PART B
Phytochemical Screening of Medicinal Plants—
*Juglans regia* and *Mimusops elengi*

CHAPTER 3
Bioeffective Strategies of *Juglans regia* stem bark

CHAPTER 4
Isolation and Characterization of Bioactive Molecules from *Mimusops elengi*
INTRODUCTION
INTRODUCTION

Ayurveda- Indigenous System of Medicines

Ayurveda has been the oldest method of healthcare. It is not only meant to cure the diseases but covers the whole gamut of human life including its spiritual aspects. Ayurveda is rich in magical practices for the treatment of diseases.

Ayurveda, the most valuable and useful Veda is a golden gift to human being by our great rishis. This system of medicine was received by Dhanvantari from Brahma and Dhanvantari was deified as the God of medicine\(^1\). The period of Vedic medicine lasted until about 800 BC. The Vedas are rich in magical practices for the treatment of diseases and in charms for the expulsion of the demons traditionally supposed to cause diseases\(^2\). It involves the physical, metaphysical and spiritual aspects of human life. Ayurveda chiefly recommends herbal preparations which are the oldest form of healthcare known to mankind. Around 1000 BC, knowledge of Ayurveda was comprehensively documented in Charak Samhita and Sushruta Samhita. The treatment in Ayurveda imparts both curative as well as preventive measures that restore the body equilibrium through various techniques, procedures, diet and medicine. This holistic system of medicine includes personal hygiene, regular daily routine, proper social behavior, use of rejuvenative food and rasayans for prevention of diseases and includes drugs, diet, exercises for curative treatment\(^3\). Natural herbs and plants are used as a part of treatment to cure diseases\(^4\).

Herbal Medicines

World Health Organization (WHO) estimates that 80% of the world population still uses herbal remedies for some aspect of primary healthcare. This has lead to a great concern in scientific community to document medicinal properties of these plants and evaluate them scientifically for their therapeutic efficacy. Indeed about 25% of the medicinal prescriptions dispensed in the USA contain at least one
active ingredient derived from plant material. Some of them are isolated from plant extracts; others synthesized to mimic a natural plant compound.

Plants are a rich, natural source of innumerable chemical substances that act individually as well as collectively upon the human body. Major pharmaceutical companies are currently conducting extensive research on plant materials gathered from the rain forests and other places for their potential medicinal value. Substances derived from them remain the basis for a large proportion of the commercial medications used today for the treatment of heart diseases, high blood pressure, pain, asthma and many other problems. Herbal medicines are more effective and less harmful as they have negligible side effects; even exhibit low mammalian toxicity and can be handled easily.

**Complementary and Alternative Medicine**

Herbal therapy is now well accepted as a part of Complementary and Alternative Medicine (CAM) all over the world. These alternative medicines are immune boosters and are essential for repair and regulation of body functions. Herbal medicines have been in practice for many years. Their history can be rooted from ancient civilization wherein their role as a primary source of medication is evident. Although today only a few of these herbs have been approved for their commendable medicinal properties, a majority of naturally occurring herbs are only considered as food supplements because of the lack of randomized controlled clinical trials. Herbal medicines definitely have future in the field of medicine. Currently studies are being conducted to know these herbs in depth. Many are hopeful about these natural substances for if ever approved, they are going to be cheap and affordable for all.

Natural products may serve to provide molecular inspiration in certain therapeutic areas for which there are only a limited number of synthetic lead compounds. A natural product lead compound may help to elucidate a new mechanism of interaction with a biological investigation. Much of synthetic medicines today have been patterned from the actions of natural substances.
Traditional Herbs – A Source of Medicine

A lot of herbal medicines are utilized in day-to-day life. *Allium sativum* L. (Garlic) has shown to reduce cholesterol in the body thus may play a role in lowering blood pressure; even it exhibits positive results in the treatment of AIDS. Research has shown that bitter gourd is effective in lowering blood sugar level in diabetic patients. It is a good source of vitamins A, B, C and minerals such as iron, folic acid, phosphorus and calcium that are essential for human being. *Ocimum sanctum* L. is one of the commonly used traditional medicines in India for the treatment of cold, fever and cough. World Health Organization has proved that methanolic extract of this plant controls the fever due to typhoid inoculation.

Preventive Medicine Research Institute California, and National Nutrition and Food Technology, Iran successfully demonstrated that fruit juice of *Punica granatum* L. possesses antibacterial, depurative, heart-strengthening, anticancer and tonic properties along with rich nutritional value. Alternative Medicine review published (2005) that *Glycyrrhiza glabra* L. roots contain triterpenes, saponins, flavonoids, polysaccharides, pectins, amino acids and mineral salts. It is effective in the ailments of lungs, throat, cough, intestinal ulcers and skin allergy.

*Tinospora cordifolia* Miers. is composed of alkaloids, glycosides, steroids naturally available and exhibits antidiabetic, anti-inflammatory, anticancer, antioxidant, antimalarial, antacid properties. Flavonoids from the flowers of *Rosa centifolia* L. offer protection from ultra-violet radiations. Antimicrobial activity study of this plant is reported. *Commiphora mukul* reduces the levels of harmful L. D. L. and enhances the efficiency of heart. Southern California University of Health Science (USA) promotes the positive applications of this plant in arthritis. Pain killer properties of *Tribulus terrestris* L. are reported by Department of Toxicology and Pharmacology in Iran.

*Aegle marmelos* L. contains active component marmesolin, which is found to be effective in dysentery and intestinal infections. *Centella asiatica* L. is useful in
psychotic problems and mental tensions. *Acorus calamus* L. provides with aromatic oils that possess antifungal, insecticidal, antituberculotic, antityphoid and antidiphtherial activities. *Asparagus racemosus* L. is an effective Ayurvedic remedy for pregnant women. Ephedra is the herb used in Traditional Chinese Medicine for more than two thousand years to treat asthma and other respiratory problems. There Traditional Chinese Medicines, Ayurvedic Herbal Medicines and Western Herbal prescriptions are three main methods all over the world through which herbal medicines are prescribed. Western herbal remedies are prepared solely from plant material, whereas traditional Chinese and Ayurvedic herbs may use some animal and mineral substances along with herbs.

**Primary and Secondary Metabolites**

All plants produce chemical compounds as part of their normal metabolic activities. These include primary metabolites such as proteins, carbohydrates and fats and secondary metabolites. In a particular genus or species they occur in various proportions of active ingredients. The medicinal plants receive attention of researchers from chemical, pharmacological and clinical fields all over the world. The studies on folk medicines through ethnobotanical surveys are gaining importance. Taking into consideration these facts, lead molecule isolated from plant can be modified by various synthetic routes to increase its potency.

Secondary metabolites produced by plants possess varied functions such as toxins used to deter predation, pheromones to attract insects for pollination etc. Bacterial and fungal attacks are prevented by means of phytoalexins whereas allelochemicals inhibit growth of the rival plants. Natural products also offer unmatched structural variety, especially to cope up with the exploration of environmental niches.

**Natural Bioactive Molecules**

Ayurvedic and Siddha systems of medicines use the fruits and seeds of *Semecarpus anacardium* for the treatment of diseases including human cancers. Most of the medicinal plants contain heterocyclic compounds. Apple
is a source of tyrosinage which can also be obtained from banana and mushrooms. Sulphur containing compound, Allicin (I) is known to be effective in preventing wounds from progressing into worse infections present in Allium sativum L. 1-(propylthio)propane (ii) from Allium cepa has antibacterial effect. Chlorothiazide (iii) containing sulphamylamide moiety is an active metabolite of a class of early diuretic compounds.

Plant-derived alkaloids have been traditionally important due to their pronounced physiological activities. Ephedrine (iv), the active ingredient in Ephedra, is used on the commercial pharmaceutical preparations for the relief of asthma symptoms and other respiratory complaints. Pterogynidine (v) was isolated from Alchornea glandulosa by Lopes F. C. et al. It can be used against pathological situations where angiogenesis is stimulated as tumor development.\textsuperscript{11}

\textit{Catharanthus roseus} L. provides with terpenoid indole alkaloids (TIAs) ajmalicine (vi) and serpentine (vii), whereas the bisindoles vinblastine (viii), vincristine (ix) and 3', 4'-anhydrovinblastine (x) are used in the treatment of many cancers.\textsuperscript{8} Dried mature fruits of \textit{Piper nigrum} contain a pungent alkaloid amide named piperine (xi) which exhibits anticonvulsant, CNS depressant, antipyretic, antioxidant and hepatoprotective properties.\textsuperscript{12}

A reputed Ayurvedic medicinal plant \textit{Adhatoda vasica} used for the treatment of cough, bronchitis, asthma and tuberculosis, contains Vasicine (xii) a pyrrolazoquinazoline alkaloid.\textsuperscript{13} Biosynthesis of peptide type ergot alkaloid, eargotamine is reported.\textsuperscript{14} Ergot alkaloids (xiii) aid in childbirth, treatment of neurological and cardiovascular disorders.\textsuperscript{15} In India \textit{Rouwolfia serpentine} is extensively used for sleeplessness, anxiety and high blood pressure.\textsuperscript{16} Reserpine (xiv) and dereserpine are alkaloids responsible for antihypertensive action.\textsuperscript{17}

A potent pain killer morphine (xv) from \textit{Papaver Somniferum} is involved in the regulation of proinflammatory mediators.\textsuperscript{18} Bioactive carbazole alkaloids Kurryam (xvi), Koenimbine (xvii) and Koenine (xviii) are isolated from \textit{Murraya Koenigii}.\textsuperscript{19}
They demonstrated a reduction in gastrointestinal mobility in charcoal meal test in Wister rats\textsuperscript{19}. Plant flavonoids, flavonoidal glycosides and phenols are also potent bioactive molecules. 3- (8’ (Z),11’(Z) - pentadecadienyl) catechol (\textit{\textsuperscript{xix}}) isolated from \textit{Semecarpus anacardium} is cytotoxic to tumor cell lines with IC\textsubscript{50} values lower than doxorubicin (\textit{\textsuperscript{xx}})\textsuperscript{20}. Rutin (\textit{\textsuperscript{xxi}}) is the quercetin glycoside abundantly spread in various plants and is useful in coronary thrombosis and for purification of blood\textsuperscript{21}. Quercetin-3-O-glucoside (\textit{\textsuperscript{xxii}}) is the flavonoid isolated from aerial parts of \textit{Prangos ferulaceae}. It exhibits cytotoxic, phytotoxic, antimicrobial and antioxidant effects\textsuperscript{22}.

Antimicrobial activity study of menthol (\textit{\textsuperscript{xxiii}}) and other isolates from leaves of \textit{Mentha longifolia} L. validate the use of this plant in treatment of minor sore throat and minor mouth or throat irritation\textsuperscript{23}. Active principle Gaultherin (\textit{\textsuperscript{xxiv}}) from \textit{Gaultheria yunnanensis} possesses analgesic and anti-inflammatory activities\textsuperscript{24}. \textit{Hippobromus pauciflorus} is commonly used for treatment of eye infections\textsuperscript{25}.

Steroidal glycosides are precious medicines used for cardiac problems. Digoxin (\textit{\textsuperscript{xxv}}) and acetyl digoxin (\textit{\textsuperscript{xxvi}}) from leaves of \textit{Digitalis lanata} possess cardio tonic and cardio toxic properties\textsuperscript{26-28}. The popular drug aspirin (\textit{\textsuperscript{xxvii}}) evolved from Egypt where extract of willow bark was used to treat inflammation. The active component of the drug was identified as the glucoside of salicylic alcohol. The severe gastric side effects associated with use of sodium salicylate prompted the synthesis of o- acetyl- derivative as a possible pro-drug.

Since ancient times a dominant role has been played by higher plants in the maintenance of human health. More than fifty percent of modern clinical drugs are of natural product origin. This is the main cause that development of pharmaceutical industries is dependent on natural products. It includes utilization of plant derived natural components or herbal preparations as alternative source of medicaments all over the world. Thus natural products have been an inevitable part of modern pharmacology and clinical practice. Natural products offer
unmatched structural variety, as new environmental niches are explored and their usefulness can be further extended.

Some Active Ingredients of plants

(i) $\text{S}^\text{O}^-\text{S}$

(ii) $\text{S}-\text{S}$

(iii) $\text{N}=\text{O}$

(iv) $\text{OH}$

(v) $\text{HN}^-\text{CH}_3$

(vi) $\text{HN}^-\text{HN}^-\text{HN}$

(vii) $\text{N}$

(viii) $\text{O}$
Family Juglandaceae

The trees of the family Juglandaceae are closely related to hickories. All the species from this family are valuable economically as well as pharmaceutically. They supply good quality, durable wood, energetic fruits, oil-rich seeds and bark composed of dyes\(^{29,30}\). Walnuts belong to the family Juglandaceae. They are deciduous trees, 10–40 meters tall with pinnate leaves 200–900 mm long.

The two most commercially important species are \textit{J. regia} and \textit{J. nigra} for timber and nuts. They are widely grown in temperate zones. Manos and Stone compared seed oil content from several species of the Rhoipteleaceae and Juglandaceae and observed that the nut oils were more unsaturated from species growing in Temperate zone than Tropical zone\(^{31}\). Juglone is a bioactive naphthaquinone, secondary metabolite of the plants from this family. It has herbicidal and pesticidal applications\(^{32}\).
Genus Juglans

The Latin name, *Juglans*, derives from *Jupiter glans*, "Jupiter's acorn": figuratively, a nut fit for a god. The twenty one species in the genus range across the north temperate Old World from southeast Europe east to Japan, and more widely in the New World from southeast Canada west to California and south to Argentina. Six species from genus juglans, including the black walnut *J. nigra* and the butternut *J. cinerea*, are native to the USA. About 15 other species occur in South America and Eurasia. English walnut *J. regia*, originally from southeastern Europe and Asia, is cultivated widely for its nut crop. Other species from this genus are *J. mandshurica J. Cordiformis (Ukraine) J. hindsii (USA) J. rupestris. J. regia var. pendula, J. californica, J. major, J. sieboldiana J.rupestris J. regia f. Laciniata, J. microcarpa. The word walnut derives from Old English *wealhnutu*, literally "foreign nut", *wealh* meaning "foreign".

*J. regia* is native of Persia and the Himalaya, and perhaps China, but was cultivated in the Mediterranean region in very early times, whence it spread to Northern Europe. It was cultivated before 1562. It is figured in Hayne xiii, t. 17, and Berg. and Schmidt, t. 86. All other species of the genus are found in the new world. The tree grows to a height of 40 - 60 feet with a large spreading top (crown) and thick, massive stem. Walnuts are light-demanding species that benefit protection from wind and hardy against drought.

The glycosides, useful as antioxidants for foods, cosmetics etc. are obtained from kernels of Juglans spp. Juglone is a naturally occurring naphthaquinone from roots, leaves and green skin of walnut. Its allelochemical properties can be used for weed control in agro chemistry. Juglone and its derivatives have a wide spectrum of applications in folk medicine, cosmetol, pharmacol and agro ecosystems, in skin coloring preparations and in hair dyes.

Family Sapotaceae

Sapotaceae family consists of large evergreen trees and very few shrubs with forty genera and six hundred species. These plants are distributed widely...
throughout the tropics of Asia, Africa and America\(^{38,39}\). The important plants from this family are *Argania, Butyrospermum, Colacarpum, Chrysophyllum, Mimusops, Payenne, Sarcospem* etc.\(^{40}\). Sapotaceae seeds are mostly nuts and their kernel fat ranges from 30\%- 50\%\(^{41}\). Members of this family are economically useful. Seeds of *Butyrospermum parkii* are used as ‘shea butter’. *Colacarpum sapota, Chrysophyllum cainite, Lucuma mammosa, Manilkara achras* etc. are valued for delicious, edible fruits. Seeds of *Madhuka butyracca* produce vegetable butter- ‘Phulwa’, used as cold cream, lip salve and luminent.

Chewing gum is made from the latex (chickle) of *Manilkara achras*. *Mimusops globosa* is the source of commercially important latex, called ‘balata’. *Chrysophyllum olivaeforme* furnishes cabinet wood\(^{39}\). Bark of Achras sapota possesses tuberculostatic principle\(^{42}\). Proteins from seeds of *Pouteria torta* having lectin like properties were found to exhibit insecticidal and antifungal activity\(^{43}\). *Pouteria campechiana, Pouteria sapota* and *Pouteria viridis* are the tropical plants from this family that bear edible fruits; among this *P. sapota* has the highest antioxidant activity\(^{44}\). *Mimusops hexandra* bark is reported to be febrifuge and general tonic. It retards fermentation.

Orally administered saponins from *Madhuka longifolia* depict antiulcerogenic as well as anti-inflammatory activities in rats. The anti-inflammatory activity was 1/5 that of phenyl butazone\(^{45}\). Argan oil from *Argania spinosa* is found to be beneficial in the treatment of hyperlipidemia and hypercholesterolema\(^{46}\). *Argania spinosa* tree has played an essential role in the micro-economy of South-western Morocco, by providing food and fuel. Traditionally the argan oil is directly eaten on toast or used for frying. The argan oil has important applications in cosmetics to cure pimples, juvenile acne and chicken pox pustules. It is reported to reduce dry skin problems and appearance of wrinkles. It is prescribed traditionally as hepatoprotective agent, or in case of hypercholesterolemia or atherosclerosis\(^{47}\).

**Genus Mimusops**

The trees of genus *Mimusops* are distributed in the tropics of Old World covering thirty species. *Mimusops elengi* is the species commonly found in India along
with *Mimusops hexandra* and *Mimusops manilkara*. All the trees are of medium height and grow throughout the country. All parts of plants have more or less similar curative properties in folk medicine. Investigations revealed that saponins are main constituents of the nuts. Saponins are pharmacodynamic group of natural products possessing significant biological activities.

*Mimusops elengi* and *Mimusops hexandra* are used in Indian traditional medicines. Most of the triterpenoid saponins have been studied and reviewed. Isolation of nine saponins from the seeds and fifteen saponins from the leaves of *Mimusops laurifolia* were reported. Saponins, hederagenin and Na-Nimbate, isolated from *Mimusops manilkara*, showed anti-inflammatory activity against carrageenin induced edema and formaldehyde induced arthritis in rats.

**Bioactive Potentials of *Juglans regia* L. and *Mimusops elengi* L.**

*J. regia* L. is a medicinally useful species. This plant from Himalayan regions in India is used in folk medicines to treat various diseases including cancer. All parts of the plant; root, stem, leaves, fruits, seeds are traditionally used as medicines. Decoction of stem bark helps to overcome dental complaints. Leaves' extracts exhibit anticancer activity. The species is used to treat 'tisis' and 'scofula' which are synonyms of tuberculosis & tuberculosis of cervical glands. Antimycobacterial activity study of hexane extract of leaves revealed that it could be an important source of potent non-polar compounds.

*J. regia* leaves contain mono and sesquiterpenes and bark is composed of bioactive juglone, regiolone, sterols and flavonoids. Antibacterial properties of plant may be due to presence of phenolics, flavonoids, terpenoids, alkaloids and sterols. Sterols play chemoprotective and cardioprotective roles. Tannins are antibacterial, anticancer, antidiarrheic, antihepatotoxic, chelators, antihypertensive, antitumor, cancer preventive, and antiulcerative. Walnuts have high phenolic and antioxidant levels. It is one of the best sources of phenolic antioxidants, a-tocopherol and unsaturated fatty acids and phytochemicals.
Bioavailability is greater when taken in an extract form. It has higher concentrations of physiologic plasma levels than dietary supplement phenolics.

*Mimusops elengi* is a potent medicinal plant having historic importance. All parts (Panchang) of the plant have been reported for their medicinal uses in Ayurvedic system of medicine. Literature survey revealed that this medicinal application of the species is due to the presence of bioactive molecules. The flowers, fruits and bark are acrid, astringent, cooling and anthelmintic\(^6\).

An important preparation of Mimusops is “Bakuladya Taila”, applied on gum and teeth for strengthening them. Leaves are helpful as an antidote for snakebite\(^69\). The flowers are considered expectorant and smoked in asthma. Pulp of ripe fruit is antidysenteric\(^70\). Saponins from seeds are spermicidal and spasmolytic\(^71\). Bhuyan et. al. extracted and identified the colour components from the bark material and suggested that it can be an alternative to synthetic dyes\(^72\).

The plant is found to be potential biomass source for hydrocarbons and other phytochemicals\(^73\). Investigation of different parts of the plant showed presence of triterpenoids, flavonoids, sterols and saponins. Many of them are responsible to cure various health complaints. Mimusopic acid depicts anti-HIV reverse transcriptase activity. A number of drugs derived from the plant contain saponins as main components. Saponins are pharmacodynamic group of natural products with a wide range of biological activities. The saponins present demonstrated to be antifungal against human pathogens\(^74\). Spasmolytic activity of saponins was studied on guinea pig ileum by Banerji et. al. It is reported that saponins having a triterpenoid moiety were more active than those with a steroid moiety\(^75\). Flavones isolated from seeds have strong antibacterial activity against gram positive and gram negative bacteria\(^76\).

These observations clearly indicate that *J. regia* L. and *M. elengi* L. are rich sources of potent bioactive components which are responsible for their valuable contribution in medicines and strongly support their applications as traditional herbal medicines.
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CHAPTER 3

Bioeffective Strategies of *Juglans regia* Stem Bark

Section I

GC-MS Study and Proximate analysis

Section II

Isolation, Characterization of a Bioactive Naphthaquinone with Quantification and Standardization

Section III

Biological Activity Study
PUBLICATIONS


9] Quantification of a phytotoxin from Walnut species by HPTLC method, communicated to journal of CRSI

10] Validation method for Standardization of a bioactive component from J. regia stem bark, communicated to Journal of Pharmacy Research
Section I

GC-MS Study and Proximate Analysis

3.1 *Juglans regia* L.

The Latin name, *Juglans*, derives from *Jupiter glans*, "Jupiter's acorn". It is also called as "Gallic nut" "English walnut" and "common walnut" in various parts of the world. In India walnuts have special mythological importance.

3.1.1 Distribution

Genus *Juglans* of family Juglandaceae covers twenty one species *Juglans regia* & *J. nigra* are grown in temperate zones including Persia, Himalaya, China and Pakistan. All other species of genus are found in new world. *Juglans* spp. extends from Greece and Asia Minor, over Lebanon, Spain, Turkey and Mexico.

3.1.2 Classification

- **Kingdom**: Plantae
- **Subkingdom**: Tracheobionta
- **Superdivision**: Spermatophyta
- **Division**: Magnoliophyta
- **Class**: Magnoliopsida
- **Subclass**: Hamamelididae
- **Order**: Juglandales
- **Family**: Juglandaceae
- **Genus**: Juglans
- **Species**: *Juglans regia*

3.1.3 Synonyms

- **English**: Walnut, Nux Juglans
- **Sanskrit**: Aksotaka, Phalasneha, Pilu.
- **Kashmiri**: Than, Kunawar-Khor, Akhar
- **Marathi**: Akroda
- **Hindi**: Akhrot

3.1.4 Description

*Juglans regia* L. is a large deciduous tree growing up to 40 to 60 feet with a broad, rounded open crown. The bark is thick, deeply furrowed with diamond
shaped pattern. Yellowish-green scented flowers bloom in June, followed by the ripening of fruits in October. The fruits are brown corrugated nuts enclosed in green, semi-fleshy husks. Tree is often grown as ornamental.

3.1.5 Shloka

Medicinal potential of *Juglans regia* L. is described in Ayurveda as:

अशोककुंक गुणो स्निःश्यं मधुरं स्नापाक्योः।
गुरुणं बृहं वृक्षं बल्यं विद्धीर्देचनम्।
हुदं श्वायास्वातवन्द्रहं कफवितलम्।

Meaning: Walnut is oily, sweet in taste and paka (post digestive action). It is heavy to digest, heat promoting, strengthening the body and constipative. It is good for heart, useful in tuberculosis, gout and irritation of body but enhances acidity and cough.

3.1.6 Uses

All parts of the plant exhibit mainly dyeing, pharmaceutical as well as agro-ecosystem protective properties⁸. The leaves, catkins and mature husks yield a brown dye that contains iodine⁹, 10, 26. The nuts, dried fruits are used as wood polish and paint¹¹, 12. Walnuts yield excellent oil and the oil cake of edible uses¹⁰-¹⁴. The crushed leaves are an insect repellent¹⁴, 15. A valuable heavy, hard and durable timber is obtained¹³. The bark and the fruit rind are dried and used as tooth cleaner. They can be used fresh¹², 13.

Therapeutics¹⁶- Bark and leaves are anthelmintic, alterative, laxative and detergent. It is used in herpes, eczema, syphilis and scrofula; Bark is used against cancer; its paste applied for toe sores. Fruits are tonic and useful in - aphrodisiac. Seed oil is used as an anthelmintic.

Homeopathy¹⁶- *J. regia* is a homeopathic remedy. Peevishness and mental indolence, exited as if intoxicated are main mental symptoms. Lancinating pains
in forehead, flatulence and bloating of abdomen, infections of spleen and liver, diarrhea are symptoms.

**Aromatherapy** - In the ancient science of aromatherapy, walnut oil exhibits history to positively influence health and to relieve some ailments. It is used in massage therapies and can be applied in a variety of ways in our everyday lives to enhance the quality of life.

**Folk medicine** - English walnuts are used in folk medicines for cancerous conditions of breast, alimentary canal, kidneys, liver, and uterus. It is reported to be alterative, anthelmintic, astringent, bactericide, depurative, digestive, diuretic, laxative, detergent, stimulant, tonic and insecticidal. It is remedy for anthrax, asthma, backache, conjunctivitis, cough, dysentery, eczema, heartburn, inflammation, rheumatism, sore and diseases of intestine, kidney and lungs.

### 3.1.7 Previous work

Literature survey revealed presence of vital amino acids, micronutrients, phenolics, flavonoids, terpenoids, fatty acids, esters & considerable percentage of proteins, fats and carbohydrates. It also contains bioactive components which exhibit biological activities like antimicrobial, antioxidant, anthelmintic etc.

**Chemical Constituents**

The literature survey revealed that the active principle of the whole tree is Nucin or Juglone (I). The kernels contain oil, mucilage, albumin, mineral water, cellulose and water. Amino acids and vitamins like Cysteine (II), tryptophan (III), thiamin (IV), riboflavin nicotinic acid (V), pantothenic acid (VI), folic acid, vitamin B6, biotin (VII), vitamin A, ascorbic acid (VIII), are found in the leaves and fruits. Kernels yield 60-70% of a drying oil known as Walnut oil. Fresh leaves, unripe fruits and green husks contain 2.5-5% ascorbic acid (vitamin C) which can be used as a vitamin supplement. Juglone, bernerine (IX), cyclotrisjuglone, β-sitosterol (X) are isolated from bark and root while oxalic acid from the fruits. Leaves consist of cyanadin (XI), kaempferol (XII), caffeic acid (XIII), p-coumaric
acid (XIV) hyperin and quercetin (XV) \(^{21}\). The seed oil contains palmitic (XVI), stearic (XVII), oleic (XVIII), linoleic (XIX) and linolenic (XX) acids. It is reported that seed contains minerals, proteins, fats and carbohydrates\(^{22}\).

The main components extracted by hydro distillation from the leaves of Juglans species identified by GC-MS were terpenoids, aromatics, esters \(^{23}\) and flavonols\(^{24}\). The presence of Cd, Co, Cu, Zn, Pb etc was detected in the leaves\(^{25}\). The principal constituents from the leaves were tannins, flavonoids, etc\(^{26}\). Gas-Chromatography detected the presence of salicylic, p-hydroxybenzoic, vanillic (XXI), gentisic, ellagic (XXII), protocatechuic (XXIII), syringic(XXIV), p-hydroxyphenyllactic, gallic (XXV), p-coumaric, ferulic (XXVI), caffeic, sinapic (XXVII),chlorogenic acids (XXVIII) \(^{27,28}\). Steam distillate of leaves detected twenty six terpenoids and eugenol (XXIX). Twenty three hydrocarbons (C\(_{19,31}\)) and twenty one fatty acids (C\(_{6,20}\)) including geranic acid (XXX) were detected\(^{29}\).

The glycosides were obtained from kernels of Juglans species\(^{30}\). The kernel oil contains triglyceride, glycerol mono & diester, sitosterol and cyclolanosterol. The saponified kernel oil is composed of hexadecanoic, octadecanoic, octadecadienoic and octadecatrienoic acids \(^{31}\). Seed extracts were assessed for antioxidant activity \(^{32}\). Serotonin (5-HT) was measured by HPLC with electrochemical detection in seeds of walnuts\(^{33}\). The nut oils were more unsaturated from species which grow in the temperate zone and more saturated for species which grow in tropical zone\(^{34}\). The walnut shell oil contains guaiacol (XXXI), 4-methylguaiacol, 4-ethylguaiacol, 4-propylguaiacol, syringol (XXXII), 4-methylsyringol,4-ethylsyringol, and 4-propylsyringol\(^{35}\). The septa of the fruit show the presence of phenolic, carboxylic acids, & aldehydes, catechins (XXXIII), proanthocyanidins, lignin and polysaccharides\(^{36}\).

Tocopherols\(^{37}\) (XXXIV), tocotrienols\(^{38}\), cucurbic acid and its 6,7-stereoisomers\(^{39}\) along with fatty acids\(^{40}\), volatile flavor components\(^{41}\), free amino acids\(^{42}\) (XXXV) were reported. 3, 3'- bisjuglone (XXXVI) (8, 8'- dihydroxy [2, 2' – binaphthalene] – 1, 1, 4, 4' – tetrone and 1, 7, 16 – trihydroxy – 5, 6, 11, 12, 17, 18 – trinaphthalenehexone\(^{43}\), 1, 4 – Dihydroxy – 1 – tetralone, betulinic acid were
isolated from stem bark of *Juglans regia*. Structures of some of them are denoted (Chart-1).

**Chart-1**

(I)  

(II)  

(III)  

(IV)  

(V)  

(VI)  

(VII)  

(VIII)  

(IX)  

(X)  

(XI)
3.1.8 Gas Chromatography- Mass Spectrometry (GC-MS)

GC-MS is a combination of two analytical techniques, Gas Chromatography (GC) and Mass Spectrometry (MS). GC is sensitive and effective in separating components with a substantial vapour pressure at the column temperature\(^*\). GC is used in the analysis of air pollutants, alcohol in blood, essential oils and food products and for the determination of heat of solution, vaporization, vapour pressure and activity coefficients\(^*\)\(^*\)\(^*\).

MS is an analytical technique used to measure relative molecular masses\(^*\). It shows various fragments of even mass suggesting rearrangement reactions or multiple bond breaking. Odd mass ions are generally produced by single bond cleavage. All even masses are consistent with fragmentation characteristic of McLafferty Rearrangements\(^*\).

3.1.8.1 Review of Literature

Aromatic plants exert a characteristic odour due to the presence of essential oils. Myrcene (i) and limonene (ii) are alkenes found in berryberry and lime oil. Terpene oil from pine tree contains α-pine (iii) while carotenoids have Lycopene (iv)\(^*\). Angelic (v) from angelica roots, tiglic (vi) from croton oil, ricinoleic from castor oil etc are naturally occurring acids\(^*\)\(^*\)\(^*\)\(^*\).

Literature survey revealed abundance of dioctyl phthalate from marine brown algae\(^*\)\(^*\), \textit{Euphorbia pulcherima}\(^*\), \textit{Pterocarpus angolensis}\(^*\) and \textit{Sterculia guttata}\(^*\). Dibutyl phthalate was isolated from marine algae, bacteria and fungi\(^*\). Occurrence of docosanol (VII) from cuticular wax of Salix, Populus hybrid\(^*\) and Nicotiana species\(^*\), \textit{Buxus arborescence} Mill\(^*\), \textit{Paliurus spina-christi} Mill fruits\(^*\), \textit{Acanthpanax sessiliflorus}\(^*\), \textit{Eucalyptus globules} wood\(^*\), \textit{Mnotheca buxifolia} seed oil\(^*\), \textit{Peruvian serdine} oil\(^*\), \textit{Pygeum africanum} bark extracts\(^*\)\(^*\), \textit{Picia obovata}\(^*\), \textit{Alpinia speciosa} K Schumann rhizome\(^*\)\(^*\), \textit{Lithospermum erythrorhizon} Sieb. Et Zucc\(^*\), \textit{Citrullus colosynthis} peels\(^*\) and \textit{Larix gemilini} \(^*\)\(^*\) etc. was reported. Compounds were isolated from fresh kaffir lime leaves such as 1-pentene-3-ol (ix), cis-2-pentanol (x), octanol (xi), L-Linalool (xii), (-)-isopulegol\(^*\)\(^*\)\(^*\)\(^*\)\(^*\)\(^*\)\(^*\)\(^*\)\(^*\)\(^*\).
trans-geraniol etc. and 1,8-Cineol (xv), (z) 3-hexenol, 3-octanol etc. from *Salvia anatolica*.

### 3.8.1.2 Biological Significance

Lipids of queen bee and royal jelly are responsible for inhibition of ovarian development. Palmitic acid esters and unsaturated triglycerides have biological activity against flour beetle and wheat germ. Polycyclic aromatic hydrocarbons inhibit HIV-1 Integrate enzyme. Naturally occurring primary alcohols may be used to reduce heart disease and as an anti-inflammatory and anti-thrombotic agent. Higher alcohols also possess antiviral activity against *Herpes simplex* virus. The compounds possess neurotrophic properties and are useful for improving male sexual activity.

### 3.8.1.3 Reports from *J. regia*

Twenty volatiles extracted by hydro distillation of leaves were identified by GC-MS. Twenty six terpenoids and eugenol were isolated from steam distillation of leaves. Twenty three hydrocarbons (C₁₉-C₃₁) and twenty one fatty acids (C₆-C₂₀) including geranic acid were detected. The kernel oil contains triglyceride, glycerol, mono and diesters, sitosterol and cyclolanosterol. The saponified kernel oil is composed of hexadecanoic, octadecanoic, octadecadienoic and octadecatrienoic acids. Major five fatty acids as hexadecylic, stearic, oleic, linoleic and linolenic acids in seeds were reported by GC. Structures of few components are reported (Chart-2).

**Chart-2**

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(i)</td>
<td>Myrecene</td>
<td></td>
</tr>
<tr>
<td>(ii)</td>
<td>Limocene</td>
<td></td>
</tr>
<tr>
<td>(iii)</td>
<td>α-pinene</td>
<td></td>
</tr>
</tbody>
</table>
3.1.9 Present Work

The plant material was collected from local market Pune, Maharashtra, India. It was authenticated at Agharkar Research Institute, Pune, India. Its voucher specimen number is AHMA/SB-2122. The air shade dried and pulverized stem bark material was refluxed with hexane to obtain crude mass, which was subjected to GC-MS. The structures were confirmed by fragmentation patterns.

3.1.10 Results and Discussion

GC-MS examination of hexane extract denoted eleven major compounds. Their structures are identified by the percentage similarity indices from NIST and Wiley
libraries (Table 1). They are confirmed by the classical fragmentation patterns (FP). This investigation reveals the presence of following components.

Mass spectra of compounds (1&4) demonstrate typical saturated straight chain hydrocarbon with a base peak at \( m/z \ 57 \). Each peak differs from other by consecutive 14 amu intervals. The retention times, molecular ion peaks and base peaks along with genesis are denoted (Table 1, FP 1). Mass spectra of compounds (2, 6 & 8) disclose the presence of aliphatic saturated acids with a base peak at \( m/z \ 43 \) must be due to \( \gamma \)-hydrogen transfer during Mc-Lafferty rearrangement followed by loss of \([\text{OH}]^+\). The fragmentation pattern confirms homologous series, indicates straight chain acids (Table 1, FP 2). Mass spectra of compounds (3, 9 & 10) exhibit the presence of aliphatic unsaturated acids with base peak at \( m/z \ 55 \). The genesis is denoted (FP 3).

Mass spectrum of compound (7) displays the presence of an alkyl halide. The peak at retention time 18.108 suggests the occurrence of 1-iodohexadecane. The molecular ion and the base peak are observed at \( m/z \ 352 \) and \( m/z \ 57 \) respectively. The mass spectrum of compound (5) shows the presence of cyclic ester (FP 4) with a base peak at \( m/z \ 57 \). An aromatic ester (11) is seen with a base peak at \( m/z \ 149 \), which is a characteristic peak of phthalate group.

**Fragmentation Patterns (FP)**

**Hydrocarbon** (FP-1)
**Saturated Acid** (FP-2)

\[ R = \text{H}_3\text{C}-(\text{CH}_2)_{11} \]

\[ m/z = 256 \text{ amu} \]

**Unsaturated Acid** (FP-3)

\[ m/z = 254 \]

\[ m/z = 254 \]

\[ m/z = 254 \]

\[ m/z = 41 \]

\[ m/z = 55 \]

**An Ollide** (FP-4)

\[ M^* = 324, \text{OE} \]

\[ 2\ e^* \text{ transfer} \]

\[ m/z = 57, \text{OE} \]
3.1.11 Proximate Analysis

Realizing medicinal importance of the bark, proximate analysis was manifested. Extractive values were calculated for the occurrence of phytochemicals like steroids, alkaloids, tannins, proteins, starch etc. Quantitative determination of volatile matter was executed by applying loss on drying along with Karl-Fischer experiment, which allotted volatile matter (0.88%). Total ash content (5.80%), acid soluble (5%), insoluble (0.46%) and silicates (0.3277%) were observed. It is reported that *J. regia* L. stem bark is utilized as chewing sticks for cleaning teeth. At physiological pH in saliva, there is an action of water soluble components and minerals. Harmful as well as useful elements which take part in such reactions were detected quantitatively (Table 2). Natural amino acids present in the plant material interact with the calcium salts and adhere to the walls of dentine tubules and the dentine surface. This process helps teeth to get a protective layer, thus the chances of bacterial infection get reduced. The detection of such vital amino acids was accomplished using paper chromatographic technique (Table 3). This analysis was brought about for the first time.

3.1.12 Conclusion

Hydrocarbons encompass the constituents of the major fossil fuels, plastics, paraffin, waxes, solvents and oils. *J. regia* L. stem bark is a rich source of hydrocarbons, saturated / unsaturated acids, esters, alcohols and elides. The occurrence of phytochemicals and proximate analysis justifies the medicinal properties. This type of analysis is executed for the first time.

3.1.13 Experimental

Air shade dried, pulverized material (50g) was refluxed with n-hexane (250ml) for 24 hours. Solvent was recovered under reduced pressure to yield crude extract (1.6%), that was analyzed by GC-MS, which exhibited complex spectrum. The compounds (Table 1) were matched with NIST and Wiley libraries and its presence was confirmed by fragmentation patterns.
GC-MS analysis was performed using a Schimadzu QP 5050A mass spectrometer coupled with a Schimadzu 17A gas chromatograph fitted with a slit-splitless injector and DB-5 fused silica capillary column (30m x 0.25mm i. d., 0.25μm film thickness) & Helium as a carrier gas at a flow rate of 1.0 ml/min. The injection port was maintained at 250°C, split ratio 40:1 & oven temperature programming was done from 50 to 280°C at 10°C/min. It was kept at 280°C for 5 min. Interference temperature was kept at 250°C. Ionization mode was El & scanning range was 40-400 amu. Mass spectra were obtained at 0.5 sec interval.

The plant material was extracted with ethanol (80 %) to get the crude extract, which was analyzed using paper chromatography for the detection of vital amino acids. n-Butanol : Pyridine : Water was selected from attempted solvent systems.

Table 1 GC-MS Analysis of Hexane Extract

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Molecular formula</th>
<th>Retention Time (min)</th>
<th>Base Peak (amu)</th>
<th>[M]+ Ion Peak (amu)</th>
<th>Compound</th>
<th>Similarity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C_{18}H_{38}</td>
<td>16.23</td>
<td>57</td>
<td>254</td>
<td>n-octadecane</td>
<td>91</td>
</tr>
<tr>
<td>2</td>
<td>C_{16}H_{32}O_{2}</td>
<td>16.60</td>
<td>43</td>
<td>256</td>
<td>Palmitic acid (A.)</td>
<td>95</td>
</tr>
<tr>
<td>3</td>
<td>C_{16}H_{36}O_{2}</td>
<td>16.88</td>
<td>55</td>
<td>254</td>
<td>9-E-hexadecanoic acid</td>
<td>91</td>
</tr>
<tr>
<td>4</td>
<td>C_{44}H_{90}</td>
<td>17.07</td>
<td>57</td>
<td>618</td>
<td>Tetra-tetracontane</td>
<td>88</td>
</tr>
<tr>
<td>5</td>
<td>C_{21}H_{40}O_{2}</td>
<td>17.25</td>
<td>99</td>
<td>324</td>
<td>4,8,12,16-olide</td>
<td>83</td>
</tr>
<tr>
<td>6</td>
<td>C_{17}H_{34}O_{2}</td>
<td>17.58</td>
<td>43, 73</td>
<td>270</td>
<td>n-heptadecanoic A.</td>
<td>93</td>
</tr>
<tr>
<td>7</td>
<td>C_{16}H_{33}I</td>
<td>18.10</td>
<td>57</td>
<td>352</td>
<td>1-iodohexadecane</td>
<td>83</td>
</tr>
<tr>
<td>8</td>
<td>C_{16}H_{32}O_{2}</td>
<td>18.81</td>
<td>43</td>
<td>284</td>
<td>Stearic acid</td>
<td>95</td>
</tr>
<tr>
<td>9</td>
<td>C_{18}H_{34}O_{2}</td>
<td>19.17</td>
<td>55</td>
<td>282</td>
<td>Oleic acid</td>
<td>95</td>
</tr>
<tr>
<td>10</td>
<td>C_{22}H_{42}O_{2}</td>
<td>19.42</td>
<td>55</td>
<td>338</td>
<td>Erucic acid</td>
<td>92</td>
</tr>
<tr>
<td>11</td>
<td>C_{24}H_{38}O_{4}</td>
<td>19.62</td>
<td>149</td>
<td>390</td>
<td>Di-n-octyl Phthalate</td>
<td>94</td>
</tr>
</tbody>
</table>
### Table 2 Elements present in stem bark of *J. regia* L.

<table>
<thead>
<tr>
<th>Sr.No</th>
<th>ELEMENT</th>
<th>METHOD</th>
<th>QUANTITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nitrogen</td>
<td>Kjeldahl method</td>
<td>0.49%</td>
</tr>
<tr>
<td>2</td>
<td>Phosphorous</td>
<td>Colorimetric method</td>
<td>0.05%</td>
</tr>
<tr>
<td>3</td>
<td>Potassium</td>
<td>Flame photometry</td>
<td>0.10%</td>
</tr>
<tr>
<td>4</td>
<td>Calcium</td>
<td>Flame photometry</td>
<td>0.089%</td>
</tr>
<tr>
<td>5</td>
<td>Copper</td>
<td>Atomic Absorption spectroscopy</td>
<td>109ppm.</td>
</tr>
<tr>
<td>6</td>
<td>Zinc</td>
<td>Atomic Absorption spectroscopy</td>
<td>59ppm.</td>
</tr>
<tr>
<td>7</td>
<td>Iron</td>
<td>Atomic Absorption spectroscopy</td>
<td>612ppm.</td>
</tr>
<tr>
<td>8</td>
<td>Manganese</td>
<td>Atomic Absorption spectroscopy</td>
<td>67ppm.</td>
</tr>
</tbody>
</table>

### Table 3 Amino acids from *J. regia* L. Stem bark

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Rf of Extract</th>
<th>Std. Rf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>0.10</td>
<td>0.12</td>
</tr>
<tr>
<td>L-isoleucine</td>
<td>0.95</td>
<td>0.93</td>
</tr>
<tr>
<td>L-ornithinemonohydrochloride</td>
<td>0.49</td>
<td>0.48</td>
</tr>
<tr>
<td>L-tyrosine</td>
<td>0.52</td>
<td>0.50</td>
</tr>
<tr>
<td>L-histidinemono hydrochloride</td>
<td>0.80</td>
<td>0.80</td>
</tr>
<tr>
<td>DL-2-amino n-butyric acid</td>
<td>0.89</td>
<td>0.89</td>
</tr>
<tr>
<td>L-hydroxyproline</td>
<td>0.80</td>
<td>0.79</td>
</tr>
<tr>
<td>DL-phenyl alanine</td>
<td>0.80</td>
<td>0.78</td>
</tr>
<tr>
<td>L-proline</td>
<td>0.70</td>
<td>0.70</td>
</tr>
<tr>
<td>DL-tryptophan</td>
<td>0.71</td>
<td>0.71</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>0.82</td>
<td>0.83</td>
</tr>
<tr>
<td>DL-arginine monohydrochloride</td>
<td>0.81</td>
<td>0.83</td>
</tr>
</tbody>
</table>
Section II

3.2 A] Isolation, Characterization of a Bioactive Naphthaquinone

Natural products play a very important role in the field of medicine as they offer a vast chemical diversity. Naphthaquinones have a variety of applications in the field of pharmacols, cosmetols and agro ecosystems. Naphthaquinones compared to other natural compounds are very toxic to mosquito larvae. Substituted naphthaquinones and derivatives can be promising larvicidal drugs. Juglone, a naphthaquinone derivative is the important component. It possesses insecticidal, herbicidal and allelochemical properties. Traditionally, it has been used as a natural dye as ink and hair dyes. Juglone exerts its effect by inhibiting certain enzymes needed for metabolic function.

3.2.1 Review of literature

5-hydroxy-1,4-naphthalenedione (Juglone) occurs naturally in the roots, husks, bark and leaves of Juglandaceae family, particularly Juglans nigra. Juglone can be synthesized and isolated from hulls of J. regia by sublimation and from Diospyros lycioides. It may be formed from glycoside precursor of leaves and in soil beneath the tree. Juglone is involved in pathogenic defense mechanisms and developmental processes, thus behaves as a phytotoxin.

Bioactivities of juglone and related naphthaquinones are reported. It is used in folk medicine to treat ringworms and found to be potent inhibitor of Herpes Simplex Virus type-1 (HSV-1). Juglone is an inhibitor of the peptidyl-prolyl isomerase Pin1. Hyperpigmentation and contact dermatitis is observed. It is a powerful fungicide. Juglone derivatives are potential inhibitors of Trypanosoma cruzi (TcTR). Induction of quinine reductase and glutathione transferase in rat tissues by juglone and plumbagin is noted. Its presence in culture of actinomycete streptoverticillium heroshimense strain 34 is demonstrated. It inhibited the growth of oral cariogenic bacteria (S. mutans and S. sanguis) and periodontal pathogens (P. gingivalis and P. intermedia). The activity tested...
against oral bacteria gave significant results\(^9\). Aqueous extract exhibits antifungal activity\(^9\). Isomeric juglones showed positive inotropic and chronotropic action on the frog heart indicating involvement of calcium channels\(^9\). Sedative effect of juglone was studied \(^9\).

### 3.2.2 Present work

In view of bioactivity of juglone, systematic analysis is planned in order to evaluate therapeutic potential. Bio guided separation of pulverized bark material is performed. Crude extract is obtained by using suitable solvent. The potent molecule (compound 18) is achieved by executing various chromatographic techniques. Purification is effected by standard methods. Elucidation of structure is followed by its quantification and validation appraisal.

### 3.2.3 Results and Discussion

Compound 18 is isolated as yellow crystalline needles. It shows sharp melting nature at 174°C. The mass spectrum (Fig 2) of the compound displays a molecular ion peak at \( m/z \) 173 [m-1]\(^+\) which suggests the molecular formula to be C\(_{10}\)H\(_{18}\)O\(_3\). The IR spectrum (Fig 3) shows absorption bands at 3444 cm\(^{-1}\) (-O-H stretching), 1717, 1653 cm\(^{-1}\) (\(\alpha\)-\(\beta\) unsaturated ketone) 1644 cm\(^{-1}\) (double bond), 1575, 1487 cm\(^{-1}\) (aromatic stretching). UV-VIS spectrum assigns \( \lambda_{\text{max}} \) at 423 nm.

\(^1\)H NMR spectrum (Fig 4, Table 4) displays a sharp singlet at \( \delta \) 11.90 (s, 1H) for hydroxy proton (peristeric effect). A merged multiplet is seen at \( \delta \) 7.62 for H-7 and H-8 protons. Another merged multiplet is observed at \( \delta \) 6.27 for H-6 proton. A strong singlet (s, 2H) at \( \delta \) 6.95 represents olefinic H-2 and H-3 protons.

\(^13\)C NMR (Fig 5, Table 4) reveals presence of ten carbon atoms. The down field singlets at \( \delta \) 190.26 and \( \delta \) 184.19 are recognized for C-4 and C-1 carbonyl carbon atoms. A singlet at \( \delta \) 161.45 is observed for C-5 (C-OH) carbon atom. Two more singlets at \( \delta \) 132.41 and \( \delta \) 114.21 display the carbon atoms C-8a and C-4a. Doublets at \( \delta \) 138.62 and \( \delta \) 138.03 are displayed for C-2 and C-3 carbon atoms. At \( \delta \) 136.54 and \( \delta \) 124.01 two doublets represent the C-7 and C-6 carbon atoms. Another doublet at \( \delta \) 118.23 is reflected for C-8 carbon atom.
**Compound 18**

Yellow crystalline solid

Melting Point.: 174°C

Mass: $m/z$ 173 [m-1]⁺

Molecular Formula C₁₀ H₆ O₃,

UV-Vis $\lambda_{max} = 423$ nm

IR: (KBr) cm⁻¹ 3444, 1717, 1653, 1575, 1487, 1466, 1345, 1287, 1133, 709, 685

---

### Table 4 $^1$H NMR & $^{13}$C NMR (CDCl₃, 500 MHz & n125 MHz)

<table>
<thead>
<tr>
<th>Protons</th>
<th>$\delta$ (ppm) reported</th>
<th>$\delta$ (ppm) observed</th>
<th>Carbons</th>
<th>$\delta$ (ppm) reported</th>
<th>$\delta$ (ppm) observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>OH</td>
<td>11.89 (m)</td>
<td>11.90 (m)</td>
<td>C-1</td>
<td>184.9 (s)</td>
<td>184.19 (s)</td>
</tr>
<tr>
<td>H-2</td>
<td>6.93 (s)</td>
<td>6.94 (s)</td>
<td>C-2</td>
<td>138.8 (d)</td>
<td>138.62 (d)</td>
</tr>
<tr>
<td>H-8</td>
<td>7.51 (s)</td>
<td>7.59 (s)</td>
<td>C-3</td>
<td>138.8 (d)</td>
<td>138.03 (d)</td>
</tr>
<tr>
<td>H-6</td>
<td>7.24 (m)</td>
<td>7.28 (m)</td>
<td>C-4</td>
<td>189.7 (s)</td>
<td>190.26 (s)</td>
</tr>
<tr>
<td>H-3</td>
<td>6.27 (s)</td>
<td>6.77 (s)</td>
<td>C-5</td>
<td>162.3 (s)</td>
<td>161.45 (s)</td>
</tr>
<tr>
<td>H-7</td>
<td>7.61 (m)</td>
<td>7.60 (m)</td>
<td>C-6</td>
<td>124.0 (d)</td>
<td>124.01 (d)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>C-7</td>
<td>136.06 (d)</td>
<td>136.32 (d)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C-8</td>
<td>119.4 (d)</td>
<td>118.23 (d)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C-8a</td>
<td>133.2 (s)</td>
<td>132.41 (s)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C-4a</td>
<td>116.7 (d)</td>
<td>114.21 (d)</td>
</tr>
</tbody>
</table>
Table 5  Broad fractionation of Acetone Extract

<table>
<thead>
<tr>
<th>Fr. No.</th>
<th>Eluent</th>
<th>Volume collected (ml)</th>
<th>Weight of fraction (g)</th>
<th>Approximate composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hexane</td>
<td>250 X 2</td>
<td>0.268</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
<td>2</td>
<td>Hexane: Toluene (1:1)</td>
<td>250 X 4</td>
<td>0.423</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
<td>3</td>
<td>Toluene</td>
<td>250 X 3</td>
<td>0.561</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
<td>4</td>
<td>Toluene: Ethyl acetate (7.5:2.5)</td>
<td>250 X 6</td>
<td>1.278</td>
<td>Mixture of unidentified compounds + Compound 18</td>
</tr>
<tr>
<td>5</td>
<td>Toluene: Ethyl acetate (1:1)</td>
<td>250 X 3</td>
<td>1.094</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
<td>6</td>
<td>Toluene: Ethyl acetate (1:3)</td>
<td>250 X 2</td>
<td>0.234</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
<td>7</td>
<td>Ethyl acetate</td>
<td>250 X 6</td>
<td>1.239</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
<td>8</td>
<td>Ethyl acetate: Ethanol (3:1)</td>
<td>250 X 5</td>
<td>0.961</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
<td>9</td>
<td>Ethyl acetate: Ethanol (1:1)</td>
<td>250 X 4</td>
<td>0.796</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
<td>10</td>
<td>Ethanol</td>
<td>250 X 5</td>
<td>0.356</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
<td>11</td>
<td>Methanol</td>
<td>250 X 3</td>
<td>0.024</td>
<td>Mixture of unidentified compounds</td>
</tr>
</tbody>
</table>

.3.2.5 Experimental

Pulverized bark material (100 g) was extracted with acetone (500 ml) by refluxing for 18 hours. Solvent was recovered under reduced pressure to yield crude extract (9.68%). This extract (7g) was adsorbed on silica gel (10 g, 60-120) and broad fractioned by stirring using non polar to polar solvents. Total eleven broad fractions were collected. The fractions were monitored by TLC. Aluminium pre-coated TLC plate (G254) exhibited various glows at 365 nm indicated the presence of various chromophores, including isolated compound 18. The Crude
fraction, (4, 25% E A in toluene) showed presence of compound 18 along with some unidentified compounds. The details are reported (Table -5). The fraction (4, 3.8 %) containing bioactive compound 18 was purified by solvent ether and repeated recrystallization using methanol as a solvent.

Spectral data of compound 18

![Fig LC-MS spectrum](image)

![Fig FTIR spectrum](image)

![Fig $^1$H NMR spectrum](image)
3.2.6 B) Quantification of Compound 18 by HPTLC Method

In past few decades compounds from natural sources have been gaining importance because of the vast diversity. Compound 18 is a substituted naphthaquinone widely distributed in Juglans species. It possesses allelochemical properties, antifungal and antimicrobial activity. The activity of naphthaquinones against oral bacteria gave significant results. The plant is also screened for anthelmintic activity. In this view a novel analytical technique for quantification of compound 18 was developed.

3.2.6.1 Review of Literature

Formerly compound 18 has been estimated using various methods. A method for its separation from chloroform extract was developed. HPLC was used for quantitative determination of juglone in fresh leaves of *J. regia*. There are no reports on High Performance Thin Layer Chromatographic (HPTLC) quantification.

3.2.6.2 Present Work

A widely used HPTLC method has been used and attempts are made to develop simple and common solvent system for it. It is an accurate and reliable technique for quantification of compound 18 from various crude stem bark extracts.

3.2.6.3 Results and Discussion

The existence of esteemed compound 18 is detected from different extracts of stem bark material. Various compositions of the mobile phases are tested and the desired resolution is achieved by a mixture of solvents, hexane: ethyl acetate (9:1). Calibration curve of standard is obtained by plotting peak areas versus concentration applied (Fig 6). It is found to be linear in the range of 1-5μg/spot. Equation of the calibration curve is *y* = 3096*x* + 4265. The correlation coefficient is 0.976 and thus exhibits good linearity between concentration and area. Scrutiny of tested extracts explored that the acetone extract is more rich (151.0μg/gm) in this bioactive compound whereas methanol extract contains...
least amount (29.34μg/gm) of it. HPTLC quantification details are indicated (Fig 7, 8, 9, 10). The results obtained by HPTLC are in accordance with the amount collected by column chromatography.

3.2.6.4 Experimental

The isolated compound 18 was assigned to be an authenticated compound, Juglone. The compound was procured from Aldrich Chemical Company Mumbai, Maharashtra, India. This compound was referred as standard for HPTLC experiment. Quantification of this compound was achieved from different extracts of stem bark material of J. regia L. The experimental conditions are described as

Chromatographic Experimental Conditions
Stationary phase: Pre-coated silica gel plates Merck60F254
(10×10cm,0.2mm )
Mobile phase: Hexane: Ethyl acetate (9:1)
Lamp: Deuterium
Wavelength: 258 nm
Application mode: CAMAG Automatic TLC Sampler III
Development mode: CAMAG Twin Trough Chamber
Scanner: CAMAG TLC Scanner 3 and CATS software
Experimental conditions: Temperature 25±2°C, relative humidity 40%

Preparation of Standard Stock Solution and Extracts
A stock solution (1mg/ml) of standard was prepared in methanol and was further diluted with methanol for working standard solution of 0.2mg/ml.

Extract Preparation
Air shade dried, pulverized plant material was extracted for 18 hours with chloroform, ethyl acetate, acetone, ethanol and methanol to capitulate the respective crude extracts (A-E).
Calibration curve for standard

The standard solution of juglone, compound 18, (1µg to 5µg per respective spot) was applied in triplicate on TLC plate. Quantitative evaluation of the plate was performed in absorption / reflection mode at 258 nm using a slit width of 6.0 × 0.30 mm, scanning speed 20 mm/s with a computerized CAMAG TLC Scanner-3 integrated with CATS - III software. The plate was developed and scanned as per the chromatographic conditions and the peak areas were recorded.

HPTLC Quantification in Test Samples

The extracts (A-E) were used for experiment. Each test sample was diluted (1 mg/ml) with respective solvent; 20µL per spot of these solutions were applied on plates in triplicates. The plates were developed by ascending mode to a distance of 10cm and scanned as per the conditions mentioned above. The content of compound 18 from various extracts was determined by comparing the area of the chromatogram with the calibration curve of working standard. The average content of juglone in different extracts was expressed as mg/g of extract. This quantification method for bioactive molecule was performed for the first time.

![Fig 6 Calibration Curve of Standard](image6.png)

![Fig 7 HPTLC Scan of Standard](image7.png)
3.2.7 Standardization of Compound 18 by UV-VIS

UV-Vis spectroscopic method has been developed for the standardization of compound 18 from different extracts of stem bark. The method was tested and validated for various parameters according to the ICH (International Conference on Harmonization) guidelines. The study was performed for the stability of the compound under neutral conditions with respect to time. Confirmation of chromophores present in the molecule, position of functional groups was studied.

3.2.7.1 Review of Literature

Juglone is a biologically active, naturally occurring naphthaquinone derivative. Since ancient times, the compound is an active ingredient of herbal extracts used for medical treatment. In order to exploit juglone various methods have been reported in the literature for the analysis. Very few reports are available on investigation of electronic transitions. Literature survey revealed the shifts in the UV-VIS absorption of the molecules with the specific arrangement of groups.

3.2.7.2 Present Work

Simple and accurate analysis of juglone is performed using UV-vis spectrophotometer. Standard solutions of Juglone (1-10mg/ml) in methanol are employed to get the calibration curve at $\lambda_{\text{max}} = 423$ nm. The spectral shifts for juglone were studied with reagents like NaOMe, NaOAc and AlCl$_3$. The analytical
parameters such as molar extinction coefficient and conductivity were performed under the same conditions.

3.2.7.3 Results and Discussion

The standard solution displays broad bands of absorption at 694 nm, 423 nm and 244 nm of which 423 nm is selected for study. By addition of sodium acetate to test sample, the absorption maxima shifts to broad plateau region showing peaks at 477nm, 467nm causing bathochromic shift. The addition of sodium hydroxide shows largest bathochromic shift showing absorption maxima at 526nm due to $\pi \rightarrow \pi^*$ transition for carbonyl compounds. For addition of sodium methoxide larger bathochromic shift at 427 nm as it is strong base and ionizes hydroxyl group usually used to detect C-5 hydroxy group. For addition of aluminium chloride bathochromic shift at 489 nm by forming stable complex between ortho- hydroxyl group and keto function. Although addition of all reagents shows bathochromic shift, intensity of the absorption has been reduced as compared to standard causing hypochromic shift. It is maximum with aluminium chloride and minimum with sodium methoxide due to distortion in geometry.

Infrared spectra of juglone exhibited band at 3403 cm$^{-1}$ that assigned to hydroxyl group. Characteristic bands for naphthaquinone compound 1715cm$^{-1}$ and 1655 cm$^{-1}$ were assigned to the free carbonyl group stretch and the conjugated carbonyl group respectively. Band at 1475cm$^{-1}$ was probably assigned to a skeletal ring stretches. In neutral compound juglone it shows intramolecular hydrogen bonding. It is proved by $^1$HNMR where a singlet for one proton is observed at $\delta$ 11.89. As intramolecular H-bonding is always strong it is very difficult to break by solvent like intermolecular H-bonding. There is no effect on O-H frequency i.e. on the polarization of $-\text{O-H}$ bonding by addition of NaOMe or NaOAc. Addition of AlCl$_3$ indicates the stronger bond formation of $-\text{O-H}$. The bond becomes more tighter which is indicated by $-\text{O-H}$ frequency. Here more energy is required for normal polarization of $-\text{O-H}$ bond. This shows that C-1 carbonyl has formed a co-ordinate covalent bond with AlCl$_3$. Due to such bond formation C-2, C-3 conjugated double bond electron cloud gets shifted towards C-1. which affects/increases deficient character of C-4 carbonyl carbon that is
intramolecularly bonded to hydroxyl proton. Thus –O-H bond becomes tighter, it requires more energy, resulting in the higher frequency 3414 cm\(^{-1}\). The change in characteristic bands for hydroxyl and carbonyl group is shifted to higher frequency indicates complex formation.

Standard parameters, Calibration curve, Linearity, Accuracy, Stability Factor and Limit of Quantification (LOQ) are performed as per standard protocols and results are reported (Table 6, 7, 8). Standard UV-Vis spectrum of Compound 18 along with comparative absorption spectra is scanned and reported (Fig 10).

Table 6  System precision study/Stability profile (n=9)

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Conc. (µg/ml)</th>
<th>Absorbance at 422 nm at time intervals in minutes</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15</td>
<td>30</td>
<td>45</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>0.129±0.001</td>
<td>0.128±0.001</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>0.163±0.001</td>
<td>0.164±0.001</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>0.198±0.002</td>
<td>0.198±0.003</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>0.229±0.003</td>
<td>0.227±0.002</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>0.272±0.001</td>
<td>0.271±0.002</td>
</tr>
<tr>
<td>6</td>
<td>60</td>
<td>0.300±0.002</td>
<td>0.303±0.001</td>
</tr>
<tr>
<td>7</td>
<td>70</td>
<td>0.347±0.001</td>
<td>0.347±0.002</td>
</tr>
<tr>
<td>8</td>
<td>80</td>
<td>0.397±0.002</td>
<td>0.398±0.003</td>
</tr>
<tr>
<td>9</td>
<td>90</td>
<td>0.407±0.003</td>
<td>0.408±0.002</td>
</tr>
</tbody>
</table>

Table 7  \(\lambda_{\text{max}}, \epsilon, \text{m\(\Omega\)^{-1}}\) for Juglone with various reagents

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Reagents</th>
<th>Absorption Maxima ((\lambda_{\text{max}}))</th>
<th>Molar Extinction Coefficient ((\epsilon))</th>
<th>Conductivity m(\Omega)^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>i</td>
<td>Juglone (J)</td>
<td>422</td>
<td>821.35</td>
<td>0.33</td>
</tr>
<tr>
<td>ii</td>
<td>J+NaOAc</td>
<td>467</td>
<td>547.83,555.94</td>
<td>6.33</td>
</tr>
<tr>
<td>iii</td>
<td>J+NaOMe</td>
<td>526</td>
<td>368.37</td>
<td>4.85</td>
</tr>
<tr>
<td>iv</td>
<td>J+AlCl(_3)</td>
<td>489</td>
<td>290.54</td>
<td>0.99</td>
</tr>
</tbody>
</table>
Table 8 Validation Parameters by calibration curve

<table>
<thead>
<tr>
<th>Validation Parameters</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>λ max</td>
<td>422 nm</td>
</tr>
<tr>
<td>Regression equation</td>
<td>y=0.004x +0.0707</td>
</tr>
<tr>
<td>Slope</td>
<td>0.004</td>
</tr>
<tr>
<td>Y- intercept</td>
<td>0.0707</td>
</tr>
<tr>
<td>Range</td>
<td>10-100 (µg/ml)</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.989</td>
</tr>
<tr>
<td>Correlation coefficient (r²)</td>
<td>0.973</td>
</tr>
</tbody>
</table>

Fig 10 Comparative UV-vis spectra

3.2.7.5 Experimental

The compound 18 procured from Aldrich Chemical Company Mumbai, Maharashtra, India was referred as standard for UV-VIS experiment. All other chemicals and solvents used were of spectroscopic grade. UV-VIS spectra were recorded on Schimadzu UV-1700 Therma spectrophotometer. The IR Spectra (4000-3500 cm⁻¹) were recorded on Shimadzu FTIR 8400S spectrophotometer. Statistical tests were performed on validation data.
A standard solution of compound 18 in methanol (1mg in 10ml) was used as stock solution. Anhydrous Sodium acetate (5%), Aluminium chloride (5%) and Sodium methoxide solution (5%) solutions were used for this experiment.

**Determination of \( \lambda \text{ max} \)**

A solution of 10\( \mu \text{g/ml} \) was prepared from the standard solution (100\( \mu \text{g/ml} \)). The maximum absorbance was recorded by taking the care of solvent error.

**Calibration curve**

The working standards in the concentration range of 10-100 \( \mu \text{g/ml} \) were used. The absorbance was measured at 423 nm against solvent blank and calibration curve was plotted and from it validation parameters were calculated (Table 8).

**Stability Profile**

Period over which absorbance value at 423 nm of juglone in methanol remained stable was investigated using above concentrations at 15 min intervals (Table 6).

**Shifts in absorbance**

Spectral shifts for juglone were studied with reagents NaOMe, NaOAc and AlCl\(_3\). The stock solution (1mg/ml) of standard was used along with above mentioned concentrations of reagents according to standard spectral methods. The shifts in absorption with respect to \( \lambda \text{max} \) of the standard were recorded and comparative spectral peak scan for different spectral reagents were noted (Fig 10). Analytical parameters were recorded under same conditions (Table 7).

**3.2.8 Conclusion**

5- Hydroxy 1, 4- Naphthaquinone (Juglone) has been isolated from the acetone extract of stem bark. This biologically active compound is estimated quantitatively from various bark extracts by HPTLC method. The proposed method is found to be simple, rapid, accurate, precise, reliable and reproducible. The UV-Vis spectral study of juglone definitely is helpful for further method development and validation study in plant extracts of medicinal value as well as drugs. This method can be utilized for detection as well as validation of known and isolated compounds. This method is reported for the first time for this compound.
Biological Activity Assessment

3.3 Antioxidant potential

Antioxidants are vital in combating free radicals which damage human cells under 'oxidative stress' conditions that result in DNA & protein damage, lipid peroxidation, cancer, ageing and inflammatory activities. Antioxidants protect living organisms from damage caused by production of reactive oxygen species (ROS). Synthetic antioxidants butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT) have toxicological effects. Natural antioxidants are disease preventing, health-promoting and anti-ageing substances. In living cells, ROS are continuously produced during normal physiologic events and removed by antioxidant defense mechanism. Attack of ROS upon proteins causes disorders of protein functions & damage in DNA strand.

Most of the natural antioxidants comprise of phenols, flavonoids etc. Plant phenolics are potential cancer preventing agents. Efficacy of natural antioxidants is evaluated by DPPH and NO methods. DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to form a stable diamagnetic hydrazine molecule, which results in color change from violet to yellow that get recorded spectrophotometrically. Nitric oxide is generated from the decomposition of sodium nitro prusside (SNP) and measured by Greiss Reagent. At physiological pH SNP spontaneously generates NO, which interacts with oxygen to produce nitrite ions that is measured by Greiss reagent. A decrease in the NO radical concentration reduces scavenging activity.

3.3.1 Review of Literature

*J. regia* L., natural antioxidant, has long history of use in folk medicine for treatment of many diseases. *In vitro* antioxidant activity in Swiss albino male mice is reported. Antioxidant effects of seeds and leaf extracts were evaluated. Quantification of phenolics and flavonoids from stem bark by
spectrophotometric method is reported\(^{118}\). Nitric Oxide (NO) has diverse biological applications in numerous diseases\(^{119}\).

### 3.3.1.1 Present Work

*J. regia.* has applications in traditional and folk medicines. With this attention antioxidant activity is executed. Stem bark extracts are screened for their antioxidant potential by the radical scavenging assays: DPPH and Nitric Oxide method. Ascorbic acid is used as a standard. Spectrophotometric estimation of phenols and flavonoids is performed using pyrocatechol, quercetin as reference.

### 3.3.1.2 Results and Discussion

Stem bark extracts of *J. regia* L. are screened to evaluate the phenolic, flavonoid content and antioxidant activity. Total phenolic and flavonoid content is expressed as pyrocatechol and quercetin equivalents. Phenolic content is obtained from calibration curve of pyrocatechol \(y = 0.0275x - 0.0278, R^2 = 0.9915\). Flavonoid content is achieved from quercetin \(y = 0.0307x - 0.0035, R^2 = 0.9978\). The results along with IC\(_{50}\) values are depicted (Table 9).

**Table 9 Phenolics, Flavonoids and IC\(_{50}\) values from Extracts**

<table>
<thead>
<tr>
<th>Extract</th>
<th>Flavonoids (mg/g)</th>
<th>Phenolics (mg/g)</th>
<th>DPPH assay (IC(_{50}))</th>
<th>Nitric Oxide Method (IC(_{50}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>3.57</td>
<td>34.50</td>
<td>352.9</td>
<td>316.3</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>44.81</td>
<td>32.81</td>
<td>186.5</td>
<td>179.6</td>
</tr>
<tr>
<td>Acetone</td>
<td>48.69</td>
<td>35.56</td>
<td>38.4</td>
<td>41.8</td>
</tr>
<tr>
<td>Ethanol</td>
<td>36.78</td>
<td>13.59</td>
<td>187.7</td>
<td>190.4</td>
</tr>
<tr>
<td>Methanol</td>
<td>20.32</td>
<td>11.48</td>
<td>227.1</td>
<td>206.2</td>
</tr>
</tbody>
</table>

Ascorbic acid exhibited 90.16% inhibition at range of concentrations under study. Acetone extract exhibited highest radical scavenging activity than other extracts. Results indicate comparable phenol and flavonoids content of ethyl acetate and acetone extracts. Antioxidant assays exhibit active nature of acetone extract. This may be due to the free phenolic hydroxy function present in it (Fig11, 12).
3.3.1.3 Conclusion

All extracts exhibit high range of radical scavenging activity. This proves that stem bark is rich in flavonoids and phenolics, which may be responsible for the antioxidant activities. Above results strongly support the antioxidant potential of *J. regia* L and its importance as a rich source of natural antioxidants.
3.3.1.4 Experimental

DPPH, sulphanilamide, naphthyl ethylenediamine dihydrochloride were obtained from Sigma Chemicals Co., USA. All other chemicals and reagents were of analytical grade. UV-VIS1700Pharma Spectrophotometer Schimadzu was used.

Sample Preparation

Ethyl acetate, acetone, ethanol, methanol and distilled water extractswere prepared using Air shade dried material. Solvents were recovered under reduced pressure to get extracts. Quath was prepared by conventional method.

Determination of Total Phenolics and Flavonoids

Total phenolics were determined by Malik and Singh method\(^{36}\). Phenol content was expressed as mg pyrocatechol equivalent of phenol per g of extract. The aluminium chloride method was used for determination of flavonoid content and expressed as mg quercetin equivalent of flavonoid per g of extract\(^{37}\). Experiments were performed in triplicate and results recorded as mean ± SEM (Table 9).

DPPH radical scavenging activity

Antioxidant potential of extracts is executed by spectroscopic method\(^{38}\). Aliquots of extracts were tested by standard protocol. Methanol served as blank and methanolic DPPH as control. Absorbance was noted at 517 nm.

Nitric Oxide scavenging activity

The absorbance of chromophores was measured at 546 nm\(^{39}\). Methanol served as blank. Test solutions were incubated at RT (27°C) for 90 minutes. This incubated solution (1.5 ml) was added to 1.5 ml of Greiss Reagent.

In both methods Ascorbic acid was used as reference compound.

The capacity of scavenging free radicals was calculated as follows:

\[
\text{Scavenging activity (\%)} = \{(\text{Control Abs.} - \text{Sample Abs.})/\text{Control Abs.}\} \times 100
\]
3.3.2 Antimicrobial activity

One of the major achievements of modern medicine has been the development of new, effective antimicrobials for the treatment of infectious diseases. Antibiotics that possess capability of ending a bacterial infection are of two types; bactericidal and bacteriostatic. There is a need of new antimicrobial components due to rapid emergence of multidrug resistant pathogens and explosive dreadful infectious diseases. Taking into account these facts several medicinal plants are being evaluated for possible antimicrobial screening and many of them exhibit significant levels of antibacterial activity.

3.3.2.1 Review of Literature

Recent reviews indicate that there is a great potential to find compounds leading to the production of new antibiotics from plant sources. J. regia L. is found all around the world, either as a wild or cultivated tree. This species, from Himalayan regions in India, is used in folk medicines to treat various diseases including cancer. Decoction of the stem bark is useful in dental complaints. The stem bark extracts are promising source of compounds with antibacterial activity. Antifungal properties of J. regia extracts against oral Candida strains have been reported. Studies on antimicrobial activity of J. regia bark extracts exhibited positive results on S. aureus, S. mutans, E. coli, P. aeruginosa and Candida albicans. There are three methods generally employed for microbial sensitivity assay as, Paper disc, Agar ditch and Turbidometric analysis.

3.3.2.2 Present Work

Air shade dried & pulverized material is refluxed with ethanol (80%) and it is fractioned using n-Hexane to n-Butanol. The fractions (i-iv) are tested against Staphylococcus aureus (ATCC6538P), Pseudomonas aeruginosa (ATCC10145) and Escherichia coli (ATCC11230). Ethyl acetate fraction (iii) exhibits more activity than other fractions and most susceptible strain is S. aureus (ATCC6538P). Minimum Inhibitory Concentration (MIC) of ethyl acetate fraction
is determined against *S. aureus* and further antimicrobial activity guided separation of EtOAc fraction is performed. Streptomycin is used as standard.

### 3.3.2.3 Results and Discussion

This is the first report showing antibacterial activity of bio guided separation of Ethanol extract. Ethanolic extract (80%) is fractionated using solvents of different polarities. Maximum percent yield (21.49%) is obtained in ethyl acetate. Antimicrobial assays denote a variable clear zone for each strain. Hexane fraction exhibits activity only against *S. aureus*. Other fractions display distinct zones for all strains. Ethyl acetate fraction is the most active against gram positive & gram negative strains. Results are presented (Table 10).

It is found that among different bacterial strains *Staphylococcus aureus* is most susceptible towards all extracts and ethyl acetate extract is the most active. Effect of various concentrations of this extract on *S. aureus* is studied. MIC results are depicted (Fig 13). It is noted to be 5μg/ml and the activity increases with increasing concentration. To isolate the bioactive component from ethyl acetate fraction further separation is performed to get sub fractions (A-E). Antibacterial activity assay of each sub fraction is executed using same concentrations and experimental conditions. Results are displayed (Table 11).

<table>
<thead>
<tr>
<th>Extract</th>
<th>Microorganisms and Zones of Inhibition (mm)*</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>S. aureus</em></td>
<td><em>P. aeruginosa</em></td>
<td><em>E. coli</em></td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>10</td>
<td>12</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Hexane</td>
<td>13</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Chloroform</td>
<td>15</td>
<td>12</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>19</td>
<td>15</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Standard</td>
<td>26</td>
<td>21</td>
<td>21</td>
<td></td>
</tr>
</tbody>
</table>

*Zone of inhibition including the diameter of well (5mm)
Table 11 Antibacterial Activity of Fractions (A-E)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition (mm)</td>
<td>--</td>
<td>--</td>
<td>11mm</td>
<td>12mm</td>
<td>--</td>
</tr>
</tbody>
</table>

Fig 13 Results for MIC of Ethyl acetate Extract

3.3.2.4 Conclusion

It is observed that fraction D of ethyl acetate is highly active as compared to other fractions against S. aureus. This activity is only due to bioactive and/or antioxidant compound/s present in this fraction. This study supports use of natural products as medicines because active biomolecules in plant extracts exhibit antibacterial activity to a considerable extent.

3.3.2.5 Experimental

Preparation of Extracts

The air shade dried and pulverized material of stem bark (50g) was refluxed with ethanol (80%, 150 ml) for 24 hrs. The extract was filtered and solvent was recovered under reduced pressure to obtain crude sticky mass (21.34%). This crude mass was broad fractioned using n-Hexane to n-Butanol. These fractions were examined for antimicrobial activity. Potent ethyl acetate extract (2.364g) was sub fractioned using silica gel (15g, 60-120) to get sub fractions (A-E).
Screening for antimicrobial activities

On the basis of pathogenic importance and literature survey following test microorganisms were selected as *Staphylococcus aureus* (ATCC6538P), *Pseudomonas aeruginosa* (ATCC10145), *Escherichia coli* (ATCC11230).

Overnight cultures of microorganisms were adjusted to ca.10 \(^6\)cfu/ml. according to McFarland turbidity standards and spread over the appropriate media (Mueller-Hinton agar Difco Laboratories, MI, USA) for bacteria in petri dishes. Wells of 5 mm. diameters were punched into the agar medium and filled with 0.5µl of plant extract. The plates were incubated at 37\(^\circ\) C for 18-48h and the inhibition zones around the wells were measured. The antibacterial activity results were calculated as a mean of three replicates. The MIC was defined as the lowest concentration that showed no growth. At the end of the period inhibition zones formed on the medium were evaluated in mm. All tests were performed under sterile conditions. Streptomycin was used as a standard.

### 3.3.3 Dental Relevance

It is well known and world-wide accepted that oral cavity is the mirror of total body health. Advancement in biological and engineering research is bringing a medical revolution to dentistry. Among several dental problems, caries process (cavitation) is deadly serious and chronic too. Different causative agents are associated with dental caries. Chlorhexidine, sanguinarine, metronidazole, phenolic antiseptics and ampicillin are the effective antibiotics currently used in the prevention of dental caries. Most of them are known to exhibit serious adverse reactions leading to systemic toxicity and other undesirable effects. At the same time there is a threat of developing resistance to these drugs by microorganisms. It becomes necessary to redesign & replace these drugs\(^\text{128}\). Plants are natural source of antibacterial agents.

### 3.3.3.1 Review of literature

Giants of dental research W. D. Miller, G. V. Black put forward Chemicoparasitic theory (1889) stating pivotal role of oral microorganisms(bacteria) present in the mouth and their interaction with the retained food particles to produce
substances capable of dissolving enamel\textsuperscript{129}. It was established that mutans group of Streptococci are the key agents causing dental caries\textsuperscript{130}. \textit{Streptococcus mutans, S. salivarius, S. mitis, S. oralis} are most actively involved in dental caries. Now-a-days extensive efforts have been made to develop anticariogenic compounds that can be incorporated into dental products\textsuperscript{131}. There is a continuous need of new antimicrobial components due to rapid emergence of multidrug-resistant pathogens and explosive infectious diseases. There is a great potential to find compounds leading to the production of new antibiotics from plant source\textsuperscript{132}. Literature suggests that \textit{J. regia} L. can definitely be the remedy for dental caries. The juice of green husks, boiled with honey, is a good gargle for sore mouth and inflamed throat. A piece of green husks put into a hollow tooth, eases pain. Decoction of stem bark is useful in dental complaints\textsuperscript{17}. The species is utilized in treatment of tuberculosis and tuberculosis of cervical glands\textsuperscript{34}. \textit{S. mutans} is non-motile and grows optimally in a range of 18-40\textdegree{}C. The ability of \textit{S. mutans} to survive in acid is one reason that this species is the main driver of dental caries and tooth decay.

### 3.3.3.2 Present Work

\textit{In-vitro} clinical studies are performed on stem bark extracts of \textit{J. regia}. against the microbes present in the saliva samples of patients suffering from dental caries. In Ayurvedia \textit{J. regia} L. is reported to have potent activity for dental complaints. Present study deals with evaluation of the effect of its acetone and aqueous extracts. The efficacy of extracts is assessed against total microflora by testing on salivary samples of patients suffering from dental caries and disc diffusion method is followed. Potency of extracts and the compound \textbf{18} is tested against \textit{S. mutans}, a gram positive facultative anaerobic bacterium found in human oral cavity.

### 3.3.3.3 Results and Discussion

All micro organisms present in the mouth cannot singly produce dental caries. These organisms have specific ability to adhere to tooth surface and to each other and results in formation of matrix and cause dental caries. Hence, the total microflora existing in saliva of patients suffering from dental caries are tested.
The results of the antimicrobial assay of the aqueous and acetone extracts of *J. regia* are presented (Table 12). Acetone extract has significant inhibitory effect on the growth of microorganisms. The degree of inhibition remains constant after 72 hours. The efficacy of extracts on salivary flora is displayed (Fig 15).

A concentration of 250 μg/disc is found to inhibit the growth of most of the test samples of saliva. The inhibition of salivary microbial flora in this study has confirmed the potency of both extracts. Compound 18 exhibits profound amount of antibacterial potential against *S. mutans* as compared to the acetone extract. It is depicted graphically (Fig 14) and blood agar plates are displayed (Fig 16).

### Table 12 Antimicrobial Assay of Aqueous and Acetone extracts

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Concentration (μg)</th>
<th>Average zone of Inhibition (mm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Aqueous Extract</td>
</tr>
<tr>
<td>1</td>
<td>150</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>200</td>
<td>1.6</td>
</tr>
<tr>
<td>3</td>
<td>250</td>
<td>1.76</td>
</tr>
<tr>
<td>4</td>
<td>300</td>
<td>1.3</td>
</tr>
<tr>
<td>5</td>
<td>Control</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* Zones of inhibition are represented excluding the diameter of disc

**Fig 14 Potency of Acetone Extract & Compound 18 against S. mutans**
3.3.3.4 Conclusion

Compound 18 of acetone extract was found to be more effective than acetone extract against the oral micro flora, thus supporting its folklore application as a preventive remedy for various microbial diseases of hard tissues in oral cavity.

3.3.3.5 Experimental

Air shade dried, powdered bark material (10g) was extracted using acetone and distilled water (50 ml) for 24 hours at room temperature. Solvents were collected under reduced pressure to obtain crude extracts (14.4% and 18%).

Criteria for selection of patients

Patients in mixed dentition period in age group of 6–12 years were selected. They had good general health with no history of antibiotic therapy and use of chemical anti plaque agents prior to six months of study initiation. The saliva samples were placed in sterile container with saline (2 ml) and used to inoculate.

Anti-Microbial assay

Paper disc diffusion method was employed. Each extracts (30mg) was dissolved in respective solvent (1ml). Sterile filter paper discs (5mm) were impregnated with extracts of concentrations ranging from 100 μg to 400 μg per disc.
The salivary flora were inoculated on nutrient broth and incubated for 24 hours at 37 ± 0.1 °C. Adequate amount of Muller Hinton Agar were dispensed into sterile plates and allowed to solidify under aseptic conditions. Test samples of saliva (0.1ml) were inoculated with a sterile spreader on the surface of solid medium in plates. Agar plates inoculated with these test samples were incubated for one hour. Sterile discs impregnated with extracts were placed on agar plates and incubated at 37 ± 0.1°C for 48 hours. After incubation, zones of inhibition were measured in mm. All tests were performed under sterile conditions.

In case of antimicrobial assay against S. mutans blood agar plates were utilized instead of Agar plates and protocol for well diffusion method was followed. Chlorhexidine and S-flo were used as positive controls.

### 3.3.4 Anthelmintic Activity

Among the most common infections of digestive system in human beings are helminth infections. Helminthiasis is one of the major prevalent diseases in the world, particularly in tropical countries. Parasites observed in India are roundworms, hookworms, thread worms, tapeworms, guinea worms and filarial worms. They cause loss of blood, nutritional deficiencies, urticaria etc. The parasites can be acquired by contact with infected water, meal and animal. Filarial worms are transmitted via blood sucking mosquitoes. Anthelmintics possess the property of ridding the body off parasitic worms. An ideal anthelmintic must have a wide margin between its toxicity to the worm and its toxic effect on the host. The drug must be effective in one dose. Ayurveda provides many herbal preparations to overcome alimentary canal infections with negligible side effects. J. regia is traditionally used as an anthelmintic.

#### 3.3.4.1 Review of literature

Anthelmintic drugs can be classified according to their chemical structure as well as to their action against the specific type of helminthes. As per WHO, only few drugs are frequently used in treatment of these parasite infections. Stem bark of J. regia is reported to be powerful anthelmintic. Tannins accomplish
anthelmintic activity\textsuperscript{136, 137}. Tannins bind to free protein in gastrointestinal tract of host animal or glycoprotein on cuticle of parasite and cause death\textsuperscript{138}.

### 3.3.4.2 Present study

Extracts of \textit{J. regia} stem bark are tested against \textit{Eicinia foetida} as test worms. Bioassay involved determination of time of paralysis and time of death control. Albendazole is included as standard reference and normal saline as control.

### 3.3.4.3 Results and Discussion

\textit{J. regia} stem bark is powerful anthelmintic and acetone extract displays profound activity than other extracts. The rate of paralysis and death is dose dependent. Phytochemical screening of the crude extracts revealed presence of flavonoid and polyphenolic components. It is possible that tannins present in extracts interfered with energy generation in helminth parasites by uncoupling oxidative phosphorylation. Results of anthelmintic activity are presented (Table 13). Potent anthelmintic activity of various extracts under investigation is viewed (Fig 17).

### 3.3.4.4 Conclusion

All extracts of \textit{J. regia} stem bark display excellent anthelmintic capacity as compared to the standard, Albendazol. Acetone extract exhibits highest activity. An active component from this extract may be responsible for this effect.

### 3.3.4.5 Experimental

Albendazole, normal saline were purchased from authorized pharmaceuticals. Solvents and other chemicals used were of analytical grade. Pulverized plant material was extracted with ethyl acetate, acetone, ethanol and methanol and screened for activity. Indian \textit{Eicinia foetida} was collected from Mahatma Phule Agri. University, Pune, India. All earthworms were nearly of equal size (14 cm).

**Anthelmintic Assay**

The anthelmintic assay was carried out as per the method of Nargund\textsuperscript{76} with minor modifications. \textit{Eicinia foetida} was selected due to its anatomical and physiological resemblance with the intestinal round worm parasite of human...
being\textsuperscript{77,78}. Different dilutions of Albendazole with normal saline were used as standard. Same dilutions of extracts in normal saline solution were used for the assay and normal saline served as control. The time taken for complete paralysis and death was recorded. External stimuli were applied to ascertain the paralysis time. The time taken by worm to become motionless was considered as paralysis time and lethal time was ascertained by death of motionless worm followed by fading away of their body color.

Table 13 Anthelmintic Activity of \textit{J. regia} extracts

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Test Sample</th>
<th>Concentration (mg/ml)</th>
<th>Paralysis Time (min)</th>
<th>Death Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>2</td>
<td>Albendazole (Standard)</td>
<td>10</td>
<td>147 ±0.192</td>
<td>230 ±0.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>136 ±0.189</td>
<td>205 ±0.213</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>115 ± 0.19</td>
<td>152 ± 0.22</td>
</tr>
<tr>
<td>3</td>
<td>Ethanol extract</td>
<td>10</td>
<td>135 ± 0.16</td>
<td>223 ± 0.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>115 ± 0.161</td>
<td>190 ± 0.145</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>96 ± 0.16</td>
<td>120 ± 0.141</td>
</tr>
<tr>
<td>4</td>
<td>Ethyl acetate extract</td>
<td>10</td>
<td>125 ± 0.181</td>
<td>192 ±0.249</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>99 ± 0.178</td>
<td>163 ± 0.243</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>50 ± 0.179</td>
<td>94 ± 0.24</td>
</tr>
<tr>
<td>5</td>
<td>Acetone extract</td>
<td>10</td>
<td>52 ±0.202</td>
<td>114 ±0.142</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>30 ±0.208</td>
<td>70 ±0.143</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>21 ±0.201</td>
<td>30 ±0.142</td>
</tr>
<tr>
<td>6</td>
<td>Methanol extract</td>
<td>10</td>
<td>100 ± 0.137</td>
<td>133 ± 0.177</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>85 ± 0.125</td>
<td>107 ± 0.175</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>42 ± 0.127</td>
<td>65 ± 0.171</td>
</tr>
</tbody>
</table>

P< 0.05 when compared to control. Values are expressed as mean ±SEM.

Fig 17 Effect of Test extract of \textit{J. regia} on paralysis time
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CHAPTER 4

Isolation and Characterization of Bioactive Molecules from *Mimusops elengi*

**Section I**

Isolation, Characterization of an Aromatic Ester and Quantification by HPTLC study

**Section II**

Isolation and characterization of an Alkene and an Aromatic Aldehyde

Publications-

1] "Isolation and Quantification of a Bioactive Molecule from Medicinal Plant – *Mimusops elengi* L." communicated to Journal of Pharmacy Research.

4.1 Introduction

Mimusops is a genus distributed in tropics of the Old World, North and Peninsular India and Andaman Islands. One of the species *M. elengi* L., is found in India. The tree attains large dimensions in moist evergreen forests of Western Ghats of Maharashtra, India. In Andaman, climate favors its height to 35 meters. It is planted as an avenue, shade tree and ornament in many parts of India\(^1\)\(^2\). It is a potent medicinal plant having historic importance. All parts of the plant (Panchang) have been reported for their medicinal uses in Ayurvedic system of medicine. From ancient times, the bark material has been used as a preventive medicine for oral gum diseases and as chewing sticks. The medicinally useful oil is extracted from the seeds.

4.2 Botanical Characterization

*M. elengi* L. belongs to family Sapotaceae. It is an evergreen tree growing up to 15 m height with dark grey fissured bark and densely spreading crown. The trunk is short and erect. Leaves are oblong, glabrous and leathery with wavy margins. Flowers are white, fragrant, axillary, solitary or fascicled. Fruits are ovoid or ellipsoid berries. Seeds are ovoid, compressed, grayish brown and shiny\(^3\).

4.3 Biological Implications

Literature survey revealed that all parts of the plant are used for treatment of various diseases. The flowers, fruits and bark are acrid, astringent, cooling and anthelmintic\(^3\). In Ayurveda, preparation of Mimusops “Bakuladya Taila”, is applied on gums and teeth for strengthening. Leaves are used as an antidote for snakebite\(^4\). Nutritive value of leaves has been evaluated\(^5\). Study of refined seed oil was compared with peanut oil in rats\(^6\). Flowers are expectorant and smoked in asthma, dried flowers are a brain tonic, a snuff to relieve cephalgia\(^3\). A lotion from unripe fruits and flowers is used for smearing on sores and wounds\(^1\). Unripe fruit is masticator and fixes loose teeth. Pulp of ripe fruit is antidysenteric\(^3\). Seeds are useful suppositories in constipation in children. Saponins from seeds are spermicidal and spasmylytic\(^1\).
Stem bark is used as a gargle for odontopathy, inflammation and bleeding of gums. Tender stems are used as tooth brushes\(^2\). It is applied as antipyretic, analgesic, tonic and febrifuge and helpful in urethrorrhoea, cystorrhoea, diarrhoea and dysentery\(^3\). Anthelmintic activity of bark was reported\(^7\). Bark and seed coat are used for strengthening the gums. “Vajradanti”, one of the famous Indian tooth powder is composed of it. Tree is lopped for medium quality fodder. Wood is used for building purpose, piles, bridges, boats, furniture, panels, cabinet work, musical instruments, walking sticks etc.\(^2,8\). Flowers are constituents of cosmetic formulations. Seed kernel oil is used for edible and lightning purposes\(^2\). The bark is used as dye\(^8,9\). Plant is potential source for hydrocarbons & phytochemicals\(^10\).

4.4 Shloka

*Mimusops elengi* L. having high medicinal value is described in Ayurveda as,

कुलस्तुवरोनुष्णः कटुपाकसो मुरः।
करपितंहस्यश्च च भ्रमित्वाण्डापहः।
महुर्जा कक्षाबुज स्निष्ठं संग्राहि या कुलम्।
स्थिरेकरुजः दन्तायां विशदं फलमुच्यते। – (भावप्रकाश)

**Meaning-** Bitter juice of Bakul is good for soothing effect, helps in cough, pitta, poisoning, worms, leprosy and dental diseases. This sweet and astringent juice prevents loose motions. Fruit reduces sensitivity of teeth and strengthens gums.

4.5 Classification\(^3\)

**Kingdom:** Plantae  
**Division:** Magnoliophyta  
**Class:** Magnoliopsida  
**Family:** Sapotaceae  
**Genus:** *Mimusops*  
**Species:** *elengi* L

4.6 Common names \(^3\)

**Bengali:** Bakul  
**Hindi:** Bakul, Maulsiri  
**English:** Bulletwood  
**Assam:** Gokul  

**Gujarathi** Barsoli  
**Oriya:** Bokulo, Baula  
**Telugu:** Pogada  
**Tamil:** Vagulam
4.7 Review of Literature

Preliminary screening as quantitative estimation of elements, presence of amino acids & carbohydrates and various phytochemicals was manifested. Reports revealed the estimation of energy content (337.38 Kcal/100g), quantitative detection of elements (potassium, calcium, magnesium, zinc, copper etc.), steam volatile matter (0.18%), qualitative exposition of amino acids (glycine, tryptophan, proline, lysine, alanine and methionine) and carbohydrates (maltose, xylose, fructose, arabinose and dextrose)\(^1\). Literature survey prompted to investigate the bark material.

4.7.1 Chemical constituents

Chemical investigations of plant revealed that it contains free triterpenoids\(^1\). Fruits and seeds were found to contain quercitol (I), ursolic acid (II), triterpene alcohol, glucose (III), dihydroquercitin (IV), \(\beta\)-D-glucoside of \(\beta\)-sitosterol (V)\(^1\). The leaves, heartwood and root were found to contain hentriacontane (VI), \(\beta\)-carotene (VII), lupeol (VIII), \(\alpha\)-spinasterol (IX), hederagenin (X), triterpenic acids, quercitol, glucose etc\(^1\). An epimer of chondrillasterol\(^1\) and 3-O-\(\beta\)-D-galactopyranoside of the sterol - (24R)-stigmasta-7,22(E)-dien-3\(\beta\)-ol (XI)\(^1\), 3\(\beta\)-hydroxy-lup-20(29)-ene-23,28-dioic acid (XII), \(\beta\)-amyrin (XIII), lupeol, \(\alpha\)-taraxerol (XIV), ursolic acid\(^1\) and its derivatives\(^1\) were reported. Bioactive 3\(\beta\), 6\(\beta\), 19\(\alpha\), 23-tetrahydroxy-urs-12-ene (XV) and 1\(\beta\)-hydroxy-3\(\beta\)-hexanoyllup-20(29)-ene-23, 28-dioic acid (XVI) were mentioned\(^1\).

An antileishmanial property of bassic acid (XVII) was carried out\(^2\). A steroidal saponin was reported from the roots\(^2\). Seventy four compounds with D-mannitol (XVIII), \(\beta\)-sitosterol and \(\beta\) - sitosterol-D-glucoside were isolated from the flowers\(^2\). The genomic study for leaf protein content was reported\(^2\). Pentacyclic triterpenic acids, mimusopic acid (XIX) and mimusopsic acid (XX) were isolated\(^2\). Mimusopic acid exhibited anti - HIV reverse transcriptase activity. Saponins showed antifungal nature against some human pathogens\(^2\). Seed kernel oil is composed of palmitic stearic, behenic, oleic, linoleic\(^2\), 9-keto-octadec-15-enoic (XXI) myristic\(^2\) and erucic acid (XXII) is from the seed oil\(^2\).
The seed oil was found to contain hydrocarbons, wax esters, triacyl glycerols, free fatty acids, 1,3-diacylglycerols, 1,2-diacylglycerols, alcohols, sterols, 2-monoacylglycerols, and 1-monoacylglycerols. Unsaponifiable lipid constituents and triterpenoid saponins were isolated. Mimusopside A and B were isolated with taxifolin (XXIII) as α-spinasterol glucoside.

Seeds contain a mixture of triterpenoid glycosides, Mimusopin, Mimusin. Elengin and pyranosyl glycosides with various sugar moieties had been isolated. Mimusic acid, 2β, 3β, 16α, 23-tetrahydroxyoleana-5,13(18)-dien-28-oicacid, ketones as mimusopgenone (XXIV) and mimugenone (XXV) were isolated from the seeds. Spasmolytic activity for triterpenoid saponins was found to be higher than a steroid moiety. Flavone glycosides had strong antibacterial activity.

Bark was found to contain α-spinasterol (XXXVI), taraxerone (XXXVII), taraxerol (XXXVIII), sodium salt of ursolic acid (XXXIX) and bitulinic acid with fatty acid ester of α-spinasterol, β-D-glucoside of β-sitosterol and quercitol. An alkaloid isolated from the bark consists of a tiglate ester of base having a mass spectrum identical with that of laburnine an isoretronecanol.

Literature survey of various bioactive glycosidic molecules present in the seeds and bark denotes the significance of the bark material in view of investigative research plan. Some isolated bioactive components are reported (Chart-1).

**Chart – 1**
(XXII)  

(XXIII)  

(XXIV)  

(XXV)  

(XXVI)  

(XXVII)  

(XXVIII)
Section I

Isolation, Characterization of an Aromatic Ester and Quantification by HPTLC study

4.8.1 Phthalates

Phthalates are widely used as additives in many plastics and consumer products like food wraps, adhesives, perfumes and cosmetics. Some of them bis-(2-ethylhexyl) phthalate (DOP), diethyl phthalate, diisobutyl phthalate, dibutyl phthalate, n-dioctyl phthalate etc are summarized (Chart 2). Dibutyl phthalate, a secondary metabolite from Mimusops elengi is isolated and characterized. R.N.Roy et. al. reported it’s strong bioactive nature against bacteria as well as unicellular and filamentous fungi. It possesses selective deterioration of leukemic cells with less harmful effect on growth of normal hemopoietic progenitors. Phthalates show numerous biological and enzymatic activities. Prolonged administration of DOP affects liver by increasing inhepatic enzymes. Phthalate regulations by U.S. government & European authorities are released as scientific evidence strongly suggests that risks to humans are low.

4.8.2 Review of Literature

Various phthalates, as secondary metabolites, are reported from soil bacteria, marine algae, bacteria, fungi and plants. Inhibition of melanogenesis by dioctyl phthalate isolated from Nigella glandulifera Freyn was studied. DOP is reported from aerial parts, fruits and roots of Nigerian Laportea aestuans L., Dracaena cochinensis, Erhesia laevis, and Sterculia guttata. Phthalates were investigated from vegetable species. Occurrence of phthalates in untreated and treated waste water is reported. Development and validation of methods for trace determination of phthalates in sludge and vegetables is tried. A non acidic catalyst QS-6 is developed and used to produce DOP. Genotoxic activity and the chronic effects of DOP on biochemical composition, survival and reproduction of Daphnia magna were indicated.
4.8.3 Present work

Air shade dried powdered bark material of *M. elengi* L is extracted with soxhlet extractor using different solvents like hexane, chloroform, ethanol and methanol for 18 hours. The solvent is recovered under reduced pressure to get respective extracts. Chromatographic separation of the components from hexane extract had been carried out. The structure of the isolated compound is elucidated by
modern spectral analysis. It is compared with reported data. It is purified by petroleum ether. The isolation of dioctyl phthalate (Compound 19) has been reported for the first time from the stem bark of *M. elengi*.

![Compound 19](image)

[1, 2-Benzenedicarboxylic acid, bis (2’-ethyl hexyl ) ester]

**Compound 19**

Colorless transparent liquid,

Molecular formula: C$_{24}$H$_{38}$O$_4$

Boiling Point: > 280°C

IR: 1728, 1600, 1579, 1464, 1285, 1124, 1074, 972, 752 cm$^{-1}$

LC-MS: m/z 391 [m+1]$^+$

### 4.8.4 Results and Discussion

The compound isolated is a colorless transparent liquid. The mass spectrum (Fig 1) of the compound exhibits a molecular ion peak at m/z 391 [m+1]$^+$, which suggests molecular formula to be C$_{24}$H$_{38}$O$_4$. Fragmentation pattern (FP1) is in agreement with the structure. The base peak at m/z 149 (100%), which is a characteristic of phthalic acid and its esters, arising as a result of the operation of the ortho effect and the peak at m/z 57 (57%) supporting the proposed branched chain structure of Compound 19.
IR spectrum (Fig 2) shows characteristic bands at 1714 cm\(^{-1}\) (ester carbonyl group), 1589 cm\(^{-1}\) (aryl moiety), 1286, 1132, 1074 cm\(^{-1}\) (-C-O- stretching) and 752 cm\(^{-1}\) (ortho-disubstituted benzene ring).

\(^1\)H-NMR (Fig 3, Table 1) shows existence of two sets of aromatic protons in the molecule suggesting that the compound must have an ortho-disubstituted benzene ring bearing the same substituents. Downfield multiplets for aromatic protons are detected at \(\delta\) 7.71 and \(\delta\) 7.51 for [H-4, H-5] and [H-3, H-6] respectively. The doublet is depicted at \(\delta\) 4.21 [d, J = 8 Hz, H-1" and H-1', 4H] for aceloxy - methylene protons. A multiplet at \(\delta\) 1.63 belongs to two methine protons [H- 2" and H- 2']. Methylene envelop is assigned at \(\delta\) 1.31 for [m, H3", H-3', H-4", H-4', H- 5", H- 5', H-7" and H-7'] protons. The resonance at \(\delta\) 0.91 shows triplet for [t, J = 8 Hz, H- 8", H- 8', 6H] and at \(\delta\) 0.88 [t, J = 8 Hz] for H- 6", H- 6'] for methyl protons. It indicates molecule possesses two sets of different types of methyl protons.

\(^{13}\)C-NMR spectrum (Fig 4, Table 1) shows presence of twelve signals due to twenty four carbon atoms. DEPT spectrum (Fig 5) depicts multiplicities of carbon signals. Compound is composed of six methine, ten methylene, four methyl carbon atoms, which confirms presence of four quaternary carbon atoms. Signal appears at \(\delta\) 168.01 (s) for ester carbonyl groups, tetra substituted carbon atoms detected at \(\delta\) 132.67 (s) for C1 & C6. A downfield multiplet at \(\delta\) 68.38 reveals attachment of ester ethereal oxygen atom [m, C 1"& C1']. Upfield peaks indicate at \(\delta\) 14.30 (q) and \(\delta\) 11.19 (q) for C8" & C8' and C6" & C6' for methyl carbons.

The COSY spectrum (Fig 6, Table 1) specifies upfield merged triplets at \(\delta\) 0.88 and \(\delta\) 0.91 (t, J = 6Hz, 6H) of H-6", H-6' and H-8", H-8' show connectivity at \(\delta\) 1.31 to H-5", H-5' (m, 4H) and H-7", H-7' (m, 4H). The spectrum indicates correlation between H-1", H-1' at \(\delta\) 4.21 (m, 4H) and H- 2", H -2' at \(\delta\) 1.63 (m, 2H). The spectrum demonstrates correlation between aromatic H-4, H-5 protons at \(\delta\) 7.71 with H-3, H-6 aromatic protons (dd, J=4, 8 Hz, 2H). It displays similar correlation of H-3, H-6 at \(\delta\) 7.51 with H-4 and H-5 protons (dd, J=4, 8 Hz, 2H).
From HMBC spectrum (Fig 8) a long range correlation of atoms is observed. The correlation of H-4, H-5 to carbons C4 & C5 at δ 131.12, δ 129.03 is noted. Similarly H-3, H-6 indicate correlation to carbons C3 & C6 at δ 129.03 and δ 131.12 and the carbonyl group at δ 168.01 is exhibited. The protons present at H-1", H-1' show correlation with the carbonyl group at δ 168.01.

4.8.5 Conclusion

The strong bioactive nature of the molecule against gram positive and gram negative bacteria as well as unicellular and filamentous fungi is observed. It is also found to be an α-glucosidase inhibitor. It has many other applications in cosmetics, cellulose plastics, food wraps, adhesives, perfumes etc. A bioactive molecule, dioctyl phthalate has been isolated for the first time from this plant.

4.8.6 Experimental

Air shade dried and pulverized bark material (300 g) was subjected to soxhlet extraction using different solvents like hexane, chloroform, ethanol and methanol for eighteen hours at their refluxing temperatures. The solvents were recovered under reduced pressure to get the respective crude masses. The number of components in each crude mass was detected by performing TLC in respective mobile phases. Hexane extract was showing presence of a prominent, intense spot along with other spots. Efforts were made for its isolation.

Hexane extract (1.36%, 4.07 g) was further purified. Broad fractionation of this crude hexane extract (4.0 g) was accomplished using gradient polarity solvents on silica gel (60-120, 160 g) to get total eleven fractions (1-11). Fractions were monitored by thin layer chromatography. The details are noted (Table 3). Fraction 1 (0.685 g) was purified by passing through silica gel column using petroleum ether as a solvent. The pure component (compound 19) was obtained as a liquid (0.89%).
<table>
<thead>
<tr>
<th>No.</th>
<th>Atom No.</th>
<th>$^1$HNMR (ppm, 400Hz)</th>
<th>$^{13}$CNMR (ppm, 100Hz)</th>
<th>COSY</th>
<th>HETCOR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1, 2</td>
<td>-</td>
<td>132.67 (s)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>4, 5</td>
<td>7.71</td>
<td>129.03 (d)</td>
<td>H-</td>
<td>129.03</td>
</tr>
<tr>
<td>3</td>
<td>3, 6</td>
<td>7.51</td>
<td>131.12 (d)</td>
<td>H-4, H-5</td>
<td>131.12</td>
</tr>
<tr>
<td>4</td>
<td>Ester Carbonyl</td>
<td>-</td>
<td>168.01 (s)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>1', 1&quot;</td>
<td>4.21</td>
<td>68.38 (t)</td>
<td>H-2', H-2&quot;</td>
<td>68.38</td>
</tr>
<tr>
<td>6</td>
<td>2', 2&quot;</td>
<td>1.63</td>
<td>36.94 (d)</td>
<td>H-1', H-1&quot;</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>3', 3&quot;</td>
<td>1.40</td>
<td>23.96 (t)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>4', 4&quot;</td>
<td>1.24</td>
<td>29.14 (t)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>5', 5&quot;</td>
<td>1.31</td>
<td>23.21 (t)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>6', 6&quot;</td>
<td>0.88</td>
<td>14.30 (q)</td>
<td>H-5', H-5&quot;</td>
<td>11.19</td>
</tr>
<tr>
<td>11</td>
<td>7', 7&quot;</td>
<td>1.40</td>
<td>30.57 (t)</td>
<td>-</td>
<td>36.94</td>
</tr>
<tr>
<td>12</td>
<td>8', 8&quot;</td>
<td>0.91</td>
<td>11.19 (q)</td>
<td>H-7', H-7'</td>
<td>14.30</td>
</tr>
</tbody>
</table>
FP 1

1. \[ m/z \ 391 = [M+1]^+ \]
   OE m/z 390

2. \[ \text{2e}^- \text{ transfer} \rightarrow \leftarrow \]
   EE m/z 113

3. \[ \text{2e}^- \text{ transfer} \rightarrow \]
   OE m/z 278

4. \[ \text{EE m/z 149 Base Peak} \]

5. \[ \text{OE m/z 166} \]

6. \[ \text{2e}^- \text{ transfer} \rightarrow \]
   EE m/z 83

7. \[ \text{EE m/z 83} \]
   \[ \text{2e}^- \text{ transfer} \rightarrow \]
   EE m/z 57
<table>
<thead>
<tr>
<th>Fr. No.</th>
<th>Eluent</th>
<th>Volume (ml)</th>
<th>Weight (g)</th>
<th>Approximate Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pet. Ether</td>
<td>250x2</td>
<td>0.685</td>
<td>Mixture of unidentified compounds + Compound 19</td>
</tr>
<tr>
<td>2</td>
<td>Hexane</td>
<td>250x3</td>
<td>0.395</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
<td>3</td>
<td>Hexane:Toluene (3:1)</td>
<td>250x3</td>
<td>1.891</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
<td>4</td>
<td>Hexane:Toluene (1:1)</td>
<td>250x3</td>
<td>0.582</td>
<td>Mixture of unidentified compounds</td>
</tr>
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<td>5</td>
<td>Toluene</td>
<td>250x3</td>
<td>0.320</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
<td>6</td>
<td>Toluene:Ethyl acetate (3:1)</td>
<td>250x3</td>
<td>0.285</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
<td>7</td>
<td>Toluene:Ethyl acetate (1:1)</td>
<td>250x3</td>
<td>1.775</td>
<td>Mixture of unidentified compounds</td>
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<tr>
<td>8</td>
<td>Ethyl acetate</td>
<td>250x3</td>
<td>0.095</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
<td>9</td>
<td>EtOAc:Ethanol (1:1)</td>
<td>250x3</td>
<td>0.336</td>
<td>Mixture of unidentified compounds</td>
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<tr>
<td>10</td>
<td>Ethanol</td>
<td>300</td>
<td>0.085</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
<td>11</td>
<td>Methanol</td>
<td>200</td>
<td>0.040</td>
<td>Mixture of unidentified compounds</td>
</tr>
</tbody>
</table>
Spectral data of compound 19

Fig 1 Mass spectrum

Fig 2 FTIR spectrum

Fig 3 $^1$HNMR spectrum
Fig 4 $^{13}$CNMR spectrum

Fig 5 DEPT

Fig 6 COSY Spectrum
Fig 7 HSQC

Fig 8 HMBC

HMBC Correlation
4.9 Quantification of DOP by HPTLC

DOP, isolated from a medicinal plant *Mimusops elengi* L. can be standardized by a simple, accurate, sensitive, precise & reproducible HPTLC method. The existence of this esteemed compound is detected from different extracts of stem bark material. Scrutiny of tested extracts explored that the hexane extract is more rich (155.46μg/gm) in this bioactive compound whereas toluene extract contains least amount (71.53μg/gm) of it.

4.9.1 Present work

Different compositions of the mobile phases are tested and the desired resolution is achieved by a mixture of solvents, hexane: ethyl acetate (9:1). Spectral characteristics of peaks of standard and that of extracts are compared for identification of compound 19. The calibration curve of standard is obtained by plotting peak areas versus concentration applied (Fig 10). It is found to be linear in the range of 1-5 μg/spot. Equation of the calibration curve is $y = 1210x + 3033$. Correlation coefficient is 0.999 and exhibits good linearity between the concentration and the area.

4.9.2 Results and Discussion

HPTLC scan for standard (Fig 9) and comparative peaks of different extracts with standard are displayed (Fig 11). The results obtained by HPTLC are in accordance with the amount collected by column chromatography

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Extract</th>
<th>Quantity (μg/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Toluene</td>
<td>71.53</td>
</tr>
<tr>
<td>2</td>
<td>Hex(sox)</td>
<td>155.46</td>
</tr>
<tr>
<td>3</td>
<td>Hexane</td>
<td>125.36</td>
</tr>
<tr>
<td>4</td>
<td>P.E.+ Hex</td>
<td>106.84</td>
</tr>
<tr>
<td>5</td>
<td>Pet. Ether</td>
<td>122.18</td>
</tr>
</tbody>
</table>

Table 4 Quantity of DOP in extracts of *M. elengi*
Fig 9 HPTLC Scan of standard

Fig 10 Calibration curve for standard

Fig 11 Comparative study of the extracts

Fig 12 HPTLC Quantification of Test Samples
4.9.3 Experimental

The isolated and purified compound was found to be an authenticated compound, DOP. The compound was procured from Aldrich Chemical Company Mumbai, Maharashtra, India. This compound was referred as standard for HPTLC experiment. Quantification of this compound was achieved from different extracts of stem bark material of *M. elengi*.

**Chromatographic Experimental Conditions**

Stationary phase: Pre-coated silica gel plates Merck60F254 (10×10cm, 0.2mm)

Mobile phase: Hexane: Ethyl acetate (9:1)

Lamp: Deuterium

Wavelength: 258nm

Application mode: CAMAG Automatic TLC Sampler III

Development mode: CAMAG Twin Trough Chamber

Scanner: CAMAG TLC Scanner 3 and CATS software

Experimental conditions: Temperature 25±2°C, relative humidity 40%

**Preparation of Standard Stock Solution**

A stock solution of DOP was prepared by dissolving 1mg/1ml in chloroform. It was diluted with chloroform for working standard solution of 0.2mg/ml.

**Extracts Preparation**

Air shade dried and pulverized plant material extracted with solvents hexane, chloroform, ethyl acetate, acetone, ethanol and methanol to capitulate crude extracts of respective solvents. Extractions were carried out by stirring for 18 hours at RT. Solvents were recovered under reduced pressure to obtain their crude extracts for High Performance Thin Layer Chromatography experiment.
Calibration curve for standard

Standard solution of DOP, (1μg to 5μg per respective spot) was applied in triplicate on TLC plate. Quantitative evaluation of the plate was performed in absorption / reflection mode at 258 nm using a slit width of 6.0 × 0.30 mm, scanning speed 20 mm/s with a computerized CAMAG TLC Scanner-3 integrated with CATS - III software. The plate was developed and scanned as per the chromatographic conditions and the peak areas were recorded.

HPTLC Quantification in Test Samples

The prepared extracts (A-E) were used for experiment. Each test sample of various extracts was accurately weighed (10mg) and dissolved in respective solvent to make up the volume of 10ml. This was further diluted to get the solutions of 1mg/ml; 20μL per spot of these solutions were applied on to pre-coated aluminium silica gel plates in triplicates.

The plates were developed by ascending mode to a distance of 10cm and scanned as per the conditions mentioned above. The content of compound 19 from various extracts was determined by comparing the area of the chromatogram with the calibration curve of working standard. Rf value of the standard was compared with the Rf values of extracts. Average content of DOP in different extracts was expressed as μg/g of extract. This quantification method for bioactive molecule was performed for the first time using this plant material.

4.9.4 Conclusion

A bioactive molecule, dioctyl phthalate has been isolated for the first time from this plant. It is a α–glucosidase inhibitor. The proposed method is simple, rapid, accurate, precise, reliable and reproducible for the quantification of DOP present in stem bark extracts of *Mimusops elengi* L. Hexane extract exhibits maximum content of this bioactive molecule.
Section II

Isolation and characterization of an Alkene and an Aromatic Aldehyde (Compounds 20 & 21)

4.10.1 Natural Alkenes

The saturated and unsaturated hydrocarbons are found mainly in petroleum and living organisms. Shall C (1892) identified undecane and eicosane in ants\(^6\) and Bryonia dioica\(^7\). They are straight chain or branched. Allenic hydrocarbons, such as 9,10-tricosadiene(i), 9,10-pentacosadiene(ii), and 9,10-heptacosadiene(iii) were isolated from melolonthine scarab beetles - Australian insects \(^7\). HDL and LDL are the significant olefins, which participate in metabolic reactions in human being and are correlated to edible oils containing unsaturated moieties.

4.10.2 Review of Literature

Several microalgae were shown to contain long-chain unsaturated alkenes from 19 to 38 carbon atoms and one to four double bonds\(^7\). They are also abundant at the outer surface of insects and several marine organisms. They are thought to serve as a barrier to water influx in the organism, to act as ant-aphrodisiacs and to affect the absorption of chemicals and microorganisms. Wild populations of Drosophila melanogaster use several cuticular hydrocarbons (mainly 7,11-heptacosene as sexual pheromone\(^7\)). In some species, mono- or di-unsaturated chains were reported. Several alkenes of 8 or 11 carbon atoms with 3 - 4 double bonds play a role in algae gamete attraction (pheromones) - cystophorene(iv), finavarrene(v) and fucoserratene(vi). Laboratory experiments have demonstrated that alkanes and alkenes appear during hydro peroxide decomposition by Fischer-Tropsch-type reactions\(^7\). These results support oxidative cleavage by peroxidase which proves theory of the origin of life in hydro-thermal systems. Isoprenoid components of marine sediments are reported\(^7\). One important member of isoprenoid polyenes is squalene (C\(_{30}\)H\(_{50}\),vii) which is metabolic precursor of steroids and triterpenoids. Squalene
is found in epicuticular wax of grapefruit, wheat, olive oil and shark liver oils. It is a component of sebaceous lipids (12-15% of sebum weight) found on human skin. It consists of isoprene units composed of 6 trans double bonds. It was discovered in shark oil\textsuperscript{79}. It was suggested that squalene and its peroxidized derivatives protect from sunburn skin\textsuperscript{80}. Squalene reduces colon\textsuperscript{81} and skin cancer\textsuperscript{82} due to its antioxidant effect\textsuperscript{83}. It has been shown that presqualene diphosphate, intermediate between farnesyl diphosphate and squalene, carries biological activity in human neutrophiles and serves as negative intracellular signal preventing superoxide anion generation\textsuperscript{84} and inhibition of phosphatidylinositol 3-kinase\textsuperscript{85}. Some important alkenes are reported (Chart 3).

**Chart 3**

(i) \[
\text{H}_3\text{C}-(\text{CH}_2)_6\text{C}=\text{(CH}_2)_{10}\text{CH}_3
\]

(ii) \[
\text{H}_3\text{C}-(\text{CH}_2)_7\text{C}=\text{(CH}_2)_{13}\text{CH}_3
\]

(iii) \[
\text{H}_3\text{C}-(\text{CH}_2)_7\text{C}=\text{(CH}_2)_{15}\text{CH}_3
\]

(iv) \[
\text{CH}_3
\]

(v) \[
\text{CH}_3
\]

(vi) \[

\]

(vii) \[

\]

**4.10.3 Present Work**

Air shade dried powdered bark material of *M. elengi* L is extracted in a soxhlet extractor using hexane, chloroform, ethanol and methanol for 18 hours. The solvent was recovered under reduced pressure to get respective extracts. Chromatographic separation of the components from chloroform extract was
performed. It was purified by petroleum ether. Structure of isolated compound was elucidated by spectral analysis. The alkene, Tetracos-1-ene, has been isolated for the first time.

\[
\text{H}_3\text{C}-\text{CH}_2-\text{(CH}_2\text{)}_{20}-\text{CH} \equiv \text{C} - \text{H} \\
\text{Ha} \quad \text{Hb}
\]

**Compound 20** (Tetracos-1-ene)

**Compound 20**

- Colorless transparent liquid,
- Molecular formula \( \text{C}_{24}\text{H}_{48} \).
- Boiling Point \( > 280^\circ\text{C} \)
- IR: 2924 cm\(^{-1}\), 2852 cm\(^{-1}\), 1645 cm\(^{-1}\), 1377 cm\(^{-1}\), 1307 cm\(^{-1}\), 751 cm\(^{-1}\)
- Mass: \( m/z 337 \text{ [m+1]}^+ \).

### 4.10.4 Results and Discussion

Compound 20 is isolated from chloroform extract using soxhlet extractor. It is a colorless transparent liquid. Mass spectrum (Fig 13) exhibits a molecular ion peak at \( m/z 337 \text{ [m+1]}^+ \) which suggests molecular formula to be \( \text{C}_{24}\text{H}_{48} \).

IR spectrum (Fig 14) displays a characteristic absorption frequency at 1645 cm\(^{-1}\) for double bond, absence of other functional groups suggests that the compound is an unsaturated hydrocarbon.

\(^1\text{H} \text{NMR spectrum (Fig 15, Table 5)}\) displays a multiplet at \( \delta 5.84 \text{ (m, 1H)} \) for \( \text{H}-2 \) methine olefinic proton. A multiplet at \( \delta 5.04 \) and \( \delta 4.95 \) is noticed for terminal olefin \( \text{Ha} \) and \( \text{Hb} \) protons. A downfield multiplet at \( \delta 2.05 \) is observed for \( \text{H}-3 \) methylene protons (m, 2H) adjacent to olefinic carbon atom. A strong methylene envelop is appeared at \( \delta 1.28 \) for \( \text{H}-4 \) to \( \text{H}-23 \) methylene protons (m, 40H). A strong triplet is displayed at \( \delta 0.90 \text{ (t, } J = 0.8 \text{ Hz, 3H)} \) for \( \text{H}-24 \) methyl protons.
\(^{13}\)CNMR (Fig 16, Table 5) reveals presence of total twenty four carbon atoms. The downfield doublets are recognized at \(\delta 139.30\) and \(\delta 114.08\) for C-2 and C-1 olefinic carbon atoms. A triplet at \(\delta 33.84\) is noted for C-3 methylene carbon adjacent to olefinic methane carbon atom. An upfield triplet is displayed at \(\delta 31.94\) for C-22 carbon atom. A strong triplet ranging from \(\delta 29.38\) to \(\delta 29.71\) is observed for (C-4 to C-21) methylene carbon atoms. An upfield triplet is seen for C-23 carbon methylene carbon atom at \(\delta 22.70\). A quartet is appeared at \(\delta 14.14\) for C-24 methyl carbon atom.

4.10.5 Conclusion

Bioactivity and other applications of the plant parts may be due to presence of this alkene which is the metabolic precursor of steroids. A straight chain hydrocarbon, the terminal olefin has been isolated for the first time from stem bark of this plant material.

4.10.6 Experimental

Air shade dried powdered bark material of \textit{M. elengi} was extracted in a soxhlet extractor with hexane, chloroform, ethanol and methanol for 18 hours. The solvent was recovered under reduced pressure to get respective extracts. Bioactive chloroform extract (1.33\%) was further purified. Broad fractionation of this extract (4.0 g) was accomplished using gradient polarity of solvents on silica gel (60-120, 160 g) to get ten fractions. Fractions were monitored by TLC. The details are noted (Table 6). Fractions 3 and 4 (1.2 g) were mixed and put for rechromatography by column using performed using gradient polarity of solvents on silica gel (60-120, 160 g) and total nine fractions (A to I) were collected by monitoring with TLC. The details are depicted (Table 7). Fraction D (125 mg) was fractioned using hexane: ethyl acetate solvent systems over silica gel (60-120, 50 g) to obtain four major fractions (D-I to D-IV) and yielded an impure compound 20. Details are presented (Table 8). Fractions D-II and D-III were mixed and purified by preparative TLC. The pure compound, Tetracos-1-ene, was obtained (0.64\%) as a liquid.
<table>
<thead>
<tr>
<th>Fr. No.</th>
<th>Eluent</th>
<th>Volume (ml)</th>
<th>Weight (g)</th>
<th>Approximate Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hexane</td>
<td>250x2</td>
<td>0.025</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
<td>2</td>
<td>Hexane:Toluene (1:1)</td>
<td>250x2</td>
<td>0.035</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
<td>3</td>
<td>Toluene</td>
<td>250x6</td>
<td>0.029</td>
<td>Mixture of unidentified compounds + <strong>Compound 20</strong></td>
</tr>
<tr>
<td>4</td>
<td>Toluene:Ethyl acetate (3:1)</td>
<td>250x8</td>
<td>1.189</td>
<td>Mixture of unidentified compounds + <strong>Compound 20</strong></td>
</tr>
<tr>
<td>5</td>
<td>Toluene:Ethyl acetate (3:1)</td>
<td>250x6</td>
<td>0.073</td>
<td>Mixture of unidentified compounds</td>
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<tr>
<td>6</td>
<td>Toluene:Ethyl acetate (1:1)</td>
<td>250x2</td>
<td>0.367</td>
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<tr>
<td>7</td>
<td>Toluene:Ethyl acetate (1:3)</td>
<td>250x2</td>
<td>0.042</td>
<td>Mixture of unidentified compounds</td>
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<tr>
<td>8</td>
<td>Ethyl acetate</td>
<td>250x2</td>
<td>0.383</td>
<td>Mixture of unidentified compounds</td>
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<tr>
<td>9</td>
<td>Ethyl acetate:Ethanol (1:1)</td>
<td>250x2</td>
<td>0.012</td>
<td>Mixture of unidentified compounds</td>
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<tr>
<td>10</td>
<td>Ethanol</td>
<td>250x2</td>
<td>0.153</td>
<td>Mixture of unidentified compounds</td>
</tr>
</tbody>
</table>
Table 7  Rechromatography of Fractions (3+4)

<table>
<thead>
<tr>
<th>Fr. No.</th>
<th>Eluent</th>
<th>Volume (ml)</th>
<th>Weight (mg)</th>
<th>Approximate Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Toluene</td>
<td>100x19</td>
<td>141</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
<td>B</td>
<td>Toluene:Ethyl acetate (9:1)</td>
<td>100x2</td>
<td>180</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
<td>C</td>
<td>Toluene:Ethyl acetate (9:1)</td>
<td>100x13</td>
<td>317</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
<td>D</td>
<td>Toluene:Ethyl acetate (4:1)</td>
<td>100x8</td>
<td>125</td>
<td>Mixture of unidentified compounds + Compd 20</td>
</tr>
<tr>
<td>E</td>
<td>Toluene:Ethyl acetate (7:3)</td>
<td>100x6</td>
<td>154</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
<td>F</td>
<td>Toluene:Ethyl acetate (1:1)</td>
<td>100x6</td>
<td>70</td>
<td>Mixture of unidentified compounds</td>
</tr>
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<td>G</td>
<td>Toluene:Ethanol (4:1)</td>
<td>100x4</td>
<td>10</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
<td>H</td>
<td>Toluene:Ethanol (1:1)</td>
<td>100x2</td>
<td>10</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
<td>I</td>
<td>Ethanol</td>
<td>100x2</td>
<td>7</td>
<td>Mixture of unidentified compounds</td>
</tr>
</tbody>
</table>

Table 8  Rechromatography of Fraction D

<table>
<thead>
<tr>
<th>Fr. No.</th>
<th>Eluent</th>
<th>Vol.(ml)</th>
<th>Wt. (mg)</th>
<th>Approximate Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-I</td>
<td>Hexane</td>
<td>100x8</td>
<td>8</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
<td>D-II</td>
<td>Hex : EtOAc (9.5:0.5)</td>
<td>100x10</td>
<td>70</td>
<td>Mixture of unidentified compounds + Compd 20</td>
</tr>
<tr>
<td>D-III</td>
<td>Hex : EtOAc(9 :1)</td>
<td>100x10</td>
<td>37</td>
<td>Mixture of unidentified compounds + Compd 20</td>
</tr>
<tr>
<td>D-IV</td>
<td>Hex : EtOAc(8.5:1.5)</td>
<td>100x6</td>
<td>27</td>
<td>Mixture of unidentified compounds</td>
</tr>
</tbody>
</table>
Table 5  \(^1\)HNMR & \(^{13}\)C-NMR of Compound 20 (CDCl\(_3\), 500 MHz & 125 MHz)

<table>
<thead>
<tr>
<th>Proton</th>
<th>Chemical Shifts δ (ppm)</th>
<th>Carbon</th>
<th>Chemical Shifts δ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H(_A)-1</td>
<td>5.04 (m)</td>
<td>C-2</td>
<td>139.3 (d)</td>
</tr>
<tr>
<td>H-2</td>
<td>5.85 (m)</td>
<td>C-1</td>
<td>114.08 (t)</td>
</tr>
<tr>
<td>H(_B)-1</td>
<td>4.95 (m)</td>
<td>C-3</td>
<td>33.84 (t)</td>
</tr>
<tr>
<td>H-3</td>
<td>2.05 (m)</td>
<td>C-22</td>
<td>31.94 (t)</td>
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<td>H-4 -20</td>
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<td>H-23</td>
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<td>C-23</td>
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<td>H-24</td>
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<td>C-24</td>
<td>14.14 (q)</td>
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</table>

Spectral data of compound 20

![Spectral data of compound 20](image1)

Fig 13 Mass Spectrum

![Fig 13 Mass Spectrum](image2)

Fig 14 FTIR spectrum

195
4.11 An Aromatic aldehyde

Vanillins

Vanillin is a phenolic aldehyde with molecular formula C₉H₆O₃. It is the primary component of vanilla bean, roasted coffee and Chinese red pine. Ethyl vanillin is used as a flavoring agent in foods, beverages, and pharmaceuticals. First commercial synthesis of vanillin started with natural compound eugenol. Artificial vanillin is made from either guaiacol or from lignin, a constituent of wood.

4.10.1 Review of Literature

Vanillin was first isolated from vanilla extract in 1858 by Nicolas-Theodore Gobley. Synthesis of vanillin from isoeugenol was reported. Vanillin was derived from eugenol found in clove oil and from petrochemical precursors. Biosynthetically, vanillin was prepared from rice bran. It flavors foodstuffs as
olive oil, butter, raspberry and lychee fruits. Aging in oak imparts vanillin to wines and spirits. Green seed pods of *Vanilla planifola* contain vanillin in the form of its β-D-glycoside. Isolation of isovanillin from Aromatic Roots of the Medicinal African Liame, *Mondia whitei* is disclosed. A benzenoid from stem of *Acanthopanax senticosus* is quoted.

Vanillin has been used as a chemical intermediate in the production of pharmaceuticals and other fine chemicals. Vanillin is an effective antimicrobial agent in fruit purees. Mode of antimicrobial action of vanillin against *Saccharomyces cerevisiae*, *Zygosaccharomyces rouxii*, *Debaryomyces hansenii* and *Z. bailii*, *Escherichia coli*, *Lactobacillus plantarum* and *Listeria innocua* is studied. Its antifungal properties are established.

### 4.10.2 Present Work

Air shed dried and pulverized plant material was refluxed in acetone for 12 hours. Solvent was collected under reduced pressure to furnish a bioactive sticky mass. In this view bio guided separation of the crude mass was performed by broad fractionation using non polar to polar solvents by increasing gradient polarity. Seven fractions are collected (i-vii, Table 10) which were subjected to antimicrobial activity. The active fraction (iii) was rechromatographed on silica gel to provide seven fractions (A-G, Table 11). Fractions were monitored by TLC. Fractions C and D were mixed and purified by repeated crystallization using mixed solvent system (Hex: EtOAc) to capitulate white crystalline solid, 3-methoxy, 4-hydroxy benzaldehyde, (Compound 21).

![Compound 21 (Vanillin)](image)
Compound 21

White crystalline solid,
Molecular formula: $\text{CaH}_8\text{O}_3$
Melting Point: $82^\circ\text{C}$ (reported $80-82^\circ\text{C}$)
IR: $3460 \text{ cm}^{-1}, 3374 \text{ cm}^{-1}, 2947 \text{ cm}^{-1}, 2728 \text{ cm}^{-1}, 1715 \text{ cm}^{-1}, 1605 \text{ cm}^{-1}, 1549 \text{ cm}^{-1}, 1512 \text{ cm}^{-1}, 1462 \text{ cm}^{-1}, 1377 \text{ cm}^{-1}, 1215 \text{ cm}^{-1}, 1022 \text{ cm}^{-1}$ and $860 \text{ cm}^{-1}$.
Mass: $m/z 153 \ [m+1]^+$.

4.10.3 Results and Discussion

Compound 21 is isolated from acetone extract of stem bark. It is in the form of white shining, needle-shaped crystals. It shows sharp melting nature at $82^\circ\text{C}$, which matches with the authentic sample. It shows $98\%$ purity by Gas chromatogram (Fig 17). The GC-MS (Fig 18) exhibits $95\%$ Similarity index factor for the occurrence of 4-hydroxy, 3-methoxy benzaldehyde (vanillin, compound 21). The mass spectrum of the compound demonstrates molecular ion peak at 152 amu (77\%) and $m/z 153 \ [M+1]^+$ (97\%). A characteristic base peak at $m/z 151[(M-1)^+ (100\%)]$ indicates the presence of an aldehydic group. Fragmentation pattern (FP 2) is in agreement with the structure and suggests molecular formula to be $\text{CaH}_8\text{O}_3$

IR spectrum (Fig 19) displays a characteristic absorption frequency at 3460, 3374 cm$^{-1}$ (-O-H stretching). A peak is noted at 2728 cm$^{-1}$ for aldehydic C-H stretching of (H-C=O) group. Conjugated aldehydic carbonyl frequency is recorded at 1715 cm$^{-1}$. Frequencies at 1605 cm$^{-1}$, 1549 cm$^{-1}$ and 1462 cm$^{-1}$ detect presence of an aromatic ring. Presence of methyl group is noted by frequency at $1377 \text{ cm}^{-1}$. Frequencies at $1215 \text{ cm}^{-1}$ and $1022 \text{ cm}^{-1}$ are detected for (C-O-C), etherial group. All frequencies are well matched with authentic sample.

$^1$H NMR spectrum (Fig 20, Table 10) displays the most downfield sharp and strong singlet for aldehydic proton at $\delta 11.90$. Chemical shift at $\delta 7.62$ represent aromatic protons (H-2 and H-6). Aromatic proton H-5 is assigned at $\delta 7.05$. A peak is designated at $\delta 3.09$ for methoxy methyl protons.
$^{13}$CNMR (Fig 21, Table 10) spectrum exhibits a characteristic downfield singlet at δ 190.29 for aldehydic carbonyl carbon. A singlet is appeared at δ 161.40 for C-4 carbon atom possessing hydroxy group. A strong singlet is noticed at δ 139.57 due to C-3 aromatic carbon having methoxy group. A singlet is seen at δ 138.65 for aromatic carbon carrying aldehydic group. Three aromatic doublets are displayed at δ 136.58, δ 124.50 and δ 119.17 for C-5, C-2 and C-6 respectively. An upfield quartet is recognized at δ 54.50 for methoxy methyl carbon atom.

**Spectral data calculations by Density Functional Theory**

The structure of the esteemed compound is examined by the use of HF(6-31G* level), density functional theory DFT (6-31 G* level) and hybrid functional B3LYP. Theoretical calculations were carried out with Gaussian 09 program using the DFT (B3LYP/ 6-31 G* level) and RHF (6-31 G*) levels of theory to predict the molecular structure and wave numbers in IR region. $^1$H NMR and $^{13}$C NMR chemical shifts are calculated by employing geometry optimization. The values of bond angles and bond lengths obtained from this computer programme are given (Table 9). A restricted Hartree Fock SCF calculation was performed using Pilay DIIS+ Geometrize Direct Minimization. The wave number values computed theoretically contain known systematic error due to the negligence of electron correlation. Therefore, the scaling factor as 0.90 is set for HF (6-31 G*) calculation of IR wave numbers. Using the optimized structure of the molecule IR, $^1$HNMR, $^{13}$CNMR data is calculated and compared with experimental data. It shows good relation between theoretically calculated IR wave numbers and observed values for IR data. The data for wave numbers and their assignments are denoted (Table 10). The chemical shifts are in good comparison for $^1$HNMR & $^{13}$CNMR spectra also. Aldehydic proton shows downfield shift by DFT calculations, which is in agreement with experimental and reported data. Aromatic protons H-7 & H-8, ortho to aldehydic, function show downfield shifts. Aromatic proton H-7 is most downfield due to disturbed planarity of ortho substituted methoxy group, where O-Me group indicates that $-I > +M$ effect. Meta substituted H-9 proton is observed with same chemical shifts like
ortho-substituted. Chemical shift of hydroxy group depends on concentration, temperature and solvent used, which indicates a little deviation in theoretical and experimental value. Same is the reason for difference of values for H-19. The details are denoted (Table 11).

Table 9 Parameters from DFT (B3LYP/6-31 G* level) for compound 21

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<th>Atom</th>
<th>Length</th>
<th>Angle</th>
<th>Dihedral</th>
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<th>Y</th>
<th>Z</th>
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Table 11  Chemical Shifts of $^1$HNMR and $^{13}$C NMR in CDCl$_3$

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<th>Exp. (ppm)</th>
<th>Reported (ppm)</th>
<th>Atom</th>
<th>Calc. (ppm)</th>
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<td>3.49</td>
<td>3.09</td>
<td>C-4</td>
<td>122.90</td>
<td>138.65</td>
<td>130.5</td>
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<td>C-6</td>
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<td>119.17</td>
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<td>54.5</td>
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</table>

FP 2

OE 152

[Diagram]

OE 152 $\rightarrow^\text{OE 152}^\text{OE 152}$

EE 137

EE 137 $\rightarrow^{2e\text{ transfer}}{\text{EE 109}}$

EE 53

EE 53 $\rightarrow{\text{EE 81}}$

EE 81

202
4.10.4 Experimental

Air shade dried, pulverized plant material (50g) was refluxed in acetone (150ml) for 12 hours. Solvent was collected under reduced pressure to furnish a bioactive sticky mass. Bio guided separation of the crude mass (7g) was performed. Broad fractionation was achieved using nonpolar hexane to polar methanol solvents. Seven fractions were collected (1-7, Table 10) and tested for their bioactivity. The active fraction (3, 450mg) was rechromatographed on silica gel (20 g, 60-120) to provide nine sub-fractions (A-I, Table 11). Fractions were monitored by TLC and bioactivity was examined. Bioactive fractions C and D were mixed and purified by repeated crystallization using mixed solvent system of hexane: ethyl acetate to capitulate white crystalline solid (0.29%), vanillin, compound 21. Structure was confirmed by modern spectral analysis. The experimental spectral data was compared with the theoretically calculated values obtained from the computerized programme ‘Gaussian 09’ DFT(B3LYP/6-31G* and RHF (6-31G*) levels of theory. The verification of spectral data using computational chemistry is performed for the first time for compound 21, isolated from natural source.

**Table 10  Broad Fractionation of Acetone Extract**

<table>
<thead>
<tr>
<th>Fr. No.</th>
<th>Eluent</th>
<th>Volume (ml)</th>
<th>Weight (g)</th>
<th>Approximate Composition</th>
</tr>
</thead>
<tbody>
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<td>Hexane</td>
<td>250x2</td>
<td>0.215</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
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<td>Toluene</td>
<td>250x6</td>
<td>0.968</td>
<td>Mixture of unidentified compounds</td>
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<td>3</td>
<td>Toluene:Ethyl acetate (9:1)</td>
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<td>0.462</td>
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<td>Toluene:Ethyl acetate (1:1)</td>
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<td>0.189</td>
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<td>Ethyl acetate</td>
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</tr>
<tr>
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<td>Ethanol</td>
<td>250x2</td>
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### Table 11  Rechromatography of Fraction 3

<table>
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<th>Weight (mg)</th>
<th>Approximate Composition</th>
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<td>100x5</td>
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<td>B</td>
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<td>0.063</td>
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<td>0.049</td>
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<td>F</td>
<td>Hexane:Ethyl acetate (1:1)</td>
<td>100x5</td>
<td>0.024</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
<td>G</td>
<td>Hexane:Ethyl acetate (7:3)</td>
<td>100x5</td>
<td>0.017</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
<td>H</td>
<td>Ethyl acetate</td>
<td>100x5</td>
<td>0.027</td>
<td>Mixture of unidentified compounds</td>
</tr>
<tr>
<td>I</td>
<td>Ethanol</td>
<td>100x4</td>
<td>0.087</td>
<td>Mixture of unidentified compounds</td>
</tr>
</tbody>
</table>

**Spectral data of compound 21**

![Spectral data of compound 21](image)

**Fig 17  GC**

![Fig 17  GC](image)

**Fig 18  GC-MS**
Fig 19 FTIR

Fig 20 $^1$HNMR spectrum

Fig 21 $^{13}$CNMR spectrum
References

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