ISOLATION AND CHARACTERIZATION OF MAJOR PHYTOCHEMICALS FROM *AZADIRACHTA INDICA* SEED CAKE

A project report submitted to

Mahatma Gandhi University, Kottayam

In partial fulfillment of the requirements for the award of

Master Degree in CHEMISTRY

GREESHMA JOSE

(Reg.No: 180011017729)

Under the supervision of

Dr. SAJIN FRANCIS K

DEPARTMENT OF CHEMISTRY

BHARATA MATA COLLEGE, THRIKKAKARA

(Affiliated to Mahatma Gandhi University, Kottayam)

2018-2020

BHARATA MATA COLLEGE

THRIKKAKARA

CERTIFICATE

This is to certify that the project report entitled "ISOLATION AND CHARACTERIZATION OF MAJOR PHYTOCHEMICALS FROM AZADIRACHTA INDICA SEED CAKE" is a bonafied work carried out by ***GREESHMA JOSE,*** *M.Sc. Chemistry student, under my supervision and guidance and that no part of this has been submitted for any degree, diploma or other similar titles of recognition under any university.*

Forwarded by

Dr. LITTY SEBASTIAN

Head of the Department

Department of chemistry

Bharata Mata College

ThrikkakaraThrikkakara

Project Guide

Dr. SAJIN FRANCIS K

Asst. Professor

Department of chemistry

Bharata Mata College

Place: Thrikkakara

Date:

DECLARATION

I **GREESHMA JOSE** hereby declare that this project report entitled **"ISOLATION AND CHARACTERIZATION OF MAJOR PHYTOCHEMICALS FROM *AZADIRACHTAINDICA* SEED CAKE"** is an authentic work carried out during my course of study under the guidance of **Dr. SAJIN FRANCIS K**, Department of Chemistry, Bharata Mata College, Thrikkakara.

Place: Thrikkakara

Date:

ACKNOWLEDGMENT

I thank God Almighty for showering his blessings on me for the successful completion of the project work undertaken by me.

I gratefully acknowledge the help and advice of a number of people in the preparation of the project work.

I express my heart full thanks to **Dr. Shiny Palatty**, Principal, Bharata Mata College, Thrikkakara for her constant encouragement for the completion of this project.

I am glad to thank **Dr. Litty Sebastian**, Head of the department, who gave valuable suggestions during the work.

I express sincere thanks to **Dr. Sajin Francis K.**, Assistant Professor, Department of Chemistry and my project guides for immense help and guidance during the course of this project work.

I owe a depth of gratitude to the lab assistants and other faculty members for their assistance and co-operation.

GREESHMA JOSE

CONTENTS

CHAPTER-1

INTRODUCTION

Human beings have depended on nature for their simple requirements. Since the early humans recognized and exploited the plants around them for use as fuel, clothing, shelter, medicines and food [1]. Plant is an important source of medicine and plays a key role in world health. Medicinal plants have provided mankind a large variety of potent drugs to eradicate infections and diseases. The use of plant based drugs increasing in all over the world. The aspirin, atropine, digoxin, ephedrine, morphine, pilocarpine, quinine, quinidine, reserpine, taxol, vincristine and vinblastine are some of the drugs believed to be obtained from plants [2].

The drugs based on allopathy are expensive and also exhibit toxic effect on normal tissues and on various biological activities. It is a largely accepted fact that numerous pharmacologically active drugs are derived from natural resources including medicinal plants [3]. Herbal medicines are natural products and proved to be safe due to their lesser side effect. Worldwide, people have a positive invention towards the herbal products and believe in "natural" rather than "synthetic" origin [4].Medicinal plants may be defined as those plants that used in treating and preventing specific ailments or diseases and that are generally considered to be harmful to humans. Medicinal plants are either "Wild plant species" or "Domesticated plants species". The use of medicinal plants not only for the treatment of diseases but also as potential material for maintaining good health conditions [2].

India has been known to be rich repository of medicinal plants. The forest in India is the principal of repository of large number of medicinal plants, which are largely collected as row materials for manufacture of drugs and perfumery products. Medicinal plants such as Aloe, Tulsi, Neem, Turmeric and Ginger cure several common ailments. These are considered as home remedies in many parts of the country [3].These plants are a rich source of compounds that can be used to develop drug synthesis. The parts of medicinal plants such as seeds, root, leaf, fruit, skin, flowers or even the whole plant is used in drug synthesis. The active compounds in most parts of the medicinal plants have direct or indirect therapeutic effects and are used as medicinal

agents. The active compounds (substances), which have physiological effects on living organisms [1]. Treatment with medicinal plants is considered very safe and there are minimal side effects. These remedies are in sync with nature, which is the biggest advantage. The golden fact is that, use of herbal treatments is independent of any age groups and the sexes. Today, according to the WHO, more than 80% of the world's population relies more often on traditional drugs, mainly plants, serving as the main source of health care [5, 1].

Plants have been used for medicinal purposes long before prehistoric period. Ancient Unani manuscripts Egyptian papyrus and Chinese writings described the use of herbs. Ayurveda, Unani, Siddha and Folk (tribal) medicines are the major systems of indigenous medicines. Among these systems, Ayurveda and Unani Medicine are most developed and widely practiced in India [5].There's no way of knowing precisely how the earliest cultures used herbs, but they had thousands of years to experiment with herbs as foods, flavorings, medicines, disinfectants, etc. [6]. Here are given just a few highlights in the history of herbalism. At the ancient times there was not sufficient information either concerning the reasons for the illnesses or concerning which plant and how it could be utilized as a cure, everything was based on experience. Until the advent of iatrochemistry in 16th century, plants had been the source of treatment and prophylaxis [4].

Evidence indicates that plants have been cultivated as drugs approximately 60000 years ago. Scripts about medicinal plants date back to almost 5000 years ago in India, China and Egypt, and at least 2500 years in Greece and Central Asia [1].Various religious documents such as Bible and Quran also supported the herbs role in health care and prevention. Islamic perspective confirms the herbs role in diseases management and Prophet Mohammed recommended various plants in the diseases cure [3].

Ancient Greek people were also familiar with the medicinal properties of some medicinal plants. Hippocrates (460-337 BC), the founder of allopathic medicine and Aristotle, pupil of Hippocrates, used medicinal plants for the treatment of diseases [1]. Greek Period, Greek civilization was an epoch of science and philosophy. The Greeks have made worthy contribution in pharmaceutical sciences, especially in phyto pharmaceuticals. Aristotle has described 500 crude drugs used in the cure of different pathological conditions. Hippocrates formulated the first scientific medical paradigm of treatment [7].

Iranians have long advanced knowledge about medicinal plants and their properties. The most prominent example of this argument is *Avesta*, the sacred book of Zoroastrianism, which explains medicinal plants and their uses in Iran. In Zoroastrianism in Iran, a plant called Ephedra was considered a sacred plant. The Iranian historical studies have shown that the history of the use of medicinal herbs in Iran dates back to the time of Aryan civilization from about 6500 to 7000 BC [1].

China was one of the first countries to have a medical culture. With a history of 5000 years, China has formed a deep and immense knowledge of medical science, theory, diagnostic methods, prescriptions, and cures [8]. The Chinese book on roots and grasses "Pen T'Sao," written by Emperor Shen Nung circa 2500 BC, treats 365 drugs (dried parts of medicinal plants), many of which are used even nowadays such as the following: Rhei rhisoma, camphor, Theae folium, Podophyllum, the great yellow gentian, ginseng, jimson weed, cinnamon bark, and ephedra [4]. Ginseng is a slow growing perennial plant which was discovered more than 5000 years ago in the mountains of Manchuria, China. It has been used as a traditional medicine in Asia and has demonstrated pharmacological effects in cancer, diabetes, cardiovascular diseases, viral infections, etc. The major bioactive components of Ginseng are the ginsenosides [8].

Ephedrine, it is a naturally occurring alkaloid obtained from Chinese herb Ephedra also called Ma-huang. In China, it has been used to treat asthma and fever for more than 5000 years. Ephedrine stimulates the central nervous system, opens blood vessels, and excites the heart. Artemisinin, the compound, which is commonly found in antimalarial drugs, has been used to treat thousands of cases of malaria, including those involving chloroquine-resistant strains of the malaria causing parasite, *Plasmodium falciparum.* It is obtained from Chinese plant Artemisia. Coptis, it is a medicinal herb found in the regions of China and Eastern Himalayas. The active element present in this plant is called berberine, an isoquinoline alkaloid. In Chinese traditional medicine, this herb occupies the leading position among the most often used herbs. This active constituent is used for lowering blood sugar levels, it inhibits and kills various bacteria, fungus, and viruses, prevents the replication of cancerous cells, can cure various heart illness, etc. [8].

Traditional Indian Medicine or Ayurveda is considered as the oldest health care system on earth. The descriptions of the system are available in ancient literatures such as Rig-Veda and Atharva-Veda, approximately 5000 years BC [7]. Ayurveda is a Sanskrit word, which is made

up of two words “Ayus”— life and “veda”—knowledge. , Hence, Ayurveda is known as “knowledge of life”. It contains traditional techniques of healing which are also influenced by Chinese, Greek, and Tibetan medicine. Therefore, ayurveda is considered “mother of healing” [9]. Indian Ayurvedic medicine has used many herbs such as turmeric is used possibly as early as 1900 B.C. Many other herbs and minerals used in Ayurveda were later described by ancient Indian herbalists such as Charaka during the 1st millennium BC. The Sushruta Samhita in the 6th century BC describes 700 medicinal plants, 64 preparations from mineral sources, and 57 preparations based on animal sources [6]. Ayurvedic products are manufactured either by herbs alone or in different combination of herbs, minerals, metals, etc. Ayurvedic medicines are versatile in nature. One medicine can be used to cure multiple diseases. The mantra of Ayurvedic medicine is to treat the disease from the root instead of just suppressing the disease [9].

As a part of Indian tradition, we can see a number of medicinal herbs at homes. Now we shall go through its importance and usage. Black pepper is one of the common and potent digestive stimulants. It burns the indigestive food particles and cleanses alimentary canal. It is used as expectorant, Carminative, Anti-helminthic and Stimulant. Coriander promotes better absorption and digestion of food. Fresh juice of coriander can be used to treat any type of skin rashes, inflammation, itching, and allergies. Disease for which the herb is used in urinary tract infection (UTI), rashes, indigestion, allergies, and vomiting. Ginger is treated as the “universal medicine”. It is mostly used with honey to treat cold and cough. It also relieves cramps and gas in the abdomen. Disease for which the herb is used—headache, heart disorder, cold and cough, abdominal pain, vomiting and arthritis. Flowers of hibiscus are used for purification of blood and heart spiritually. Hibiscus enhances complexion of skin and promotes hair growth. Mint is widely used for calming the nerves, relaxing mind, and bringing clarity to mind and senses. Mint has soothing action on the nervous system [9].

Earlier studies reported that plants and their constituents show inhibitory effects on the growth of malignant cells via modulation of cellular proliferation, tumour suppressor gene, and various other molecular pathways [3]. Some of the Ayurvedic medicine show anticancer activity. Turmeric, the rhizome of the turmeric is traditionally used in cooking. The active ingredient of this plant is curcumin (diferuloylmethane), a polyphenol derived from the rhizome of the plant. Turmeric is used for both cancer prevention and treatment. The anticancer potential of curcumin

is associated with its ability to inhibit proliferation in a wide variety of tumor cells. Chiretta (*Andrographis paniculata)* is plant also known as the king of bitters. The roots and leaves are generally used for medicinal purposes. It is a potent chemo protective agent and is effective against a variety of infectious and oncogenic agents. Andrographolide is the active compound present in Chiretta shows cytotoxic activity against a variety of cancer cells especially leukemia [10].

Pennywort is another plant that has shown potential as an anticancer agent. This plant is commonly found in India. The whole plant or its leaves are being traditionally used for their therapeutic properties. The mechanism underlying the antitumor activity of pennywort is suggested to be a direct inhibition of DNA synthesis [10].*Azadirachta indica* and their active compounds play important role in the prevention of cancer development and progression. Azadirachta indica, popularly known as neem tree has been an ancient source of herbal medicine against a variety of human health problems. Neem contains flavanoids and various other constituents that play an important role in inhibition of cancer development. These constituents activate the tumour suppressor genes and inactivate the activity of several genes involved in the cancer development [3].

The Ashok, Aswagandha, Brahmi, pepper, Sandalwood, Tulsi and Neem are the important plants where the secondary metabolites are widely used for health-related problems. Among these, neem has gained the distinction of being the most researched tree in the world [11].

NEEM:

Neem is a member of the mahogany family. It is today known by the botanic name Azadirachta indica A. Juss. It is often said that it is from the Persian word *azad- "*free" and *drakhat- "*tree", that means free tree, and when the specific name *indica* is added to it, the meaning of the botanical name becomes "*the free tree from India*" [12].Neem is native to India and Burma. Presently neem trees can be seen growing successfully in about 72 countries worldwide, in Asia, Africa, Australia, North, Central and South America [11, 12]. The tree is said to grow "almost anywhere" in the lowland tropics. However, it generally performs best in

areas with annual rainfalls of 400–1200 mm. It can be planted using seeds, seedlings, saplings, root suckers, or tissue culture [11].

TAXONOMICAL CLASSIFICATION OF NEEM

Kingdom	Plantae
Division	Mangoliophyta
Order	Sapindales
Family	Meliaceae
Genus	Azadirachta
Species	A.indica

Neem ingredients are applied in Ayurveda, Unani, Homeopathy, and modern medicine for the treatment of many infectious, metabolic, or cancer diseases. Different types of preparation based on plants or their constituents are very popular in many countries diseases management [3]. Neem products have been used for centuries in the field of agriculture and medicine, and the plant extracts of neem have enormous potential to influence modern agrochemical research [11].

The plant Nimba or Neem has been used in Ayurvedic medicine for more than 4500 years, due to its medicinal and healing properties it is known as “The Village Pharmacy”. The benefits of neem are listed in the earliest documents of Ayurveda “Charak Samhita” and “Susruta-Samhita” (1500 BC–400 CE), which forms the foundation of the Indian system of natural treatments, Ayurveda [11, 12]. It is interesting to note that the neem tree has more than 50 Sanskrit synonyms in Ayurvedic literature. Neem is also called arista because it eradicates diseases [11]. Neem is present, in one form or another, in 75% of Ayurvedic formulations. The Vedas called Neem as, “*SARVA ROGA NIVARINI*”, which means ‘one that cures all ailments and ills [13].

Neem is a fast growing tree that usually reaches a height of up to 20m, and under very favorable conditions up to approximately 30-35 m. The branches spread widely. The fairly dense crown may reach a diameter 15-20 m in old free standing trees. The trunk is relatively short, straight and may reach a girth of 1.5-3.5 m. The bark is hard fissured or scaly and whitish-gray to reddish-brown. The sap wood is grayish-white and the heart wood reddish. Neem trees are attractive broad-leaved evergreens. The leaves are unpaired, pinnate, medium to dark green leaflets, with each comprising 5–15 leaflets, are approximately 3-8 cm long. The shape of mature leaflets is more or less asymmetric [3, 13].Neem tree flowers between February and May. The white flowers are arranged in axillary, normally more or less drooping panicles which are up to 25 cm long [12,13].

A Neem tree normally begins bearing fruit after 3–5 years and becomes fully productive in 10 years and it may live for more than two centuries [11]. Neem fruits are green drupes that turn golden yellow on ripening in the month of June-August in India. It can produce up to 50 kg of fruits annually, after attaining tenth years and onwards [12]. Fruits are green when young and yellowish-green to yellow, rarely reddish when mature. The fruit skin (exocarp) is thin and the bitter-sweet pulp (mesocarp) is yellowish-white and very fibrous and 0.3-0.5 cm thick. The seeds are about 2 cm long and 1.5cm diameter, kernel of about 1.5 cm length containing about 30 to 40 percent oil [13, 14].

Neem takes about five years to produce the first fruit crop. The fruit yield is extremely variable ranging from 10 to 50kg per tree. A full grown tree can produce 30-100 kg fruit depending on rainfall, soil type and ecotype. 50 kg of fruits yield 30 kg of seed giving 6 kg of oil and 24 kg of seed cake [14]. India stands first in neem seed production and about 4, 42,300 tons of seeds are produced annually yielding 88,400 tons of neem oil and 3, 53,800 tons of neem seed cake [12]. In agriculture, neem can be utilized efficiently by using its products like Neem oil, fruit and byproducts like seed cake are used as bio pesticides, fungicides, and organic manures [11].

The neem seed contain tignic acid (5methyl-2-butanic acid) responsible for the distinctive odour of the oil. Neem cake is the residue left after oil has been pressed from neem seeds. It is used as organic manure resulting in high yield of crops and plants. Neem seed cake is very unpalatable due to the presence of salanolide meliacin which has been found to be one of the

active principles of neem seed oil. These compounds are natural products called triterpenoids [11, 14].

Neem contains maximum useful non wood products (leaves, bark, flowers, fruits, seed, gum, oil, and neem cake). Over thousands of years, millions of Asians have used neem medicinally [14].

MEDICINAL USE OF VARIOUS PARTS OF NEEM

PARTS	M**EDICINAL PROPERTIES**
Leaf	Treatment of anorexia, biliousness, skin ulcers, leprosy, eye problems, intestinal worms
Flower	Elimination of intestinal worms and phlegm, bile suppression,
Fruit	Treatment of leprosy and wounds, diabetes, urinary disorder, relieves piles, intestinal worms, eye problems
Bark	Antipyretic and Analgesic
Twig	Relieves cough, asthma, piles, urinary disorder, diabetes
Seed pulp	Treatment of leprosy and intestinal worms
Oil	Treatment of rheumatism, leprosy, intestinal worms
Root, bark, leaf, flower and fruit together	Treatment of leprosy, skin ulcer, burning sensation, blood morbidity, itching, and rheumatism**.**

ACTIVE COMPOUNDS OF AZADIRACHTA INDICA

Plants are known to contain many kinds of nutrients, including macronutrients (carbohydrates, proteins, and lipids) and micronutrients (minerals and vitamins), that shows positive effects on human health. There are non-nutritive substances have also been found in

plants, such as phenols, flavonoids, alkaloids, tannins, and terpenoids, collectively known as phytochemicals which helps plants to survive in its environment. The phytochemicals are known to contain multifunctional properties like anti-oxidation, anti-inflammation, cardio-protection, antimalarial and anticarcinogenic activities. Plants are the only major source of drugs for the majority of the world population. There are approximately 500,000 plant species occurring worldwide, of which only 1% has been phytochemically investigated [11].

The Neem research was started in the 1920s by the Indian scientists. Neem's ability to repel insects was reported in the scientific literature in 1926–1929. Neem has one of the richest sources of secondary metabolites in nature, and over 140 compounds have been isolated from different parts of the Neem plant [11]. In 1963 an Indian scientist extensively examined the chemistry of the active principles of neem. Several compounds have been isolated and characterized [13].

The compounds found in Azadirachta indica divided into two groups:

a) Isoprenoids like diterpenoids and triterpenoids including protomeliacin, limonoids, azadirone and its derivatives, vilasinin type compounds, nimbin, salanin and azadirachtin.

b) Non isoprenoids which include proteins, aminoacids, carbohydrates, sulphur compounds, polyphenolics such as flavonoids and their glycosides, dihydrochalcones, coumarins, tannins, aliphatic compounds and phenolic acids [17].

Limonoid, are the most important and well-studied class of triterpenoids and about one-third of phytochemicals isolated from neem belong to this class, which are the major cause for these widespread activities of neem. These compounds have a low toxicity against non-target and beneficial organisms and cause less disruption to ecosystems than conventional insecticides. Azadirone, azadiradione, epoxyazadiradione (E- Azadiradione), isonimolide, azadirachtin, salanin and nimbolide are few representative limonoids. Limonoids are concentrated in the seed despite their presence in other parts of neem tree [11, 14].

The most important active constituent is Azadirachitin and the others are nimbolinin, nimbin, nimbidin, nimbidol, sodium nimbinate, gedunin, salanin, and quercetin. Azadiradione is

present in very high concentration in neem plant as compared to other metabolites. It seems to be the most abundant metabolite in the neem leaf, bark and seed extracts [11].

Nimbidin, a major crude bitter principle extracted from the oil of seed kernels of A. indica, demonstrated several biological activities such as anti-inflammatory, antipyretic, hypoglycemic and antiulcer effects [15]

Nimbolide has been shown to exert antimalarial activity by inhibiting the growth of *Plasmodium falciparum*. Nimbolide also shows antibacterial activity. Gedunin, isolated from neem seed oil, has been reported to possess both antifungal and antimalarial activities [15].

Leaves of neem maily yield quercetin (flavonoid) and nimbosterol (β- sitosterol) as well as number of liminoids (nimbin and its derivatives). Quercetin (a polyphenolic flavonoid) is known to have antibacterial and antifungal properties [13].

The flowers contain nimbosterol and flavonoids. Flowers also yield a waxy material consisting of several fatty acids, viz., behenic (0.7%), arachidic (0.7%), palmitic (13.6%), oleic (6.5%) and linoleic (8.0%). The pollen of neem contains several amino acids like glumatic acid, tyrosine, arginine, methionion, phenylalanine, histidine and isoleucine [13].

The trunk bark contains nimbin (0.04%), nimbinin (0.001%), nimbidin (0.4%), nimbosterol (0.03%), essential oil (0.02%) and tannins (6.0%). The stem bark contains tannins (12-16%) and non-tannin (8-11%). The bark also yields an antitumor polysaccharide [13, 16].

The tree exudes a gum, which is on hydrolysis yields, L-arabinose, D-galactose and D-glucoronic acid. The older tree exudes a sap containing free sugars (glucose, fructose, mannose and xylose), amino acids (alanine, aminobutyric acid, arginine, asparagines, aspartic acid, glycine, praline, etc.) and organic acids (citric, malonic, succinic and fumaric) [13].

Seed is very important both because of its high lipid content as well as the occurrence of a large number of bitter principles (azadirachtin, azadiradione, nimbin, etc.) in considerable quantities. Azadirachtin has proven effectiveness as a pesticide against about 300 insect species and is reported as non-toxic to humans. Neem seeds are also described as antihelminitic, antileprotic and antipoisonous [13, 16].

PHARMACOLOGICAL ACTIVITIES OF *AZADIRACHTA INDICA*

The Neem tree is now gaining the importance due to its wide scope of commercialization in the areas of agriculture, veterinary, cosmetics, medicine and various industries. It is an ecofriendly natural source of phyto- chemicals and nutrients. However, there are several reports on the biological activities and pharmacological actions of neem based on modern scientific investigations [13, 15].

Neem has been well known for various medicinal properties like anti-inflammatory, antipyretic, antihistamine, antifungal, anti-bacterial, anti-ulcer, analgesic, antiarrhythmic, anti-tubercular, antimalarial, diuretic, anti-arthritic, anti-protozoal, insect repellent, anti-feedant, and anti-hormonal, anti-oxidant, antimalarial, and anticarcinogenic activities [11].

1) ANTIOXIDANT ACTIVITY

Neem has the capacity to control the hyperactivity and free radical activities of the chemicals normally created within the body. These cause ageing and may also cause cancer under hyperactive condition. A series of disorders including cardiovascular disease, eye health, macular degenerations, age-related neuron degeneration, etc. are caused due to the hyperactivity of these free radicals. Neem products protect against the chemically induced carcinogens and liver damage by boosting antioxidant levels [15].

Leaf and bark extracts of *A. indica* have been studied for their antioxidant activity and results of the study clearly indicated that all the tested leaf and bark extracts of neem have significant antioxidant properties. Antioxidant capacity of different crude extracts of neem was as follows: chloroform > butanol > ethyl acetate extract > hexane extract > methanol extract. Result of the current finding suggested that the chloroform crude extracts of neem could be used as a natural antioxidant [3].

ANTI-CANCEROUS ACTIVITY

Cancer is multifactorial disease and major health problem worldwide. The alteration of molecular or genetic pathways plays role in the development and progression of cancer. The treatment module based on allopathic is effective on one side but also shows adverse effect on the normal cell. Neem contains flavonoids and various other ingredients that play an important role in inhibition of cancer development. Large number of epidemiological studies proposes that high flavonoid intake may be correlated with a decreased risk of cancer [3]. Neem has been found to be effective in the a variety of human cancer cells that include the colon, stomach, lung, liver, skin, oral, prostrate, and breast [15].

2) ANTI-INFLAMMATORY ACTIVITY

A study result has confirmed that extract of *A. indica* leaves at a dose of 200 mg/kg, showed significant anti-inflammatory activity in cotton pellet granuloma assay in rats. Other study results revealed that neem leaf extract showed significant anti-inflammatory and study results suggest that nimbidin suppresses the functions of macrophages and neutrophils relevant to inflammation. Earlier finding showed the anti-inflammatory effect of bark and leave extracts of neem and seed oil shows antipyretic and anti-inflammatory activities [3].

3) ANTI-DIABETIC ACTIVITY

Leaves extracts of Azadirachta indica have significant anti-diabetic activity and could be a potential source for treatment of diabetes mellitus**.** Aqueous extract of neem leaves significantly decreases blood sugar level and prevents adrenaline as well as glucose-induced hyperglycemia. The effect is possibly due to the presence of a flavonoid, quercetin [15].

A study was undertaken to evaluate the 70% alcoholic neem root bark extract (NRE) in diabetes and results showed that neem root bark extract showed statistically significant results in 800 mg/kg dose [3].

4) WOUND HEALING EFFECT

Leave extracts of *Azadirachta indica* promote wound healing activity through increased inflammatory response and neovascularization [3].

5) ANTIMICROBIAL EFFECT

Neem and its ingredients play role in the inhibition of growth of numerous microbes such as viruses, bacteria, and pathogenic fungi. Azadirachtin, complex triterpenoids limonoid present in seeds, it is an important constituent which cause toxic effect on the insects. The role of neem in the prevention of microbial growth is described individually as follows [3].

6.1) Antibacterial Activity: Oil from the leaves, seeds, and bark possesses a wide spectrum of antibacterial action against Gram-negative and -positive microorganisms, including M. tuberculosis and streptomycin-resistant strains. Recently, the antibacterial activity of neem seed oil was assessed in vitro against 14 strains of pathogenic bacteria [15].

6.2) Antiviral Activity**:** Leaves extract of neem has shown virucidal activity, interfering at an early event of fungi's replication cycle [3]. The antiviral and virucidal effect of methanolic extract fraction of neem (NCL-11) leaves was observed regarding its activity and possible mechanism of action against Coxsackie B group of viruses. NCL-11 inhibited plaque formation in six antigenic types of Coxasackie virus B. Aqueous leaf extract offers antiviral activity against Vaccinia virus, Chikungunya, and measles virus in vitro [15].

6.3) Antifungal Activity**:** Alcoholic extract of neem leaf was most effective as compared to aqueous extract for retarding the growth of both fungal species. The antimicrobial role of aqueous extracts of neem cake in inhibition of spore germination against sporulating fungi. The methanol and ethanol extract of *Azadirachta indica* showed growth inhibition against *Aspergillus flavus, Alternaria solani,* and *Cladosporium*. Aqueous extracts of various parts of neem such as neem oil and its chief principles have antifungal activities and have been reported by earlier investigators [3].

6.4) Antimalarial Activity: Neem seed and leaf extracts are effective against malarial parasites. Components of the alcoholic extracts of leaves and seeds are effective against both chloroquin-resistant and -sensitive strains of malarial parasite. Recently, neem seed extract and its purified fractions have been shown to inhibit growth and development of asexual and sexual stages of the human malarial parasite. Azadirachtin and other limonoids available in neem extracts are active on malaria vectors [15, 3].

AIM:

Isolation and characterization of major phytochemicals from Azadirachta indica seed cake.

OBECTIVES:

Among the thousands of plants used in ayurveda, neem tree has intense traditional uses. The studies based on neem tree illustrates that traditional wisdom can guide the efforts of modern science in discovering remedies for human aliments. The seed, bark, leaf and flower of A. indica contain compounds with proven antiseptic, antiviral, antipyretic, anti- inflammatory, anti-cancer, anti-ulcer and antifungal activities. An extensive research should be undertaken on neem and its products for their better economic and therapeutic utilization. So in this project aims to the **"ISOLATION AND CHARACTERIZATION OF MAJOR PHYTOCHEMICALS FROM *AZADIRACHTAINDICA* SEED CAKE".**

CHAPTER - 2

Extraction, Isolation and Characterization of Phytochemical Constituents from *Azadirachta indica* seeds cake

EXTRACTION

About 1kg of *Azadirachta indica* seeds cake was collected from registered fertilizer shop in Ernakulam, Kerala. 150 g of the powdered material was subjected to extraction three times with chloroform (500 mL) at room temperature for about 24 hrs. Then the extract filtered in to RB flask using filter paper. The chloroform present in it was removed under pressure on a rotary evaporator. The crude extract thus obtained was used for further works. It yielded 70 g of chloroform extract.

Isolation of compounds from chloroform extract

The 70 g chloroform extract transferred into a clean conical flask containing methanol and hexane mixture. This solution is subjected to magnetic stirrer for 24 hours. Then it is separated using a separating funnel in to hexane layer and methanol layer. It yielded 50 g of hexane extract and 20 g of methanol extract. The extraction using hexane is removing the oil content in the extract. The active compounds are completely transferred in to the methanol layer. Removal of methanol under reduced pressure gave to extract.

Thin layer chromatography of the extracts was carried out using solvents of polarity starting from 100% hexane to 100% ethyl acetate. After studying the TLC of methanol extract, the total extract was subjected for column chromatographic separation. For this, a column of 50 ml capacity was used. It was then loaded with 100 g of column chromatographic grade silica gel using hexane solvent. 500 mg methanol extract was dissolved in minimum amount of hexane and

loaded on the top of the column. It was kept for several minutes for adsorption over silica gel. Elution was started with 100% hexane and increases the polarity of eluting solvent by increasing the amount of ethyl acetate in hexane-ethyl acetate mixtures. Final elution was carried out with 100% ethyl acetate.

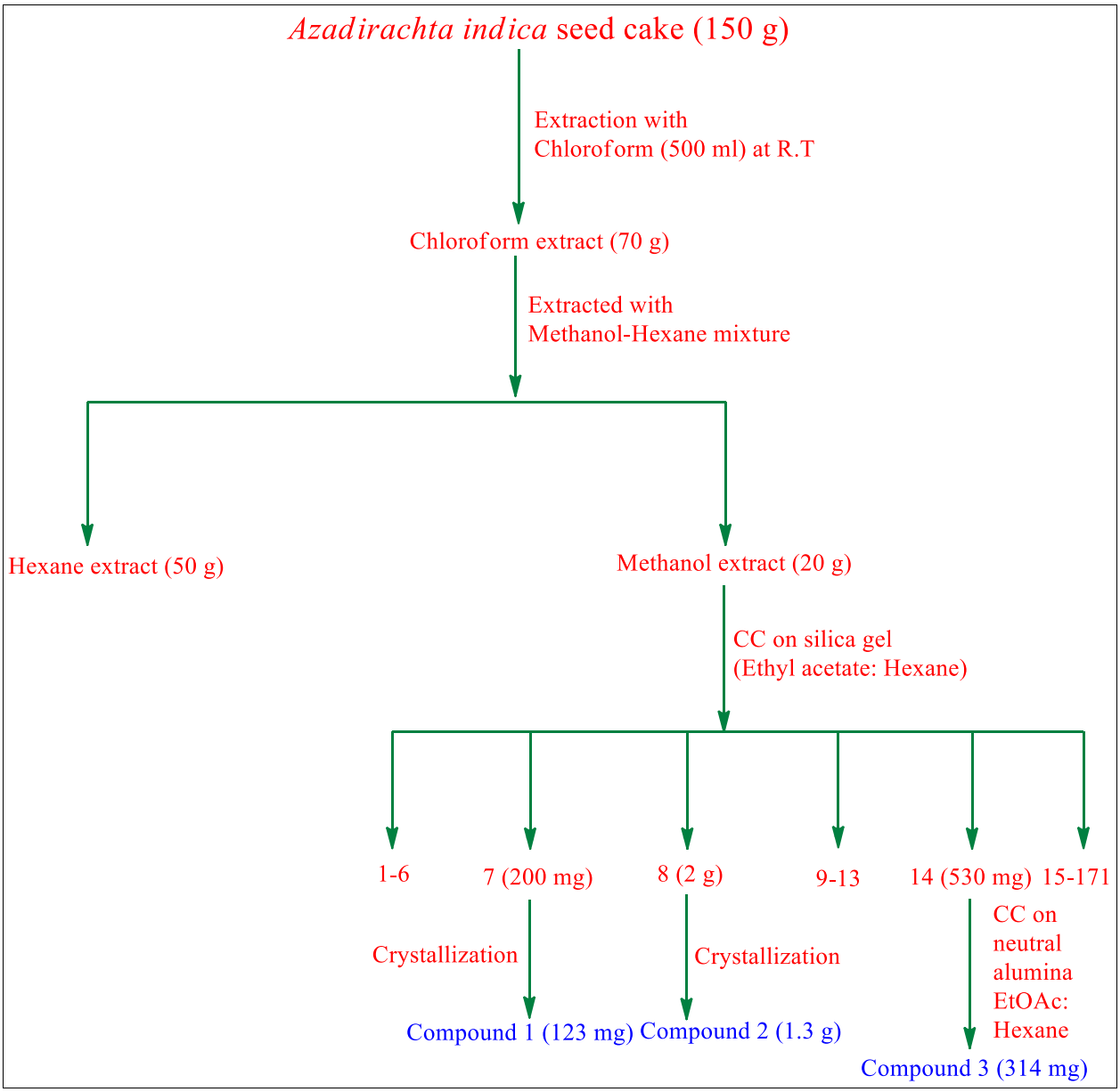
Different fractions of approximately 50 ml volume were collected in conical flasks. A total of 171fractions were collected. TLC of each fraction was checked and those fractions whose TLC profile was alike were pooled together to get 22 different fraction pools. Each of these pooled fractions was concentrated by removing the solvent under reduced pressure using rotary evaporator.

The fraction pool 7 (200 mg) showed single UV active spot with minor impurities. It was subjected to crystallization to yield colourless crystals. This compound obtained in 123 mg yield was named as compound 1.

The fraction pool 8 (2 g) showed a UV active spot with some impurities, was subjected to crystallization to yield colourless crystals. This compound obtained in 1.3 g yield was named as compound 2.

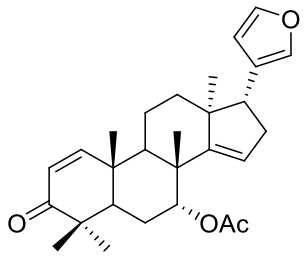
The fraction pool 14 (530 mg) showed a UV active spots. The fraction was subjected to purification on neutral alumina using hexane and ethyl acetate as eluent. This compound obtained in 314 mg yield was named as compound 3. Thus through extraction and isolation of neem seed cake, sufficient quantity of the above compounds were obtained.

PICTORIAL REPRESENTATION OF CHEMICAL COMPOUNDS FROM *A. INDICA* SEED CAKE



Characterization of Compound 1

The compound 1 was analyzed using various spectroscopic techniques. Compound 1 showed a strong absorption at 1732 cm^{-1} in IR spectrum indicating the presence of an ester carbonyl and a strong absorption at 1662 cm^{-1} suggesting the presence of six membered α, β unsaturated ketone. A doublet observed at δ 7.16 in the ^{1}H NMR (**Figure 2.1**) spectrum, integrating for one proton indicates β hydrogen and another at δ 5.85 indicates an α hydrogen of an α, β unsaturated ketone. The signals at δ 0.79, 1.08, 1.20 and 1.23 each integrating for three, six, three and three protons each respectively indicated the presence of five methyl groups. The three proton singlet at δ 1.96 could be attributed to acetyl group. The peaks at δ 204.7 and 170.2 in ^{13}C NMR (**Figure 2.2**) spectrum confirmed the presence of α, β unsaturated ketone and an ester carbonyl. Peaks in between δ 111.1-158.8 (8C) in ^{13}C NMR spectrum are due to alkenyl carbons. On analyzing these spectral data, structure of the compound was confirmed as azadirone.



Structure of Compound - 1

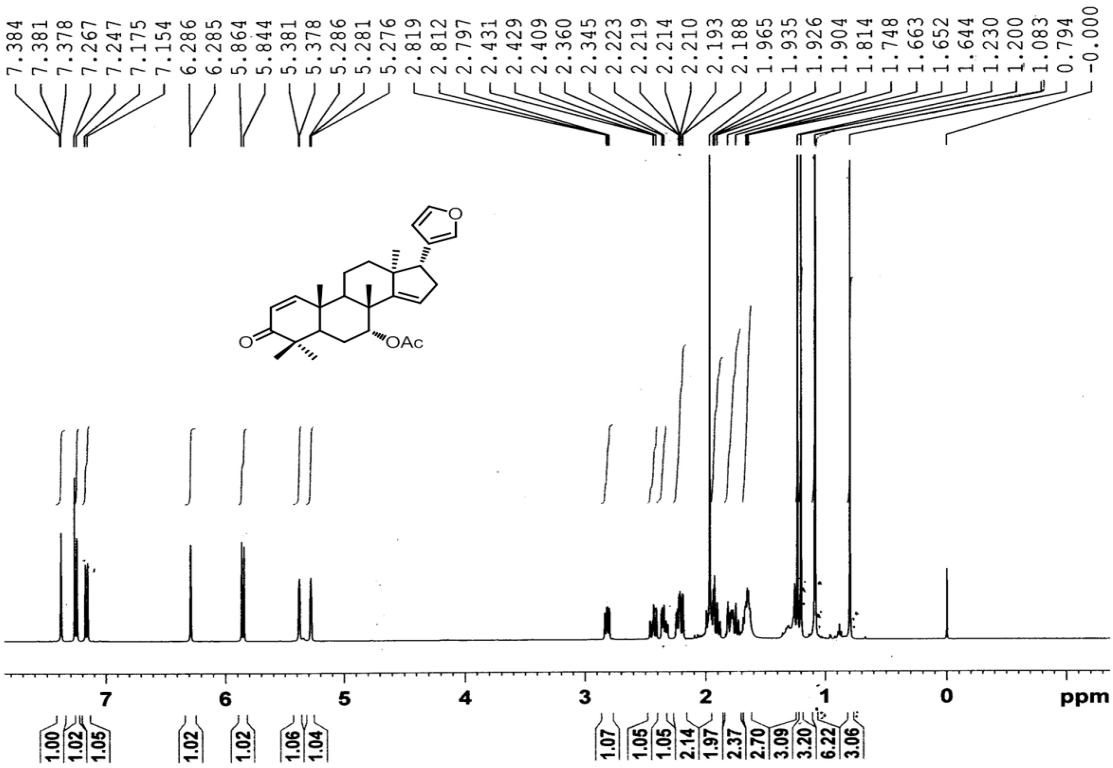


Figure 2.1:[1]H NMR spectrum of Azadirone

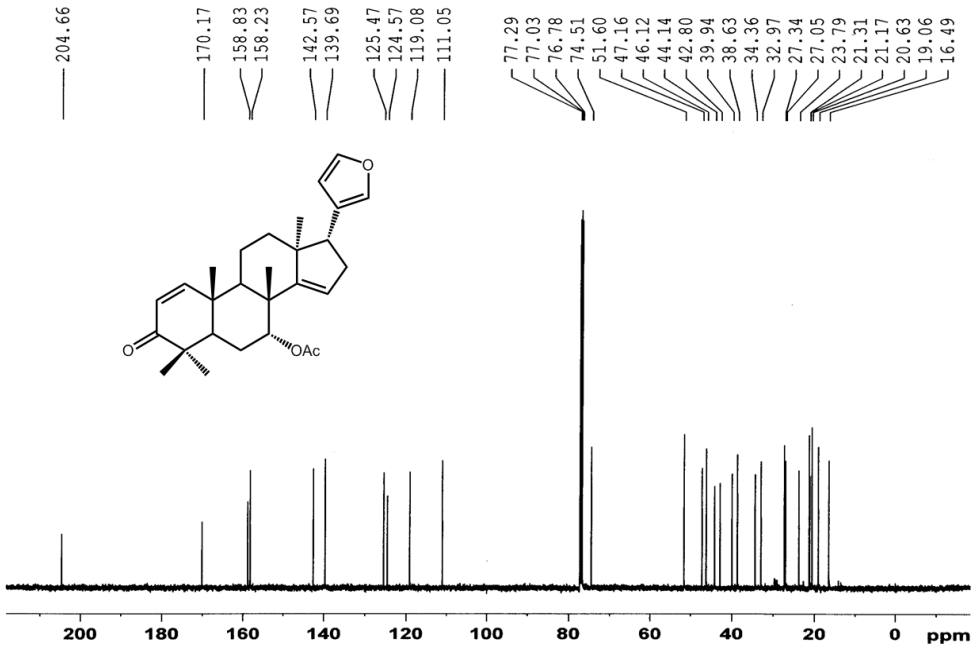
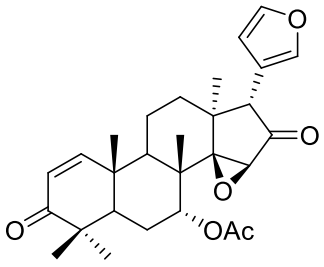


Figure 2.2 :[13]C NMR spectrum of Azadirone

Characterization of compound 2

The compound 2 was analyzed using various spectroscopic techniques. IR spectrum of the compound 2 showed a strong absorption at 1730 cm^{-1} indicating the presence of carbonyl group and a strong absorption at 1668 cm^{-1} suggesting the presence of six membered α, β unsaturated ketone. The strong absorption at 1747 cm^{-1} in IR spectrum indicated the presence of an ester carbonyl. A doublet present at δ 7.17 in the ^{1}H NMR (**Figure 2.3**) spectrum integrating for one proton could attributed to β hydrogen and another at δ 5.88 to an α hydrogen of the α, β unsaturated ketone. The signals at δ 1.04, 1.07, 1.21 and 1.22 each integrating for three, six, three and three protons respectively indicated the presence of five methyl groups. The three protons corresponds to acetyl group appeared at δ 2.03. The peaks at δ 208.4, 204.3 and 169.8 in ^{13}C NMR (**Figure 2.4**) spectrum confirmed the presence of a ketone, α, β unsaturated ketone and ester carbonyl respectively. Peaks in between δ 110.9-157.5 (6C) in ^{13}C NMR spectrum due to alkenyl carbons too were observed. Further, by comparison of the spectroscopic data and with the values reported earlier it was confirmed that the compound 2 was epoxyazadiradione.



Structure of Compound –2

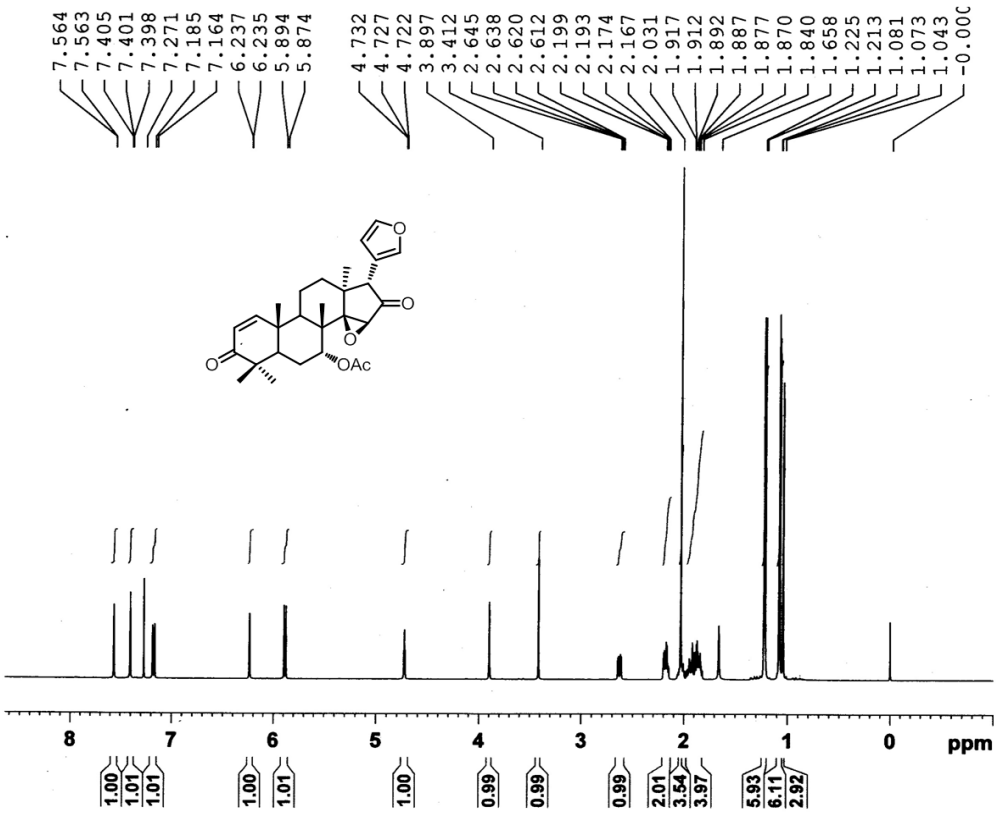


Figure 2.3 : ^{1}H NMR spectrum of Epoxyazadiradione

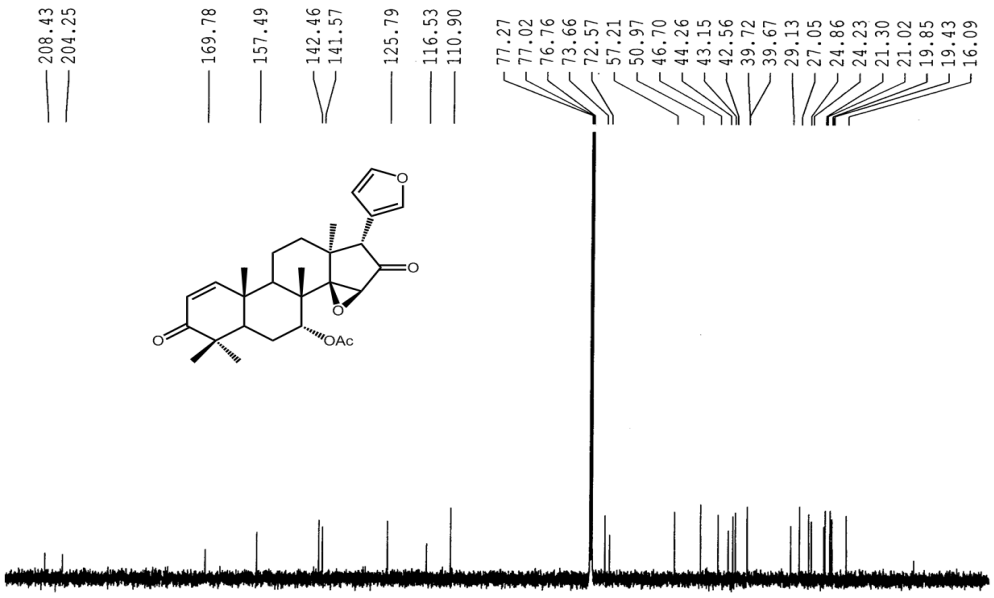
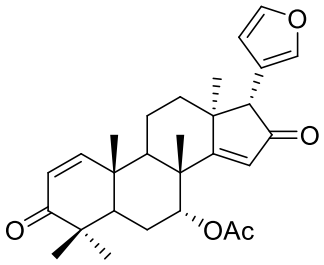


Figure 2.4 : ^{13}C NMR spectrum of Epoxyazadiradione

Characterization of compound 3

The compound 3 was analyzed using various spectroscopic techniques. IR spectrum of the compound 3 showed strong absorption at 1728 cm^{-1}, which indicated the presence of an ester carbonyl. A strong absorption at 1691 cm^{-1} in IR spectrum indicated the presence of five membered α, β unsaturated ketone and a strong absorption at 1666 cm^{-1} was suggestive of six membered α, β unsaturated ketone. The ^{1}H NMR (**Figure 2.5**) spectrum showed singlets at δ 1.03, 1.25, 1.10, 1.09 and 1.34 each integrating for three protons was indicative of five methyl groups. The doublet at δ 7.12 in the ^{1}H NMR spectrum integrating for one proton indicated the presence β hydrogen and another at δ 5.89 indicated an α hydrogen of an α, β unsaturated ketone. Singlet appearing at δ 1.95 in ^{1}H NMR spectrum which integrating for three proton corresponds to protons of acetyl group. ^{13}C NMR (**Figure 2.6**) spectrum showed 28 peaks. The peaks at δ 205.1 and 204.1 indicated the presence of two keto carbonyls and the peak at 169.6 showed the presence of ester carbonyl. The peaks at δ 20.9, 19.0, 26.9, 23.4, 15.8 and 26.3 could be attributed to methyl carbons. The peaks observed at δ 125.9, 192.5, 118.4, 123.2, 141.61, 111.2 and 142.8 shows the presence of alkenyl carbons. On analyzing these spectral data the compound was confirmed as azadiradione.



Structure of Compound –3

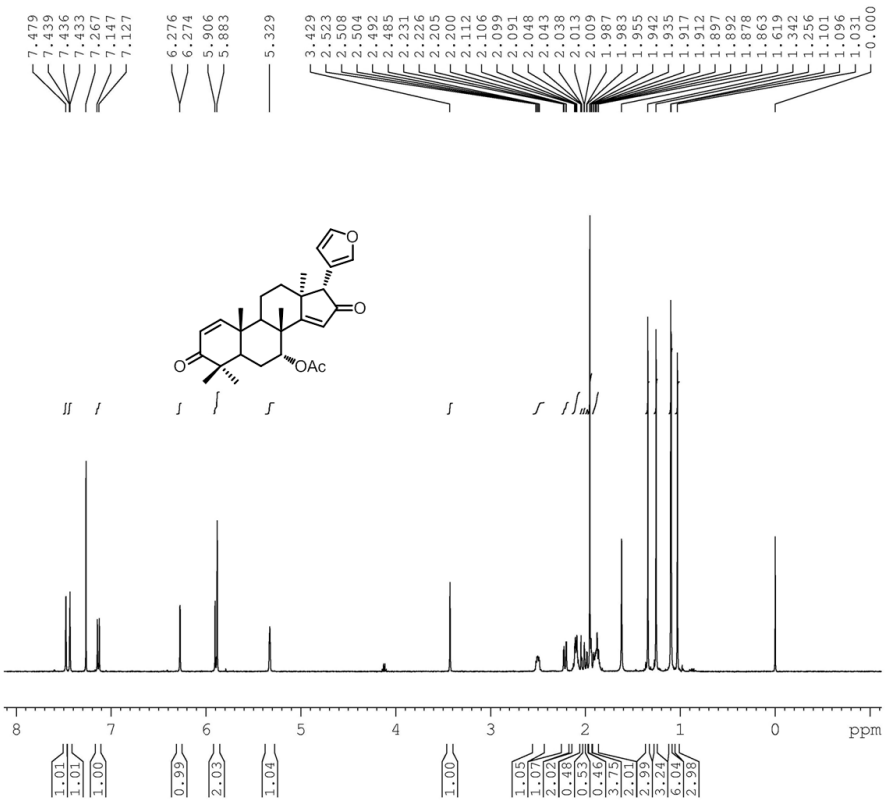


Figure 2.5 : ^{1}H NMR spectrum of Azadiradione

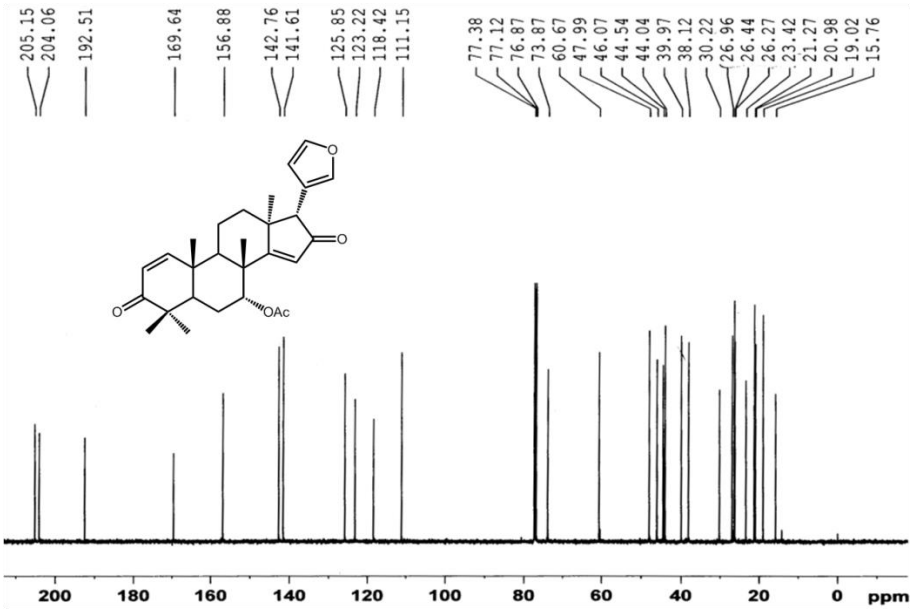
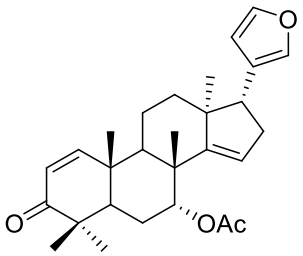


Figure 2.6 : ^{13}C NMR spectrum of Azadiradione

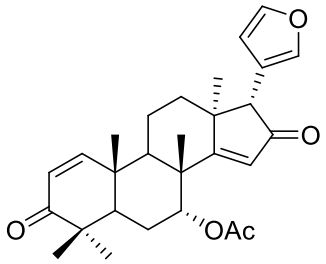
CONCLUSION:

The neem tree is one of the most potent herbal trees with untapped natural potentials. The whole plant could be of use as a good source of antioxidant. So the A. indica have greater attention of the researchers for designing new drug molecules with higher efficacy and least toxicity. Neem and its ingredients have therapeutics implication and have been traditionally used worldwide especially in Indian Subcontinent since ancient time. Researchers are exploring the therapeutic potential of this plant as it has more therapeutic properties which are not known.

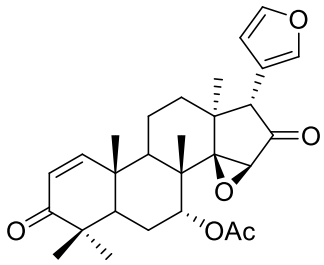
We have carried out phytochemical investigation of *Azadirachta indica*seed cake from that we have isolated threemajor compounds named such as Azadirone, Epoxyazadiradione and Azadiradione. We have successfully characterized these compounds using various spectroscopic methods such as ^{1}H NMR, ^{13}C NMR and IR. The structure of isolated compounds are given below.



Azadirone



Azadiradione



Epoxyazadiradione

REFERENCES

1) Fatemeh Jamshidi-Kia1, Zahra Lorigooini1, Hossein Amini-Khoe, Medicinal plants: Past history and future perspective, Journal of Herbmed Pharmacology, 2018, Volume: 7 (1), 1-7.
2) OladeJi O, The characteristics and role of medicinal plants; some important medicinal plants in Nigeria, Natural products: An Indian Journal, 2016, Volume: 12, 1-3.
3) Mohammad A. Alzohariry, Therapeutics Role of *Azadirachta indica* (Neem) and Their Active Constituents in Diseases Prevention and Treatment, Evidence- based complementary and alternative medicine. 2016, 1-7.
4) Akhileshwar Kumar Srivastava, Significance of medicinal plants in human life, Synthesis of medicinal agents from plants, 2018, 1-7.
5) Importance of Medicinal Plants and Herbs,Wikipedia. [Online] Available from:https://www.nhp.gov.in/introduction-and-importance-of-medicinal-plants-and-herbs_mtl
6) Special Supplement on Organic Herbs - The Importance of Herbs. [Online] Available from:www.nofa.org/tnf/Summer2012B.pdf
7) Haroon Khan, Medicinal Plants in Light of History: Recognized Therapeutic Modality, Topical Review Article, 2014, Vol.19 (3), 216-219.
8) Anuj Thakur and Seema R Pathak, Introduction to medicinally important constituent from Chinese medicinal plants, Synthesis of medicinal agents from plants, 2018, 333-345.
9) Manisha Mann and Seema R. Pathak, Ayurveda: A new dimension in the era of modern medicine, Synthesis of medicinal agents from plants, 2018, 283-303.
10) Avni G. Desai, Ghulam N. Qazi, Medicinal Plants and Cancer Chemoprevention, HHS publication access, 2018, Volume: 9 (7) 581-591.
11) Malali Gowda, Ambardar Sheetal and Chittaranjan Kole.The Neem Genome published by Compendium of Plant Genomes, 2019, 1-31.
12) Manoj Kumar, Jhariya Abhishek Raj, K. P. Sahu and P. R. Paikra, Neem- A Tree for Solving Global Problem, Research Paper – Agriculture, 2013, volume-3, 1-2.

13) Neem foundation. All about neem. Mumbai: Neem Foundation; 2012. [Online] Available from:https://neemfoundation.org

14) A. Aruwayo1 and S. A. Maigandi, Neem (Azadirachta indica) Seed Cake/Kernel as Protein Source in Ruminants Feed, American Journal of Experimental Agriculture, 2013, volume-3, 483-485.

15) Rakesh Kumar, Simpi Mehta and Seema R. Pathak, Bioactive constituents of neem, Synthesis of medicinal agents from plants, 2018, 75-93.

16) Neem (Azadirachta indica),[Online] Available from:http://www.frienvis.nic.in/WriteReadData/UserFiles/file/pdfs/Neem.pdf

17) Sunday E. Atawodi and Joy C. Atawodi, Azadirachta indica (neem): a plant of multiple biological and pharmacological activities, Phytochem Rev (2009) 8:601–620.