KUVEMPU UNIVERSITY

Isolation and characterization of pharmacologically active components from *Cryptocarya stocksii* and *Nardostachys jatamansi*

Thesis submitted to Faculty of Science and Technology
Kuvempu University
for the award of the degree of

DOCTOR OF PHILOSOPHY
in
BIOCHEMISTRY

By

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DECLARATION

I, **Veena M.E.,** hereby declare that the thesis entitled **"Isolation and characterization of pharmacologically active components from *Cryptocarya stocksii* and *Nardostachys jatamansi*"** embodies the results of bonafide research work carried out by me under the guidance of **Dr. P. Niranjana,** Assistant Professor, Department of Biochemistry, Kuvempu University, Jnana Sahyadri, Shankaraghatta, Shivamogga District, Karnataka and co-guidance of **Dr. Rajeshwara N. Achur,** Associate Professor, Department of Biochemistry, Kuvempu University, Jnana Sahyadri, Shankaraghatta, Shivamogga District, Karnataka. I, further declare that this or part thereof has not been the basis for the award of any other degree or diploma either in this or any institution or university.

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Dr. G.J. Sathisha

Dedicated to my Beloved Family & Friends

for their love, support and encouragement throughout my career…..

ACKNOWLEDGMENTS

First and foremost, I express my profound sense of gratitude to my guide **Dr. P. Niranjana,** Assistant Professor, Department of Biochemistry, Kuvempu University, Jnana Sahyadri, Shankaraghatta, for giving me an opportunity to pursue my doctoral degree. His guidance and support over the past few years have been an invaluable part of my progress and education. I would also like to thank my co-guide **Dr. Rajeshwara N. Achur,** Associate Professor, Department of Biochemistry, Kuvempu University, Jnana Sahyadri, Shankaraghatta, for his inspiration and great efforts to explain things clearly and simply, that helped me to understand and practice good science. The motivating and critical discussions that we had are duly acknowledged.

It gives me an immense pleasure to thank Dr. G.J. Sathisha, Associate Professor and Chairman, Dr. Neelgund, S.E., Assistant Professor, Department of Biochemistry, Kuvempu University for their moral support and encouragement.

I wish to express my gratitude to the Dr. Tulasiram, Senior Scientist, National Chemical Laboratory, Pune, for providing lab facility to carry out this work.

I extend my gratitude to Dr. Sharanappa, Associate Professor, Department of P.G. Studies in Botany, Hassan, Mysore University, for suggesting me to choose the plant for my work.

I would also like to thank Dr. Krishnaswamy, K., Dr. Latha, K.P., Dr. Vagdevi, Assistant Professors, Department of Chemistry, Dr. Pramod, Assistant Professor, Department of Biochemistry, Dr. N.B. Thippeswamy,

Department of Microbiology, Dr. Manjunath, H., Assistant Professor, Department of Biotechnology, Dr. Manjunath, K.R., Assistant Professor, Institute of Management Studies, Kuvempu University and Dr. Subbarao, for their support in completion of my research work.

In this occasion, I remember the splendid days passed with my dear friends Asha, Shwetha, Jayanna, Anitha, Sameer, Vasanth Raj, Raghu, Raghavendra, Xablon Kaerima, Priya, Fayaj Mulani, Ajish, Ramesh and Jagadeesh for their support and encouragement.

I thank the Non-teaching Staff of the Department of Biochemistry, Librarian and Staff of Kuvempu University, for their support.

I thank all those, who directly or indirectly helped me in completion of my research work.

…….. any omission in this short note of acknowledgement does not indicate lack of gratitude.

Veena M.E.

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ABBREVIATIONS USED

%	-	Per cent
µg	-	Micrograms
AAE	-	Ascorbic acid equivalent
AIDS	-	Acquired immune deficiency syndrome
ANOVA	-	Analysis of variance
CFU	-	Colony forming unit
COX	-	Cyclooxygenase
DMSO	-	Dimethyl sulfoxide
DPPH	-	2,2-diphenyl-1-picrylhydrazyl
et al.	-	Co-authors (Others)
GABA	-	Gamma amino butyric acid
GAE	-	Gallic acid equivalent
IUCN	-	International union for conservation of nature and natural resources
MDR	-	Multiple-drug resistant
mg	-	Milligram
NF-kB	-	Nuclear factor kappa B
NMPB	-	National medicinal plants board
NSAIDS	-	Non-steroidal inflammatory drugs
PBS	-	Phosphate buffer saline
PUFA	-	Poly unsaturated fatty acids
ROS	-	Reactive oxygen species
SD	-	Standard deviation
SEM	-	Standard error of mean
TAC	-	Total antioxidant capacity
TCA	-	Tri chloro acetic acid.
TCAM	-	Traditional, complementary and alternative medicine
TNF	-	Tumor necrosis factor
WHO	-	World health organization

Chapter-1

General Introduction

1.1 Introduction

Earth is a green planet. The green plants are fundamental to all other life. The oxygen, nutrients, fuels and many of the most important materials we use are produced by plants. Therefore, plants represent the first stage in the evolution of living organisms (Tewari, 2000). All around the world there is talk about 'health for all'. Natural products obtaining from plants, animals, and minerals have been the basis of treatment of diseases from time immemorial. Herbs are staging a comeback and herbal 'renaissance' is happening all over the globe. The herbal products today symbolise safety in contrast to the synthetics that are regarded unsafe to human and environment. People are increasingly returning to the naturals with hope of safety and security. Herbal medicine is now regarded as important but underutilized tool against disease. The World Health Organization (WHO) recognized this fact in the early 1970s and encouraged governments to effectively utilize local knowledge of herbal medicines for disease prevention and health promotion (Gupta, 2010). Traditional systems of medicine continue to be widely practiced on many accounts. Population rise, inadequate supply of drugs, prohibitive cost of treatments, side effects of several allopathic drugs and development of resistance to currently used drugs for infectious diseases have led to increased emphasis on the use of plant materials as a source of medicines for a wide variety of human ailments.

Over three-quarters of the world population relies mainly on plants and plant extracts for health care (Joy, 1998). The primary health care of 70-80 per cent of the world's population is based on the use of medicinal plants derived from traditional systems of medicine and local health practices. During the past few decades public interest in traditional, complementary and alternative medicine (TCAM) and use of herbal medicines has increased dramatically in industrialized countries.

Current problems associated with the use of antibiotics, increased prevalence of multiple-drug resistant (MDR) strains of a number of pathogenic bacteria such as *Staphylococcus aureus*, *Helicobacter pylori* and *Klebsiella pneumonia* have received the interest in plants with antimicrobial properties (Voravuthikunchai and Kitpipit, 2003). In addition, the increase in cases of opportunistic infections and the advent of Acquired Immune Deficiency Syndrome (AIDS) patients and individuals on immunosuppressive chemotherapy, toxicity of many antifungal and antiviral drugs have imposed pressure on the scientific community and pharmaceutical companies to search alternative and novel drug source.

Ayurveda, Siddha, Unani and Folk (tribal) medicines are the major systems of indigenous medicines. Among these systems, Ayurveda is most developed and widely practised in India since ancient time. The Ayurvedic system of medicine is purely of Indian origin and development. More than 2400 medicines have been known from Indian medicinal flora. Ayurveda dating back to 1500-800 BC has been an integral part of Indian Culture. As the name implies, it is not only the

science of treatment of the ill but covers the whole gamut of happy human life involving the physical, metaphysical and the spiritual aspects.

A major lacuna in Ayurveda besides drug standardisation is the lack of information and quality control of herbal drugs. Most of the Ayurvedic medicines are in the form of crude extracts, which are a mixture of several ingredients and the active principles when isolated individually fail to give desired activity. Ayurvedic pharmacopoeia combined with modern medicine and updated periodically is an urgent requirement. A combination therapy integrating ayurveda and allopathy whereby the side effects and undesirable reactions could be controlled can be thought of. Studies show that the toxic effects of radiations and chemotherapy in cancer treatment could be reduced by Ayurvedic medication and similarly surgical wound healing could be accelerated by Ayurvedic medicines (Sharma *et al.*, 1997).

Of the 2,50,000 higher plant species on earth, more than 80,000 are known to have medicinal uses. India is home to about 1,30,000 species of plants belonging to 120 families, earning the tag "The Botanical Garden of the World". India has a rich heritage of indigenous drugs from the Vedic times, which is endowed with a variety of natural resources. It has been known to be rich repository of medicinal plants. The forest in India is the principal repository of large number of medicinal and aromatic plants, which are largely collected as raw materials for manufacture of drugs and perfumery products. India is one of the world’s 12 mega biodiversity centres with 2 hotspots are Western Ghats and north eastern regions. Approximately 60% of the world's population relies almost

entirely on plants for medication (Farnsworth, 1994). According to WHO, around 21,000 plant species have the potential for being used as medicinal plants.

The entire Western Ghats is known for its biodiversity, richness and endemism of different plant species. The hill chain of the Western Ghats, runs majestically parallel to the West coast of India from 8° N to 21° N latitudes, 73° E to 77° E longitudes for around 1600 km. The hills reach up to a height of 2800 m before they merge to the east. The average width of this mountain range is about 100 km and this mountain range contains a large proportion of the country's plant and animal species, many of which are endemic. The Western Ghats are well-known for harbouring a rich variety of medicinal plants. The forests are house for over 700 medicinal plants, several of which are utilized in Ayurvedic medicine production firms, while others are exploited commercially for their active enzymes. Medicinal plant species of Western Ghats include algae, climbers, lichen, herbs, shrubs and trees.

Conservation of the rich biological wealth of Western Ghats turns out to be a priority. The richness of the Western Ghats is further increased by the exclusive varieties of medicinal plants that the Ghats has to its credit. The tropical climate complimented by heavy precipitation from southwest monsoon and favourable edaphic factors create an ideal condition for the luxuriant growth of plant life, which can be seen only in few parts of the world. With its rainfall regime, the western slopes of the Ghats have a natural cover of evergreen forest, which changes to moist and then dry deciduous type as one comes to the eastern slopes. The vegetation reaches its highest development towards the southern tip in Kerala

with rich tropical rain forests. The plant species known to be from the Western Ghats is about 4,500 species out of which 35 per cent are endemic. Levels of endemism in this area are high, including nearly 2000 species of higher plants (Daniel *et al*., 1997).

Western Ghats with its species diversity is a treasure house of medicinal plants. Out of the large variety of species available in the Western Ghats, about 50 species hold a very high value in the folk and herbal health forms for the treatment of different forms of ailments, such as *Ochreinauclea missionis, Nothopodytes foetida, Glycosmis macrocarpa* and *Coscinum fenestratum*. Plants like *Anona squamosa, Buchanania lanzan, Semecarpus anacardium, Dioscorea bulbifera* and *Aphanamixis polystachya* are recommended for various forms of tumors. Frequent doses of medicinal plant extracts of *Rhincanthus nasuta, Momordica dioica, Cinnamomum zeylanicum* and *Ophiorhiza mungos* used for cancer treatment. The spread of knowledge on the natural wealth is more important for a country like India, at a time when the synthetic drugs are stealing the economy rates. Appropriate conservation strategies have to be implemented immediately to protect the fragile habitats of many such medicinal plants.

Higher plants are ‘treasure houses’ for a repertoire of phytochemicals. Medicinal plants form a sizeable component of traditional medicine and are a mainstay for 80% of the people in developing nations**.** Plants have been used as healers and health rejuvenators since time immemorial. Even now, WHO recognizes that medicinal plants plays an important role in the health care of about 80 percent of World population in developing countries and depend largely on traditional medicines, of which herbal medicines constitutes the most prominent

part (Farnsworth *et al*., 1988). The rest of the 20 percent also depend substantially on the plant-based medicines.

According to the World Health Organization, a medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi synthesis. Plant parts including leaves, roots, rhizomes, stems, barks, flowers, fruits, grains or seeds, employed in the control or treatment of a disease condition and therefore contains chemical components that are medically active (Van den Bogaard *et al*., 2000; Smolinski *et al*., 2003). In addition, almost 25% of the entire compounds of current prescription drugs were derived originally from plant sources (Balandrin, *et al.*, 1985). Some of the useful plant derived compounds are vinblastine, vincristine, taxol, podophyllotoxin, camptothecin, digitoxigenin, gitoxigenin, digoxgenin, tubocurarine, morphine, codeine, aspirin, atropine, pilocarpine, capscicine, allicin, curcumin and artemesin. Of the estimated 25,000 flowering plant species in the world today, only about 10% have been scientifically examined for their medical application, mostly in rudimentary way. Undoubtedly, many more plant-derived medicinal substances await discovery (Akerale, *et al*., 1991). The information on the medical wealth of these examined 10% of plant species is scattered in different sources.

Medicinal plants are used as raw materials for extraction of active constituents in pure form (e.g. alkaloids like quinine and quinidine from cinchona bark, emetine from ipecacuanha root, glycosides from digitalis leaves, sennosides from senna leaves), as precursors for synthetic vitamins or steroids, and as preparations for herbal and indigenous medicines. Products such as ginseng,

and valerian roots are part of the herbal and health food market, as well as the food flavours, fragrance and cosmetic industries. Certain plant products are industrially exploited like liquorice in confectionery and tobacco, papaine as meat tenderiser, quinine as soft drink tonic and cinchona as wine flavour.

A large quantity of medicinal plant materials are used in the preparation of herbal and medicinal teas and other formulations. These herbal and food uses are of great importance, also to the exporters from developing countries. Hundreds of medicinal plants are items of commerce, however small countries are used in formulated herbal remedies. Several formulations like herbal teas, extracts, decoctions, infusions, tinctures, etc., are prepared from medicinal plants (Kraisintu *et al.,* 1997).

Most of the medicinal plants are found to occupy forest types like deciduous forests, evergreen forests and they are found in shallow lands and wayside. It can be noted that because of commercial explitation, habitat destruction many plants have become endangered and extinct and included in IUCN list. *Rauvolfia serpentina*, *Saraca asoca*, *Gymnema sylvestre*, *Gloriosa superba*, *Strycnosnux-vomica* and *Oroxylum indicum* are included in the list which are very rich in their medicinal strength but are on the edge of extinction. The Western Ghats also host many medicinal plants that are endemic to the area. Appropriate conservation strategies have to be implemented immediately to protect the fragile habitats of many such medicinal plants. Western Ghats was and will remain an area where the therapeutics of nature can be exploited. Hence, efforts have to be taken to bring to limelight the use of nature's doctors, methods to conserve them and to sustain their existence for the future generations.

1.1.1 Uses of phytochemicals

Plants have played a crucial role in maintaining human health and improving the quality of human life for thousands of years. The World Health Organization has estimated that 80% of the earth's inhabitants rely on traditional medicine for their health care needs, and most of this therapy involves the use of plants extracts or their active components. Therefore, therapeutic approach of several traditional medicines is rather more holistic. Majority of fundamental concepts of their medicinal systems still cannot be explained using modern tools. Medicinal plants sector has traditionally occupied an important position in the socio-cultural, spiritual and medicinal area of rural and tribal lives of India. The global thirst areas for drugs from medicinal plants include disease conditions, whose incidence is unavailable or unsatisfactory.

Different mechanisms of action of phytochemicals have been elucidated. Phytochemicals may inhibit growth of microorganisms, interfere with some metabolic processes or may modulate gene expression and signal transduction pathways (Kris-Etherton *et al.*, 2002; Manson *et al*., 2003). Phytochemicals may either be used as chemotherapeutic or chemo preventive agents with chemoprevention referring to the use of agents to inhibit, reverse, or retard tumorogenesis.

Plant extracts and essential oils may exhibit different modes of action against bacterial strains, such as interference with the phospholipids bilayer of the cell membrane which has as a consequence of permeability increase and loss of cellular constituents, damage of the enzymes involved in the production of cellular energy and synthesis of structural components, and destruction or inactivation of

genetic material. In general, the mechanism of action is considered to be the disturbance of the cytoplasmic membrane, disrupting the proton motive force, electron flow, active transport, and coagulation of cell contents (Kotzekidou *et al.*, 2008).

1.2 The Genus *Cryptocarya*

The word natural refers to something that is present in or produced by nature and not artificial or man made. Natural products today commonly refers to herbs, herbs concoctions, dietary supplements, traditional medicine. Natural products have been an integral part of the ancient traditional medicine systems, e.g. Chinese, Ayurvedic and Egyptian (Sarker *et al.*, 2007). The family Lauraceae belongs to the Order Laurales that is classified in the subclass Magnolidae. The family is pantropical, including about 50 genera with 350 species (Rohwer *et al.*, 1993).

Cryptocarya is a genus of evergreen tree belongs to the family Lauraceae and is the 5th largest genus in the family Lauraceae. This family comprises more than 350 species distributed mostly in tropical and subtropical regions of South America, Mauritius, India, China, Java, New Guinea and Africa (Ayu Afiqah Nasrullah *et al.*, 2014).

Cryptocarya can be distinguished from other Laurel genera by its bisexual and trimerous flowers and fruit morphology, which are very typical in shape (usually slender, urceolate, apically narrowed tube and immersed ovary, six equal to sub equal petals, nine fertile stamens with disporangiate anthers), and by the characteristic fruit that is enclosed by the accrescent flower tube (Pedro and Moraes, 2007). The leaves are alternate, simple, pinnate nerved, rarely trinerved,

hairy and whitish underneath. Inflorescenses in axillary or sub terminal panicles. The fruits entirely included in the enlarge perianth tube leaving only a minute opening at apex.

Due to high medicinal values of Lauraceae members, these plants have been explored. Herbalism and medicine, ancient and modern, have been the source of much useful therapy for field of pharmacology. Important Cryptocarya species reported from Western Ghat region are *Cryptocarya stocksii, Cryptocarya beddomei, Cryptocarya anamallayana* and *Cryptocarya wightiana*.

1.2.1 Medicinal value

Proper management of traditional medicinal plant resources is important, not only because of their value as a potential source of new drugs, but due to reliance on traditional medicinal plants for health (Cunningham *et al*., 1993). Natural products have been the single most important productive source of most of the active ingredients of medicines, leads for the development of drugs. Plants have always been the single most productive source of lead compounds (e.g. morphine, cocaine, digitalis, quinine, tubocurarine, nicotine and muscarine). Many of these lead compounds are useful as drugs (e.g. morphine and quinine), and others have been the basis for synthetic drugs (e.g. local anaesthetics developed from cocaine). Clinically useful drugs which have been isolated from plants include the anticancer agent paclitaxol from the *Taxus* sp. and the antimalarial agent artemisinin from *Artemisia annua*. Plants provide a large bank of rich, complex and highly varied structures which are difficult to be synthesized in laboratories. With the rapid evolution of screening process many plants compounds have screened. Even today, the number of plants that have been

extensively studied are relatively very few and the vast majorities have not been studied at all (Corner, 1988).

During the 19th century, organic chemists took up the study of many plant principles, the physiological effect of which have been recognized. A new importance was given to the search for medicinal plant principles. By the discovery of the clinical usefulness of alkaloids of *Rauwolfia* species and during the past dozen years this has provided initiation for an enlarged and concentrated search upon the still unexplored botanical resources of the world.

Cryptocarya massoy is a native of New Guinea. In Peninsular Malaysia, the bark of this species is used by the Women after childbirth, and also is added to tonics and cigarettes. In Indonesia, it is used against diarrhoea and spasmodic bowel trouble, usually in combination with a *Cinnamon*. Furthermore, it is an ingredient of various native medicines and has a characteristic odour. In New Guinea, the aromatic bark of *Cryptocarya massoy* is employed to treat fever, and often it is used by the natives to treat bad cases of tuberculosis of the lungs, externally to relieve sore muscles and head ache and internally used against fever and diarrhoea. A small portion of the bark is placed so as to close a fresh wound. It is also chewed and the saliva rubbed over the limbs to easier muscular pains. In small quantities, mostly in combination with other vegetable drugs, it is ingested for violent headache, pain in the joints, puerperal infection, distension of the stomach, vomiting and chronic constipation. It is also mixed with cloves and sandalwood and used by the natives as rubefacient. *Cryptocarya latifolia* used as substitute for *Ocotea bullata*, a well known medicinal plant of Lauraceae in Africa. *O. bullata* has good reputation of medicinal uses i.e., for head ache,

morning sickness, pulmonary diseases, tuberculosis and having antifungal and antibacterial properties. Hence same medicinal uses can be expected in *C. latifolia*. In India, leaves of *Cryptocarya wightiana* are used for the treatment of rheumatism and elephantiasis.

1.2.2 Occurrence of compounds across *Cryptocarya* species

The previous phytochemical investigations of the genus established the presence of various secondary metabolites such as Flavonoids (Chou *et al.,* 2010; Feng *et al*., 2013), Pavines (Lee *et al*., 1990; Lin *et al*., 2002; Wu and Lin, 2001; Feng *et al*., 2013) and aporphines (Feng *et al*., 2013), terpenoids, lignans and steroids many of which exhibited cytotoxic activities (Chou *et al*., 2010; Dumontet *et al*., 2001), nuclear factor-kB (NF-kB) inhibitory (Feng *et al*., 2012; Meragelman *et al*., 2009) and antitrypanosomal (Davis *et al*., 2010) activities. These chemical compounds also important to economic and nutrition field. Besides alkaloids, they produced a wide range of non-alkaloidal compounds including carbohydrate, proteins, lipid, amino acids, terpenes, essential oils, acetogenins, polyphenols and aromatic compounds (Thomson *et al*., 1993).

Past studies of *Cryptocarya chinensis* have found that it contains many Pavine and Proaphorpine alkaloids. The Pavine alkaloids have been noted to possess antiviral and immunological activity, behavioural and electrophysiological effects, and antiarrhythmic potential (Varadinova *et al*., 1996). Several other compounds were isolated from the leaves and bark of the South African plant. *Cryptocarya latifolia*, which has long been noted for its medicinal properties (Drewes *et al.,* 1995). These range from the treatment of headaches and morning sickness to that of cancer, pulmonary diseases and various bacterial and fungal

infections. However in India local people use the leaves of *Cryptocarya stocksii* for the skin infection. A number of flavonoids have been isolated from some *Cryptocarya* species include dihydrochalcone, chalcone, dihydroflavanone and biflavonoid derivatives (Govindachari *et al.*, 1973; Fu *et al.*, 1993; Juliawaty *et al.*, 2000; Dumontet *et al.*, 2004).

1.2.3 Pharmacological importance

The exploration of Lauraceous plants have been studied many decades ago either as crude drug or bioactivity of isolated compounds such as alkaloids, essential oils, polyphenols, aromatic compounds carbohydrates, lipids amino acids etc. In Brazilian Atlantic rain forest, the fruits of the *Cryptocarya mandioccana* Meisner are distinct by their aroma and pungent flavour, being carminative useful in the treatment stomach ache. Its bark is bitter in taste and scented which is helpful in fighting colic and diarrhoea. The tea from its seeds is used against stomach ache and it's crushed leaves against aches and colic (Trager *et al.*, 1976).

A phytochemical study of stem bark and leaves of *Cryptocarya moschata* from Atlantic Rain Forest shows the occurrence of pyrones, stylopyrones, flavonoids and glycosides (Nehme *et al.*, 2002). Some of the pyrones and stylopyrones isolated from this species have shown larvicidal and antifertility activities, as well as inhibition of breast cancer cell lines (Hawariah Stanlas *et al.*, 1998). Phytochemical relationship of *Cryptocarya libertiana* and *Ocoteabullata* was studied (Siegfried Drews *et al.*, 1997).

A dichloromethane extract of the stem bark of *Cryptocarya nigra* showed strong *in vitro* inhibition of *Plasmodium falciparum* growth (Ayu Afiqah Nasrullah *et al.*, 2013).

Studies on the organic extract of stems of *C. maclueri* confirms the presence of five new flavanones such as cryptogiones and a new polyketide, cryptomaclurone. These compounds exhibit cytotoxic activity on KB (human nasopharyngeal carcinoma), SGC-7901 (human gastriccancer) and SW1116 (human colon cancer) cells (Feng *et al.*, 2013).

Fu-Wen Lin *et al*. (2002) isolated three new pavine*N*-oxide alkaloids, isocaryachine-*N*-oxide, caryachine-*N*-oxide and a new isoquinoline alkaloid, 6,7-methylenedioxy-*N*-methylisoquinoline together with 11 known alkaloids and characterized from the stem bark of *Cryptocarya chinensis*.

Usman (2005) isolated cytotoxic Chalcones and Flavones from *Cryptocarya costata*. Stem bark extract which showed strong inhibition activity.

Usman (2006) isolated a new flavanone, 7-hydroxy-5,6-dimethoxyflavanone, together with three other flavonoids, didymocarpin, 2-,4-dihydroxy-5-,6-dimethoxychalcone, and isodidymocarpin, had been isolated from the methanol extract of the tree bark of *Cryptocarya costata*. Cytotoxic properties of these compounds were evaluated against murine leukemia P-388 cell lines. The results showed that these compounds strongly inhibited the growth of the cells.

Topul Rali (2007) reported comparative chemical analysis of essential oil from the bark, heartwood and fruits of *Cryptocarya massoy* Heart wood, bark confirms the presence of C-10 massoia lactone as the major constituent, correlating with its presence as a mode of chemical defense.

Aline Miranda de Oliveira (2007) evaluated micronuclei frequency in *Tradescantia pallida* pollen mother cells treated with ethanolic extracts isolated from *Cryptocarya mandioccana* and *Cryptocarya moschata*.

Overall *Cryptocarya* genus has proven to be valuable for the discovery and utilization of medicinal natural products, particularly alkaloids, flavonoids glycosides tanins. The information provides a means to understand the latest information and developments in the pharmacological and phytochemistry of these plants. In view of this the present study on the *Cyptocarya stocksii* plant extracts, isolation and characterization of pure compounds from extracts and their biological activities has been undertaken to systematically evaluate its pharmacological potential.

1.3 *Nardostachys jatamansi*

Nardostachys jatamansi DC is a plant belongs to the family Valerianaceae and is commonly known as Indian spikenard or musk root. The herb *N. jatamansi* is known to be a popular medicinal and aromatic plant. It is a reputed Ayurvedic herb and used in various multiple formulations. It has also been mentioned in the Holy Bible and Quran. In Ayurveda, *N. jatamansi* is used for nervous headache, excitement, menopausal symptoms, flatulence, epilepsy and intestinal colic. In combination with cold water, the oil is considered to be effective against nausea, stomach ache, flatulence, liver problems, jaundice and kidney complaints, insomnia and headache. Externally, the oil is added to a steaming bath to treat inflammation of the uterus. The oils are also used in eye compounds and as poison antidotes. Oil is reported to be useful in the treatment of atrial flutter. The roots and rhizomes of *N. jatamansi* have been used to treat epilepsy, hysteria and mental weakness. It also exhibits cardio protective activity and used in the treatment of neural diseases. The essential oil obtained from the roots of *jatamansi* showed

various pharmacological activity including antimicrobial, antifungal, hypotensive, antiarrhythmic and anticonvulsant activity.

1.3.1 Phytochemistry

The roots of the plant contain essential oil, rich in sesquiterpenes and coumarins. Jatamansone or valeranone is the principal sesquiterpene. Other sesquiterpenes include nardostachone, dihydrojatamansin, jatamansinol, jatamansic acid, jatamansinone, jatamansinol, oroseolol, oroselone, seselin, valeranal, nardostachyin, nardosinone, spirojatamol, jatamol A and B, calarenol, seychellene. Sesquiterpene is the major component of this plant, others include jatamansone, nardostachone. But this reputed plant has become critically endangered and requires various conservation strategies. *N. jatamansi* has been discovered with both volatile and non-volatile constituents. Sesquiterpenes contribute to the major portion of the volatile compounds while sesquiterpenes, coumarins, lignans, neolignans, alkaloids and steroids form the major components of the non-volatile extracts. Sesquiterpenes include nardin, nardal, nardol, valerenal, jatamnsic acid, b-malineandpatchouli alcohol. Chatterjee *et al*. (2005) undertook the chemical examination of the rhizomes in detail leading to the isolation of a new terpenoidester, nardostachysin. Principal sesquiterpene of this plant named jatamansone orvaleranone. Various other sesquiterpenes known are nardostachone, dihydrojatamansin, jatamansinol, jatamansicacid, jatamansinone, jatamansinol, oroseolol, oroselone, seselin, valeranal, nardostachyin, nardosinone, spirojatamol, jatamol A and B4, calarenol, seychellene, seychelane, coumarin : jatamansin or xanthogalin. An alkaloid named actinidine has also been reported

(Sastry, 1967; Hoerster *et al.*, 1977; Rucker, 1978; Bagchi *et al.*,, 1990; 1991; Rűcker, 1993; Harigaya, 2000).

In view of this, the present study on the extraction, isolation and characterization of *Nardostachys jatamansi* compounds and their antiproliferative activities has been undertaken systematically to evaluate its pharmacological potential.

For the systematic presentation, the present thesis has been divided into following chapters:

Chapter-1 : General Introduction

Chapter-2 : Isolation and characterization of bioactive compounds from stem bark of *Cryptocarya stocksii*

Chapter-3 : Pharmacological activity of *Cryptocarya stocksii*

Chapter-4A : Isolation and characterization of bioactive compounds from *Nardostachys jatamansi*

Chapter-4B : *In vitro* anti-proliferative activity of bioactive compounds from *C. stocksii* and *N. jatamansi*

Chapter-5 : Summary

References

Chapter-2

Isolation and characterization of bioactive compounds from stem bark of *Cryptocarya stocksii*

2.1 Introduction

Natural product medicine originating from terrestrial plants, terrestrial microorganisms, marine organisms, terrestrial vertebrates and invertebrates have played an important role worldwide in treating and preventing various human diseases from time immemorial. Among them, medicinal plants are the richest resources of drugs for traditional systems of medicine, modern medicines, neutraceuticals, food supplements, folk medicines, pharmaceutical intermediates as well as chemical entities for synthetic drugs (Sukhdev Swami Handa, 2008). The biggest obstacle to natural product chemistry is the shortage in continuous supply of large amounts of medicinal plants required for further biological evaluation.

The term Materia Medica was coined for the study of natural products. Materia Medica is defined as the knowledge of natural history, physical characteristics, and chemical properties of drugs. The classical texts of Ayurveda, Charaka Samhita and Sushruta were written around 1000 B.C. The Ayurvedic Materia Medica includes 600 medicinal plants along with therapeutics. It has been estimated that only 5 to 15% of the approximately 2,50,000 species of higher plants have been systematically investigated for the presence of bioactive compounds. Global market for medicinal plants stands at US $ 60 billion per year, which is growing at the rate of 7% annually (Cuong *et al*., 2002). In India, herbal medicine is a common practice, about 960 plant species are used by the Indian herbal industry, of which 178 are of higher magnitude exceeding 100 metric tons

per year (Sahoo *et al.*, 2010). The World Health Organization (WHO) has also recognized the importance of traditional medicine and has created strategies, guidelines and standards for usage.

Indispensable association between human beings and plants led to emergence of scientific approaches for the systematic study. The phytochemical research based on ethno pharmacological information is generally considered as an effective approach in the discovery of new anti-infective agents from higher plants (Chetri *et al*., 2008). Extracts from herbs/plants are mostly formulated as tablet/capsule and to some extent as oral liquid preparations. The science of pharmacognosy and traditional knowledge of drugs based on experimental database can provide new functional leads to reduce time, money and toxicity and also provide a disciplined scientific description of natural materials used in medicine (Soumya Prakash *et al*., 2009). The search of global thirst areas for drugs from medicinal plants include disease conditions, its availability and satisfaction.

Phytomedicine almost went into extinction during the first half of the 21st century owing to the use of more powerful and potent synthetic drug. Soon after the discovery and isolation of active principles from medicinal plants, prescription began to be substituted rapidly with pure substances which were more powerful, safe and easy to administer. A major advantage of botanicals over conventional single-component drugs is the presence of multiple active compounds that together can provide a potentiating effect that may not be achievable by any single compound. For the first time pharmacologically active pure compound morphine was produced from opium extracted from seed pods of poppy *Papaver somniferum*, about 200 years ago. In 1920, quinine was isolated from *Cinchona* by the French pharmacist, Peletier and Caventou. In the mid-nineteenth century, a

German chemist, Hoffmann, obtained salicylic acid a precursor of aspirin from the bark of the Willow, an antipsychotic and antihypertensive drug from *Rauwolfia* species and antimalarials such as quinine from *Cinchona* bark and lipid-lowering agents (Rousseaux and Schachter, 2003; Hartmann, 2007). With the advances in methodology and the development of more sophisticated isolation and analytical techniques, many more of these phytochemicals should be identified (Mamta Saxena *et al*., 2009).

Current research in drug discovery from medicinal plants involves an all-round approach combining botanical, phytochemical, biological and molecular techniques. Health authorities and governments of various nations have taken an active interest in providing standardized botanical medications. Indian government has also plunged into this opportunity and initiated some regulations in this sector. Around 6000 species of medicinal plants are documented in published medical and ethno-botanical literature. Wild populations of several hundreds of these species are under threat in their natural habitats. In the Tenth Plan, a National Medicinal Plants Board (NMPB) was established for supporting conservation of gene pools and large scale cultivation of medicinal (Five Year Plan). The rich heritage of traditional systems of medicine and vast repository of natural resources put India at a prominent position in global scenario of herbal sector.

This natural pharmacy is endangered by rapidly moving destruction of the biosystems by emerging uncontrolled timber logging for wood production and generation of new farm land. Time seems to run out for many species and making needs to awake and stop the elimination of natural resources. In tropical rain forests, there are still many plant species awaiting for a thorough phytochemical investigation.

The *Cryptocarya stocksii* is an endemic plant that is exclusively present in the Western Ghats of Karnataka. The species belongs to the family Lauraceae. The other species of this plant are used to cure many diseases such as stomach ache, headache, dysentery, antimicrobial activity, anticancer etc. There is no data regarding the isolation and characterization of active principles present in this plant. An attempt has been made to systematically study the extracts of *Cryptocarya stocksii* plant for its application in the field of pharmacy.

2.2 Materials and methods

2.2.1 Plant profile

Cryptocarya stocksii is an evergreen tree frequently occurs in Shola forest of the hills. The bark of the plant material was collected from Western Ghats of nearly about altitude of 1600 meter. The classification for *C. stocksii* is as follows:

Kingdom : Plantae

Division : Magnoliophyta

Class : Magnoliopsida

Order : Laurales

Family : Lauraceae

Genus : *Crypyocarya*

Species : *stocksii*

The *C. stocksii* plant is an evergreen tree, grows up to 30-40 feet height, with alternate leaves, oblong, emarginated at tip, golden brown hairs present on veins in lower surface of leaves. Infloresence is panicle short and congested, rufuous hairy. Flowers are bisexual, stamens 9 in three whorls, ovary sessile. Fruit is berry and enclosed in perianth tube.

2.2.2 Collection and identification of plant material

The bark of the *Cryptocarya stocksii* was collected in the month of November 2012 from the Bababudangiri hills of Chikkamagaluru District, Western Ghats, Karnataka, India and it was taxonomically identified by Dr. P. Sharanappa, Assistant Professor, Department of Bioscience, University of Mysore, PG Centre, Hasan, Karnataka. The voucher specimen was deposited in the same department.

2.2.3 Preparation of the plant extracts

Fresh bark was collected, washed with distilled water to remove adhering substance, shade dried and mechanically pulverized into fine powder. A known weight of the powdered material was subjected to soxhlet extraction using solvents

in the increasing order of their polarity viz., petroleum ether < chloroform < ethanol. The extraction was ran for 24 cycles until the final extracted solvent in the thimble becomes colourless. The solvent extract was concentrated under reduced pressure and stored at 4ºC until further use. The extracts were used for analyzing phytochemical constituents, isolation of pure compounds and determination of biological activity.

2.2.4 Phytochemical analysis

The phytochemical analysis of the plant extracts was carried out according to the procedure of Indian pharmacopoeia (Indian Pharmacopoeia, 1985). The stem bark extracts were subjected to phytochemical analysis such as alkaloids, flavonoids, steroids, tannins, phenolics, saponins, proteins and amino acids, qualitatively as described earlier (Mallikarjuna *et al*., 2007; George *et al*., 2010; Ugochukwu *et al*., 2013).

2.2.5 Total phenols

The total phenolic content of stem bark extracts were determined by Folin-Ciocalteu Reagent (FCR) (Slinkard and Singletyon, 1977) method. Briefly, diluted extracts (0.5 ml) were mixed with 0.5 ml of FC reagent (1:1) and 1 ml of sodium carbonate (20%). The reaction mixture was allowed to stand for 30 min. and optical density was read at 765 nm using UV-visible Spectrophotometer. A standard curve was plotted using Gallic acid (0-200 μg/ml) and the phenolic content was expressed as mg of gallic acid equivalents (GAE/100mg) of dried extracts (Kekuda *et al*., 2011).

2.2.6 Total Flavonoids

Total flavonoids of stem bark extracts were determined by adopting aluminium chloride method (Zhishen *et al*., 1999). Briefly, 0.5 ml of stem bark extracts were separately mixed with 4.5 ml of distilled water. To this, 0.3 ml of 1 M sodium nitrite (Na NO_2) was added, followed by 0.3 ml of 10% aluminium chloride and kept at room temperature for 6 min. Further, 2.0 ml of 1 M NaOH and 3.4 ml of distilled water was added and kept at room temperature for 30 min. The absorbance of the reaction mixture was then measured at 510 nm. The total flavonoid content was calculated as catechol equivalents/100 mg of extract (10-100 µg/ml).

2.2.7 Gas Chromatography and Mass Spectrometry analysis (GC-MS)

1 µl of petroleum ether extract or chloroform extract was injected into the GC-MS instrument. GC analyses were carried out on an Agilent 7890 instrument equipped with a hydrogen flame ionization detector and HP-5 capillary column (30 m × 0.32 mm × 0.25 µm, J and W Scientific). Nitrogen was used as carrier gas at a flow rate of 1 ml/min. Initially, the column temperature was maintained at 60°C for 2 min, followed by a temperature gradient from 60 to 120°C at 2°C/min. and held constant for 5 min. at 120°C, then raised to a temperature of 150°C at 1°C/min. and finally to a temperature of 200°C with a rise of 5°C/min. and maintained for 10 min. at 200°C. The injector and detector temperatures were maintained at 250°C and operated in split mode (split ratio 1:10). GC-MS was performed on an Agilent 5975C mass selective detector interfaced with an Agilent 7890A gas chromatograph. GC-MS analyses was performed under similar

conditions using an HP 5-MS capillary column (30 m × 0.32 mm × 0.25 μm, J and W Scientific).

Thin-layer chromatography was performed on silica gel G-coated plates (0.25 mm) developed with hexane (system I), hexane–ethyl acetate (98.5:1.5; system II), dichloromethane (system III), and dichloromethane–methanol (98.5:1.5; system IV). Compounds were visualized by spraying with a solution of 3.0% anisaldehyde, 2.8% H_2SO_4 and 2% acetic acid in ethanol followed by heating for 1-2 minutes.

2.2.8 Isolation and Characterization

2.2.8.1 Column Chromatography

In order to select the best mobile phase for eluting the Chloroform fraction, small amount of extract was dissolved and spotted on the TLC and run with the combination of the solvents. The solvent system in which most favourable separation of compounds tookplace was chosen as a mobile phase.

2.2.8.2 Column Packing

Glass column with a length of 55 cm and diameter of 3 cm was cleaned, dried and used for separating the constituents from plant extracts. A known weight of silica gel G (Merck, 60-120 mesh size) was activated for 24-48 hrs in an oven and transferred to beaker containing solvent with continuous shaking. The slurry was poured into the column and the solvent was continuously eluted allowing slurry to make uniform packing of column. 3 g of chloroform extract was dissolved in chloroform and loaded to the column. The elution was carried out with solvent mixtures of increasing order of their polarity. Fractions were

collected in 100ml portions and monitored by TLC and fractions with similar spots were pooled together. *C. stocksii* stem bark extracts were subjected separation by using petroleum ether and ethyl acetate mixture of various proportions as mobile phase. The eluted fraction was analysed with TLC. TLC was carried out on readymade silica plates (Merck, Mumbai, India) using capillary tube approximately 1 µL of column eluted fraction was loaded on silica gel G plates 3mm above from the bottom. Fractions with similar TLC pattern ware pooled together and concentrated at reduced pressure and temperature using rotatory vacuum evaporator. The concentrated components were further dried in vacuum desiccators. The structure of isolated compounds were determined using ^{1}H, ^{13}C-NMR, IR, Mass spectral analysis.

2.2.8.3 Infrared spectroscopy

The infrared spectra of the compound was recorded using K Brinthe range of 4000-400 cm^{-1} on unicam FTIR (Research Spectrometer series).

2.2.8.4 Nuclear Magnetic Resonance (NMR)

The magnetic spectrum of the compounds were recorded on AV 400 FT NMR spectrophotometer (400 MHZ) using TMS as an internal standard. Samples were dissolved by preparing solvents dissolved in $CDCl_3$. The chemical shifts were expressed in ppm.

2.2.8.5 Mass spectrometry

The mass spectrum was recorded in Auto spec E_1 mass spectrometer at 70 eV ionisation energy with direct inlet system. The mass spectrum confirms the molecular weight of the compound.

2.3 Results

2.3.1 Phytochemical analysis

The preliminary phytochemical analysis confirmed the presence of steroids, flavonoids, terpenoids and quinines in all three extracts of *C. stocksii* stem bark. Phenolics, tanins and alkaloids were present only in chloroform and ethanol extract. Coumarins were detected in petroleum ether and chloroform extracts but absent in ethanol extract. Saponins were absent in all the three extracts including proteins and amino acids. Ethanol extract has significant amount of alkaloids, phenols and tannins. While chloroform extract possesses moderate quantity of same molecules, they are found to be lacking in petroleum ether extract. Proteins and amino acids were absent in all the three extracts (Table 2.2).

Table 2.1. Soxhlet extraction of stem bark of *C. stocksii*

Stem Bark Quantity	Solvents	Color	% Yield (gms)
1.850 Kg	Petroleum ether (60-80°C)	Yellow paste	15
	Chloroform	Dark yellow paste	25
	Ethanol	Dark brown semisolid	30

Table 2.2. Phytochemical analysis of *C. stocksii* extracts

Tests	Petroleum ether	Chloroform	Ethanol
Alkaloids			
a. Mayer's test	-	++	+++
b. Dragendorff's test	-	++	+++
Flavonoids			
a. Ferric chloride test	+	++	+++
b. Lead acetate test (NaOH)	+	++	+++
Steroids			
a. Salkowski's test	++	+	+++
b. Libermann- Burchard's test	++	+	+++
c. Sulphur test	++	+	+++
Terpenoids			
a. Salkowski's test	+	+	+++
b. Libermann-Burchard's test	+	+	+
Tannins and Phenols			
a. Ferric chloride test	-	+	++
b. Gelatin test	-	+	++
Saponins			
a. Foam test	-	-	-
Proteins and amino acids			
a. Ninhydrin test	-	-	-
B. Millons test	-	-	-
Quinones	+	+	+
Coumarins	+	+	-

The phytochemical constituents present in different extracts of *C. stocksii*

(-Absent; + present; ++ significant; +++ highly significant)

2.3.2 Total phenolic and flavonoid content

Pharmacologically important polyphenols are widely found in medicinal plants and food products derived from plant sources, and they have been shown to possesses significant antioxidant activities and antimicrobial activity. The total phenolic content of *C. stocksii* was found to be 0.85±0.05 mg GAE/100mg, 2.0 mg±0.5 GAE/100mg and 20±0.8 mg GAE/100mg in petroleum ether, chloroform and ethanol extract, respectively (Table 2.3). Flavonoids are secondary metabolites that include flavonols, flavones and condensed tannins. Similar to phenols flavonoid content vary with the type of solvent used for extraction. The flavonoid content of ethanol extract was found to be 10.9±0.4 mg CE/100 mg, which was found to be higher compared to chloroform and petroleum ether extract (0.475±0.5 mg CE /100 mg and 0.15mg±0.2 mg CE /100 mg).

Table 2.3. Total Phenolic and Flavonoid content of the *C. stocksii* extracts

Extracts	mg of Gallic acid equivalents/ 100mg of dried extracts	mg of Catechol equivalents/ 100mg of dried extracts
Petroleum ether	0.85	0.15
Chloroform	2.0	0.475
Ethanol	20	10.93

2.3.3 GC-MS analysis

The compounds which were present in the stem bark extracts were identified based on the spectrum obtained using database of National Chemical Laboratory (NCL), Pune, India. The percentage of each component was calculated by comparing its average

peak area to the total areas. The spectrum of unknown compounds was compared with spectrum of known compounds stored in NCL library Pune. The R-match, retention time and probability are used to identify the compound from NCL library Pune.

Phytochemicals present in petroleum ether and chloroform extract of *C. stocksii* stem bark were identified by GC-MS analysis and the GC-MS chromatogram with peak area are shown in the Figure 2.1 and 2.2, respectively. GC-MS analysis of petroleum ether and chloroform extract led to identification of compounds. The active principles present in petroleum ether and chloroform extract were confirmed based on the R-match and retention time (RT) and probability and peak area in percentage (%) are shown in Tables. Petroleum ether extract of stem bark contains thirty compounds and the first compound identified with less retention time was 2-Methylheptane (4.453 min), whereas the last compound to be identified was nonacosane. Results revealed that 1-Trifluoroacetoxy-5-methoxy-3-phenylpentane (30.71%) was found as a major component followed by 4-Acetyl-2-phenyl-9,10-dihydro-2H, 9aH-2,3,4a-triazaanthracen-1-one (28.44%), 1,4-Cyclohexadienylbenzene (2.51%), Heptacosane (2.20%) and biphenyl (1.50%). Phytocomponents identified by GC-MS analysis of petroleum ether extract showed many biological activities are presented in the Table 2.4.

Fourteen compounds were identified in the chloroform stem bark extract of *C. stocksii* and the first compound to be identified with least retention time was Benzaldehyde (8.43 min) and last compound identified was 1-Monolinoleoyl glyceroltrimethylsilyl ether with a retention time of 35.9 min. The prevailing compounds were 4-Acetyl-2-phenyl-9,10-dihydro-2H,9aH-2,3,4a-triazaanthracen-1-one (41.43%), 1-Trifluoroacetoxy-5-methoxy3phenylpentane (32.37%), 5,8,11-Heptadecatriynoic acid (1.72%), Biphenyl (1.80%) and Azulene (0.97%) (Table 2.5).

Table 2.4. Phytochemicals identified in petroleum ether extract of *C. stocksii* stem bark by GC-MS analysis

Sl. No.	Retention time	Total %	Compound name
1.	4.453	0.07	2-Methylheptane
2.	8.427	0.15	Benzaldehyde
3.	12.833	0.11	Disulfide, bis(1-methylpropyl)
4.	14.332	0.25	Benzene, 2,5-cyclohexadien-1-yl-
5.	15.513	0.29	á-Cubebene
6.	15.37	1.50	Biphenyl
7.	15.936	0.47	(+)-Sativen/Azulene
8.	16.033	0.86	Caryophyllene
9.	16.229	0.16	Thujopsen
10.	16.446	0.50	à-Bulnesene
11.	17.08	0.31	Pentacyclo[7.5.0.0(2,7).0(3,5).0(4,8)]tetradeca-10,12-diene
12.	17.358	0.09	Caryophyllene oxide
13.	17.984	0.33	p-Cymen-8-ol
14.	28.165	0.70	Emersol 140
15.	28.824	0.45	Phthalic acid, butyl octyl ester
16.	28.793	28.44	4-Acetyl-2-phenyl-9,10-dihydro-2H, aH-2,3,4a-triazaanthracen-1-one
17.	30.025	0.69	Propanedinitrile
18.	30.911	30.711	1-Trifluoroacetoxy-5-methoxy-3-phenylpentane
19.	31.057	1.34	Benz[a]anthracene
20.	31.629	0.51	Stearic acid
21.	33.682	0.43	2-Methyl-3-nitro-4-phenyloctahydrochromen-8a-ol
22.	33.953	0.36	Octadecane
23.	34.206	0.41	5,8,11-Heptadecatriynoic acid
24.	34.686	1.02	Heptacosane
25.	34.953	0.66	Cyclopropanecarboxylic acid
26.	35.083	0.30	Hexa-t-butylselenatrisiletane
27.	35.339	2.20	Heptacosane
28.	37.452	4.03	Nonacosane

Table 2.5. Phytochemicals identified in chloroform extract of *C. stocksii* stem bark by GC-MS analysis

Sl. No.	Retention Time	Total %	Compound name
1.	14.986	0.84	1-(2Vinylphenyl)ethanone
2.	15.36	1.80	Biphenyl
3.	15.937	0.97	Azulene
4.	16.035	0.65	4,7-Methano-1H-indene, octahydro-2-(phenylmethylene)-
5.	28.13	0.58	Palmitic acid
6.	28.735	41.43	4-Acetyl-2-phenyl-9,10-dihydro-2H,9aH-2,3,4a-triazaanthracen-1-one
7.	30.021	0.80	Propanedinitrile
8.	30.865	32.37	1-Trifluoroacetoxy-5-methoxy-3-phenylpentane
9.	31.041	1.72	5,8,11-Heptadecatriynoic acid
10.	34.684	0.45	Stearic acid
11.	34.954	0.51	1-Monolinoleoylglycerol trimethylsilyl ether

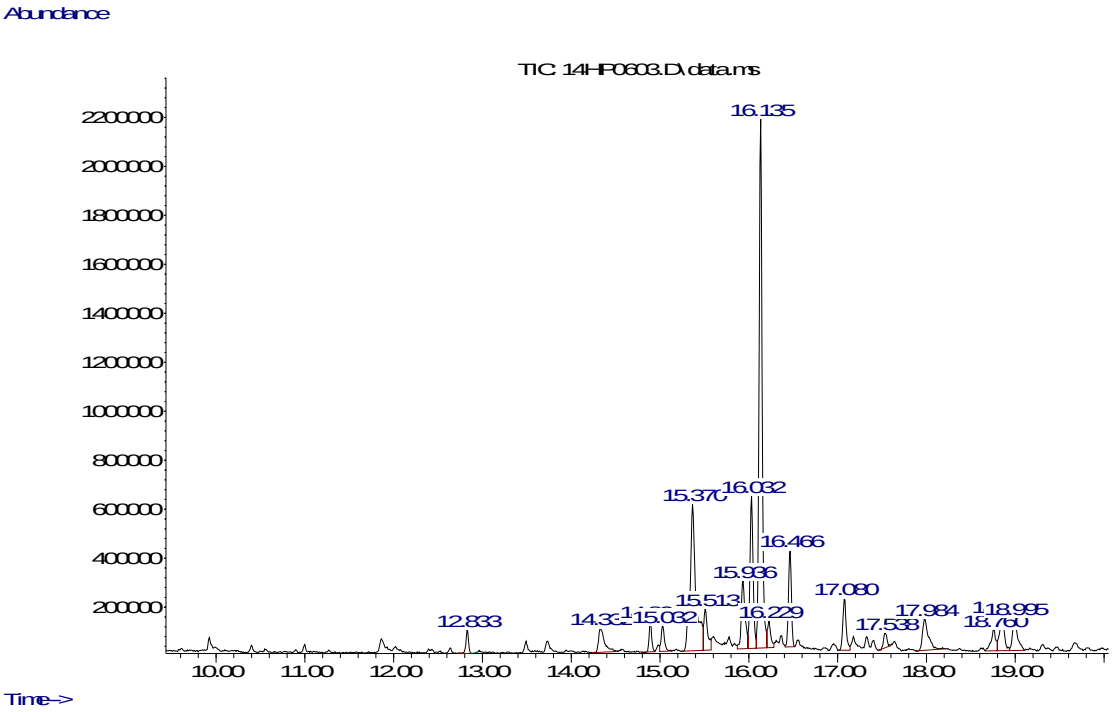


Fig. 2.1. GC-MS chromatogram of the petroleum ether extract of the *C. stocksii*

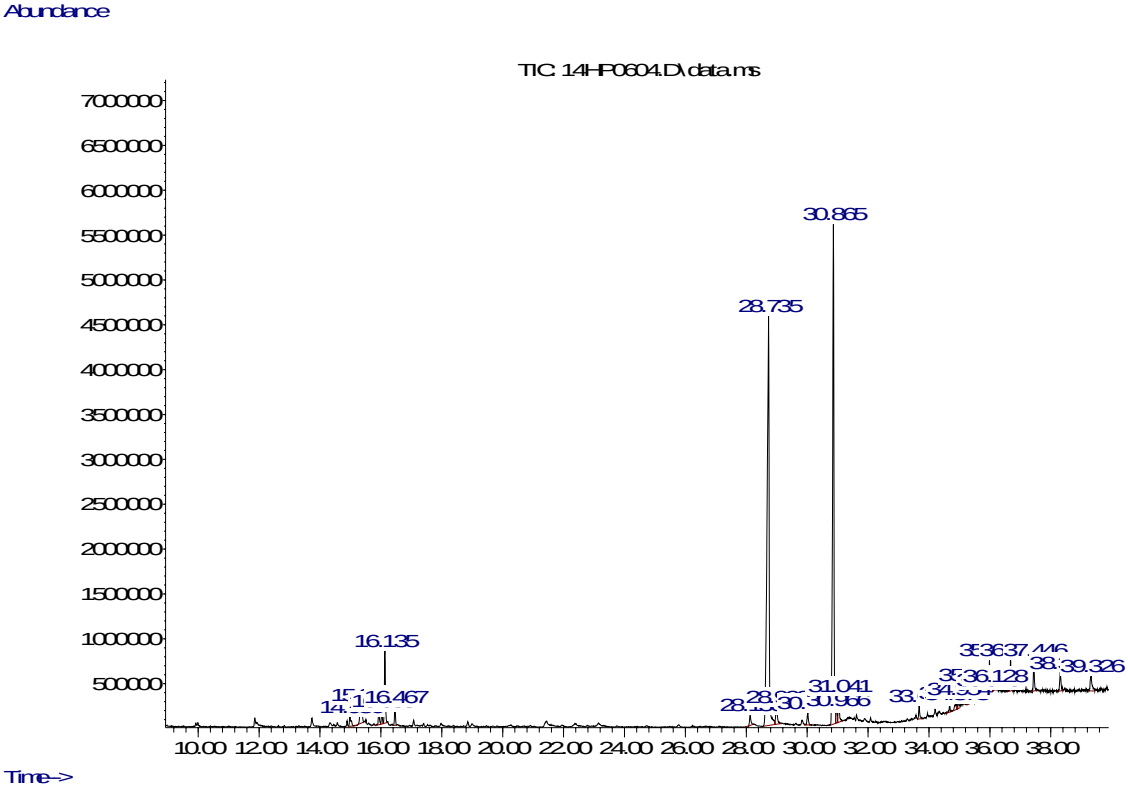


Fig. 2.2. GC-MS chromatogram of the chloroform extract of the *C. stocksii*

2.3.4 Isolation and characterization of compounds from chloroform stem bark extract

Based on preliminary phytochemical screening and GC-MS analysis, chloroform stem bark extract was selected for further purification and characterization of compounds from *C. stocksii*. The chloroform extract (3g) was chromatographed on silica gel G (mesh size 60-120) column packed in petroleum ether and eluted with gradient of petroleum ether and ethyl acetate in increasing order of polarity, affording 4 major fractions. Among them, fraction 1, fraction 2 and fraction 3 contain the compounds CS1, CS2 and CS3, respectively. They were further characterized by IR, ^{1}H NMR, ^{13}C NMR and mass spectral analysis.

Compound CS1

Molecular Formula-$C_{17}H_{36}$, IR [(KBr): v_{max}/cm^{-1}]: 1645 (C=C stretching),1462 (C-H Stretching) and 1376 (C-H Stretching), Molecular Weight: 240.46, molecular ion peak M-at m/z [M^{+}], Column specification :Auto Spec E_1mass Spectrometer. ^{1}H NMR ($CDCl_3$, 400 MHz), δ 0.725 (3H, s), δ 1.18 –δ 1.5 (2H, s,), δ 2.0 (2H,s), δ 4.86 (2H,s), 5.6 (2H, s,b). ^{13}CNMR(CDCl3—D_6, 400MHz), δ 10.86(d), δ 14.11(s), δ 19.75(s), δ 22.69(s), δ 27.09(s), δ 27.46(s), δ 28.93(s), δ 29.34(s), δ 29.67(s), δ 32.76(s), δ 37.08(s), δ 45.13(s), δ 45.32(s), δ 114.03(s), δ 139.24(s,d).

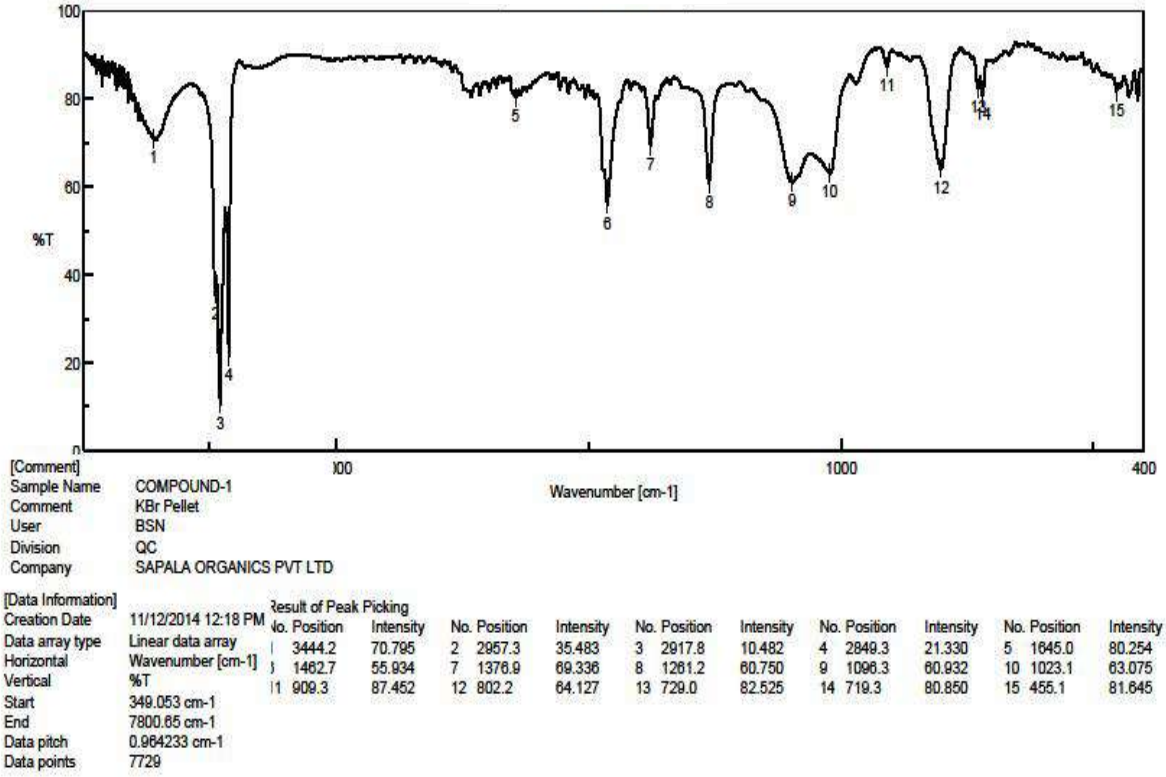


Fig. 2.3. IR spectrum of *C. stocksii* compound CS1

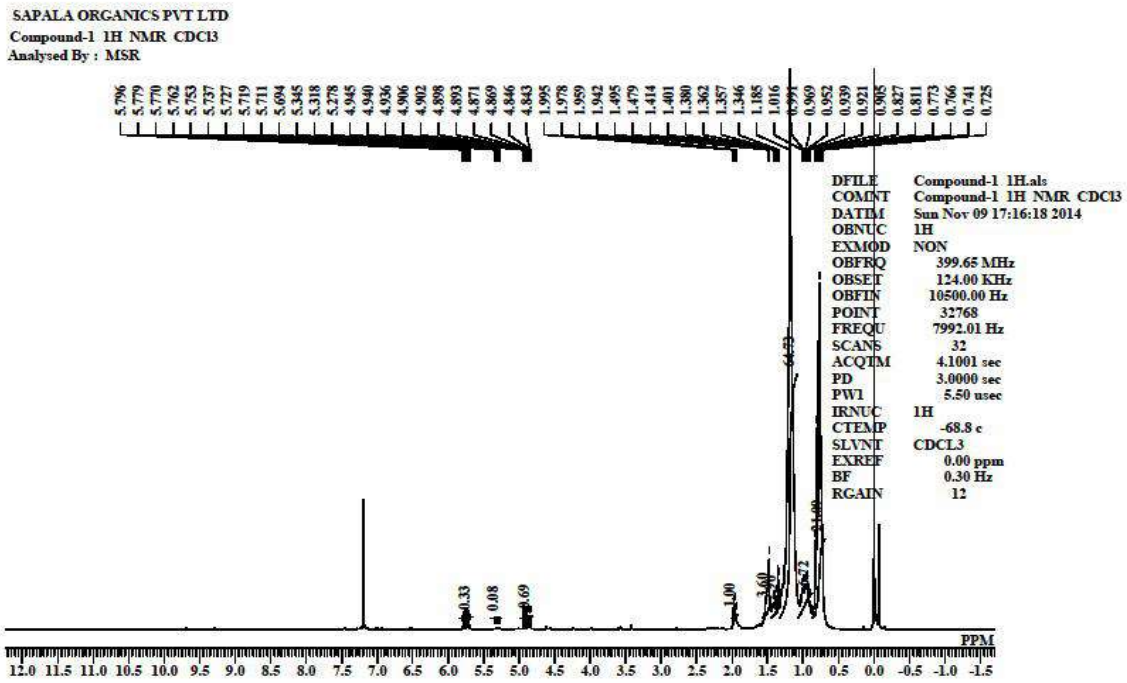


Fig. 2.4. ^{1}H NMR spectrum of *C. stocksii* compound CS1

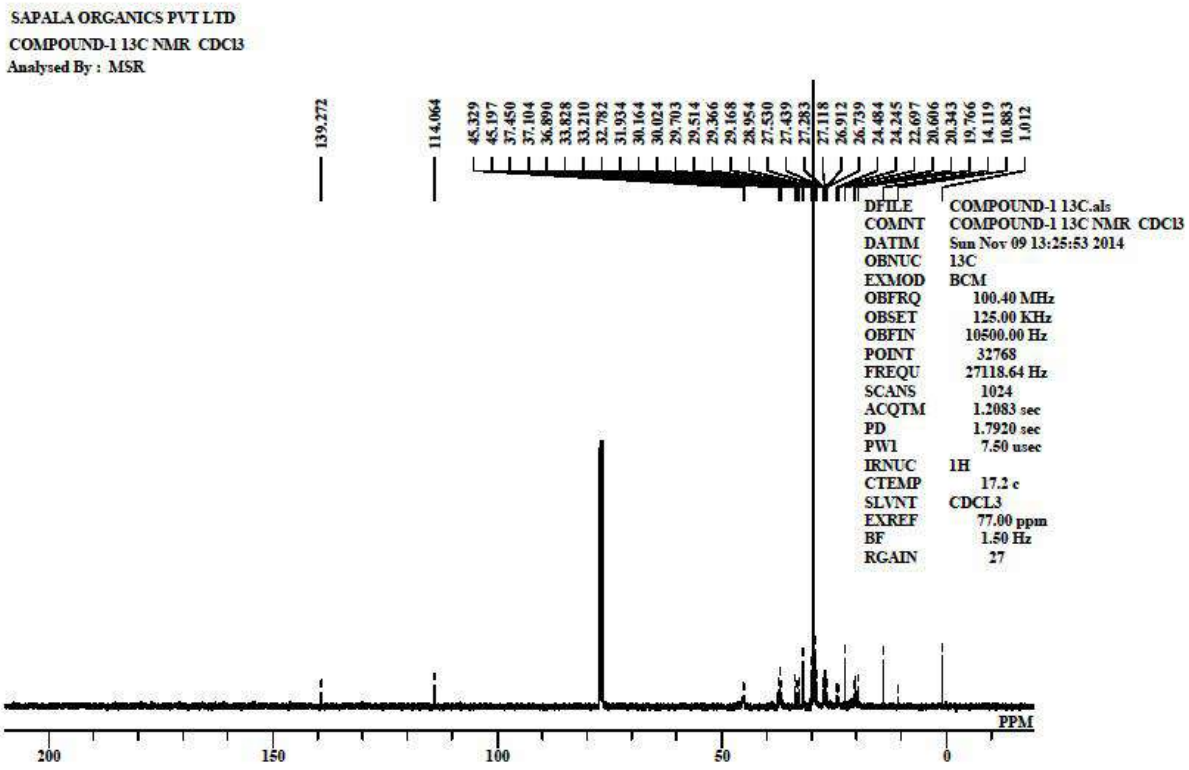


Fig. 2.5. ^{13}C spectrum of *C. stocksii* compound CS1

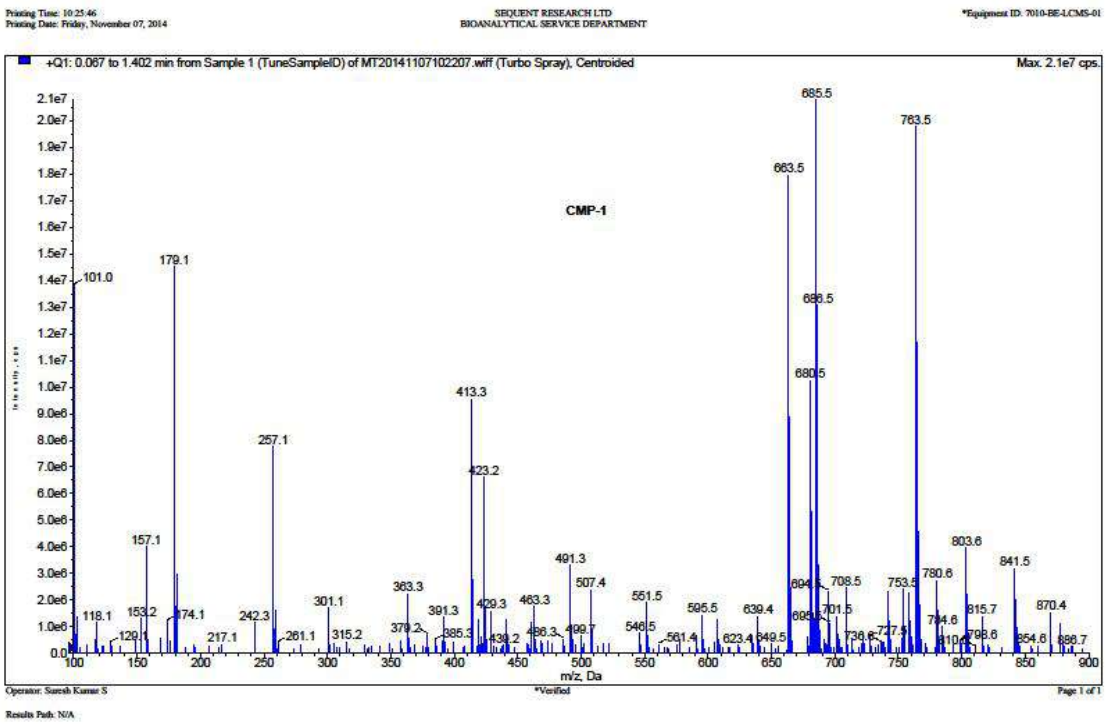
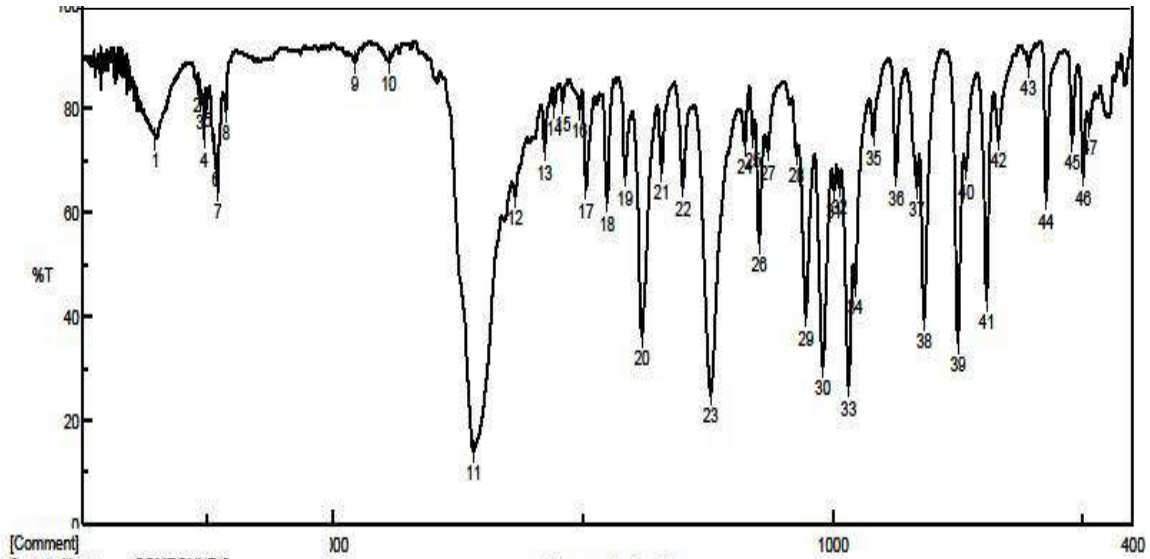


Fig. 2.6. Mass spectrum of *C. stocksii* compound CS1

Compound CS2

Molecular Formula- $C_{15}H_{14}O_3$,IR[(KBr): v_{max}/cm^{-1}]: 3418 cm^{-1}(OH group) and 1718cm^{-1}(C=O group), Molecular Weight : 242.3, molecular ion peak M-at m/z [M^+], $^+$], Column specification : Auto Spec E_1 mass Spectrometer. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.56 (2H,s), δ 5.10 (2H,s), δ 6.10 (1H,m), δ 6.26 (1H,dd), δ 6.71 (1H, d), δ 6.90(1H,m), δ 7.409 (5H,s).^{13}CNMR ($CDCl_3$-D_6, 400 MHz) δ 29.86(s) δ 77.93 (d), δ 121.67(s), δ 125.59(s), δ 126.67(s), δ 128.34(s), δ 133.11(s), δ 144.60(s), δ 135.73(s).



[Comment]
Sample Name COMPOUND-2
Comment KBr Pellet
User PSR
Division QC
Company SAPALA ORGANICS PVT LTD

[Data Information]
Creation Date 11/12/2014 12:10 PM
Data array type Linear data array
Horizontal Wavenumber [cm-1]
Vertical %T
Start 349.053 cm-1
End 7800.65 cm-1
Data pitch 0.964233 cm-1
Data points 7729

Result of Peak Picking

No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity
	3418.2	74.104	2	3082.7	84.978	3	3060.5	81.381	4	3027.7	74.398	5	3000.7	81.934
	2939.0	70.463	7	2917.8	64.172	8	2853.2	79.569	9	1955.5	89.033	10	1888.0	89.006
1	1718.3	13.980	12	1635.3	63.042	13	1576.5	71.874	14	1557.2	80.342	15	1539.9	81.183
6	1507.1	79.824	17	1493.6	64.559	18	1451.2	62.179	19	1415.5	66.986	20	1381.7	36.144
1	1342.2	67.978	22	1299.8	65.036	23	1244.8	24.938	24	1176.4	73.002	25	1160.0	74.434
6	1147.4	54.190	27	1130.1	71.975	28	1073.2	71.326	29	1053.9	40.000	30	1020.2	30.288
1	998.0	64.204	32	987.4	65.060	33	969.1	26.425	34	955.6	45.806	35	918.9	74.851
6	873.6	66.975	37	832.1	64.688	38	817.7	39.341	39	750.2	34.932	40	733.8	68.220
41	692.3	43.014	42	669.2	74.027	43	609.4	88.063	44	573.7	62.466	45	521.7	73.695
46	499.5	67.005	47	486.9	76.506									

Fig. 2.7. IR spectrum of *C. stocksii* compound CS2

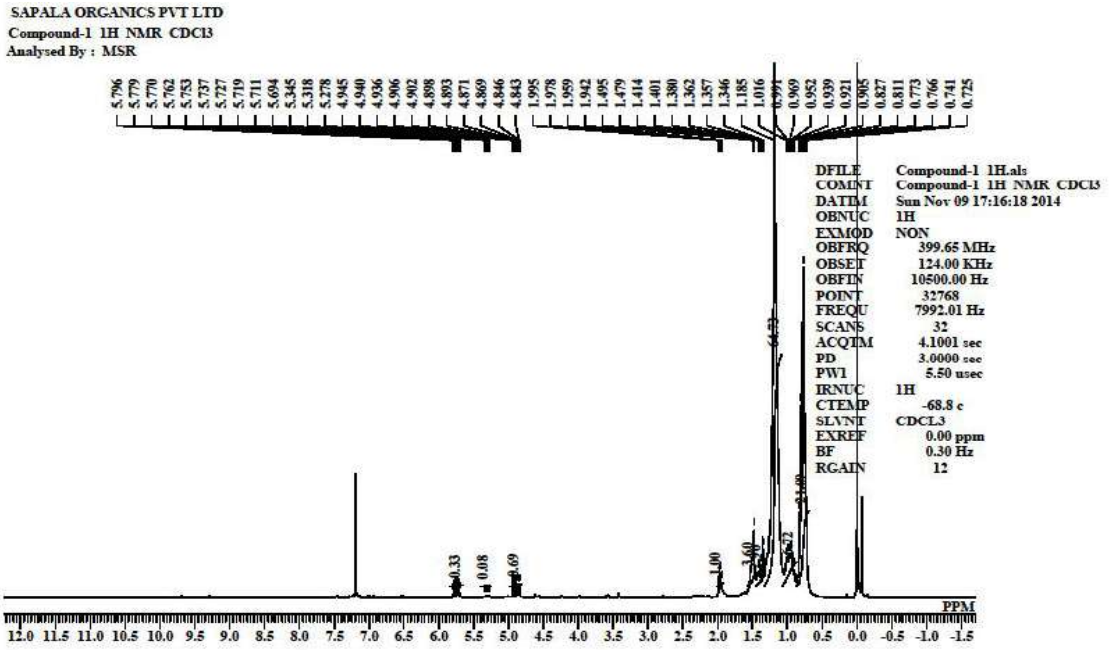


Fig. 2.8. ^{1}H NMR spectrum of *C. stocksii* compound CS2

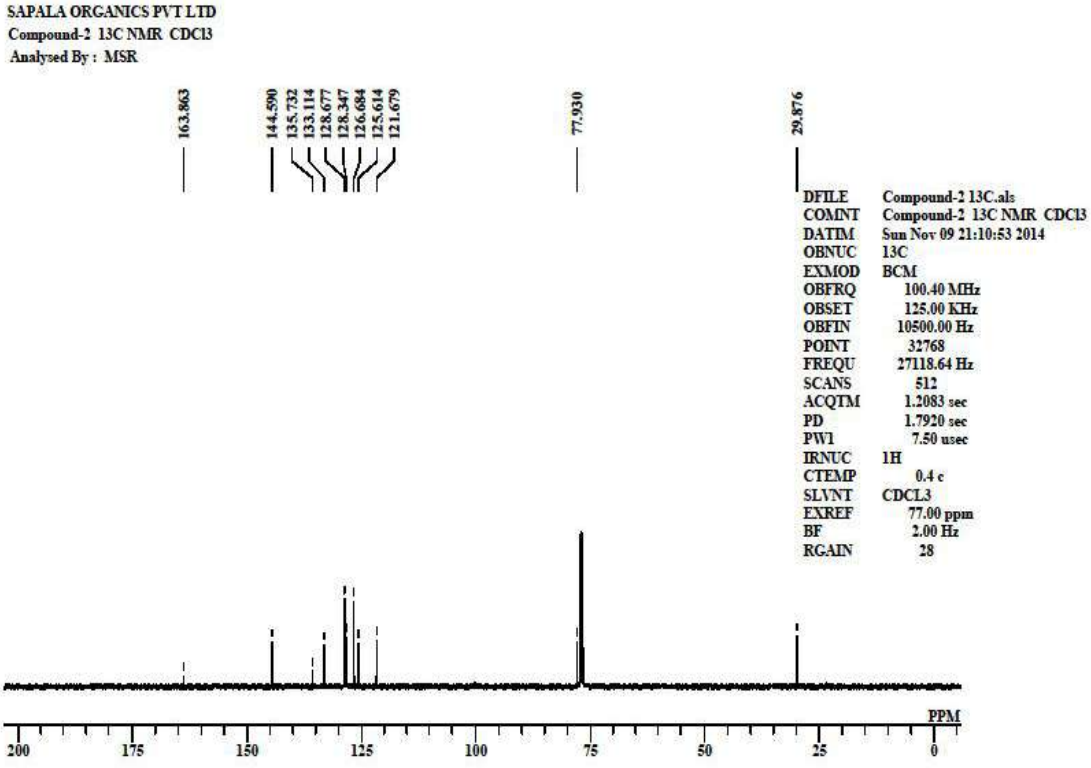


Fig. 2.9. ^{13}C NMR spectrum of *C. stocksii* compound CS2

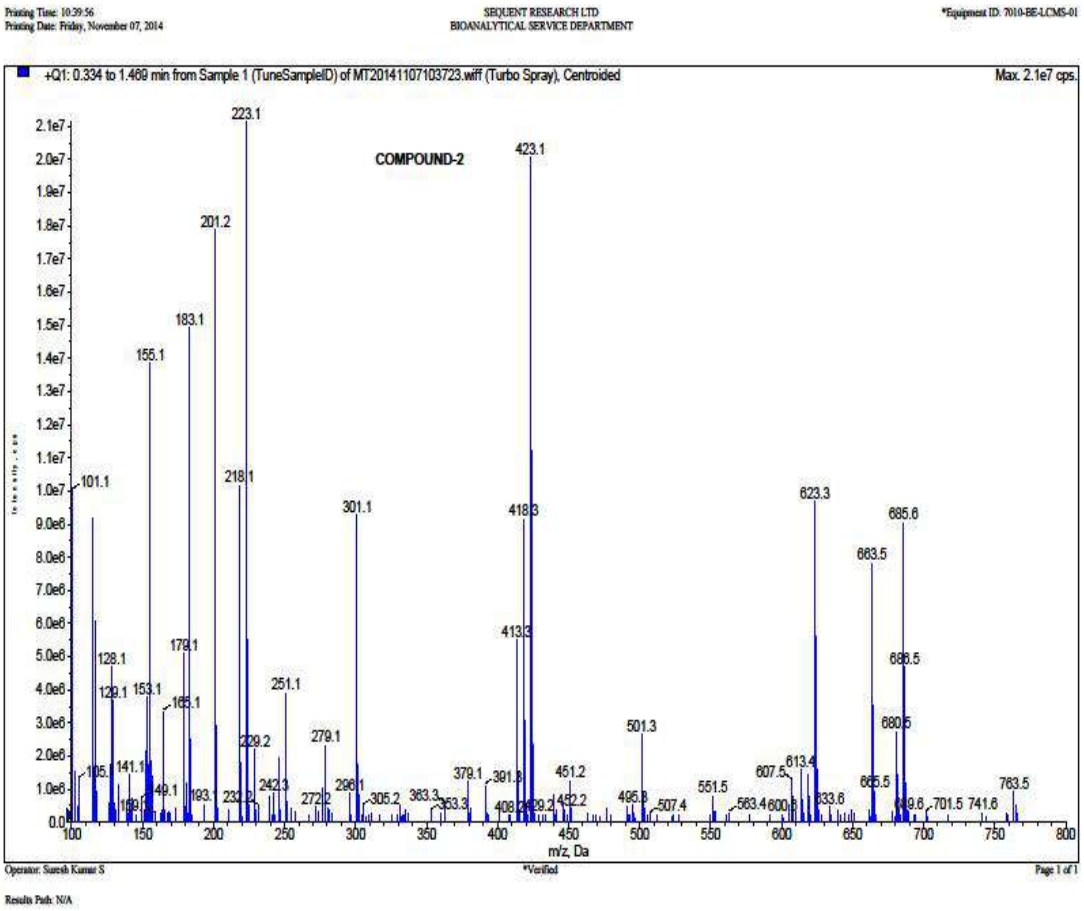


Fig. 2.10. Mass spectrum of *C. stocksii* compound CS2

Compound CS3

Molecular Formula- $C_{12}H_{16}O_2$, IR[(KBr): v_{max} /cm^{-1}]: 1643 cm^{-1}(C=C stretching group), Molecular Weight : 192.2, molecular ion peakat m/z value of 194[M+2H], Column specification : Auto Spec E_1 mass Spectrometer. ^{1}H NMR (CDCl3, 400 MHz) δ 1.45 and 1.489 (2H ,s), δ 1.63, δ 4.85, δ 1.89, δ 7.31). ^{13}CNMR (CDCl3—D_6, 400MHz) δ 128.86, δ 129.03 and δ 133.0, δ 182.77 δ 76.51, δ 77.14, δ 77.77.

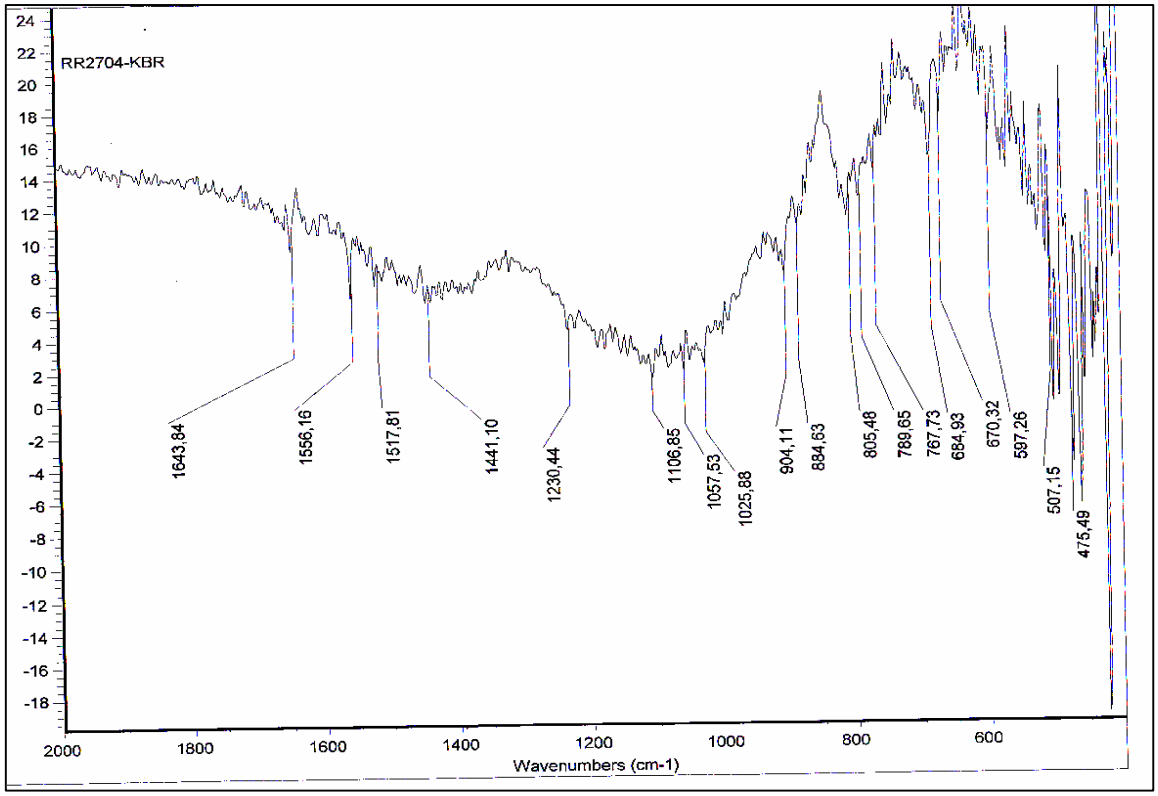


Fig. 2.11. IR spectrum of *C. stocksii* compound CS3

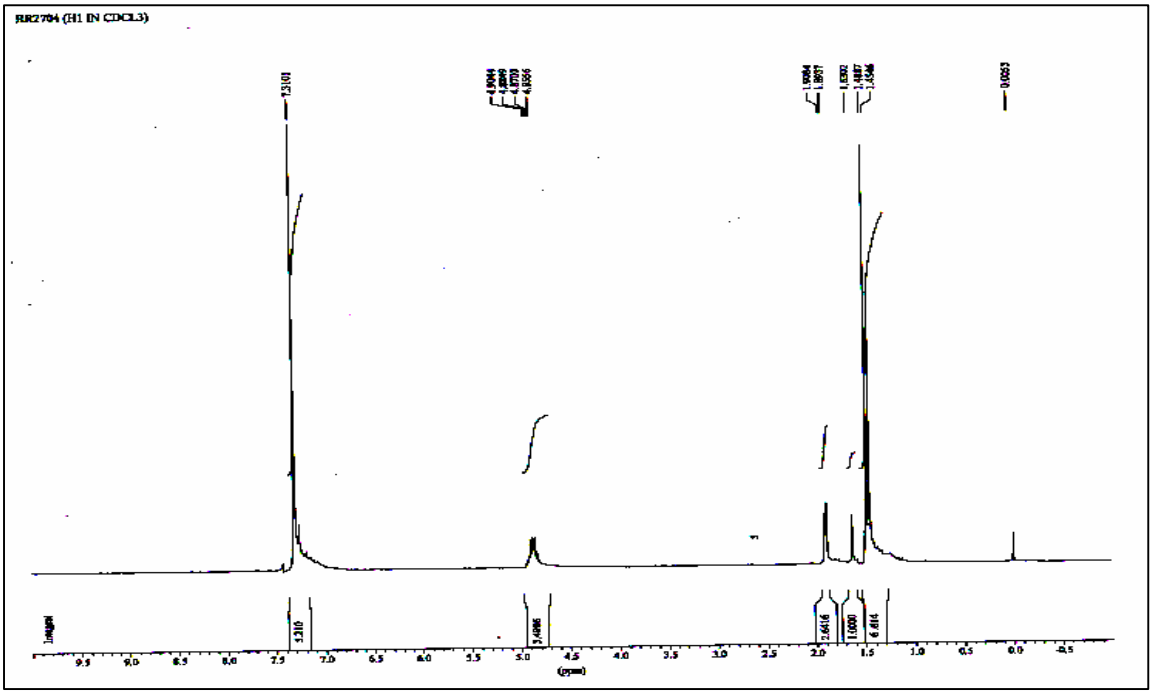


Fig. 2.12. ^{1}H NMR spectrum of *C. stocksii* compound CS3

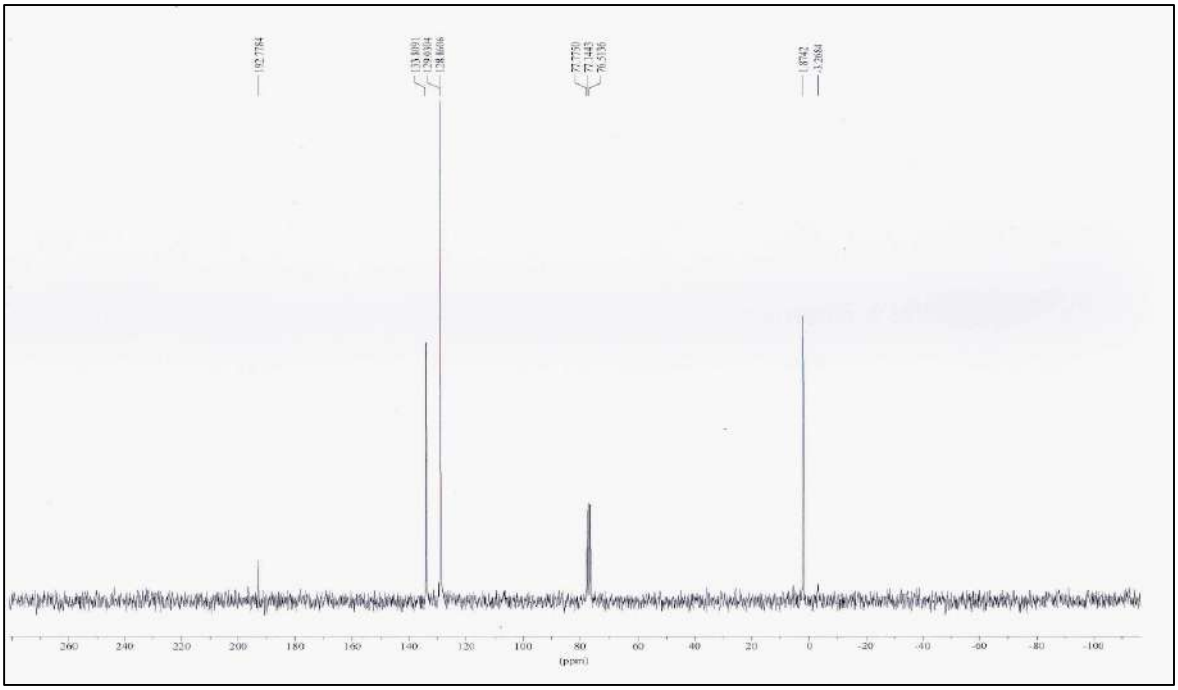


Fig. 2.13. ^{13}C NMR spectrum of *C. stocksii* compound CS3

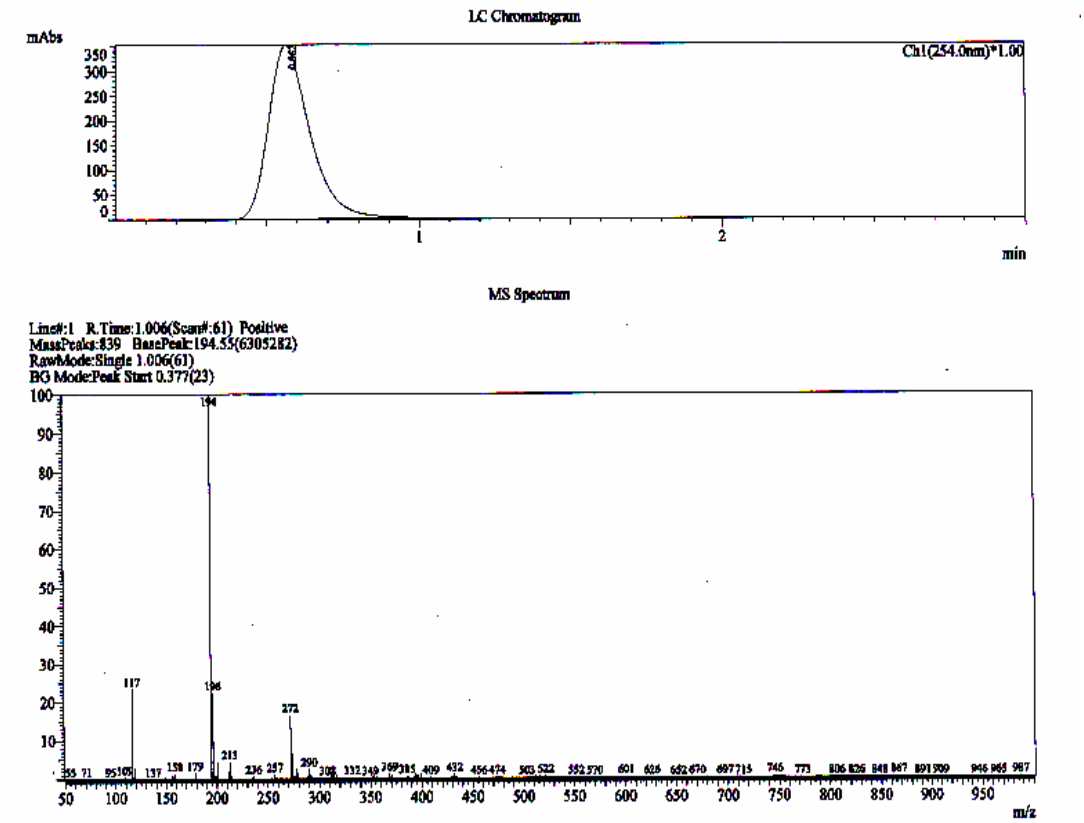
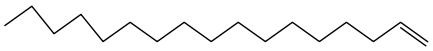
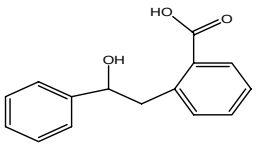
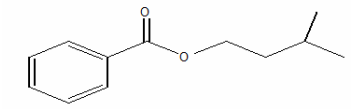


Fig. 2.14. LCMS spectrum of *C. stocksii* compound CS3

Table 2.6. Molecular structure of compounds from chloroform extract of *C. stocksii* stem bark

Sl. No	Compound Name	Structure	Nature	Solubility
1	Hepta dec-1-ene		White amorphous solid	Chloroform, ethanol
2	2-(2-hydroxy-2-phenyl ethyl) benzoic acid		White amorphous solid	Chloroform ethanol
3	3-methyl butyl benzoate		White crystal	Chloroform, ethane

2.4 Discussion

The medicinal properties of plants are mainly due to secondary metabolites produced by plants such as alkaloids, flavonoids, steroids and other phenolic compounds. Alkaloids are the polyphenols, which constitute the biggest class of secondary metabolites produced by plants which protects the plant by pathogens. Their potent biological activity includes analgesics, anticancer, antiasthma, antibacterial, aneasthetics and CNS stimulants (Madziga *et al.*, 2010). Structurally, they are made of more than one benzene ring in its structure and numerous reports support their use as antioxidants or free radical scavengers (Kar, 2007). Polyphenols are chemical components that occur ubiquitously as natural colour pigments responsible for the colour of fruits of plants. The most important role

may be in plant defence against pathogens and herbivore predators, and thus are applied in the control of human pathogenic infections (Pupponen Pimiä *et al*., 2008). The presence of steroids, flavonoids, terpenoids and quinones in all the three extracts of *C. stocksii* stem bark can be attributed to various pharmacological as well as physiological activities (Sofowa, 1993) of the plant. Tannins are heterogenous group of molecules with high molecular weight are segregated in ethanol extract of *C. stocksii* stem bark, which are known to form irreversible complex with proteins, polysaccharides and alkaloids. Phytochemical analysis of *C. stocksii* bark gives the overview of the secondary metabolites in different extracts of bark. Presence of these secondary metabolites in the stem bark extract of *C. stocksii* confirms the usage of plant for various pharmacological activities. Further investigation has been carried out to know the active phytoconstituents and their pharmacological activity.

Among *C. stocksii* stem bark extracts, the chloroform and ethanol extract are richest sources of phenols. The total amount of phenols in different extracts of *C. stocksii* varied significantly accounting for highest amount in ethanol extract followed by chloroform and petroleum ether extract. The presence of these molecules in the extract could be considered as important as they have been shown to possess various biological properties such as antitumor, anti-mutagenic an antioxidant and anti-microbial property (Feng-Lin Song *et al*., 2010).

In Aluminium chloride method, flavonoids present in the bark extract reacts with aluminium chloride forming acid labile complex. Presence of greater amount

of these flavonoids in the ethanol extract of *C. stocksii* stem bark extract compared to petroleum ether and chloroform may have significant health benefits. Because of the extensive biological property of flavonoids which promote human health, help and reduce risk of chronic diseases. This type of quantitative estimation gives approximate amount of phenols and flavonoids in different extract of *C. stocksii* stem bark extracts.

The GC-MS analysis of *C. stocksii* stem bark extract of petroleum ether and chloroform indicated the presence of various phytochemicals which have diverse use. The profile of *C. stocksii* stem bark comprises thirty compounds and the chloroform extract consists of fourteen compounds. The phytochemicals are identified based on peak area, retention time and molecular formula. Biphenyl is found in both the extracts comprises nearly 1.50 and 1.80% in petroleum ether and chloroform stem bark extract respectively. It exhibits antimicrobial activity and used as preservative in food industry especially during the transportation of citrus fruits. In addition, Caryophyllene and caryophyllene oxides are sesquiterpenoids found in both the extracts. Caryophyllene has anti-inflammatory activity, whereas caryophyllene oxide functions as antitumor, anaesthetic, antibacterial, anti-inflammatory, analgesic and antioxidant activity. In both the extracts two major compounds,4-Acetyl-2-phenyl-9,10-dihydro-2H,9aH-2,3,4a-triazaanthracen-1-one (28.44 and 41.31%) and 1-Trifluoroacetoxy-5-methoxy-3-phenylpentane (30.10 and 32.37%) were present. Presence of fluorine can exhibit florescent activity. The identified compound possesses many biological properties. Among the identified

phytochemicals, heptacosane and nonacosane palmitic acids have the property of antioxidant, hypocholesteromic, nematocide, pesticide and lubricant activities and hemolytic 5-alpha exhibit reductase inhibition (Jegadeeswari *et al*., 2012; Upgade *et al*., 2013).

The squalene (triterpenes) is a phenolic compound and that the terpenes are found in latex and resins of some plants and physiological function of these compounds are generally believed to be a chemical in defense against certain pathogens causing human and animal diseases (Scortichini and Rossi, 1991). Among the identified phytochemicals, Squalene has the property of antioxidant activity (Lalitharani *et al*., 2009; Sutha *et al*., 2013) and anticancer activity (Purushoth *et al*., 2013). It has been found that squalene possess chemopreventive activity against colon cancer (Rao *et al*., 1998; Alagammal *et al*., 2012). Squalene has been reported in *Aloe vera* (Arunkumar *et al*., 2009) and *Vitexnegundo* (Praveen Kumar *et al*., 2010). Squalene is used in cosmetics as a natural moisturizer (Sermakkani *et al*., 2012). Their activity is due to function of the lipophilic properties of the constituent terpenes, the properties of their functional groups and their aqueous solubility (Mahato and Sen, 1997; Ezhilan and Neelamegam, 2012). The squalene was also reported to have anticancer, antioxidant, chemopreventive, gastropreventive, hepatoprotective, pesticide, anti-tumor and sunscreen properties (Sunitha *et al*., 2001; Ukiva *et al*., 2002; Katerere *et al*., 2003).

Based on the GC-MS analysis the chloroform extract of *C. stocksii* has been selected for the isolation and characterization following three compounds.

Compound CS1

IR spectrum of compound CS1 exhibited absorption band at 1645 cm^{-1} which is due to C=C stretching, another absorption band at 1462 cm^{-1} for C-H stretching frequency. The ^{1}H NMR spectrum of the compound displayed an upfield signal for three protons for a terminal methyl group, a broad singlet at δ 1.18 and a multiplet at δ 1.5 for a long chain of methylene groups and a signal at δ 2.0 for a methylene group connected to the unsaturated group. Further a pair of multiplets at δ 4.86 for two protons and a multiplet at δ 5.6 for an unsaturated proton suggests the presence of a terminal methylene group. ^{13}CNMR spectrum of the compound is in agreement with the assigned structure. The mass spectrum confirms the molecular weight of the compound to be 238.

Compound CS2

IR spectrum of compound CS2 exhibited characteristic absorption band at 3418 cm^{-1} which is due to hydroxy group, another absorption band at 1718 cm^{-1} for C-H carbonyl carbon stretching frequency. The ^{1}HNMR spectrum of the compound displayed an upfield signal at δ 2.56 for two protons for a methylene group, a signal at δ 5.10 for a proton under oxygen function, and a bunch of signals at 6.10 (^{1}H,m), 6.26 (^{1}H,dd), 6.71 (^{1}H, d), 6.90 (^{1}H,m), and at δ 7.40 for five protons which amounts to nine aromatic protons. The ^{13}CNMR spectrum of the compound displayed signals at δ 29.86 for an aliphatic methylene group, a

signal at δ 77.93 for a carbon under oxygen function, a signal at δ 163.56 for a carbonyl carbon and a bunch of signals between δ 121.67, 125.59, 126.67, 128.34, 133.11, 144.60 and δ 135.73 for aromatic carbon atoms. The mass spectrum confirms the molecular weight of compound to be 242.3 as revealed by the presence of molecular ion peak M-at m/z.

Compound CS3

IR spectrum showed absorption band at 1643 cm^{-1} indicating the presence of C=C group. ^{1}H NMR spectra showed strong singlets at δ 1.45 and 1.48 for methyl groups attached to an unsaturated system. The multiplet peaks at δ 1.63 suggests a proton signal at δ 4.85 and δ 1.89 suggests the presence of a methylene protons attached to an oxygen function. Apart from this a strong multiplet at δ 7.31 suggests the presence of aromatic protons. In the ^{13}CNMR spectra it showed strong signals at δ 128.86, 129.03 and 133.0 for unsaturated carbon atoms. The signal at δ 182.77 is attributed to the presence of a carbonyl group. The signal at δ 76.51, 77.14 and 77.77 corresponds to aliphatic carbons. The mass spectrum confirms the molecular weight of compound to be 192 as revealed by the presence of molecular ion peak M-at m/z 194[M+2H].

Chapter-3

Pharmacological activity of *Cryptocarya stocksii*

3.1 Introduction

In 1956 for the first time, Denham Harman introduced free radicals in to the world of biological systems and their role in aging process (Gerschman, 1954). These free radicals are molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals, highly reactive, due to unpaired electron(s) they are short-lived, ranging from microseconds to seconds (Halliwell and Gutteridge, 1993) few of them are reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive sulphur species (RSS). In the cell, small number of electrons leak to oxygen prematurely during energy transduction, forming the oxygen free radical superoxide, which leads to the pathophysiology of a variety of diseases (Kovacic and Pozos, 2005). Oxygen is essential for all the cells but when it loses single electron, it becomes deleterious to cells (Lobo *et al*., 2010).

The process of "redox regulation" protects living organisms from various oxidative stresses and maintains "redox homeostasis" by controlling the redox status *in vivo* (Droge, 2002). Production of superoxide radical occurs within the mitochondria of a cell during cellular metabolism and functional activities, and has important roles in cell signalling, apoptosis, gene expression and ion transportation. Excessive accumulation of ROS resulting to a phenomenal disturbance in the equilibrium status of pro-oxidant/ antioxidants reactions (Valko *et al*., 2006) causes deleterious effects on proteins, lipids, nucleic acids and carbohydrates. ROS can attack bases in nucleic acids, amino acid side chains in

proteins and double bonds in unsaturated fatty acids, in which hydroxyl radical (▪OH) is the strongest oxidant and contributes to the pathogenesis of inflammatory disease, cardiovascular disease, cancer, diabetes, Alzheimer's disease, cataracts, autism and aging. In order to prevent or reduce the ROS-induced oxidative damage, the human body and other organisms have developed an antioxidant defence system that includes enzymatic, metal chelating and free radical scavenging activities to neutralize these radicals after they have formed. In addition, intake of dietary antioxidants may help to maintain an adequate antioxidant status in the body.

3.1.1 Antioxidants

Antioxidants are molecules that are capable of neutralizing the harmful effects of the ROS through the endogenous enzymatic defense system such as the superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) in human system. Antioxidants may decrease the cellular level of free radicals either by inhibiting the activities or expression of free radical generating enzymes such as NAD(P)H oxidase and xanthine oxidase (XO) or by enhancing the activities and expression of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) (Muthuselvam *et al*., 2009; Praveen Kumar *et al*., 2010). Many antioxidant compounds have been shown to increase the average or mean life span of an organism. The prevention of lipid peroxidation is an essential process in all the aerobic organisms, as lipid peroxidation products can cause DNA damage. Increased lipid peroxidation and decreased antioxidant protection frequently occurs: epoxides may spontaneously react with nucleophilic centers in the cell and thereby covalently bind to DNA,

RNA and protein (Mate´s and Sa´nchez-Jime´nez, 1999). Such a reaction may lead to cytotoxicity, allergy, mutagenicity or carcinogenicity, depending of the properties of the epoxide. The beneficial effect of antioxidants on promoting health is believed to be achieved through several possible mechanisms, such as directly reacting with and quenching free radicals, chelating transition metals, reducing peroxides, and stimulating the antioxidative defense enzyme system (Maity *et al*., 2010).

3.1.2 Plants as a source of antioxidants

Herbs contain some of the most powerful natural antioxidants and are highly valued for their antioxidant and anti-ageing effects. Synthetic antioxidants such as *tert*-butylhydroxy-toluene, *tert*-butylhydroxyanisole and *tert*-butylhydroquinone have been widely used in the food industry to retard lipid oxidation. Synthetic antioxidants are not preferred for pharmacologic use primarily due to toxicological concerns. Thus, increased interests have focused on identifying plant extracts to use as dietary antioxidant supplements. Most of these natural antioxidants are derived from fruits, vegetables, spices, grains and herbs such as ginseng, curcuma, ginkgo, rosemary, green tea, grape, ginger and garlic. They contain a wide variety of antioxidant compounds, such as phenolics (phenol and polyphenols), flavonoids, carotenoids, steroids and thiol compounds (Krishnaiah *et al*., 2010).

Several biologically important compounds have been reported to have antioxidant functions. These include vitamin C, vitamin E, vitamin A, β-carotene, metallothionein, polyamines, melatonin, NADPH, adenosine, coenzyme Q-10, urate, ubiquinol, polyphenols, flavonoids, phytoestrogens, cysteine, homocysteine,

taurine, methionine, *s*-adenosyl-L-methionine, resveratrol, nitroxides, GSH, glutathione peroxidase (GPX), superoxide dismutase (SOD), catalase (CAT), thioredoxin reductase, nitric oxide synthase (NOS), heme oxygenase-1 (HO-1) and eosinophil peroxidase (EPO) (Krishna *et al.,* 1996; Chanvitayapongs *et al.*, 1997; Evans *et al*., 1997; Beyer *et al*., 1998; Devamanoharan *et al*., 1998; Jourd'heuil *et al.,* 1998).

3.1.3 Antimicrobial activity

The interaction between plants, animals and microorganisms is natural and constant. The ecological role of microorganisms in biogeochemical cycle is well documented. Microorganisms are so small that they can be visualized only with the aid of high resolution microscopes. Pathogenic microorganisms affect the health of individuals by causing infectious disease. Failure of chemotherapy, rapid increase in the number of antibiotic resistant pathogenic microorganisms and the low rate of development, and introduction of new antimicrobials represent a serious threat to human health on a global scale (WHO, 2014) as it reduces the effectiveness of treatments and increases morbidity, mortality, and health care cost (Coast *et al*.,1996). Epidemics of infectious and degenerative diseases remains largest cause of global death each year, accounting for approximately one- half of death in tropical countries (Avery *et al*., 2006; Tekwu *et al.,* 2012). There is no specific effective therapy and vaccines for many of these diseases which are difficult to treat (Hamer *et al.,* 2010).

In the nineteenth century, there was rapid decrease in public health hazard of bacterial infection due to discovery, development and clinical use of antibiotics. Discovery of new natural antibacterial agents for treating infectious disease is essential to prevent the spread of disease and improve their treatment. But

antibiotics are occasionally associated with few side effects, hypersensitivity, allergic reaction, and immune suppression (Ahmad *et al*., 1998; Sumaira Sahreen *et al*., 2015). Developed and developing countries alike have embarked to natural products for their health care services because they are natural, non-narcotic, and in most cases easily available at affordable price, with least side effects. Evaluation of natural resources is also useful in food preservation as the long historic use of herbs has proved their safety efficacy in various traditional medicinal systems. Modern usage of antimicrobials as natural remedies has accelerated their applications in food, cosmetics, pharmaceutical product (Krishnaiah *et al*., 2007).

Natural medicine constitute valuable source of new drug molecule, derived from prokaryotic bacteria, eukaryotic microorganisms, plants and various animal organisms. Plant products occupy major part of antimicrobials discovered till now with possibly new modes of action (Balouiri *et al*., 2016). Plant extracts and essential oils have long been known to possess antimicrobial activity and were frequently studied and reviewed (Bakkali *et al*., 2008; Burt 2004; Sahreen *et al*., 2015). Biologically active compounds and secondary metabolites present in plants have a significant role and are becoming part of integrative health care system due to therapeutic property. Recent studies have reported increased interest in plant materials for their diverse pharmacological and biological properties, including antibacterial activity in treating various diseases.

3.1.4 Analgesic activity

The molecular mechanism involved in the action of analgesics involves the inhibition of enzymatic activity of a homogeneous class of isoenzyme cyclooxygenase (COX) or prostaglandin endoperoxidase synthase (PGHS). COX

enzyme occurs in different isoforms such as COX-1, COX-2 and COX-3 in injured tissue. Opiods exhibit analgesic activity by activating opiod receptor in CNS and thus cause changes in ion conduction leading to a decrease in signal transmission from the periphery to the CNS, thereby producing analgesia (Hanna Skubatz *et al*., 2009).

Majority of human population is affected by chronic painful condition like gout rheumatoid arthritis etc. Synthetic drugs available in the market possess only symptomatic relief and their toxic effects pose limitations to continuous use. Many medicinal plants have been used since ages without any adverse effects. The success of salicylic acid from willow bark prompted the pharmaceutical manufacturing house of Frederick Bayer to actively search for a derivative of comparable or better efficacy to salicylic acid. Many analgesics drugs of plant origin have been discovered and search for better analgesic drug is ongoing. Herbal medicines derived from the plant extracts are being increasingly utilized to treat a wide variety of clinical diseases, though relatively little knowledge about their mode of action is available (Pramod *et al*., 2015).

3.1.5 Antipyretic activity

Pyrexia or fever is defined as elevation in body temperature above normal range due to tissue damage, inflammation, malignancy or graft rejection. Cytokines, interleukin, interferon and Tumor Necrosis Factor α (TNF-α) are formed in large amount under this condition, which increase PGE2 which in turn triggers hypothalamus to elevate body temperature (Rajani *et al*., 2011). Fever is associated with symptoms of sickness behaviour which consist of lethargy, depression, anorexia, sleepiness, & inability to concentrate. Cytokines interleukin

and Tumor Necrosis Factor α (TNF- α) are formed in large amount under this condition, which in turn increases hypothalamus to elevate body temperature. Antipyretics are the drugs used to reduce body temperature. Most antipyretics drugs work by inhibiting COX-2 expression levels primarily by reducing PGE biosynthesis, whose action is elevated in an elevated temperature (Umesh *et al*., 2010). Some other agents used as medications include NSAIDs, opioid etc. chronic usage of most these agents results in, renal, gastrointestinal, hepatic, central nervous system, dermatological diseases and other several side effects (Arun Vijayan *et al.*, 2007).

Acetaminophen or paracetamol are well-known and widely used antipyretic drug. Though it was discovered 100 years ago, exact mechanism of action of paracetamol has not been resolved till now. It suppresses prostaglandin production like NSAIDs. These NSAIDs act by inhibiting the conversion of arachidonic acid (AA) into prostaglandin H (PGH2). The studies on the mechanism of paracetamol action require further verification - they should concern not only on the therapeutic action of this drug but also more frequently reported poisoning, especially strong hepatotoxicity resulting from the drug overdose since numerous preparations containing paracetamol are available without a prescription.

3.1.6 Anti-inflammatory activity

Several mechanism of action have been proposed to explain the anti-inflammatory action of phytoconstituents, it consists of: (1) Antioxidative and radical scavenging activities; (2) Modulation of cellular activities of inflammation-related cells (mast cells, macrophages, lymphocytes, and neutrophils); (3) Modulation of proinflammatory enzyme activities such as phospholipase A2

(PLA2), cyclooxygenase (COX), and lipoxygenase (LOX) and the nitric oxide (NO) producing enzyme, nitric oxide synthase (NOS); (4) Modulation of the production of other pro-inflammatory molecules; (5) Modulation of proinflammatory gene expression.

Phytochemicals have been shown to modulate various points in these inflammatory processes. These modulations serve as controlling points where the amplification of the inflammatory processes can be disconnected and thereby reduce subsequent diseases risk. They probably have multiple cellular mechanisms acting on multiple sites of cellular machinery. The continual efforts will provide new insight into the anti-inflammatory activities of phytochemicals, and eventually lead to development of a new class of anti-inflammatory agents.

3.1.7 Wound healing activity

Research on drugs that increase wound healing is a developing area in modern biomedical sciences. Herbal medicines in wound management involve disinfection, debridement and providing a moist environment to encourage the establishment of the suitable environment for natural healing process (Purna and Babu, 2000). Several drugs obtained from plant sources are known to increase the healing of different types of wounds (Biswas and Mukherjee, 2003). Herbal medicine has become an integral part of standard healthcare, based on a combination of time honoured traditional usage and ongoing scientific research.

As previously described, there is no data available regarding the antioxidant and antimicrobial activities of *C. stocksii* plant extract. Present work is undertaken to systematically study the antioxidant and antimicrobial activity of different plant extracts by different antioxidant methods.

3.2 Materials and methods

3.2.1 Chemicals and reagents

2-diphenyl-1 picrylhydrazyl (DPPH), Sodium nitrite, Ammonium molybdate, Sodium phosphate, Ascorbic acid, Ferrous chloride, Ferrozine (1-napthyl) ethylene diamine dihydrochloride were purchased from Merck Chemical Co. All other chemicals and reagents were of analytical grade procured from Himedia labs, Mumbai. The solvents used were distilled prior to use.

3.2.2 DPPH scavenging activity

The antioxidant activity of the plant extracts and the standard were assessed on the basis of the radical scavenging effects of the stable DPPH free radical. The conventional DPPH Scavenging capacity assay was performed according to previously described method (Braca *et al.*, 2001). About 10-100 µl of each extract or standard was added to 3 ml of DPPH in methanol (0.33%) in a test tube and make up to final volume of 4 ml with methanol. Ascorbic acid was used as reference standard and dissolved in methanol to make the same concentration as remaining extracts. The reaction mixture was incubated at 37ºC for 30 min. The absorbance of each reaction mixture at 517 nm was measured against blank using a UV-visible Spectrometer (Shimadzu UV-800). The percentage of DPPH radical absorption for each reaction was calculated using the following equation,

$$\text{Scavenging Activity (\%)} = A_{o-} A_1 / A_o \times 100$$

where, A_0 is the absorbance of the control and A_1 is the absorbance of the sample. The inhibition curve was plotted for triplicate experiments and IC_{50} values were calculated.

3.2.3 FRAP method

The reductive potentials of different extracts of *C. stocksii* were determined according to the method of Oyaizu *et al.*, (1986). Different concentration of extracts of *C. stocksii* (20–100 μg/ml) in 1.0 ml of methanol was mixed with equal volume of 2 M phosphate buffer, pH 6.6 and 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. An equal volume of 10% TCA was added to the mixture and centrifuged at 3000 rpm for 10 min. The supernatant was mixed with distilled water and 0.1% ferric chloride, and absorbance was measured at 700 nm.

3.2.4 The total antioxidant capacity

The total antioxidant capacity of different extracts of *C. stocksii* was evaluated by the method of Prieto *et al.* (1999). 0.5 ml of extract was combined with 5 ml of reagent solution (0.6M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The absorbance of reaction mixture was measured at 695 nm against blank after cooling to room temperature. Methanol (0.5 ml) in the place of extract was used as the blank. The antioxidant activity is expressed as the number of gram equivalent of ascorbic acid. The calibration curve was prepared by mixing ascorbic acid (20, 40, 60, 80 and 100 μg/ml) with methanol.

3.2.5 Bacterial strains and antibacterial assay

Bacillus subtilis (MTCC 441), *Staphylocccus aureus* (ATCC 25923), *Escherichia coli* (MTCC 443) and *Pseudomonas aeruginosa* (ATCC 27853) were used for determining the antibacterial activity. All the bacterial strains were maintained on nutrient agar at 37°C. The *in vitro* antibacterial activities of the

different extracts of *C. stocksii* were studied comparatively with that of standard streptomycin by using agar well diffusion method. 0.1 ml of the 24 h old bacterial suspension (10-100 cfu/ml) was spread on the nutrient agar plate and wells were made by using sterile cork borer. The extracts dissolved in DMSO (100 µL) were added to respective wells and incubated for 24 h at 37°C. After incubation, zone of inhibition was measured in mm.

3.2.6 Fungal strains and antifungal assay

Pencillium chrysogenum MTCC-11709, *Aspergillus clavatus* MTCC-16604 and *Aspergillus terrus* MTCC-20542 were used for determining the antifungal assay. All the fungal strains were maintained on potato dextrose agar at 37°C. The *in vitro* antifungal activities of different extracts of *C. stocksii* were studied comparatively with that of standard Flucanazole by using agar well diffusion method. 0.1 ml of 24 h old fungal suspension spread on the potato dextrose agar plate and wells were made by sterile cork borer. The petroleum ether, chloroform and ethanol stem bark extracts dissolved in DMSO (100 µL) were added to respective wells and incubated for 48 h at 27°C. After incubation the zone of inhibition was measured in mm. The minimum inhibitory concentration (MIC) is the lowest concentration of the extract able to inhibit the visible bacterial growth was calculated and measured.

Animals

Adult *Wistar albino* mice of either sex, weighing 20-25 g were procured from National College of Pharmacy, Shivamogga, Karnataka, India. The animals were housed in polypropylene cages in standard environmental conditions of

temperature (21±2°C), humidity (55±10%) and a 12 h light-dark cycle. The mice were given a standard laboratory diet (Commercial pelleted food from Hindustan Lever Ltd., Bengaluru) and water *ad libitum*. Food was withdrawn 12 h before and during the experimental duration. All the experimental protocols were approved by the institutional animal ethical committee having registration no. 144/1999/ CPCSEA/dated: 10/04/2000.

3.2.7 Analgesic activity

The analgesic activity of *C. stocksii* was examined using hot plate method. Animals were divided into five groups, each group containing six animals. Group I served as the positive control. Group II animals received the standard drug of aspirin 150 mg/kg body weight, whereas group III to V animals were orally administered various plant extracts viz., petroleum ether, chloroform and ethanol at the dose of 500 mg/kg body weight, respectively. The temperature of the hot plate was maintained at 55±1°C. The mice were placed on the hot plate and time in seconds for paw licking or jumping was recorded as basal reaction time. Cut off time in the absence of response was 15 sec to prevent the animals being burnt. The reaction time (seconds) was observed on hot plate for the time taken by mouse to react to the thermal pain by licking its paw or attempting to jump out. Observations were made before and after administration of respective drugs at an interval of 60 minutes.

3.2.8 Antipyretic activity

The antipyretic activity was evaluated using Swiss mice (25-30 g) of either sex. The animals were fasted overnight and divided into V groups consisting of six

animals each. The normal body temperature of each mouse was recorded using digital thermometer and then pyrexia was induced in all mice by injecting 20% of aqueous suspension of Brewer's yeast. The induction of pyrexia was confirmed by rise in body temperature of 0.5°C. The control group received a 1% Tween 80 (10 ml/kg), standard group animal received a paracetamol 150 mg/kg body weight. Animals in the test group received a different extracts at the dose of 500 mg/kg body weight. After drugs administration, rectal temperature was recorded periodically at 1, 2 and 3 h intervals. The percentage reduction in pyrexia was calculated by the following formula,

$$\text{Percent reduction} = B\text{-}C_n/B\text{-}A \times 100$$

where, B represents temperature after pyrexia induction, Cn temperature after 1, 2 and 3 hrs and A is the normal body temperature.

3.2.9 Anti-inflammatory activity

The anti-inflammatory activity of *C. stocksii* was examined using carrageenan induced rat paw edema method. Thirty experimental animals were randomly selected and divided into five groups denoted as Group I, Group II, Group III, Group IV, and Group V, considering six rats in each group. Each group received a particular treatment i.e. Control (Group I), Standard (Group II), and three doses of the extract. Prior to any treatment, each rat was weighed properly and the doses of the test samples were adjusted accordingly. Group III to Group V received a stem bark extracts orally at a dose of 500 mg/kg body weight. Group II received a intraperitoneal administration of indomethacin at a dose of 5 mg/kg body weight as a standard for anti-inflammatory activity, while Group I received 0.1% Tween 80.

The anti-inflammatory activity of stem bark extracts were evaluated in Wistar rats. The animals were fasted overnight and divided into control, standard and test groups consisting of six animals each. The different test extracts were administered to test group at the dose of 500 mg/kg body weight by oral route. Animals in the standard group received Indomethacin at the dose of 4 mg/kg body weight. Control group received 1% Tween 80. Thirty minutes after administration of the respective drugs, all the animals challenged with 0.1 ml of 1% carrageenan into subplantar region of the left hind paw. Paw volume was measured using digital pleythsmometer after administration of carrageen at an interval of 30 min, 1, 2, 3 and 4 hrs. The efficacy of the drug was tested on its ability to inhibit paw edema as compared to the control group. The Percentage inhibition of paw edema was calculated by the formula as below:

$$\% \text{ of inhibition of paw edema} = [(V_c - V_T)/V_c] \times 100$$

where, Vc = Paw edema of control group and VT = Paw edema of treated group.

3.2.10 Wound healing activity

Excision method

The rats were inflicted with excision wounds. The rats were anaesthetized prior to creation of the wounds using diethyl ether. The dorsal fur of the animal was shaved with an electric clipper and the area of the wound to be created was outlined on the back of the animals with methylene blue using a circular stainless steel stencil. A full thickness of the excision wound of 2.5 cm in width (circular area = 4.90 cm^2) and 0.2 cm depth was created along the markings using toothed forceps, a surgical blade and pointed scissors. The entire wound left open. The animals were divided into five groups of 6 each. The group 1 animals were left

untreated and considered as the control. Group 2 animals served as reference standard and treated with Silver nitrate ointment. Animals of groups 3, 4 and 5 were treated with different extract of C. *stocksii* stem bark extract of petroleum ether, chloroform and ethanol respectively, for 14 days. The measurements of the wound areas of the excision wound model were taken on 4^{th}, 8^{th} 12^{th} and 14^{th} day following the initial wound using transparent paper and a permanent marker. The recorded wound areas were measured with graph paper. The period of epithelialisation was calculated as the number of days required for falling off of the dead tissue remains without any residual raw wound.

Incision method

The rats were anaesthetized prior to and during creation of the wounds with mild diethyl ether. The dorsal fur of the animals was shaved with an electric clipper. A longitudinal paravertebral incision of 6 cm long was made through the skin and cutaneous tissue on the back as described by Ehrlich and Hunt. After the incision, the parted skin was sutured 1 cm apart using a surgical thread and curved needle. The wounds were left undressed. Stem bark extracts were topically applied to the wound once a day. The sutures were removed on 8^{th} post wound day and continued the application of the stem bark extract. The skin-breaking strength was measured on the 10^{th} day evening after the last application. The granulation tissue was taken on the 11^{th} day for further studies.

3.2.11 Statistical analysis

All the results were analysed using one way ANOVA followed by Dunnets multiple test. P value of < 0.05 were considered to be significant. The mean value ± SEM was calculated for each parameter. All the test samples and standard drug's

parameter were compared with control group (control P < 0.01 and standard P < 0.05 considered to be statistically significant). Finally, the stem bark extracts were compared with standard drug to know the significance difference between two groups at respective time.

3.3 Results

3.3.1 DPPH scavenging activity

The DPPH method has been widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant capacity. In this assay, standard ascorbic acid showed strong scavenging activity, while the ethanol extract scavenges DPPH radical in concentration dependent manner with an IC_{50} of 41µg. Among all the extracts ethanol extract showed 81% at 100 µg and chloroform extracts showed 67.7% of scavenging activity followed by petroleum ether extracts (35%) compared with standard ascorbic acid (96.2%).

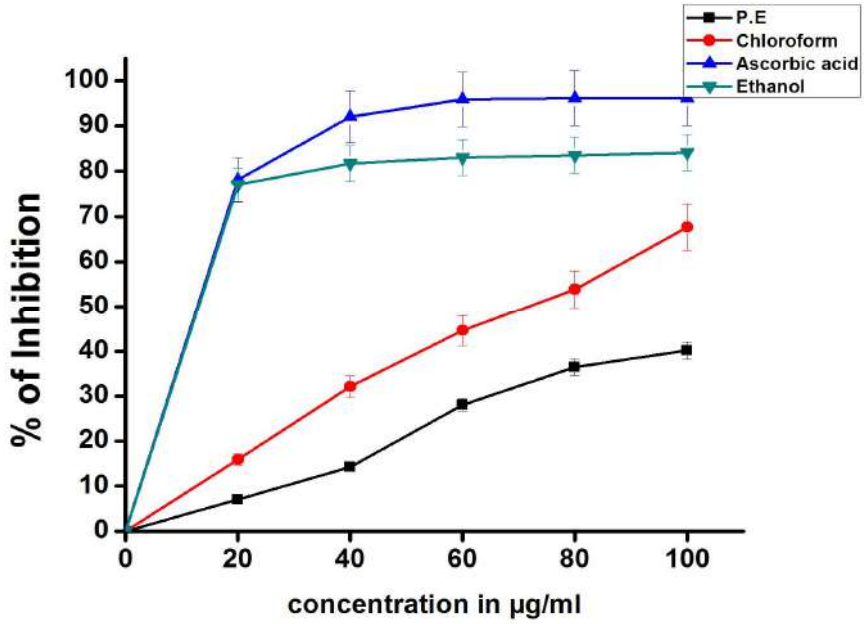


Fig. 3.1. DPPH activity of *C. stocksii* stem bark extract

3.3.2 Reducing power assay

In reducing power assay, the presence of antioxidants in the samples would result in reducing of Fe^{3+} to Fe^{2+} by donating an electron. The presence of reductants in *C. stocksii* stem bark causes the reduction of ferricyanide (Fe^{3+}) complex to the ferrous (Fe^{2+}) form. Therefore, the Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. The increase in the absorbance of reaction indicated the increased reducing power. From figure 3.2 it is clear that the absorbance of ethanol stem bark extract of *C. stocksii* increases with increase in concentration of reaction mixture as compared to chloroform and petroleum ether stem bark extract.

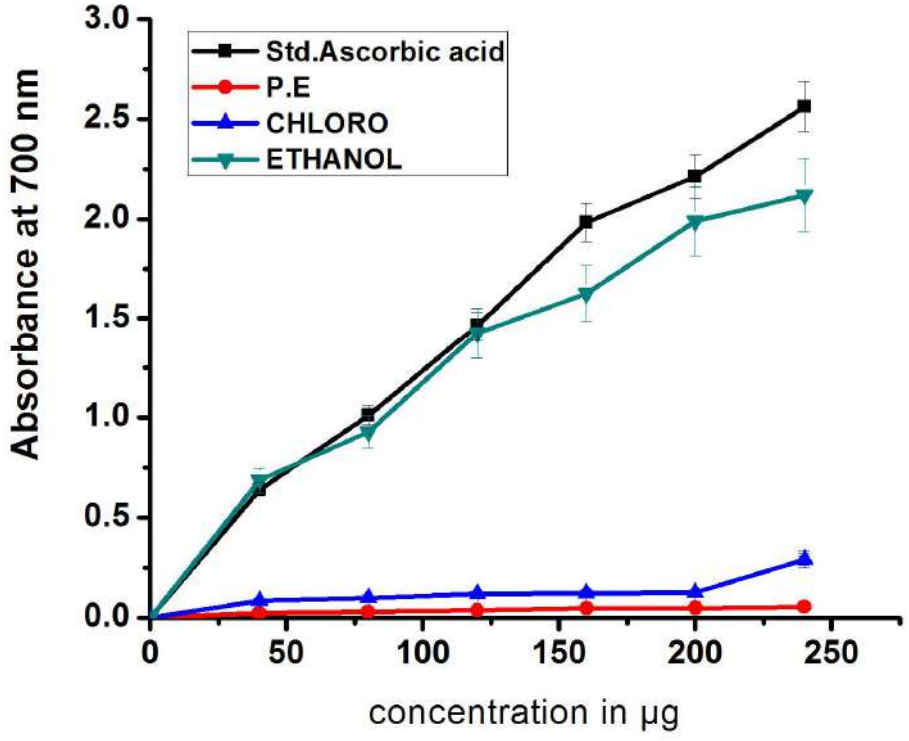


Fig. 3.2. Reducing power assay of *C. stocksii* stem bark extracts

3.3.3 Total antioxidant activity

The results of total antioxidant capacity are expressed as equivalents of ascorbic acid. The phosphomolybdenum method is an important antioxidant assay based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of green phosphate/Mo (V) complex with a maximal absorption at 695 nm. The ethanolic extract showed high antioxidant capacity of 0.9 mg/100 mg of extract followed by chloroform stem bark extract of 0.43 mg/100 mg and petroleum ether stem bark extract of 0.29 mg/100 mg of extract. This is due to the presence of high content phenols in the ethanol extract, as polyphenols play an important role as antioxidants in living systems due to the presence of hydroxyl groups in ortho and para positions of benzene ring (Table 3.1).

Table 3.1. Total antioxidant activity of *C. stocksii* stem bark extracts

Stem bark Extracts	**mg of ascorbic acid equivalents/100 mg of dried extracts**
Petroleum ether	0.29 ± 0.34
Chloroform	0.430 ± 0.21
Ethanol	0.90 ± 0.41

3.3.4 Antibacterial activity

The antibacterial activity of different extracts of *C. stocksii* was tested against different gram positive and gram negative strains and the results are tabulated in the Table 3.2. Zone of inhibition was measured and results were expressed in mm. Among the test organisms *E. coli* was the most insensitive among all the bacteria used in this study. The Chloroform stem bark extract of the plant was found to be active against all bacterial strains (MIC 200 µg/ml). The

petroleum ether stem bark extract showed moderate activity (MIC 800 µg/ml) than ethanol stem bark extract. While the chloroform stem bark extract's maximum inhibitory concentration was close to standard streptomycin.

Table 3.2. Total antibacterial activity of *C. stocksii* stem bark extracts

Stem bark extracts / Standard	Concentration (mg/ml)	Zone of inhibition (mm)			
		E. coli	*K. pneumonia*	*P. aeruginosa*	*B. subtilis*
Control (DMSO)		0±0	0±0	0±0	0±0
Petroleum ether	10	10±0.2	12±0.3	12±0.1	11±0.2
Chloroform	10	19±0.4	19±0.4	19±0.3	16±0.4
Ethanol	10	14±0.2	ND	14±0.2	ND
Streptomycin	1	22±0.5	24±0.4	23±0.3	25±0.5

ND = None detected.

Table 3.3. MIC values for antibacterial assay

Extracts (µg/ml)	*E. coli*	*K. pneumonia*	*P. aeruginosa*	*B. subtilis*
Petroleum extract	800	800	700	600
Chloroform	200	400	600	400
Ethanol extract	400	300	400	200
Streptomycin (Standard)	1.5	1.8	1.4	1.4

3.3.5 Antifungal activity

The antifungal assays of different extracts of stem bark of *C. stocksii* were tested against pathogenic fungi (Table 3.3). From the result, it is clear that chloroform extract of the stem bark showed promising activity (MIC 200 µg/ml) when compared against standard drug flucanazole, while petroleum ether (MIC 600 µg/ml) and ethanol (MIC 400 µg/ml) stem bark extracts showed considerable antifungal activity.

Table 3.4. Total antifungal activity of *C. stocksii* stem bark extracts

Stem bark extracts / Standard	Concentration (mg/ml)	Zone of inhibition (mm)		
		Pencillium chrysogenum	***A. terrus***	***A. clavatus* (0198)**
Control (DMSO)		0±0	0±0	0±0
Petroleum ether	10	20±0.4	14±0.3	13±0.2
Chloroform	10	28±0.3	19±0.2	20±0.4
Ethanol	10	18±0.2	0	14±0.1
Flucanazole	1	36±0.5	34±0.1	33±0.3

Table 3.5. MIC values for antifungal assay

Extracts (μg/ml)	***Pencillium chrysogenum***	***Aspergillus clavatus***	***Aspergillus niger***
Pet ether extract	600	400	600
Chloroform	400	200	400
Ethanol extract	500	400	400
Streptomycin (Standard)	0.8	0.6	1.0

3.3.6 Analgesic activity

In acute toxicity evaluation, albino rats did not exhibit any mortality or adverse effect at 5000 mg/kg body weight after 24 h of observation which was fixed as Lethal dose (LD_{50}), based on which dose were fixed for further study.

The present investigation reveals that the chloroform stem bark extract of *C. stocksii* exhibited a maximum analgesic activity of 72.7%, by hot plate method at the given dose of 500 mg/kg, and it was significant when compared with control and standard group. The ethanol stem bark extract showed a moderate analgesic activity of 35.3% when compared with control and standard drug group. But petroleum ether stem bark extract showed 33.5% of activity, which is insignificant compared to standard drug aspirin treated group. The graph represented the percentage of analgesic activity of ethanol chloroform, petroleum ether stem bark extract, standard and control.

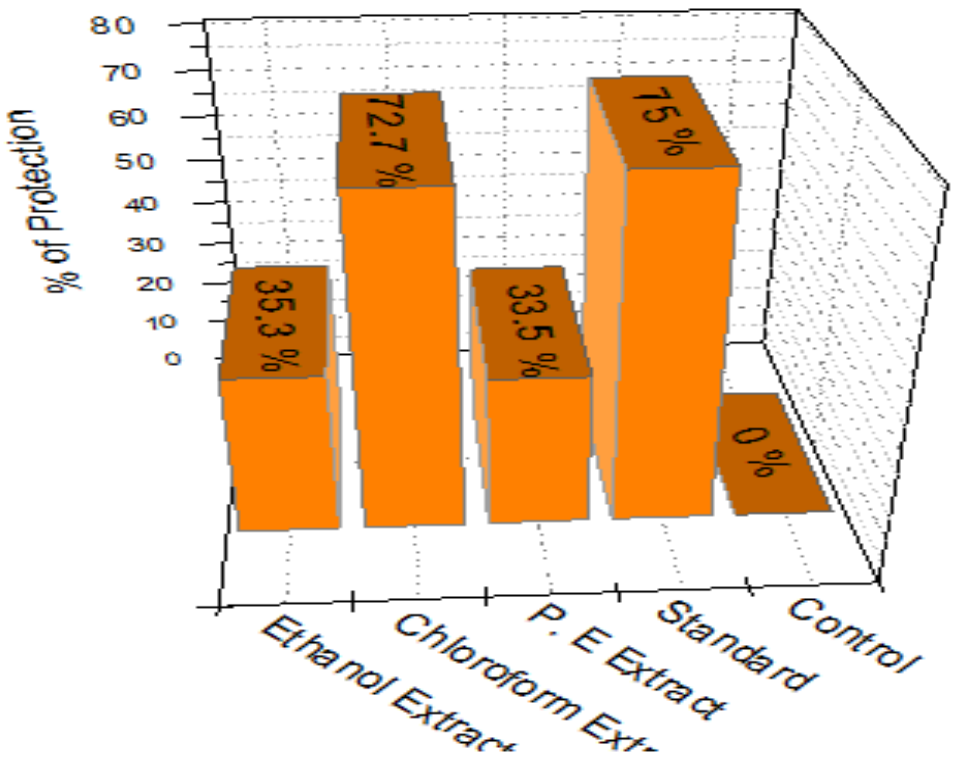


Fig. 3.3. Analgesic activity of *C. stocksii* by hot plate method

3.3.7 Antipyretic activity

In vivo antipyretic activity of stem bark extract of *C. stocksii* was carried out by yeast induced pyrexia method. Based on the observation of the data obtained from the present study, we found that there was significant inhibition of hyperthermia induced by the yeast. Chloroform stem bark extract showed significant activity ($P<0.05$) when compared with standard at a dose of 500 mg/kg body weight. However, petroleum ether stem bark extract was found to be not effective when compared to standard. The standard drug paracetamol, shows maximum inhibition at the end of 3 h. Among the extracts, chloroform stem bark extract showed maximum inhibition followed by ethanol and petroleum ether stem bark extract.

Most of the antipyretics drugs exert their action by inhibiting the enzymatic activity of cyclooxygenase and consequently reducing the levels of prostaglandin E2 within the hypothalamic region. The administration of *C. stocksii* extract attenuated the rectal temperature in yeast induced *Swiss albino* mice, indicating that *C. stocksii* contains pharmacologically active principles that interfere with release of prostaglandins. The chloroform stem bark extract showed more pronounced effect in lowering the hyperthermia than petroleum ether and ethanol stem bark extract. These extracts are likely to reduce pyrexia by reducing brain concentration of PGE2 especially in the hypothalamus through its action on COX-3 or by enhancement of bodies own antipyretic substances like vasopressin and arginine. The results of the present study indicate that the chloroform stem bark extract of *C. stocksii* exhibits significant antipyretic activity.

Table 3.6. Antipyretic activity of *C. stocksii* stem bark extracts

Groups/ Stem bark extract	Dose ml or mg/kg b w	Initial Rectal temperature (ºC) before Yeast injection	Rectal temperature (ºC) after 18 h of yeast injection (±SEM)			
			0 h	1 h	2 h	3 h
Control/ 1% Tween-80	10	36.8±0.11	38.0±0.11	38.1±0.15	38.2±0.16	38.2±0.12
Standard Paracetamol	15	37.1±0.08**	38.5±0.04**	36.2±0.01**	35.8±0.03**	35.4±0.02**
Petroleum ether	500	37.7±0.02*	37.9±0.11*	39.7±0.15*	40.1±0.12*	39.2±0.18*
Chloroform	500	37.2±0.12**	39.1±0.11*	38.6±0.31**	38.0±0.12*	37.0±0.44**
Ethanol	500	37.5±0.14*	39.6±0.11*	39.3±0.02*	38.3±0.25*	37.7±0.45*

Values are expressed as Mean ± SEM. n = 6 in each group
'*' $P < 0.05$ compared to control; '**' $P < 0.01$

3.3.8 Anti-inflammatory activity

During the search of anti-inflammatory activity of different extracts of *C. stocksii* using carrageenan induced paw edema method it was observed that there is gradual increase in volume of edema till 3rd hour after the carrageenan formation and then declined. The standard drug Indomethacin at a dose of 4 mg/kg body weight showed highly significant activity (P<0.0001) as compared to control group at 1, 2 and 3 h, respectively.

The chloroform stem bark extract exhibited significant (P<0.05) activity at a dose 500 mg/kg body weight. In the carrageenan test, the maximum inhibition elicited by chloroform stem bark extract (42.9%) which was comparable to that of indomethacin at a dose of 4 mg/kg (60.50%). Petroleum ether stem bark extract showed negligible inhibition while ethanol stem bark extract showed a moderate inhibition of decrease in paw edema in rats when compared to standard indomethacin.

Table 3.7. Anti-inflammatory activity of *C. stocksii* stem bark extracts by carrageenan induced paw edema method

Groups/ Stem bark extract	Dose ml or mg/kg	0 h	1 h	2 h	3 h	% inhibition
Control	10	1.12±0.14	1.12±0.14	1.12±0.03	1.12±0.04	---
Standard	4	1.25±0.06**	0.90±0.06**	0.48.±0.01**	0.10±0.03**	60.5
Petroleum ether	500	1.12±0.06	1.04±0.14	0.88±0.04	0.80±0.02	15.9
Chloroform	500	1.10±0.11	0.94±0.08**	0.58.±0.03	0.36±0.05**	42.9
Ethanol	500s	1.12±0.00*	1.02±0.04*	0.82±0.02*	0.72±0.04*	23.9

Values are expressed as Mean ± SEM. n = 6 in each group
'*' P < 0.05 compared to control; '**' P < 0.01

3.3.9 Wound healing activity

In both the models, significantly improved wound-healing has been observed with the *C. stocksii* stem bark extract, compared to that of reference standard and control group of animals. In the excision wound model *C. stocksii* treated animals showed significant reduction in the wound area ($P < .001$), faster rate of epithelialization when compared with the control group. But the animals treated with *C. stocksii* petroleum ether and ethanol stem bark extract have showed moderate reduction in the wound area ($P < .05$) and slower rate of epithelialisation (11.7 ± 0.15) than chloroform stem bark extract. Table 3.4 shows the wound area and other biochemical observations of all the five group of animals in excision wound model. In an incision wound model, *C. stocksii* treated animals demonstrated significant skin-breaking strength up to (532±2.8) when compared to control animals (320.13 ± 3.23).

Table 3.8. Wound healing activity of *C. stocksii* stem bark extracts by excision method

Groups /Treatment	4^{th} day	8^{th} day	12^{th} day	16^{th}day	Epithelialization days
I/control	22.7±0.54	43.8±0.53	64.9±0.63	75.6±0.41	24
II/silver nitrate	55.7±0.93	77.2±1.83*	95.9±1.62*	100*	14*
III/Petroleum Ether extract	37.0±1.48	65.6±0.84	84.4±1.27*	93.1±1.59*	18*
IV/Chloroform extract	53.7±0.45*	75.0±1.46*	93.4±1.36*	100*	16*
V/Ethanol	23.7±0.43	45.9±0.80	67.3±1.11	77.6±0.7	20

Group I-Control, Group II-Standard, Group III-Petroleum ether extract, Group IV-Chloroform extract, Group V-Ethanol extract. Values are mean ± S.E.; * $P < 0.01$ vs. Control; n = 6 albino rats per group, tabular value represents mean±

S.E. Effect of topical application of *C. stocksii* stem bark extracts and standards ointment on % of wound contraction and epithelialization period of incision wound model in rats.

Table 3.9. Wound healing activity of *C. stocksii* stem bark extracts by incision method

Treatment	Tensile strength in g ± SEM
I/Control	320.13 ± 3.23
II/Silver nitrate	556±1.8*
III/Petroleum Ether extract	385.21±1.1
IV/Chloroform extract	532±2.8*
V/Ethanol	440.0 ± 4.53*

Values are mean ± SE; *P< 0.01 Vs control by Student's test

3.4 Discussion

3.4.1 *In vitro* antioxidant activity

Potential antioxidant compounds from various medicinal plant sources have been investigated (Ramarathnam *et al.*, 1995). These secondary metabolites may be effective in preventing disease due to their antioxidant effect (Farombi *et al.*, 1998). The stable free radical DPPH is the most widely used method to evaluate antioxidant activity in relatively short time (Soares *et al.*, 1997). DPPH when used as substrate; the odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm and purple in colour (Gülçin *et al.*, 2003). The colour turns from purple to yellow as the molar absorptivity of the DPPH radical at 517 nm

reduces, due to quenching of colour by antioxidants. The DPPH radical reduced to non-radical diphenyl-picrylhydrazine (DPPH-H). This method has been useful to assess both polar and non polar molecule. The more rapid absorbance decrease indicates the more potent antioxidant activity in terms of hydrogen donating ability.

The DPPH activity of *C. stocksii* stem bark extracts showed dose response curve compared with standard ascorbic acid, which perhaps due to their electron donating ability. Extracts have proton donating ability and could serve as free radical scavengers, acting possibly on primary antioxidants. The capacity of each extracts to scavenge DPPH radical has been investigated (Fig. 3.1). From the figure, it is clear that ethanol stem bark extract of *C. stocksii* has more scavenging activity than chloroform and petroleum ether stem bark extract. The decrease in absorbance of DPPH radical caused by antioxidants due to reaction between antioxidant molecule and DPPH radical. Higher scavenging activity of *C. stocksii* ethanol stem bark extracts against DPPH radical was attributed to higher concentration of phenolic and flavonoid contents.

$$DPPH^{\bullet} + AH \rightarrow \quad DPPHH + A\bullet$$

The reducing power of *C. stocksii* stem bark extracts is monitored by potassium ferricyanide. The ferrous reducing power of *C. stocksii* is related to the electron donating ability and serves as indicator of antioxidant activity. In potassium ferricyanide assay, Fe^{3+} ferricyanide is reduced to ferrous Fe^{2+} by the reductants which are present in the extract. Amount of ferrous ion produced was monitored by the formation of Perl's Prussian blue and the absorbance was

measured at 700 nm (Nabavi *et al*., 2009). The reducing power of the stem bark extracts increases with the increase in concentration, which is compared with ascorbic acid. The reducing power of ethanol stem bark extract is high almost equal to ascorbic acid followed by chloroform and petroleum stem bark extract. The reducing capacity of stem bark extracts of *C. stocksii* is mainly due to flavonoid and phenolic compounds present in the plant.

Evaluation of total antioxidant by phosphomolybdenum based on the reduction of MO (VI) to MO(V) by the extract and formation of green phosphomolybdenum (V). From the results, it was clear that the extract retains reductive potential and could serve as electron donor for terminating the radical chain reactions. Chloroform and ethanol stem bark extracts showed higher level of antioxidant activity than petroleum ether stem bark extract.

3.4.2 Antibacterial activity

Plants have got the ability to produce wide variety of metabolites which are used as chemotherapeutic agent or serve as ingredient for the development of modern drugs. Huge number of plants have been screened for their antimicrobial activities (Tilak *et al*., 2004; Kirbag *et al*., 2009). The inhibitory activity of bark extracts of *C. stocksii* against Gram positive and Gram negative bacteria were tested using agar well diffusion method. Appearance of zone of inhibition around the well was considered positive. All the test bacteria were susceptible to extracts. The chloroform stem bark extract of *C. stocksii* induced a growth inhibition of all Gram positive strains and slightly reduced inhibition of gram negative strains. The result showed that gram positive bacteria were more sensitive than gram negative

bacteria. This is mainly due to the significant difference in outer layers in gram negative and gram positive bacteria (Gill and Holley, 2006; Shan *et al*., 2007; Gao *et al*., 2011). The presence of hydrophilic surface on the outer membrane of gram negative bacteria, which is rich in lipopolysaccharide molecules and most impermeable to lipophilic compounds results in insensitivity of gram negative bacteria to antibacterial substance.

Stem bark extract significantly exhibited potent antibacterial activity against all the test organisms. Among the extracts, chloroform stem bark extract showed a potent activity against other test organisms, as marked by zone of inhibition. Moderate zone of inhibition can be observed in petroleum ether and ethanol stem bark extracts. Inhibition caused by standard streptomycin was higher when compared with stem bark extracts.

3.4.3 Antifungal activity

Petroleum ether, chloroform, and ethanol stem bark extract of *C. stocksii* were assayed for antifungal activity against *Pencillium chrysogenum*, *Aspergillus terrus* and *Aspergillus clavatus*. The extracts inhibited the growth of test microorganisms and expressed as zone of inhibition in mm. The stem bark extracts were compared with standard flucanazole. Chloroform stem bark extract showed highest zone of inhibition followed by petroleum ether and ethanol extracts. The antimicrobial activity of the extract may be due to the formation of complex with bacterial cell wall and inhibiting the growth of microorganisms (Sumaira Sahreen *et al*., 2015). Many plant species and herbs exert antimicrobial activity due to their essential oil fractions. Plants are rich in a wide variety of secondary metabolites,

such as phenols, tannins, terpenoids, alkaloids, and flavonoids, which have been found to have antimicrobial properties.

Mortality rate of infectious disease in India is mainly due to *Salmonella aureus, Pseudomonas aeruginosa, Klebsiella pneumonia, Escherchia coli, Salmonella typhi* and *Salmonella paratyphi* (Muktanjali Arya *et al*., 2005; Faiyaz Ahmad *et al*., 2012). It has become difficult to manage and treat these infectious diseases. The studies on *C. stocksii* stem bark extract was undertaken to test the extracts for potent antimicrobial activity. The highest antimicrobial activity of chloroform stem bark extract may be due to some active properties of molecules like flavonoids, phenolic compounds and other secondary metabolites. Antimicrobial activity of flavonoids is mainly due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell wall. Phenols and polyphenols present in the plants are known to be toxic to microorganisms (Mason *et al*., 1987). Tannins may inactivate microbial adhesion enzymes, cell envelopes transport proteins and can also form complex with polysaccharides. Flavonoids may also disrupt microbial membrane (Suchiya *et al*., 1996). Phenol and polyphenols present in the plants are known to be toxic to microorganism. Antibacterial activity of tannins may be related to their ability to inactivate microbial adhesions, enzymes and cell envelope transport proteins, they also complex with polysaccharides (Gaffney *et al*., 1998). The broad spectrum of antimicrobial activity of *C. stocksii* stem bark may be due to various active molecules present in extract. This could be of considerable interest in the development of new drugs.

3.4.4 Analgesic activity

From the present study, it is clear that the *C. stocksii* stem bark has showed significant analgesic and antipyretic effect with a reasonable safety profile. Hot-plate test model used for testing analgesic agents on neurologic pain and centrally acting property, which can increase reaction time in hot-plate test through their action on spinal cord level (Wigdor *et al*., 1987; Vongtau *et al*., 2004). The study revealed that the central stimulating and analgesic activities of *C. stocksii*, which strongly supports use of this plant as a central stimulant and analgesic traditional medicine. The inhibition of prostaglandin synthesis could be the possible mechanism for antipyretic action as that of paracetamol and the inhibition of prostaglandin, which can be achieved by blocking the cyclooxygenase enzyme activity. Thus, it can be postulated that *C. stocksii* contained pharmacologically active principle(s) that interfere with the release of prostaglandins. The significant pain reduction of the plant extracts might be due to the presence of analgesic principles acting with the prostaglandin pathways.

The different extracts of the plant and Aspirin (150 mg/kg) also presented a longer latency time than the control group in the hot plate test in a dose related manner. It is an established fact that any agent that causes a prolongation of the hot plate latency using this test must be acting centrally (Ibironke and Ajiboye, 2007). Therefore, the chloroform extracts of the plants must have a central activity. In a condition when body temperature increases, the temperature regulatory system dilates the blood vessels causing increased sweating to lower the temperature by nervous feedback mechanism.

3.4.5 Antipyretic activity

The major action of antipyretics is their potential to inhibit the enzyme cyclooxygenase causing the synthesis of inflammatory prostaglandins to be interrupted (Jong Chanapong *et al*., 2010). They are known to act by suppressing the production of pyrogenic cytokines such as TNF-α and IL-β (Aronoff *et al*., 2001). This study examined the antipyretic potential of different extract of *C. stocksii* in experimental animal models. Chloroform stem bark extract significantly reduced the rectal temperature similar with standard antipyretic drug paracetamol in a dose dependent fashion. On the basis of observation of data obtained from our study, it is clear that the chloroform stem bark extract showed more significant antipyretic activity at a higher dose of 500 mg/kg body weight compared to ethanol and petroleum ether stem bark extract. Thus, it can be inferred that *C. stocksii* inhibits the synthesis of prostaglandins. It seems that the extract decreased production of prostaglandins, leading to relief pain (Payan, 1992). Consequently, these results indicate that the mechanism of analgesic and antipyretic effects of the extract are modulated by COX2 activity. It is not clear which parts of upper spinal cord were active or suppressed by the extract to reduce the pain resulting from the hot-plate. However, Silva Brum *et al.* (2001) have shown that linalool increases GABA ergictonus and decreases glutamate content in mice brains. Since it is well established that nearly 60% of the extract component is linalool (Hajhashemi and Ghannadi, 2003), and linalool has a major analgesic effect in case of the hot-plate test, although this has not been experimentally assessed. Barocelli *et al.* (2004) reported that the administration of *L. officinalis* leaves blocked pain in hot-plate tests. According to our results, it looks like the hydro-alcoholic extract of *L. officinalis* could block

the chronic pain and the inflammation induced by formalin in mice by inhibiting COX1 and COX2 activity. Furthermore, the intraperitoneal administration of this extract inhibited pain in the hot-plate test. Many peripheral or centrally acting analgesics have been isolated from plants and thus require extensive studies to explore more analgesic agents from natural sources. Results of the present study revealed that *C. stocksii* has centrally acting analgesic and antipyretic activity.

Furthermore, flavonoids and tannins are known to inhibit prostaglandin synthesis as reported by Ramaswamy *et al.* (1985). We might therefore say that the flavonoids, tannins and other chemical compounds present in the plant's extract are the components responsible for the observed antipyretic effects.

3.4.6 Anti-inflammatory activity

The study was undertaken to evaluate the anti-inflammatory activity of the different extracts of *Cryptocarya stocksii* bark using established acute inflammatory in vivo animal models by carrageenan rat paw edema method. Results showed that the extract could significantly reduced the cutaneous vascular permeability caused by topical administration of carrageenan in rat paw, revealing its anti-inflammatory effect. The potency of *C. stocksii* was similar to that of standard indomethacin (4 mg/kg). Carrageenan-induced inflammation is a suitable method for evaluation of the anti-inflammatory effects of the agents (Winter *et al*., 1962). The extract was found to significantly inhibit the paw edema by inhibiting mediators of acute inflammation such as TNF-α, IL-1β, and nitrite, which facilitates vascular permeability in the carrageenan induced rats. The development of carrageenan induced edema is believed to be biphasic (Vinegar *et al*., 1969).

The initial phase (1-2 h) is attributed to the release of histamine and serotonin bradykinin or vascular permeability (Maity *et al*., 1998). The edema produced at the peak 3rd hr is thought to be due to the release of kinin-like substances, especially bradykinin (Crunkhon and Meacock, 1971). The second phase of edema is due to the release of prostaglandins, protease and lysosomes and it is sensitive to most antiinflammatory drugs (Vinegar *et al.*, 1969; Di Rosa *et al.*, 1971).

The results of this study indicate that the chloroform stem bark extract of *C. stocksii* significantly reduced carrageenan induced paw edema in rats. Therefore, the mechanism of action may be by inhibition of histamine, serotonin or prostaglandin synthesis. Usually most anti-inflammatory and analgesic drugs possess antipyretic activity.

During the exploration of anti-inflammatory activity of different extracts of *C. stocksii* using carrageenan induced paw edema method it was observed that there is a gradual increase in volume of edema till 3rd h after the carrageenan formation and then declined. The standard drug Indomethacin at a dose of 4 mg/kg body weight showed highly significant activity ($P<0.0001$) as compared to control group at 1, 2 and 3 h respectively.

The chloroform extract exhibited significant ($P<0.05$) activity at a dose 500 mg/kg body weight. In the carrageenan test, the maximum inhibition elicited by chloroform extract (42.9%) was comparable to that of indomethacin at a dose of 4 mg/kg (60.5%). Petroleum ether stem bark extract showed negligible inhibition while ethanol stem bark extract showed a moderate inhibition of decrease in paw edema in rats when compared to standard indomethacin. The analgesic effect of

the plants in both models suggests that they have been acting through central and peripheral mechanism (Sabina *et al.*, 2009). Brewer's yeast forms a linkage to an immunological protein called Lipopolysaccharide-Binding Protein (LBP). It is well accepted that COX2 could be inhibited by non-steroid anti-inflammatory drugs such as indomethacin and aspirin (Simmons *et al.*, 2004). In the present study, the stem bark extract also decreased formalin evoked-edema and added to the concept that the aquatic-alcoholic extract of *L. officinalis* has analgesic effects.

3.4.7 Wound healing activity

The present study was carried out to evaluate different extracts of *C. stocksii* stem bark extract on the healing of experimentally induced wounds in rats using excision and incision method. Wound healing is a biological process of damaging tissue that begins with trauma is restored as closely as possible to its normal state and wound contraction is the process of shrinkage of area of the wound and ends with scar. It mainly depends upon the repairing ability of the tissue type and extent of damage and general state of the health of the tissue. The inflammation, macrophagia, fibroblasia and collagenation are interconnected to one another. By using drugs to any of these phases could either promote or inhibit one or all the phases of wound healing (Vinothapooshan and Sundar, 2010).

The wound-healing property of *C. stocksii* may be attributed to the phytoconstituents present in it, which may be either due to their individual or additive effect that fastens the process of wound healing. Wound healing activities of petroleum ether stem bark extract, chloroform and ethanol stem bark extract were observed, but at different time points. Animals treated with different extract

had a significantly higher degree of healing on 4, 8 and 12th days compared to the vehicle treated group. However, on Day 14th, there was a similar degree of wound healing in all the groups but chloroform stem bark extract showed faster healing than the other extracts such as petroleum ether and ethanol stem bark extract. The wound healing activity of chloroform stem bark extract was almost equal to that of standard silver nitrate. All extract-treated wounds appeared to heal better than the controls based on gross examination, degree of wound healing, The results of this study support that *C. stocksii* can promote wound healing by inhibiting inflammation, inducing collagen synthesis, promoting angiogenesis, inducing vasodilation, and reducing wound oxidative stress. In addition, *C. stocksii* extracts have been shown to affect cellular growth and proliferation in injured tissues, mainly contributed by identified chemical constituents (Biswas *et al*., 2003; Natubhai *et al*., 2015).

Chapter-4A

Isolation and characterization of bioactive compounds from *Nardostachys jatamansi*

4A.1 Introduction

The genus *Valeriana* (Valerianaceae), containing about 200 species, is distributed throughout the world. In the history, the early uses of valerian were in the most part due to its bitter and aromatic qualities (Hobbs, 1994). The Greek physician and pharmacist, Galen (131-201 A.D), was the first to identify the sedative qualities of valerian. It was given to those who are bitten or stung by any venomous creature, boiled in wine. It was specially used to treat against plague, the decoction being given to drink and the roots used for smelling it. It has an excellent property to heal any inward sores or wounds, outward hurts or wounds, drawing away splinters or thorns out of the flesh, reducing frequency of pulse, and for increased flow of urine. It is useful in cases of irregular nervous action, when not connected with inflammation, or an excited condition of the system. Among the complaints in which it has been particularly recommended are hysteria, hypochondriasis, epilepsy, low forms of fever, restlessness, morbid vigilance, or other nervous disorder. It has also been used inacute rheumatism in combination with Peruvian bark. It has been used as the perfect herbal tranquilliser during First World War to treat soldiers suffering from shell shock (Howard, 1987).Valerian has been mentioned in the Bible a number of times under the name of spikenard. It is used generally in slight cuts, wounds, and small hurts.

Rhizomes (underground stems) can be crushed and distilled into an intensely aromatic amber-coloured essential oil, which is very thick in consistency. Nard oil is used as a perfume, incense, sedative, and an herbal

medicine said to fight insomnia, birth difficulties, and other minor ailments. The oil was known in ancient times and was part of the Ayurvedic herbal tradition of India. It was used as a luxury in ancient Egypt, the near East. In Rome, it was the main ingredient of the perfume *nardinum* derived from the Hebrew. Bethany, Mary, sister of Lazarus used a pound of pure nard to anoint Jesus's feet. The powdered root of spikenard is also mentioned in some Islamic traditions as the fruit which Adam ate in Paradise, which God had forbidden him to eat (Anthony Dewek, 1996).

Spikenard was used as season food in Medieval European cuisine, especially as a part of the spice blend used to flavour hypocras, a sweetened and spiced wine drink. From the 17th century it was one of the ingredients for a strong beer called Stingo. Today, oil of spikenard is not used as widely as that of its many valerian relatives. Spikenard is still used in many Tibetan healing incenses and is used in the herbal medicine of India, Tibet and the rest of China as a nerve tonic and sedative for sleep disorders, a property it shares with the closely related valerian, *Valeriana officinalis*. Spikenard is known as healing oil and is grown in India and China. The essential oil is obtained through steam distillation and it is a base note with an earthy/musty scent. Physically Spikenard essential oil is used as a diuretic, useful for rashes and skin allergies, anti-fungal agent and has a balancing effect on the menstrual cycle. Emotionally this oil is reserved for deep seated grief or old pain. It is used in palliative care to help ease the transition from life to death (Dalby Andrew, 2000).

Chemical investigations of *Valeriana* species have mainly focused on low-polarity components, leading to the isolation of two major groups of constituents,

essential oil sesquiterpenes and valepotriates and are responsible for sedative, cytotoxic, antitumor, and antifungal activities (Bach *et al*., 1993). The roots and rhizomes of some *Valeriana* species have been used in traditional medicine as a sedative for centuries. A large number of species in the genus *Valeriana* have been studied so far, with many iridoids associated with the sedative activity of *Valeriana* preparative having been isolated from the roots and rhizomes of species in this genus (Handjieva *et al*., 1978; Kucaba *et al*., 1980; Becker *et al*., 1984; Mikhova *et al*., 1987; Bach *et al*., 1993). Some of the *Valerian* sp. used in traditional medicines are *Valeriana officinalis*, *Valeriana jatamansi*, *Valeriana walachi***,** *Valeriana mexicana, Valeriana alliariifolia*, *Valeriana fauriei* and *Valeriana dioica.*

A considerable number of investigations on *Valeriana* species have yielded iridoids, sesquiterpenoids, lignans alkaloids, with pharmacological properties including sedative, cytotoxic, antitumor, antioxidant and vasorelaxnt activities (Piccinelli *et al*., 2004; Bach *et al.*, 1993; Thies *et al.*, 1984; Bounthanh *et al*., 1981).

4A.1.1 Medicinal uses of selected *Valerian* genus

Valeriana officinalis is the official species categorized as plant extract in the European Pharmacopoeia and is commonly referred to as valerian, and the rhizomes and roots of this plant exhibit anxiolytic, antidepressant, antispasmodic, sedative, and anti-HIV activities (Wang *et al*., 2011; Waldmann *et al*., 2012).

Investigations on the roots of *V. fauriei* Briq resulted in the isolation and characterization of phenols, flavones, and terpenoids. Some of the isolated

compounds were evaluated for their antidepressant-activity potential based on recording the total duration of immobility of the forced-swim test on mice.

Valeriana wallichii DC (Valerianaceae) is an indigenous medicinal plant used in ethno medicine for the treatment of habitual constipation and insomnia. *Valeriana wallichii* has considerable reputation for its traditional use in inflammatory conditions such as scorpion stings and jaundice (Nadkarni, 1976), in pain conditions (Vohora and Dandiya, 1992), epilepsy, insomnia, neurosis, and sciatica (Nadkarni, 1976; Marder *et al*., 2003). The plant is widely used in the treatment of anxiety and depression either alone or in combination with other herbs specifically St. John's Wort (Leathwood and Chauffard, 1982; Panijel, 1985; Ron *et al*., 2000). The plant is also used in habitual constipation (Baquar, 1989), antispasmodic (Gilani *et al*., 2005) and as cytotoxic (Bos *et al*., 1986). An herbal preparation (Dhanya Panchaka Kashaya) containing *Valeriana wallichii* has been found to be effective in dyspeptic symptoms (Tripathi *et al*., 1982). Its essential oil exhibited antimicrobial activity against large number of pathogenic bacteria and potent antifungal activity against different human and plant fungal pathogens (Suri and Thind, 1978). *Valeriana wallichii* showed antiinflammatory properties, similar to those observed for non-steroidal anti inflammatory drugs, such as aspirin.

4A.1.2 Phytochemistry

The phytochemical constituents of *Valerian genus* has been extensively studied since early 1950s. The prominent compounds present in the *valerian genus* are sesquiterpenes, flavonoids, phenolic compounds, valepotriates, iridoids, lignans, flavone, glycosides, rhizome and root contains volatile oil (valerianic oil) which is composed of alkaloids, bornyl isovalerianate, chatinine, formate,

glucoside, isovalerenic acid, 1-camphene, 1-pinene, resins, terpineol and valerianine (Nadkarni, 1976). Phytochemical investigation has also shown the presence of esters, phenolic compounds, sesquiterpene ketones and coumarins.

The plants are known to be rich in sesquiterpenes (Xiao *et al*., 2002), which have been found to exhibit antimalarial, antinociceptive and cytotoxic activities, as well as to enhance the action of nerve growth factor. It also has other ayurvedic applications such as in complexion, kidney stones, jaundice, removes blood impurities, spasmodic hysteria and other nervous convulsive ailments, heart palpitations, nervous headache, flatulence, epilepsy, convulsions, respiratory and digestive diseases, skin diseases, typhoid, gastric disorders (Itokowa *et al.,* 1993; Takaya *et al.*, 2000; Li Matsunga and Yamakuni *et al*., 2003).

The plant is reported for hypolipidemic effects in triton-induced hyperlipidemic rats, for inhibitory activity of methanolic and water extracts against acetyl cholinesterase, anticonvulsant, and neurotoxicity profile in rats, as well as protective effect in rat cerebral ischemia (Dixit *et al*., 1998; Raov *et al*., 2005; Vinutha *et al*., 2007).

4A.1.3 Pharmacological activity

Pharmacological activity refers to study of plant material either in purified form or crude extract for different biological activity. Many species of the plant were studied for different pharmacological activity.

A methanol extract of the roots of *Valeriana fauriei* exhibited antidepressant activity in mice (Oshima, 1995). The psychotropic effects of roots of Japanese valerian were compared with those of diazepam and imipramine

(Sakamoto *et al*., 1992). Both the ethanolic extract of valerian root and diazepam significantly prolonged hexobarbital-induced sleep in mice. These results indicate that valerian extract acts on the central nervous system and may be an antidepressant.

Valeriana officinalis also has the properties to relieve smooth muscle spasm and has a powerful vasodilation effect (Yang, 1994).

Based on the above information, the present work has been undertaken to systematically study the rhizome of *Nardostachys jatamansi* which is also called as Spikenard by subjecting it to GC-MS analysis followed by the compound isolation.

4A.1.4 *Nardostachys jatamansi*

Nardostachys grandiflora DC or *Nardostachys jatamansi* DC, commonly known as Jatamansi, Bhootajata (Kannada), Balchara (Hindi), Mamsi (Sanskrit), Jatamaji (Tamil) and Manchi (Malayalam). The botanical description of the plant is as follows:

Kingdom : Planate

Division : Mangnoliophyta

Class : Mangnoliopsida

Order : Dispsacales

Family : Valerianaceae

Genus : *Nardostachys*

Species : *jatamansi*

Botanical name : *Nardostachys jatamansi* DC

Colour : Dark grey rhizomes are crowned with reddish brown tufted fibers. Internally they are reddish brown in colour.

Odour : Highly agreeable, aromatic.

Taste : Acrid, slightly bitter and aromatic.

Narrdostachys jatamansi DC is a small perennial herbaceous, important flowering plant of the Valerian family. It is commonly known as Indian spikenard and found in Himalayas, also found growing in the northern region of India and Nepal. Rootstock covered with fibres of old leaves and flowering stems 5-30 cm length. Rhizomes of 2.5 to 7.5 cm occurs in short pieces of reddish brown colour with elongated cylindrical shape, has dark grey colour and typical smell. Leaves are sessile and ovate. The leaves are rosy, slightly pink or blue in dense cymose. Flowers are slightly pink in colour. It has an agreeable odour with bitter aromatic taste and is used as substitute for valerian. The plant grows to about 1 m in height and has pink, bell-shaped flowers. Spikenard rhizomes (underground stems) occur in short pieces, has dark grey colour and typical smell. Leaves are sessile and ovate.

Rhizome of *Jatamansi* has high medicinal value and the essential oil extracted from the rhizomes is used in perfumery. The leaves of *Jatamansi* are elliptic-lanceolate or spathulate of about 5-20 cm mostly basal and arising from the stout and woody rootstock covered with dark fibres of old leaves. The root of this taxon consists of short, thick, dark grey rhizomes crowned with reddish brown tufted fibrous remains of the petioles of the radical leaves. Flowers are rose-purple to whitish in dense head borne in terminal. Calyx is coloured, 5-lobed, the lobes enlarge in fruit and become papery. Corolla tube is 6-20 mm long with 5-rounded spreading lobes. The whole plant has a distinct lingering smell. The underground part of *N. jatamansi* is used as a substitute for valerian and the extracts find use in over 26 Ayurvedic preparations (Prasheed Kekuda *et al*., 2009).

4A.2 Materials and methods

4A.2.1 Collection and identification of plant material

The *N. Jatamansi* is perennial herb growing at higher elevations up to 17,000 ft on Alpine Himalaya, in Nepal, Bhutan and Sikkim, rhizome and roots is mainly used as drug. The plant is about 10 to 60 cm in height and with stout and long woody root stocks. The rhizome of the plant was collected from the local shops of Udupi, Karnataka, India and authenticated. The collected plant material was thoroughly washed with distilled water, shade dried and kept mechanically grinded into fine powder and stored in a dry place until further use.

4A.2.2 Extraction of Plant material

A known weight of powdered rhizomes of *N. jatamansi* was subjected to Soxhlet's extraction procedure in the increasing order of their polarity of solvents,

i.e. petroleum ether, chloroform, and ethanol. The apparatus was made to run for 24 cycles or until the solution in the timble becomes colourless. The extract was concentrated under reduced pressure using rotary evaporator. The weight of the extracts were recorded and stored at -20º C until use.

4A.2.3 GC-MS Analysis

GC combined with mass spectroscopy is a preferable methodology for routine analysis of compounds. 1 µl of petroleum ether extract, chloroform extract, and ethanol extract was injected into the GC-MS instrument.

GC analyses were carried out on an Agilent 7890 instrument equipped with a hydrogen flame ionization detector and HP-5 capillary column (30 m × 0.32 mm × 0.25 µm, J and W Scientific). Nitrogen was used as carrier gas at a flow rate of 1 mL/minute. Initially, the column temperature was maintained at 60°C for 2 min, followed by a temperature gradient from 60°C to 120°C at 2°C/min and held constant for 5 minutes at 120°C, then raised to a temperature of 150°C at 1°C/minutes and finally to a temperature of 200°C with a 5°C/min rise and maintained for 10 minutes at 200°C. The injector and detector temperatures were maintained at 250°C and operated in split mode (split ratio 1:10). GC-MS was performed on an Agilent 5975C mass selective detector interfaced with an Agilent 7890A gas chromatograph. GC-MS analyzes were performed under similar conditions using an HP- 5-MS capillary column (30 m × 0.32 mm × 0.25 µm, J and W Scientific).

Thin-layer chromatography was performed on silica gel G-coated plates (0.25 mm for analytical) developed with hexane (system I), hexane–ethyl acetate

(98.5:1.5; system II), dichloromethane (system III), and dichloromethane–methanol (98.5:1.5; system IV). Compounds were visualized by spraying with a solution of 3.0% anisaldehyde, 2.8% H_2SO_4, and 2% acetic acid in ethanol followed by heating for 1-2 minutes.

4A.2.4 Identification of compounds

Interpretation of the mass spectrum GC-MS was conducted using the database of National Chemical Laboratory (NCL), Pune. The spectrums of the unknown compounds were compared with the spectrum of known compounds stored in the NCL library.

4A.2.5 Isolation and Characterization

4A.2.5.1 Column Chromatography

Many separation techniques are available to accomplish purification and isolation of components from crude extracts of plants. Column chromatography is widely used technique which can also used to determine the quantity of different compounds in mixture (analytical chromatography). Theory of column chromatography is analogues to thin layer chromatography. In order to select best mobile phase TLC of *N. jatamansi* extract was spotted on TLC plate run with combination of solvents. The solvent which exhibited maximum separation of the compounds from mixture was chosen.

4A.2.5.2 Chromatographic Separation using Silica gel

The ethanol extract of (4 g) of *N. jatamansi* was subjected to column chromatography by using Dichloromethane and methanol mixture of various

proportions as mobile phase. For the chromatographic separation, column of length of 55 cm and diameter of 3.5 cm was cleaned and dried. Stationary phase was prepared from silica gel (60-120 mesh) in freshly distilled Dichloro methane and loaded into the column.

The ethanol extract was (4 g) was dissolved in minimum amount of distilled Dichloro methane and mixed with silica gel and loaded to the column. Elution was carried out using solvent mixtures of increasing polarities. Fractions were collected in 100ml portions and monitored by TLC. TLC was carried out on readymade silica plates (Merck Mumbai). Using capillary tube, approximately 5 µL of the eluted fraction was loaded on silica gel 3 mm from above from the bottom. Samples with similar spots were pooled together and concentrated at reduced pressure using rotatory vacuum evaporator. Completely dried components were weighed to calculate the total mass extracted. The isolated compounds were subjected for spectral studies.

4A.2.5.3 Nuclear Magnetic Resonance spectra (^{1}H NMR and ^{13}C NMR)

The magnetic resonance spectra of the compounds were recorded using Shimadzu Spectrophotometer. Samples were dissolved by dissolving in $CDCl_3$ and DMSO. The Chemical shifts were expressed in delta ppm.

4A.3 Results

4A.3.1 GC-MS analysis

GC-MS analysis of the phytochemicals in different extracts (pet ether, chloroform, and ethanol) of *N. jatamansi* showed the presence of 61 compounds.

The active principles with their retention time (RT) probability, R-match, and relative percentages and compound name are presented in Tables 4A.1 to 4A.3. The GC-MS chromatogram of the petroleum ether extract, chloroform extract, ethanol extract is shown in the Figure 4A.1 to 4A.3, respectively.

About 61 compounds were found in the petroleum ether extract. The results revealed that valence (8.8%), globulol (8.2%), beta-patacholene (8.4%), aristolene (7.9%), and alpha-gurjunene (5.4%) were found as major components in petroleum ether extract. A total of 50 compounds found in chloroform extract in which tetrachloroethane (16.6%), allylphenol (5.8%), and valeranone (3.4%) are the major compounds. Eleven compounds are reported from ethanol extract out of which m-ethyl toluene (27.9%), indane (28.8%), and actinidine (11.8%) were major compounds.

4A.3.2 Isolation and Characterization

Based on the GC- MS analysis of the crude extracts, we selected ethanol extract for further purification and isolation of compounds. The ethanol extract was chromatographed on silica gel (Mesh size 60-120) packed in DCM and eluted with a gradient of DCM and methanol of increasing polarity which afforded a total of 16 major fractions. Each fraction was purified by a combination of column chromatography, TLC and recrystallization using suitable solvent system after elution. Column chromatography of fraction IV yielded compound 1(220 mg). Compound 1 was characterized by IR, ^{1}H NMR, ^{13}C NMR and mass spectral studies.

Table 4A.1. Compounds detected in the petroleum ether extract of *N. jatamansi*

Rt	Prob	R-Match	Percent	Compound
7.359	39.7	835	0.22	3-Methylbutanoic acid
10.899	15.3	920	0.054	(-)-β-Pinene
13.634	11.9	801	0.016	1,8-Cineol
26.569	3.07	837	0.11	3-Methyl-3-hexen-2-ol
26.962	9.41	765	0.024	Cyclopentaneacetaldehyde,2-formyl-3-methyl-α-methylene-
27.078	2.71	884	0.018	Bicyclo|5.1.0|octane, 8-(1-methylethylidene)-
28.097	3.87	800	1.18	Beta-pinenoxide
28.594	40.4	870	0.22	Cyclopentaneacetaldehyde,2-formyl-3-methyl-α-methylene-
29.316	8.86	796	0.113	D-Verbenone
29.628	9.58	879	0.08	Carveol
30.072	5.2	769	0.049	7'-Oxaspiro|cyclopropane-1,4'-tricyclo|3.3.1.0(6,8)|nonan-2'-one|
30.75	18.5	812	0.017	(+)-β-Guaiene
30.888	18.5	866	0.037	(R)-(+)-Limonene
32.345	10.3	854	0.221	τ-Gurjunene
33.018	17.9	914	0.18	(-)-β-Elemene
33.636	9.52	851	0.099	(-)-α-Gurjunene
33.805	6.9	918	1.672	β-Maaliene
34.191	12.3	934	1.163	(+)-9-Aristolene
34.664	202	32.8	0.178	β-Vatirenene
34.916	26.7	953	7.917	(+)-1[10]-Aristolene or (+)-Calarene
35.016	11.6	884	0.176	Eudesma-3,7[11]-diene
35.257	53.6	972	2.691	Seychellene
35.35	20.8	928	0.314	α-Guaiene
35.586	9.25	920	5.466	(-)-α-Gurjunene
35.954	39.6	916	2.569	α-Patchoulene
36.292	15.1	950	0.444	(+)-Aromadendrene
36.419	22.3	928	3.961	(-)-α-Gurjunene
36.565	12.1	743	0.478	5β,7βH,10α-Eudesm-11-en-1α-ol
37.527	6.43	912	1	τ-Gurjunene
37.705	14.6	843	0.636	(E)-β-Ionone
37.981	26.3	953	8.887	(+)-Valencene
38.246	5.71	873	0.469	Seychellene

Contd….

38.566	25.7	819	2.05	8-Camphenemethanol
39.06	28.5	910	3.677	Eudesma-3,7[11]-diene
39.448	36.3	920	0.272	(-)-β-Cadinene
39.572	4.78	843	0.479	Globulol
40.438	5.42	856	0.548	β-Vatirenene
41.15	3.34	785	0.611	Thujopsene
41.77	21.6	835	0.671	2-Butenal, 2-methyl-4-[2,6,6-trimethyl-cyclohexen-1-yl]-
42.034	8.58	900	4.367	Seychellene
42.284	9.25	865	1.433	Isoledene
42.727	7.4	859	2.156	Cubenol
43.039	3.51	876	1.259	(-)-Alloaromadendrene
44.309	6.93	863	6.067	(+)-Aromadendrene
45.388	4.16	892	8.402	β-Patchoulene
46.121	74.7	946	4.679	(+)-Valeranone or 1[2H]-Naphthalenone, octahydro-4a,8a-dimethyl-7-[1-methylethyl]-,|4aR-[4aα,7β,8aα]|-
48.202	15.2	947	2.014	Valerenal
50.27	18.6	865	0.773	2,4a-Methanonaphthale-7[4aH]-one, 1,2,3,4,5,6-hexahydro-1,1,5,5-tetramethyl-,[2s-cis]-
50.538	10.1	840	8.197	Globulol
50.759	35.1	782	0.934	Methylhinokiate
51.231	39.1	747	1.898	Butanal,3-hydroxy-2-methyl-4-|4-t-butyl|-
51.617	12	955	1.233	9-Acetyl-2,6-dimethyl-10-hydroxybicyclo|4.4.0|deca-1,4-dien-3-one
52.546	3.8	826	2.755	6-Methyl-5-[1-methylethylidene]-3,6,9-decatrien-2-one
53.603	25.7	823	0.493	6-[1-Hydroxymethyl-vinyl]-4,8a-dimethyl-3,5,6,7,8,8a-hexahydro-1H-naphthalen-2-one
53.981	5.35	828	0.275	2[1H]Naphthalenone, 3,5,6,7,8,8a-hexahydro-4,8a-dimethyl-6-[1-methylethenyl]-
54.151	7.21	832	0.221	Phenol, 2-methoxy-4-[1-propenyl]-
55.241	5.87	868	0.224	Verrucarol
59.412	21.3	722	0.982	3-[6,6-Dimethyl-5-oxohept-2-enyl]-cycloheptanone
60.902	22.1	861	0.73	4,4-Dimethyl-3-[3-methylbut-3-enylidine]-2-methylenebicyclo|4.1.0|heptane
61.194	21.7	873	1.133	Dehydroaromadendrene
67.263	4.91	822	0.807	1,4-Butanediol,1-phenyl-

Table 4A.2. Compounds detected in the chloroform extract of *N. jatamansi*

Rt	Prob	R-Match	Percent	Compound
8.287	98.5	978	16.589	1,1,2,2-Tetrachloroethane
10.871	85.5	866	0.262	Pentachloroethane
13.513	20.1	760	0.868	1,1,2-Trichloro-2-methylpropane
13.863	62.1	767	0.27	2,3-Dichloro-2-methylbutane
15.655	98.9	979	3.413	1,1,1,2,2,2-Hexachloroethane
17.194	1.45	919	0.37	4-Chloro-2-[chloromethyl]-1-butene
18.888	96.4	927	2.859	1,1,2,3,3-Pentachloropropane
25.009	57.6	679	2.855	1,1,2,2,3,3-Hexachloropropane
29.528	33.1	861	2.22	(S)-(-)-Actinidine
30.066	5.25	780	2.715	1-Isopropenyl-3-propenyl-cyclopentane
32.28	11.3	903	1.166	β-Patchoulene
32.616	27.5	784	0.297	4-Iodo-2-oxaadamantan-6-one
32.828	7.18	774	0.143	Bicyclo|4.1.0|heptane, 7-bicyclo|4.1.0|hept-7-ylidene
34.161	17.1	818	0.478	β-Guaiene
34.594	3.48	853	0.532	(-)-Alloaromadendrene
34.689	6.91	813	1.037	Cyclopentaneacetaldehyde, 2-formyl-3-methyl-α-methylene-
34.853	7.5	775	3.824	8-Camphenemethanol
35.009	28.7	875	1.171	β-Vatirenene
35.187	31.6	943	1.973	Seychellene
35.903	5.47	854	2.272	Viridiflorol
36.358	5.48	870	2.233	Cyperene
37.093	2.63	804	0.267	α-Selinene
37.195	11.5	793	0.167	(+)-β-Cedrene
37.427	7.38	889	0.86	τ-Gurjunene
37.494	8.22	856	0.918	(+)-Aromadendrene
37.649	21.8	922	0.867	Seychellene
37.846	13.7	842	4.78	(±)-Cadinene
38.115	60.9	731	2.04	Dehydrocyclolongifolene oxide
39.436	37.6	962	1.404	15-Methyltricyclo|6.5.2[13,14].0[7.15]| pentadeca-1,3,5,7,9,11,13-heptene
39.923	33.8	806	0.396	4,5,9,10-Dehydroisolongifolene
40.158	4.1	703	0.82	(+)-Longifolene
40.604	11	786	0.587	Nerolidol-epoxyacetate

Contd….

41.736	14.1	806	0.459	2-Butenal,2-methyl-4-[2,6,6-trimethyl-1-cyclohexen-1-yl]-
42.699	8.77	760	4.431	Caryophyllene oxide
42.86	12.6	813	0.828	1-Nonadecene
43.005	18.4	731	0.863	Bicyclo|4.2.0|oct-1-ene, 2-methyl-7-endo-phenyl
44.215	14.2	834	4.095	τ-Himachalene
44.356	5.71	866	3.282	Isoledene
45.293	3.64	876	1.421	Seychellene
45.415	12.9	855	0.716	β-Vatirenene
46.028	42.9	923	3.385	(+)-Latamansone or (+)-Valeranone
46.629	2.96	912	2.726	(+)-Valencene
48.039	6.08	909	2.674	β-Patchoulene
48.655	3.25	900	3.763	9-Acetyl-2,6-dimethyl-10-hydroxybicylco|4.4.0|deca-1,4-dien-3-one
48.973	8.03	817	0.839	Murolan-3,9[11]-diene-10-peroxy
50.111	8.17	792	0.849	2,2,7,7-Tetramethyltricyclo|6.2.1.0(1,6) |undec-4-en-3-one
50.71	4.51	860	0.475	Verrucarol
51.555	19	774	1.535	1-Chloro-7-heptadecene
59.474	2.28	869	1.212	1-Eicosene
70.653	14.9	824	5.795	4-Allylphenol

Table 4A.3. Compounds detected in the ethanol extract of *N. jatamansi*

Rt	Prob.	R-Match	Percent	Compound
10.271	44	907	3.737	n-Propylbenzene
10.622	26.3	937	27.892	m-Ethyltoluene
10.927	15.8	940	2.08	1,3,5-Trimethylbenzene
11.399	19.2	927	8.213	p-Ethyltoluene
12.032	22.4	946	9.219	1,3,5-Trimethylbenzene
13.371	16.9	879	1.467	1,2,4-Trimethylbenzene
13.962	17.6	928	28.787	Indane
29.426	14.1	938	11.729	(±)-Actinidine
46.023	4.19	850	1.957	(+)-Valeranone
59.532	73.7	816	1.897	Ethylpalmitate
65.522	23.4	784	1.092	Ethyllinoleate
65.749	11.1	795	1.929	Ethyloleate

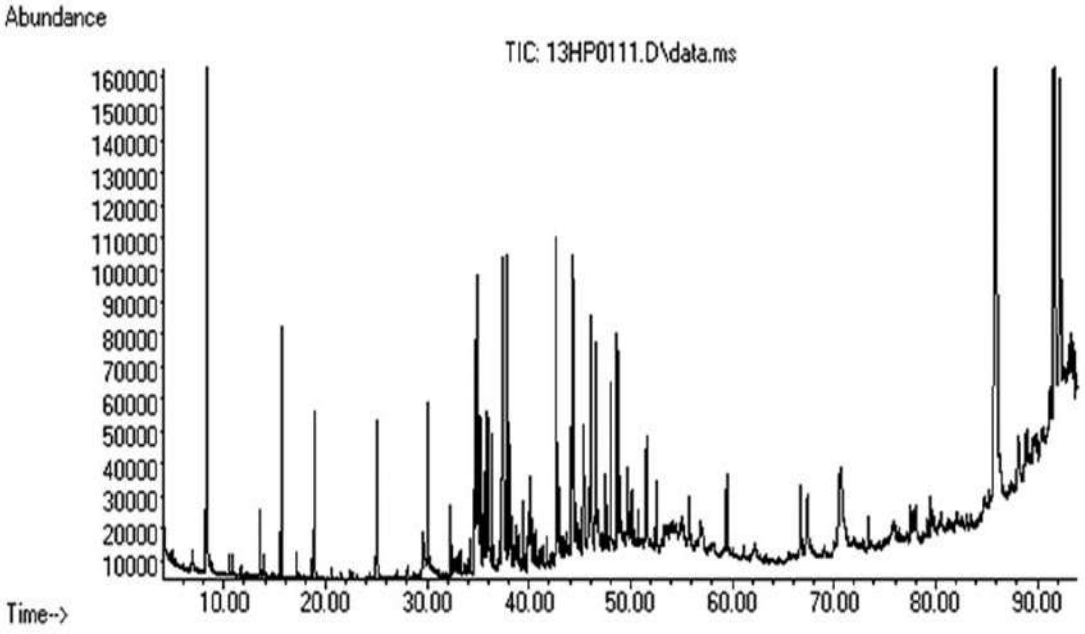


Fig. 4A.1. Gas chromatography-mass spectrometry chromatogram of the petroleum ether extract of the *Nardostachys jatamansi*

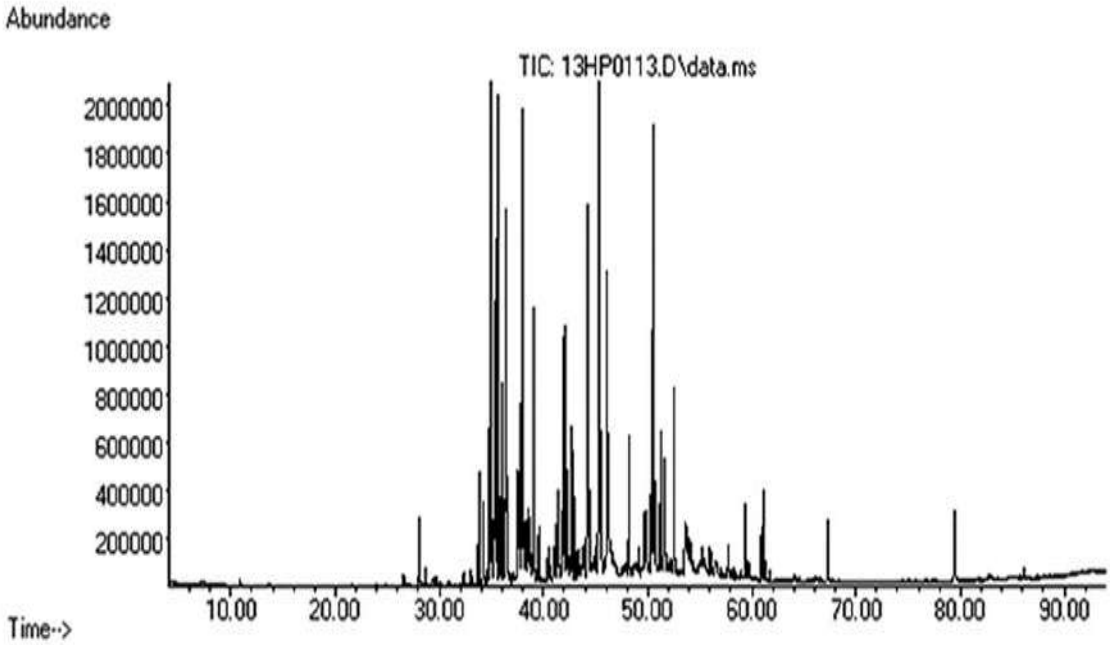


Fig. 4A.2. Gas chromatography-mass spectrometry chromatogram of the chloroform extract of the *N. jatamansi*

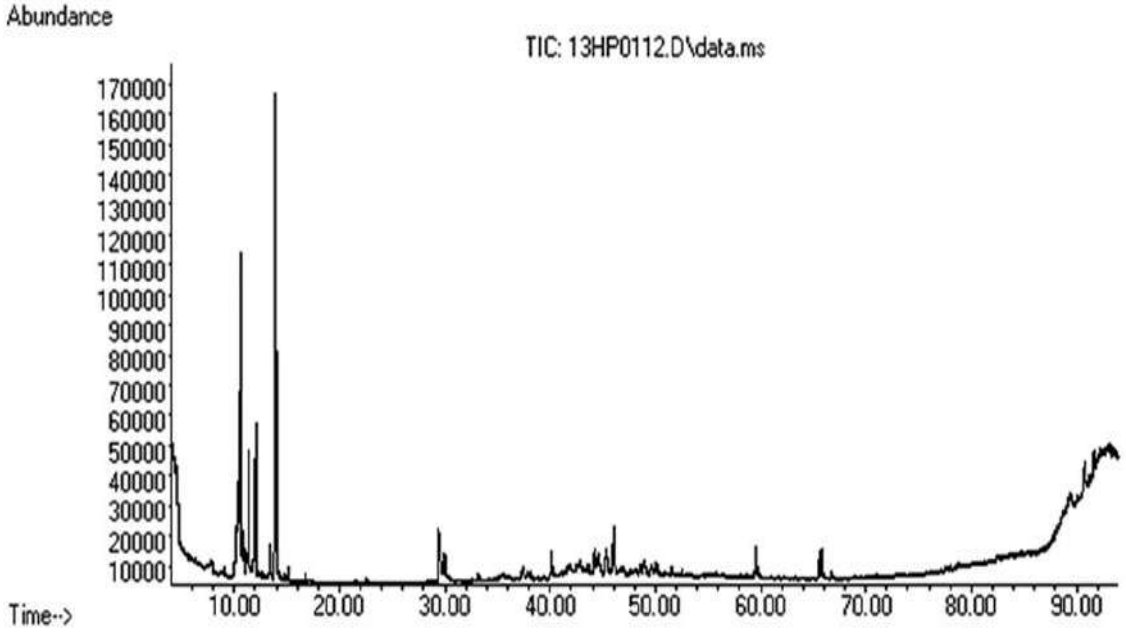


Fig. 4A.3. Gas chromatography-mass spectrometry chromatogram of the ethanol extract of the *N. jatamansi*

Compound 1

Molecular Formula-$C_{15}H_{22}O_2$, IR [(KBr): v_{max} /cm^{-1}]: 1633 (C=C stretching), 1423 (C-H Stretching) and 1378 (C-H Stretching), 1650cm^{-1} (C=O group), Molecular Weight:234.2, molecular ion peak M-at m/z [M^+], Column specification : Agilient mass Spectrometer. ^{1}H NMR (DMSO, 400MHz) δ 12.35(1H,), δ 6.900 (2H, dd), δ 3.55 (1H,q), δ 2.810 (1H,t), δ 2.184 (2H, t). δ 1.969 (1H,d), δ 1.844 (6H,m), δ 1.618 (3H,s), δ 1.551 (1H,m), δ 1.404 (2H,m), δ 1.235 (1H,S), δ 0.760 (3H,d), ^{13}CNMR (DMSO-400MHz), δ 12.35(d), δ 12.66(d), δ 24.52(d), δ 25.52.80(s), δ 28.14(s), δ δ29.46(d), 32.99(s), 34.09(s), 37.43(s), δ 126.64(s), δ 130.65(s), δ 133.75(s), δ 142.48(s), δ169.59(s).

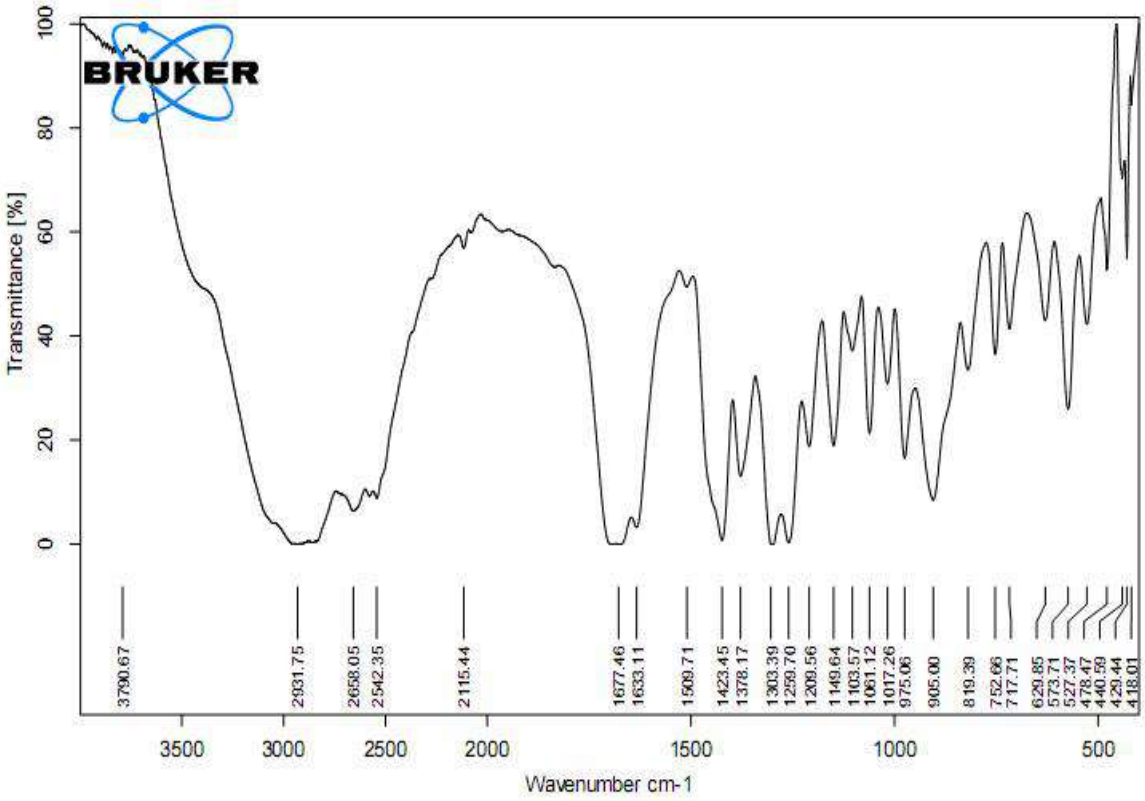


Fig. 4A.4. IR spectrum of the compound 1 isolated from *N. jatamansi*

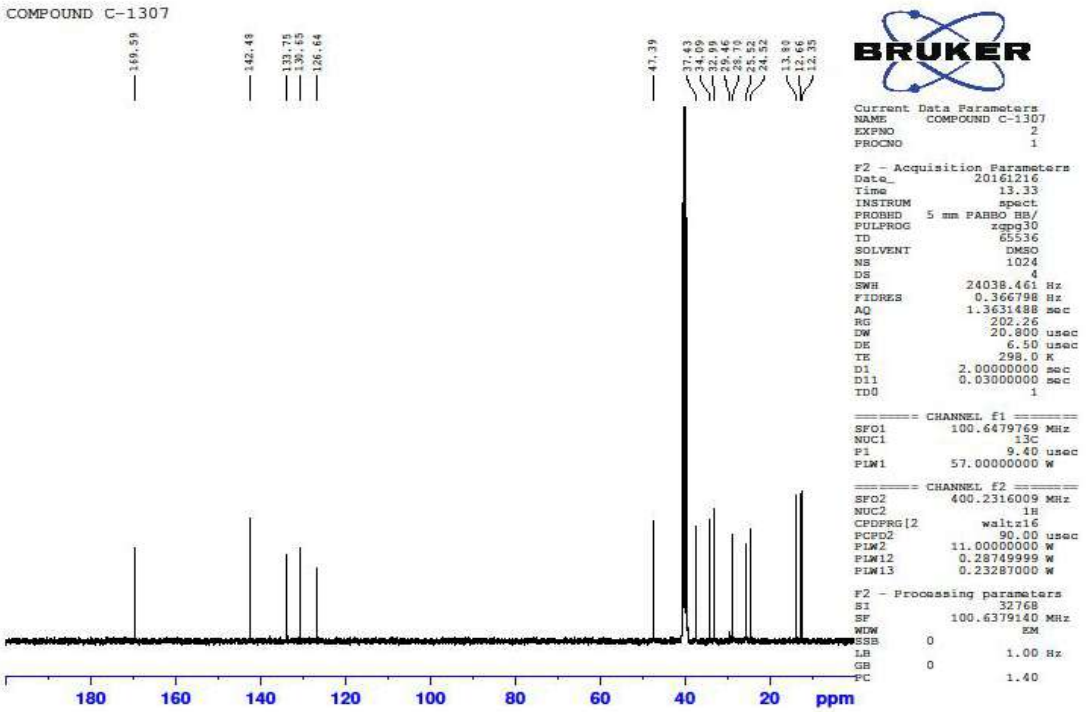


Fig. 4A.5. ^{13}C spectrum of the compound 1 isolated from *N. jatamansi*

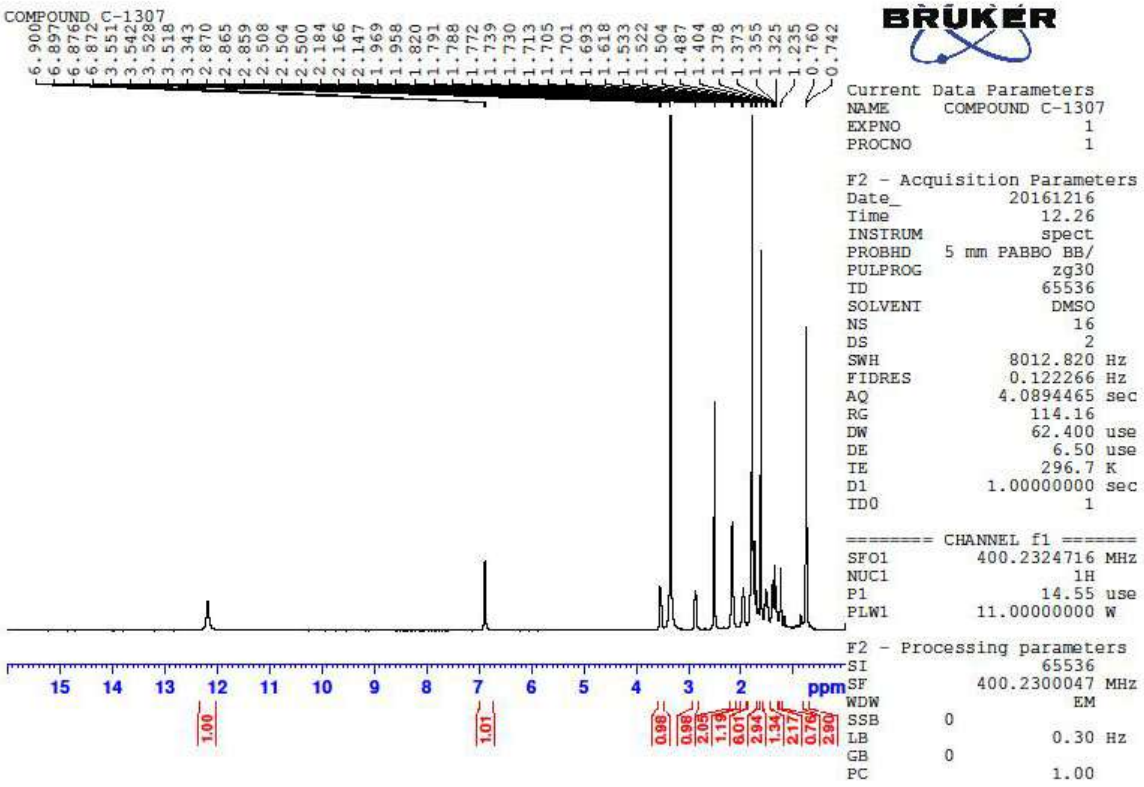


Fig. 4a.6. ^{1}H spectrum of the compound 1 isolated from *N. jatamansi*

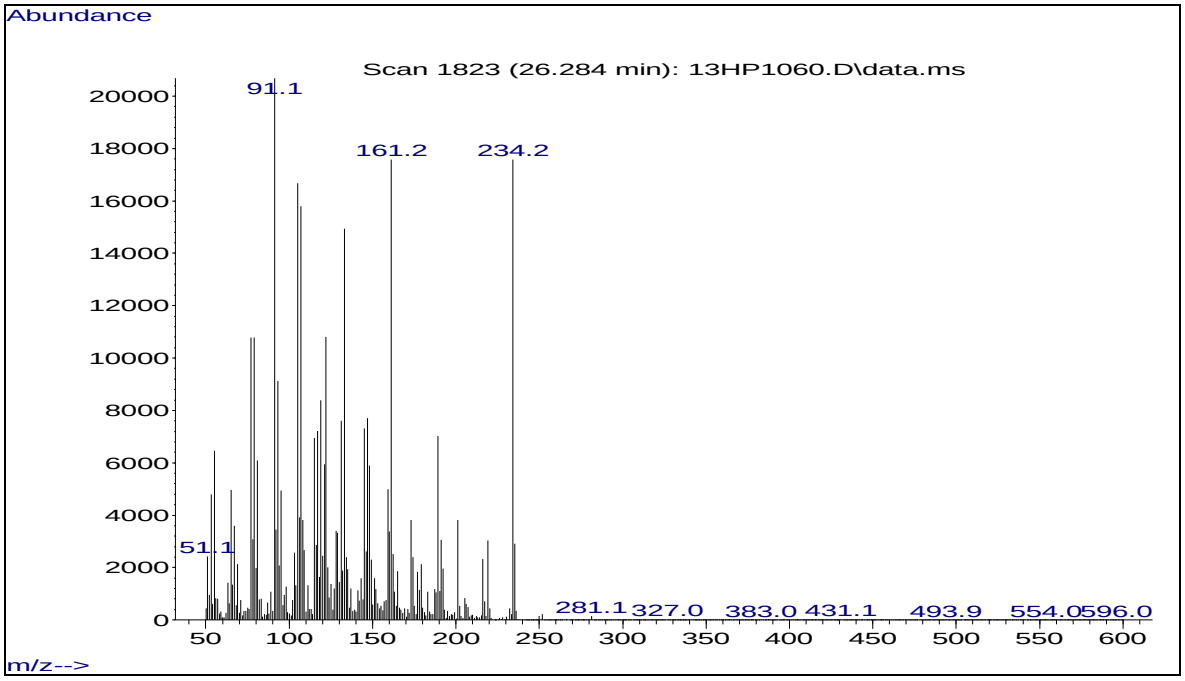


Fig. 4A.7. GC-MS of compound 1 from *N. jatamansi*

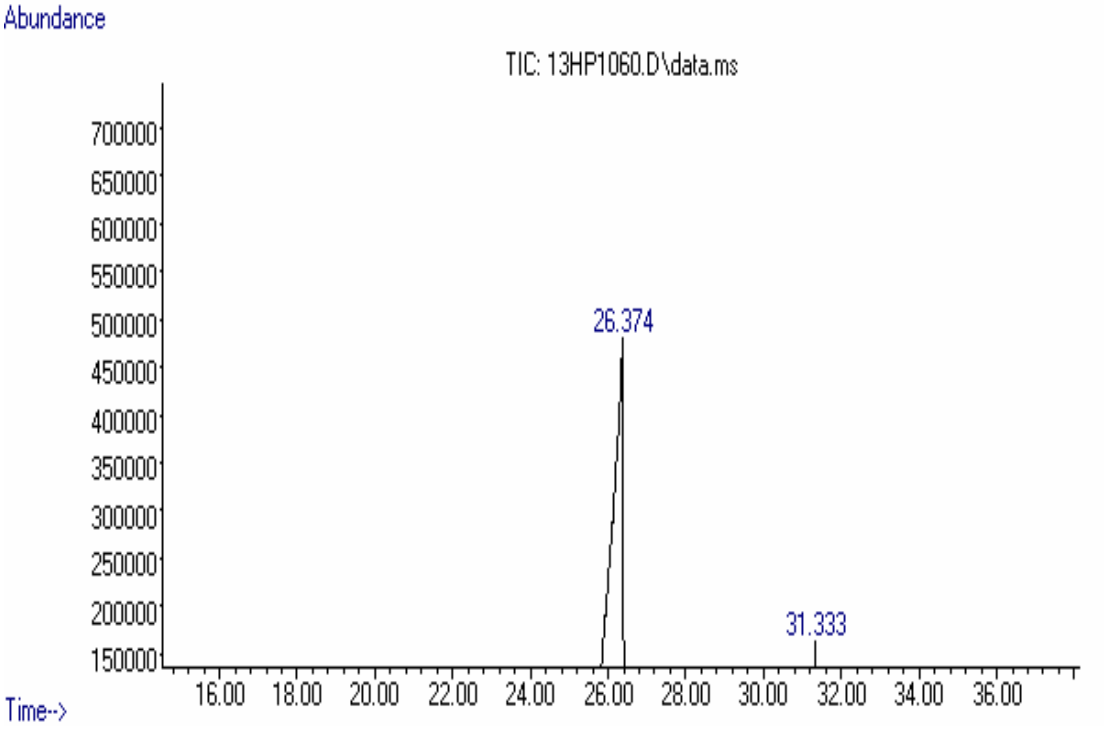
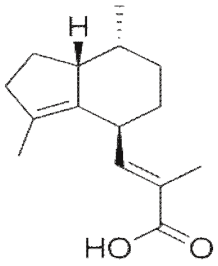


Fig. 4A.8. GC-MS chromatogram of the crystallized compound 1 from the ethanol extract of the *N. jatamansi*

By considering all the above spectral studies, the following structure has been assigned to the compound 1 is



IUPAC Name: 2-Propenoic acid, 3-(2,4,5,6,7,7A-Hexahydro-3,7-DimethylI-1H-INDEN-4-YL)-2-Methyl-, (4S-(4alpha(E),7Beta,7A,alpha)).

Formula: $C_{15}H_{22}O_2$

Common name : Valerenic acid

4A.4 Discussion

The bioactive compounds of *N. jatamansi* have been evaluated using GC-MS. The chemical composition of *N. jatamansi* was investigated using Agilent 7890 GC-MS instrument. It has been reported that secondary metabolites exert a wide range of biological activities on physiological systems. Among the identified chemicals globulol, gurjunene, and aristolene have a role in antioxidants and antimicrobial activities. Similarly, the presence of alkaloids such as indane and actinidine in the ethanol extract of *N. jatamansi* are reported to have a role in the antidiabetic anticancer activity and antifungal activity. Actinidine is a monoterpenoid alkaloid.In traditional medicine, the rhizome of *N. Jatamansi* used to treat epilepsy, hysteria, coryza, convulsions, internal colic, palpitations of the heart, and mental disorders. Thus, this type of GC-MS analysis is the fruitful step towards understanding active principles in medicinal plants, and this type of study will be helpful for further detailed study and isolation of active components present in the plant extract.

The sedative effect which has been well established in the literature by the phytocompound, Valerenic acid or Pentanoic acid has been identified here by GCMS analysis. Neurotransmitters carry nerve impulses across synapse and are small molecules that incorporate a positively charged nitrogen atom. They include several amino acids, peptides and monoamines. The amines acid, glutamine, glycine and gamma amino butyric acid (GABA) serve as neurotransmitters at most CNS synapse (Squire *et al*., 2003). Caryophyllene oxide, an oxygenated terpenoid, well known as preservative in food, drugs and cosmetics, has been tested *in vitro*

for antibacterial, antifungal activity (Guillen *et al.,* 1996; Yang *et al.,* 1999). It is also suggested as potential anticarcinogenic agent that exhibit cytotoxic activity against several solid tumor cell lines (Kubo *et al.,* 1996). The caryophyllene oxide in medicinal plants is reported to be potentially useful for diuretic activity (Ratnasooriya *et al.,* 2004). Plant phyto sterols such as Beta-sterols have connected with inhibition of cancer cell growth, angiogenesis, and apoptosis of cancer in lung, stomach, prostrate, ovarian and breast cancer (Badford *et al.,* 2007; Rubis *et al*., 2008; Wayengo *et al*., 2009).

The infrared spectrum of the compound was recorded in the range of 4000-400 cm^{-1} exhibited characteristic absorption bands for a C=C bond at 1643 cm^{-1}, at 1423 and 1378 cm^{-1} for C-H group. The magnetic resonance spectra of the compound were recorded. The samples were prepared by dissolving in ethanol. The chemical shifts are expressed in δ ppm. The different peaks observed in proton magnetic resonance spectrum are δ 12.35 (1H, s), δ 6.900 (2H, dd), δ 3.55 (1H,q), δ 2.810 (1H,t), δ 2.184 (2H, t), δ 1.969 (1H,d), δ 1.844 (6H,m), δ 1.618 (3H,s), δ 1.551 (1H,m), δ 1.404 (2H,m), δ 1.235 (1H,S), δ 0.760 (3H,d), for protons which accounts for aliphatic and aromatic protons. The data obtained by ^{1}HNMR was complemented by recording ^{13}CNMR. The ^{13}C NMR spectrum of the compound displayed signals at δ 12.35, δ 12.66, δ 24.52, δ 25.5, δ 2.80, δ 28.14, δ 29.46, 32.99, 34.09, 37.43, δ 29.46. Bunch of signals between δ 126.64, δ 130.65, δ 133.75, δ 142.48, δ 169.59 for aromatic carbon atom. The mass spectrum confirms the molecular weight of compound to be 234.2 as revealed by the presence of molecular ion peak.

Chapter-4B

In vitro anti-proliferative activity of bioactive compounds from *C. stocksii* and *N. jatamansi*

Chapter 4B

In vitro anti-proliferative activity of bioactive compounds from *C. stocksii* and *N. jatamansi* DC

4B.1 Introduction

Cancer is a major public health burden in both developed and developing countries. WHO (2002) reported that malignant neoplasm is the third (12.4%) leading cause of death worldwide, the first (30%) being cardiovascular disease, and the second (18.8%) being infectious diseases (Mathers *et al*., 2001). With changing standard of living and food habits and also due to availability of curative treatment for many infectious diseases, cancer is surpassing other illnesses as a principle cause of morbidity and mortality (Noting, 2001). Cancer can be defined as a disease in which a group of abnormal cells grow uncontrollably by breaking the normal rule of cell division. Normal cells are usually subject to signals whether cell should divide or differentiate or as to die. But cancer cell develop degree of autonomy from these signals resulting in uncontrolled proliferation of cells. They may also spread through the blood stream and lymphatic systems to other parts of the body to form metastatic tumours in distant organs.

Normally mammalian cells are interconnected through similar molecular network that control cell proliferation, cell differentiation and cell death. Cancer results in a change in molecular biochemical and cellular level network. Cancer cells may become invasive and transform normal adjacent cells into malignant cells. They may also spread through the blood stream and lymphatic systems to other parts of the body to form metastatic tumours in distant organs. Cancer is caused by abnormalities in the genetic material of the transformed cells. The

cancerous cells may occur in liquid as in leukemia. Most cancerous cells occur in solid tumours that originally appear in various tissues in various parts of the body. Cancer cells have defect in regulatory circuits that govern normal cell proliferation and homeostasis.

Initiation and progression of cancer occurs through both external and internal factors in the environment. Death of the cells in any case is mediated by an intracellular activity either of two distinct mechanisms, necrosis or apoptosis. Cancers cause annually more than 13% of human deaths. More than 70% of all cancer deaths occur in low and middle income countries. Deaths from cancer worldwide are projected to continue rising with an estimated 12 million deaths in 2030 (WHO) (Sidambaram *et al*., 2011).

There are more than 100 distinct types of cancer and subtypes of tumours can be found with specific organs. Tumours can be benign or malignant. Benign tumours are non-cancerous and they are made up of cells that are quite similar to normal cells. Malignant tumours are made up of cancer cells which usually grow faster than benign tumours. These tumours spread through other parts of the body and destroy surrounding tissues. Dubick, (1986) reported that the medical use of herbs is deeply rooted in human history and folklore, and incorporated into the historical medicine of virtually all human cultures. The medicinal plants derived from folklore are huge, *Vincarosea* (Sun and Zeng, 2005), *Podophyllum peltatum* (Gordaliza *et al*., 1994) and *Taxus* spp. (Wani *et al*., 1980) are classical examples of anticancer drugs. These plants and many others lead to discover important drugs including vincristine, vinblastine, podophyllotoxin, 10-hydroxycamptothecin and Taxol (Wani *et al*., 1980; Coker *et al*., 2003). The

objective of cancer chemotherapy is to kill cancer cells with as little damage as possible to normal cells (Halliwell and Gutteridge, 1988). Therefore, any discovery of anticancer agents must be related to novel molecular targets; *i.e.* they should be effective against specific types of cancer cells but less toxic to normal cells, or have a unique mechanism of action for specific types of cancer (Pezzuto, 1997).

Cancer is the second leading cause of death in the United States (Hoyert *et al.*, 2005), where one in four deaths is due to cancer. The National Cancer Institute collected about 35,000 plant samples from 20 countries and screened around 1, 14, 000 extracts for anticancer activity (Shoeb, 2005). 92 anticancer drugs commercially available prior to 1983 in the US and among worldwide approved anticancer drugs between 1983 and 1994, 60% are of them are natural origin (Cragg *et al*., 1997). In this instance, natural origin is defined as natural products, derivatives of natural products or synthetic pharmaceuticals based on natural product models (Jaspars and Lawton, 1998). Between 2000 and 2020, the total number of cases of cancer is predicted to increase by 73% in the developing world and by 29% in the developed world (Parkin, 2001).

4B.1.1 Cancer therapy

Chemotherapy is a treatment with anticancer drugs while the radiotherapy is a treatment using high energy rays to kill cancer cells. Bone marrow or stem cell transplants are used to treat a variety of cancer. Fortunately, research in this area is widespread, and, as for cancer, natural products can be considered one of the main sources of new drugs (Riad Naim Younes *et al.*, 2007).

The use of complementary and alternative medicine (CAM) by patients with cancer survivors has increased (Adams and Jewell, 2007). Most patients use CAM to complement the conventional therapies of radiotherapy, chemotherapy and surgery (Leis and Millard, 2007). Among the promising bioactive components being investigated by natural cancer institute to reduce cancer risk are Indole-3-carbinol sulforaphane, phytoestrogen, isoflavones, perillyl alcohol and green tea polyphenols etc.

4B.1.2 Plant as a source of antitumor compound

Bio-prospection of natural resources using screening procedure is not a `new technique. Plants have played an important role as a source of effective antitumor agents (Hartwell, 1982). It is a significant fact that over 62% of currently used antitumor agents are derived from natural sources such as plants, marine organisms and micro-organisms and about 60% of the currently used anti-cancer agents are derived from plants. Plants have a long history of use in treatment of cancer. More than 3000 plant species that have reportedly been used in the treatment of cancer. First agents to advance into clinical use were Vinca alkaloids, Vinblastine (VLB) and Vincristine (VCR) isolated from *Madagascar periwinkle* (Cragg and Newman, 2006) *Catharanthus roseus* (Apocyanaceae).

The Triterpenoid acids such as oleanolic and ursolic acid which are common plant constituents that is known to possess anti-inflammatory and antitumor activities. Recently, B-lapachone is also known to possess significant activity against a range of tumor cell line including breast leukemia and prostrate lines. Other important compounds are Taxol and camptothecin derivatives

topotecan and irinotecan which exert their cytotoxic action through inhibition of topoisomerase-I, a fundamental enzyme complex involved in DNA "winding unwinding" (Cragg and Newman, 2005). Flavonoids and Quercetin has been shown to exert antitumor effect through blocking cell cycle progression at G_0 and G_1 interface consistent with cdk inhibition (Kellard *et al.*, 2003).

4B.1.3 Plant-Derived Anti-Cancer Agents in Clinical Use

Plants have long been used in the treatment of cancer (Hartwell, 1982). Plants have been a prime sources of highly effective conventional drugs for the treatment of many forms of cancer. With the rapid identification of new proteins having significant regulatory effects on tumor cell cycle progression, and their conversion into targets for high throughput screening, molecules isolated from plants and other natural organisms are proving to be an important source of novel inhibitors to these key proteins, and have the potential for development into selective anti-cancer agents.

Ethnopharmacological or traditional use of plants often results in the discovery of new biologically active molecules (Houghton, 1995). However, it is important that the investigators understand the principles of folk medicine or mode of action of folk herbs (Nakanishi, 1999).The US National Cancer Institute (NCI) has had an established program for the development of methods for initial screening of anticancer compounds since 1955.

The first agents to advance clinical use were the so-called Vinca alkaloids, Vinblastine (VLB) and Vincristine (VCR) isolated from the Madagascar periwinkle, *Catharanthus roseus* G. Don. (Apocynaceae), which was used for the

treatment of diabetes. These drugs were first discovered during an investigation of the plant as a source of potential oral hypoglycemic agent. More recent semi-synthetic analogues of these agents are Vinorelbine (VRLB) and Vindesine (VDS). These agents are primarily used in combination with other cancer chemotherapeutic drugs for the treatment of a variety of cancers. The two clinically-active agents, etoposide (VM 26) and teniposide (VP 16-213), which are semi-synthetic derivatives of the natural product, epipodophyllotoxin, may be considered as being more closely linked to a plant originally used for the treatment of "cancer". The *Podophyllum* species (Podophyllaceae), *P. peltatum* Linnaeus (commonly known as the American mandrake or Mayapple) and *P. Emodii* Wallich from the Indian subcontinent, have a long history of medicinal use, including the treatment of skin cancers and warts. *P. Peltatum* was used by the Penobscot Native Americans of Maine for the treatment of "cancer", and promoted by the observation in the 1940 s that venereal warts could be cured by topical application of an alcohol extract of the dried roots (called podophyllin).

An early example of a natural compound class that ultimately led to Cdk inhibition, was the moderately anti-tumor active flavonoid and quercetin. This flavonoid can be thought of as an ATP-mimic where the planar bicyclic chromone ring system is an isostere of adenine. Quercetin has been shown to exert its anti-tumor effect through blocking cell cycle progression at the G_0/G_1 interface, consistent with Cdk inhibition. However, a close analogue myricetin, shows an IC_{50} close to 10 μM versus Cdk2. Flavopiridol showed 100 fold more selectivity for Cdks compared to its activity for tyrosine kinases, and was the first compound identified by the NCI as a potential anti-tumor agent that subsequently was proved

to be a relatively specific Cdk inhibitor. Other examples mentioned above include olomucine and roscovitine, and the indirubins. Another important addition to the anti-cancer drug armamentarium is the class of clinically-active agents derived from camptothecin, which is isolated from the Chinese ornamental tree, *Camptotheca acuminate* Decne (Nyssaceae), known in China as the tree of joy.

Other important examples are the camptothecin derivatives, topotecan and irinotecan, which exert their cytotoxic action through inhibition of topoisomerase I, a fundamental enzyme complex involved in DNA “winding and unwinding”. In spite of significant efforts on the part of many research groups, few structural classes of compounds demonstrated topoisomerase I inhibitory activity. New classes of topoisomerase I inhibitors in preclinical development are the 2-aryl-quinoline derivatives (indenoquinolines), 3-aryl-isoquinoline derivatives (indeno-isoquinolines), and the naphthyridines which can be traced to the proto-berberine alkaloids, such as nitidine, isolated from *Zanthoxylum* and *Fagara* species.

Cyclin-dependent kinases (Cdks), together with their cyclin partners, play a key role in the regulation of cell cycle progression, and inhibition of their activity delays or arrests progression at specific stages of the cell cycle. An early example of a natural product compound class that ultimately led to Cdk inhibiton, was the moderately anti-tumor active flavonoid, quercetin. Quercetin was shown to exert its anti-tumor effect through blocking cell cycle progression at the G_0/G_1 interface, consistent with Cdk inhibition; and was the first compound identified by the NCI as a potential anti-tumor agent that subsequently was proved to be a relatively specific Cdk inhibitor. Other examples mentioned above include olomucine and roscovitine and indirubins.

4B.2 Materials and Methods

DMEM media, CO_2 incubator, Dimethyl sulfooxide, Fetal Bovine Serum Albumin, Trichloro acetic acid (TCA), SRB dye, Tris base, Acetic acid. All other Chemicals and reagents were analytical grade obtained from sigma Aldrich.

4b.2.1 Anti-proliferative assay

The anti-proliferative assay, Sulphorhodamine B (SRB) assay, performed according to the method of Skehan *et al.* (1990) was used to assess growth inhibition. This colorimetric assay estimates cell number indirectly by staining total cellular protein with the dye SRB (Skehan *et al.*, 1990). Anticancer activities of the compounds of different extract of *C. stocksii* and *N. Jatamansi* were determined using the SRB assay according to Skehan *et al.* (1990). 5×10^3 lung cancer cell lines and the colon cancer cell lines plated in 96 well plates in 100 µl of DMEM media and incubated for 48 h in CO_2 incubator at 37°C. Appropriate dilutions of the compounds were made with DMEM media containing 10% FBS from the stock solutions dissolved in DMSO. 100 µl of the compounds with media was added to the cells and incubated for 24 and 48 h to determine the cytotoxicity. After the incubation time the cells were fixed with 50% TCA for 1 h at 4°C followed by staining with 0.4% SRB dye for 30 min. The unbound dye was washed off with 1% acetic acid and the bound dye was solubilized in 10 mM Tris base and read at 490 nm. Cell survival was measured as the percentage of the absorbance compared with the control. The experiment was conducted in triplicates and results were expressed as percentage of inhibition of cancer cell lines.

4B.3 Results

The anticancer activity of compounds isolated from different extracts of two plants viz., chloroform stem bark extract of *C. stocksii* plant and ethanol stem bark extract of *N. jatamansi* plant as tested on the A549 lung and the HCT-116 colon cancer cell lines at different concentration and exposed at 24 and 48 h, which exhibited a dose dependent and time dependent activity are summarized in Tables 4B.1 to 4B.3. Valerenic acid of *N. jatamansi* exhibited better anticancer activity on HCT-116 cells with 50.9 and 74.8% inhibition at 24 and 48 h while the inhibition on A549 cells was found to be 19.5 and 46.8% at 24 and 48 h respectively. Among the compounds of *C. stocksii* CS2 and CS3, the CS3 (3-methyl butyl benzoate) exhibited better anticancer activity on both HCT-116 and A549 cell lines with 28.7, 18.3, 45.6, 36.0 % inhibition at 48 h. In conclusion, the C1 from *N. jatamansi* and CS3 (3-methyl butyl benzoate) from *C. stocksii* was found to have better anticancer activity on colon and lung cancer cell lines.

4B.4 Discussion

Traditionally many medicinal plants possess the ability to prevent and even to stall the progress of cancer. Although there have been great advances in recent decades in the treatment of commonly seen cancers (Eg; lung and colon cancer). Lung cancer still represents a major challenge and remains the most deadly cancer diagnosis (Siegel *et al*., 2012). Plants possess certain chemicals, which have the ability to modify the physiological function of cells and hence act as anti-cancer drugs to arrest the proliferation of cancer cells. The mode of action of extract is unknown but successfully integrating our documented knowledge of plant properties and modern technological tools, effective anti-cancer drugs can be

derived from and their mechanism can be elucidated (Jaiprakash *et al*., 2006; Liu *et al*., 2007).

Many of the naturally derived anti-cancer agents originally discovered using different assays, have been shown to exert their cytotoxic action through interaction with tubulin. Thus agents, such as vinblastine, vincristine, colchicine, combretastatin and maytansine promote the de-polymerization of tubulin, while in the case of taxanes, microtubules are bundled as a result of stabilization against de-polymerization. The discovery of taxol assumed an added measure of importance through the ground-breaking discovery of its unique mechanism of action. The pervilleines isolated from the Madagascar plant, *Erythroxylum pervillei* (Erythroxylaceae), have shown promising MDR activity both *in vitro* and *in vivo*, and pervilleine A is currently in preclinical development.

Various studies have shown that curcumin modulates numerous targets. These include the growth factors, growth factor receptors, transcription factors, cytokines, enzymes, and genes regulating apoptosis. Numerous growth factors have been implicated in the growth and promotion of tumors. Curcumin has been shown to down-regulate the expression of several cytokines including TNF, IL-6, IL-8, IL-12, and fibroblast growth factor receptors. Curcumin has been shown to down regulate both epithelial growth factor receptor (EGFR) and HER2/neu receptors. It also modulates and rogen receptors, transcription factors. Curcumin may also operate through suppression of various transcription factors including NF-kB, STAT3, Egr-1, AP-1, PPAR-k, and betacatenin activation. These transcription factors play an essential role in various diseases. The constitutively active form of NF-kB has been reported in a wide variety of cancers. NF-kB is

required for the expression of genes involved in cell proliferation, cell invasion, metastasis, angiogenesis, and resistance to chemotherapy. Bharti *et al.*, demonstrated that curcumin inhibited IL-6–induced STAT3 phosphorylation curcumin suppresses a number of protein kinases including mitogen-activated protein kinases, JNK, PKA, PKC, src tyrosine kinase, phosphorylasekinase, JAK kinase, and the growth factor receptor protein tyrosinekinases. Cell Cycle. Curcumin modulates cell-cycle–related gene expression. Specifically, curcumin induced G0/G1 and/or G2/M phase cell cycle arrest, up regulated CDKIs, p21WAF1/CIP1, p27KIP1, and p53, and slightly down regulated cyclin B1 and cdc2. We found that curcumin can indeed down regulate cyclin D1 expression at the transcriptional and posttranscriptional levels.

The present need is to develop drugs that can potentially target cancer cells by means of their inherent difference to normal cells. The development of such drugs with differential action will be very valuable in cancer chemotherapy without the observed side effects. The methodology involves use of cancer cell lines to test the efficacy of the plant extracts *in vitro*.

A number of flavonoids have been isolated from some *Cryptocarya* species. They include dihydrochalcone, chalcone, dihydroflavanone, flavanone and biflavonoid derivatives (Dumontet *et al.*, 2001, 2004; Fu *et al.*, 1993; Govindachari *et al.*, 1973; Juliawaty *et al.*, 2000). *C. costata* represents two pairs of flavanone-chalcone containing highly oxygenated functionality in ring A, which is the first time in *Cryptocarya*. Compounds didymocarpin and isodidymocarpin have been isolated previously from an Indian plant.

A phytochemical study of *C. maclurei* led to isolation of five flavanones, cryptogiones G–H, polyketide and Cryptomaclurone. Cytotoxicity of these compounds evaluated against KB (human nasopharyngeal carcinoma), SGC-7901 (human gastric cancer) and SW1116 (human colon cancer) cell lines using MTT assay. Cryptomaclurone exhibiting moderate cytotoxicity against all three cell lines (Ruifeng *et al*., 2013). A new flavanone and flavonoids, didymocarpin, 2,4-dihydroxy-5,6dimethoxychalcone and isodidymocarpin, has been isolated from the methanol extract of the tree bark of *C. costata*. Cytotoxic properties of compounds were evaluated against murine leukemia P-388 cells. The chalcones were found to have substantial cytotoxicity with IC_{50} of 5.7 and 11.1 μm respectively (Hanapi Usmana *et al*., 2006).

A valerenic acid from the *N. jatamansi* exhibited better anticancer activity. This is the first report of Valerenic acid on two different cancer cell lines along with its neurotransmitter activity. The results of the two plants *C. stocksii* and *N. jatamansi* against two different cell lines indicating that plant can be used as a potential source of antitumor agent.

The two compounds purified from *C. stocksii* chloroform stem bark extract 2-(2 hydroxy-2 phenylethyl benzoic acid), 3-methyl butyl benzoate were subjected to cytotoxic studies against two different colon and lung cancer cell lines. The results are tabulated in the table and graphically represented. 3(3-methyl benzoate) exhibited better anticancer activity for both colon and lung cancer cell lines than the 3-methyl butyl benzoate. In this study purified compounds were found to possess significant activity at micromolar concentration. Thus our findings have important implication for phenolics and alkaloids in the inhibition of cancer cell lines by ethanol and chloroform stem bark extracts of *N. jatamansi* and *C. stocksii*.

Table 4B.1. Anticancer activity of valerinic acid from *N. jatamansi*

Cancer cell lines	Period of incubation (hrs)	Concentration of plant extracts in ug/ml				
HCT-116	24	300	150	75	37.5	18.75
		50.92642	33.9333	16.14611	18.21069	6.643727
	48	74.80891	70.7544	67.0987	65.86906	34.92855
A-549	24	19.51872	16.04278	-2.94118	-2.13904	-7.55348
	48	46.83544	42.44229	17.72152	7.222636	3.909159

Table 4B.2. Anticancer activity of compound CS2 from *C. stocksii*

Cancer cell	Period of incubation (hrs)	Concentration of plant extracts (µg/ml)				
HCT-116	24	100	50	25	12.50	6.25
		25.62	15.78	4.659	1.059	1.958
	48	28.68	9.771	13.23	7.777	5.916
A-549	24	0.936	-5.348	-6.149	-12.17	-14.17
	48	18.32	10.65	4.393	-1.042	-0.149

Table 4B.3. Anticancer activity of CS3 isolated from *C. stocksii*

Cancer cell lines	Period of incubation (hrs)	Concentration of plant extracts (µg/ml)				
HCT-116	24	400	200	100	50	25
		82.37	71.88	45.79	20.01	16.57
	48	72.61	59.65	45.63	28.25	25.06
A-549	24	23.39	18.04	13.77	3.875	-1.079
	48	51.97	39.98	35.96	21.52	10.57

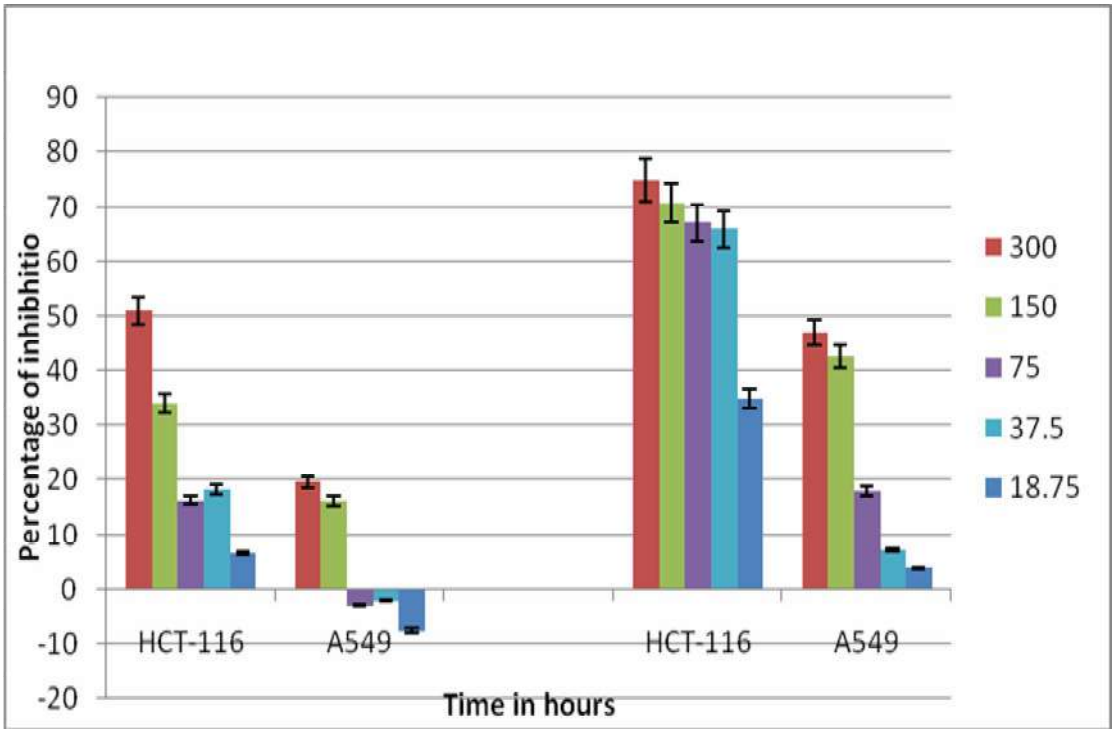


Fig. 4B.1. Anticancer activity of valerinic acid from *Nardostachys jatamansi*

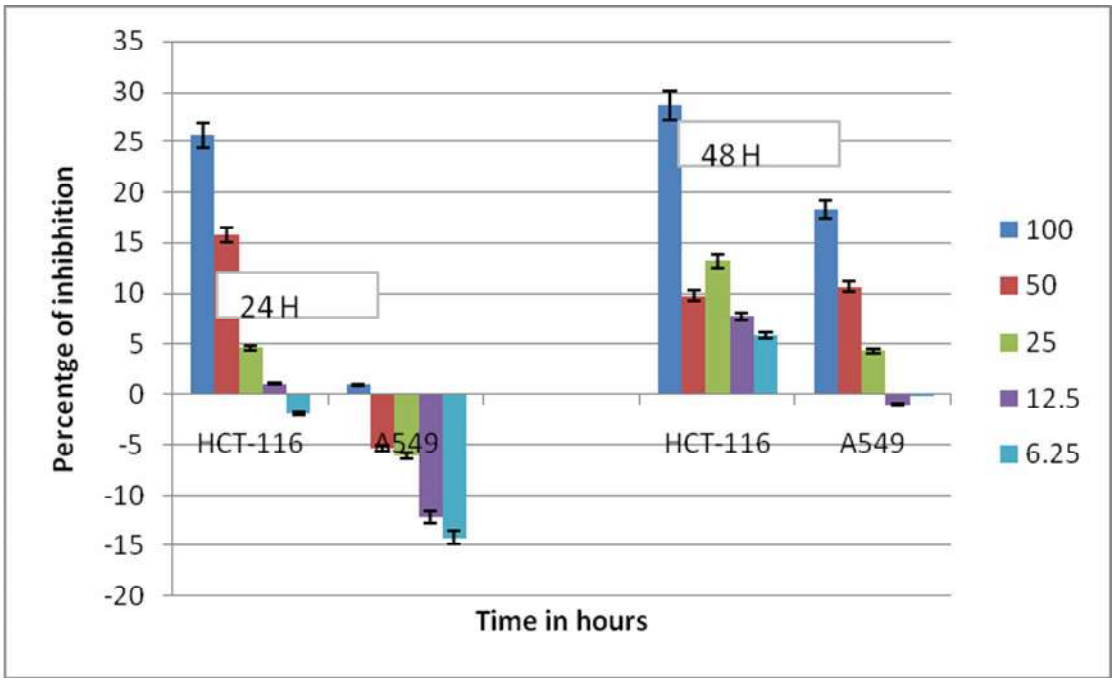


Fig. 4B.2. Anticancer activity of CS2 from *Cryptocarya stocksii*

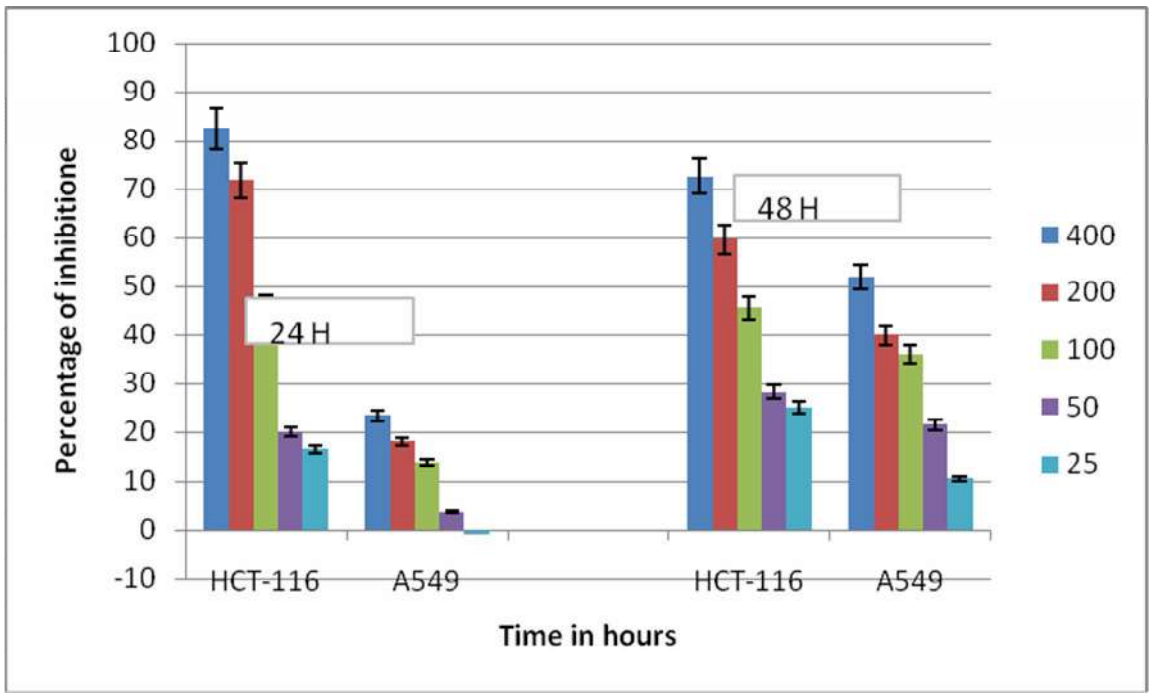


Fig. 4B.3. Anticancer activity of CS3 from *C. stocksii*

Chapter-5

Summary

Cryptocarya stocksii is an endangered plant which is listed in red hot list located in Bababudangiri hills of Chikkamagaluru district, Western Ghats of Karnataka. Hence, the systematic study of *C. stocksii* has been undertaken in the present study to know its phytochemical content and pharmacological activity. Three different extracts of plant were prepared and subjected for phytochemical analysis and various biological activities. Chloroform and ethanolic extracts of *C. stocksii* stem bark, shows promising activity which may be due to large amount of alkaloids, phenolics, flavonoids and tannins which is evident from phytochemical analysis. The presence of these phytochemicals in different extract of plant directly related to its biological activity.

The phytochemical quality of *C. stocksii* different stem bark extracts were evaluated by chromatographic fingerprints. This has been carried out using GC-MS analysis. Nearly twenty compounds have been identified from the chloroform and petroleum ether stem bark extract. The approximate phytochemicals present in the plant extracts and their relative percentage obtained. This study helps to identify various components present in petroleum ether stem bark extract and Chloroform stem bark extract.

Three different plant extracts were subjected for antimicrobial activity against different strains of bacteria and fungi. The results showed that chloroform stem bark extract showed good zone of inhibition than petroleum ether and ethanol stem bark extract. The present study is important in search of new biologically

active antimicrobial agent and its usage can be tested in various fields such as food and pharmaceutical industry. Thus further work can be carried out on the isolation procedure for finding out the exact moiety responsible for the biological activity

To test the scavenging ability of different extracts of *C. stocksii* were subjected to different antioxidant assay. Among the extracts ethanol stem bark extract shows good free radical scavenging activity. It also showed reduction of ferrous to ferric in dose dependent manner. The total antioxidant capacity of ethanol stem bark extract is expressed as ascorbic acid equivalents followed by chloroform and petroleum ether stem bark extract.

From this study, it is clearly indicated that the *C. stocksii* extract has powerful antioxidant activity against different oxidative mechanism *in vitro*. Hence *C. stocksii* can be used as source of natural antioxidant in food and pharmaceutical industry. The various antioxidant activity of *C. stocksii* extract are mainly due to strong electron donating, reducing ability which may be due to the presence of phenols and flavonoids. The hydroxyl group of phenolic compounds in the ethanolic extract of *C. stocksii* are responsible for strong scavenging activity. Today, almost all processed foods have synthetic antioxidants incorporated, which are reported to be safe, although some studies indicate otherwise.

In the present study the central analgesic activity of different extracts of *C. stocksii* was carried out using hot plate method. Chloroform stem bark extract showed significant analgesic activity almost equal to standard. The good analgesic activity of chloroform stem bark extract was mainly due to the presence of

polyphenolic compound which was responsible for reducing pain in experimentally induced mice. The exact mechanism by which *C. stocksii* exerts its analgesic activity is not determined yet and needs further investigation to elucidate the other active compounds and underlying mechanism(s). The ethanol extract of *C. stocksii* exhibited potent analgesic effect against thermal noxious stimuli indicating the extract acting as a peripheral analgesic agent. Present research work demonstrates scientific rationale for traditional use of this plant as a central stimulant and analgesics.

Antipyretic activity of plant extracts were carried out by brewer's yeast method. Different extracts of *C. stocksii* plant extracts were studied. Chloroform extract significantly decreased the elevated rectal temperature after the administration of a dose. By observing the graphical representation, the plant moderately inhibits pyretics.

Carageenan induced hind paw edema models were used to evaluate the anti-inflammatory activity and the results indicate the efficiency of chloroform extract of *C. stocksii* in reducing inflammation and exhibited in a dose dependent manner. The comprehensive analysis of data demonstrated that anti-inflammatory activity of three different extracts of *C. stocksii* decreased in the order chloroform extract > ethanol > petroleum ether extract. The anti-inflammatory activity is mainly related to inhibition of prostagalandin synthesis, as described for anti-inflammatory process induced by carrageenan.

Wound healing is a biological process that starts with trauma and ends with scar formation. The present review clearly revealed that nature provides huge

number of plants that show significant wound healing activities. All the three extracts of *C. stocksii* were analysed for wound healing activity using excision and incision method. This has given fruitful result where chloroform extract possesses good healing power than petroleum ether and ethanolic stem bark extract. The good healing power of *C. stocksii* extract is mainly due to presence of secondary metabolites present in the extract.

In the present study isolation and characterization of biologically active phytochemicals from chloroform extract of *C. stocksii* stem bark has been undertaken. The compounds include heptadecane (CS1), 2(2-hydroxy phenyl ethyl benzoic acid (CS2) and 3(3-methyl butyl benzoate) (CS3).

The two purified compounds CS2 2-(2-hydroxy phenyl ethyl benzoic acid) and CS3 3(3-methyl benzoate) were subjected for *in vitro* studies using colon and lung cancer cell lines. The results showed that the compound CS3 showed better anticancer activity for lung and colon cancer cell lines. This is the first report from this plant where the plant extracts and purified compounds together have shown significant activity.

N. jatamansi is another plant taken for study which is growing in Himalayan region. The plant has usage since from ancient times. Hence, present work is undertaken to study the detailed phytochemicals present in the plant extracts. The quality of herbal medicine can be evaluated by the construction of chromatographic fingerprints. In the present study, 60 compounds have been identified from the rhizome of *N. jatamansi* DC by GC-MS analysis. There for GC-MS method is a direct and fast analytical approach for identification of

terpenoids and steroids and only a few grams of plant material is required. The presence of various bioactive compounds with different biological activity such as anti-inflammatory, antipyretics, antibacterial, antifungal, skin conditioning have been identified, justifying the use of the whole plant for various ailments by traditional practitioners.

In this study the ethanolic extract of plant is subjected for purification using column chromatography. Valerenic acid was isolated and characterized. This compound was studied for anticancer activity using two cancer cell lines i.e., colon and lung cancer cell line. Valerenic acid showed good cytotoxic activity. This justifies the use of plant in the treatment of cancer. The genus *Cryptocarya* was used by traditional healers to treat various diseases. There were no reports and data available about this plant. The present work is undertaken to provide scientific proof for the better usage of plants in different fields such as food and pharmaceutical industry for different purpose.

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