.D. Cuong, B.S. Min, J.H. Lee, *J. Cell Biochem*., 113, 2866, **2012**.
13. K.J. Pallavi, R. Singh, S. Singh, K. Singh, M. Farswan, V. Singh, *J. Chem. Pharm. Res*. 3, 911, **2011**.
14. H.K. Dhamija, B. Parashar, J. Singh, *J. Chem. Pharm. Res*., 3, 725, **2011**.
15. B.S. Patro, A.K. Bauri, S. Mishra, S. Chattopadhyay, *J. Agric. Food Chem*., 53, 6912, **2005**.
16. Y.M. Chong, W.F. Yin, C.Y. Ho, M.R. Mustafa, A. Hamid, A. Hadi, K. Awang, P. Narrima, C.L Koh, D.R. Appleton, K.G. Chan, *J. Nat. Prod*., 74, 2261, **2011**.
17. A. Maia, I. Schmitz-Alfonso, M.T. Martin, K. Awang, O. Laprevote, F. Garitte, M. Litaudon, *Planta Med*., 74, 1, **2008.**
18. H. Shinzo, K. Fumiyuki, N. Norio, I. Masaaki, A.A. Mohammad, S. Youhei, T. Eriko, T. Yoshiyuki, K. Kaoru, T, Yoshisuke, *Chem. Pharm. Bull*., 47, 37, **1999.**
19. V.C. Pham, A. Jossang, T. Se´venet, B. Bodo, *Tetrahedron.*, 56, 1707, **2000.**

20. P.V. Cham, A. Jossang, T. Se´venet, B. Bodo, *Tetrahedron.*, 58, 5709, **2002**.

21. N.F. Cooray, E.R. Jansz, S. Wimalasena, T.P. Wijesekera, B.M. Nair, *Phytochem.*, 26, 3369, **1987**.

22. Megawati, Akhmad Darmawa, Resorcinol compounds isolated from the bark of *Myristica fatua houtt. Indonesian J. Pharm*. 28, 82, **2017**.

23. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, Clinical and Laboratory Standards Institute, January, **2012.**

CHAPTER-VII

Biological activities of pure compounds

7. Biological Activities of Pure Compounds

7.1 Introduction

The emergence of bacterial resistance to various antibiotics has increased the rate of infectious diseases worldwide, accounting for more than 41% of the global disease. The emergence of multidrug-resistant organisms has forced the scientist to search for new antimicrobial substance from various sources including medicinal plants. Herbal extracts are found to be more efficient, safer and better-quality drugs with improved antibacterial and antifungal activities. Based on the literature survey the compound I and II found to exhibit similar biological activities, which lead to the selection of pure compound II (Malabaricone C) of *Myristica dactyloides gaetrn* and compound III (Viniferin) of *Hopea ponga (Dennst.)* for carrying out the biological activities such as, antimicrobial, antioxidant and docking studies.

7.2 Antimicrobial activity of Compound II and III

7.2.1.1 Antibacterial activity by Agar diffusion method

The antibacterial activity of compound II and III was studied comparatively with that of standard Ciprofloxacine by Agar diffusion method. The results of antibacterial activity of compound II and III are summarized in table 7.1.

Table 7.1: Antibacterial activity of compound II and III.

Organisms	Std					Compound-II					Compound-III				
	50	100	250	500	1000	50	100	250	500	1000	50	100	250	500	1000
S. aureus	13	18	21	25	27	0	3	5	6	8	0	7	12	10	17
P. vulgaris	9	13	18	21	25	0	2	4	4	5	0	4	7	7	10

Result and Discussion

In the present study, compound II exhibited significant activity against *Staphylococcus aureus* and moderate activity against *Proteus vulgaris*. Whereas compound III, exhibited significant activity against *Staphylococcus aureus,* and moderate activity against *Proteus vulgaris*.

7.2.1.2 Antibacterial activity by MIC, Micro dilution method

The antibacterial activity of compound II and III was studied by MIC method, methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically[36]. The results of antibacterial activity are summarized in table 7.2.

Table 7.2: Antibacterial analysis by MIC, Micro dilution method of compound II and III.

Sample	Bacterial strains		Standard
	S. aureus(MIC in μg)	*P.vulgaris*(MIC in μg)	Ciprofloxacin
Compound II	25	25	25
Compound III	100	25	

Result and Discussion

The results of Minimum Inhibitory Concentration (MIC) recorded in table 7.2 indicates that, the compound III was more potent to *S.aureus* and *p.vulgaris.*

7.3 Antifungal activity by MIC method

The antifungal activity of compound II and III was studied by MIC method, methods for dilution antimicrobial susceptibility tests for fungi that grow aerobically. The results of antibacterial activity by MIC of compound II and III are summarized in table 7.3.

Table 7.3: Antifungal analysis by mic method of compound II and III.

Sample	*T. viride* MIC (μg)
Compound II	250
Compound III	250
Fluconazole	100

Result and Discussion

The results of Minimum Inhibitory Concentration (MIC) recorded in table 7.3 indicates that, the MIC of compound II and III was 250μg/ml.

7.4 Antioxidant activity

The antioxidant activity of extracts was carried out by DPPH (2,2-diphenyl-picryl-hydrazyl radical) scavenging assay. The results of antioxidant DPPH scavenging activity was summarized in table 7.4.

Table 7.4: Free radical scangenging activity of compound II and III.

Concentration in ml	% of free radical scavenging		
	Compound II	Compound III	BHA
0.01	9.15	7.92	54.27
0.05	13.63	13.96	70.10
0.1	23.83	22.16	91.82

Result and Discussion

The compound II has showed a moderate antioxidant activity of 23.83% inhibition, while the compound III with 22.83% inhibition at the concentration of 0.1ml.

7.5 Molecular Docking Studies

7.5.1 Antibacterial Docking

In this study, docking simulation is carried out for all the synthesized derivatives with the protein receptor SEC2 in order to gain information related to their appropriate binding orientation and to understand the nature of interaction between them. Docking of the compounds in the active site of the protein receptors are performed using HEX 8.0 software and discovery studio visualizer tools, The crystal structure of the protein receptor SEC2 (PDB code: 1STE) in Staphylococcus aureus were downloaded from RCSB protein data bank.

7.5.1.1 Antibacterial docking of Compound II and III.

Docking score -238.26 KJ/mol^{-1} Docking score -254.25 KJ/mol^{-1}

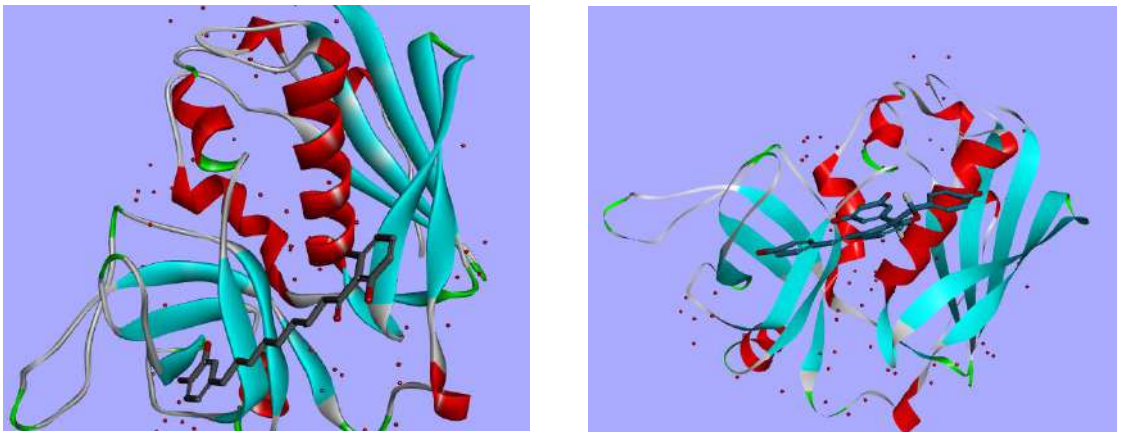


Fig: 7.1 and 7.2. 3D docking interactions with the protein receptor SEC2 (PDB code: 1STE) in *Staphylococcus aureus* for compound II and III.

Result and Discussion

Molecular Docking In association with *in-vitro* antimicrobial activity, it is useful to carry out in silico studies to predict the orientation and binding affinity at the active site of the receptor. The 3D docking interactions with the protein receptor SEC2 (PDB code:1STE) in *Staphylococcus aureus* is shown in Figure 7.1 and 7.2. Among them the compound III exhibited better docking efficiency with the least binding affinity -254.25 KJ/mol^{-1} and hence is considered as the best dock conformation.

7.5.2 Antioxidant docking

In this study, docking of the compounds in the active site of the protein receptors are performed using HEX 8.0 software and discovery studio visualizer tools, The human antioxidant enzyme in complex with the competitive inhibitor DTT (PDB: 3MNG) were taken from the Protein Data Bank (http:// www.rcsb.org/pdb).

7.5.2.1 Antioxidant docking of Compound II and III.

Docking score -314.65 KJ/mol^{-1} Docking score -311.02 KJ/mol^{-1}

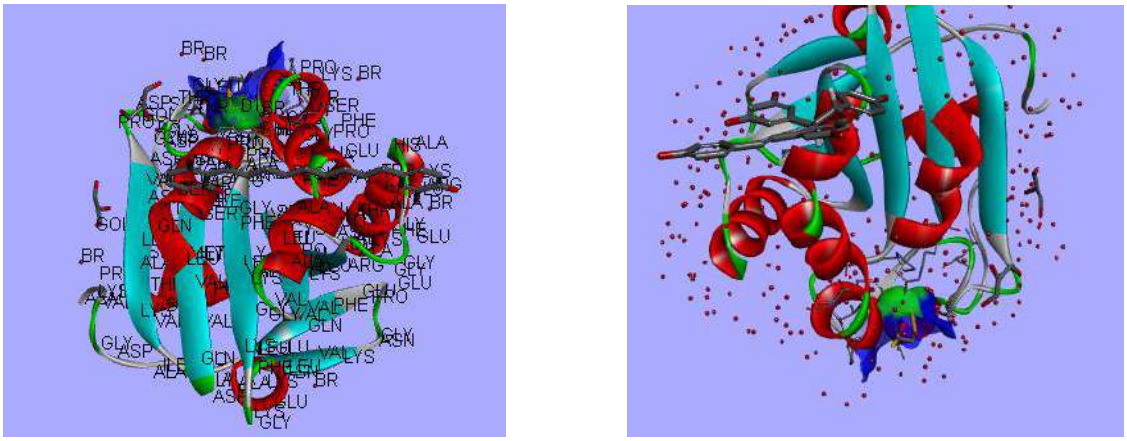


Fig: 7.3 and 7.4. 3D docking interactions with the protein receptor SEC2 (PDB code: 1STE) in *Staphylococcus aureus* for compound II and III.

Result and Discussion

Molecular Docking In association with *in-vitro* antioxidant activity, it is useful to carry out in silico studies to predict the orientation and binding affinity at the active site of the receptor. 3D docking interactions with the protein receptor SEC2 (PDB code:1STE) in *Staphylococcus aureus* is shown in Figure 7.3 and 7.4. Among them the compound II exhibited better docking efficiency with the least binding affinity -314.65 KJ/mol^{-1} and hence is considered as the best dock conformation.

7.5.3 Antitubercular Docking

In this study, docking of the compounds in the active site of the protein receptors are performed using HEX 8.0 software and discovery studio visualizer tools, receptor protein from m.tuberculosis, cAMP-free form (PDB:33D0S) were taken from the Protein Data Bank (http:// www.rcsb.org/pdb).

7.5.3.1 Antitubercular docking of Compound II and III.

Docking score -288.41 KJ/mol^{-1} Docking score -261.44 KJ/mol^{-1}

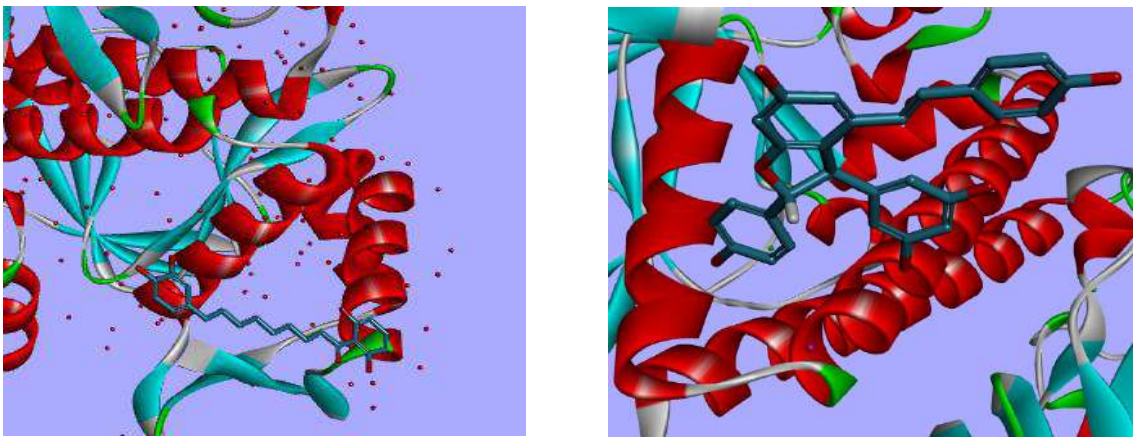


Fig: 7.5 and 7.6. 3D docking interactions with the protein receptor of *M.tuberculosis,* cAMP-free form (PDB code: 3D0S) for compound II and III.

Result and Discussion

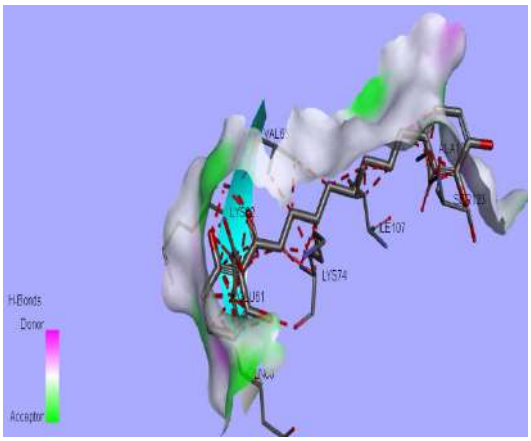
Molecular Docking In association with antitubercular activity, it is useful to carry out in silico studies to predict the orientation and binding affinity at the active site of the receptor. 3D docking interactions with the protein receptor of *M.tuberculosis*, cAMP-free form (PDB code: 3D0S) is shown in Figure 7.5 and 7.6. Among them the compound II exhibited better docking efficiency with the least binding affinity -288.41 KJ/mol^{-1} and hence is considered as the best dock conformation.

7.4.4. Cytotoxicity docking

In this study, docking of the compounds in the active site of the protein receptors are performed using HEX 8.0 software and discovery studio visualizer tools, dimeric variant of human pancreatic ribonuclease with high cytotoxic and antitumor activities (PDB:3F8G) were taken from the Protein Data Bank (http:// www.rcsb.org/pdb).

7.4.4.1.Cytotoxicity docking of Compound II and III.

Docking score -358.12 KJ/mol^{-1} Docking score -341.22 KJ/mol^{-1}



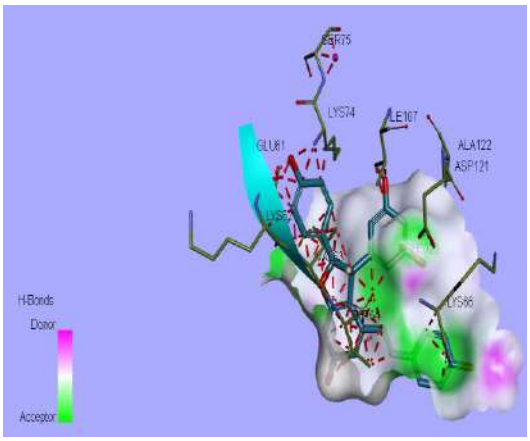


Fig: 7.7 and 7.8. 3D docking interactions with the protein receptor (PDB code: 3F8G) for compound II and III.

Result and Discussion

Molecular Docking In association with anticancer activity, it is useful to carry out in silico studies to predict the orientation and binding affinity at the active site of the receptor. 3D docking interactions with the protein receptor (PDB code: 3F8G) is shown in Figure 7.7 and 7.8. Among them the compound II exhibited better docking efficiency with the least binding affinity -358.12 KJ/mol^{-1} and hence is considered as the best dock conformation.

Altogether, this pioneering study was used to preliminarily investigate the potential compounds (drug candidates) from natural products and conventional docking study to analyze the best docked ligands permitted us to know the binding mode of compounds. Binding energies of the drug target interactions are important to describe how fit the drug binds to the target. Further studies are essential to explore the target specific effect of these natural compounds on various signalling pathways, mode of action in various brain regions, the ability to cross the blood brain barrier and the mechanism behind the synergistic action of the antimicrobial, antioxidant, antitubercular and anticancer agents on the target.

CHAPTER-VIII

Conclusion

Conclusion

Natural product once served as the only source of medicines for mankind. Screening of natural products, especially those having medicinal use, structure determination and biological activity has been an important aspect in chemistry. A good number of bioactive compounds isolated from medicinal plants and their semi synthetic and synthetic analogs have found to have wide application in chemotherapy. Some of the synthetic derivatives have by far surpassed the naturally occurring moieties due to their applicability in various fields. In recent years, the chemistry of natural products have been extended to enormous length and as a result large number of bioactive natural products, their lead and synthetic derivatives have been synthesized. The following conclusions can be drawn from the results obtained from the investigation on medicinal plants ***Myristica dactyloides Gaertn*** and ***Hopea ponga (Dennst.)***

The results of phytochemical investigations and biological activities of *Myristica dactyloides Gaertn* bark extracts revealed that,

- The phytochemical investigation of *Myristica dactyloides Gaertn* revealed the presence of alkaloids, steroids, carbohydrates, flavonoids and glycosides.
- The methanol extract showed a potent antibacterial activity against all the organisms, whereas, pet ether and ethyl acetate extracts showed moderate antibacterial activity.
- The MIC values of antibacterial activity suggested that, the pet-ether extract inhibited the growth of *S. aureus* and *P. fluorescens* at the lowest concentration of 25μg/ml and 50μg/ml.
- The antifungal activity results showed that, the fungal strain *T.viride* was highly susceptible to pet ether extract.
- The pet ether extracts of *Myristica dactyloides Gaertn* was more effective towards *M. tuberculosis* at a concentration of 50μg/ml, H_{37}Rv than ethyl acetate and methanol extracts.
- The pet ether extract of *Myristica dactyloides Gaertn* exhibited strong DPPH radical scavenging activity.

- The pet ether and ethyl acetate extract was highly toxic to SiHa cell line, whereas methanol extract was toxic to Hela cell line MDA-MB cell lines respectively.
- The DNA cleavage activity demonstrates that ethyl acetate and methanol extract cleaved the DNA whereas pet ether extract has shown partial cleavage
- Malabaricone B and Malabaricone C were isolated from pet ether extract of *Myristica dactyloides Gaertn*, which is known for its anticancer, antioxidant, antimalarial and anti-quorum sensing activities.
- The results of the antimicrobial, antioxidant and molecular docking studies shows that the compound II is a potent biologically active molecule.

The results of phytochemical investigations and biological activities of *Hopea ponga (Dennst.)* bark extracts revealed that,

- The phytochemical investigation of *Hopea ponga (Dennst.)* revealed the presence of alkaloids, steroids, saponins, flavonoids, steroids, carbohydrates, tannins and phenolics.
- The ethyl acetate extract has shown excellent antibacterial activity on both Gram positive and Gram negative bacteria, whereas, pet ether and methanol extracts showed moderate antibacterial activity.
- The MIC values of antibacterial activity suggested that, the ethyl acetate extract inhibited the growth of *S. aureus* and *B.cereus* at the lowest concentration of 100µg/ml.
- The antifungal activity results showed that, the fungal strain *C.tropicalis* was highly susceptible to ethyl acetate extract.
- The ethyl acetate extract was more effective towards *M. tuberculosis*, H_{37}Rv than pet ether and methanol extracts.
- The ethyl acetate extract of exhibited strong DPPH radical scavenging activity.
- The pet ether and ethyl acetate extract was highly toxic to HeLa cell line but methanol extract was highly toxic to SiHa cell line.
- DNA cleavage activity demonstrates that pet ether and ethyl acetate cleaved the DNA, the methanol extract has shown partial cleavage.

- Viniferin was isolated from ethyl acetate extract of *Hopea ponga (Dennst.)*, which is known for its anticancer, antioxidant and anti-inflammatory activities.
- The results of the antimicrobial, antioxidant and molecular docking studies shows that the compound III is a potent biologically active molecule.

All the extracts of ***Myristica dactyloides Gaertn*** and ***Hopea ponga (Dennst.)*** possessed one or the other biological activities. The potent antimicrobial and antioxidant activity of methanol extract of ***Myristica dactyloides Gaertn*** may be due to the presence of Malabaricone B and Malabaricone C potent cytotoxic activity of ethyl acetate extract of *Hopea ponga (Dennst.)* may be attributed due to the presence of Viniferin. However, there is a need for screening of isolated molecules in both *in-vitro* and i*n-vivo* models to confirm their biological activities. The present study reveals that the crude drug posse's prominent antimicrobial, anthelmintic antioxidant, antitubercular, anticancer properties, which supports its folk claim. Phytochemical studies portray the presence of several biologically active secondary metabolites. Therefore, there is no doubt that these plants are a reservoir of potentially useful chemical compounds, which serve as drugs, provide newer leads and clues for modern drug design.

Publications

1. **Ajish A.D,** Vagdevi H.M, Asha K, and Jayanna N.D. Potential Antimicrobial, Anthelmintic and Antioxidant Activities of *Myristica dactyloides Gaetrn* Bark. *International Journal of Pharmacy and Pharmaceutical Sciences*. Vol 7, Issue 5, 2015.

2. **Ajish A.D**, Vagdevi H.M, Sunitha B.J Evaluation of Antimicrobial, Anthelmintic and Antioxidant Activities of *Hopea ponga (Dennst.)* Bark. Accepted for publication in Vol. 10, Issue 4; April, 2019 Issue of International Journal of Pharmaceutical sciences and Research.

3. **Ajish A.D**, Vagdevi H.M, Chemical constituents and *in-vitro* Anticancer activity of bark extracts of *Myristica dactyloides Gaetrn* Bark. Communicated to International Journal of Pharma research and Health sciences.

4. **Ajish A.D**, Vagdevi H.M, Dr. Yallappa Shiralgi, *In-vitro* screening of cytotoxic, antitubercular and DNA cleavage activities of bark extracts of *Hopea ponga (Dennst.).*Communicated to Tropical Journal of Pharmaceutical Research.

5. **Ajish A.D**, Vagdevi H.M, Evaluation of antitubercular activity of bark extracts of *Myristica dactyloides.* Communicated to International Journal of Herbal Medicine.

National conferences - Papers presented

1. Participated and presented paper in one day National conference on **"An overview of medicinal value of plants"** held on 21st April 2014 organized by Department of Chemistry, Sahyadri Science College (Autonomous), Shivamogga.

2. Participated and presented paper in two days National conference on **"Recent Advances in Chemical Biology and Material Science for Industry and Society"** held in Department of Chemistry, Kuvempu University, Jnana Sahyadri during 9th and 10th February 2018.

International conferences - Paper presented

1. Participated and presented paper in three days International conference on **"Importance of Herbal Medicine in the Era of Globalization-A live Demonstration"** held on 21st to 23rd December-2016 organized by Department of Chemistry, Sahyadri Science College (Autonomous), Shivamogga.