PHYTOCHEMICAL AND BIOLOGICAL INVESTIGATIONS OF *NYCTANTHES ARBORTRISTIS*, *TINOSPORA TOMENTOSA* & *MUSA SAPIENTUM VAR. SYLVESTRIS*

A Dissertation Submitted to the Department of Pharmacy
The University of East West in partial fulfillment of
Requirements for the degree of Bachelor of Pharmacy
(B. PHRM)

Submitted By

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2008-3-70-057

East West University

DEDICATED

TO MY

PARENTS & FRIENDS……..

Certificate

This is to certify that the thesis In-vitro antioxidant, Biomedical & antimicrobial assay of different plants such as ***Nyctanthes arbortristis, Tinospora tomentosa & Musa sapientum Var. sylvestris*** submitted to the department of pharmacy, East West University,Aftabnagar,Dhaka, in partial fulfillment of the requirements for the degree of bachelor of pharmacy (B.Phrm) was carried out by Srijan Acharya (ID#2008-3-70-057) under our guidance and supervision and that no part of the thesis has been submitted for any other degree. We further certify that all the sources of information and laboratory facilities availed in this connection is duly acknowledged.

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Dr. Repon Kumer Saha

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Contents

LIST OF TABLES:

LIST OF FIGURE:

Acknowledgement

All praise is for God who has given me the ability to complete my B. PHRM thesis & the opportunity to study in this subject.

It is my pleasure and proud privilege to express my heartiest regards and gratitude to my respected teacher and supervisor Dr. Repon Kumer Saha, Department of Pharmacy, Associate Professor of Pharmacy, East West University; for his mastermind direction, constant supervision and support, optimistic counseling and continuous backup to carry out the research work as well as to prepare this dissertation.

I feel proud to express my heartiest gratitude to my reverend teachers, Dr. Chowdhury Faiz Hossain, Dean Faculty of Science and assistant Professor Dr. Sufia Islam, Chairperson, Department of Pharmacy, East West University, for extending their helping hands & affectionate attitude whenever I needed.

Finally I would like to extend my thanks to the office staffs of the Faculty of Pharmacy who have cooperated with and all the research students in the lab for their help and assistance, friendly behavior and earnest cooperation, which enabled me to work in a very congenial and comfortable atmosphere.

I remember here the inspiring words of my family members and to all my well wishers. I say many thanks to them for their wholehearted inspiration during my thesis work.

Abstract

The crude methanolic extracts and buffer extracts derived from the Nyctanthes arbortristis, Tinospora tomentosa & Musa sapientum Var. sylvestris were screened in vitro for possible phytochemical, antibacterial and biomedical activities. Under phytochemical analysis, antioxidant test & Chemical screening was done whereas in biomedical analysis haemolytic activity & haemagglutination test was performed.

Moderate antioxidant property found in case of banana & night jasmine, whereas for Tinospora tomentosa it was very poor.

The crude methanolic extract of banana & night jasmine showed good antibacterial activity against different type of bacterias. The aqueous fractions of Tinospora tomentosa showed high activity against different type of bacterias.

High haemagglutination activity was observed against human red blood cells (RBCs) of all positive group bloods by crude methanolic extract of banana & night jasmine and aquous fraction of Tinospora tomentosa.

The crude methanolic extract of banana & night jasmine also showed to have prevented haemolysis of RBCs.

Hence the plant species can be a source of antibacterial agent(s) and antioxidant agents as well as these plants (methanolic extract of banana & night jasmine) have good property to prevent haemolysis & have high haemagglutination activity.

RATIONALE AND OBJECTIVE OF THE WORK

Higher plants represent a rich source of new molecules with pharmacological properties, which are lead compounds for the development of new drugs. During the last decades, the renewed interest in investigating natural products has led to the advent of several important drugs, such as the anticancer substances vinblastine, vincristine and taxol, or the anti-malarial agent artemisinin. Success in natural products research is conditioned by a careful plant selection, based on various criteria such as chemotaxonomic data, ethnomedical information, field observations or even random collection. One main strategy in the isolation of new leads consists of the so-called bioactivity-guided isolation, in which pharmacological or biological assays are used to target the isolation of bioactive compounds. The work described in this dissertation is an attempt to isolate the chemical constituents of the *Nyctanthes arbor-tristis* Linn and to evaluate their possible pharmacological and microbiological profiles. Although some mono-, di-, triterpenes, limonoids, flavonoids and benzopyranoids were reported earlier, yet except for insect feeding deterrent property, no extensive chemical and biological investigations have been carried out on this plant. There is medical flow about this plant and that is the plant can be used as analgesic and anti-pyretic. Therefore, the objective of this work is to explore the possibility of developing new drug candidates from this plant for the treatment of various diseases.

INTRODUCTION

Earth is a planet dominated by plants. The green plant is fundamental to all other life. The oxygen we bnsthe, the nutrients we consume, the fuels we bum and many of the most important materials we use wen produced by plants. Plants represent the first stage in the evolution of living things. In the process of the growth of nature, plants multiplied in number, variety and types. Human has identified as many as 7.5 lakh species of plants on earth, of which 5 lakhs are classified as "higher plants" and 2.5 lakhs as "lower plants" **[3].** The association between plant and man is an age-old process starting from human civilization. There has always been a race between nature and human knowledge. The plants sustain nature and nature sustains them. The interdependence of man and nature increases day by day. If human race makes sensible use of nature, posterity is bound to be prosperous **[3].**

A medicinal plant is any plant which, in one or more of its organ, contains substance that can be used for therapeutic purpose or which is a precursor for synthesis of useful drugs. The plants that possess therapeutic properties or exert beneficial pharmacological effects on the animal body are generally designated as “Medicinal Plants” **[1].** Although, there are no apparent morphological characteristics in the medicinal plants growing with them, yet they possess some special qualities or virtues that make them medicinally important. It has now been established that the plants which naturally synthesis and accumulate some secondary metabolites, like alkaloids, glycosides, tannins, volatiles oils and contain minerals and vitamins, possess medicinal properties termed as medicinal plants **[1].**

Plants are the natural reservoir of many antimicrobial, anticancer agents, analgesics, anti-diarrheal as well as various therapeutic activities. Bangladeshi people have traditional medical practice as an integral part of their culture. A lot of medicinal plants are available for the

treatment of various diseases. However, scientific studies have been conducted on only a relatively few medicinal plants, and then only to a superficial extent **[2].**

Medicinal plants constitute an important natural wealth of a country. They play a significant role in providing primary health care services to rural people. They serve as therapeutic agents as well as important raw materials for the manufacture of traditional and modern medicine. Substantial amount of foreign exchange can be earned by exporting medicinal plants to other countries. In this way indigenous medicinal plants play significant role of an economy of a country **[2].**

Plants are a source of large amount of drugs comprising to different groups such asantispasmodics, emetics, anti-cancer, antimicrobials etc. A large number of the plants are claimed to possess the antibiotic properties (e.g.; *Penicillium notatum*) in the traditional system and are also used extensively by the tribal people worldwide. It is now believed that nature has given the cure of every disease in one way or another. Plants have been known to relieve various diseases in Ayurveda. Therefore, the researchers today are emphasizing on evaluation and characterization of various plants and plant constituents against a number of diseases based on their traditional claims of the plants given in Ayurveda. Extraction of the bioactive plant constituents has always been a challenging task for the researchers **[1, 2].**

PHYTOCHEMICALS

Phytochemistry is the study of phytochemicals produced in plants, describing the isolation, purification, identification, and structure of the large number of secondary metabolic compounds found in plants. Effect of extracted plant phytochemicals depends on:

- The nature of the plant material
- Its origin

- Degree of processing
- Moisture content
- Particle size **[5]**

A phytochemical chart is given below to understand the term in an easy way **[5].**

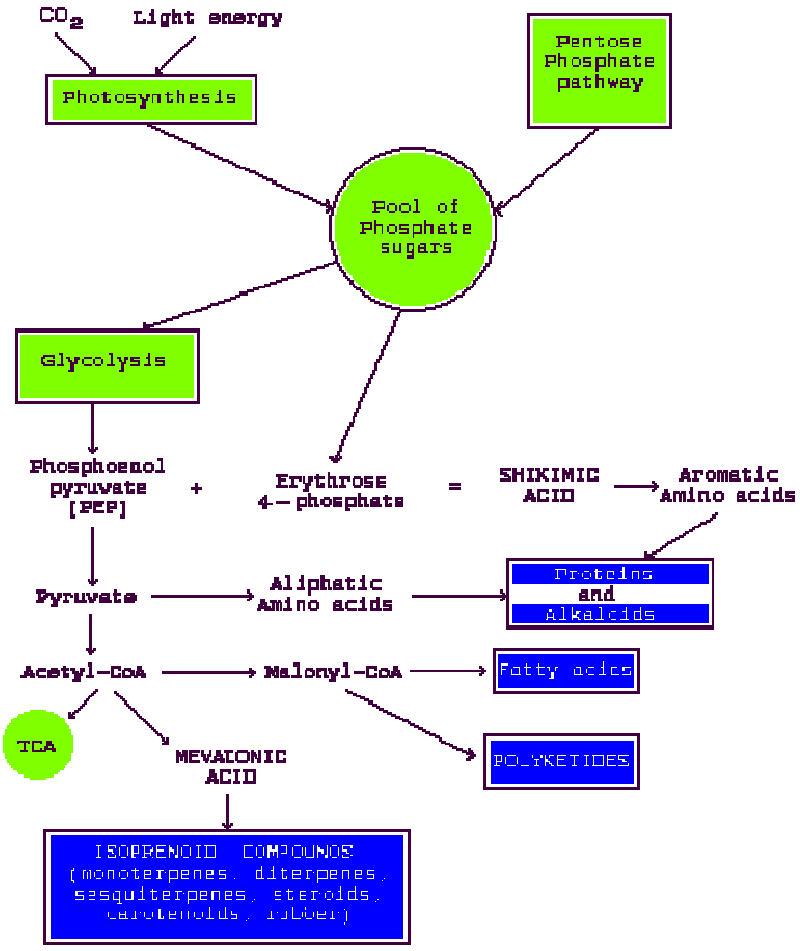


Figure 1.1: Formation of general phytochemicals

Carbon dioxide gas deals with the photosynthesis process in plants in the presence of light energy. Photosynthesis and pentose pathway together pools the phosphate group present in the sugar molecules of plants which leads to glycolysis process and which is accounted for

producing many of phytochemicals of plants, such as, shikimic acid, proteins, aliphatic and aromatic acids, mevalonic acids, fatty acids, flavanoids, terpinoids, steroids etc **[5].**

There are lots of medicinal plants which contain a number of phytochemicals and those phytochemicals are used medicine purpose to treat various kinds of diseases. In the following table a list is shown of phytochemicals having medicinal values **[5].**

Table a: Structural features and activities of various phytochemicals from plants **[5]**

Phytochemicals	**Structural features**	**Example(s)**	**Activities**
Phenols and Polyphenols	C3 side chain, -OH groups, phenol ring	Catechol, Epicatechin, Cinnamic acid	Antimicrobial, Anthelmintic, Antidiarrhoeal
Quinones	Aromatic rings, two ketone substitutions	Hypericin	Antimicrobial,
Flavones Flavonoids Flavonols	Phenolic structure, one carbonyl group Hydroxylated phenols, Flavones + 3-hydroxyl group	Abyssinone Chrysin, Quercetin, Rutin Totarol	Antimicrobial, Antidiarrhoeal
Tannins	Polymeric phenols (Mol. Wt. 500-3000	Ellagitannin	Antimicrobial, Anthelmintic
Coumarins	Phenols made of fused benzene and α-pyrone rings	Warfarin	Antimicrobial,
Terpenoids and essential oils	Acetate units + fatty acids, extensive branching	Capsaicin	Antimicrobial, Antidiarrhoeal

Alkaloids	Heterocyclic nitrogen compounds	Berberine, Piperine, Palmatine, Tetrahydropalmatine	Antimicrobial, Anthelmintic, Antidiarrhoeal
Lectins and Polypeptides	Proteins	Mannose-specific agglutinin, Fabatin	Antidiarrhoeal
Glycosides	Sugar+non chaobohydrate moiety	Amygdalin	Antidiarrhoeal

NECESSITY OF STUDYING OF MEDICINAL PLANTS

- Medicinal plants have played an essential role in the development of human culture, for example religions and different ceremonies. (E.g. *Dutura has long been associated with the worship of Shiva, the Indian god*).
- Many of the modern medicines are produced indirectly from medicinal plants, for example aspirin.
- Plants are directly used as medicines by a majority of cultures around the world, for example Chinese medicine and Indian medicine.
- Many food crops have medicinal effects, for example garlic.
- Medicinal plants are resources of new drugs. It is estimated there are more than 250, 000 flower plant species **[4].**
- Studying medicinal plants helps to understand plant toxicity and protect human and animals from natural poisons.
- Cultivation and preservation of medicinal plants protect biological diversity, for example metabolic engineering of plants.
- Plant resources (E.g. *Angiosperm, Gymnosperm, Seedless vascular plants, Bryophytes*) for new medicine.

- The medicinal plants find application in pharmaceutical, cosmetic, agricultural and food industry **[4].**
- With onset of scientific research in herbals, it is becoming clearer that the medicinal herbs have a potential in today's synthetic era, as numbers of medicines are becoming resistant. According to one estimate only 20% of the plant flora has been studied and 60%of synthetic medicines owe their origin to plants. Ancient knowledge coupled with scientific principles can come to the forefront and provide us with powerful remedies to eradicate the diseases.
- To identify alternative and complementary medicine.
- To reduce the toxicity of drug therapy especially toxicity reduction of synthetic and semi synthetic drugs.
- To find the lead compound diversification to treat various diseases **[4].**

HISTORY OF TRADITIONAL HERBAL MEDICINE IN BANGLADESH

Traditional Medicine is the medicine or treatment based on traditional uses of plants, animals or their products, other natural substances (including some inorganic chemicals), religious verses, cultural practices, and physical manipulations including torture. As this system of medicine has been in use almost unchanged generation after generation throughout the ages for the treatment of various physical and psychological diseases, it is called traditional. Most of the times, the type, preparation, and uses of traditional medicines are largely influenced by folklore customs and the cultural habits, social practices, religious beliefs and, in many cases, superstitions of the people who prescribe or use them **[1, 6].**

The earliest mention of traditional medicine is found in *Rigveda,* the oldest repository of knowledge in this subcontinent. Later *Ayurveda,* developed from the *Vedic* concept of life,

became the important source of all systems of medical sciences. In course of time it became a part of culture and heritage of the people of the Indian subcontinent **[6].**

Traditional medicine involves the use of both material and non-material components. The material components invariably comprise parts or organs of plants and their products. They also consist of animal organs, minerals and other natural substances. The non-material components, which constitute important items of religious and spiritual medicines, include torture, charms, magic, incantations, religious verses, amulets and rituals like sacrifices, appeasement of evil spirits, etc. **[1, 6].**

Treatments in traditional medicine are carried out by internal and external application of medicaments, physical manipulation of various parts of the body, performing rituals, psychological treatment, and also by minor surgery. *Ayurvedic* medicinal preparations consist mainly of plant materials in the form of powders, semi-solid preparations, decoctions, elixirs and distillates. Many of them also contain inorganic chemical substances, minerals and animal products. Alcoholic extracts and alcoholic solutions of the ingredients, tinctures and elixirs are also frequently used in *Ayurvedi*c medicine **[1, 6].**

Whole plants or their powders or pastes or products and their extracts, infusions, decoctions and distillates constitute the major constituents of *Unani* medicine. Minerals, inorganic chemicals and animal products are also frequently used in preparing these medicines **[1].**

For hundreds of years, the medical knowledge in Bangladesh is termed as Ayurveda. Ayurveda remains an important system of medicine and drug therapy in Bangladesh. Plant alkaloids are the primary active ingredients of Ayurvedic drugs. Today the pharmacologically active ingredients of many Ayurvedic medicines are being identified and their usefulness in drug therapy being determined. As only a certain percentage of plants are used in traditional medicines, it is roughly

estimated that of the discovered 17,000 species, nearly 3,000 species are used in medicinal field **[1, 6].** Some crude drugs used as medicine in Bangladesh are reported in following table.

Table 1.1: Some crude drugs used as medicine in Bangladesh **[1, 6]**

Common name	**Botanical name**	**Uses**
Amla	*Emblica officinalis*	Vitamin - C, Cough, Diabetes, cold, Laxative, hyper acidity.
Ashok	*Saraca asoca*	Menstrual Pain, uterine, disorder, Deiabetes.
Bael / Bilva	*Aegle marmelous*	Diarrrhoea, Dysentry, Constipation.
Chiraita	*Swertia chiraita*	Skin Desease, Burning, censation, fever.
Kalmegh/ Bhui neem	*Andrographis paniculata*	Fever, weekness, release of gas.
Long peeper / Pippali	*Peeper longum*	Appetizer, enlarged spleen , Bronchities, Cold, antidote.
Pashan Bheda / Pathar Chur	*Coleus barbatus*	Kidny stone, Calculus.
Sandal Wood	*Santalum album*	Skin disorder, Burning, sensation, Jaundice, Cough.
Satavari	*Asparagus racemosus*	Enhance lactation, general weekness, fatigue, cough.
Senna	*Casia augustifolia*	General debility tonic, aphrodisiac.

Tulsi	*Ocimum sanclum*	Cough, Cold, bronchitis,expectorand
Pippermint	*Mentha pipertia*	Digestive, Pain killer
Henna/Mehd	*Lawsennia iermis*	Burning, Steam, Anti Imflamatary
Gritkumari	*Aloe verra*	Laxative, Wound healing, Skin burns & care,Ulcer
Sada Bahar	*Vincea rosea*	Leaukamia, Hypotensiv, Antispasmodic , Atidot
Vringraj	*Eclipta alba*	Anti-inflamatory, Digestive, hairtonic
Neem	*Azardirchata indica*	Sdedative, analgesic, epilepsy, hypertensive
Anantamool/sariva	*Hemibi smus indicus*	Appetiser, Carminative, aphrodisiac, Astringent
Kantakari	*Solanum xanthocarpum*	Diuretic, Antiinflamatory, Appetiser, Stomachic
Shankhamul	*Geodorum denciflorum*	Antidiabetic

PLANT METABOLITES

Metabolites are compounds synthesized by plants for both essential functions, such as growth and development (primary metabolites), and specific functions, such as pollinator attraction or defense against herbivory (secondary metabolites). Metabolites are organic compounds

synthesized by organisms using enzyme-mediated chemical reactions called metabolic pathways. Primary metabolites have functions that are essential to growth and development and are therefore present in all plants **[7].** In contrast, secondary metabolites are variously distributed in the plant kingdom, and their functions are specific to the plants in which they are found. Secondary metabolites are often colored, fragrant, or flavorful compounds and they typically mediate the interaction of plants with other organisms. Such interactions include those of plant-pollinator, plant-pathogen, and plant-herbivore **[7].**

Primary Metabolites

Primary metabolites comprise many different types of organic compounds, including, but not limited to, carbohydrates, lipids, proteins, and nucleic acids. They are found universally in the plant kingdom because they are the components or products of fundamental metabolic pathways or cycles such as glycolysis, the Krebs cycle, and the Calvin cycle. Because of the importance of these and other primary pathways in enabling a plant to synthesize, assimilate, and degrade organic compounds, primary metabolites are essential. Examples of primary metabolites include energy rich fuel molecules, such as sucrose and starch, structural components such as cellulose, informational molecules such as DNA (deoxyribonucleic acid) and RNA (ribonucleic acid), and pigments, such as chlorophyll. In addition to having fundamental roles in plant growth and development, some primary metabolites are precursors (starting materials) for the synthesis of secondary metabolites **[7].**

Secondary Metabolites

Secondary metabolites are organic compounds that are not directly involved in the normal growth, development, or reproduction of an organism. Unlike primary metabolites, absence of secondary metabolites does not result in immediate death, but rather in long-term impairment of

the organism's survivability, fecundity, or aesthetics, or perhaps in no significant change at all. Secondary metabolites are often restricted to a narrow set of species within a phylogenetic group. Secondary metabolites often play an important role in plant defense against herbivory and other interspecies defenses. Humans use secondary metabolites as medicines, flavorings, and recreational drugs **[7].**

Most of the secondary metabolites of interest to humankind fit into categories which classify secondary metabolites based on their biosynthetic origin. Since secondary metabolites are often created by modified primary metabolite synthases, or "borrow" substrates of primary metabolite origin, these categories should not be interpreted as saying that all molecules in the category are secondary metabolites **[7].**

Secondary metabolites largely fall into three classes of compounds: alkaloids, terpenoids, and phenolics. However, these classes of compounds also include primary metabolites, so whether a compound is a primary or secondary metabolite is a distinction based not only on its chemical structure but also on its function and distribution within the plant kingdom. Many thousands of secondary metabolites have been isolated from plants, and many of them have powerful physiological effects in humans and are used as medicines. It is only since the late twentieth century that secondary metabolites have been clearly recognized as having important functions in plants **[7].**

Some examples of secondary metabolites include alkaloids (Hyoscyamine, Atropine, Cocaine, Scopolamine, Codeine, Morphine etc.), terpinoids (Steroid, Saponins), glycosides, natural phenols, phenazines, biphenyls, dibenzofurans, phytoalexines, fatty acids etc. **[7].**

C h a p t e r A

N y c t a n t h e s a r b o r t r i s

Chapter – 1

INTRODUCTION

1.1 OVERVIEW OF FAMILY *OLEACEAE*

Oleaceae are a family containing 24 extant genera and around 600 species of mesophytic shrubs, trees and occasionally vines. As shrubs, members of this family may be twine climbers, or scramblers **[8].**

Table 1.2: Some genera with common names of *Oleaceae* family **[8]**

Genera	**Common name**
Abeliophyllum	White Forsythia
Chionanthus	Fringetree
Forestiera	Swamp-privet
Forsythia	Forsythia
Fraxinus	Ash
Jasminum	Jasmine
Ligustrum	Privet
Osmanthus	Osmanthus
Olea	Olive
Phillyrea	Mock-privet
Syringa	Lilac

1.1.1 Leaves

The family is characterized by opposite leaves that may be simple or compound (either pinnate or ternate), without stipule. Alternate or whorled arrangements are rarely observed, with some *Jasminum* species presenting spiral configuration. The laminas are pinnately-veined and can be serrate, dentate or entire at margin. Domatia are observed in certain taxa. The leaves may be

either deciduous or evergreen, with evergreen species predominating in warm temperate and tropical regions, and deciduous species predominating in colder regions **[8].**

1.1.2 Flowers

The flowers are most often bisexual and actinomorphic, occurring in racemes or panicles, and often fragrant. The calyx, which may or may not be present, and the corolla are gamosepalous and four-lobed. The androecium has tow stamens inserted in the perigynous zone and alternate with the lobes. The stigmas are two-lobed. The gynoecium consists of a compound pistil with two carpels. The ovary is superior with two locules, each of which bearing two axillary ovules. Sometimes the base of the ovary is circled by a nectary disk. The plants are most often hermaphrodite but sometimes polygamomonoecious **[8].**

1.1.3 Fruits

Oleaceae fruit can be berries, drupes, capsules or samaras **[8].**

1.1.4 Uses

Many members of the family are economically significant. The olive (*Olea europaea*)(Fig: 2.1) is important for its fruit and the oil extracted from it, the ashes (*Fraxinus*)(Fig;2.2) are valued for tough wood, and forsythia, lilacs, jasmines, osmanthuses, privets, and fringetrees are valued as ornamental plants in gardens and landscaping. Forsythia fruit is used to treat colds and viral infections and fevers in traditional Chinese medicine. *Nyctanthes arbortristis* is used in the treatment of piles **[8].**

1.2 COMMON NAME OF *NYCTANTHES ARBOR-TRISTIS* LINN

There are a number of common names of *Nyctanthes arbortristis* in the language of Bengali, Hindi, Marathi, and English. Some of them are listed below: (Table: 2.2) **[9]**

Figure 1.2: *Olea_europaea*

Figure 1.3: *Syringa vulgaris*

Table 1.3: Common name of *Nyctanthes arbortristis* (sad tree) **[9]**

Common name	**Area**
Seri gading	Malaysia
Sheuli or Shefali	West Bengal, Bangladesh
Night-flowering Jasmine, Coral Jasmine	English
Harashringara, Harsingar	Hindi
Kannika	Thailand
Parijata, Paghala	Nepal

1.3 BROAD OR TAXONOMICAL CLASSIFICATION

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Lamiales

Family: Oleaceae

Genus: *Nyctanthes*

Species: *arbor-tristis*

Binomial name: *Nyctanthes arbor-tristis* **[9]**

1.4 DISTRIBUTION AND HABITAT

Nyctanthes arbor-tristis Linn is native to India, distributed widely in sub-Himalayan regions and southward to Godavari. It is also widely distributed in Bangladesh, Indo-Pak subcontinent and South-East Asia, tropical and sub-tropical South East Asia. It grows in Indo-Malayan region and distributed across Terai tracts as well as Burma and Ceylon. It tolerates moderate shade and is often found as undergrowth in dry deciduous forests. It is also found in Thailand **[8, 9].**

1.5 BOTANICAL DESCRIPTION OF PLANT PARTS

Nyctanthes arbor-tristis Linn is a large shrub growing to 10 m tall, with flaky grey bark, stiff whitish hair, young branches and rough leaves. The flowers are fragrant, with a five to eightlobed white corolla with an orangered centre; they are produced in clusters of two to seven together, with individual flowers opening at dusk and finishing at dawn. Calyx is 6-8 mm long, narrowly campanulate, hairy outside, glaborous inside truncate or obscurely toothed or lobed, ciliated. Corolla glaborous and is more than 13 mm long; tube is 6-8 mm long, orange coloured, about equalling the limbs; lobes are white and unequally obcordate and cuneate. The leaves are opposite, simple, 6–12 cm long and 2–6.5 cm broad, with an entire margin. The fruit is a flat brown heart-shaped to round capsule 2 cm diameter, with two sections each containing a single seed. These are long and broad, obcordate or nearly orbicular, compressed, 2-celled. Seeds are exalbuminous, testa are thick, outer layer of large transparent cells is heavily vascularised **[8, 9].**

1.6 USES OF *NYCTANTHES ARBOR-TRISTIS* LINN

1.6.1 Traditional Uses

Traditionally the flowers are gathered for religious offerings and to make garlands. The orange heart is used for dyeing silk and cotton, a practice that started with Buddhist monks whose orange robes were given their colour by this flower. The Parijata is regarded in Hindu mythology as one of the five wish-granting trees of Devaloka **[9].**

Different parts of *Nyctanthes arbortristis* Linn are known to possess various ailments by tribal people of Indian subcontinent with its use in Ayurveda, Unani systems of medicines **[9].**

1.6.2 Flowers

The flowers are used as stomachic, carminative, astringent to bowel, antibilious, expectorant, hair tonic and in the treatment of piles and various skin diseases and in the treatment of ophthalmic purposes. The bright orange corolla tubes of the flowers contain a coloring substance nyctanthin, which is identical with ά-Crocetin from Saffron. The corolla tubes were formerly used for dyeing silk, sometimes together with Safflower or turmeric **[9].**

1.6.3 Stems

Traditionally the powdered stem bark is given in rheumatic joint pain, in treatment of malaria and also used as an expectorant. The bark is used for the treatment of snakebite and bronchitis. The stem bark pounded with *Zingiber officinale* and *Piper longum* is boiled in water and the resultant liquid is taken for two days for the treatment of malaria. The resulting paste on mixing with Arjuna bark is rubbed on the body to treat internal injury and for joint broken bones **[9].**

1.6.4 Leaves

The leaves of *Nyctanthes arbor-tristis* Linn are used extensively in Ayurvedic medicine for the treament of various diseases such as sciatica, chronic fever, rheumatism, and internal worm

infections, and as a laxative, diaphoretic and diuretic. Leaves are used in cough reducion. Leaf juice is mixed in honey and given thrice daily for the treatment of cough. Paste of leaves is given with honey for the treatment of fever, high blood pressure and diabetes. Juice of the leaves is used as digestives, antidote to reptile venoms, mild bitter tonic, laxative, diaphoretic and diuretic. Leaves are also used in the enlargement of spleen. The leaf juice is used to treat loss of appetite, piles, liver disorders, biliary disorders, intestinal worms, chronic fever, obstinate sciatica, rheumatism and fever with rigors. The extracted juice of leaves acts as a cholagogue, laxativeand mild bitter tonic. It is given with little sugar to children as a remedy for intestinal ailments **[9].**

1.6.5 Seeds

The seeds are used as anthelmintics and in alopecia. It is antibilious and an expectorant, and is also useful in bilious fevers. The powdered seeds are used to cure scurfy affections of scalp, piles and skin diseases **[9].**

A B

C D E

Figure 1.4: *Nyctanthes arbor-tristis* Linn (A→ Whole plant, B→ Flowers, C→ Leaves, D→ Bark, E→Fruits)

1.7 Chemical constituents of *Nyctanthes arbor-tristis* Linn

1.7.1 Leaves

Leaves contain D-mannitol, β-sitosterole, Flavanol glycosides, Astragaline, Nicotiflorin, Oleanolic acid, Nyctanthic acid, Tannic acid, Ascorbic acid, Methyl salicylate, Amorphous glycoside, Amorphous resin, Trace of volatile oil, Carotene, Friedeline, Lupeol, Mannitol, Glucose, Fructose, Iridoid glycosides, Benzoic acid **[9].**

1.7.2 Flowers

Flowers contain Essential oil, Nyctanthin, d-mannitol, Tannin, Glucose, Carotenoid, Glycosides, β-monogentiobioside ester of α-crocetin (or crocin-3), β-monogentiobioside, β-D monoglucoside ester of α-crocetin, β-digentiobioside ester of α-crocetin (or crocin-1) **[9].**

1.7.3 Seeds

Seeds contain Arbortristoside A&B, Glycerides of linoleic acid, oleic acid, lignoceric acid, stearic acid, palmitic and myristic acids, nyctanthic acid, 3-4 secotriterpene acid, a water soluble polysaccharide composed of D-glucose and D mannose **[9].**

1.7.4 Bark

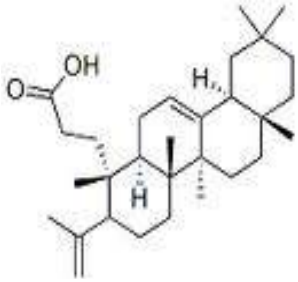
Bark contains Glycosides and alkaloids **[9].**

1.7.5 Stem

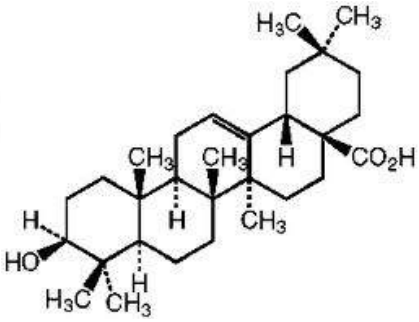
Stem contains Glycoside-naringenin-4-0-β-glucapyranosyl-α-xylopyranoside and β-sitosterol **[9].**

1.7.6 Flower oil

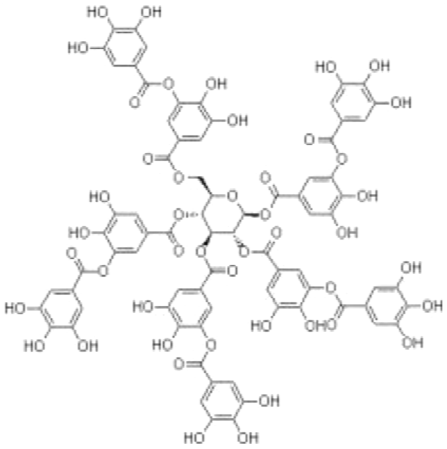
Flower oil contains α-pinene, p-cymene, 1- hexanol methyl heptanone, phenyl acetaldehyde, 1-deconol and anisaldehyde **[9].**



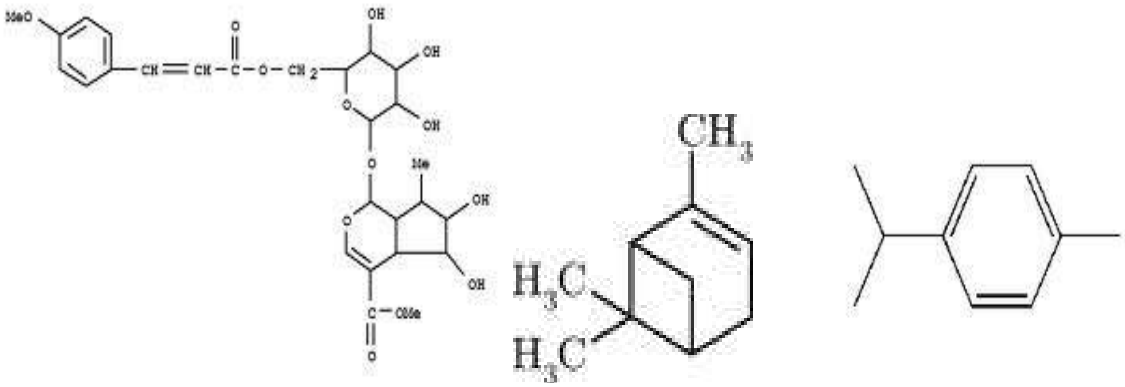
Nycthintic acid



Oleanolic acid



Tannic acid



Arbortristoside A α-Pinene p-cymene

Figure 1.4: Some chemical constituents of *Nyctanthes arbor-tristis* Linn with structure

Chapter – 2

LITERATUE REVIEW

2.1 REVIEWS ON PHYTOCHEMICAL INVESTIGATION

2.1.1 Phytoconstituents from Leaves

Three new benzoic esters of Loganin and 6-β-hydroxy loganin, namely Arborside-A, Arborside-B, and Arborside-C were found to be present in the leaves. From leaves 10-Benzoylnyctanthoside named as Arborside-D were isolated. Other iridoid glycosides that were reported are 6, 7-Di-*O*-benzoyl nyctanthoside, 6-*O*-transcinnamoyl- 6-β-hydroxy loganin and 7-*O*-trans cinnamoyl-6-β-hydroxy loganin from the leaves. A phenyl propanoid glucoside Desrhamnosylverbascoside was reported from the leaves. Leaves also contain the alkaloid Nyctanthine along with Mannitol, β-Amyrin β-Sitosterol, Hentriacontane, Benzoic acid, Astragalin, Nicotiflorin, Oleanolicacid, Nyctanthic acid, Friedelin and Lupeol **[10].**

2.1.2 Phytoconstituents from Stems

A glycoside Naringenin-4′-O-β-glucopyranosyl- α-xylopyranoside was screened from the stem chromatographed the chloroform extract of the stem over silica gel column and reported the presence of β-Amyrin, Arbortristoside-A, Oleanolic acid, Nyctoside-A, Nyctantic acid and 6- β-hydroxyloganin **[10].**

2.1.3 Phytoconstituents from Seeds

Seeds give a water soluble polysaccharide containing D-Glucose and D-Mannose, indicating that the polysaccharide is a glucomannan. Iridoid glucosides Arbortristoside-A, Arbortristoside B, Arbortristoside-C and 6-β-hydroxyloganin have been isolated. Further examination of the seeds led to the isolation and identification of two minor iridoid glucosides, Arbortristoside-D and Arbortristoside-E together with the previously reported Arbortristoside-B. Other iridoid glucosides reported are Nyctanthoside, A phenyl propanoid glucoside, Nyctoside-A was isolated from the methanolic extract of the seeds **[10].**

2.1.4 Phytoconstituents From Flowers

Ethanolic extract from the flowers led to the isolation of an antiplasmodial cyclohexylethanoid, Rengyolone, a new iridoid glucoside 6-*O* -trans-cinnamoyl-7-*O*-acetyl-6-β-hydroxyloganin and three known iridoid glucosides, Arborside-C, 6-β-hydroxyloganin and Nyctanthoside. Rengyolone was first isolated from Forsythia suspansa (Oleaceae), an important plant of the crude drug "rengyo". It was also isolated, as a Halleridone from the African medicinal plant Halleria lucida (Scrophulariaceae) and as a cytotoxic constituent from Cornus controversa (cornaceae). It has been isolated from the flowers of the Thai medicinal plant, Millingtonia hortensis (Bignoniaceae) but has not previously been isolated from Nyctanthes. It was found that after several months the compound Arborside-C has changed to the isomeric structure with the benzoate group shifted to C-6-OH. This structure is named as Isoarborside-C **[10].**

2.1.5 Phytoconstituents From Roots

The root part of the plant composed of alkaloids, tannins and glucosides. From the chloroform extract of the root β-Sitosterol and Oleanolic acid has been isotated **[10].**

2.1.6 Structure Of Various Constituents Of *Nactanthes Arbor-Tristis*

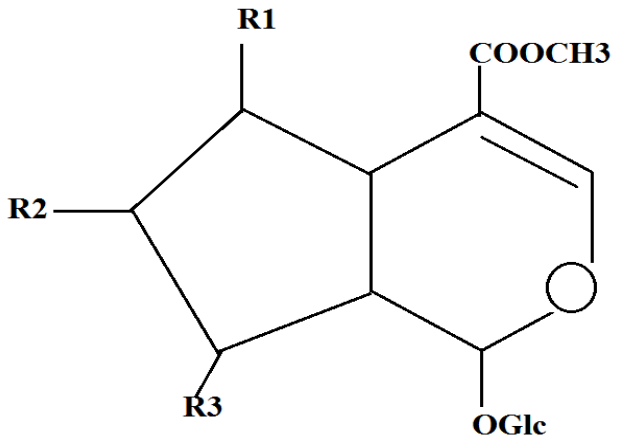


Figure 2.1: General structure of various chemical constituents of *Nactanthes arbor-tristis*

Table 2.1: Structure of the phytoconstituents isolated from different parts (leaves,stem,seed,flowers and root) of *Nyctanthes arbortristis* **[10]**

Chemical constituents	**R_1**	**R_2**	**R_3**
Loganin	H	OH	CH_3
6-β -hydroxyloganin	OH	OH	CH_3
Arborside-A	OBz	OBz	CH_3
Arborside-B	H	OBz	CH_3
Arborside-C	OH	OBz	CH_3
Arborside-D	OH	OH	CH_2OBz
6,7-Di-*O*-benzoyl nyctanthoside	OBz	OBz	CH_2OH
6-O-trans-cinnamoyl-6-β-hydroxyloganin	*O*-cinnamoyl	OH	CH_3
7-O-trans-cinnamoyl-6-β-hydroxyloganin	OH	*O*-cinnamoyl	CH_3
Arbortristoside-A	*O,P*-methoxy cinnamoyl	OH	CH_3
Arbortristoside-B	*O*-caffeyol	OH	CH_2OH
Arbortristoside-C	*O,P*-caumaroyl	OH	CH_3
Arbortristoside-D	OH	OH	CH_2O-trans caffeoyl
Nyctanthoside	OH	OH	CH_2OH
6-*O*-trans cinnamoyl-7-*O*-acetyl-6-β -hydroxyloganin	*O,P*-cinnamoyl	O-acetyl	CH_3
Isoarborside-C	OBz	OH	CH_3

2.2 REVIEWS ON PHARMACOLOGICAL INVESTIGATION

2.2.1 Anti Anxiety

Hydroalcoholic extracts of *Nyctanthes arbour tristis linn.*(NAT) have anxiolytic potential. Using hydro-alcoholic mixture, dried plant parts of *Nyctanthes arbor tristis linn.* was extracted, concentrated by distilling off the solvent and then evaporated to dryness on the water bath and then stored in an air tight container in a refrigerator till used **[11].**

2.2.2 Anti-Trypanosomal Potential

Antitrypanosomal potential of a crude 50% ethanolic extract of *Nyctanthes arbor-tristis* leaves was evaluated in vitro and in vivo. The extract exhibited trypanocidal activity at the highest concentration (1000 µg/ml) tested. In vivo studies revealed that the extract exerted antitrypanosomal effects at doses of 300 and 1000 mg/kg, intraperitoneally and significantly prolonged the survival period of the *Trypanosoma evansi* infected mice. However, as soon as the treatment with the extract was discontinued, the parasitaemia increased and resulted in the death of the experimental animals **[12].**

2.2.3 Sedative Effects

Hot infusion of *Nyctanthes arbo-tristis.* Linn. (Oleaceae) flowers have potential sedative effect. Different concentrations of hot flower infusion was made (3.7, 7.5, 12.5, or 18.7 mg/kg) and orally administered. Sedative potential was assessed 2 h post-treatment. The infusion had a moderate dose-dependent conscious sedative activity in male but, surprisingly, not in female rats. The infusion was well tolerated (in terms of overt toxic signs, liver or kidney functions) even following subchronic treatments and also did not show any overt signs of dependence (classical sign of withdrawal reactions) **[13].**

2.2.4 Immuno-Pharmacological Investigation

Table 2.2 Immuno pharmacological investigation with findings **[14]**

Test name	**Findings**
Leukocyte Count	*Nyctanthes arbor-tristis* extract helps to increase white blood cell count and also has a positive effect on spleen weight and spleen leukocyte count.
Heamagglutination Antibody Titer	It was observed that with five doses of *Nyctanthes arbor-tristis* extract, a dose related increase in heamagglutination antibody titer was observed.
Plaque Forming Cell Assay	*Nyctanthes arbor-tristis* extract administration for 5 daysbeginning from the day of sensitization with SRBC produced 30–35% increase in antibody secreting cells in mouse spleen.
Active Systemic Anaphylaxis	*Nyctanthes arbor-tristis* extract has a positive effect on systemic anaphylaxis.

2.2.5 Anti-Aggressive Activity in Rodents

The fresh juice obtained from the leaves of the plant was found to have anti malarial activity. The 50% ethanolic extract of the seeds, leaves, roots, flowers and stem of the plant has been proved to posses antiamoebic and anti allergic properties. Leaf extract of the plant showed anti-inflammatory, analgesic, antipyretic and ulcerogenic activities. The leaves, seeds and flowers of

the plant were reported to exert immunostimulant activity. The water soluble fraction of the ethanolic extract has been proved to posses tranquilizing, antihistaminic, purgative effects and depletion of tumor necrosis factor- a. The arbortristoside A isolated from the seeds was found to have antitumor acivity **[15].**

2.2.6 Analgesic, Antipyretic and Ulcerogenic Activity

Besides being used in the treatment of sciatica and arthritis, the leaves of Nyctanthesarbortristis are advocated for various kinds of fevers and painful conditions. In the present study, the water-soluble portion of an ethanol extract of the leaves was screened for analgesic, antipyretic and ulcerogenic activities. The extract exhibited significant aspirin-like antinociceptive activity but failed to produce morphine-like analgesia. It was also found to possess antipyretic activity against brewer's yeast-induced pyrexia in rats **[16].**

2.2.7 Depletion of Tumor Necrosis Factor-A (TNF-A) In Mice

The effect of the water soluble fraction of the ethanol extract of Nyctanthesarbor-tristis (NAT) on tumor necrosis factor-α (TNF-α) level in plasma of arthritic and soluble protein A (SpA)-treated Balb/c mice has been studied. Oral administration of this fraction in arthritic mice showed a consistent depletion of TNF-α from the host plasma. A similar depletion of TNF-α in the plasma of SpA-treated mice has been observed. The extract also reduces plasma interferon-γ level but the plasma IgM and IgG levels are not affected. The implications of these observations are discussed in the light of management of TNF-α in clinical disorders **[17].**

2.2.8 Anti-Inflammatory Activity

The water soluble portion of the alcoholic extract of the leaves of *N. arbor-tristis* was screened

for the presence of anti-inflammatory activity. *N. arbor-tristis* inhibited the acute inflammatory oedema produced by different phlogistic agents, viz. carrageenin, formalin, histamine, 5-hydroxytryptamine and hyaluronidase in the hindpaw of rats. The acute inflammatory swelling in the knee joint of rats induced by turpentine oil was also significantly reduced. In subacute models, *N. arbor-tristis* was found to check granulation tissue fornation significantly in the granuloma pouch and cotton pellet test. Acute and chronic phases of formaldehyde induced arthritis were significantly inhibited. *N. arbo- tristis* was also found to inhibit the inflammation produced by Freund's adjuvant arthritis and PPD induced tuberculin reaction **[18].**

2.2.9 In The Treatment of Piles, Gout, Dry Cough

The seeds of *N. arbor-tristis* are used in treatment of piles. The decoction of *N. arbo- tristis* flowers are used in treatment of gout. Leaves are used against dry cough. The aqueous paste of leaves is used externally in treatment of skin related troubles specifically in treatment of ring worm. The young leaves are used as female tonic. *N. arbor-tristis* also has hypoglycaemic effect, potentiating action of exogenous insulin and streptozotocin- induced diabetic rat model **[19].**

2.2.10 Anti-Spermatogenic Effects

Methanol extracts of *Nycthantes arbortristis stem bark* was found to possess anti spermatogenic activity in male albino rats. Stem bark of *Nycthantes arbortristis* was collected. Then shade dried stem bark (3kg) was crushed and coarsely powered and then extracted in a soxhlate apparatus with 70% methanol for 40-45hrs. The extract was filtered and methanol was removed under reduce pressure to obtain dark viscous brown mass. The concentrated extract (40g) was treated with acetonitrile (CH_3CN) to remove fats. The non-fatty portion was then treated with charcoal

to remove colored impurities. The filtrate was concentrated (9g) and a part of the methanol extract of *Nycthantes arbortristis* stem bark was administered orally to adult male albino rats at the dose level of 100mg/kg b.wt. for 2 months. Then the rats were autopsied, the testes and accessory reproductive organs were removed and weighed. The organs were processed for biochemical estimations and histopathological observations along with assessment of sperm motility and sperm density in cauda epididymis **[20].** The results revealed that the *Nycthantes arbortristis* stem bark extract treatment caused a significant decrease in weight of testes and accessory reproductive organs whereas body weight did not show any significant change when compared to control. Total suppression of fertility resulted due to significant reduction in sperm motility as well as sperm density. Significant reduction was also observed in biochemical estimation of testicular protein, sialic acid, g;ycogen and seminal vescular fructose whereas cholesterol content of testes was increased. A marked diminution in the germ cell population was noticed. Production of spermatids declined by was 64.29% **[20].** Seminiferous tubular diameter and Leydig cell nuclear area showed notable reduction. The Sertoli cells count and its cross-sectional surface area was also significantly reduced. There was no significant alteration in the blood and serum parameters throughout the course of investigation. In conclusion *Nycthantes arbortristis* methanol stem extract causes impairment of testicular function and effects spermatogenesis in male rats **[20].**

2.2.11 Cytotoxic Evaluation

The potential cytotoxic effect of a new benzofuran derivative, 4-hydroxy hexahydrobenzofuran-7-one isolated from *Nycthantes arbortristis* was evaluated on Ehrlich Ascite Carcinoma Cells (EAC) in Swiss Albino Mice. The fresh flowers (500gm) were taken in an aspirator and soaked in 2.5litres rectified spirit at room temperature for 15days with continuous changing the fresh

flowers with the old ones every three days interval. The rectified spirit extract after evaporation of the solvent was fractionated with petroleum ether, chloroform, ethyl acetate and finally with methanol. The compound that has the ability to suppress the growth of tumour cells more than 75% is considered to be highly anticarcinogenic **[21].** The compound 4-hydroxy hexahydrobenzofuran-7-one was found to inhibit the cell growth only 43.27%. Non significant difference was observed between the average no. of cells counted between experimental and control animals. It was also observed that there was no abnormality in the behaviour in either control or experimental animals indicating that 4-hydroxy hexahydrobenzofuran-7-one has no adverse effect on central nervous system. Therefore it can be concluded that 4-hydroxy hexahydrobenzofuran-7-one has no significant cytotoxicity on EAC cells in mice at the dose and duration used in the study **[21].**

2.2.12 Amelioration of CCl_4-Induced Hepatosuppression in Wistar Albino Rats

A study was conducted which dealt with the amelioration by *Nyctanthes arbortristis* Linn. Leaves extract against hepatosuppression induced by carbon tetrachloride (CCl_4), which was evaluated in terms of serum marker enzymes like viz. GOT, GPT, Alkaline phosphate, glucose, cholesterol, and total protein concentration in blood. At 40 ^{o}C, leaves were dried and pulverized were extracted with 70% ethanol at room temperature for 72hrs and dried at 60 ^{0}C to give a yellow colored residue. A portion of the residue was dissolved in distilled water, filtered and dried to determine the amount of the water-soluble fraction in the residue. Prior to the experiment residue was dissolved in a saline/Cremophor (0.025%v/v) solution and diluted to desired concentration to give a water soluble fraction (AFSC) **[22].**

The findings indicated the hepatoprotective potential of Nyctanthes arbortristis Linn. Against hepatosuppression possibly involving mechanism related to its ability to block the P-450 mediated CCl_4 bioactivation through selective inhibitors of ROS (reactive oxygen species) like

antioxidants brought about significant inhibition of TBARS suggesting possible involvement of O_2 , HO_2, HO_2 , H_2O_2 and OH. Thus *Nyctanthes arbortristis* Linn. showing protection in liver may prove promising as a rich source of antioxidants because its use is cost effective, especially for peoples in adverse and hazardous circumstances, who are living in poverty **[22].**

Based on the present findings, it can be concluded that the probable mechanism by which the *Nyctanthes arbor-tristis* Linn leaves exerts its protective action against CCl_4-induced hepatocellular metabolic alterations could be by the stimulation of hepatic regeneration through an improved synthesis of proteins, or due to its ability to block the bioactivation of CCl_4 by inhibiting the P-450 2E1 activity and/or its accelerated detoxification and the potential to minimise the deleterious effects of free radicals including the peroxy radicals and its antioxidant activity in combination with the inhibition of lipid peroxidation, thereby the Nyctanthes Arbor – Tristis Linn leaves can be ranked as hepatoprotective agent by the combined synergistic effect of its constituents and micronutrients rather than to any single factor through free radicals scavenging activity **[22].**

2.2.13 Anti-Malarial Activity

In a recent study with *Nyctanthes arbor-tristis* in patients with malaria, the parasite cure was confirmed by the disappearance of the parasitic DNA with polymerase chain reaction; the marked clinical improvement within 48 hours was accompanied by a significant drop in Tumor Necrosis Factor-α (TNF- α) **[23].**

2.2.14 In The Treatment Of Rheumatoid Arthritis

Harshingar (*Nyctanthes arbor tristis* Linn., NAT) has been used widely as a decoction for the treatment of arthritis and sciatica in Indian ayurvedic system of medicine since centuries. The plant has its origin from the Bengal region of India while it is distributed all over sub tropical

regions of country. Arbortristosides, nyctanthic acid, and crocetin are the main active principals of NAT. Water soluble ethanolic extract of NAT leaves have been reported to reduce significantly the levels of inflammatory cytokines (IL-1, TNF-α) in experimental arthritis **[24].**

2.2.15 Antidiabetic Activity

The anti-diabetic activity of methanol extract of root of *Nyctanthes arbor-tristis* Linn is comparable to that of diabetic control animals. The extract poses safe and strong anti-diabetic activity. The extract was prepared by extracting 50g root powders with 400mL of methanol for 18 hours by hot continuous extraction method. The methanolic extract was filtered and partitioned by using petroleum ether to remove impurities **[25].** The solvent was evaporated under pressure and dried in vacuum. The dried extract *Nyctanthes arbor-tristis* Linn thus obtained was used for the assessment of hypoglycaemic activity. It reduces blood glucose level after seven days at the 500 mg/Kg in rats compare with standard drug. It was found that methanolic extract of *Nyctanthes arbor-tristis* Linn roots were more effective in reducing the blood glucose level compare to the standard drug (Glibenclamide) **[26].**

2.2.16 Wound Healing Activity

Study was conducted to evaluate the wound healing activity of Nyctanthes *arbor-trisitis* Lin. The extract was prepared from the plant material for the wound healing activity testing. Initially, the powdered plant material was kept in n-hexane for removing the fatty material of the plants then, extract (Yield; 11.89%) was obtained from the dried powdered leaves (750 gm) of Nyctanthes arbor-tristis using 100% methanol by cold percolation method as described in folk medicine for the assessment of wound healing activity, then, evaporated to dryness to give semi solid crude using vacuum evaporator **[23].**

The dried extract was stored at 2-8°C in refrigerator. The extract was further used for the evaluation of wound healing activity. The study was aimed to minimize tissue damage and provide an adequate tissue perfusion and oxygenation, proper nutrition and moist wound healing environment to restore the anatomical continuity and function of the affected part. In the present study, rats were treated with *Nyctanthes arbor-tristis* methanolic extract 2% w/w ointment for 16 days period, taking observation in every 4th day. It was noticed that complete epithelization of both the wound models takes about 16 days. This is the period of complete healing of wound. Thus, it can be conclude that the plant extract at 300 mg dose/kg b.w. can be a good solution for the healing of both the wounds. Thus, the folklore claim for the use of *Nyctanthes arbor-tristis* leaves in healing of the wounds can be justified by the present study **[23].**

2.2.17 Antibacterial Activity

The antibacterial potential of NAT was evaluated for gram positive (*Staphylococcus aureus*) and gram negative (*Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa*) bacteria. The dried leaf, flower, fruit and seed extracts prepared in ethylacetate and chloroform were used to assess their antibacterial potential in terms of zone of inhibition of bacterial growth. These activities of plant parts were due to the presence of various plants secondary metabolites viz. glycosides and phenolics. The ethanolic and hydro-alcoholic extracts of the leaves were also investigated for its antibacterial performance against both antibiotic resistant and nonresistant strains of Staphylococcus aureus. A benzofuranone, 3, 3a, 7, 7a-tetrahydro-3ahydroxy- 6 (2H) benzo-furanone, was isolated from the flowers. The compound showed significant antibacterial activity against both gram positive and gram negative bacteria **[10].**

2.2.18 Antiviral Activity

Several Iridoid glucoside have been isolated from the different parts of the plant. These compounds belong to the class of cyclopentapyran and possess a wide array of activities.the study showed that the effect of few iridoid glucosides (Arbortristoside-A and C) isolated from the n-butanol fraction of the seeds of the plant against Encephalomyocarditis virus (EMCV) and Semlikiforest virus (SFV) in mice and it was found that Arbortristoside-A and C showed maximum 75% cytopathic inhibition effect against EMCV and SFV **[10].**

2.2.19 Antifungal Activity

Leaves extract has also found its use in antifungal activity. The activity was evident from their inhibitory effects on mycelial growth of dermatophytes and related keratinophilic fungi **[10].**

2.2.20 Tranquilizing, Antistaminic And Purgative Activity

An intensive research was done with water soluble portion of the alcoholic extract of the leaves of this plant for some CNS activities (viz. hypnotic, tranquilizing, local anaesthetic, hypothermic, anticonvulsant), antihistaminic and purgative activities. The results were found in accordance with major standard tranquilizers and thus supported the usage of the plant by Ayurvedic physicians in aforementioned conditions **[10].**

2.2.21 Antianemic Activity

A research was performed as hematological study on the ethanolic extracts of the flowers, seeds and leaves of the plant and noticed the dose dependent rise in hemoglobin content and red blood cells count in rats. The extracts also protect the decline of hemogram profile in anemic rats **[10].**

2.2.22 Membrane Stabilizing Activity

From a research work of *Nactynthes arbor-tristis* Lin isolated a carotenoid aglycone Ag-NY1 from the orange colored tubular calyx of flowers. Elucidation of the structure revealed that the

carotenoid molecule is crocetin, which is the major aglycone present in the stigma of Crocus sativus. The compound exhibited a good membrane stabilizing activity as compared to the corresponding glycoside crocin **[10].**

CHAPTER 3

PREPARATION OF PLANT EXTRACT FOR EXPERIMENT

3.1 PLANT SELECTION

There are thousands of medicinal plants in Bangladesh and in this Indian subcontinent. Among these plants it was not easy to select a few plants for the research purpose. The selection of plant greatly affects the research work if there is carelessness takes place. Plant secondary metabolites often accumulate in specific plant parts. Thus, unless it is already known which parts contain the highest level of the compounds of interest, it is important to collect multiple plant parts, or the whole plant to ensure the extracts prepared representative of the range of secondary metabolites. For drug discovery from plants, sample may be selected using a number following criteria by which the research work will run smoothly.

From the literature review it is seen that there are lots of work on the plant *Nyctanthes arbor-tristis* Linn about the pharmacological activity of the plant. But there is a least of work has been found about chemical investigation of this plant, especially about the leaves of this plant. So I got a chance to select the leaves of *Nyctanthes arbor-tristis* Linn for my research work to see whether the leaves have antipyretic and analgesic activity or not.

3.2 PLANT COLLECTION

After selection of plant it is must to collect the plant parts for the research purpose. Throughout Bangladesh the plant *Nyctanthes arbor-tristis* Linn is available. From the garden of Dhanmondi lake, Dhaka, Bangladesh the plant leaves were collected.

3.3 PLANT IDENTIFICATION

It is advisable to attempt field identification of sample collected. To aid taxonomic experts in confirming the field identification and to get a permanent scientific record (accession number) a voucher specimen was prepared on 20th December, 2011. In the voucher specimen the dried leaves of sample plant were attached and some information like local name, medicinal use, location of the sample plant were also written on that voucher specimen. Finally, from BNH

(Bangladesh National Herbarium) I got the identification or accession number of collected sample on 12th May, 2012, and the accession number is 37509 with *Nyctanthes arbor-tristis* Linn and *Oleaceae* scientific name and family name of the plant respectively.

3.4 DRYING OF PLANT SAMPLE

After the collection of sample it needs to be dried to make the sample extract. In general the plant material should be dried at temperature below 30^0C to avoid the decomposition of thermolabile compounds. So sun drying can be very effective but drawback is sometimes water molecules are absorbed by the sample and hence microbial growth can affect the phytochemical study. The leaves were dried in the sun light thus chemical decomposition can not take place.

3.5 GRINDING OF DRIED SAMPLE

Small amount of plant material can be milled using grinder or blender. But if the sample is in high amount then it is easier to get powdered sample by grinding from a spice mill. Grinding improves the efficiency of extraction by increasing surface area. It also decreases the amount of solvent required for the extraction. The dried samples were ground to coarse powder with a mechanical grinder (Blender) and powdered samples were kept in clean closed glass containers pending extraction. During grinding of sample, the grinder was thoroughly cleaned to avoid contamination with any remnant of previously ground material or other foreign matter deposited on the grinder.

3.6 MACERATION OF DRIED POWDERED SAMPLE

3.6.1 Principle

The choice of extraction procedure depends on the nature of the plant material and the components to be isolated. The principle of solid-liquid extraction is that when a solid material comes in contact with a solvent, the soluble components in the solid material move to the solvent

[27]. Thus, solvent extraction of plant material results in the mass transfer of soluble active principle (medicinal ingredient) to the solvent, and this takes place in a concentration gradient. The rate of mass transfer decreases as the concentration of active principle in the solvent increases, until equilibrium is reached, i.e. the concentrations of active principle in the solid material and the solvent are the same. Thereafter, there will no longer be a mass transfer of the active principle from plant material to the solvent. Since mass transfer of the active principle also depends on its solubility in the solvent, heating the solvent can enhances the mass transfer. Moreover, if the solvent in equilibrium with the plant material is replaced with fresh solvent, the concentration gradient is changed **[27].**

3.6.2 Procedure

After getting the sample as dried powdered, the sample (500 Gram) was then soaked in 1000mL of Methanol for five days, the process is known as maceration technique. A glass made jar with plastic cover was taken and washed thoroughly with ethanol and dried. Then the dried powder sample was taken in the jar. After that Methanol (10000mL) was poured into the jar up to 1-inch height above the sample surface as it can sufficiently cover the sample surface. The plastic cover with aluminium foil was closed properly to resist the entrance of air into the jar. This process was performed for five days. The jar was shaked in several times during the process to get better extraction.

3.7 FILTRATION OF THE EXTRACT

After the extraction process the plant extracts was filtered with sterilized cotton filter. The cotton was rinsed with ethanol and fitted in a funnel. The filtrate was collected in a beaker. Then again it was filtered and this time What man's filter was used for getting more clear extract which would be useful making the sample more concentrate in Rotary Evaporation Technique. Then the

filtrate was taken into a volumetric flask and covered with alumina foil paper and was prepared for rotary evaporation.

3.8 SAMPLE CONCENTRATION BY ROTARY EVAPORATION TECHNIQUE

3.8.1 Principle

A rotary evaporator is a device used in chemical laboratories for the efficient and gentle removal of solvents from samples by evaporation. Rotary evaporators are also used in molecular cooking for the preparation of distillates and extracts **[28].** A rotary evaporator consists of following parts-

- A motor unit that rotates the evaporation flask or vial containing the user's sample.
- A vapor duct that is the axis for sample rotation, and is a vacuum-tight conduit for the vapor being drawn off of the sample.
- A vacuum system, to substantially reduce the pressure within the evaporator system.
- A heated fluid bath (generally water) to heat the sample.
- A condenser with either a coil passing coolant, or a "cold finger" into which coolant mixtures such as dry ice and acetone are placed.
- A condensate-collecting flask at the bottom of the condenser, to catch the distilling solvent after it re-condenses.
- A mechanical or motorized mechanism to quickly lift the evaporation flask from the heating bath **[28].**

Figure 3.1: Rotary Evaporator in EWU Laboratory (IKA ®RV05 Basic, Biometra)

3.8.2 Affecting Factors

There are following factors, omission of one of the following factor may interfere the sample concentration procedure and thus which may interfere the phytochemical investigation. Remove the flask from the heat bath.

- Opening the stopcock
- Halting the rotor
- Turning off the vacuum/aspirator
- Disconnecting the flask
- Dropping flask in heat bath **[28].**

3.8.3 Procedure

After the filtration process two parts were obtained namely ‘residue part’ and ‘filtered part or filtrate’. The filtered part, which contains the substance soluble in ethanol, was putted into a1000ml round bottom flask (BOROSIL), and then the flask was placed in a rotary evaporator. The evaporation was done at 45 degree Celsius temperature. The number of rotation per minute

was selected as 130 RPM. The pressure of the vacuum pumper machine (Biometra) was 6bar. The water flow through the distillation chamber was also provided in a satisfactory flow rate. When the evaporation seemed to be satisfactory, then the ethanolic extract was collected in a 50mL beaker. The evaporator flask was rinsed by diethyl ether. Then the beaker was covered with aluminum foil paper and kept on the water bath for 60 minutes and the temperature of water bath maintained as 50^0C. Finally the concentrated ethanolic plant extract was found and stored in the laboratory refrigerator from which the extract was used for many chemical investigations.

CHAPTER 4

METHOD

4.1 CHEMICALS AND OTHER REAGENTS

DPPH (2,2-diphenyl-1-picrylhydrazyl), Sulfuric acid, Folin reagent, Ciocalteu reagent, prolein amino acid (protein), 1-butanol, glacial acetic acid, Ninhydrine solution, Glucose, Galactose, Maltose, Lactose, Acetone, Phosphate buffer, Anisaldehyde, L-Ascorbic acid, potassium ferricyanide,Ttrichloro acetic acid(TCA), Ferric chloride, Sodium carbonate, deionized water, Gallic acid, Sodium nitrite, Aluminium chloride, Sodium hydroxide, Hydrogen peroxide, Normal saline, Wagner's reagent, Hydrochloric acid, Glacial acetic acid, Ammonia, Phoshomolybdic acid, Acetic anhydride, Alcoholic ferric chloride, 5-aqua copper sulphate, Sodium potassium tartrate.

4.2 EQUIPMENTS AND OTHER NECESSARY TOOLS

In case of the extraction procedure and for various phytochemical tests many equipments and materials were used. Some of them are analytical balance, beaker (in various size), pipette, micro-pipette, rotary evaporator, hot air oven, dryer, storage cabinet, spatula, test tube, volumetric flask, conical flask, test tube holder, test tube rack, aluminum foil paper, scotch tape, refrigerator, water bath, electronic shaker, ultra violate lamp, mask, gloves, lab coat, sprayer, reagent bottle, TLC plate, TLC tank, scale, pencil, TLC plate cutter, capillary tube, mortar and pestle, laminar air flow cabinet, loop, burner, micropipette tip, petri dishes, glass rod, cotton, filter paper, funnel, hot plate, centrifugal machine, autoclave, glassware washers, stirrer, UV spectroscopy, knife, ephedrine tube, Whatman’s filter paper, paper disc, incubator, vortex machine, P^H meter.

4.3 SOLVENTS FOR EXPERIMENTS

Dimethylsulfoxide(DMSO), Acetone, Chloroform, Distilled water, Ethanol, Methanol, Diethyl ether, Acetic acid, n-Hexane, Ethyl acetate, Dichloromethane, Benzene, Ammonium hydrooxide, Formic acid.

4.4 THIN LAYER CHROMATOGRAPHY (TLC)

4.4.1 Principle

The extracts were analyzed by performing TLC to determine the composition of each extract. TLC was done under three solvent systems. Successively the polarity of the solvent systems was increased to get a clear graph of all the possible compounds present in the extract. Most organic solvents are nonpolar in nature and so organic compounds such as benzene was used to elute the non polar compounds present in the extract. Again, since Alkaloid and terpenoid type compounds are basic in nature, ammonium base is used in the solvent system to help them run over TLC plate **[44].**

Table 4.1: The compositions of various solvent systems for TLC **[44].**

Nonpolar Basic solvent	**Intermediate polar Basic Solvent**	**Polar Basic solvent**
Benzene 9mL Ethanol 1mL Ammonium hydroxide 0.1mL	Chloroform 5Ml Ethyl acetate 4mL Formic acid 1mL	Ethyl acetate 8mL Ethanol 1.2mL Water 0.8mL

4.4.2 Apparatus

1. TLC tank
2. Pencil
3. Scale
4. UV-lamp
5. Spray bottle
6. Heat gun
7. Petri dish
8. Capillary tube

4.4.3 Reagents

1. Benzene
2. Ethanol
3. Ammonium hydrooxide
4. $CHCl_3$
5. Formic acid
6. Ethyl acetate
7. EtOH
8. Water
9. Nutrient agar
10. H_2SO_4
11. DPPH
12. Folin & ciocalteu solution
13. 1-butanol
14. Glacial acetic acid
15. 2% ninhydrine solution
16. Acetone
17. Phosphate Buffer
18. Methanol
19. N-Hexane
20. Standard flavonoid solution

4.4.4 Procedure

- Firstly, above three solvent systems were prepared.
- In the next step, TLC plates were prepared. For this nine TLC plates were prepared, three for each solvent system to run with. The TLC plates were labeled in the following maner:

  1-C, 1-D, 1-F

  2-C, 2-D, 2-F

  3-C, 3-D, 3-F

Where,

1denoted run with nonpolar solvent system, 2 denoted run with intermediate polar solvent system, 3 denoted run with polar solvent system and C denoted treatment with 10% Sulphuric acid,D denoted treatment with 0.04%DPPH solution, F denoted treatment with 10% Folin & ciocalteu solution **[44].**

- On each plate four spots were spotted. The spotting patterns were as follows:

  1^{st} spot was spotted with Solvent; in this case methanol was used.

  2^{nd} & 3^{rd} spots were spotted with the extracts of shefali leaf.

  4^{th} spot was the control, spotted with standard flavonoid solution

- After spotting the respective TLC plate was exposed to the respective solvent system. Like all plates that were marked 1 were run with nonpolar solvent system, those that were marked 2 were run with intermediate polar solvent system and the 3 marked plates run with polar solvent system **[44].**
- Upon completion of TLC, the plates were exposed to reagent for compound detection and identification. For this the C marked plates were exposed to 10% sulphuric acid solution, dried and then heated to $80\text{-}90^0$ C for charring purpose. This helped in the fixation of spot and allowed it to be prominently visible.
- Likewise, the D marked plates were dipped into 0.04% DPPH solution and dried while keeping in a dark place.
- The F marked plates were washed with Folin & ciocalteu reagent and dried **[44].**

4.5 PROTEIN DETECTION

4.5.1 Protein Detection by TLC

4.5.1.1 Apparatus

TLC plate	TLC tank
Pencil	UV lamp
Capillary tube	

4.5.1.2 Reagents

Prolein amino acids	Water
1-butanol	Ethanol
Glacial acetic acid	2% ninhydrine

4.5.1.3 Procedure

- A TLC plate was taken and to both of its ends lines were drawn using pencil at 1cm from the edge. The bottom line was for spotting the samples, while the upper edge line was solvent line.
- To the bottom line two sample spots were made using capillary tube. One was the positive control having prolein amino acid and the other another was sample extracts. All the spots were labeled using pencil and they equidistant apart from each other **[45].**
- The TLC tank was saturated with mobile phase having the composition of 1-butanol, glacial acetic acid and water in the ratio of 4:1:1 respectively.
- To the saturated tank the previously spotted plate was placed and the mobile phase was allowed to run through the spots until the solvent line was reached.
- The plate was then taken out of the tank, dried and then visualized under UV light in dark room.
- After marking the florescent compounds, the plate was sprayed with 2% ninhydrine in ethanol solution, dried and then heated using heat gun to make the protein or amino acid component spots visible **[45].**

4.5.2 Protein Detection by Lowry Method

4.5.2.1 Principle

A standard curve of absorbance as a function of initial protein concentration was made by preparing solution of varying protein concentration using a stock solution of standard protein (e.g., bovine serum albumin fraction V). The stock solution had a concentration of 200mcg/ml & 500mcg/ml protein in distilled water, stored frozen at -20^0C. Using it different concentration of standard protein was made by diluting it with distilled water as follows: **[46].**

Table 4.2: Different concentration of standard protein in Lowry method **[46].**

Stock Solution(µL)	0	2.5	5	12.5	25	50	125	250	500
Water(µL)	500	498	495	488	475	450	375	250	0
Protein conc(µg/ml)	0	10	20	50	100	200	500	1000	2000

4.5.2.2 Apparatus

Refrigerator

Conical flask

Beaker

Pipette

Glass rod

Vortex mixer

Test tube

Water bath

UV spectrometer

4.5.2.3 Reagents

2% (w/v) Na_2CO_3

1 % (w/v) $CuSO_4.5H_2O$

Bovine serum albumin fraction V

2N NaOH

Distilled water. 1N Folin reagent

2% (w/v) sodium potassium tartrate

4.5.2.4 Procedure

- In this process complex forming reagent used was prepared just before using. Complex forming reagent consisted of 3 solutions (Solution A, Solution B, Solution C) in the ratio of 100: 1:1 respectively and those having following composition:

  Solution A had 2% (w/v) Na_2Co_3 in distilled water.

  Solution B had 1 % (w/v) CuSO4.5H2O in distilled water.

  Solution C had 2% (w/v) sodium potassium tartrate in distilled water **[46].**

- 0.1ml of each standard protein conc. solution was taken in a test tube.
- To it 0.1ml of 2N NaOH solution was added and the resulting solution as let to hydrolyse at 100^0C for 10min in boiling water bath.
- The resulting solution or hydrolysate was cooled to room temperature and to it 1ml of freshly mixed complex forming reagent was added and the solution was left to stand for 10mins in room temperature.
- After 10 minutes to the solution 0.1 ml of 1N conc. Folin reagent was added and using a vortex mixer it was thoroughly mixed. The mixture was left to stand for 30-40minutes at room temperature.
- Then the absorbance reading of all the resulting mixture was taken at 750nm **[46].**

4.6 PRELIMINARY PHYTOCHEMICAL INVESTIGATION OF SECONDARY METABOLITES

4.6.1 Principle

The secondary metabolites in the plant sample are the main concern of research work. There are many tests available for this purpose. In the following table these tests are shown **[36].**

Table 4.3: Preliminary phytochemical investigations of secondary metabolites **[36]**

Secondary metabolite	**Name of test**	**Methodology**	**Observation**
Alkaloid	Wagner test	Add 2ml filtrate with 1% HCl + steam. Then add 1ml of the solution with 6 drops of Wagner's reagent	Brownish-red precipitate
Anthraquinone	Borntrager's test	Add 1 ml of dilute (10 %) ammonia to 2 ml of chloroform extract	A pink-red color in the ammoniacal (lower) layer
Cardiac glycosides	Kellar - Kiliani test	Add 2ml filtrate with 1ml of glacial acetic acid, 1ml ferric chloride and 1ml concentrated sulphuric acid	Green-blue coloration of solution
Flavonoid	NaOH test	Treat the extract with dilute NaOH, followed by addition of dilute HCl	A yellow solution with NaOH, turns colorless with dilute HCl
Reducing sugar	Fehling test	Add 25ml of diluted sulphuric acid (H_2SO_4) to 5ml of water extract in a test tube and boil for 15mins. Then cool it and neutralize with 10% sodium hydroxide to pH 7 and 5ml	Brick red precipitate

		of Fehling solution	
Saponin	Frothing test/ Foam test	Add 0.5ml of filtrate with 5ml of distilled water and shake well	Persistence of frothing
Steroid	Liebermann-Burchardt test	To 1ml of methanolic extract, add 1ml of chloroform, 2–3ml of acetic anhydride, 1 to 2 drops of concentrated sulphuric acid	Dark green coloration
		To 1 ml of extract, add 2 ml acetic anhydride and 2 ml concentrated sulphuric acid	Color change to blue or green
Terpenoid	Liebermann-Burchardt test	To 1ml of methanolic extract, add 1ml of chloroform, 2–3ml of acetic anhydride, 1 to 2 drops of concentrated sulphuric acid	Pink or red coloration

4.6.2 Apparatus

Test tubes	Beaker
Conical flask	Filter paper
Pipette	Dropper
Electric balance	Hot plate
Shaker	Hot water bath

4.6.3 Reagents

1% HCl	Dilute sulphuric acid
Wagner's reagent	Water
Dilute (10 %) ammonia	Fehling solution
Glacial acetic acid	Chloroform
Ferric chloride	Acetic anhydride
Concentrated sulphuric acid	10% alcoholic ferric chloride
Dilute NaOH	Dilute HCl

Phoshomolybdic acid reagent **[36]**

4.6.4 Procedure

- Extract of different solvents were prepared.
- According to above table the tests were done.
- Detection of various secondary metabolites present in the plant was done according to above principle.

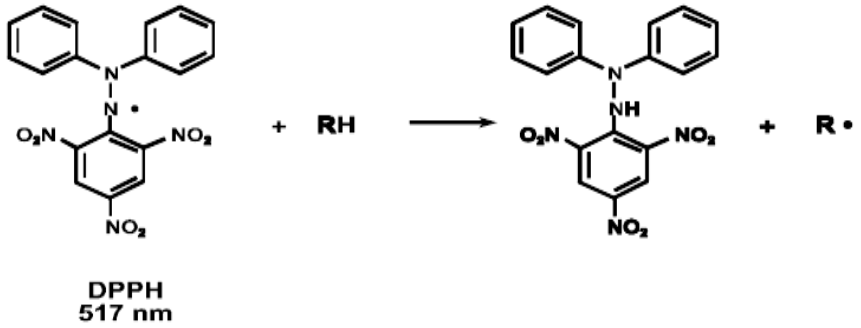
4.7 ANTI-OXIDANT TESTS

4.7.1 DPPH Test (1, 1-diphenyl-2-picrylhydrazyl radical)

4.7.1.1 Principle

The 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) has been widely used to evaluate the free radical scavenging capacity of antioxidants. DPPH free radical is reduced to the corresponding hydrazine when it reacts with hydrogen donors. DPPH can make stable free radicals in aqueous or methanol solution. With this method it was possible to determine the antiradical power of an antioxidant activity by measurement of the decrease in the absorbance of DPPH at 517 nm. Resulting from a color change from purple to yellow the absorbance decreased when the DPPH

was scavenged by an antioxidant, through donation of hydrogen to form a stable DPPH molecule. In the radical form this molecule had an absorbance at 517 nm which disappeared after acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule **[29].**



4.7.1.2 Apparatus

Test tube	Uv-spectrophotometer
Racker	Spatula
Beaker	Analytical balance

4.7.1.3 Reagents

DPPH	L-ascorbic acid
Methanol	Water

4.7.1.4 Procedure

- The DPPH radical scavenging method was used for the determination of the antioxidant capacity of the extracts. Different concentrations of the plant extract (2, 4, 6, 8 & 10

µg/ml, in methanol) were added at an equal volume (10ml) to methanol solution of DPPH (400 µg /ml, 100 µg/m l).

- Different concentrations of L-Ascorbic acid (2-10 mg/ml) were used as the standard antioxidant.
- After 30 min at room temperature, the absorbance values were measured at 517 nm on a spectrophotometer and converted into the percentage antioxidant activity using the equation, DPPH antiradical scavenging capacity (%) = [1 – (Abof sample – Abof blank)/Abof control] × 100.
- Methanol plus different concentration of plant extract solution was used as a blank, while DPPH solution plus methanol was used as a control **[34].**
- The IC_{50} values were calculated by the sigmoid non-linear regression model using plots, where the abscissa represented the concentration of tested plant extracts and the ordinate the average percent of scavenging capacity. IC_{50}values denote the concentration of the sample required to scavenge 50% of DPPH radicals **[34].**

4.7.2 Reducing Power Activity Test

4.7.2.1 Principle

In this assay, the yellow color of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant samples. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The presence of reductants such as antioxidant substances in the antioxidant samples causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm **[38].**

4.7.2.2 Apparatus

Test tube

Spatula

Analytical balance

Hot air oven

Centrifuge machine

Uv-spectrometer

Vortex mixer

Pipette & pumper

4.7.2.3 Reagents

Potassium ferricyanide

Distilled water

TCA **[32]**

$FeCl_3$

L-ascorbic acid

4.7.2.4 Procedure

- The reducing power of the extracts of night jasmine leaves was measured using the potassium ferricyanide reduction method.
- Various amounts of extracts & standard collected in different test tubes.

Table 4.4: Test tube designation for reducing power activity test **[32]**

Test tube no	Type of compound	Amount in gram
1	L-ascorbic acid	0.12
2	L-ascorbic acid	0.27

3	L-ascorbic acid	0.5
4	L-ascorbic acid	0.77
5	L-ascorbic acid	1
6	Night jasmine leaves	0.05
7	Night jasmine leaves	0.10
8	Night jasmine leaves	0.16
9	Night jasmine leaves	0.20

- Then all test tubes added with 2.5 ml of pH 7 distilled water & 2.5 ml of potassium ferricyanide[K_3Fe_3 $(CN)_6$] 1% solution & vortexed.
- After incubation at 50^0 C for 20 min, 2.5 ml of trichloro acetic acid(TCA)[10% w/v] was added to all test tubes & centrifuged at 3000 rpm for 10 min.
- Afterwards, upper layer of solution (5ml) was mixed with distilled water.
- Then 1ml of $FeCl_3$ was added on each test tube.
- Then from each test tube 1ml of solution was collected and mixed it with 9 ml of distilled water.
- After that it was incubated at 35^0C for 10 min **[32].**
- The formation of perls Prussian color was measured at 700 nm in a UV-VIS spectrometer.
- Increased absorbance of the reaction mixture indicated increase reducing power. The percentage (%) Reducing capacity was calculated from the following equation.

$\{(A_m - A_b)/A_b\}$ X 100

Where,

A_m is the absorbance of the reaction mixture

A_b is the absorbance of the blank **[32].**

4.7.3 Total Phenolic Assay

4.7.3.1 Principle

The content of total phenolic compounds in plant methanolic extracts was determined by Folin–Ciocalteu Reagent (FCR). The FCR actually measures a sample's reducing capacity. The exact chemical nature of the FC reagent is not known, but it is believed to contain heteropolyphosphotunstates - molybdates. Sequences of reversible one- or two-electron reduction reactions lead to blue species, possibly $(PMoW_{11}O_{40})^{4-}$. In essence, it is believed that the molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reductants and Mo (VI): **[39]**

$$Mo\ (VI) + e \rightarrow Mo\ ()$$

4.7.3.2 Apparatus

Test tube	Pipette
Spatula	Analytical balance
Vortex	Uv-spectrophotometer

4.7.3.3 Reagents

Folin-Ciocalteu	Ethanol or Methanol
deionized water	Galic acid (Analytical or Reagent grade)
Na_2CO_3 **[39]**	

4.7.3.4 Procedure

- 1.0 ml of plant extract 200µg/ml or standard of different concentration solution was taken in a test tube.
- 5 ml of Folin – ciocalteu (Diluted 10 fold) reagent solution was added into the test tube.
- 4 ml of Sodium carbonate solution was added into the test tube.
- The test tube was incubated for 30 minutes at 20^0C to complete the reaction. (only applicable for standard) **[39].**

  **Incubated the test tube for 1 hour at 20^0C to complete the reaction (Applicable for extract).

- Then the absorbance of the solution was measured at 765 nm using a spectrophotometer against blank.
- A typical blank solution contained ethanol or methanol was taken.
- The Total content of phenolic compounds in plant methanol extracts in gallic acid equivalents (GAE) was calculated by the following formula equation

$$C = (c \times V)/m$$

Where:

C = total content of phenolic compounds, mg/g plant extract, in GAE;

c = the concentration of gallic acid established from the calibration curve, mg/ml;

V = the volume of extract, ml;

m = the weight of pure plant methanolic extract, g.

- By the equation, y = 0.0162x+0.0215, R^2=0.9972 equivalent amount are neasured **[39].**

4.7.4 Total Flavanoid Assay

4.7.4.1 Apparatus

Uv-spectrophotometer Spatula

Test tube Pipette

Analytical balance

4.7.4.2 Reagents

De ionized water $AlCl_3$

$NaNO_2$ NaOH **[32]**

4.7.4.3 Procedure

- An aliquot (1.5 ml) of methanolic extract was added to 6ml of deionized water.
- Then 0.45 ml 5% (w/v) $NaNO_2$ and incubated for 6 min.
- 0.45 ml 10% (w/v) $AlCl_3$ and 6 ml 4% (w/v) NaOH was added and the total volume was made up to 15 ml with distilled water.
- The absorbance was measured at 510 nm by using visible spectrophotometer.
- The results were expressed as mg rutin equivalents/g DW. The analyses were done in three replications **[32].**

4.7.5 Hydrogen Peroxide Radical Scavenging Assay

4.7.5.1 Apparatus

Test tube	Analytical balance
Beaker	Spatula
Pipette	Uv-spectrophotometer

4.7.5.2 Reagents

- Hydrogen peroxide
- Phosphate buffer
- L-ascorbic acid **[33]**

4.7.5.3 Procedure

- A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (50mM, pH 7.4).
- The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer.
- Extract (4 - 20 µg/ml) in phosphate buffer was added to hydrogen peroxide and absorbance at 230 nm is determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide.
- L-ascorbic acid was used for comparison.
- The percentage of hydrogen peroxide scavenging was calculated as follows:

  % scavenged (H_2O_2) = (1– A_1 / A_0) X 100 Where; A_0 is the absorbance of control and A_1 is the absorbance of test.

- Ascorbic acid was used as a positive control. **[33]**

4.8 ANTI-MICROBIAL ASSAY

4.8.1 Determination of Antimicrobial activity by Disc Diffusion Method

Worldwide, infectious disease is one of main causes of death accounting for approximately one-half of all deaths in tropical countries. Perhaps it is not surprising to see these statistics in developing nations, but what may be remarkable is that infectious disease mortality rates are actually increasing in developed countries, such as the United States **[35].**

Death from infectious disease, ranked 5th in 1981, has become the 3rd leading cause of death in 1992; an increase of 58% .It is estimated that infectious disease is the underlying cause of death in 8% of the deaths occurring in the US **[30].** This is alarming given that it was once believed that we would eliminate infectious disease by the end of the millenium. The increases are attributed to increases in respiratory tract infections and HIV/AIDS. Other contributing factors are an increase in antibiotic resistance in nosicomial and community acquired infections. Furthermore, the most dramatic increases are occurring in the 25–44 year old age group **[30].**

These negative health trends call for a renewed interest in infectious disease in the medical and public health communities and renewed strategies on treatment and prevention. It is this last solution that would encompass the development of new antimicrobials **[40].**

The antimicrobial screening which is the first stage of antimicrobial drug research is performed to ascertain the susceptibility of various fungi and bacteria to any agent. This test measures the ability of each test sample to inhibit the *in vitro* fungal and bacterial growth. This ability may be estimated by disc diffusion method **[40].**

But there is no standardized method for expressing the results of antimicrobial screening **[40].** Some investigators use the diameter of zone of inhibition and/or the minimum weight of extract to inhibit the growth of microorganisms. However, a great number of factors viz., the extraction methods, inoculum volume, culture medium composition, p^H, and incubation temperature can influence the results **[41].**

Among the above mentioned techniques the disc diffusion is a widely accepted in vitro investigation for preliminary screening of test agents which may possess antimicrobial activity. It is essentially a quantitative or qualitative test indicating the sensitivity or resistance of the microorganisms to the test materials. However, no distinction between bacteriostatic and bactericidal activity can be made by this method **[41].**

4.8.1.1 Principle of Disc Diffusion Method

In this classical method, antibiotics diffuse from a confined source through the nutrient agar gel and create a concentration gradient. Dried and sterilized filter paper discs (6 mm diameter) containing the test samples of known amounts are placed on nutrient agar medium uniformly seeded with the test microorganisms.

Standard antibiotic discs and blank discs are used as positive and negative control. These plates are kept at low temperature (4°C) for 24 hours to allow maximum diffusion of the test materials to the surrounding media **[41].** The plates are then inverted and incubated at 37°C for 24 hours for optimum growth of the organisms. The test materials having antimicrobial property inhibit microbial growth in the media surrounding the discs and thereby yield a clear, distinct area defined as zone of inhibition. The antimicrobial activity of the test agent is then determined by measuring the diameter of zone of inhibition expressed in millimeter **[41].** In the present study the crude extracts, fractions as well as some pure compounds were tested for antimicrobial

activity by disc diffusion method. The experiment is carried out more than once and the mean of the readings is required **[30].**

4.8.1.2 Apparatus and Reagents

1. Filter paper discs
2. Autoclave
3. Nutrient Agar Medium
4. Laminar air flow hood
5. Petri dishes
6. Spirit burner
7. Sterile cotton
8. Refrigerator
9. Micropipette
10. Incubator
11. Inoculating loop
12. Ethanol
13. Sterile forceps
14. Nosemask and Hand gloves
15. Screw cap test tubes

4.8.1.3 Test Materials of *Nyctanthes arbor-tristis*

Methanolic extract of *Nyctanthes arbor-tristis* leaves were taken as test sample.

4.8.1.4 Test Organisms

The bacterial strains used for the experiment were collected as pure cultures from the East West University microbiology laboratory. Both gram positive and gram-negative organisms were taken for the test and they are listed in the following table.

Table 4.5: List of micro-organisms used

Gram positive Bacteria	**Gram negative Bacteria**
Bacillus cereus *Staphylococcus aureus*	*Salmonella typhi* *Shigella dysentery* *Vibrio mimicus*

4.8.1.5 Culture Medium and Their Composition

The Nutrient Agar medium was used normally to demonstrate the antimicrobial activity and to make subculture of the test organisms. Nutrient Agar medium contains following things

Table 4.6: Composition of nutrient agar medium **[41]**

Ingredients	**Amount**
Bacto peptone	0.5 gm
Sodium chloride	0.5 gm
Bacto yeast extract	1.0 gm
Bacto agar	2.0 gm
Distilled water q.s.	100 ml

Agar medium having this composition was directly brought from the market.

4.8.1.6 Preparation of the Medium

To prepare required volume of this medium, calculated amount of agar medium was taken in a bottle with a cap and distilled water was added to it to make the required volume. The contents were then autoclaved to make a clear solution.

4.8.1.7 Sterilization Procedure

In order to avoid any type of contamination and cross contamination by the test organisms the antimicrobial screening was done in Laminar Hood and all types of precautions were highly maintained. UV light was switched on one hour before working in the Laminar Hood. Petridishes and other glassware were sterilized by autoclaving at a temperature of 121^0C and a pressure of 15-lbs/sq. inch for 20 minutes. Micropipette tips, cotton, forceps, blank discs etc. were also sterilized.

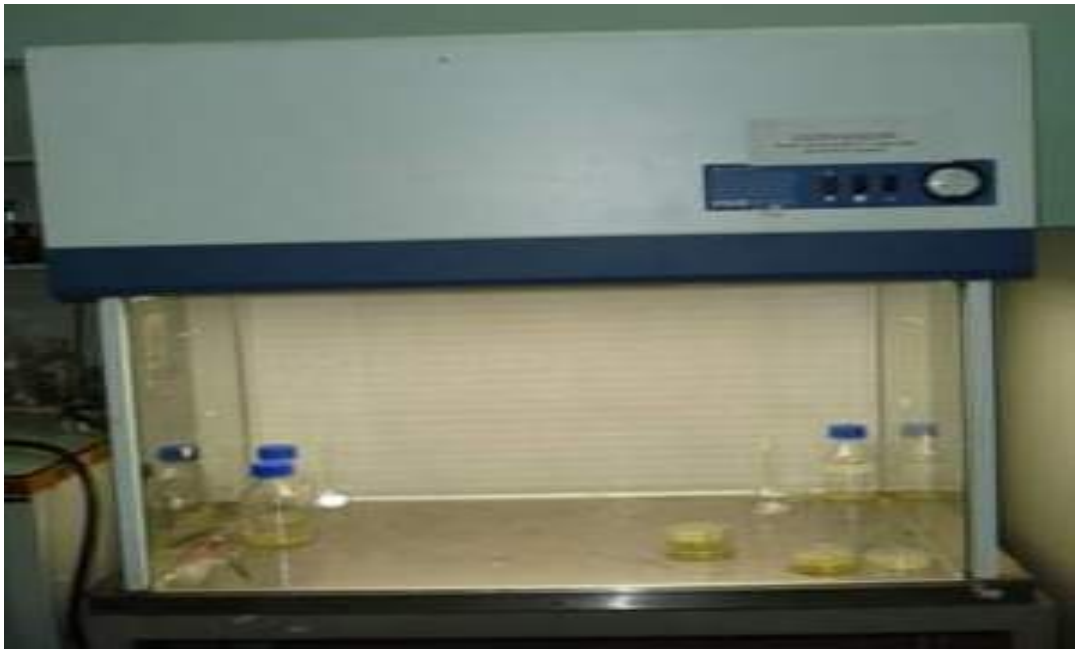


Figure 4.1: Laminar hood

4.8.1.8 Preparation of Subculture

In an aseptic condition under laminar air cabinet, the test organisms were transferred from the pure cultures to the agar slants with the help of a transfer loop to have fresh pure cultures. The inoculated strains were then incubated for 24 hours at 37^0C for their optimum growth. These fresh cultures were used for the sensitivity test.

Figure 4.2: Incubator

4.8.1.9 Preparation of the Test Plate

The test organisms were transferred from the subculture to petridish containing about 10 ml of melted and sterilized agar medium.

The bacterial and fungal suspension was taken by a loop a mixed with normal saline with the help of vortex machine. Then a sterilized cotton bud was taken and dipped into the bacterial/ fungal suspension. Then the bacterial/fungal sample is applied to the petridish with the help of this cotton bud.

4.8.1.10 Preparation of Discs

4.8.1.10.1 Standard Discs

These were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of the test sample. In this investigation, Azithromycin (30μg/disc) standard disc was used as the reference.

4.8.1.10.2 Blank Discs

These were used as negative controls which ensure that the residual solvents (left over the discs even after air-drying) and the filter paper were not active themselves.

4.8.1.10.3 Preparation of Sample Discs with Test Sample

Measured amount of each test sample was dissolved in specific volume of solvent to obtain the desired concentrations in an aseptic condition. Sterilized metrical (BBL, Cocksville, USA) filter paper discs were taken in a blank Petri dish under the laminar hood. Then discs were soaked with solutions of test samples and dried.

4.8.1.11 Diffusion and Incubation

The sample discs, the standard antibiotic discs and the control discs were placed gently on the

previously marked zones in the agar plates pre-inoculated with test bacteria and fungi. The plates were then kept in a refrigerator at 4^0C for about 24 hours upside down to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at 37^0C for 24 hours.

4.8.1.12 Determination of Antimicrobial Activity by Measuring the Zone of Inhibition

The antimicrobial potency of the test agents are measured by their activity to prevent the growth of the microorganisms surrounding the discs which gives clear zone of inhibition. After incubation, the Antimicrobial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale.

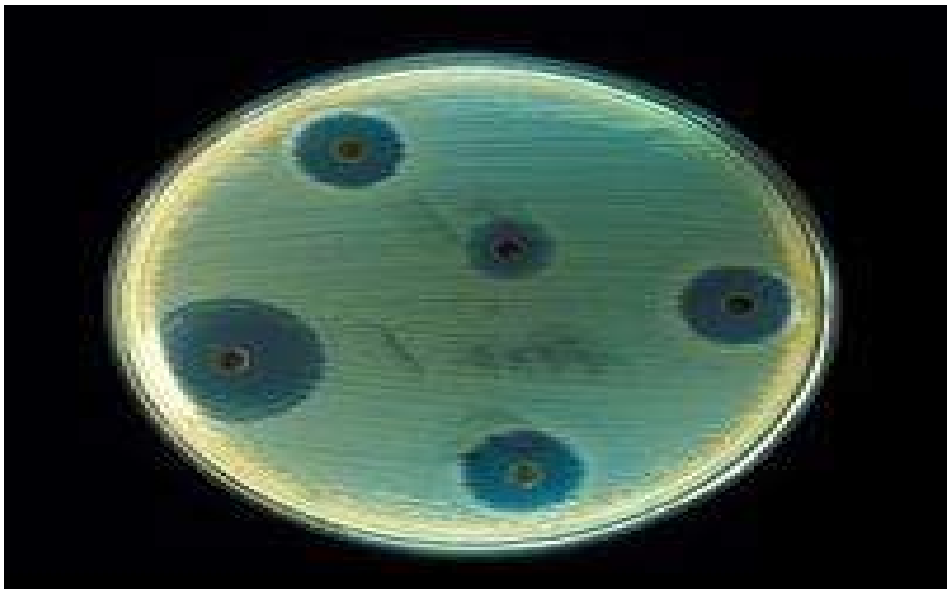


Figure 4.3: Zone of inhibition

4.8.2 Determination of MIC (Minimum Inhibitory Concentration)

The aim of this study was to determine the minimum inhibitory concentration (MIC) of the fraction methanolic extract required to kill microorganism. In the experiment, medicaments were added to bacterial species into eppendrof tube, in concentrations. The MIC was the lowest concentration of the drug at which bacterial growth could not be observed.

4.8.2.1 Principle of MIC

The disc diffusion method, which is a 'semi-quantitative' method, determination of MIC values provides a quantitative measure for the level of resistance expressed by the test organism. The minimal inhibitory concentration (MIC) values, which represent the lowest extract concentration that completely inhibits the growth of microorganisms. Next to the agar dilution method, the broth dilution method is one of the most frequently used methods to determine MIC's **[35].** The method is based on the inoculation of a standardized liquid culture of the test strain in a dilution series of the extract for which the MIC is determined. The first concentration in the dilution series at which no visual growth can be determined is then considered as the MIC **[41].**

4.8.2.2 Apparatus and Reagents

Nutrient Agar Medium	Autoclave
Petridishes	Laminar air flow hood
Sterile cotton	Spirit burner
Micropipette	Refrigerator
Inoculating loop	Incubator
Sterile forceps	Ethanol
Nutrient Broth Medium	Screw cap test tubes
Eppendrof tube	Nosemask and Hand glove

4.8.2.3 Test Materials of *Nyctanthes arbor-tristis*

Methanolic extract of *Nyctanthes arbor-tristis* leaves were taken as test sample.

4.8.2.4 Test Organisms

The bacterial strains (*Vibrio mimicus*) used for the experiment were collected as pure cultures

from the East West University microbiology laboratory.

4.8.2.5 Culture Medium and Their Composition

The Nutrient Agar medium was used normally to demonstrate the antimicrobial activity and to make subculture of the test organisms. Nutrient Agar medium contains following things

Table 4.7: Composition of nutrient agar medium **[35]**

Ingredients	**Amount**
Bacto peptone	0.5 gm
Sodium chloride	0.5 gm
Bacto yeast extract	1.0 gm
Bacto agar	2.0 gm
Distilled water q.s.	100 ml

Table 4.8: Composition of nutrient broth medium **[35]**

Ingredients	**Amount**
Bacto beef extract	0.3 gm
Bacto peptone	0.5 gm
Distilled water q.s.	100 ml

Agar and broth medium having this composition was directly brought from the market and the PH = 7.2 + 0.1 at 25^0C was maintained.

4.8.2.6 Procedure

- The agar diffusion method was employed for the determination of antibacterial activities of *Nyctanhes arbor-tristis.*

- The leave extracts were dissolved in 100% methanol to a final concentration of 100 mg/ml **[35].**
- The bacterial strains were cultured in a nutriment broth for 24 hours.
- Then, previously prepared 1ml of suspension bacteria (30 X 1012 CFU estimated) was spread on nutrient Broth agar.
- Disks were made by using a sterile filter paper and were loaded with 20 µl of each sample extract.
- Methanol was used as negative control and Azithromycin (30 mcg/disk) as positive reference standard.
- All the plates were incubated at 37°C for 24 hours.
- Antibacterial activity was evaluated by measuring the zone of inhibition in millimetres.
- All experiments were done in triplicates **[35].**

4.8.3 TLC Bioautography

4.8.3.1 Apparatus

TLC plate	Laminar airflow
Pencil	Incubator
Nutrient Agar	

4.8.3.2 Reagents

Chloroform	Hexane
Methanol	*E. coli* bacterial inoculums

4.8.3.3 Procedure

- TLC bioautography assay was performed by agar overlay bioautography technique.
- Plant extract samples were applied 1 cm from the base of the silica plate.

- After drying, the plates were developed using solvent chloroform: methanol (8.2: 1.8) and chloroform : hexane (5.4 : 6.6) **[37].**
- Developed TLC plates were carefully dried for complete removal of solvents. Aliquot of 50 ml of nutrient agar was prepared by adding *Vivrio mimicus* bacterial inoculums.
- Now, the dried TLC plate was overlaid on inoculums containing agar under aseptic condition in laminar airflow. The plates were incubated at 37°C and examined for the zone of inhibition **[37].**

4.9 BIOMEDICAL TEST

4.9.1 Haemagglutination Assay (HA)

4.9.1.1 Principle

Many viruses attach to molecules present on the surface of RBCs. A consequence of this is that at certain concentrations, a viral suspension may bind together (agglutinate) the RBCs, thus preventing them from settling out of suspension. Since agglutination is rarely linked to infectivity, attenuated viruses can therefore be used in assays. By serially diluting a virus suspension into an assay tray (a series of wells of uniform volume) and adding a standard amount of blood cells, an estimation of the number of virus particles can be made **[42].**

While less accurate than a plaque assay, it is cheaper and quicker (taking just 30 minutes). This assay may be modified to include the addition of an antiserum. By using a standard amount of virus, a standard amount of blood cells, and serially diluting the antiserum, one can identify the concentration of the antiserum (the greatest dilution which inhibits hemagglutination) **[42].**

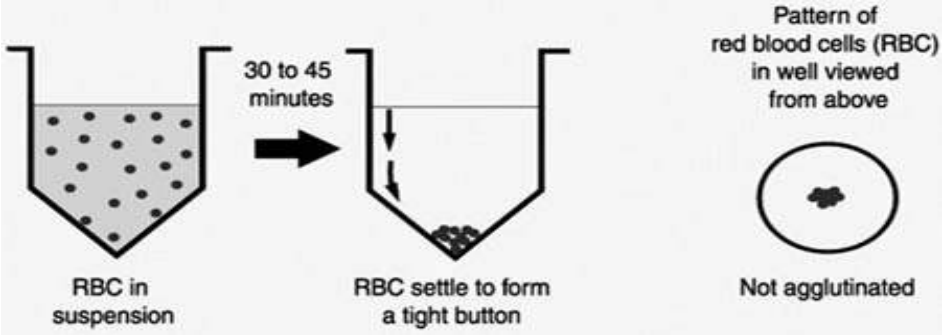


Figure 4.4: Formation of tight button with no agglutination

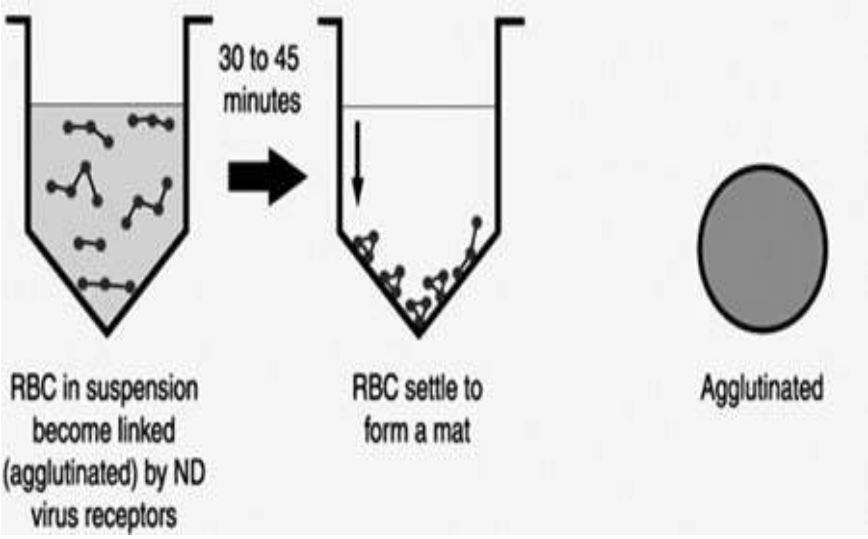


Figure 4.5: Formation of agglutination

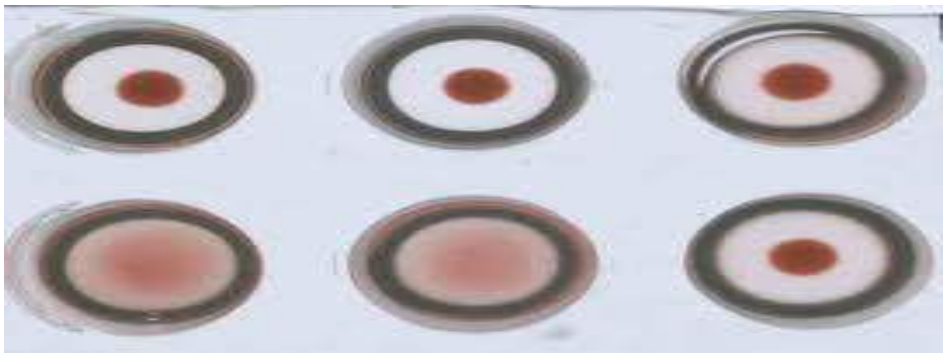


Figure 4.6: Overview of HA and not HA

4.9.1.2 Apparatus

Centrifuge machine	Refrigerator
Ephindorf tube	Test tube
Micro pipette	Eppendorf tube box
Syringe	CD marker
Cotton	

4.9.1.3 Reagents

Isotonic phosphate buffer	Blood

4.9.1.4 Procedure

- Stock solution of the test sample was prepared at concentration of 5 mg/ml and each solution was serially diluted.
- Fresh blood from healthy person was collected only for the test of Haemagglutination Assay (HA). The blood group A^+, B^+, AB^+, O^+ were collected from healthy person for this test of east West University students **[30].**
- Then the all bloods were centrifuged and the erythrocytes were separated.
- 4% erythrocyte suspension was prepared in phosphate buffer (pH 7.4) of all blood groups.
- 1 ml of the test sample dilution was taken with 1 ml of 4% erythrocyte and incubated at 25°C.
- After incubation, the results were noted. Smooth button formation in bottom indicated negative activity, while a rough granular deposition at bottom showed positive activity.
- The intensity of haemagglutination was determined from the extent of deposition **[30].**

4.9.2 Anti-Hemolytic Activity

4.9.2.1 Apparatus

Centrifuge machine	Refrigerator
Micro pipette	Test tube
Syringe	CD marker
Cotton	Uv-spectrophotometer

4.9.2.2 Reagents

H_2O_2

Isotonic sodium phosphate buffer

Blood samples **[43]**

4.9.2.3 Procedure

- The erythrocytes from man blood were separated by centrifugation and washed with saline or isotonic sodium phosphate buffer (pH 7.4) until the supernatant was colorless.
- The erythrocytes were then diluted with saline or phosphate buffer to give a 4% suspension **[43].**
- Varying amounts of sample (1000,500,250,125,62.5 □g/ml) with saline or buffer were added to 2 ml of the suspension of erythrocytes and the volume was made up to 3.5 ml with saline or buffer.
- This mixture was pre-incubated for 120 min and then 0.5 ml H_20_2 solutions of appropriate concentration in saline or buffer were added.
- The concentration of H_20_2 in the reaction mixture was adjusted so as to bring 90% hemolysis of blood cells after 120 min incubation.
- About 80-90% hemolysis of rat erythrocytes was obtained after 4-6 h.

- Incubation was concluded after these time intervals by centrifugation during 5 min at Xl000 g and the extent of hemolysis was determined by measurement of the absorbance at 540 nm corresponding to hemoglobin liberation.
- Antihemolytic activity was expressed as the inhibition percentage and was calculated using the following formula:

  Antihemolytic activity (%) = 1 − Sample $_{540\ nm}$ /Control $_{540\ nm}$ X 100, where, Sample $_{540\ nm}$ was the absorbance of the sample and Control $_{540\ nm}$ was the absorbance of the control **[43].**

4.10 IR Analysis

After doing all of the above tests then the sample were run to the IR spectrometer. From the data obtained was required to analysis the probable functional group present in the sample.

CHAPTER 5

RESULT & DISCUSSION

5.1 Thin Layer Chromatography (TLC)

The results obtained after TLC of the methanolic extract of the *Nyctanthes arbor-tristis* leaf in solvent system 1 is given below-

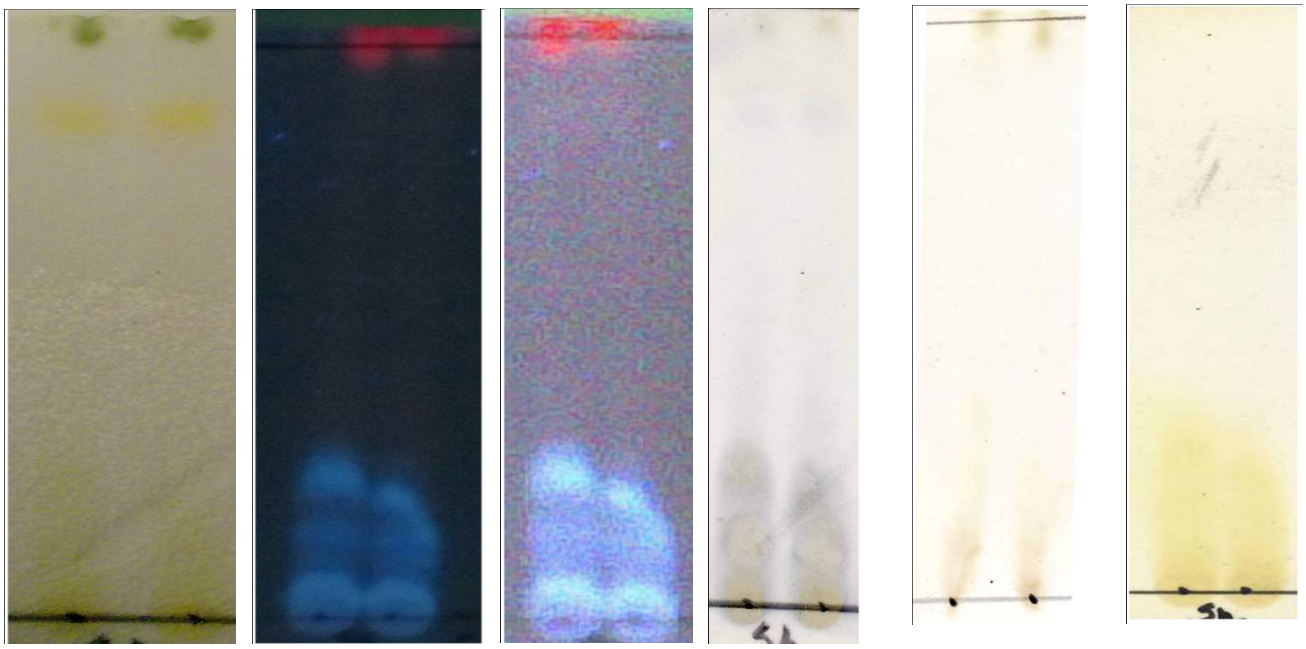
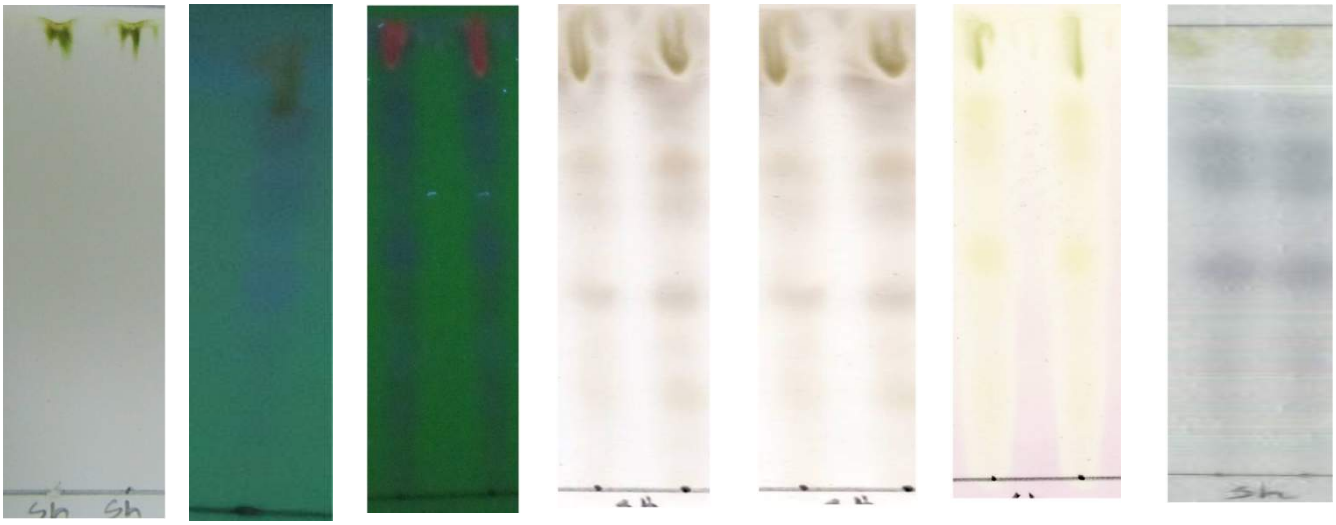


Figure 5.1: Results for TLC in nonpolar basic solvent (1= naked eye view; 2 & 3 = UV light view; 4= after application of FC reagent; 5= after charing; 6= after application of DPPH)

The naked eye view of the TLC was mentioned in the plate 1 which did not show any clear spot. Then the plate was observed under UV which is shown in the plate 2 and 3. It showed some spots which indicate the presence of different compounds in that sample. Folin & ciocalteu solution was used to determine the presence of the phenolic compouns in the sample. The formation of intense violet color after application of the Folin & ciocalteu solution in the sample indicates the presence of phenolic compound in the sample. Spraying of the Folin & ciocalteu solution in the TLC plate showed moderate violet color which indicates the presence of phenolic

compound in that fraction (plate 4). After charing of the TLC plate with sulfuric acid has showed (plate 5) two spots at the bottom as well as at the top of the TLC plate.

Spraying of DPPH solution on the TLC plate did not show any significant color changes which indicate the less free radical scavenging property of that fraction.



1 2 3 4 5 6 7

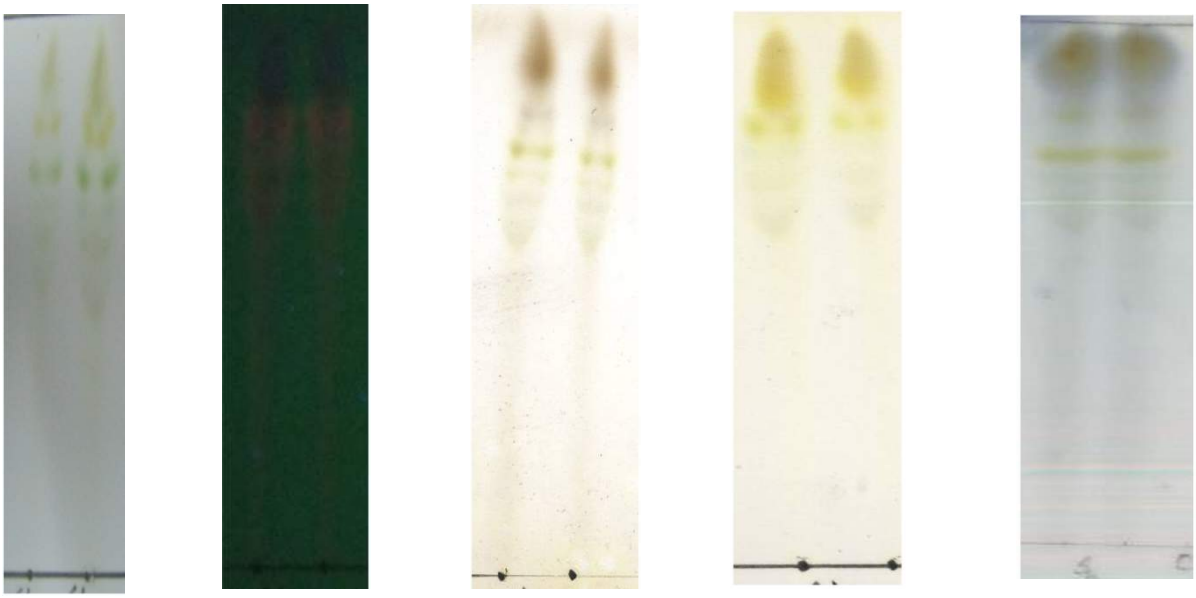
Figure 5.2: Results for TLC in intermediate polar basic solvent (1= naked eye view; 2 & 3 = UV light view; 4& 5= after charing; 6= after application of DPPH solution; 7= after application of FC solution)

The naked eye view of the TLC is mentioned in the plate 1 which showed two clear spots at the top of the plate. Then the plate was observed under UV which is shown in the plate 2 and 3. It showed some additional spots which indicate the presence of different compounds in that sample. After charing of the TLC plate with sulfuric acid (plate 4 & 5) has revealed many spots in the TLC plate.

Spraying of DPPH solution on the TLC plate have shown significant formation of pale yellow color (plate 6) in the place of the spots which indicates significant free radical scavenging

property of that fraction.

Folin & ciocalteu solution was used to determine the presence of the phenolic compouns in the sample. The formation of intense violet color after application of the Folin & ciocalteu solution in the sample indicates the presence of phenolic compound in the sample. Spraying of the Folin&ciocalteu solution in the TLC plate shows formation of intense violet color which indicates the presence of phenolic compound in that fraction (plate 7).



1 2 3 4 5

Figure5.3: Results for TLC in polar basic solvent (1= naked eye view; 2 = UV light view; 3= after charing; 4= after application of DPPH solution; 5= after application of FC solution)

The naked eye view of the TLC was mentioned in the plate 1 which showed clear spot. Then the plate was observed under UV which is shown in the plate 2. It showed some spots which indicate the presence of different compounds in that sample. After charing of the TLC plate with sulfuric acid (plate 3) has revealed many spots in the TLC plate.

Spraying of DPPH solution on the TLC plate did not show significant formation of pale yellow color (plate 4) in the place of the spots which indicates less significant free radical scavenging

property of that fraction.

Folin & ciocalteu solution was used to determine the presence of the phenolic compouns in the sample. The formation of intense violet color after application of the Folin & ciocalteu solution in the sample indicates the presence of phenolic compound in the sample. Spraying of the Folin&ciocalteu solution in the TLC plate shows moderate violet color which indicates the presence of phenolic compound in that fraction (plate 5).

5.2 Protein detection

5.2.1 Protein Detection by TLC

To detect protein (amino acid) prolein was used as positive control. After TLC, it was found that the extract made some spots which were very much visible when it was sprayed by Ninhydrin solution under UV light. The brown spots indicate that there may be amino acids present in the extract thus we may go further test for the protein detection.

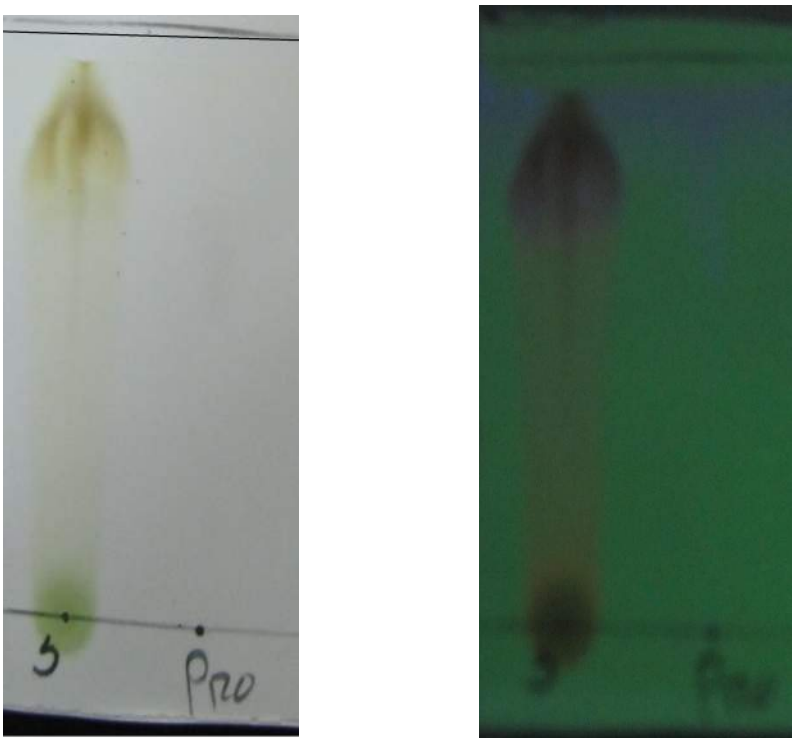


Figure 5.4: Protein detection eye view (left) and UV view (right)

5.2.2 Protein Detection by Lowry Method

Table 5.1: Total protein content equivalent to BSA

Standard/Sample	Concentration µg/ml	Absorbance	Total Protein Content (Gram of BSA /mg of dried extract)
BSA	10	0.011	
	20	0.012	
	50	0.034	
	100	0.114	
	200	0.122	
	500	0.25	
	1000	0.328	
	2000	0.456	
MENA	200	0.06	202
	500	0.1	130

N.B: BSA= Bovine Serum Albumin; MENA= Methanolic Extract of Nycthanthes arbor-tristis

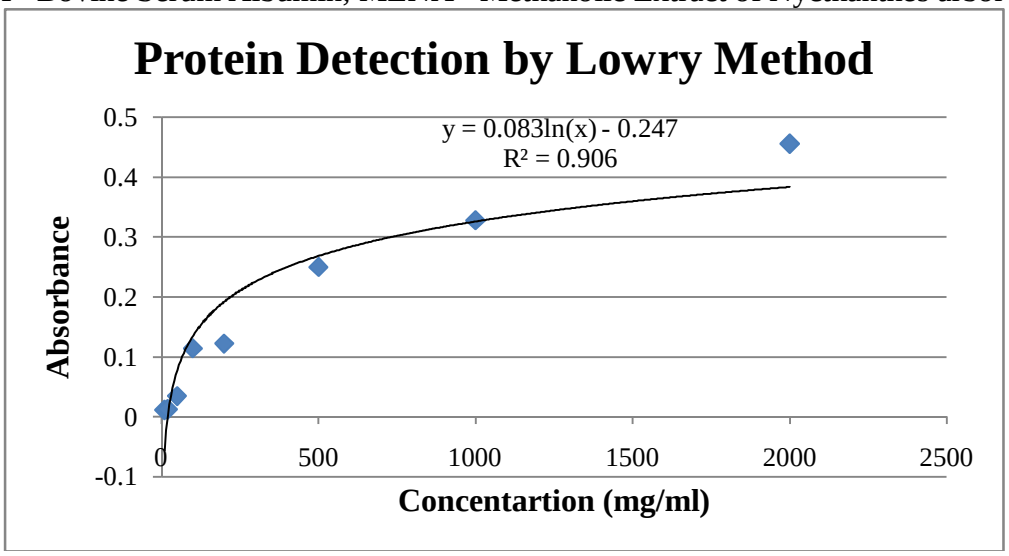


Figure 5.5: Graph of absorbance versus concentration in protein detection by Lowry method

As, from the protein detection by TLC method it was found that there might be protein in the sample thus Lowry method had been introduced to establish the protein in the extract. From the

above curve it is clearly seen that the regression coefficient value is under the acceptable range. The equation found from the curve was used to determine the equivalent amount of protein present in the sample to the BSA (Bovine Serum Albumin).

When the concentration of the dried extract was 200µg/ml then total protein content found as 202 Gram of BSA /mg of dried extract, and when the concentration of the dried extract was 500µg/ml then total protein content found as 130 Gram of BSA /mg of dried extract.

This test suggests that there must be protein in the extract.

5.3 Preliminary Phytochemical Investigation

Secondary metabolites are very important for the plant. In this test I searched for whether my plant contains some kind of secondary metabolites like alkaloid, flavanoid, steroid, rducing sugar etc. Simply by following standard methods and by the observation of different color change of the solution the following results were made. From the tests it was concluded that there might be alkaloid, flavanoid, steroid, cardiac glycoside present in the sample because of test of these compound has given the positive result by showing specific color change or making specific precipitation. In addition, some of the secondary metabolites like anthra-quinone, terpe-noid, reducing sugar, saponin were absent as their test showed negative result.

Table 5.2: Result of preliminary phytochemical investigation of secondary metabolites

Sample	Phytochemical Test	Result
MENA	Alka-loid	+
	Flavonoid	+
	Anthra-quinone	-

MENA	Terpe-noid	-
	Reducing sugar	-
	Saponin	-
	Steroid	+
	Cardiac Glycoside	+

N.B: MENA= Methanolic Extract of Nycthanthes arbor-tristis;+ = presence of compound; - = absence of compound

5.4 Anti-oxidant Tests

Anti oxidant tests were classified by various sections like DPPH Test, Reducing power activity test, Total phenolic assay, Total flavanoid assay, Hydrogen peroxide radical scavenging assay. Samples were subjected as methanolic extract to various standard methods to determine various scavenging capacity and amount that is equivalent to various standards like ascorbic acids, gallic acids.

5.4.1 DPPH Test

The crude methanolic extract of *Nyctanhes arbor-tristis* (leaves) was subjected to free radical scavenging activity and it showed significant free radical scavenging activity with an IC_{50} value of 0.0425 mg/ml whereas IC_{50} value of the standard was 6.493 mg/ml at higher concentration. The percentage inhibition of free radical DPPH and the IC_{50} value of crude methanolic extract of *Nyctanthes arbor-tristis* (leaves) are given in table 5.4 and figure 5.9.

Table 5.3: Determination of free radical scavenging capacity for the standard (ascorbic acid)

Standard	Concentration mg/ml	Absorbance	%Inhibition	IC $_{50}$ mg/ml
Blank (water)	0	0.3846667	0	6.493
Ascorbic acid	0.2	0.3393333	11.7851	
	0.4	0.3046667	20.7972	
	0.6	0.257	33.1889	
	0.8	0.2223333	42.201	
	1	0.2153333	44.0208	

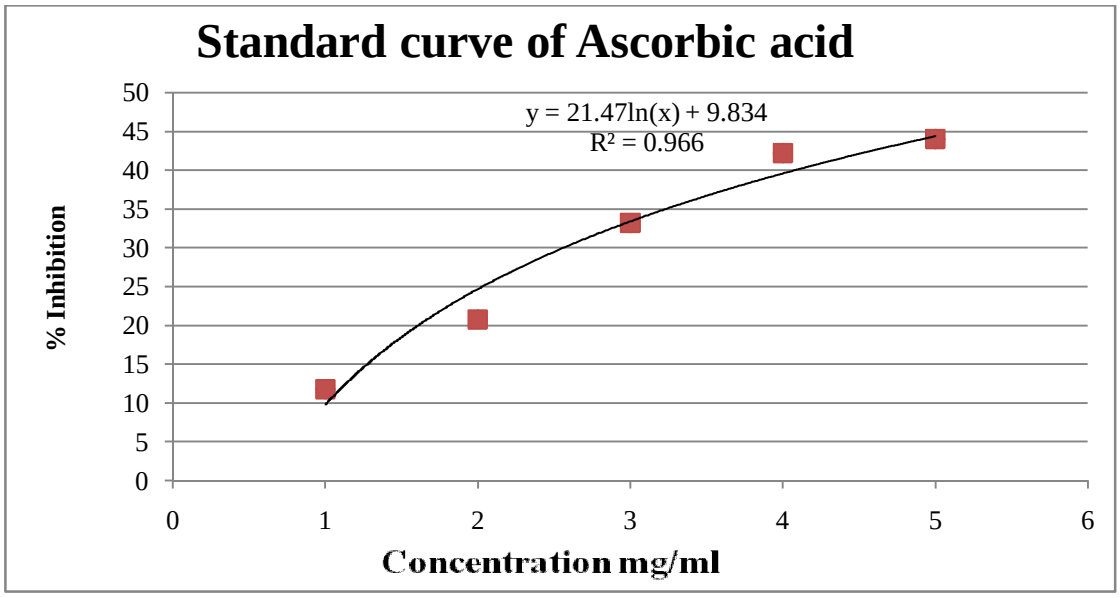


Figure 5.6: DPPH scavenging potential & IC_{50} value of ascorbic acid

Table 5.4: Determination of free radical scavenging capacity for MENA

Sample	Concentration mg/ml	Absorbance	%Inhibition	IC $_{50}$ mg/ml
Blank	0	0.523	0	0.0425
MENA1	0.02	0.349	33.2696	
MENA2	0.04	0.27433	47.54621	
MENA3	0.06	0.21367	59.14595	
MENA4	0.08	0.19333	63.03442	
MENA5	0.10	0.15767	69.85277	

N.B: MENA= Methanolic extract of *Nyctanthes arbortristis*, IC_{50} = Inhibitory Concentration 50

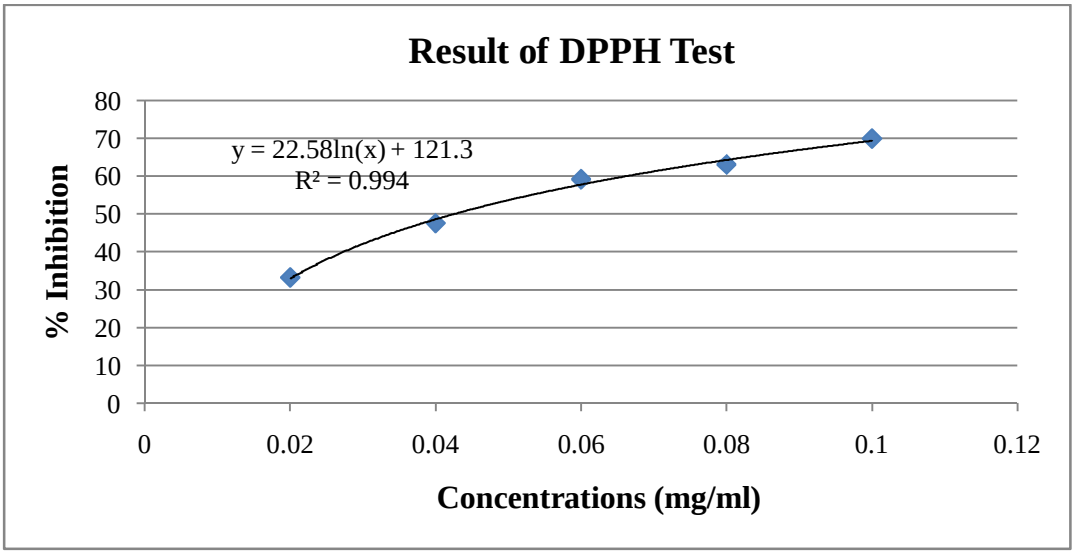


Figure 5.7: DPPH scavenging potential & IC_{50} value of crude methanolic extract of of *Nyctanthes arbor-tristis* (leaves)

5.4.2 Reducing Power Activity Test

The reducing properties are generally associated with the presence of reductanes which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen

atom. The reducing power ability of crude methanolic extract of *Nyctanthes arbortristis* (leaves) was determined using L-ascorbic acid as positive control. In case of reducing power, the higher the concentration of the test samples, the higher the absorbance. The higher the absorbance, the higher the inhibition. The reducing power of various concentrations of crude methanolic extract of *Nyctanthes arbortristis* (leaves) are given in table 5.5 & figure 5.10. The avearge % increase in reducing power was observed of crude ethanolic extract and it was 68.36799 %.

Table 5.5: Determination of Average % Reducing Capacity

Sample	**Concentration Mg/ml**	**Absorbance**	**% Reducing Capacity**	**Average % Reducing Capacity**
Blank	0	0.529	0	68.36799
MENA	20	0.652	23.25142	
	40	0.9055	71.17202	
	64	1.1145	110.6805	

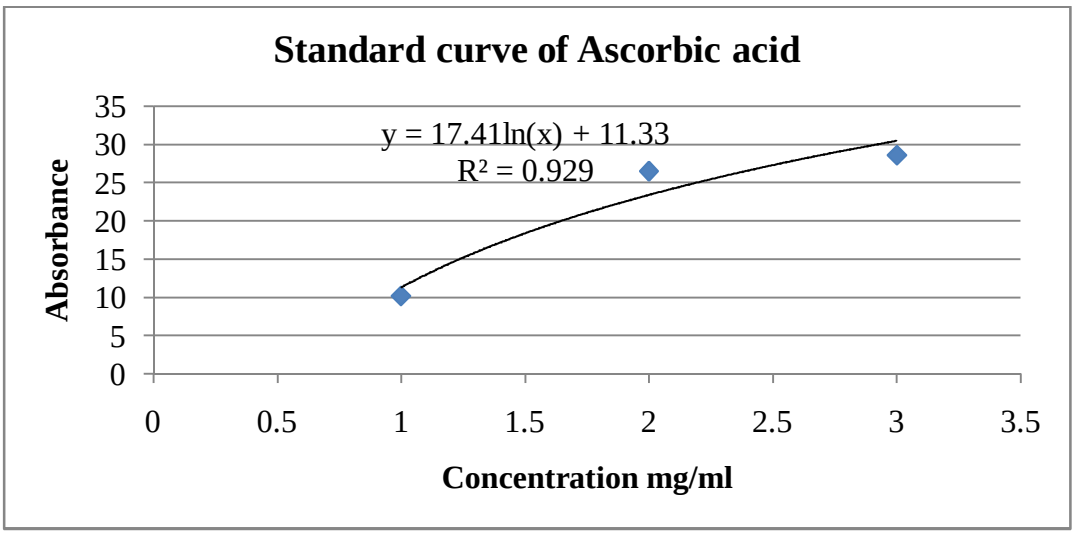


Figure 5.8: Standard curve of ascorbic acid showing absorbance versus concentration relationship

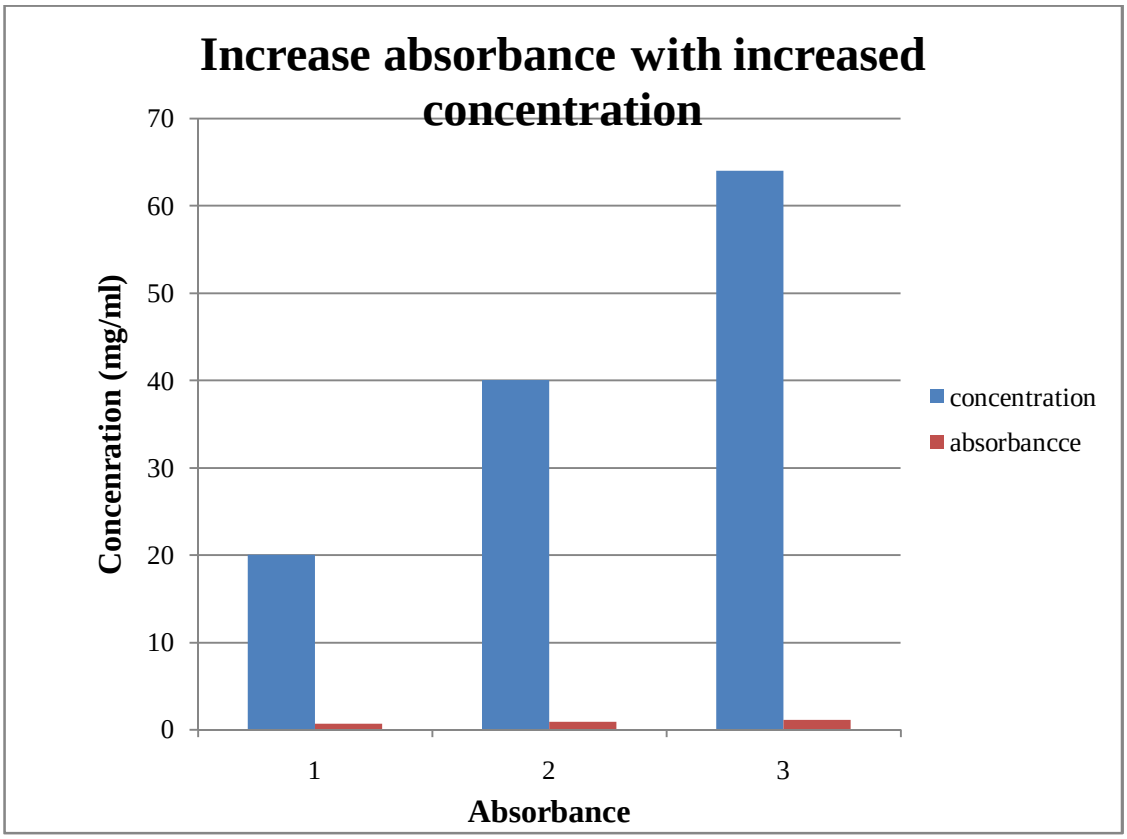


Figure 5.9: Bar diagram of showing higher the concentration higher the absorbance

6.4.3 Total Phenolic Assay

The crude methanolic extract of *Nyctanthes arbortristis* (leaves) was subjected to total phenolic content determination. Based on the absorbance values of the extract solution, reacted with Folin-Ciocalteu reagent and compared with the standard solutions of gallic acid (table 5.6) equivalents, result of the colorimetric analysis of the total phenolics are given in table 5.7. Total phenolic content of the samples are expressed as mg of GAE (gallic acid equivalent) per mg of dried extract. The phenolic content found in the crude methanolic extract of *Nyctanthes arbortristis* (leaves) was 32.81038 mg of gallic acid (GAE) per mg of dried extract.

Table 5.9: Standard curve preparation by using gallic acid

SL. No.	Concentration (µg	Absorbance	Regression line	R^2
1	100	1.620	y = 0.0162x + 0.0215	0.9985
2	50	0.866		
3	25	0.450		
4	12.5	0.253		
5	6.25	0.120		
6	3.125	0.059		
7	1.5625	0.034		
8	0.78125	0.022		
9	0.3906	0.020		
10	0	0.011		

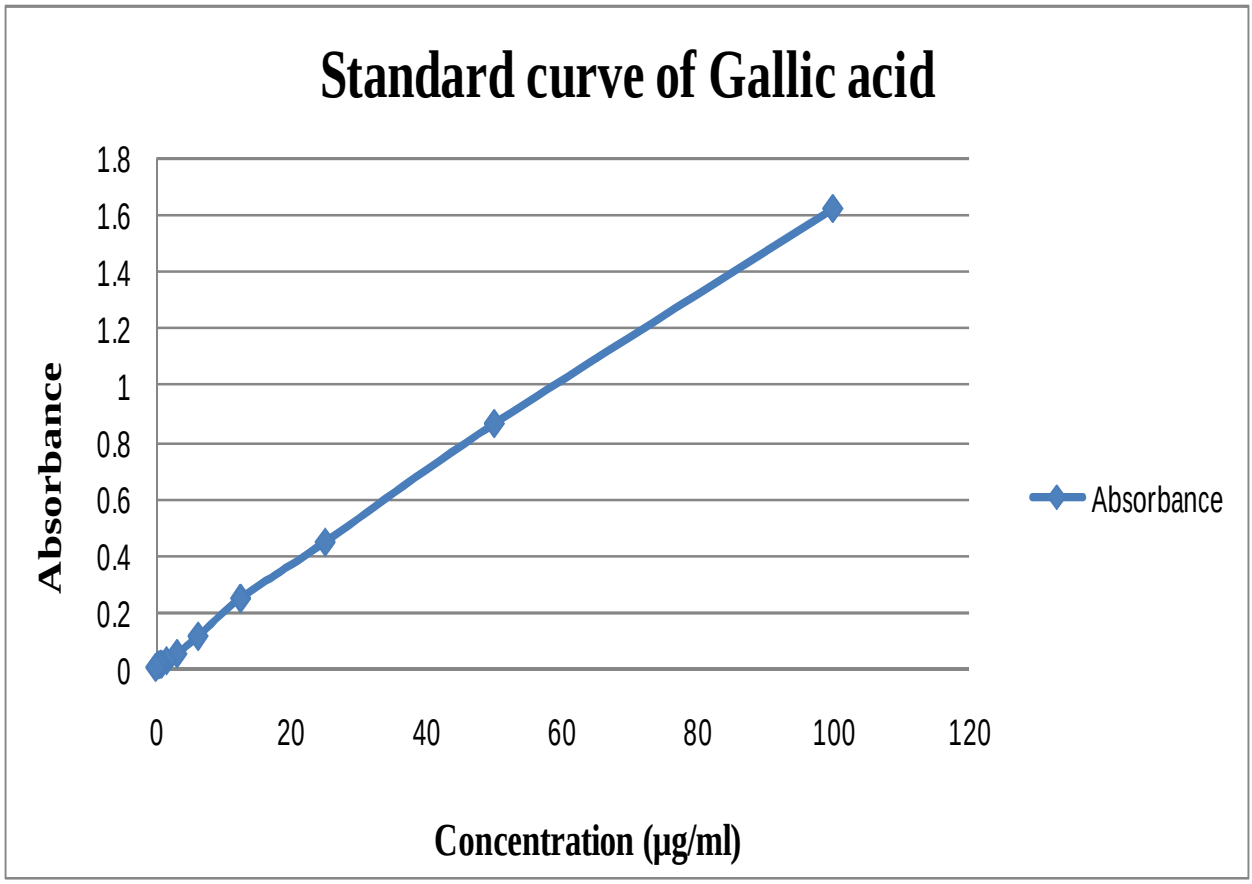


Figure 5.10: Standard curve of gallic acid

Table 5.10: Determination of total phenolic content (mg of GAE/mg of dried extract)

Sample	**Concentration** mg/ml	**Absorbance**	**Total Phenolic content(mg of GAE/mg of dried extract)**	**Average**
MENA	20	0.374	32.28377	32.81038
	20	0.396	34.29864	
	20	0.369	31.82584	
	20	0.38	32.83328	

N.B: MENA= methanolic extract of *Nyctanthes arbortristis*

5.4.4 Total Flavanoid Assay

To determine the total flavonoids content of crude methanolic extract of *Nyctanthes arbortristis* (leaves), a standard curve is needed which is obtained from a series of different quercetin concentrations (table 5.8). The total flavonoids content of the sample is expressed as mg of quercetin per gm of dried extract in table 5.9 by using the standard curve equation of quercetin ($y = 0.002x + 0.0318$, $R^2 = 0.9989$). Where y is absorbance at 415 nm and x is flavonoid content of crude plant extract. The total flavonoids content found in the crude methanolic extract of *Nyctanthes arbortristis* (leaves) was 3144.333 mg (average) of quercetin per mg of dried extract.

Table 5.11: Standard curve preparation by using quercetin

SL. No.	Concentration (µg /ml)	Absorbance	Regression line	R^2
1	2.5	0.0365	y = 0.002x + 0.0318	0.9989
2	5	0.0417		
3	10	0.0521		
4	20	0.0735		
5	30	0.0906		
6	40	0.1127		

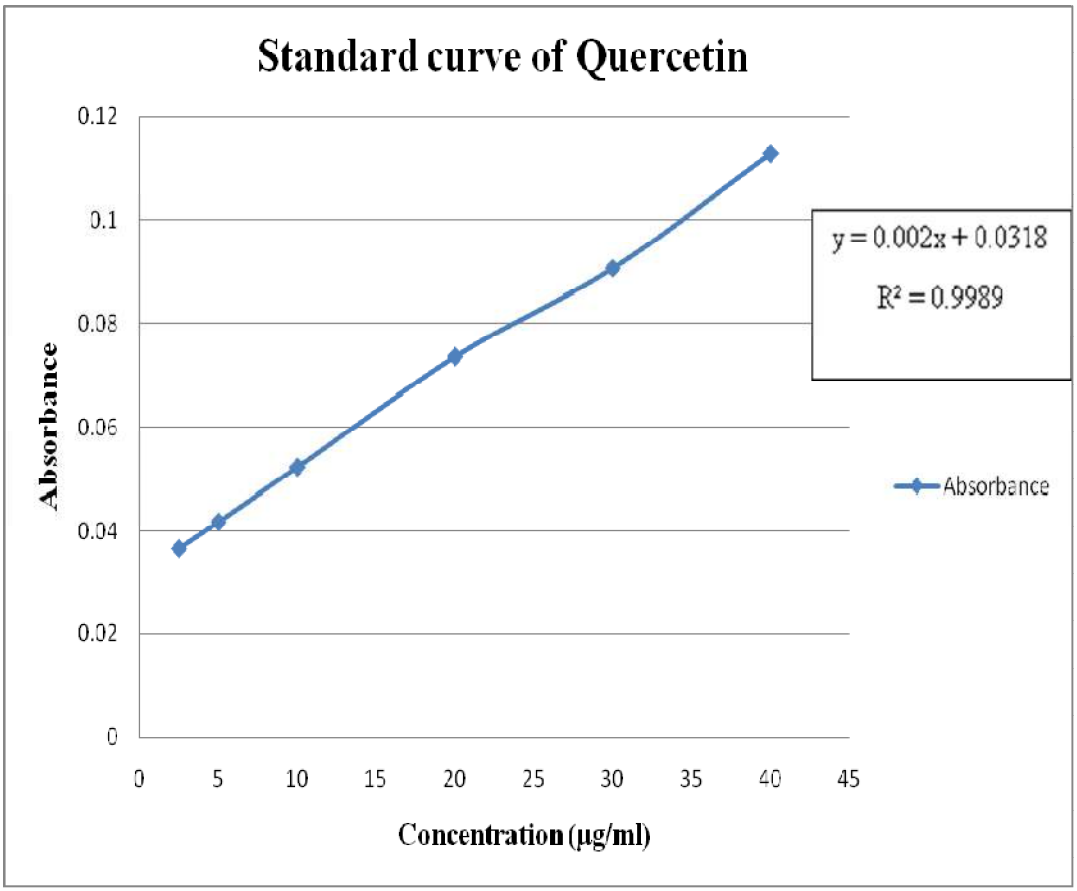


Figure 5.11: Standard curve of Quercetin

Table 5.12: Determination of total flavanoid assay

Sample	Concentration	Absorbance	Total Phenolic content(mg of GAE/mg of dried extract)	Average
MENA	1 mg/ml	0.658	3131	3144.333
	1 mg/ml	0.672	3201	
	1 mg/ml	0.652	3101	

N.B: MENA= methanolic extract of *Nyctanthes arbortristis*

5.4.5 Hydrogen Peroxide Radical Scavenging Assay

The crude methanolic extract of *Nyctanhes arbor-tristis* (leaves) was subjected to free radical scavenging activity and it showed significant free radical scavenging activity with an IC_{50} value of 3.0868 mg/ml whereas IC_{50} value of the standard was 418.407 mg/ml at higher concentration. The percentage inhibition of free radical hydrogen peroxide and the IC_{50} value of crude methanolic extract of *Nyctanthes arbor-tristis* (leaves) are given in table 5.11 and figure 5.15.

Table 5.13: Determination of free radical scavenging capacity for the standard (ascorbic acid)

Standard	Concentration Mg/ml	Absorbance	%Inhibition	IC_{50} mg/ml
Blank	0	2.046	0	418.407
Ascorbic acid	0.2	1.857	9.24	
	0.4	1.787	12.66	
	0.6	1.783	12.85	
	1.0	1.696	17.11	

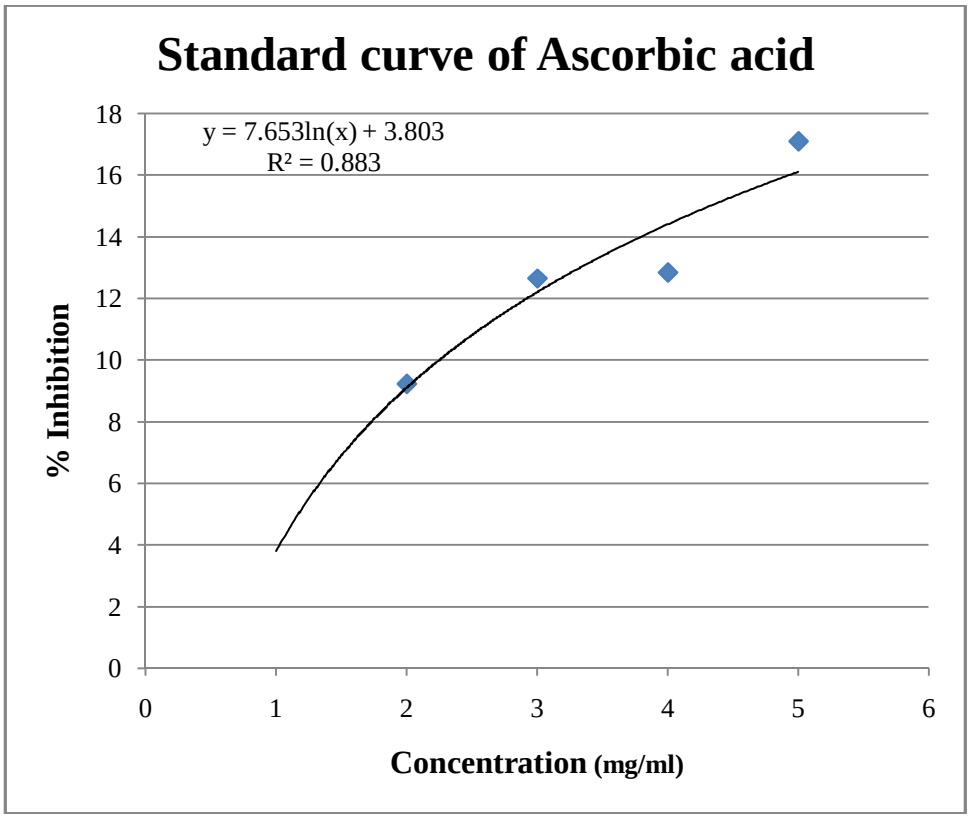
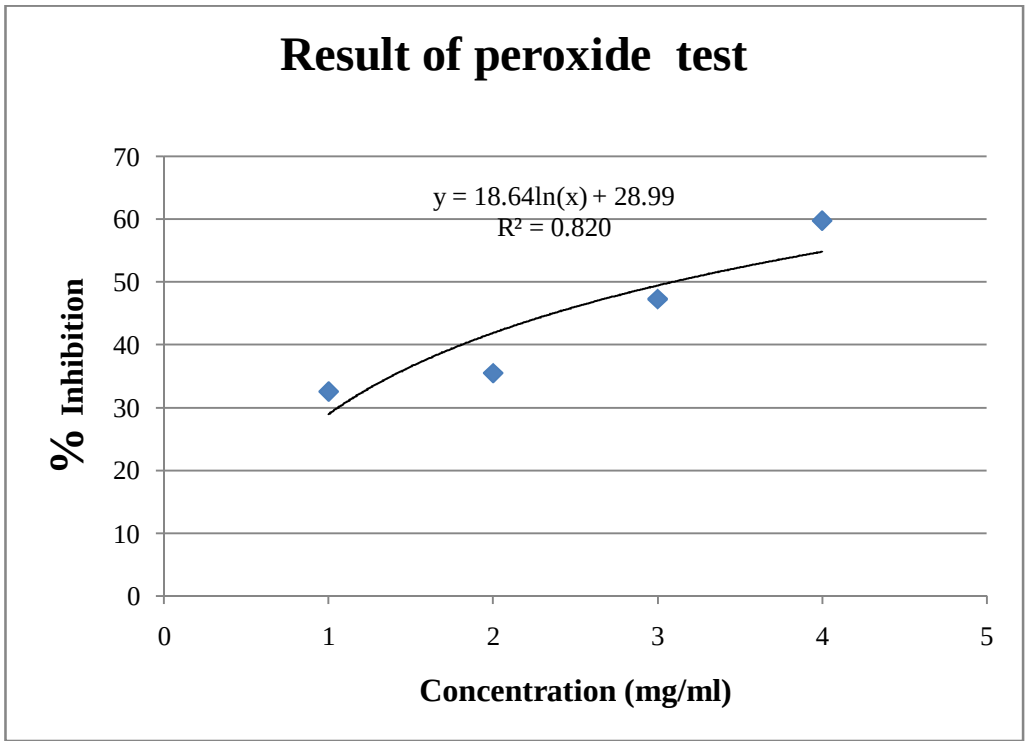


Figure 5.13: H_2O_2 scavenging potential & IC_{50} value of ascorbic acid

Table 5.14: Determination of free radical scavenging capacity for MENA

Sample	Concentration Mg/ml	Absorbance	%Inhibition	IC $_{50}$ mg/ml
Blank	0	1.615	0	
MENA	0.008	1.088	32.63	
	0.012	1.041	35.54	
	0.016	0.851	47.31	3.0868
	0.020	0.65	59.75	



N.B: MENA= methanolic extract of *Nyctanthes arbortristis*

Figure 5.13: H_2O_2 scavenging potential & IC_{50} value of crude methanolic extract of of *Nyctanthes arbor-tristis* (leaves)

5.5 Anti-microbial Assay

The methanolic crude extract of *Nyctanhes arbor-tristis* (leaves) was subjected to the various bacterial cultures and from that zones of inhibition were measured. There was a positive control called Azithromycin was used as 30mcg/disk. And no zone of inhibition was found in the negative control which was conducted by methanol. Zones of inhibition by the mehanolic extract of 1 mg/ml and 10 mg/ml are given table 5.12.

With the values obtained from the table are plotted in the figure 5.16 which shows the zones of inhibition against specific microorganisms. In case of 1mg/ml of extract he maximum zone of inhibition found against *Shigella dysentery* as 10mm and no zone of inhibition found against *Bacillus cereus.* In case of 10mg/ml of extract he maximum zone of inhibition found against

Staphylococcus aureus as 25mm and minimum zone of inhibition found against *Salmonella typhi* as 21mm. From the graph it is clearly seen that in case of 10mg/ml of extract the zone of inhibition found which is greater than the positive control (zone of inhibition by extract=22mm and the zone of inhibition by Azithromycin= 21mm both against *Salmonella typhi)*

Table 5.15: Zones of inhibition of methanolic extract of *Nyctanhes arbor-tristis* (leaves)

Microorganisms	**ZMENA (1mg/ml)**	**ZMENA (10mg/ml)**	**Positive control Azithromycin (30 mcg/disk)**	**Negative control**
Vibrio mimicus	7 mm	17 mm	22 mm	0 mm
Salmonella typhi	6 mm	22 mm	21 mm	0 mm
Shigella dysentery	10 mm	17 mm	23 mm	0 mm
Staphylococcus aureus	8 mm	18 mm	25 mm	0 mm
Bacillus cereus	0 mm	14 mm	23 mm	0 mm

N.B: ZMENA= zone of inhibition of methanolic extract of *Nyctanhes arbor-tristis*

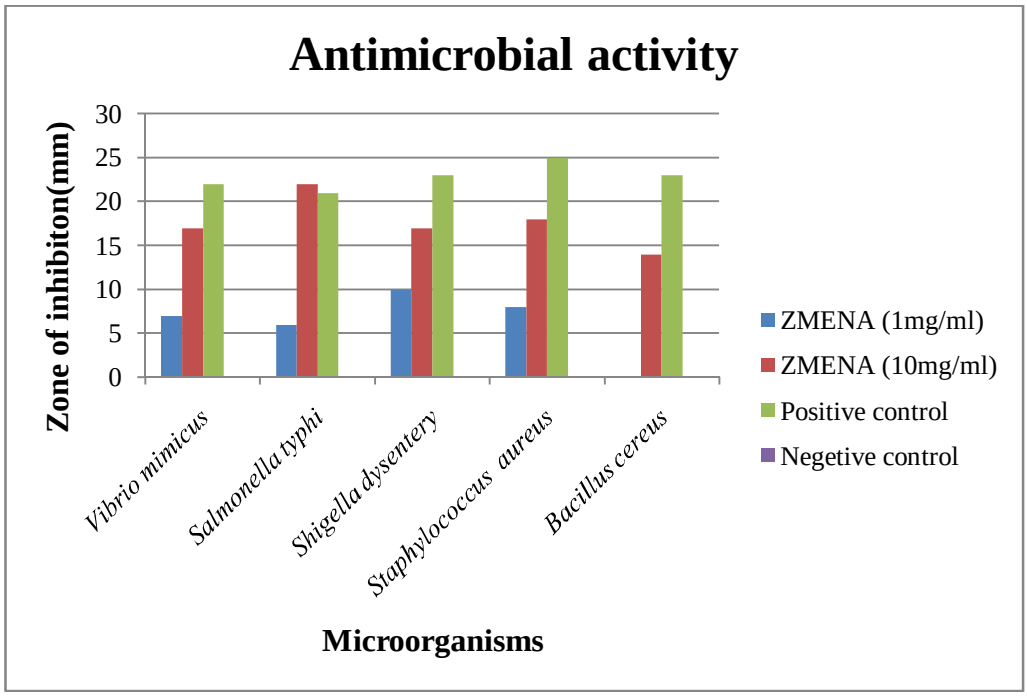


Figure 5.14: Zones of inhibition of methanolic extract of *Nyctanhes arbor-tristis* (leaves) against various microorganisms

N.B: ZMENA= zone of inhibition of methanolic extract of *Nyctanhes arbor-tristis*

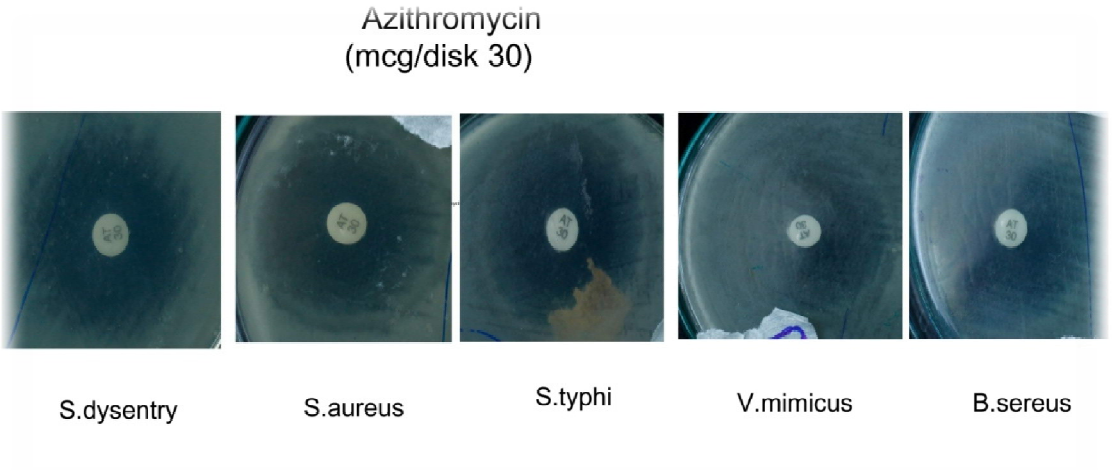


Figure 5.15: Positive control test against microorganisms

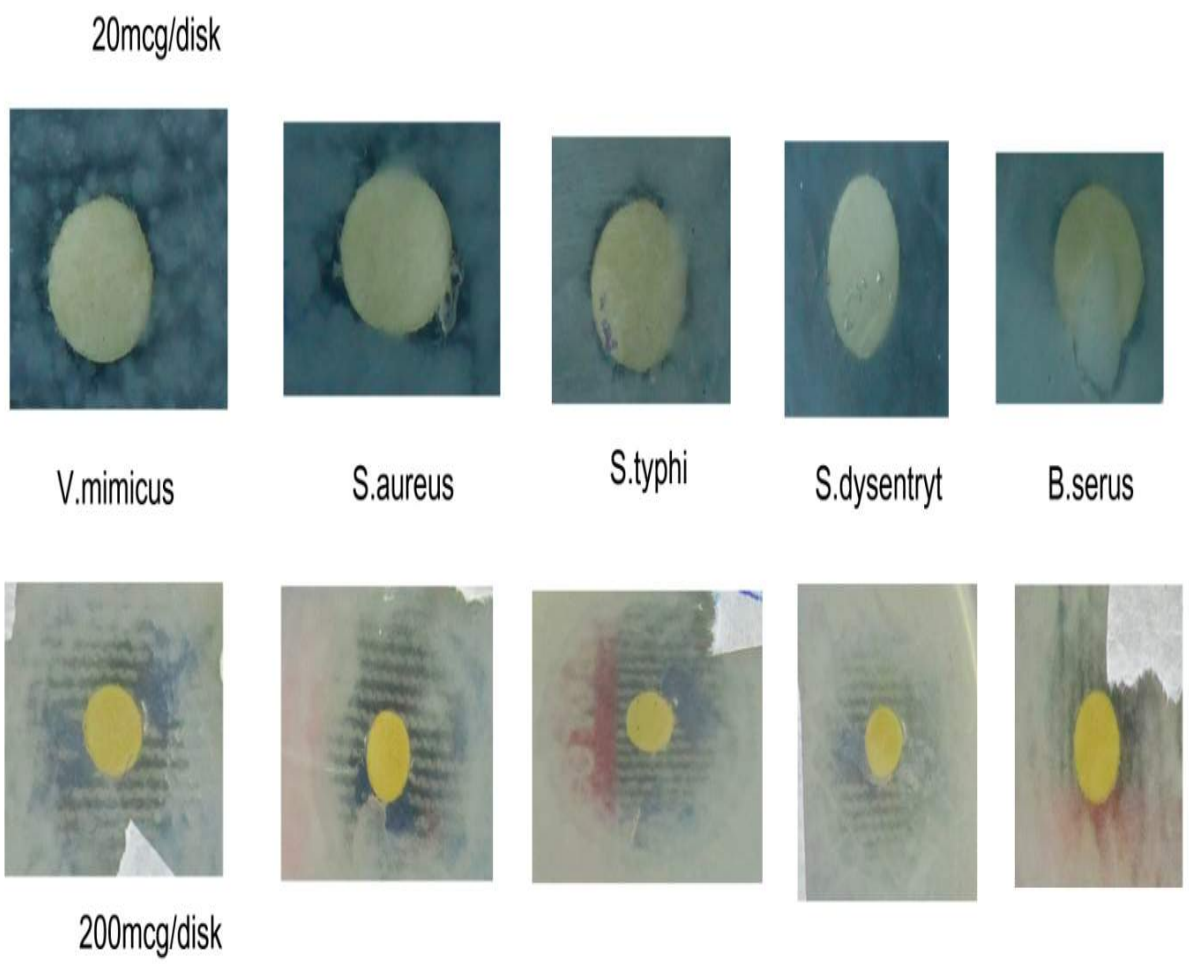


Figure 5.16: Zone of inhibition against microorganisms of both 20 mcg and 200 mcg per disc

5.6 MIC

Zone of inhibition by MIC method, the results obtained are shown in the table 5.13 and in the figure 5.19. When the concentration was 2000 and 200 µg/disc, then the zone of inhibition found 8 and 6 mm respectively. In case of 20, 2, 0.2 µg/disc no zone of inhibition was found.

Table 5.16: Zone of inhibition by MIC method

Sample	**Concentration** µg/disc	**Microorganism**	**MIC in Zone of Inhibition Method**
MENA	2000		8
	200		6

MENA	20	*Vibrio mimicus*	0
	2		0
	0.2		0

N.B: ZMENA= zone of inhibition of methanolic extract of *Nyctanhes arbor-tristis*

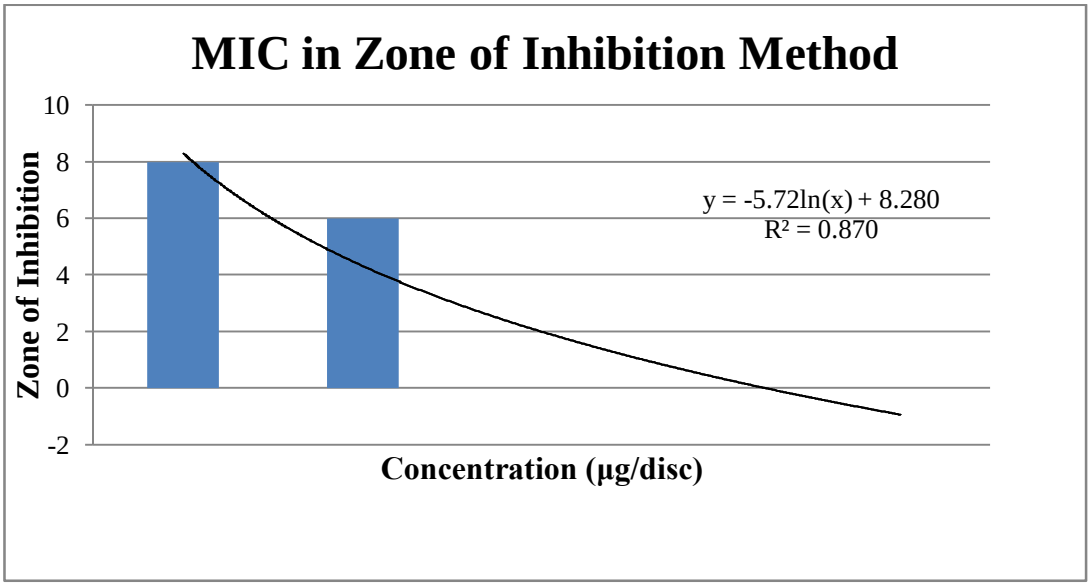


Figure 5.17: Zone of inhibition by MIC method

5.7 TLC Bioautography

When this test was run then a clear zone of inhibition was found against *E.coli.* From the following figure white circle shows that a clear zone of inhibition was found.

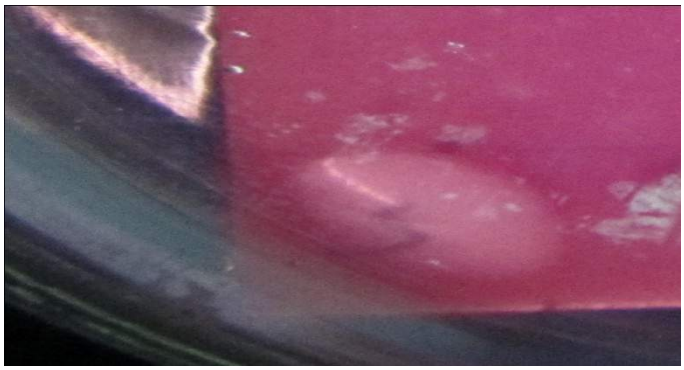


Figure 5.18: Zone of inhibition by TLC bioautography method

5.8 Biomedical test

5.8.1 Haemagglutination Assay (HA)

From the table 6.14 and figure 6.21 it is seen that in case of A^+, B^+ blood group maximum Haemagglutination activity was found of 5mg/ml extract. In case of AB^+ blood group no activity was found. From the table it is also seen that increase the activity with the increased concentration thus in case of concentration 2.5mg/ml moderate activity found.

Table 5.19: Haemagglutination test for methanolic extract of plant

Blood group	Sample			Positive control
	MENA 5mg/ml	MENA 2.5mg/ml	MENA 1.25mg/ml	
A^+	+++	++	+	- - -
B^+	+++	++	+	- - -
O^+	++	++	+	- - -
AB^+	++	+	-	- - -

N.B: ZMENA= zone of inhibition of methanolic extract of *Nyctanhes arbor-tristis*

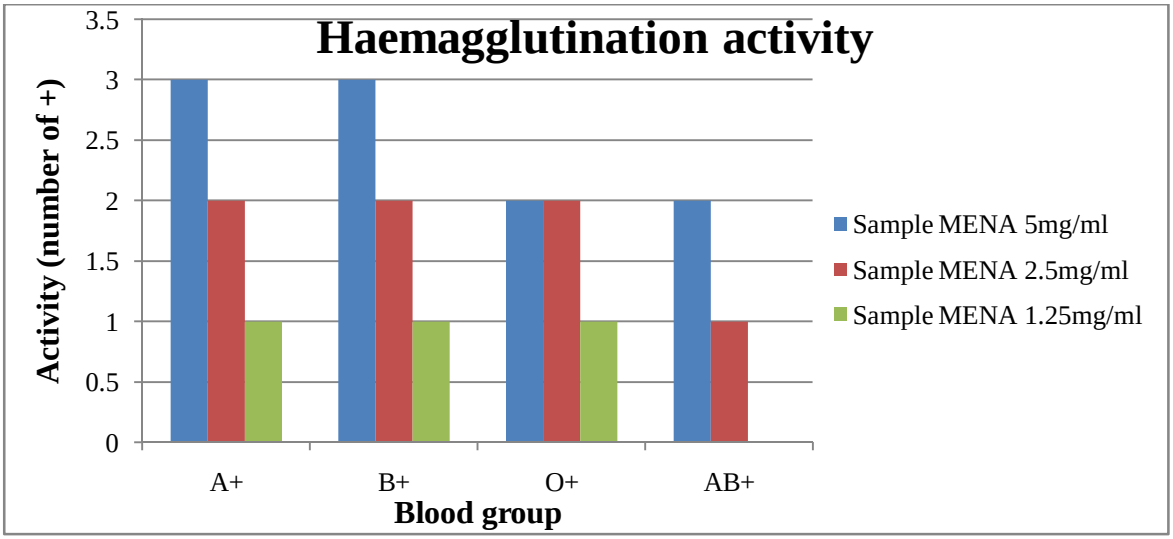


Figure 5.19: Haemagglutination activity against various blood groups

5.8.2 Anti-Hemolytic Activity

The crude methanolic extract of *Nyctanhes arbor-tristis* (leaves) was subjected to free radical scavenging activity and it showed significant free radical scavenging activity with an IC_{50} value of 1.48 mg/ml. The percentage inhibition of free radical hydrogen peroxide and the IC_{50} value of crude methanolic extract of *Nyctanthes arbor-tristis* (leaves) are given in table 6.15 and figure 5.22.

Table 5.18: Determination of free radical scavenging capacity for the MENA

Sample	Concentration	A	%Inhibition	IC_{50} mg/ml
Positive control		0.853	0	1.48
MENA	0.3125 mg/ml	0.4855	43.08324	
	0.6250 mg/ml	0.405	52.52052	
	1.250 mg/ml	0.2895	66.06096	
	2.500 mg/ml	0.1895	77.78429	

N.B: ZMENA= zone of inhibition of methanolic extract of *Nyctanhes arbor-tristis*

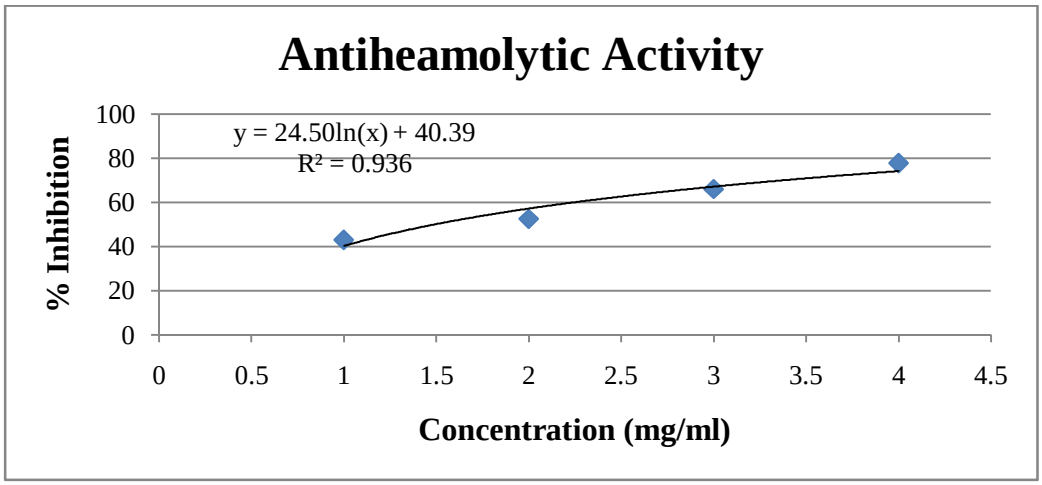


Figure 5.20: Anti heamolytic scavenging potential & IC_{50} value of crude methanolic extract of of *Nyctanthes arbor-tristis* (leaves)

5.9 IR Data Analyses

IR Data Analysis

Results obtained from IR spectrum are given in the figure 5.23 There are lots of stretching found in the IR spectrum as broad and intense spectrum. Those peaks are easy to describe given in the figure 5.24 and in the table 5.16

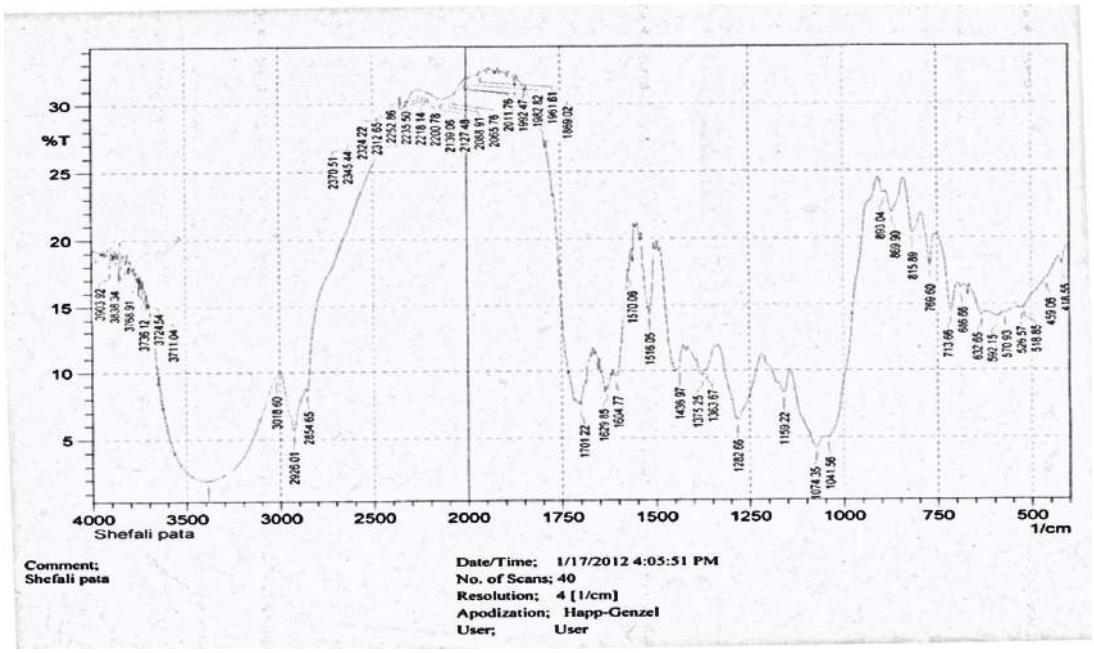


Figure 5.21: IR spectrum of Nyctanthes arbortristis Lin (leaves)

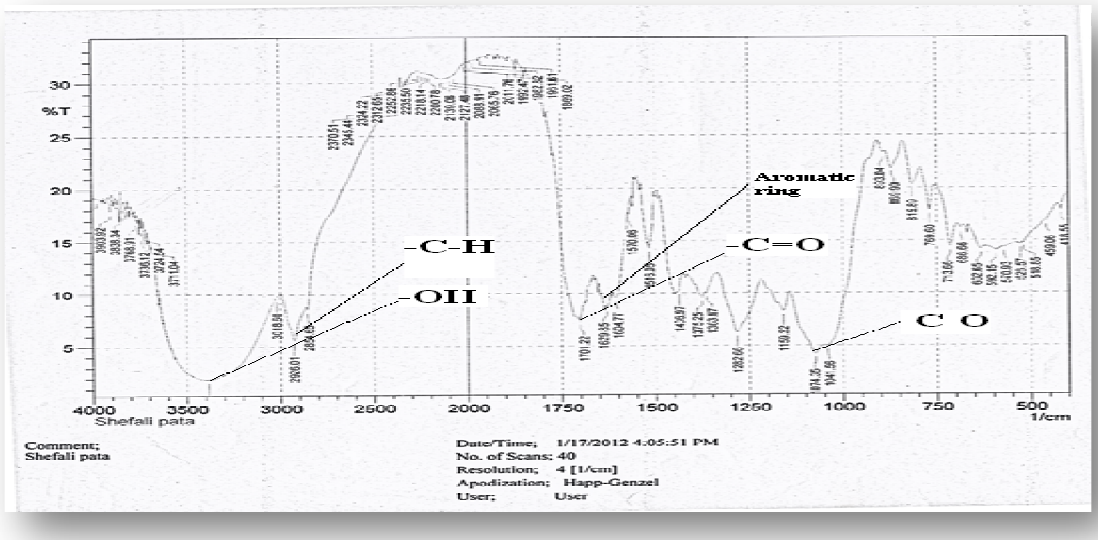


Figure 5.22: Analysis of IR spectrum Nyctanthes arbortristis Lin (leaves)

The figure above shows the IR spectrum of the methanolic extract of the *Nyctanthes arbor-tristis.* As we know a plant extract contains several compounds and IR spectrum gives the idea about the functional group present in the compounds. So, we can get idea about the functional groups present in compounds present in the sample.

By interpreting the IR spectrum, presence of following group may be identified-

1. Alcohol/ Phenolic group: Presence of broad absorption near 3400-3300 cm-1 which is confirmed by C-O absorption near 1300-1000 cm-1.

   Alcoholic group may be of aliphatic alcohol, phenolic or carboxylic alcoholic group.
2. Carbonyl group: Strong absorption in the region 1820-1660 cm-1
3. Aromatic ring: Strong absorption at 1600-1450 cm-1

So, from the above discussion we can say that the compounds present in the sample mostly contain three functional groups i.e. alcoholic, carbonyl and aromatic ring. As we know these groups play an important role in receptor binding and phenolic groups are also responsible for the antioxidant property of the plant. So, the plant extract can be subjected to test for determining antioxidant capacity.

Table 5.19: Probable functional group presnt in the sample obtained from IR data analysis**[110]**

Peak (Cm^{-1})	Intensity	Probable group
1375-1436	Medium	OH group in blending(within plane)
686.66	Weak	OH group in blending(out of plane)
1041.56	Medium	Sulfoxide

1550	Strong	amino acid zwitterions
1670	Strong	Aromatic carboxylic acid, aromatic ketone
1200	Strong	Phenol
1282	Medium	Carboxylic acid(any)
1150	Strong	Tertiary alcohol
3018	Weak	Benzene/subs. Benzene.
750	Strong	Mono substituted benzene / ortho-disubstituted benzene
800	Strong	meta-disubstituted benzene/ para-disubstituted benzene

CHAPTER 6

CONCLUSION

CONCLUSION

For the plant physiologist, work on medicinal plants opens up a wide range of research possibilities, and plant physiological studies would indeed have a major role to play in this burgeoning field. With only a few exceptions, many widely used medicinal plants have not received the extensive plant physiological characterization received by food crops or model plant systems. Although active phytochemicals may have been identified, in general, many pathways for the biosynthesis of specific medicinal compounds and the factors (biotic and abiotic) regulating their production remain unclear. At present, a major concern with the use of phytomedicines regards the maintenance of consistent medicinal quality in botanical medicines.

Therefore, plant materials can be potential sources of chemically interesting and biologically important drug entrant. And for this purpose the plant can be further screened against various diseases in order to find out its unexplored efficacy with a gaze to the future with a great deal of expectancy.

Crude extract of *Nyctanthes arbor-tristis* leaves of the family *Oleaceae* is traditionally used in various disease conditions. There are many established research reports regarding the phytochemical and pharmacological properties of this product. Still there are plenty of scopes to establish a variety of properties which are significantly beneficial to mankind.

C h a p t e r B

M u s a s a p i e n t u m V a r . s y l v e s t r i s

Chapter – 1

PLANT REVIEW

1.1 OVERVIEW OF FAMILY *MUSACEAE*

Musaceae**,** the banana family of plants, consisting of 2 genera, *Musa* and *Ensete*, with about 50 species native to Africa, Asia, and Australia. **[47]**

a) Musa - Type genus of the Musaceae: bananas

b) Ensete - Old World tropical herbs: Abyssinian bananas

Musaceae is in the major group *Angiosperms* (Flowering plants). The common banana (*M. sapientum*) is a subspecies of the plantain (*M. paradisiaca*). Both are important food plants.

Figure 1.1: Plant and fruit

The slender or conical false trunk of Musaceae herbs may rise to 15 meters (50 feet). **[47]**

The “trunk” is formed by the leaf sheaths of the spirally arranged leaves, which form a crown at the top. The large leaves may be up to three meters long and half a meter wide. The prominent midrib of the leaf is joined at right or slightly oblique angles with parallel veins. When the plant grows in an unsheltered place, wind and rain easily tear the leaves between the veins, giving the

leaves a fringed or ragged appearance. The large, leathery bracts (leaf like structures) are red to purple. The yellow flowers have five fertile stamens and are rich in nectar.

Some species of wild bananas, such as *M. coccinea,* have ornamental scarlet flowers but inedible fruit. *M. textilis* from the Philippines furnishes Manila hemp, also called abaca fiber. The genus *Ensete* of Africa produces no edible bananas, but the flower stalk of one species, *E. ventricosa,* is edible after cooking. Species of *Ensete* are distinguished from those of *Musa* by their larger seeds. *See also* abaca; banana; plantain.

Figure 1.2: Bunch/Puso of Banana

1.1.1 Statistics

Species of Musaceae contained within The Plant List belong to 2 plant genera.

The Plant List includes 235 scientific plant names of species rank for the family Musaceae. Of these 74 are accepted species names.

The Plant List includes a further 182 scientific plant names of intraspecific rank for the family Musaceae. **[48]**

1.1.1.3 Common names of *Musaceae* family

Table 1.1: Some plant with common names of *Musaceae* family[49]

Plant	**Common name**
Musa cheesmanii	Cheesman's Banana
Musa nana	Dwarf Banana
Musa bauensis	Bau Banana
Musa borneensis var. flavida	*Angkadan Banana*
Musa campestris var. campestris	Swamp Banana
Ensete glaucum	Snow Banana
Musella lasiocarpa	Chinese Yellow Banana, Golden Lotus Banana
Musa textilis	Abaca
Ensete superbum	Banana
Ensete perrieri	Madagascar Banana
Musa acuminata subsp. microcarpa	Monkey Fingers Banana
Musa sapientum	French plantain banana
Musa acuminata	Dwarf Brazilian Banana 'Dwarf Brazilian'
Ensete ventricosum	False Banana, Red Abyssinian Banana, Wild Banana 'Maurelii'
Musa siamensis	Banana 'Thai Gold'

1.1.1.4. Size

Banana (Musaceae) is a large, perennial, monocotyledonous herb 2–9 m (6.6–30 ft) in height that arises from large, subterranean rhizomes (usually called "corms).**[50]**

1.1.1.5. Flowers

Upon flowering, the true stem or growing point emerges from the center of the tightly rolled bunch of leaves. This odd-looking "flower cluster" is actually an elongated, plump, purple to green "bud" (sometimes called the "bell" or "heart"), which at first displays large female flowers (whose ovaries ripen into fruit). As the "bud" elongates, it exposes semicircular layers of female flowers, then neutral flowers, and finally small, generally non-functional (with no viable pollen) male flowers. Each group of flowers is arranged radially on the stem in nodal clusters. Each flower cluster is borne on a prominence on the stem bearing the fruit (peduncle) and covered by a bract.About 12–20 flowers are produced per cluster. Collectively, the flowering parts and fruit are referred to as the bunch.Individual clusters of fruits are known as hands,

and individual fruits are known as fingers. **[50]**

1.1.1.6 Leaves

The entire above-ground portion of the plant is not a true woody trunk, as in other trees, but a "false trunk" or "false stem" that consists of leaves and their fused petiole bases, referred to as a pseudostem. The pseudostem supports a canopy consisting of 6–20 (or more) leaves. **[50]**

1.1.1.7 Fruit

Musa fruits are variable in size, shape, and color. They are generally elongate-cylindrical, straight to strongly curved, 3–40 cm (1.2–16 in) long, and 2–8 cm (0.8–3 in) in diameter.The fruit apex is important in variety identification; it may be tapered, rounded, or blunt. The skin is thin and tender to thick and leathery, and silver, yellow, green, or red in color. Inside the ripe fruit, the flesh ranges from starchy to sweet, and in color from

white, cream, yellow, or yellow-orange to orange. Bananas also vary in peel thickness. Some variet-ies have a thin peel and are more susceptible to damage in transport, whereas others have a comparably thicker peel. **[50]**

1.1.1.8 Seeds

Cultivated varieties are typically seedless. When seeds are present, they vary among species in shape and morphology. Seeds of Musa balbisiana, parent of many commercial edible banana varieties, are dark brown, ovoid, about 4mm (0.2 in) long, with a conspicuous white, powdery endosperm. **[50]**

1.2. Common name of *Musa sapientum* [50]

ASSAMESE :	Kala
BENGALI :	Kala
BURMESE :	Taw nget byaw .
CHINESE :	Da jiao, Fen ba jiao.
ENGLISH :	Dessert banana, Sweet-fruited banana.
FRENCH :	Banane cultivée, Bananier des sages, Bananier commun.
GERMAN :	Adamsfeige, Dessertbanane, Jamaicabanane, Obstbanane.
ITALIAN:	Banana comune, Banano comune, Fico d'Adamo.
JAPANESE :	Banana.
LAOTIAN :	Kwàyz khauz.
MALAY :	Biu (Bali), Cau (Sunda), Gedang (Java), Puti (Lampung), Kulo (Ambon), Pisang.
SPANISH :	Banana, Bananeira, Guineo, Plátano (Mexico).
TAGALOG:	Saging.

TAMIL : Vaazhai, Vaazhaipoo (flower).

TELUGU : Arati, Artipandu, Kadala.

THAI : Kluai.

CHAPTER - 2

PREPARATION OF PLANT EXTRACT FOR EXPERIMENT

2.1 Plant Selection

There are thousands of medicinal plants in Bangladesh and in this Indian subcontinent. Among these plants it was not easy to select a few plants for the research purpose. The selection of plant greatly affects the research work if there is carelessness takes place. Plant secondary metabolites often accumulate in specific plant parts. Thus, unless it is already known which parts contain the highest level of the compounds of interest, it is important to collect multiple plant parts, or the whole plant to ensure the extracts prepared representative of the range of secondary metabolites. For drug discovery from plants, sample may be selected using a number following criteria by which the research work will run smoothly.

From the literature review it is seen that there are lots of work on the plant *Musa sapientum* Linn about the pharmacological activity of the plant. But there is a least of work has been found about chemical investigation of this plant, especially about the leaves of this plant. So I got a chance to select the leaves of *Musa sapientum* Linn for my research work to see whether the leaves have antipyretic and analgesic activity or not.

2.2 Plant Collection

After selection of plant it is must to collect the plant parts for the research purpose. Throughout Bangladesh the plant *Musa sapientum* Linn is available. The plant leaves were collected from the rural area near Dhaka, Bangladesh.

2.3 Plant Identification

It is advisable to attempt field identification of sample collected. To aid taxonomic experts in confirming the field identification and to get a permanent scientific record (accession number) a voucher specimen was prepared on 20th December, 2011. In the voucher specimen the dried leaves of sample plant were attached and some information like local name, medicinal use, location of the sample plant were also written on that voucher specimen. Finally, from BNH

(Bangladesh National Herbarium) I got the identification or accession number of collected sample on 12th May, 2012, and the accession number is 36560 with *Musa sapientum* Linn and *Musaceae* scientific name and family name of the plant respectively.

2.4 Drying of Plant Sample

After the collection of sample it needs to be dried to make the sample extract. In general the plant material should be dried at temperature below 30^0C to avoid the decomposition of thermolabile compounds. So sun drying can be very effective but drawback is sometimes water molecules are absorbed by the sample and hence microbial growth can affect the phytochemical study. The leaves were dried in the sun light thus chemical decomposition can not take place.

2.5 Grinding of Dried Sample

Small amount of plant material can be milled using grinder or blender. But if the sample is in high amount then it is easier to get powdered sample by grinding from a spice mill. Grinding improves the efficiency of extraction by increasing surface area. It also decreases the amount of solvent required for the extraction. The dried samples were ground to coarse powder with a mechanical grinder (Blender) and powdered samples were kept in clean closed glass containers pending extraction. During grinding of sample, the grinder was thoroughly cleaned to avoid contamination with any remnant of previously ground material or other foreign matter deposited on the grinder.

2.6 Maceration of Dried Powdered Sample

2.6.1 Principle

The choice of extraction procedure depends on the nature of the plant material and the components to be isolated. The principle of solid-liquid extraction is that when a solid material comes in contact with a solvent, the soluble components in the solid material move to the

solvent. Thus, solvent extraction of plant material results in the mass transfer of soluble active principle (medicinal ingredient) to the solvent, and this takes place in a concentration gradient. The rate of mass transfer decreases as the concentration of active principle in the solvent increases, until equilibrium is reached, i.e. the concentrations of active principle in the solid material and the solvent are the same. Thereafter, there will no longer be a mass transfer of the active principle from plant material to the solvent. Since mass transfer of the active principle also depends on its solubility in the solvent, heating the solvent can enhances the mass transfer. Moreover, if the solvent in equilibrium with the plant material is replaced with fresh solvent, the concentration gradient is changed.

2.6.2 Procedure

After getting the sample as dried powdered, the sample (500 Gram) was then soaked in 1000mL of Ethanol for five days, the process is known as maceration technique. A glass made jar with plastic cover was taken and washed thoroughly with ethanol and dried. Then the dried powder sample was taken in the jar. After that Ethanol (10000mL) was poured into the jar up to 1-inch height above the sample surface as it can sufficiently cover the sample surface. The plastic cover with aluminium foil was closed properly to resist the entrance of air into the jar. This process was performed for five days. The jar was shaked in several times during the process to get better extraction.

2.7 Filtration of the extract

After the extraction process the plant extracts was filtered with sterilized cotton filter. The cotton was rinsed with ethanol and fitted in a funnel. The filtrate was collected in a beaker. Then again it was filtered and this time What man's filter was used for getting more clear extract which would be useful making the sample more concentrate in Rotary Evaporation Technique. Then the

filtrate was taken into a volumetric flask and covered with alumina foil paper and was prepared for rotary evaporation.

2.8 Concentrated sample by rotary evaporation

2.8.1 Principle

A rotary evaporator is a device used in chemical laboratories for the efficient and gentle removal of solvents from samples by evaporation. Rotary evaporators are also used in molecular cooking for the preparation of distillates and extracts. A rotary evaporator consists of following parts-

- A motor unit that rotates the evaporation flask or vial containing the user's sample.
- A vapor duct that is the axis for sample rotation, and is a vacuum-tight conduit for the vapor being drawn off of the sample.
- A vacuum system, to substantially reduce the pressure within the evaporator system.
- A heated fluid bath (generally water) to heat the sample.
- A condenser with either a coil passing coolant, or a "cold finger" into which coolant mixtures such as dry ice and acetone are placed.
- A condensate-collecting flask at the bottom of the condenser, to catch the distilling solvent after it re-condenses.
- A mechanical or motorized mechanism to quickly lift the evaporation flask from the heating bath.

2.8.2 Affecting Factors

There are following factors, omission of one of the following factor may interfere the sample concentration procedure and thus which may interfere the phytochemical investigation.

-Remove the flask from the heat bath.

-Opening the stopcock

-Halting the rotor

-Turning off the vacuum/aspirator

-Disconnecting the flask

-Dropping flask in heat bath.

2.8.3 Procedure

After the filtration process two parts were obtained namely ‘residue part’ and ‘filtered part or filtrate’. The filtered part, which contains the substance soluble in ethanol, was putted into a1000ml round bottom flask (BOROSIL), and then the flask was placed in a rotary evaporator. The evaporation was done at 45 degree Celsius temperature. The number of rotation per minute was selected as 130 RPM. The pressure of the vacuum pumper machine (Biometra) was 6 bar. The water flow through the distillation chamber was also provided in a satisfactory flow rate. When the evaporation seemed to be satisfactory, then the ethanolic extract was collected in a 50mL beaker. The evaporator flask was rinsed by diethyl ether. Then the beaker was covered with aluminum foil paper and kept on the water bath for 60 minutes and the temperature of water bath maintained as 50^0C. Finally the concentrated ethanolic plant extract was found and stored in the laboratory refrigerator from which the extract was used for many chemical investigations.

Chapter – 3

Phytochemical and Pharmacological review (Literature Review) of *Musa Sapientum*

3.1 Phytochemical Review

Carbohydrates have been isolated from M. Sapientum. **[51]** Catecholamines such as norepinephrine, serotonin, dopamine **[52] & [53]**, tryptophan, indole compounds **[54]**, pectin have been found in the pulp. Several flavonoids andrelated compounds (Leucocyanidin, quercetin and its 3-O-galactoside, 3-O-glucoside, and 3-O-rhamnosyl glucoside) wereisolated from the unripe pulp of plantain **[55] & [56].** Serotonin, nor-epinephrine,tryptophan, indole compounds, tannin, starch, iron, crystallisableand non crystallisable sugars, vitamin C, B-vitamins, albuminoids,fats, mineral salts have been found in the fruit pulp of M. Paradisiaca and M. Sapientum **[57]**.

Acyl steryl glycosides such as sitoindoside-I,sitoindoside-II, sitoindoside-III, sitoindoside-IV and sterylglycosides such as sitosterol gentiobioside, sitosterol myo -inosityl- β -D-glucoside have been isolated from fruits of M. Paradisiaca a bicyclic diarylheptanoid, rel - (3S, 4aR, 10bR) – 8 - hydroxy-3-(4-hydroxyphenyl) – 9 –methoxy - 4a,5,6,10b – tetrahydro - 3H - naphtho [2,1-b] pyran, and 1,2-dihydro-1,2,3-tri hydroxy -9 - (4 - methoxyphenyl) phenalene, hydroxyanigorufone, 2 - (4 - hydroxyphenyl) naphthalic anhydride, 1,7 – bis (4-hydroxyphenyl) hepta – 4 (E), 6 (E) – dien -3 - one. **[58]**

The isolation of severaltriterpenes such as cyclomusalenol, cyclomusalenone, 24 methylenecycloartanol, stigmast-7-methylenecycloartanol, stigmast-7 en-3-ol, lanosterol and β – amyrin was reported**[59].** An antihypertensive principle, 7,8-dihydroxy-3-methylisochroman-4-one was isolated from the fruit peel of M. Sapientum **[60]**Cycloartanetriterpenes such as 3-epicycloeucalenol, 3-epicyclomusalenol, 24 methylen epollinastanone, 28-norcyclo musalenone, 24-oxo-29-norcycloartanone have been isolated from the fruit peel of M.sapientum **[61]**.

Cellulose, hemicelluloses, arginine, aspartic acid,glutamic acid, leucine, valine, phenylalanine and threonine havebeen isolated from pulp and peel of M. Paradisiaca **[62].** Hemiterpenoid glucoside (1,1-

dimethylallyl alcohol), syringin, (6S, 9R)-roseoside, benzyl alcohol glucoside,(24R)-4α,l4 α,24-trimethyl-Sacholesta-8,25(27)-dien-3β o1 havebeen isolated from flower of M. Paradisiaca [30].Structures of some important isolatedchemicals-

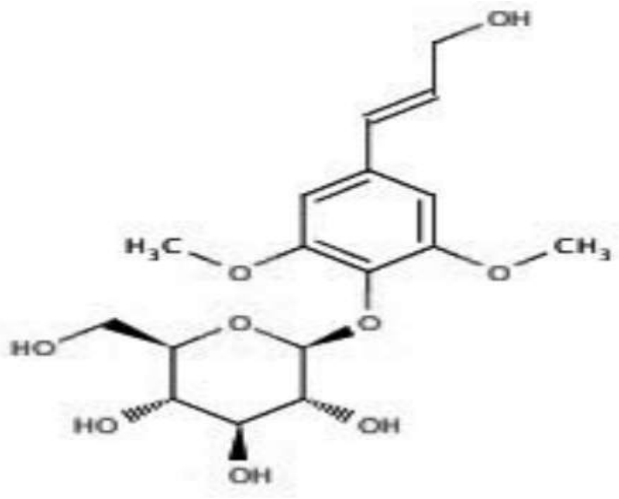


Figure1: Syringin

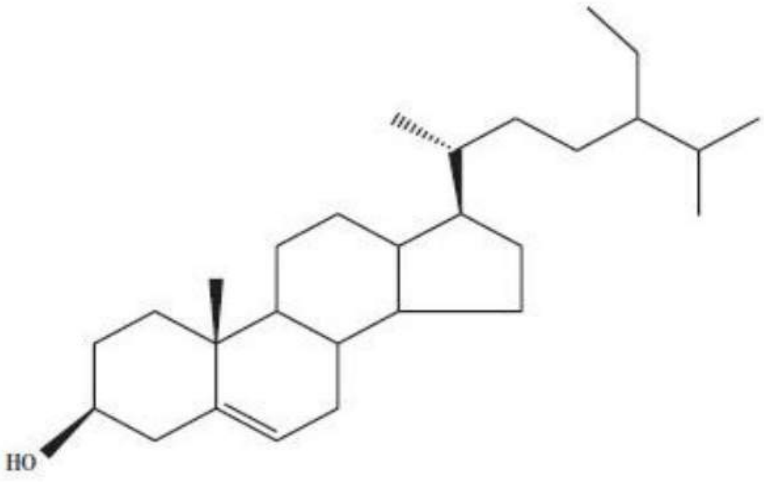


Figure2: β-Sitosterol

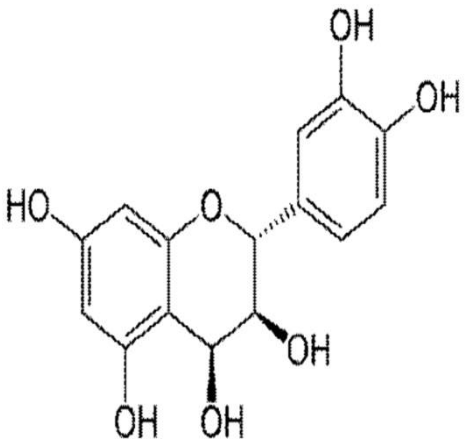


Figure3: Leucocyanidin

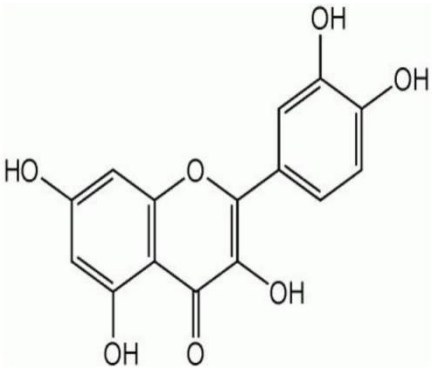


Figure4: Quercetin

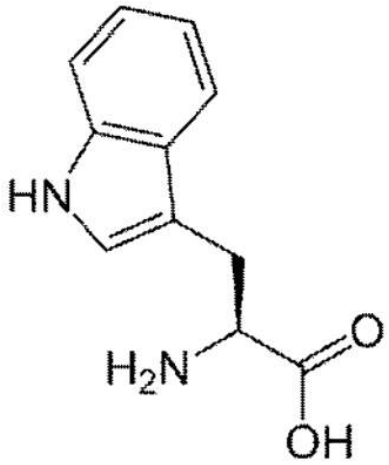


Figure5: L-tryptophan

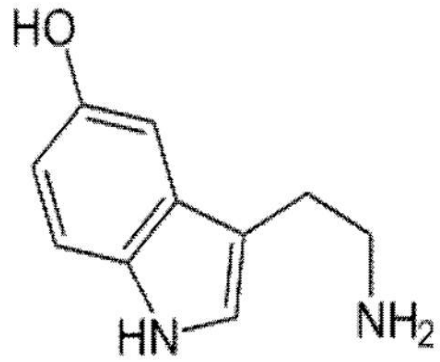


Figure6: 5-Hydroxytryptamine

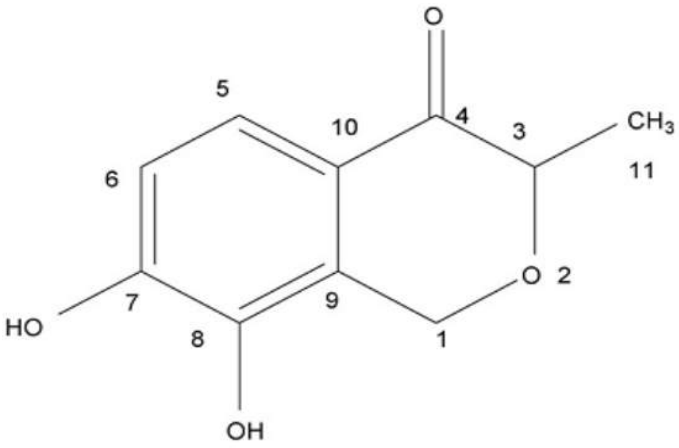


Figure7: 7, 8-dihydroxy-3-methyl isochroman-4-one

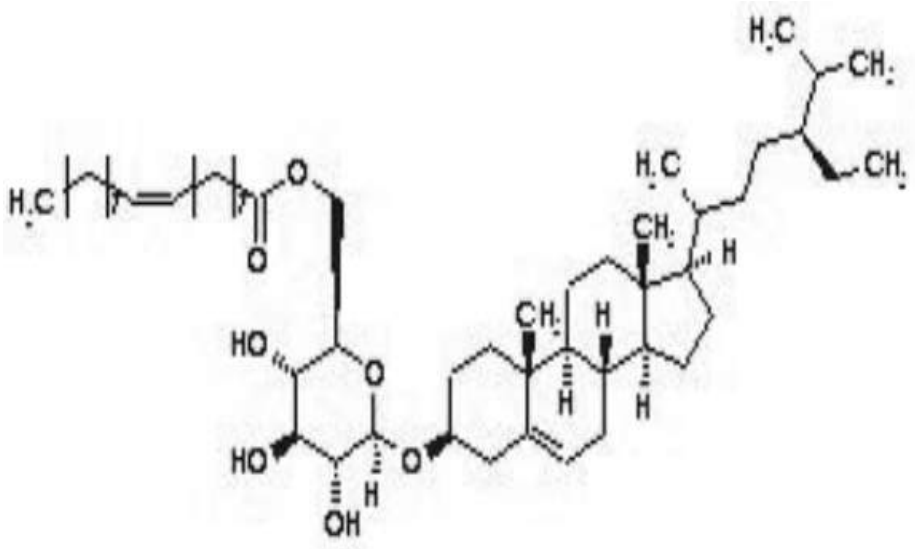


Figure8: Sitoindoside-II

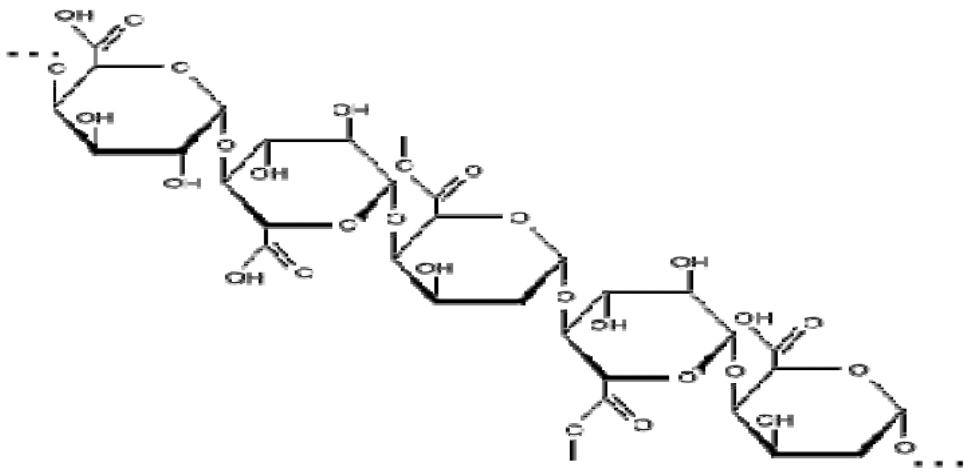


Figure9: Pectin

Structures of some phytochemicals isolated from Musa paradisiaca and Musa sapientum.

3.1.1 Chemical composition of of *Musa sapientum*

Musa sapientum peels were analyzed for minerals, nutritional and anti – nutritional contents. The result of mineral content indicate the concentrations (mg/g) of potassium, Calcium, sodium, iron, manganese, bromine, rubidium, strontium, zirconium and niobium to be 78.10, 19.20, 24.30, 0.61, 76.20, 0.04, 0.21, 0.03, 0.02 and 0.02 respectively. The percentage concentrations of protein, crude lipid, carbohydrate and crude fiber were 0.90, 1.70, 59.00 and 31.70 respectively **[63].** The results indicate that if the peels are properly exploited and process, they could be a high-quality and cheap source of carbohydrates and minerals for livestock.

Table 3.1 Minerals composition of Musa sapientum peel

Element	**Concentration (mg/g)**
Potassium	78.10 + 6.58
Calcium	19.20 + 0.00
Sodium	24.30 + 0.12

Iron	0.61 + 0.22
Manganese	76.20 + 0.00
Bromine	0.04 + 0.00
Rubidium	0.21 + 0.05
Strontium	0.03 + 0.01
Zirconium	0.02 + 0.00
Niobium	0.02 + 0.00

Values are Mean ± SE

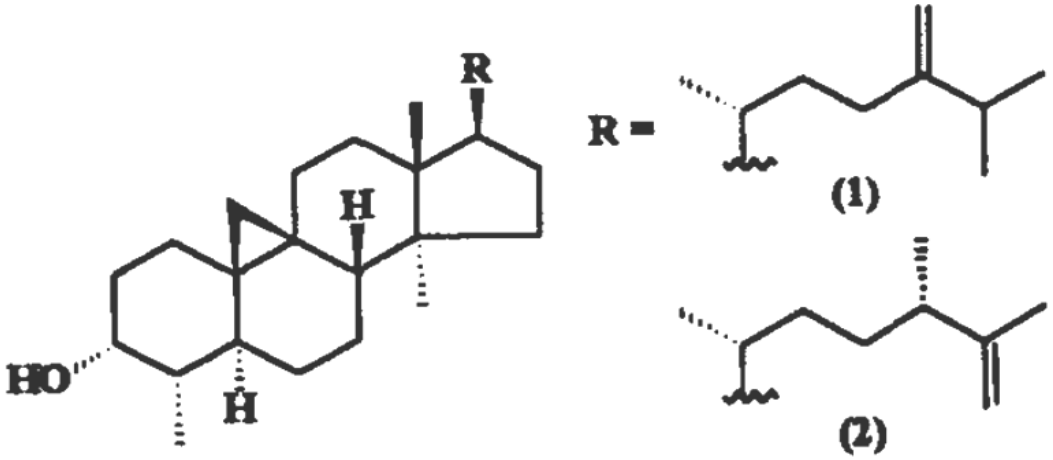
Table 3.2 Proximate composition and anti - nutritional content of Musa sapientum peel

Parameter	**Concentration**
Moisture (%)	6.70 + 02.22
Ash (%)	8.50 +1.52
Organic matter (%)	91.50 + 0.05
Protein (%)	0.90 + 0.25
Crude Lipid (%)	1.70 + 0.10
Carbohydrate (%)	59.00 + 1.36
Crude Fibre (%)	31.70 + 0.25
Hydrogen cyanide (mg/g)	1.33 + 0.10
Oxalate (mg/g)	0.51 + 0.14
Phytate (mg/g)	0.28 + 0.06
Saponins (mg/g)	24.00 + 0.27

Values are Mean ± SE

Plantain sheath (Musa sapientum) contained 6.4% dry matter and 3.4% crude protein, 31.4%

crude fiber **[64]**, 34.6% cellulose, 15.5% hemicellulose and 6% lignin in dry matter. Five novel cycloartane-type triterpenes were isolated from the nonsaponifiable lipids obtained from the methanol extract of the fruit peel of Musa sapientum L. **[61]**. Their structures were determined to be 3-epicycloeucalenol, 3-epicyclomusalenol, 24-methylenepollinastanone, 28-norcyclomusalenone and 24-oxo-29-norcycloartanone by spectroscopic and chemical methods.



(1) 3-epicycloeucalenol and (2) 3-epicyclomusalenol

A new isochroman-4-one, 7, 8-dihydroxy-3-methylisochroman-4-one was isolated from water soluble fraction of Musa sapientum L.**[63]**. Its structure was determined by spectroscopic evidences and its total synthesis has also been reported. The compound showed potent antihypertensive activity.

Alkali treatment coupled with high pressure defibrillation and acid treatment have been tried on banana fibers obtained from the pseudo stem of the banana plant Musa sapientum **[62]**. The structure and morphology of the fibers have been found to be affected on the basis of the concentration of the alkali and acid and also on the pressure applied. Steam explosion in alkaline medium followed by acidic medium is found to be effective in the depolymerization and defibrillation of the fiber to produce banana nanowhiskers. The chemical constituents of raw and

steam exploded fibers were analyzed according to the ASTM standards. Structural analysis of steam exploded fibers was carried out by FTIR and XRD. The fiber diameter and percentage crystallinity of the modified fibers were investigated using X-ray diffraction studies. Characterization of the fibers by SFM and TEM supports the evidence for the development of nanofibrils of banana fibers.

An enzyme system isolated from the pulp of banana fruit (Musa sapientum) was capable of 177atalyzing the hydroxylation of the monophenol, tyramine, to the diphenol, dopamine (3,4-dihydroxyphenylethylamine)**[67]**. Unlike some tyrosinases, the reaction was not stimulated by catalytic amounts of diphenolic reaction product. Ascorbic acid, however, reduced the initial lag period in the oxidation of tyramine, stimulated the reaction rate and promoted the accumulation of dopamine during the first few minutes of the reaction. The hydroxylation of tyramine was apparently dependent upon molecular oxygen. On the basis of these observations it is tentatively suggested that the enzyme is a tyramine hydroxylase which may be responsible for the formation of dopamine in the banana.

3.2 Pharmacological Review

3.2.1 Antidiarrhoeal activity

The antidiarrhoeal activity of banana in rats was observed as early as in 1930s. This effect in the intestinal diseases was attributed to the pectin content of banana. Later banana diet was reported to be effective and advantageous in bacillary dysentery in a proctoscopic study on 127 patients of age nine month to forty eight years **[68]**. Banana flakes has also been tested and found effective in the treatment for diarrhoea in critically ill patients receiving enteral feedings **[69].** The antidiarrhoeal activity of green banana diet was found very effective in children with diahorea **[70]**.

3.2.2 Antiulcerative activity

Banana is used in herbal medicin to treat peptic ulcer disease. The use of M. Sapientum in peptic ulcer as a component of herbal medicine has been evaluated and found effective **[71].** reported that pectin and phosphatidylcholine in green banana strengthens the mucous-phospholipid layer that protects the gastric mucosa**.** They also reported that the gastric mucosa protective activity of the banana is due to multiple active components. It was reported that a natural flavonoid from the unripe banana (M. sapientumvar. paradisiaca) pulp, leucocyanidin, protects the gastric mucosa fromerosions**[72].** Leucocyanidin and the synthetic analogues, hydroxyethylated leucocyanidin and tetraallyl leucocyanidin werefound to protect the gastric mucosa in aspirin-induced erosions inrat by increasing gastric mucus thickness**[72].**Goel et al. (1986) reported that banana pulp powder (M. Sapientum var. Paradisiaca) showed significant antiulcerogenic activity inaspirin, indomethacin-, phenylbutazone-, prednisolone-inducedgastric ulcers and cysteamine- and histamine-induced duodenalulcers in rats and guinea-pigs, respectively**[71].** The authors attributedthe effect to increased mucosal thickness and increased [3H] thymidine incorporation into mucosal DNA that results in mucosal cellular proliferation and healing. Mukhopadhyaya in (1987) also found that the same effects like Goel (1986) in rat after orally administering banana pulp powder as aqueous suspension at 0.5g/kg twice daily dose for 3 days. They also reported a significant decrease in gastric juice DNA content after the treatment. It was reported that the antiulcerative effect ofbanana may vary depending on different varieties of banana **[73]**. The article showed that the ethanolic extract of both M. Sapientum and M. Paradisiaca have significant gastroprotective effect but only M. Paradisiaca promotes ulcer healing by a similar mechanism like prostaglandins **[73]**. It was reported that acid neutralizing capacity of M. Sapientum fruit peel ash in rats **[74]**.

A group of scientists showed that Musa sapientum contain a monomrtic flavanoid compound

leuococyanidin which has anti-ulcerogenic activity by High - Performance Thin Layer Chromatography **[75]**. They used aqueous extract of Musa sapientum as the sample and esomeprazole as the standard drug.

It was studied that methanolic extract of plantain banana pulp (BE) was evaluated for its (i) antiulcer and antioxidant activities in 2 hr cold restraint stress and (ii) anti-H.pylori activity in vitro**[71].** The extract (BE, 50 mg/kg, twice daily for 5 days) showed significant antiulcer effect and antioxidant activity in gastric mucosal homogenates, where it reversed the increase in ulcer index, lipid peroxidation and super oxide dismutase values induced by stress. However it did not produce any change in catalase values, which was significantly decreased by stress. Further, in the in vitro study. BE (0.32-1,000 microg/ml) did not show any anti-H.pylori activity. The results suggest absence of anti-H. pyloric activity of methanolic extract of banana in vitro and its antioxidant activity may be involved in its ulcer protective activity.

3.2.3 Analgesic activity

The pill extract of Musa sapientum was found to have analgesic property **[76]**. The investigation was undertaken to study analgesic activity of aqueous (AMS) and ethanolic (EMS) extract of stem of Musa sapientum Linn. using hot plate method and tail immersion method. AMS and EMS (100mg/kg and 200mg/kg, i.p) significantly increased reaction time as compared to vehicle treated group. Maximum analgesic effect was observed at 30 min. interval for 100 mg/kg and 200 mg/kg, i.p. (P = 0.01) **[76]**. The study indicates that AMS & EMS have central analgesic action.

3.2.4 Antimicrobial activity

Aqueous extract of unripe fruit peels and leaves of M. Paradisiaca var. Sapientum has been reported to show antimicrobial activity against Staphylococcus And Pseudomonas species in

dehydrogenase assay. The IC50 of the aqueous fruit peel extract were 143.5 and 183.1 µg/ml against Staphylococcus and Pseudomonas species respectively and in case of leaf extract were 401.2 and 594.6µg/ml respectively **[77]**. In this assay the fruit peel extract showed better activity against both the bacteria than leaf extract while the peel extract was more active against Staphylococcus (Gram-positive) than Pseudomonas species(Gram-negative). However, the alcoholic extract of stem of M. Paradisiaca showed no activity against Staphylococcus aureus,Salmonella paratyphi, Shigella dysenteriae, Escherichia coli, Bacillus subtilis, Candida albicans. It has been reported that both ethanolic and aqueous extract of unripe M. Sapientum fruit showed good activity against S. Aureus ATCC 25921, S. aureus, Salmonella paratyphi, Shigella flexnerii, E. Coli ATCC 25922, E. coli, Klebsiella pneumoniae, B. Subtilis and Pseudomonas aeruginosa. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration(MBC) of unripe banana ranged 2-512 mg/ml and 32-512 mg/mlrespectively considering both the solvents.Though both ethanolic and water extracts showed significant activity against the organisms, the activity of ethanolic extract was stronger indicating that ethanol can dissolve the active phytochemicals than water. The aqueous extract of banana pureehas also reported to have bacteriostatic activity against B. cereus, B. coagulans, B. stearothermophilus, and Clostridium sporogenes **[78]**.

3.2.5 Hypoglycemic activity

The green fruit of M. Paradisiaca has been reported to have hypoglycemic effect due to stimulation of insulin production and glucose utilization **[79]**. Its high potassium (K) and sodium (Na) content has been correlated to the glycemic effect **[80]**. Fibers from M. Paradisiaca fruit increased glycogenesis in the liver and lowered fasting blood glucose **[81]**. Antihyperglycemic effect of the hydromethanolic extract of M. Paradisiaca root has been found significant **[82]**. Musasapientum showed antihyperglycemic effect in hyperglycemic rabbit. The chloroform extract of flowers of

M.Sapientum showed blood glucose and glycosylated haemoglobin reduction and total hemoglobin increase after oraladministration in rats. It also controls lipid peroxidation in diabetes **[83]**. However, M. Paradisiaca stem juice showed hyperglycemic activity. Isolated pectin from the juice of the inflorescence stalk of M. Sapientum increases the glycogensynthesis, decreases glycogenolysis and gluconeogenesis**[84]**.

Oral administration of 0.15, 0.20 and 0.25 g/kg body weight of the chloroform extract of the flowers for 30 days resulted in a significant reduction in blood glucose and glycosylated haemoglobin and an increase in total haemoglobin **[83]**. The extract prevented a decrease in body weight, and also resulted in a decrease in free radical formation in the tissues. Thus the study shows that banana flower extract has an anti hyperglycemic action. The decrease in thiobarbituric acid reactive substances and the increase in reduced glutathione, glutathione peroxidase, superoxide dismutase and catalase clearly show the antioxidant property of banana flower extract. The effect of banana flower extract was more prominently seen in the case of animals given 0.25 g/kg body weight **[83].** Banana flower extract was more effective than glibenclamide.

It was found that the antihyperglycemic effect of ethanolic extract of flowers of Musa sapientum in alloxan induced diabetic rats **[85]**. Oral administration of the ethanolic extract showed significant ($p < 0.001$) blood glucose lowering effect at 200 mg/kg in alloxan induced diabetic rats (120 mg/kg, i.p.) and the extract was also found to significantly ($p < 0.001$) scavenge oxygen free radicals, viz., superoxide dismutase (SOD), catalase (CAT) and also protein, malondialdehyde and ascorbic acid in vivo. Musa sapientum induced blood sugar reduction may be due to possible inhibition of free radicals and subsequent inhibition of tissue damage induced

by alloxan **[85]**. The antidiabetic activity observed in this plant may be attributed to the presence of flavonoids, alkaloids, steroid and glycoside principles.

3.2.6 Hypocholesterolaemic activity

Hemicellulose and other neutral detergent fibers (NDF) from the unripe M. Paradisiaca fruit showed low absorption of glucose and cholesterol and low serum and tissue levels of cholesterol and triglycerides **[81]**. Flavonoids isolated from unripe fruits showed hypolipidemic activity evidenced by decrease in cholesterol, triglycerides (TG), free fattyacids and phospholipids levels in serum, liver, kidney and brain ofrats. The cholesterol lowering effect was attributed to a higherdegradation rate of cholesterol than synthesis **[86].** Methanolic root extract of M. Paradisiaca showed total cholesterol (TC), triglyceride (TG), LDLc and VLDLc lowering effect in diabetic rats.The pectin content to the juice of the inflorescence stalk of M. Sapientum has also been reported to possess cholesterol and triglyceride lowering activity in rats **[87]**.

A Study Showed that soluble and insoluble components of dietary fibre participate in the hypocholesterolaemic effect of banana pulp **[88]**. The pulp of banana fruit (Musa sapientum L. var. Cavendishii) was examined for its cholesterol-lowering effect with male rats fed on a diet containing lard (50 g/kg) and cholesterol (5 g/kg). Freeze-dried banana pulp showed a marked cholesterol-lowering effect when incorporated into a diet at the level of 300 or 500 g/kg, while the banana pulp dried in a hot-air current (65 degrees) did not **[88]**. Starch and tannin prepared from banana pulp were not responsible for the cholesterol-lowering effect. The results also suggest that banana lipids did not affect the concentration of serum cholesterol. Feeding of dopamine, n-epinephrine and serotonin tended to raise the concentration of serum cholesterol. Thus, all the substances tested which were thought to be susceptible to influence by hot-air drying were unlikely to be responsible for the hypocholesterolaemic effect. However, both

soluble and insoluble fibers fractionated from banana pulp had a cholesterol-lowering effect, with the exception of cellulose**[88]**. It was assumed that a browning reaction undergone during hot-air drying might be related to the disappearance of the hypocholesterolaemic effect of banana pulp dried in a hot-air current.

3.2.7Antihypertensive activity

The antihypertensive effect of M. Paradisiaca in albinorats was reported . Later same group reported that banana diet has a mean arterial blood pressure lowering as well as onset preventing effect in rats with elevated blood pressure induced by desoxycorticosterone acetate (DOCA)administration. It was reported that the antihypertensive effect of ripe banana pulp in deoxycorticosteroneenantate-induced hypertensive rats which may be due to the high tryptophan and carbohydrate content of banana that increase esserotonin levels and gives serotonin-mediated natriorexic effect. However, another group reported that serotonin produced acontraction in place of relaxation in isolated rat aortic rings. The aqueous extract of the ripe M. Paradisiacal fruit was found to give aconcentration dependent hypotensive effect in both noradrenaline-and potassium chloride-contracted aortic rings isolated from rat. The effect was due to the non-specific interference in calcium ion availability needed for the smooth muscle contraction that results in relaxation **[89] & [90].**

3.2.8 Effect in atherosclerosis

Saraswathi and Gnanam (1997) reported that M. Paradisiaca inhibits cholesterol crystallization in vitro which mayhave an effect on atherosclerosis plaque and gallstones in vivo. Parmar and Kar (2007) tested the peel extract of M. Paradisiaca inrats with diet-induced atherosclerosis. This study reports the protective role of the extract in atherosclerosis and thyroids function though it was not very effective like other plants tested. Yin X. and Quan J (2008) further studied the

effect of banana inhuman and found that plasma oxidative stress was significantly reduced and the resistance to oxidative modification of LDL was enhanced only after a single banana meal. The effect may be due to the presence of dopamine, ascorbic acid and other antioxidants present in banana**[81].**

3.2.9 Antioxidant activity

Plasma oxidative stress is significantly reduced only after a single banana meal in healthy human due to the presence of dopamine, ascorbic acid and other antioxidants present in banana [**60].** Antioxidant activity was also reported with aqueous acetone extract of banana peel by β –carotene bleaching method, DPPH free radical scavenging and linoleic acid emulsion method. Glycosides and monosaccharide components are mainly responsible for the antioxidant activity **[91]**. Vijayakumar (2008) reported the antioxidant activityof the extracted flavonoids from M. Paradisiaca in rats **[86]**. This group found that the flavonoids from banana stimulated the activities of superoxide dismutase (SOD) and catalase which might beresponsible for the reduced level of peroxidation products such asmalondialdehyde, hydroperoxides and conjugated dienes.

3.2.10 Diuretic activity

Ash of the peel of M. Sapientum showed an increase in urine volume and K + as well as other electrolyte excretion than normal saline in a study in rats. Successive ethanolic extract also give this diuretic effect. Phytochemicals such assaponin, flavonoids and terpenoids are known to be responsible for this effect **[92]**

3.2.11 wound healing activity

Agarwal (2009) reported the wound healing activityof both methanolic and aqueous extract of plantain banana (M.sapientum var. 184aradisiacal) in rats. Both extracts were found to increase hydroxyproline, hexuronic acid, hexosamine, super oxidedismutase as well the wound breaking

strength and reduced glutathione level. They also decreased the wound area, scar area and lipid peroxidation. The effects were attributed to the antioxidant property of the plantain **[63].**

3.2.12 Anti-allergic activity

The water extract of pulp of ripe M. Sapientum has been reported to have significant anti-allergic activity on antigen-induced degranulation in RBL-2H3 cells with an IC50 value of 13.5±2.4 **[93]**.

3.2.13 Antimalarial activity

The decoction of the leaves of M. Paradisiaca added to Ocimum americanum and Ocimum gratissimum is used as to treat malarial. But in vitro study using Plasmodium falciparum chloroquine-resistant strain proves this plant ineffective in malarial **[94]**.

3.2.14 Effects on Muscle

The stem juice of plantain banana tree (M. Sapientum vs paradisiaca) has been found to induce contraction in skeletal muscles by enhancing excitation-contraction coupling and transmembrane Ca 2+ fluxes. Later, Benitez in (1991) reported the trunks juice of M. Sapientum vs. Cavendishi has muscle paralyzing effect in rat and attributed the effect to monopotassium oxalate present in the juice**[64].**

3.2.15 Anti-snake venom activity

Borges in (2005) reported the in vitro neutralizing capacity of bothrops jararacussu and Bothrops neuwiedi snakevenoms by the stem juice of M. Paradisiaca .The phospholypase A 2(PLA 2) and hemorrhagic activities induced by the venom was inhibited by the extract as it forms unspecific complex with the venom protein. However, the in vivo activity of the extract in mice was not significant to protect against the venom **[95]**.

3.2.16 Mutagenecity

A report describes that the mutagenic effect of M. paradisiacal fruit peel extract in mice assessed by

the single-cell gel electrophoresis (SCGE) and micronucleus assays. The experiments showed DNA damaging property in peripheral blood leukocytes for 1500 and 2000 mg/kg body weight [**96].**

3.2.17 Digestion

The digestion and absorption from the small bowel of the carbohydrate of banana has been studied by feeding ileostomy subjects banana from six batches of different ripeness and measuring the amounts excreted in the effluent **[97]**. Starch content of bananas depended on the ripeness being 37% of dry weight in the least ripe and 3% in the most ripe. Excretion of carbohydrate from banana in ileostomy effluent ranged from 4-19 g/day and was directly related to the starch content (r = 0.99). Up to 90% of the starch could be accounted for in the effluent. Complete recovery of non starch polysaccharides NSP (dietary fiber) was obtained. The amount of banana starch not hydrolyzed and absorbed from the human small intestine and therefore passing into the colon may be up to 8 times more than the NSP present in this food and depends on the state of ripeness when the fruit is eaten **[97]**.

Chapter – 4

Methods

4.1 CHEMICALS AND OTHER REAGENTS

DPPH (2,2-diphenyl-1-picrylhydrazyl), Sulfuric acid, Folin reagent, Ciocalteu reagent, prolein amino acid (protein), 1-butanol, glacial acetic acid, Ninhydrine solution, Glucose, Galactose, Maltose, Lactose, Acetone, Phosphate buffer, Anisaldehyde, L-Ascorbic acid, potassium ferricyanide,Ttrichloro acetic acid(TCA), Ferric chloride, Sodium carbonate, deionized water, Gallic acid, Sodium nitrite, Aluminium chloride, Sodium hydroxide, Hydrogen peroxide, Normal saline, Wagner's reagent, Hydrochloric acid, Glacial acetic acid, Ammonia, Phoshomolybdic acid, Acetic anhydride, Alcoholic ferric chloride, 5-aqua copper sulphate, Sodium potassium tartrate.

4.2 EQUIPMENTS AND OTHER NECESSARY TOOLS

In case of the extraction procedure and for various phytochemical tests many equipments and materials were used. Some of them are analytical balance, beaker (in various size), pipette, micro-pipette, rotary evaporator, hot air oven, dryer, storage cabinet, spatula, test tube, volumetric flask, conical flask, test tube holder, test tube rack, aluminum foil paper, scotch tape, refrigerator, water bath, electronic shaker, ultra violate lamp, mask, gloves, lab coat, sprayer, reagent bottle, TLC plate, TLC tank, scale, pencil, TLC plate cutter, capillary tube, mortar and pestle, laminar air flow cabinet, loop, burner, micropipette tip, petri dishes, glass rod, cotton, filter paper, funnel, hot plate, centrifugal machine, autoclave, glassware washers, stirrer, UV spectroscopy, knife, ephedrine tube, Whatman’s filter paper, paper disc, incubator, vortex machine, P^H meter.

4.3 SOLVENTS FOR EXPERIMENTS

Dimethylsulfoxide(DMSO), Acetone, Chloroform, Distilled water, Ethanol, Methanol, Diethyl ether, Acetic acid, n-Hexane, Ethyl acetate, Dichloromethane, Benzene, Ammonium hydrooxide, Formic acid.

4.4 THIN LAYER CHROMATOGRAPHY (TLC)

4.4.1 Principle

The extracts were analyzed by performing TLC to determine the composition of each extract. TLC was done under three solvent systems. Successively the polarity of the solvent systems was increased to get a clear graph of all the possible compounds present in the extract. Most organic solvents are nonpolar in nature and so organic compounds such as benzene was used to elute the non polar compounds present in the extract. Again, since Alkaloid and terpenoid type compounds are basic in nature, ammonium base is used in the solvent system to help them run over TLC plate **[44].**

Table 4.1: The compositions of various solvent systems for TLC **[44].**

Nonpolar Basic solvent	**Intermediate polar Basic Solvent**	**Polar Basic solvent**
Benzene 9mL Ethanol 1mL Ammonium hydroxide 0.1mL	Chloroform 5Ml Ethyl acetate 4mL Formic acid 1mL	Ethyl acetate 8mL Ethanol 1.2mL Water 0.8mL

4.4.2 Apparatus

9. TLC tank
10. Pencil
11. Scale
12. UV-lamp
13. Spray bottle
14. Heat gun
15. Petri dish
16. Capillary tube

4.4.3 Reagents

21. Benzene
22. Ethanol
23. Ammonium hydrooxide
24. $CHCl_3$
25. Formic acid
26. Ethyl acetate
27. EtOH
28. Water
29. Nutrient agar
30. H_2SO_4
31. DPPH
32. Folin & ciocalteu solution
33. 1-butanol
34. Glacial acetic acid
35. 2% ninhydrine solution
36. Acetone
37. Phosphate Buffer
38. Methanol
39. N-Hexane
40. Standard flavonoid solution

4.4.4 Procedure

- Firstly, above three solvent systems were prepared.
- In the next step, TLC plates were prepared. For this nine TLC plates were prepared, three for each solvent system to run with. The TLC plates were labeled in the following maner:

  1-C, 1-D, 1-F

  2-C, 2-D, 2-F

  3-C, 3-D, 3-F

Where,

1denoted run with nonpolar solvent system, 2 denoted run with intermediate polar solvent system, 3 denoted run with polar solvent system and C denoted treatment with 10% Sulphuric acid,D denoted treatment with 0.04%DPPH solution, F denoted treatment with 10% Folin & ciocalteu solution **[44].**

- On each plate four spots were spotted. The spotting patterns were as follows:

  1^{st} spot was spotted with Solvent; in this case methanol was used.

  2^{nd} & 3^{rd} spots were spotted with the extracts of shefali leaf.

  4^{th} spot was the control, spotted with standard flavonoid solution

- After spotting the respective TLC plate was exposed to the respective solvent system. Like all plates that were marked 1 were run with nonpolar solvent system, those that were marked 2 were run with intermediate polar solvent system and the 3 marked plates run with polar solvent system **[44].**
- Upon completion of TLC, the plates were exposed to reagent for compound detection and identification. For this the C marked plates were exposed to 10% sulphuric acid solution, dried and then heated to $80\text{-}90^0$ C for charring purpose. This helped in the fixation of spot and allowed it to be prominently visible.
- Likewise, the D marked plates were dipped into 0.04% DPPH solution and dried while keeping in a dark place.
- The F marked plates were washed with Folin & ciocalteu reagent and dried **[44].**

4.5 PRELIMINARY PHYTOCHEMICAL INVESTIGATION OF SECONDARY METABOLITES

4.5.1 Principle

The secondary metabolites in the plant sample are the main concern of research work. There are many tests available for this purpose. In the following table these tests are shown **[36].**

Table 4.3: Preliminary phytochemical investigations of secondary metabolites **[36]**

Secondary metabolite	**Name of test**	**Methodology**	**Observation**
Alkaloid	Wagner test	Add 2ml filtrate with 1% HCl + steam. Then add 1ml of the solution with 6 drops of Wagner's reagent	Brownish-red precipitate
Anthraquinone	Borntrager's test	Add 1 ml of dilute (10 %) ammonia to 2 ml of chloroform extract	A pink-red color in the ammoniacal (lower) layer
Cardiac glycosides	Kellar - Kiliani test	Add 2ml filtrate with 1ml of glacial acetic acid, 1ml ferric chloride and 1ml concentrated sulphuric acid	Green-blue coloration of solution
Flavonoid	NaOH test	Treat the extract with dilute NaOH, followed by addition of dilute HCl	A yellow solution with NaOH, turns colorless with dilute HCl
Reducing sugar	Fehling test	Add 25ml of diluted sulphuric acid (H_2SO_4) to 5ml of water extract in a test tube and boil for 15mins. Then cool it and neutralize with 10% sodium hydroxide to pH 7 and 5ml	Brick red precipitate

		of Fehling solution	
Saponin	Frothing test/ Foam test	Add 0.5ml of filtrate with 5ml of distilled water and shake well	Persistence of frothing
Steroid	Liebermann-Burchardt test	To 1ml of methanolic extract, add 1ml of chloroform, 2–3ml of acetic anhydride, 1 to 2 drops of concentrated sulphuric acid	Dark green coloration
		To 1 ml of extract, add 2 ml acetic anhydride and 2 ml concentrated sulphuric acid	Color change to blue or green
Terpenoid	Liebermann-Burchardt test	To 1ml of methanolic extract, add 1ml of chloroform, 2–3ml of acetic anhydride, 1 to 2 drops of concentrated sulphuric acid	Pink or red coloration

4.5.2 Apparatus

Test tubes	Beaker
Conical flask	Filter paper
Pipette	Dropper
Electric balance	Hot plate
Shake	Hot water bath

4.5.3 Reagents

1% HCl	Dilute sulphuric acid
Wagner's reagent	Water
Dilute (10 %) ammonia	Fehling solution
Glacial acetic acid	Chloroform
Ferric chloride	Acetic anhydride
Concentrated sulphuric acid	10% alcoholic ferric chloride
Dilute NaOH	Dilute HCl
Phoshomolybdic acid reagent **[36]**	

4.5.4 Procedure

- Extract of different solvents were prepared.
- According to above table the tests were done.
- Detection of various secondary metabolites present in the plant was done according to above principle.

4.6 BIOMEDICAL TEST

4.6.1 Haemagglutination Assay (HA)

4.6.1.1 Principle

Many viruses attach to molecules present on the surface of RBCs. A consequence of this is that at certain concentrations, a viral suspension may bind together (agglutinate) the RBCs, thus preventing them from settling out of suspension. Since agglutination is rarely linked to infectivity, attenuated viruses can therefore be used in assays. By serially diluting a virus

suspension into an assay tray (a series of wells of uniform volume) and adding a standard amount of blood cells, an estimation of the number of virus particles can be made **[42].**

While less accurate than a plaque assay, it is cheaper and quicker (taking just 30 minutes). This assay may be modified to include the addition of an antiserum. By using a standard amount of virus, a standard amount of blood cells, and serially diluting the antiserum, one can identify the concentration of the antiserum (the greatest dilution which inhibits hemagglutination) **[42].**

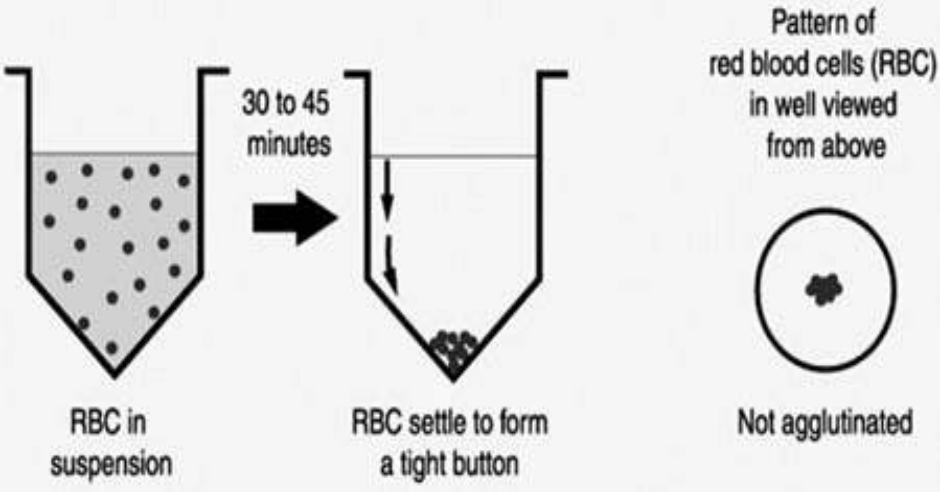


Figure 4.1: Formation of tight button with no agglutination

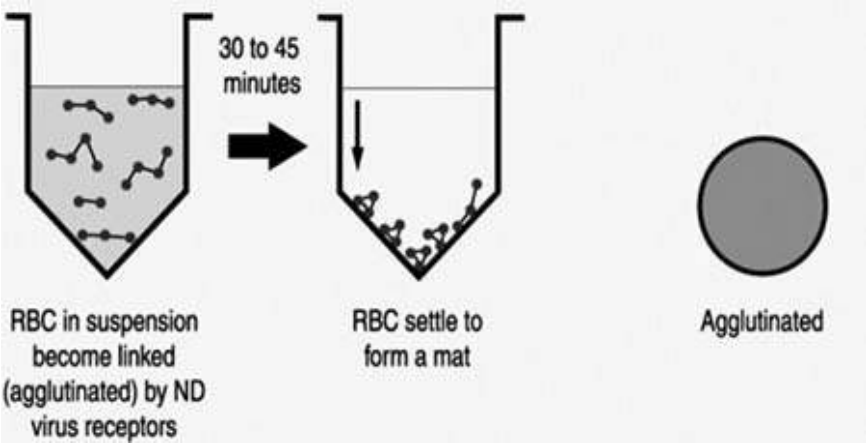


Figure 4.2: Formation of agglutination

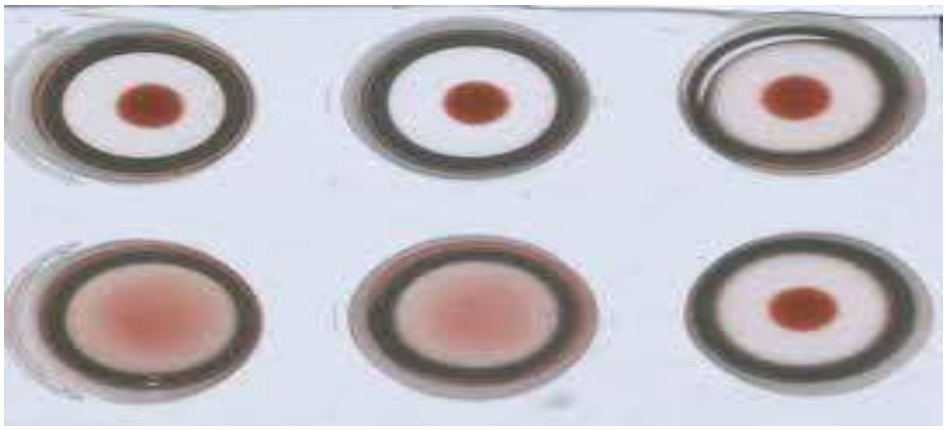


Figure 4.3: Overview of HA and not HA

4.6.1.2 Apparatus

Centrifuge machine	Refrigerator
Ephindorf tube	Test tube
Micro pipette	Eppendorf tube box
Syringe	CD marker
Cotton	

4.6.1.3 Reagents

Isotonic phosphate buffer	Blood

4.6.1.4 Procedure

- Stock solution of the test sample was prepared at concentration of 5 mg/ml and each solution was serially diluted.

- Fresh blood from healthy person was collected only for the test of Haemagglutination Assay (HA). The blood group A^+, B^+, AB^+, O^+ were collected from healthy person for this test of east West University students **[30].**
- Then the all bloods were centrifuged and the erythrocytes were separated.
- 4% erythrocyte suspension was prepared in phosphate buffer (pH 7.4) of all blood groups.
- 1 ml of the test sample dilution was taken with 1 ml of 4% erythrocyte and incubated at 25°C.
- After incubation, the results were noted. Smooth button formation in bottom indicated negative activity, while a rough granular deposition at bottom showed positive activity.
- The intensity of haemagglutination was determined from the extent of deposition **[30].**

4.6.2 Anti-Hemolytic Activity

4.6.2.1 Apparatus

Centrifuge machine	Refrigerator
Micro pipette	Test tube
Syringe	CD marker
Cotton	Uv-spectrophotometer

4.6.2.2 Reagents

H_2O_2

Isotonic sodium phosphate buffer

Blood samples **[43]**

4.6.2.3 Procedure

- The erythrocytes from man blood were separated by centrifugation and washed with

saline or isotonic sodium phosphate buffer (pH 7.4) until the supernatant was colorless.

- The erythrocytes were then diluted with saline or phosphate buffer to give a 4% suspension **[43].**
- Varying amounts of sample (1000,500,250,125,62.5 □g/ml) with saline or buffer were added to 2 ml of the suspension of erythrocytes and the volume was made up to 3.5 ml with saline or buffer.
- This mixture was pre-incubated for 120 min and then 0.5 ml H_20_2 solutions of appropriate concentration in saline or buffer were added.
- The concentration of H_20_2 in the reaction mixture was adjusted so as to bring 90% hemolysis of blood cells after 120 min incubation.
- About 80-90% hemolysis of rat erythrocytes was obtained after 4-6 h.
- Incubation was concluded after these time intervals by centrifugation during 5 min at Xl000 g and the extent of hemolysis was determined by measurement of the absorbance at 540 nm corresponding to hemoglobin liberation.
- Antihemolytic activity was expressed as the inhibition percentage and was calculated using the following formula:

  Antihemolytic activity (%) = 1 − Sample $_{540\ nm}$ /Control $_{540\ nm}$ X 100, where, Sample $_{540\ nm}$ was the absorbance of the sample and Control $_{540\ nm}$ was the absorbance of the control **[43].**

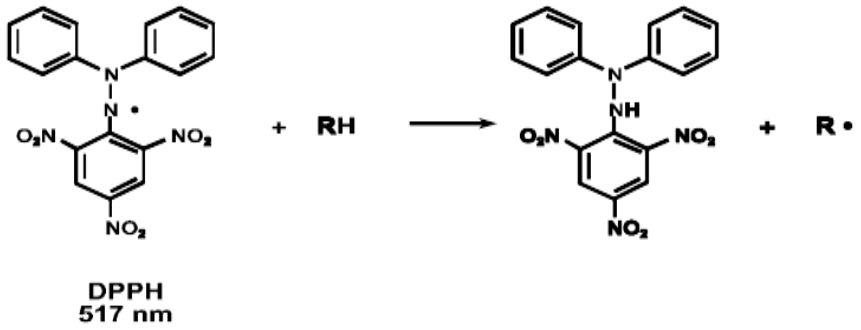
4.7 ANTI-OXIDANT TESTS

4.7.1 DPPH Test (1, 1-diphenyl-2-picrylhydrazyl radical)

4.7.1.1 Principle

The 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) has been widely used to evaluate the free

radical scavenging capacity of antioxidants. DPPH free radical is reduced to the corresponding hydrazine when it reacts with hydrogen donors. DPPH can make stable free radicals in aqueous or methanol solution. With this method it was possible to determine the antiradical power of an antioxidant activity by measurement of the decrease in the absorbance of DPPH at 517 nm. Resulting from a color change from purple to yellow the absorbance decreased when the DPPH was scavenged by an antioxidant, through donation of hydrogen to form a stable DPPH molecule. In the radical form this molecule had an absorbance at 517 nm which disappeared after acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule **[29].**



4.7.1.2 Apparatus

Test tube	Uv-spectrophotometer
Racker	Spatula
Beaker	Analytical balance

4.7.1.3 Reagents

DPPH	L-ascorbic acid

Methanol Water

4.7.1.4 Procedure

- The DPPH radical scavenging method was used for the determination of the antioxidant capacity of the extracts. Different concentrations of the plant extract (2, 4, 6, 8 & 10 µg/ml, in methanol) were added at an equal volume (10ml) to methanol solution of DPPH (400 µg /ml, 100 µg/m l).
- Different concentrations of L-Ascorbic acid (2-10 mg/ml) were used as the standard antioxidant.
- After 30 min at room temperature, the absorbance values were measured at 517 nm on a spectrophotometer and converted into the percentage antioxidant activity using the equation, DPPH antiradical scavenging capacity (%) = [1 – (Abof sample – Abof blank)/Abof control] × 100.
- Methanol plus different concentration of plant extract solution was used as a blank, while DPPH solution plus methanol was used as a control **[34].**
- The IC_{50} values were calculated by the sigmoid non-linear regression model using plots, where the abscissa represented the concentration of tested plant extracts and the ordinate the average percent of scavenging capacity. IC_{50}values denote the concentration of the sample required to scavenge 50% of DPPH radicals **[34].**

4.7.2 Reducing Power Activity Test

4.7.2.1 Principle

In this assay, the yellow color of the test solution changes to various shades of green and blue

depending on the reducing power of antioxidant samples. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The presence of reductants such as antioxidant substances in the antioxidant samples causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm **[38].**

4.7.2.2 Apparatus

Test tube	Centrifuge machine
Spatula	Uv-spectrometer
Analytical balance	Vortex mixer
Hot air oven	Pipette & pumper

4.7.2.3 Reagents

Potassium ferricyanide	$FeCl_3$
Distilled water	L-ascorbic acid
TCA **[32]**	

4.7.2.4 Procedure

- The reducing power of the extracts of night jasmine leaves was measured using the potassium ferricyanide reduction method.
- Various amounts of extracts & standard collected in different test tubes.

Table 4.4: Test tube designation for reducing power activity test **[32]**

Test tube no	Type of compound	Amount in gram
1	L-ascorbic acid	0.12
2	L-ascorbic acid	0.27
3	L-ascorbic acid	0.5
4	L-ascorbic acid	0.77
5	L-ascorbic acid	1
6	Night jasmine leaves	0.05
7	Night jasmine leaves	0.10
8	Night jasmine leaves	0.16
9	Night jasmine leaves	0.20

- Then all test tubes added with 2.5 ml of pH 7 distilled water & 2.5 ml of potassium ferricyanide[K_3Fe_3 $(CN)_6$] 1% solution & vortexed.
- After incubation at 50^0 C for 20 min, 2.5 ml of trichloro acetic acid(TCA)[10% w/v] was added to all test tubes & centrifuged at 3000 rpm for 10 min.
- Afterwards, upper layer of solution (5ml) was mixed with distilled water.
- Then 1ml of $FeCl_3$ was added on each test tube.
- Then from each test tube 1ml of solution was collected and mixed it with 9 ml of distilled water.
- After that it was incubated at 35^0C for 10 min **[32].**
- The formation of perls Prussian color was measured at 700 nm in a UV-VIS spectrometer.

- Increased absorbance of the reaction mixture indicated increase reducing power. The percentage (%) Reducing capacity was calculated from the following equation.

$$\{(A_m - A_b)/A_b\} \text{ X } 100$$

Where,

A_m is the absorbance of the reaction mixture

A_b is the absorbance of the blank **[32].**

4.7.3 Total Phenolic Assay

4.7.3.1 Principle

The content of total phenolic compounds in plant methanolic extracts was determined by Folin–Ciocalteu Reagent (FCR). The FCR actually measures a sample's reducing capacity. The exact chemical nature of the FC reagent is not known, but it is believed to contain heteropolyphosphotunstates - molybdates. Sequences of reversible one- or two-electron reduction reactions lead to blue species, possibly $(PMoW_{11}O_{40})^{4-}$. In essence, it is believed that the molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reductants and Mo (VI): **[39]**

$$Mo\,(VI) + e \rightarrow$$

4.7.3.2 Apparatus

Test tube	Pipette
Spatula	Analytical balance

Vortex	Uv-spectrophotometer

4.7.3.3 Reagents

Folin-Ciocalteu	Ethanol or Methanol
deionized water	Galic acid (Analytical or Reagent grade)
Na_2CO_3 **[39]**	

4.7.3.4 Procedure

- 1.0 ml of plant extract 200µg/ml or standard of different concentration solution was taken in a test tube.
- 5 ml of Folin – ciocalteu (Diluted 10 fold) reagent solution was added into the test tube.
- 4 ml of Sodium carbonate solution was added into the test tube.
- The test tube was incubated for 30 minutes at 20^0C to complete the reaction. (only applicable for standard) **[39].**

  **Incubated the test tube for 1 hour at 20^0C to complete the reaction (Applicable for extract).

- Then the absorbance of the solution was measured at 765 nm using a spectrophotometer against blank.
- A typical blank solution contained ethanol or methanol was taken.
- The Total content of phenolic compounds in plant methanol extracts in gallic acid equivalents (GAE) was calculated by the following formula equation

$$C = (c \times V)/m$$

Where:

C = total content of phenolic compounds, mg/g plant extract, in GAE;

c = the concentration of gallic acid established from the calibration curve, mg/ml;

V = the volume of extract, ml;

m = the weight of pure plant methanolic extract, g.

- By the equation, y = 0.0162x+0.0215, R^2=0.9972 equivalent amount are neasured **[39].**

4.7.4 Total Flavanoid Assay

4.7.4.1 Apparatus

Uv-spectrophotometer Spatula

Test tube Pipette

Analytical balance

4.7.4.2 Reagents

De ionized water $AlCl_3$

$NaNO_2$ NaOH **[32]**

4.7.4.3 Procedure

- An aliquot (1.5 ml) of methanolic extract was added to 6ml of deionized water.
- Then 0.45 ml 5% (w/v) $NaNO_2$ and incubated for 6 min.

- 0.45 ml 10% (w/v) $AlCl_3$ and 6 ml 4% (w/v) NaOH was added and the total volume was made up to 15 ml with distilled water.
- The absorbance was measured at 510 nm by using visible spectrophotometer.
- The results were expressed as mg rutin equivalents/g DW. The analyses were done in three replications **[32].**

4.7.5 Hydrogen Peroxide Radical Scavenging Assay

4.7.5.1 Apparatus

Test tube	Analytical balance
Beaker	Spatula
Pipette	Uv-spectrophotometer

4.7.5.2 Reagents

- Hydrogen peroxide
- Phosphate buffer
- L-ascorbic acid **[33]**

4.7.5.3 Procedure

- A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (50mM, pH 7.4).
- The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer.

- Extract (4 - 20 µg/ml) in phosphate buffer was added to hydrogen peroxide and absorbance at 230 nm is determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide.
- L-ascorbic acid was used for comparison.
- The percentage of hydrogen peroxide scavenging was calculated as follows:

  % scavenged (H_2O_2) = (1– A_1 / A_0) X 100 Where; A_0 is the absorbance of control and A_1 is the absorbance of test.

- Ascorbic acid was used as a positive control. **[33]**

4.8 ANTI-MICROBIAL ASSAY

4.8.1 Determination of Antimicrobial activity by Disc Diffusion Method

Worldwide, infectious disease is one of main causes of death accounting for approximately one-half of all deaths in tropical countries. Perhaps it is not surprising to see these statistics in developing nations, but what may be remarkable is that infectious disease mortality rates are actually increasing in developed countries, such as the United States **[35].**

Death from infectious disease, ranked 5th in 1981, has become the 3rd leading cause of death in 1992; an increase of 58% .It is estimated that infectious disease is the underlying cause of death in 8% of the deaths occurring in the US **[30].** This is alarming given that it was once believed that we would eliminate infectious disease by the end of the millenium. The increases are attributed to increases in respiratory tract infections and HIV/AIDS. Other contributing factors are an increase in antibiotic resistance in nosicomial and community acquired infections. Furthermore, the most dramatic increases are occurring in the 25–44 year old age group **[30].**

These negative health trends call for a renewed interest in infectious disease in the medical and public health communities and renewed strategies on treatment and prevention. It is this last solution that would encompass the development of new antimicrobials **[40].**

The antimicrobial screening which is the first stage of antimicrobial drug research is performed to ascertain the susceptibility of various fungi and bacteria to any agent. This test measures the ability of each test sample to inhibit the *in vitro* fungal and bacterial growth. This ability may be estimated by disc diffusion method **[40].**

But there is no standardized method for expressing the results of antimicrobial screening **[40].** Some investigators use the diameter of zone of inhibition and/or the minimum weight of extract to inhibit the growth of microorganisms. However, a great number of factors viz., the extraction methods, inoculum volume, culture medium composition, p^H, and incubation temperature can influence the results **[41].**

Among the above mentioned techniques the disc diffusion is a widely accepted in vitro investigation for preliminary screening of test agents which may possess antimicrobial activity. It is essentially a quantitative or qualitative test indicating the sensitivity or resistance of the microorganisms to the test materials. However, no distinction between bacteriostatic and bactericidal activity can be made by this method **[41].**

4.8.1.1 Principle of Disc Diffusion Method

In this classical method, antibiotics diffuse from a confined source through the nutrient agar gel and create a concentration gradient. Dried and sterilized filter paper discs (6 mm diameter) containing the test samples of known amounts are placed on nutrient agar medium uniformly seeded with the test microorganisms.

Standard antibiotic discs and blank discs are used as positive and negative control. These plates are kept at low temperature (4°C) for 24 hours to allow maximum diffusion of the test materials to the surrounding media **[41].** The plates are then inverted and incubated at 37°C for 24 hours for optimum growth of the organisms. The test materials having antimicrobial property inhibit microbial growth in the media surrounding the discs and thereby yield a clear, distinct area defined as zone of inhibition. The antimicrobial activity of the test agent is then determined by measuring the diameter of zone of inhibition expressed in millimeter **[41].** In the present study the crude extracts, fractions as well as some pure compounds were tested for antimicrobial activity by disc diffusion method. The experiment is carried out more than once and the mean of the readings is required **[30].**

4.8.1.2 Apparatus and Reagents

1. Filter paper discs
2. Autoclave
3. Nutrient Agar Medium
4. Laminar air flow hood
5. Petri dishes
6. Spirit burner
7. Sterile cotton
8. Refrigerator
9. Micropipette
10. Incubator
11. Inoculating loop
12. Ethanol
13. Sterile forceps
14. Nosemask and Hand gloves
15. Screw cap test tubes

4.8.1.3 Test Materials of *Nyctanthes arbor-tristis*

Methanolic extract of *Nyctanthes arbor-tristis* leaves were taken as test sample.

4.8.1.4 Test Organisms

The bacterial strains used for the experiment were collected as pure cultures from the East West

University microbiology laboratory. Both gram positive and gram-negative organisms were taken for the test and they are listed in the following table.

Table 4.5: List of micro-organisms used

Gram positive Bacteria	**Gram negative Bacteria**
Bacillus cereus *Staphylococcus aureus*	*Salmonella typhi* *Shigella dysentery* *Vibrio mimicus*

4.8.1.5 Culture Medium and Their Composition

The Nutrient Agar medium was used normally to demonstrate the antimicrobial activity and to make subculture of the test organisms. Nutrient Agar medium contains following things

Table 4.6: Composition of nutrient agar medium **[41]**

Ingredients	**Amount**
Bacto peptone	0.5 gm
Sodium chloride	0.5 gm
Bacto yeast extract	1.0 gm
Bacto agar	2.0 gm
Distilled water q.s.	100 ml

Agar medium having this composition was directly brought from the market.

4.8.1.6 Preparation of the Medium

To prepare required volume of this medium, calculated amount of agar medium was taken in a bottle with a cap and distilled water was added to it to make the required volume. The contents were then autoclaved to make a clear solution.

4.8.1.7 Sterilization Procedure

In order to avoid any type of contamination and cross contamination by the test organisms the antimicrobial screening was done in Laminar Hood and all types of precautions were highly maintained. UV light was switched on one hour before working in the Laminar Hood. Petridishes and other glassware were sterilized by autoclaving at a temperature of 121^0C and a pressure of 15-lbs/sq. inch for 20 minutes. Micropipette tips, cotton, forceps, blank discs etc. were also sterilized.

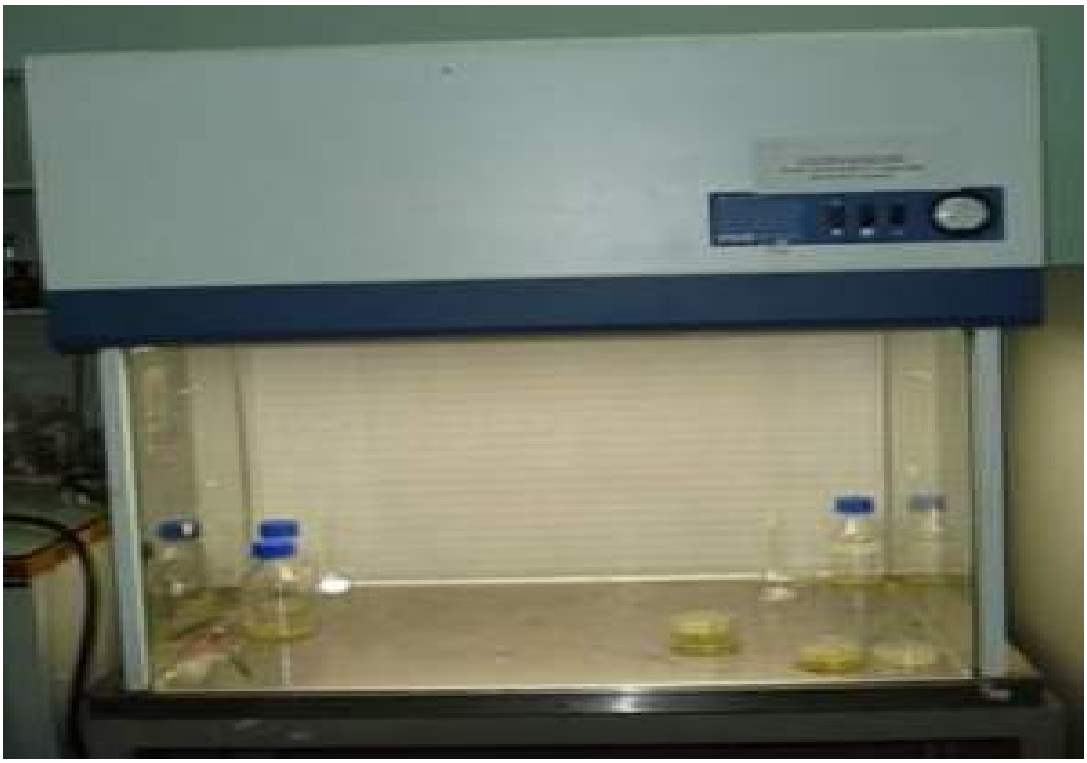


Figure 4.4: Laminar hood

4.8.1.8 Preparation of Subculture

In an aseptic condition under laminar air cabinet, the test organisms were transferred from the pure cultures to the agar slants with the help of a transfer loop to have fresh pure cultures. The

inoculated strains were then incubated for 24 hours at 37^0C for their optimum growth. These fresh cultures were used for the sensitivity test.

Figure 4.5: Incubator

4.8.1.9 Preparation of the Test Plate

The test organisms were transferred from the subculture to petridish containing about 10 ml of melted and sterilized agar medium.

The bacterial and fungal suspension was taken by a loop a mixed with normal saline with the help of vortex machine. Then a sterilized cotton bud was taken and dipped into the bacterial/ fungal suspension. Then the bacterial/fungal sample is applied to the petridish with the help of this cotton bud.

4.8.1.10 Preparation of Discs

4.8.1.10.1 Standard Discs

These were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of the test sample. In this investigation, Azithromycin (30μg/disc) standard disc was used as the reference.

4.8.1.10.2 Blank Discs

These were used as negative controls which ensure that the residual solvents (left over the discs even after air-drying) and the filter paper were not active themselves.

4.8.1.10.3 Preparation of Sample Discs with Test Sample

Measured amount of each test sample was dissolved in specific volume of solvent to obtain the desired concentrations in an aseptic condition. Sterilized metrical (BBL, Cocksville, USA) filter paper discs were taken in a blank Petri dish under the laminar hood. Then discs were soaked with solutions of test samples and dried.

4.8.1.11 Diffusion and Incubation

The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test bacteria and fungi. The plates were then kept in a refrigerator at 4^0C for about 24 hours upside down to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at 37^0C for 24 hours.

4.8.1.12 Determination of Antimicrobial Activity by Measuring the Zone of Inhibition

The antimicrobial potency of the test agents are measured by their activity to prevent the growth of the microorganisms surrounding the discs which gives clear zone of inhibition. After

incubation, the Antimicrobial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale.

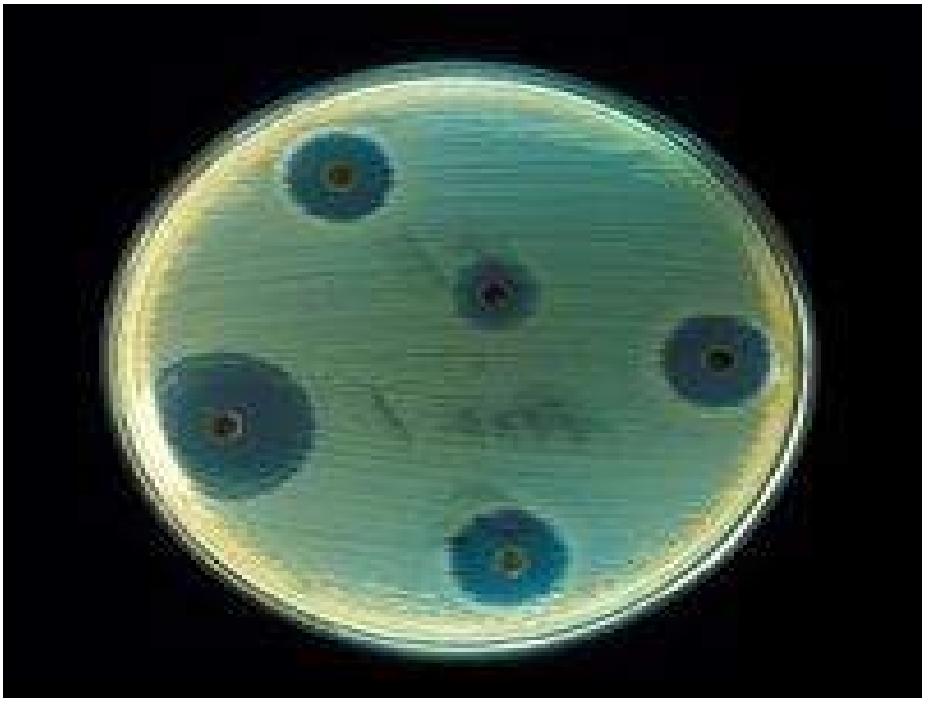


Figure 5.3: Zone of inhibition

4.8.2 Determination of MIC (Minimum Inhibitory Concentration)

The aim of this study was to determine the minimum inhibitory concentration (MIC) of the fraction methanolic extract required to kill microorganism. In the experiment, medicaments were added to bacterial species into eppendrof tube, in concentrations. The MIC was the lowest concentration of the drug at which bacterial growth could not be observed.

4.8.2.1 Principle of MIC

The disc diffusion method, which is a 'semi-quantitative' method, determination of MIC values provides a quantitative measure for the level of resistance expressed by the test organism. The minimal inhibitory concentration (MIC) values, which represent the lowest extract concentration that completely inhibits the growth of microorganisms. Next to the agar dilution method, the broth dilution method is one of the most frequently used methods to determine MIC's **[35].** The

method is based on the inoculation of a standardized liquid culture of the test strain in a dilution series of the extract for which the MIC is determined. The first concentration in the dilution series at which no visual growth can be determined is then considered as the MIC **[41].**

4.8.2.2 Apparatus and Reagents

Nutrient Agar Medium	Autoclave
Petridishes	Laminar air flow hood
Sterile cotton	Spirit burner
Micropipette	Refrigerator
Inoculating loop	Incubator
Sterile forceps	Ethanol
Nutrient Broth Medium	Screw cap test tubes
Eppendrof tube	Nosemask and Hand glove

4.8.2.3 Test Materials of *Nyctanthes arbor-tristis*

Methanolic extract of *Nyctanthes arbor-tristis* leaves were taken as test sample.

4.8.2.4 Test Organisms

The bacterial strains (*Vibrio mimicus*) used for the experiment were collected as pure cultures from the East West University microbiology laboratory.

4.8.2.5 Culture Medium and Their Composition

The Nutrient Agar medium was used normally to demonstrate the antimicrobial activity and to make subculture of the test organisms. Nutrient Agar medium contains following things

Table 4.7: Composition of nutrient agar medium **[35]**

Ingredients	**Amount**
Bacto peptone	0.5 gm
Sodium chloride	0.5 gm
Bacto yeast extract	1.0 gm
Bacto agar	2.0 gm
Distilled water q.s.	100 ml

Table 4.8: Composition of nutrient broth medium **[35]**

Ingredients	**Amount**
Bacto beef extract	0.3 gm
Bacto peptone	0.5 gm
Distilled water q.s.	100 ml

Agar and broth medium having this composition was directly brought from the market and the $P^H = 7.2 + 0.1$ at 25^0C was maintained.

4.8.2.6 Procedure

- The agar diffusion method was employed for the determination of antibacterial activities of *Nyctanhes arbor-tristis.*
- The leave extracts were dissolved in 100% methanol to a final concentration of 100 mg/ml **[35].**
- The bacterial strains were cultured in a nutriment broth for 24 hours.
- Then, previously prepared 1ml of suspension bacteria (30 X 1012 CFU estimated) was spread on nutrient Broth agar.

- Disks were made by using a sterile filter paper and were loaded with 20 µl of each sample extract.
- Methanol was used as negative control and Azithromycin (30 mcg/disk) as positive reference standard.
- All the plates were incubated at 37°C for 24 hours.
- Antibacterial activity was evaluated by measuring the zone of inhibition in millimetres.
- All experiments were done in triplicates **[35].**

Chapter – 5

RESULT & DISCUSSION

The aim of this chapter is to illustrate the results and discussions of crude methanolic extract of *Musa sapientum* (leaf). This chapter will include the following results and discussions of the test samples of *Musa sapientum* (leaf):

1. In vitro antimicrobial screening 2. Minimum inhibitory concentration (MIC)

3. In vitro antioxidant activity

3.1. Total phenolic content

3.2. DPPH radical scavenging assay (Quantitative analysis) and IC_{50}

3.3. Reducing power assay

3.4. Hydrogen Peroxide radical scavenging assay and IC_{50}

3.5. Total flavonoids content

4. Haemagglutination test 5. Antihaemolytic Activity

6. Sensityvity test 7. Preliminary phytochemical investigations

8. Thin Layer Chromatography (TLC)

Besides the results of various tests are represented through suitable graphical representation methods. All the data are expressed as mean ± standard deviation (n=3).

5.1 In vitro antimicrobial screening

The antimicrobial activities of extracts were examined in the present study. The crude extract produced strong activity against a number of the test organisms. The results are given in table 5.1. The zones of inhibitions produced by the crude methanolic extract of the fruit of *Musa sapientum* (leaf) were ranged from 6-11 mm at a concentration of 40µg/disc. While the zones of inhibition produced by the crude methanolic extract of the fruit of *Musa sapientum* (leaf) were ranged from 7-17 mm at a concentration of 400µg/disc. The methanolic extract showed greatest activity against *Staphylococcus aureus* having the zone of inhibition of 11 mm (40µg/disc) and

16 mm (400µg/disc). Besides the extract showed strong activity against *Shigella dysentery* (7mm for 2mg/disc and 16 mm for 20mg/disc), *Salmonella typhi* (7 mm for 40µg/disc and 16 mm for 400µg/disc), *Vibrio mimicus* (7 mm for 40µg/disc and 17 mm for 400µg/disc). Moderate activity was found against *Bacillus cereus* (6 mm for 40µg/disc and 7 mm for 400µg/disc). Among the tested Gram positive bacteria, the extract showed greatest activity against *Staphylococcus aureus* having the zone of inhibition of 11 mm (40µg/disc) and 16 mm (400µg/disc). Among the tested Gram negative bacteria, the extract showed strong activity against *Shigella dysentery* (7mm for 40µg/disc and 16 mm for 400µg/disc), *Salmonella typhi* (7 mm for 40µg/disc and 16 mm for 400µg/disc) and, *Vibrio mimicus* (7 mm for 40µg/disc and 17 mm for 400µg/disc). In brief, the mehanolic extract of *Spondias pinnata* fruit showed a potent inhibitory activity against *Staphylococcus aureus.*

Table 5.1 Antimicrobial activity of crude methanolic extract *Musa sapientum* (leaf)

Test microorganisms	Diameter of zone of inhibition (mm)		
	Crude extract (Dose 40µg/disc)	Crude extract (Dose 400µg/disc)	Control Azithromycin (30 µg/disc)
Gram positive bacteria			
Bacillus cereus	6	7	23
Staphylococcus aureus	11	16	25
Gram negative bacteria			
Shigella dysentery	7	16	23
Salmonella typhi	7	16	21
Vibrio mimicus	7	17	22

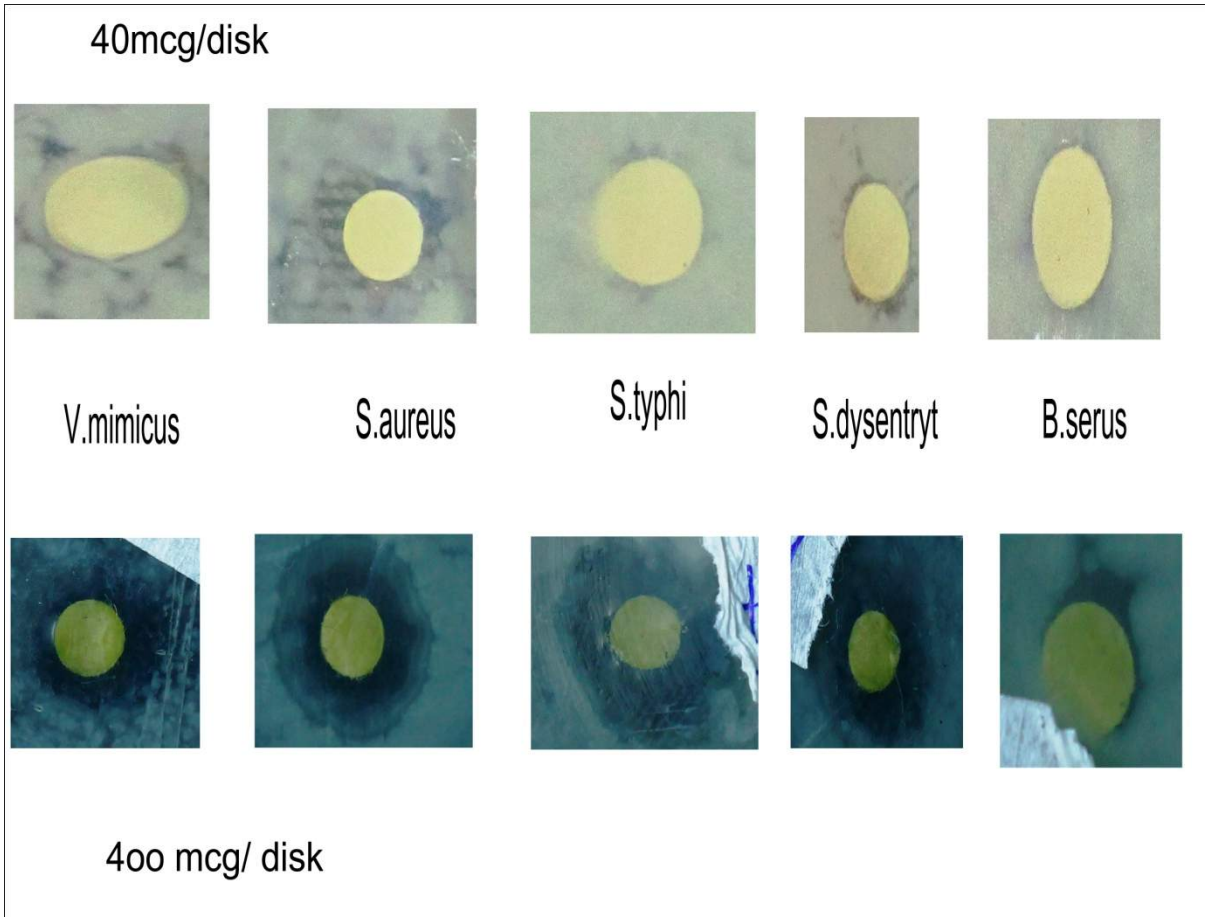


Figure 5.1: Antimicrobial activity of crude methanolic extract *Musa sapientum* (leaf)

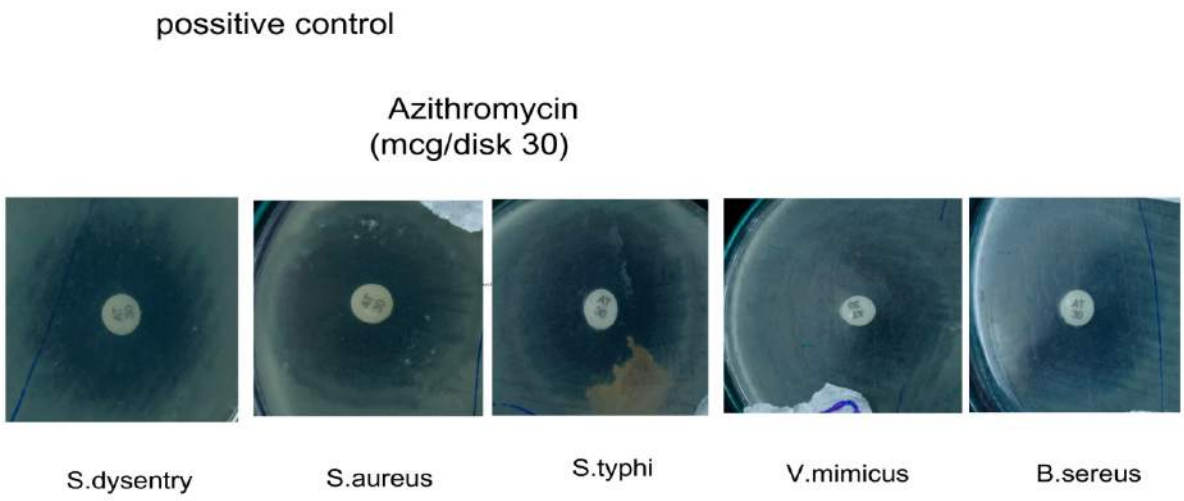


Figure 5.2 Positive control test against microorganisms

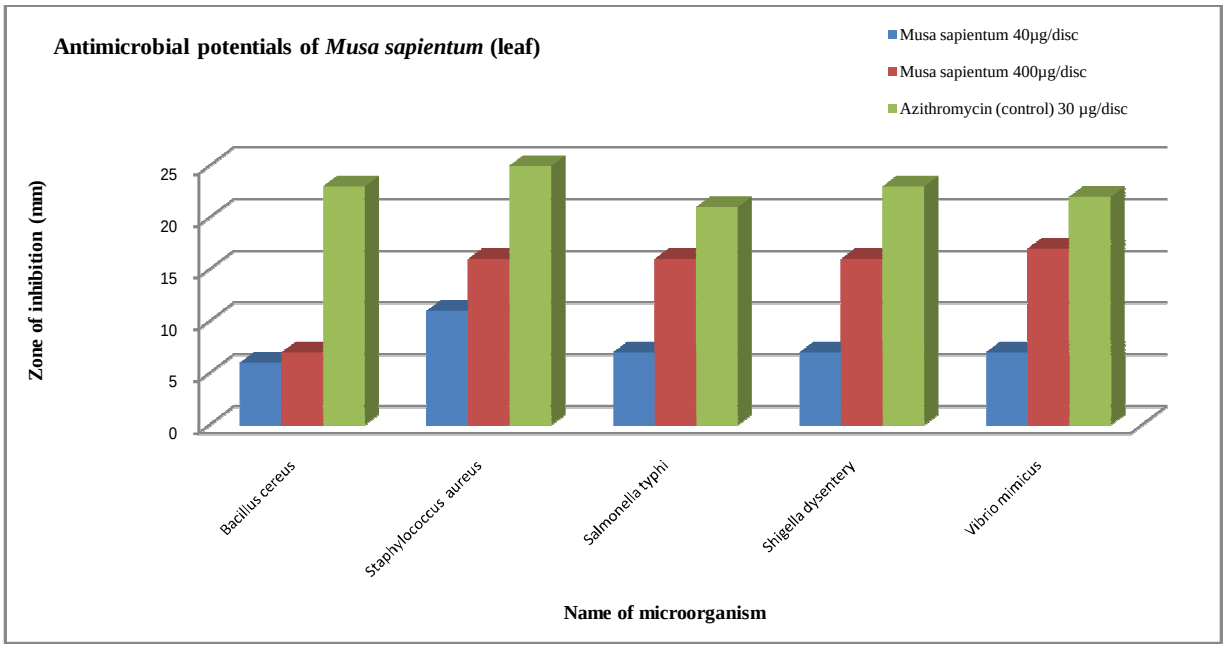


Figure 5.3: Comparison between the antimicrobial activities (zone of inhibition) of the crude methanolic extract of *Musa sapientum* (leaf) and positive control Azithromycin

5.2 Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of the crude extract was determined for *Vibrio mimicus* against which the crude methanolic extract of *Musa sapientum* (leaf) showed promising and potent antimicrobial activity in the preliminary in vitro antimicrobial screening test. The minimum inhibitory concentration (MIC) of crude methanolic extract of *Musa sapientum* (leaf) for the test microorganisms is expressed in table 5.2 and is shown by figures (Figure 5.4).

Table 5.2 Result of MIC

Concentration (µg/disc)	Zone of Inhibition (mm)
0.2	10
2	11
20	14
200	14
2000	16

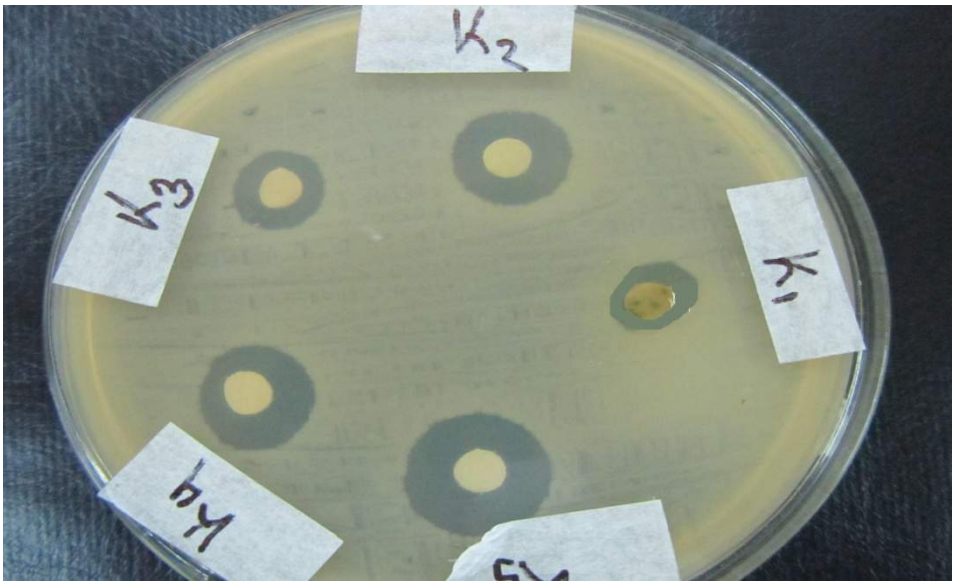


Figure 5.4: Zone of inhibition of *Musa sapientum* (leaf) against *vibrio mimicus* by MIC

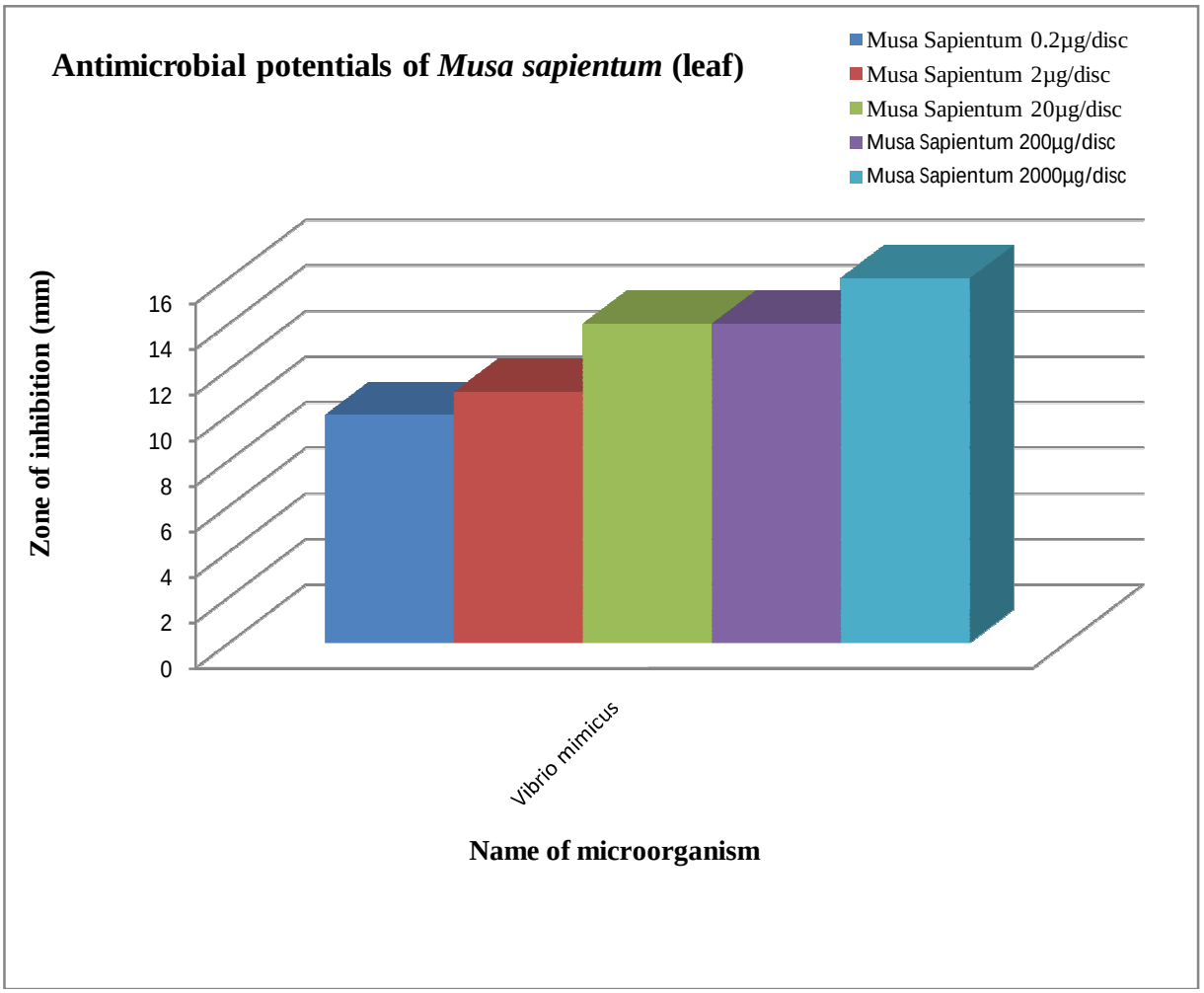


Figure 5.5: MIC (zone of inhibition) of the crude methanolic extract of *Musa sapientum* (leaf)

5.3 Antioxidant Test

5.3.1 Total phenolic content

The crude methanolic extract of *Musa sapientum* (leaf) was subjected to total phenolic content determination. Based on the absorbance values of the extract solution, reacted with Folin-Ciocalteu reagent and compared with the standard solutions of gallic acid (table 6.3) equivalents, result of the colorimetric analysis of the total phenolics are given in table 6.4. Total phenolic content of the samples are expressed as mg of GAE (gallic acid equivalent) per gm of dried extract. The phenolic content found in the crude ethanolic extract of *Musa sapientum* (leaf) was 8.564±0.716 mg of gallic acid (GAE) per gm of dried extract. Absorbance Values are expressed as average ± SD (n=4).

Table 5.3 Standard curve preparation by using gallic acid

SL. No.	Concentration (µg /ml)	Absorbance	Regression line	R^2
1	100	1.620	y = 0.0162x + 0.0215	0.9985
2	50	0.866		
3	25	0.450		
4	12.5	0.253		
5	6.25	0.120		
6	3.125	0.059		
7	1.5625	0.034		
8	0.78125	0.022		
9	0.3906	0.020		
10	0	0.011		

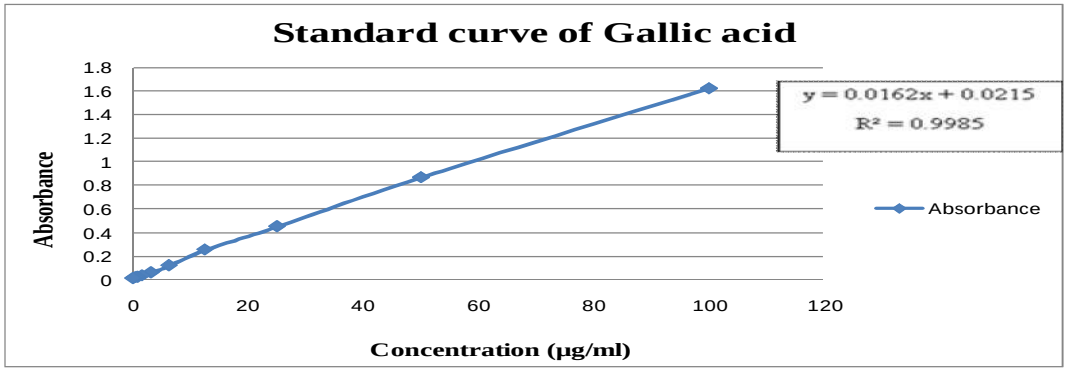


Figure 5.6: Standard curve for gallic acid

Table 5.4 Total phenolic content assay

Sample	Conc (mg/ml)	Absorbance	mg of Gallic acid equivalent (GAE) per gm of dried extract)
Crude ethanolic extract of *Musa sapientum* (leaf)	20mg/ml	0.115±0.011	8.564±0.716

5.3.2 DPPH radical scavenging assay (Quantitative analysis) and IC_{50}

The crude methanolic extract of *Musa sapientum* (leaf) was subjected to free radical scavenging activity and it showed significant free radical scavenging activity with an IC_{50} value of 10.527µg/ml. The percentage inhibition of free radical DPPH and the IC_{50} value of crude ethanolic extract of *Musa sapientum* (leaf) are given in table 5.5 and figure 5.6. Absorbance Values are expressed as average ± SD (n=3).

Table 5.5 IC_{50} value of crude methanolic extract of *Musa sapientum (leaf)*

SL no	A_{Blank}	Concentration (µg/ml)	A_{Sample}	% inhibition of free radical DPPH = $(1 - A_{Sample}/A_{Blank}) \times 100$	IC_{50} µg/ml
1	0.689	2	0.489±0.480	29.028	10.527
2		4	0.434±0.381	36.961	
3		6	0.395±0.327	42.622	
4		8	0.340±0.246	40.605	
5		10	0.314±0.231	54.378	

Here, A_{Blank} and A_{Sample} are the absorbance of blank and sample respectively.

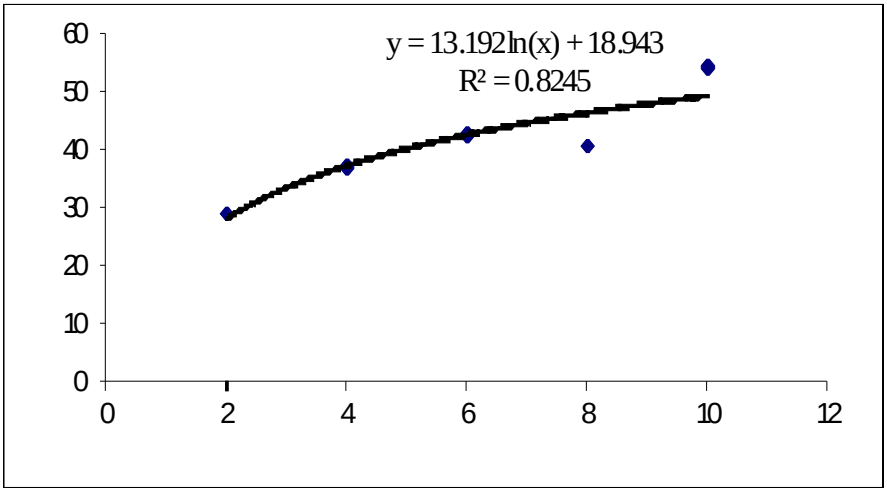


Figure 5.7: DPPH scavenging potential & IC_{50} value of crude ethanolic extract of *Musa sapientum* (leaf)

5.3.3 Reducing power assay

The reducing properties are generally associated with the presence of reductanes which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Duh *et al.*, 1999). The reducing power ability of crude ethanolic extract of *Musa sapientum* (leaf) was determined using L-ascorbic acid as positive control. In case of reducing power, the higher the concentration of the test samples, the higher the absorbance. The higher the absorbance, the higher the inhibition. The reducing power of various concentrations of crude ethanolic extract of *Musa sapientum* (leaf) are given in table 5.6 & figure 5.7. The percentage increase in reducing power of various concentrations of test material and EC_{50} value of the extract is represented in figure 5.8. The highest % increase in reducing power was observed for 84 mg/ml concentration of crude ethanolic extract and it was 63.433%. The EC_{50} value of the extract was found to be 42.52 mg/ml for crude ethanolic extract of *Musa sapientum* (leaf).

Table 5.6 EC_{50} value of crude ethanolic extract of *Musa sapientum (leaf)*

SL no	Absorbance of blank, A_{Blank}	Concentration (mg/ml)	Absorbance of test, A_{Test}	% increase in reducing power = (A_{Test} / A_{Blank}) -1 X 100	EC_{50} mg/ml
1	0.402	12	0.475	18.159	42.52
2		36	0.594	47.761	
3		56	0.64	59.204	
4		84	0.657	63.433	

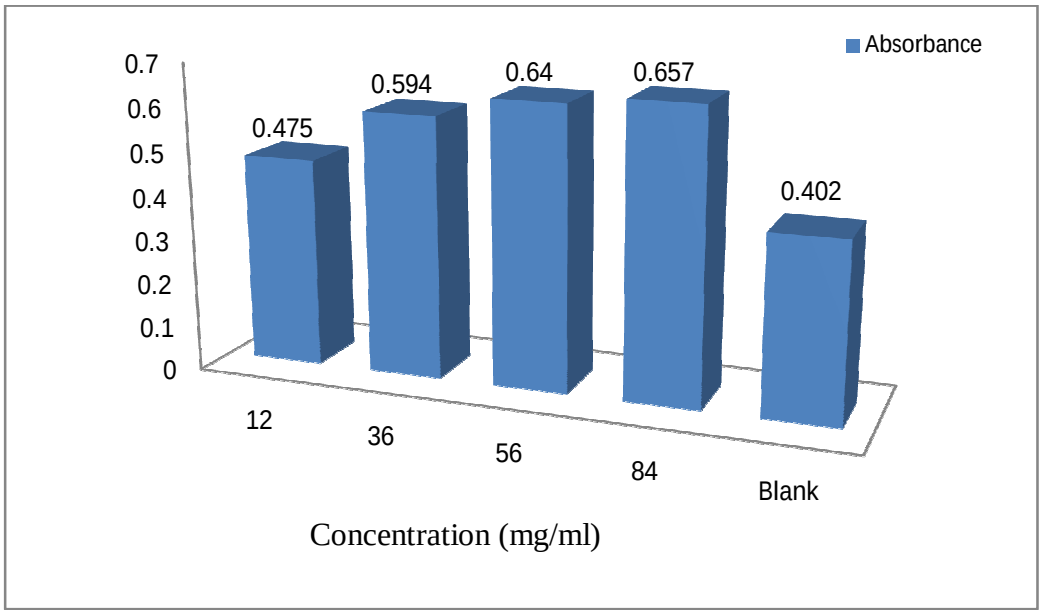


Figure 5.8: Bar diagram of Reducing power assay of various concentration of crude extract of *Musa sapientum* (leaf)

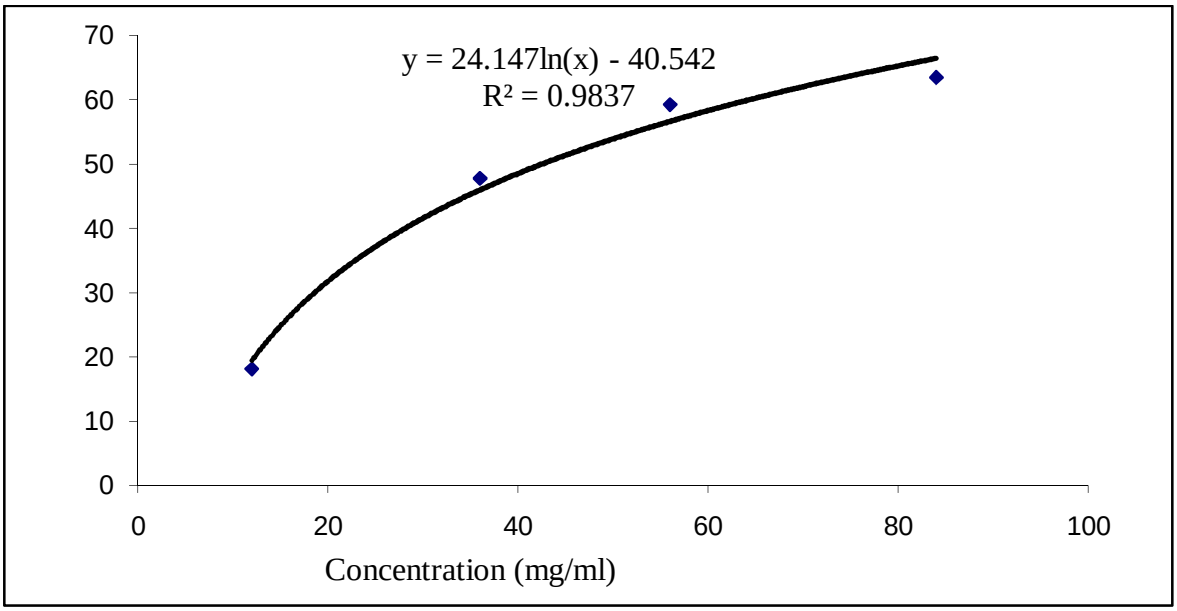


Figure 5.9 : reducing power & EC_{50} value of crude extract of *Musa sapientum* (leaf)

5.3.4 Hydrogen Peroxide radical scavenging assay and IC_{50}

The crude ethanolic extract of *Musa sapientum* (leaf) was subjected to free radical scavenging activity and it showed significant free radical scavenging activity with an IC_{50} value of

311.06µg/ml. The percentage inhibition of free radical Hydrogen peroxide and the IC_{50} value of crude ethanolic extract of *Musa sapientum* (leaf) are given in table 5.7 and figure 5.9.

Table 5.7 IC_{50} value of crude Methanolic extract of *Musa sapientum (leaf)*

SL no	A_{Blank}	Concentration (µg/ml)	A_{Sample}	% inhibition of free radical DPPH = $(1 - A_{Sample}/A_{Blank}) \times 100$	IC_{50} µg/ml
1	1.615	4	1.13	30.031	311.06
2		8	1.11	31.269	
3		12	1.07	33.746	
4		16	1.039	35.666	
5		20	1.003	37.895	

Here, A_{Blank} and A_{Sample} are the absorbance of blank and sample respectively.

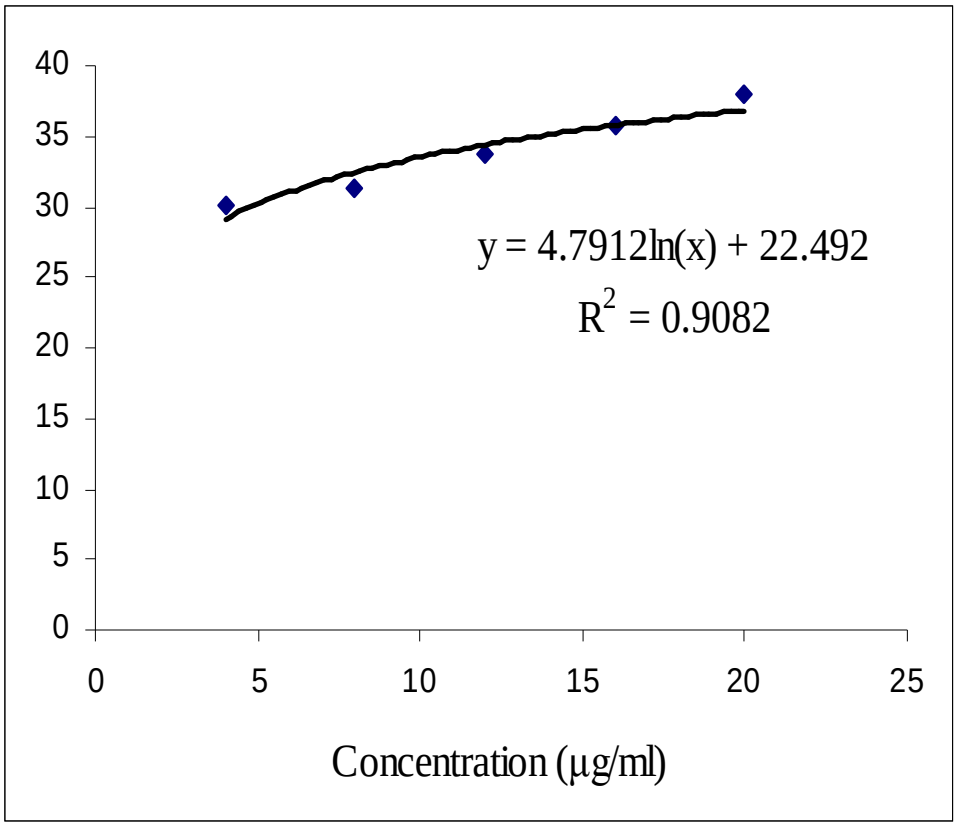


Figure 5.10: Hydrogen Peroxide scavenging potential & IC_{50} value of crude ethanolic extract of *Musa sapientum* (leaf)

5.3.5 Total flavonoids content

To determine the total flavonoids content of crude ethanolic extract of *Musa sapientum* (leaf) by using partially modified method of Ismail et al., a standard curve is needed which is obtained from a series of different rutin concentrations (table 6.8). The total flavonoids content of the

sample is expressed as mg of rutin per gm of dried extract in table 6.9 by using the standard curve equation of rutin (y = 0.002x + 0.0318, R^2 = 0.9989). Where y is absorbance at 510 nm and x is flavonoid content of crude plant extract. The total flavonoids content found in the crude ethanolic extract of *Musa sapientum* (leaf) was 1431±65.57 mg of rutin per gm of dried extract. Absorbance Values are expressed as average ± SD (n=3).

Table 5.8 Standard curve preparation by using rutin

SL. No.	Concentration (µg /ml)	Absorbance	Regression line	R^2
1	2.5	0.0365	y = 0.002x + 0.0318	0.9989
2	5	0.0417		
3	10	0.0521		
4	20	0.0735		
5	30	0.0906		
6	40	0.1127		

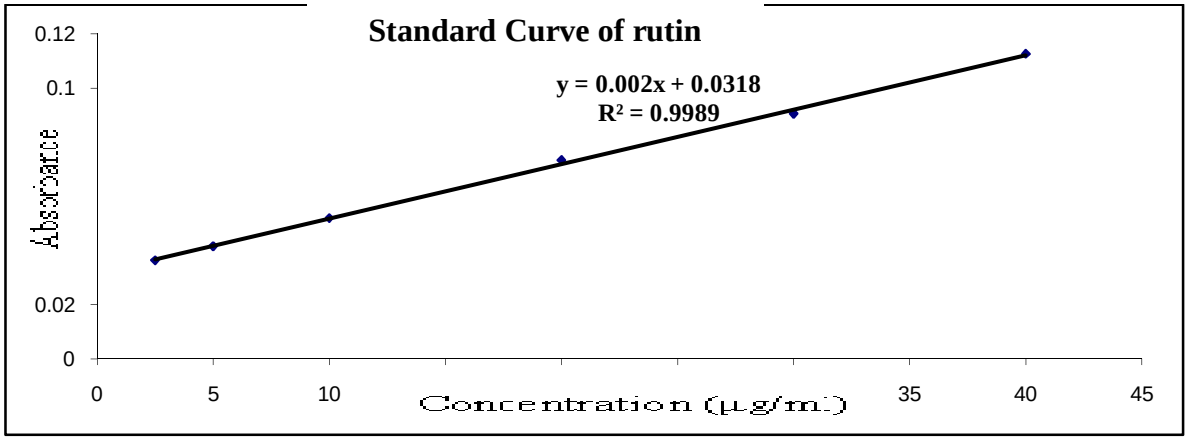


Figure 5.11: Standard curve for rutin

Table 5.9 Total flavonoids content assay

Sample	Conc (mg/ml)	Absorbance	mg of rutin equivalent per gm of dried extract
Crude methanolic extract of *Musa sapientum* (leaf)	1 mg/ml	0.318±0.013	1431±65.57

5.4 Haemagglutination test for methanolic extract of *Musa sapientum* (leaf)

From the table 5.10 it is seen that in case of A^+, B^+ blood group maximum Haemagglutination activity was found of 5mg/ml extract. In case of AB^+ blood group no activity was found. From the table it is also seen that increase the activity with the increased concentration thus in case of concentration 2.5mg/ml moderate activity found

Table 5.10 Haemagglutination test for methanolic extract of *Musa sapientum* (leaf)

Blood group	Banana leaves		
	K1	**K2**	**K3**
	5mg/ml	**2.5mg/ml**	**1.25mg/ml**
A+	+++	+	+
B+	+++	++	+
O+	++	++	+
AB+	+	+	-

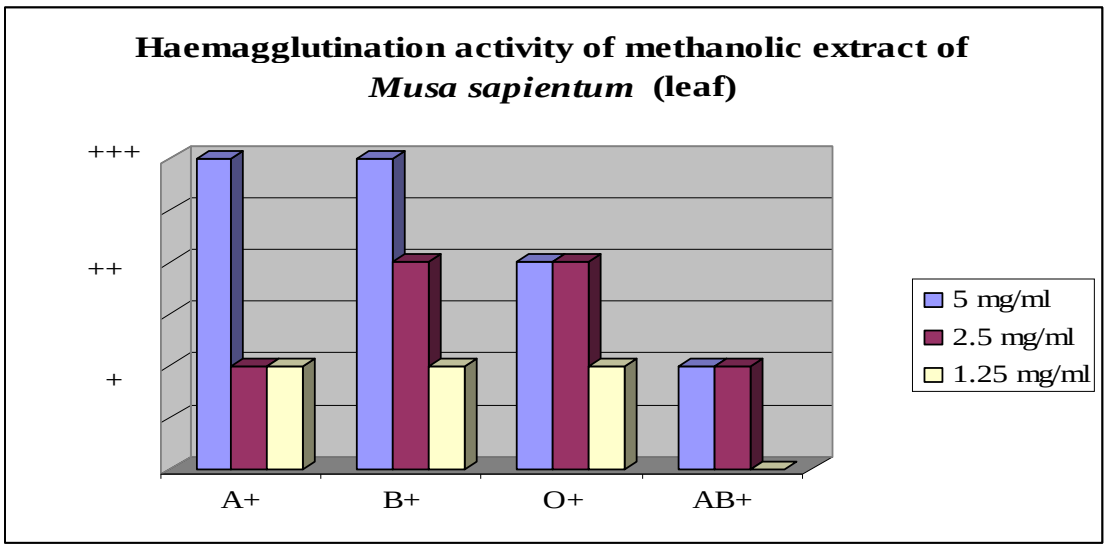


Figure 5.12: Bar diagram of Haemagglutination test of *Musa sapientum* (leaf)

6.5 Antihaemolytic test for methanolic extract of *Musa sapientum* (leaf)

The crude methanolic extract of *Musa sapientum* (leaf) was subjected to antihaemolytic activity and it showed significant antihaemolytic activity with an IC_{50} value of 3.107mg/ml. The

percentage inhibition of haemolysis and the IC_{50} value of crude methanolic extract of *Musa sapientum* (leaf) are given in table 5.11 and figure 5.13.

Table 5.11 Antihaemolytic test for methanolic extract of *Musa sapientum* (leaf)

Sample	concetration	Absorbance	Sample	concetration	Absorbance	% Antihemolytic activity
Musa sapientum (leaf) crude extract	0.3125mg/ml	0.8615	Positive control	0.3125mg/ml	0.896	3.850446
	0.625mg/ml	0.537		0.625mg/ml	0.853	37.04572
	1.25mg/ml	0.3675		1.25mg/ml	0.753	51.19522
	2.5mg/ml	0.275		2.5mg/ml	0.683	59.73646
	5mg/ml	0.2225		5mg/ml	0.629	64.62639

IC50 = 3.107 mg/ml

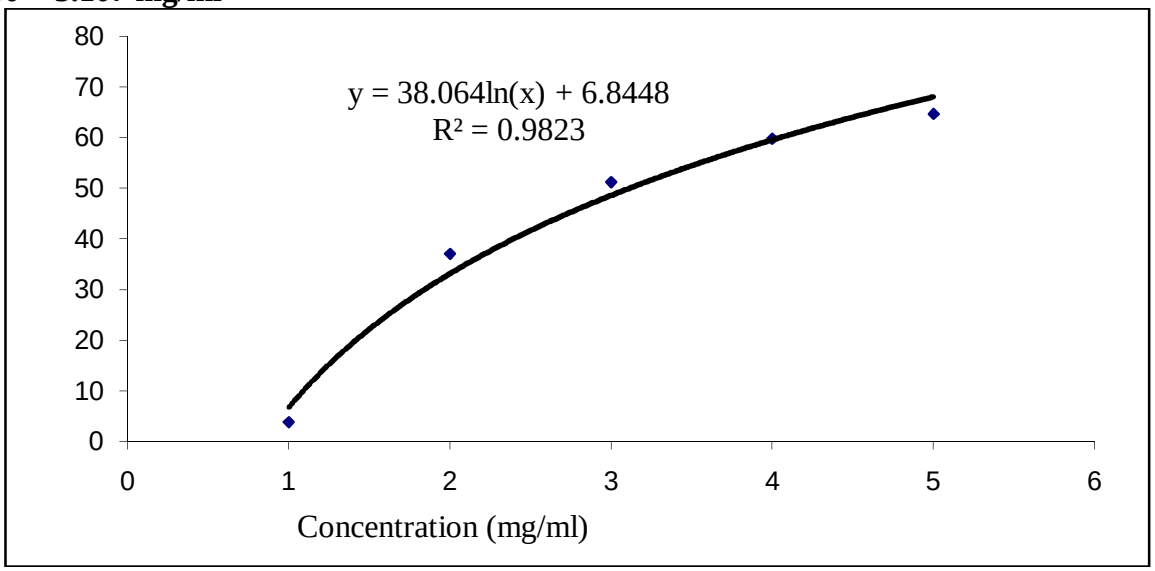


Figure 5.13: Concetration Vs % Antihemolytic activity

5.6 Sensitivity test

From the table 6.12 it is seen that the crude methanolic extract of *Musa sapientum* (leaf) (200 µg/disc) show sensityvity against *Shigella dysentery*. But the crude methanolic extract of *Musa sapientum* (leaf) (200 µg/disc) show no sensityvity against *Sacharomyces cerevacae* and

Escherichia coli. Figure 5.14 show the Sensitivity test for methanolic extract of *Musa sapientum* (leaf).

Table 5.12 Sensitivity test for methanolic extract of *Musa sapientum* (leaf)

Sample & concentration	Name of Microorganism	Zone of Inhibition (mm)
Musa sapientum (leaf) crude extract (200 µg/disc)	*Sacharomyces cerevacae*	0
	Escherichia coli	0
	Shigella dysentery	6

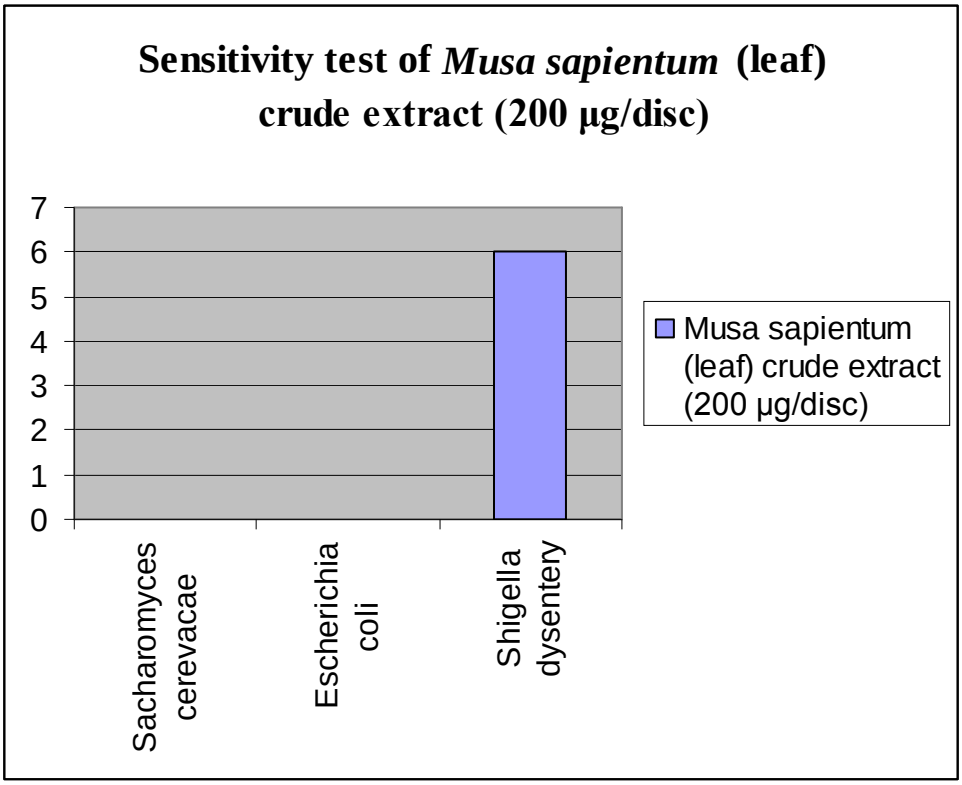


Figure 5.14: Sensitivity test for methanolic extract of *Musa sapientum* (leaf)

5.7 Preliminary phytochemical investigations

Secondary metabolites are very important for the plant. In this test I searched for whether my plant contains some kind of secondary metabolites like alkaloid, flavanoid, steroid, rducing sugar etc. Simply by following standard methods and by the observation of different color change of the solution the following results were made. From the tests it was concluded that there might be alkaloid, flavanoid, tanin, steroid, cardiac glycoside present in the sample because of test of these

compound has given the positive result by showing specific color change or making specific precipitation. In addition, some of the secondary metabolites like anthra-quinone, terpe-noid, reducing sugar, saponin were absent as their test showed negative result.

Table 5.13 Result of preliminary phytochemical investigation of secondary metabolites

Sample name	Phytochemical composition	Result
Musa sapientum (leaf)	Alkaloid	+
	Flavonoid	+
	Anthraquinone	-
	Terpe-noid	-
	Reducing sugar	-
	Saponin	-
	tanin	+
	Steroid	+
	Cardiac Glycolside	+

5.8 Thin Layer Chromatography (TLC)

The results obtained after TLC of the methanolic extract of the *Musa sapientum* (leaf) in solvent system 1 is given below-

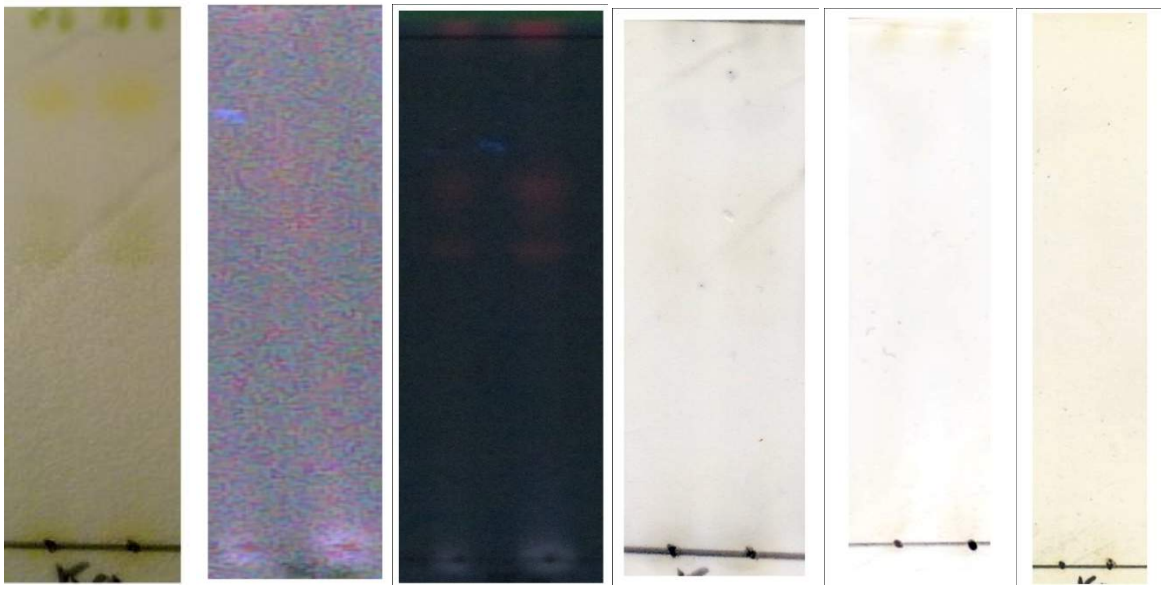
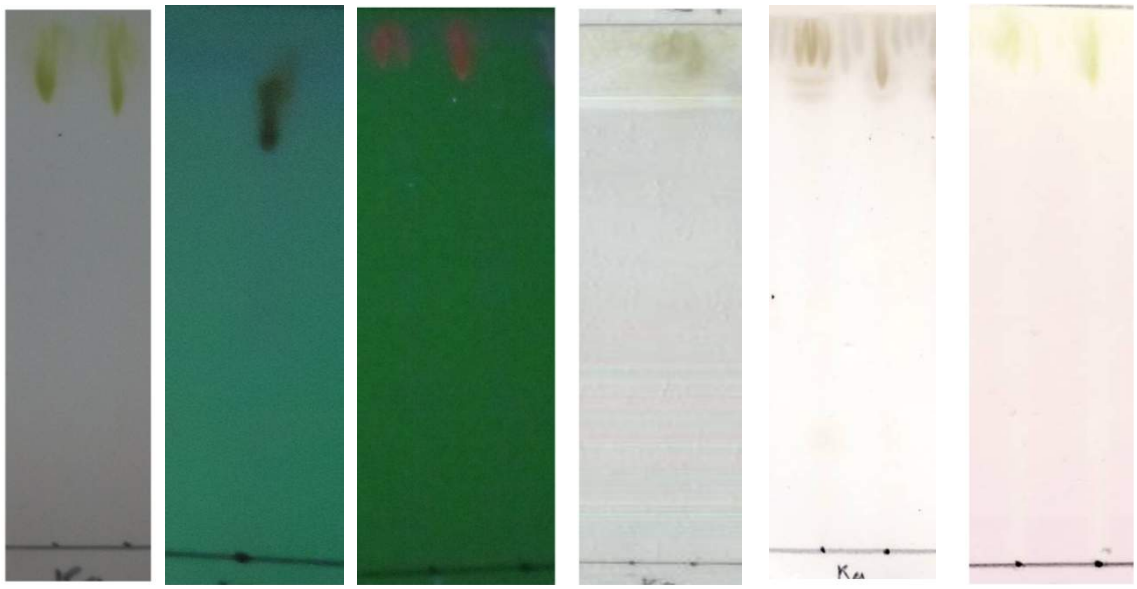


Figure 5.15*:* Results for TLC in nonpolar basic solvent (1= naked eye view; 2 & 3 = UV light view; 4= after application of FC reagent; 5= after charing; 6= after application of DPPH)

The naked eye view of the TLC was mentioned in the plate 1 which did not show any clear spot. Then the plate was observed under UV which is shown in the plate 2 and 3. It showed some spots which indicate the presence of different compounds in that sample. Folin & ciocalteu solution was used to determine the presence of the phenolic compouns in the sample. The formation of intense violet color after application of the Folin & ciocalteu solution in the sample indicates the presence of phenolic compound in the sample. Spraying of the Folin & ciocalteu solution in the TLC plate showed moderate violet color which indicates the presence of phenolic compound in that fraction (plate 4).

After charing of the TLC plate with sulfuric acid has showed (plate 5) two spots at the bottom as well as at the top of the TLC plate. Spraying of DPPH solution on the TLC plate did not show any significant color changes which indicate the less free radical scavenging property of that fraction



1 2 3 4 5 6

Figure 5.16: Results for TLC in intermediate polar basic solvent (1= naked eye view; 2 & 3 = UV light view; 4= FC reagent view; 5= after charing view 6= after application of DPPH solution

From the figure 5.16 we see that the naked eye view of the TLC is mentioned in the plate 1

showed two clear spots at the top of the plate. Then the plate was observed under UV which is shown in the plate 2 and 3. It showed some additional spots which indicate the presence of different compounds in that sample. Folin & ciocalteu solution was used to determine the presence of the phenolic compouns in the sample. The formation of intense violet color after application of the Folin & ciocalteu solution in the sample indicates the presence of phenolic compound in the sample. Spraying of the Folin&ciocalteu solution in the TLC plate shows formation of intense violet color which indicates the presence of phenolic compound in that fraction (plate 4). After charing of the TLC plate with sulfuric acid (plate 5) has revealed many spots in the TLC plate. Spraying of DPPH solution on the TLC plate have shown significant formation of pale yellow color (plate 6) in the place of the spots which indicates significant free radical scavenging property of that fraction.

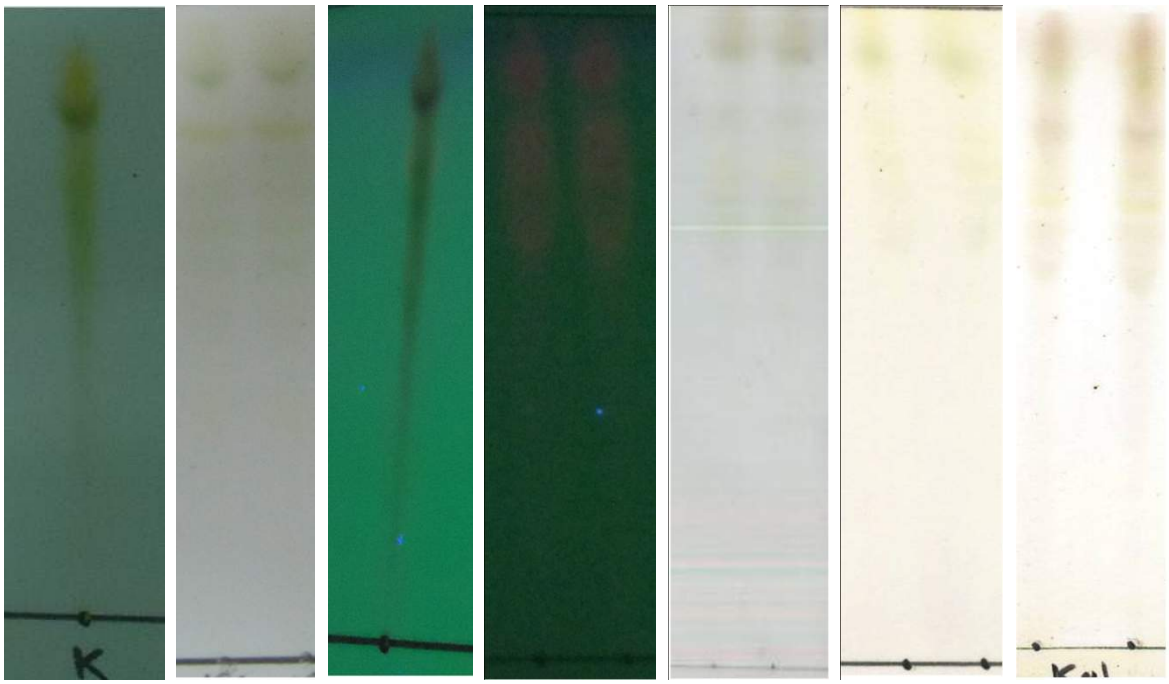


Figure 5.17: Results for TLC in polar basic solvent (1 & 2= naked eye view; 3 & 4 = UV light view;5= after application of FC solution, 6= after application of DPPH solution; 7= after

charing;

From the figure 5.18 we see that the naked eye view of the TLC was mentioned in the plate 1 which showed clear spot. Then the plate was observed under UV which is shown in the plate 3 and 4. It showed some spots which indicate the presence of different compounds in that sample. After charing of the TLC plate with sulfuric acid (plate 7) has revealed many spots in the TLC plate.

Spraying of DPPH solution on the TLC plate did not show significant formation of pale yellow color (plate 6) in the place of the spots which indicates less significant free radical scavenging property of that fraction.

Folin & ciocalteu solution was used to determine the presence of the phenolic compouns in the sample. The formation of intense violet color after application of the Folin & ciocalteu solution in the sample indicates the presence of phenolic compound in the sample. Spraying of the Folin&ciocalteu solution in the TLC plate shows moderate violet color which indicates the presence of phenolic compound in that fraction (plate 5)

Chapter – 6

CONCLUSION

CONCLUSION

For the plant physiologist, work on medicinal plants opens up a wide range of research possibilities, and plant physiological studies would indeed have a major role to play in this burgeoning field. With only a few exceptions, many widely used medicinal plants have not received the extensive plant physiological characterization received by food crops or model plant systems. Although active phytochemicals may have been identified, in general, many pathways for the biosynthesis of specific medicinal compounds and the factors (biotic and abiotic) regulating their production remain unclear. At present, a major concern with the use of phytomedicines regards the maintenance of consistent medicinal quality in botanical medicines.

Therefore, plant materials can be potential sources of chemically interesting and biologically important drug entrant. And for this purpose the plant can be further screened against various diseases in order to find out its unexplored efficacy with a gaze to the future with a great deal of expectancy.

Crude extract of *Musa sapientum Var. sylvestris* leaves of the family *Musaceae* is traditionally used in various disease conditions. There are few established research reports regarding the phytochemical and pharmacological properties of this product. So there are plenty of scopes to establish a variety of properties which are significantly beneficial to mankind.

C h a p t e r C

T i n o s p o r a t o m e n t o s a

Chapter – 1

INTRODUCTION

1.1Natural products:

Natural products such as plants play an important source of new drug discovery and development. Plants act as a basis for about 25% of the drugs prescribed worldwide. Now a days, about 121 active compounds which obtained from plants are used in the current market. According to the World Health Organization (WHO), 252 Drugs are considered as basis and essential for current use among which, 11% are only obtained from plant origin and additional are synthetic drugs, but especially obtained from natural precursors. Examples of important drugs obtained from plants are digoxin from *Digitalis* spp., quinine and quinidine from *Cinchona* spp., vincristine and vinblastine from *Catharanthus roseus*, atropine from *Atropa belladonna* and morphine and codeine from *Papaver somniferum*. Moreover, compounds such as muscarine, physostigmine, cannabinoids, yohimbine, forskolin, colchicines and phorbol esters which obtained from plants, are also important for used in pharmacological, physiological and biochemical studies. It is reported that about 60% of anti-tumour and anti-infectious drugs already on the market or under clinical trial are of natural origin. For the design and planning of new drugs, biomimetic synthetic development and the discovery of new therapeutic properties, natural compounds can be lead compounds. In recent years, there is growing interest in the therapeutic use of natural products, especially those derived from plants, due to several reasons including 1.Conventional medicines have more side effects and ineffective in therapeutical use, 2.Abusive or incorrect use of synthetic drugs may cause many problems and side effects, 3.A huge number of population in the world not depend on conventional pharmacological treatment, and 4.Folk medicine and ecological awareness suggest that “natural” products are harmless.

The approach for development of drugs from plants depends on the aim and objective.

The selection of a suitable plant for an isolation of a new drug that is pharmacologically active is

very much important consideration. There are several ways for the selection of plants including traditional use, used of plants by the folk medicinar, used of plants for the isolation of drugs, chemical contents, toxicity, randomized selection or a combination of several criteria.

The most common strategy is careful observation of the use of natural resources in folk medicine in different cultures; this is known as ethanobotany or ethanopharmacology. Information on how the plant is used by an ethnic group is extremely important. The preparation procedure may give an indication of the best extraction method.

On the basis of above selection ways, I select padmaguruj or padmagulancha for phytochemical research.

1.2 Phytochemistry

Phytochemistry is in the strict sense of the word the study of phytochemicals. These are chemicals derived from plants. In a narrower sense the terms are often used to describe the large number of secondary metabolic compounds found in plants. Many of these are known to provide protection against insect attacks and plant diseases. They also exhibit a number of protective functions for human consumers. These compounds perform different functions. For example, some enable plants to store energy n the form of sugar, whilst others are protective against disease or predators.

The phytochemistry unit functions as a laboratory for plant collection and processing, sample preparation and chromatographic analysis of extracts, fractions and compounds isolated from herbs and medicinal plants.

The phytochemistry unit plays a very important role in the collection of plants and has capabilities in the following areas:

- Plant sample collection and processing.

- Plant sample preparation for bioassay studies. Phytochemical screening.
- Fractionation and isolation of bioactive compounds using analytical techniques.
- Preparation and analysis of standardized extracts by HPLC. **[98]**

1.3 Primary metabolites

Primary metabolites include compounds such as carbohydrates and lipids - substances essential to the structure and life of the plant, as well as essential for human nutrition. Carbohydrates are largely made up of sugars – saccharides. Glucose and fructose are examples of monosaccharides – they consist of a single saccharide molecule. Polysaccharides consist of several saccharide molecules linked together. Lipids – commonly known as fats – provide a reservoir of fuel for cells. They also form a major component of cell membranes in both plants and animals. A group of lipids known as fatty acids are important for human health. There are some fatty acids that the body cannot produce, and which must be sourced through the diet. These are known as essential fatty acids. **[99]**

1.4 Secondary metabolites

Using the primary metabolites, plants produce secondary metabolites, which are largely responsible for the plant individual properties such as aroma, flavor, colour and medicinal actions. Secondary metabolites include terpenes, polyphenols, alkaloids and some glycosides.

The medicinal actions of herbs are largely due to these groups of chemicals. Secondary metabolites include antioxidants, which defend the body against the effects of reactive free radicals. A large group of secondary metabolites known as terpenes provide us with many medicinal compounds, such as anti-inflammatory agents, expectorants and sedatives. The carotenoids – precursors of vitamin A – belong to the terpene group of compounds. Flavanoids – an important sub group of polyphenols – contain a number of important antioxidant compounds.

In addition, certain of them are know to protect against heart disease and cancer. Plants rich in isoflavones, such as soy, exhibit marked hormone balancing activity. The alkaloid groups of secondary metabolites include caffeine (a stimulant) and ephedrine (a decongestant). Many alkaloids, such as mescaline and cocaine, have hallucinogenic effects. Glycosides are a diverse group of chemicals particularly important in the study of herbal medicine. Cardiac glycosides improve the efficiency of the hearts without increasing its need for oxygen**[99]**.

1.5 *Tinospora tomentosa Miers.:*

Tinospora tomentosa Miers**.** known as padmaguruj is a large deciduous climbing

shrub mainly found in the tropical thickets of Bengal and almost throughout India, being presence of many noxious constituents it is composition of many traditional remedies Padmaguruj has occupied a pivotal position in Indian culture and folk medicine. It has been used in all most all the traditional system of medicine viz., ayurveda, unani and sidha. The wide therapeutic application of stem can made researcher to study this plant in detail. The present paper enumerates all the various aspects of the Padmaguruj.

Figure 1.1: botanical prints of *Tinospora tomentosa*

1.6 Scientific classification :

Kingdom	*Plantae*
Subkingdom	*Viridaeplantae*
Superdivision	*Tracheophyta*
Division	*Euphyllophytina*
Class	*Magnoliopsida*
Subclass:	*Ranunculidae*
Order	Ranunculales
Family	Menispermaceae
Genus	Tinospora
Species	*tomentosa*

Botanicalname *Tinospora tomentosa* Miers

Figure 1.2: different parts of *Tinospora tomentosa Miers.*

1.7 Growth and development:

Tinospora cardifolia is a deciduous plant that grows to 1.0 meters (3.3 feet) high by 0.5 meters (1.65 feet) wide and prefers many types of soil ranging from acid to alkaline and partial to full sun with moderate moisture. Stems of *Tinospora cardifolia are succulent and having long filliform fleshy aerial roots, which arise from the branches. Bark is thin, greyish or creamy white in colour, when peeled fleshy* stem is exposed. It often attains a great height and mostly climbs up the trunks of large neem trees. Leaves of Tinospora cardifolia are heart shaped membranous, juicy and cordate. Wood of this plant is porous soft and white in colour. Tinospora *cardifolia has greenish flowers, which are unisexual and Bloom in summer. Male flowers are small, yellow or green coloured occur in clusters whereas female flower occur singly. Fruits are Pea shaped, fleshy, shiny turn red when boiled and occur in winter. Seeds of Tinospora cardifolia are curved and pea sized.***[113]**

1.8 Medicinal properties:

A variety of constituents have been isolated from T. cardifolia belonging to different classes such as alkaloids, glycosides, diterpenoid lactones, sesquiterpenoids and steroids *T. cardifolia contains about 11.2 percent protein and rich in calcium and phosphorus.*

Tinospora cordifolia is used for diabetes, high cholesterol, allergic rhinitis (hay fever), upset stomach, gout, lymphoma and other cancers, rheumatoid arthritis (RA), hepatitis, peptic ulcer disease (PUD), fever, gonorrhea, syphilis, and to boost the immune system.

The root is a powerful emetic and is used for visceralobstructions; its water extract is used in leprosy. The root also exhibit antidiabetic effect. The extracts of stem, leaves, barks and rootsshow strong antioxidant activities. The bitterprinciple present in the stem is used in the treatment of debility, dyspepsia, fever and urinary disease and the decoction of the leavesis used

for the treatment of gout. Thepharmaceutical significance of this plant is mainly due to thepresence of various bioactive compounds, such as glucosides andalkaloides including berberine **[114].**

It has been reported that extract of *Tinospsora cardifolia has free radical scavenging* and antioxidant effect. Alcoholic root extract has antioxidant defence mechanism in alloxan induced diabetic rats and it is reported that there is significant increase in the concen tration of thiobarbituric acid reactive substances (TBARS) in liver and kidney in diabetic rats. Decreased concentration of glutathione (GSH) and decreased activities of superoxide dismutase (SOD) and catalase in liver and kidney of diabetic rats were also noted. Alcoholic Tinospora cardifolia root extract (TCREt) administered at a dose of 100 mg/kg body weight to diabetic rats orally for six weeks normalized the antioxidant status of liver and kidney. He also reported that effect of Tinospora cardifolia root extract was more potent than glibenclamide.

Aqueous extract of T. cardifolia inhibited ferrous sulphate mediated lipid peroxidation in a dose-dependent manner with an IC50 value of 1300 microg/ml and maximally (70%) at 2000 microg/ml. The results reveal that the direct and indirect antiocidant actions of T. cardifolia probably act in corroboration to manifest the overall radioprotective effects.

1.8.1 Hypoglycemic effect

In Indian Ayurvedic medicine, *Tinospora cardifolia* is widely used for diabetes mellitus. Oral administration of n aqueous *T. cardifolia* root extract (TCREt) to alloxan

diabetic rats caused a significant reduction in blood glucode and brain lipids. The extract caused an increase in body weight and total haemoglobin and hepatic hexokinase. The root extract also lowers hepatic glucose-6-phosphate and serum acid phosphate, alkaline phosphatase,

and lactate dehydrogenase ln diabetic rats. Thus TCREt has hypoglycaemic and hypolipidaemic effect. Oral administration of the extract of *Tinospora cardifolia* (TCREt) roots for 6 weeks resulted in a significant reduction in blood and urine glucose in alloxan diabetic rats. The extract also prevented a decrease in body weigh. **[115].**

1.8.2 ***Immuno-modulatory activity***

The plant is used in ayurvedic medicine to improve the immune system and the body resistance against infections. It is reported that T. cardifolia benefits the immune system in a variety of ways. Both alcoholic and aqueous extracts of T. cardifolia have been tested successfully for immuno-modulatory activity. **[115].**

1.8.3 Hepatoprotective activity

The hepatoprotective action of T.cardifolia was reported in one of the experiment in which goats treated with T.cardifolia have shown significant clinical and hemato-biochemical improvement in CCl4 induced hepatopathy. Extract of T.cardifolia has also exhibited in vitro inactivating property against Hepatitis B and E surface antigen in 48-72 hrs. **[116].**

1.8.4 Other properties

It has been reported that guduchi killed the HeLa cells ver y effectively in vitro and thus it indicates that guduchi needs attention as an anti neoplastic agent. Anti inflammatory potency of water extract of T. cardifolia has been proved by the study on induced oedema arthritis and on human arthritis. The effect was comparable with indomethacin and its mode of action appeared to ressemble that of a non-steroidal anti inflammatory agent. The dried stem of T. cardifolia produced significant anti-inflammatory effect in both acute and sub acute models of inflammation. T. cardifolia has found to be more effective than acetylsalicylic acid in acute inflammation. It has also antipyretic action. The aqueous extract of the stem antagonizes the

effect of agonists such as 5-hydroxytryptamine, histamine, bradykinin and prostaglandin E1 and E2 on the rabbit smooth muscle, relaxes the intestinal, uterine smooth muscle and inhibits the constrictor response of histamine and acetylcholine on smooth muscle.

Ethanol extract of root of T.cardifolia induced a marked protection against restrain stress induced ulcerization. This activity was comparable to that of diazepam. Antiamoebic effect of crude drug formulation containing T. cardifolia against entamoeba histolytica was also observed.

T. cardifolia is widely used in ayurvedic medicine for the treatment of various ailments. It is reported that extract of Tinospora cardifolia has good immunomodulating effect. It also has the ability to scavenge free radicals and to block free radicals and to inhibit radical induced membrane damage. It also has the hypoglycemic activity and hypolipidemic activity. It also has ability to protect the liver from various diseases. It is found that it is non-toxic in acute toxicity studies. Various types of studies, which have been done on T. cardifolia, reveal that it is an excellent drug, which could be a good remedy for various ailments of animals as well as human beings yet the safety and the potential indications in human beings and animals have to be established using modern techniques **[111].**

1.9 Antibacterial susceptibility test

Antibiotic sensitivity is a term used to describe the susceptibility of bacteria to antibiotics. Antibiotic susceptibility testing (AST) is usually carried out to determine which antibiotic will be most successful in treating a bacterial infection *in vivo*. Testing for antibiotic sensitivity is often done by the Kirby-Bauer method. Small wafers containing antibiotics are placed onto a plate upon which bacteria are growing. If the bacteria are sensitive to the antibiotic, a clear ring, or zone of inhibition, is seen around the wafer indicating poor growth. Other methods to test antimicrobial susceptibility include the Stokes method, E-test (also based on antibiotic

diffusion). Agar and Broth dilution methods for Minimum Inhibitory Concentration determination.

Ideal antibiotic therapy is based on determination of the aetiological agent and its relevant antibiotic sensitivity. Empiric treatment is often started before laboratory microbiological reports are available when treatment should not be delayed due to the seriousness of the disease. The effectiveness of individual antibiotics varies with the location of the infection, the ability of the antibiotic to reach the site of infection, and the ability of the bacteria to resist or inactivate the antibiotic. Some antibiotics actually kill the bacteria (bactericidal), whereas others merely prevent the bacteria from multiplying (bacteriostatic) so that the host's immune system can overcome them **[100].**

1.9.1 Kirby-Bauer antibiotic testing

Kirby-Bauer antibiotic testing (KB testing or disk diffusion antibiotic sensitivity testing) is a test which uses antibiotic-impregnated wafers to test whether particular bacteria are susceptible to specific antibiotics. A known quantity of bacteria is grown on agar plates in the presence of thin wafers containing relevant antibiotics. If the bacteria are susceptible to a particular antibiotic, an area of clearing surrounds the wafer where bacteria are not capable of growing (called a zone of inhibition).

This along with the rate of antibiotic diffusion is used to estimate the bacteria's sensitivity to that particular antibiotic. In general, larger zones correlate with smaller (MIC) of antibiotic for that bacteria. This information can be used to choose appropriate antibiotics to combat a particular infection.

Useful consideration for kirby-bauer antibiotic testing

The culture used in this test has to be the Mueller-Hinton agar because it is an agar that is thoroughly tested for its composition and its pH level. Also, using this agar ensures that zones of inhibitions can be reproduced from the same organism, and this agar does not inhibit sulfonamides. The agar itself must also only be 4mm deep. This further ensures standardization and reproducibility.

The size of the inoculated organism must also be standardized (using barium sulfate standards, McFarland standards). The reasons are because if the size of the inoculum is too small, the zone of inhibition will be larger than what it is supposed to be (the antibiotics will have a distinct advantage) and if the inoculum is too large, the zone of inhibition will be smaller**[115].**

1.9.2 Factors that influence zones of inhibition:

- Concentration of bacteria spread onto agar plate
- Pathogen susceptibility
- Antibiotic diffusion effects
- Agar depth
- Growth temperature
- Nutrient availability
- Drug antagonists

1.9.3 Factors that influence the diffusion of the antibiotic:

- Concentration of antibiotic
- Molecular weight of antibiotic

- Water solubility of antibiotic
- pH and ionization
- Binding to agar

1.9.4 Interpretation of results:

After the plates have been incubated, there should be a noticible “clearing” zone around each of the antibiotic discs. The diameter of each zone should be measured and recorded in millimeters (mm).Each measurement can be compared to a zone-size interpretive chart. Using the chart, the organism can be characterized as being resistant, intermediate or susceptible to the specific antibiotic. Intermediate susceptiblity means that some inhibition from the antibiotic occurred but not sufficiently enough to inhibit the growth of the organism in the body.

Chapter – 2

Literature Review

2. Literature Review:

2.1 Phytochemical Evaluation:

Tinospora cordifolia which is also known as Giloe, belongs to the family Menispermaceae. It is an important medicinal plant used in ayurvedic system of medicine. The stem of the plant is greyish brown-black in colour and bitter in taste. The hydroalcoholic and aqueous extracts of *Tinospora cordifolia* were subjected to qualitative photochemical screening for the detection of phytoconstituents like carbohydrates, alkaloids, proteins, amino acids, tannins, phenolics, saponins, flavonoids, triterpenoids, steroids, glycosides, fixed oils, gums and mucilages. As shown in Table 1, the results revealed the presence of alkaloids, steroids, carbohydrates, glycosides, proteins, saponins, gums and mucilages.

Table-1.1: Qualitative Phytochemical constituents of *Tinospora cordifolia* stem extracts

S. No.	Phytoconstituents	Hydroalcoholic extract	Aqueous extract
1.	**Alkaloids**		
	Dragendroff's test	+	+
	Wagner's test	+	+
	Mayer's test	+	+
	Hager's test	+	+
2.	**Carbohydrates**		
	Benedict's test		
	Fehling's test	+	+
	Molisch test	+	+
	Barfoed's test	-	-
3.	**Glycosides**		
	Legal test	+	+
	Baljet test	+	+
	Borntrager's test	-	-
	Keller Kiliani test	-	-
4.	**Steroids**		
	Libermann Burchard	+	+
	Test Salkowski test	+	+
	Liebermann's test	+	+
5.	**Triterpenoids**	-	-
6.	**Proteins & Amino acids**		
	Biuret test	+	-
	Xanthoprotein test	+	+
	Lead Acetate test	-	-
	Ninhydrin test	+	+
7.	**Fixed oils and Fats**		
	Spot test	+	-
	Saponification test	-	-
8.	**Tannins & Phenolics**		
	Ferric Chloride test	+	+
	Potassium dichromate test		
9.	**Saponins**		
	Foam test	+	+
10.	**Flavonoids**		
	Shinoda test	-	-
11.	**Gums**		
12.	**Mucilages**		
	Ruthenium Red Test	+	+

(+) : Indicates the presence of chemical constituents;
( ) : Indicates the absence of chemical constituents

The plant *Tinospora tomentosa* Miers. (Menispermaceae) is a large deciduous climbing shrub mainly found in the tropical thickets of Bengal and almost throughout India, ascending to an altitude of 1000 meter. It is locally known as ìPadmagulanchaì and has long been used in Ayurvedic medicine.

The powdered plant material was extracted successively with redistilled, analytical grade petroleum ether (40-60^{o}C), chloroform and methanol using Soxhlet apparatus. The solvents were removed under reduced pressure to obtain greenish-yellow (PE), brownish-black (CE) and reddish-brown (ME) colored solid residues (yield 2.9%, 3.4% and 14.1% w/w on dried plant material basis, respectively). Then, (with same plant material obtained after successive extraction with petroleum ether, chloroform and methanol) aqueous extract was prepared by decoction process using double distilled water. Then it was filtered, evaporated and dried under reduced pressure to give solid residue (AE) with yield of 22.5%, w/w on dried plant material basis. Phytochemical investigations were performed on all four extracts which was shown in the following table: **[111].**

Table 1.2: Qualitative phytochemical evaluation of the *Tinospora tomentosa* Miers. Extract

Constituents	Observations			
	PE	CE	ME	AE
Alkaloids	-	+	+	-
Flavonoids	-	-	+	-
Tannins	-	+	+	+
Saponins	-	-	-	+
Sugars	+	+	+	+
Protein	+	-	-	+
Organic acids	+	-	-	-
Glycoside	-	-	-	+

An accurately weighed amount of the ash of the plant was digested with 5 mL of 10% HCl. This was filtered through Whatman No. 41 filter paper and the residue was washed with hot water, cooled and made to volume. The sample solution was then compared in the flame photometer against standard solutions of NaCl, KCl and CaCO3 containing the same amount of HCl. The concentrations of thesodium, potassium and calcium ions were calculated by extrapolation method.

Table-1.3: Determination of calcium, potassium and sodium levels of *Tinospora tomentosa* Miers.

Calcium	Potassium	Sodium
82.65	79.20	UD

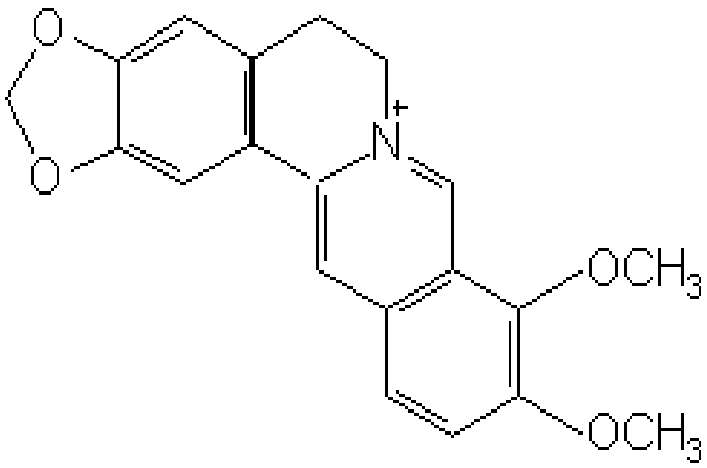
The major phytoconstituent in *Tinospora cordifolia* include tinosporine, tinosporide, tinosporaside, cordifolide, cordifol, heptacosanol, clerodane furano diterpene, diterpenoid furanolactone tinosporidine, columbin and b-sitosterol. Berberine, Palmatine, Tembertarine, Magniflorine, Choline, and Tinosporin are reported from its stem. Tinocordiside, a cadinane

sesquiterpene glycoside consisting of a tricyclic skeleton with a cyclobutane ring, has been isolated and reported from this plant. A new clerodane furano-diterpene has been reported from the stems of *Tinospora cordifolia.* Tinocordifolin, a new daucane-type sesquiterpene, along with tinocordifolioside and N-trans-feruloyl tyramine has been isolated from the stem of the plant.

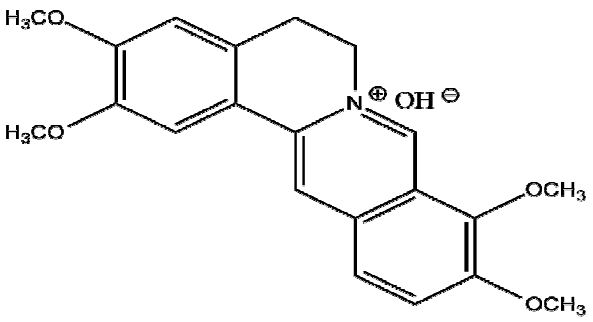
A variety of constituents have been isolated from *Tinospora cordifolia* plant and their structures were elucidated. They belong to different classes such as alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds and polysaccharides. **[103].**

Table-1.4: Different types of alkaloids isolated from various parts of *Tinospora cordifolia*

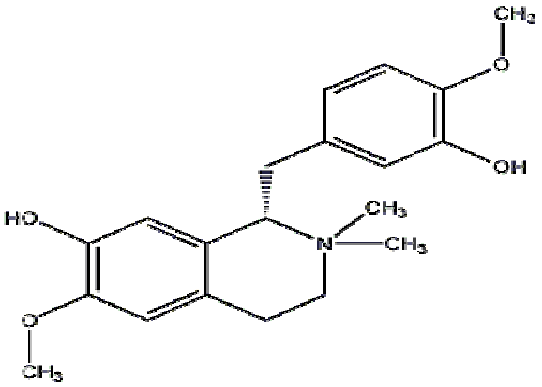
Type of Chemical	**Active principle with references**	**Part in which present**
Alkaloids	Berberine, Palmatine, Tembetarine , Magnoflorine , Choline , Tinosporin, Isocolumbin, Palmatine, Tetrahydropalmatine, Magnoflorine	Stem Root



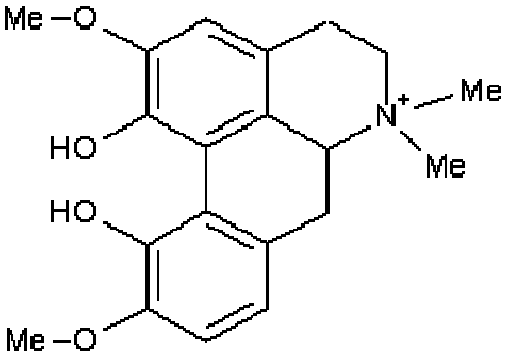
Berberine



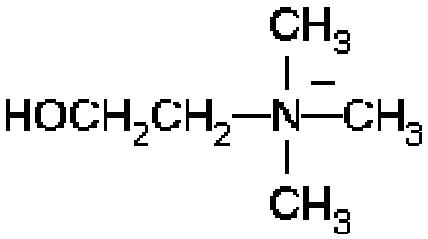
Palmatine



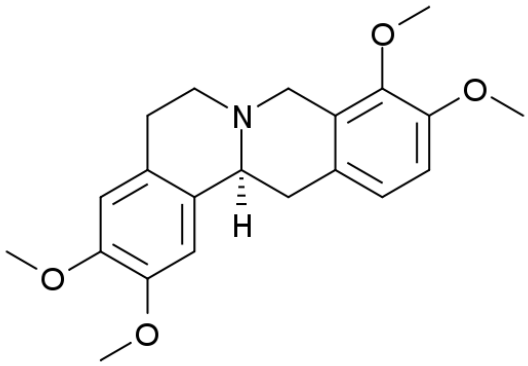
Tembetarine



Magnoflorine



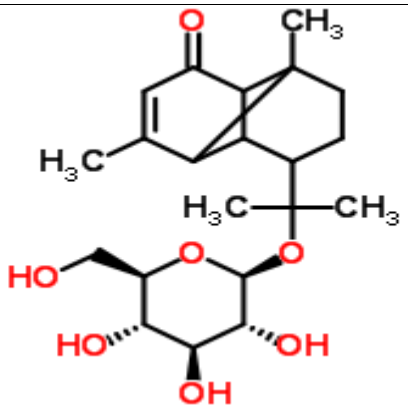
Choline



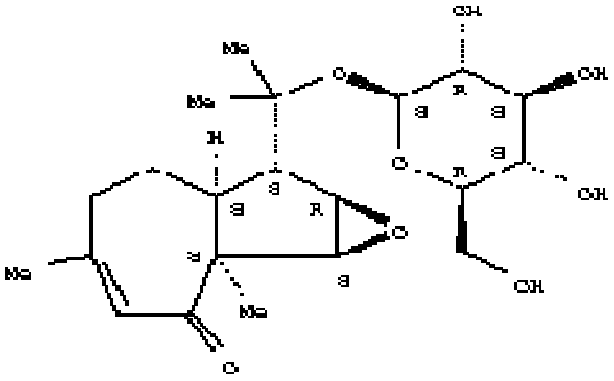
Tetrahydropalmatine

Table-1.5:Different types of Glycosides isolated from various parts of *Tinospora cordifolia*

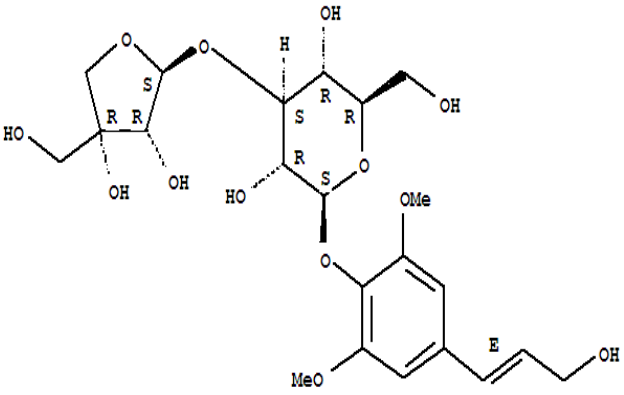
Type of Chemical	Active principle with references	Part in which present
Glycosides	18-norclerodane glucoside, Furanoid diterpene glucoside, Tinocordiside, Tinocordifolioside, Cordioside, Cordifolioside A, Cordifolioside, Syringin, Syringinapiosylglycoside, Palmatosides C, Palmatosides P, Cordifoliside A, Cordiofoliside B, Cordifoliside C, Cordifoliside D, Cordifoliside E	Stem



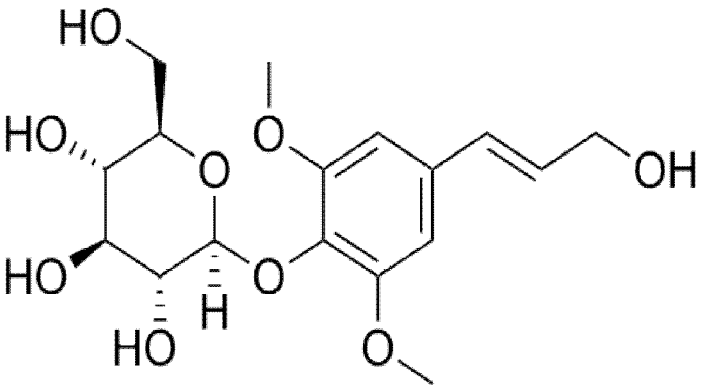
Tinocordiside



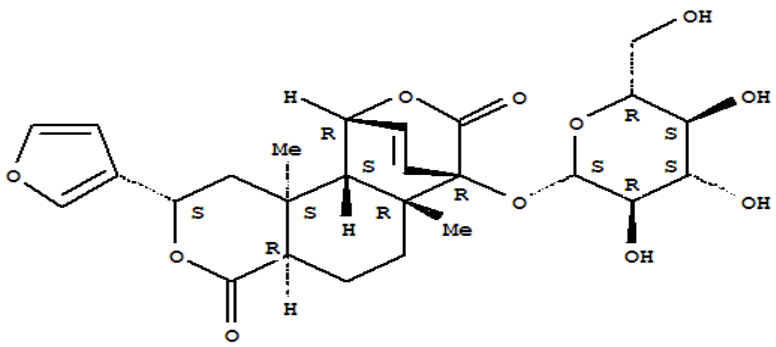
Tinocordifolioside



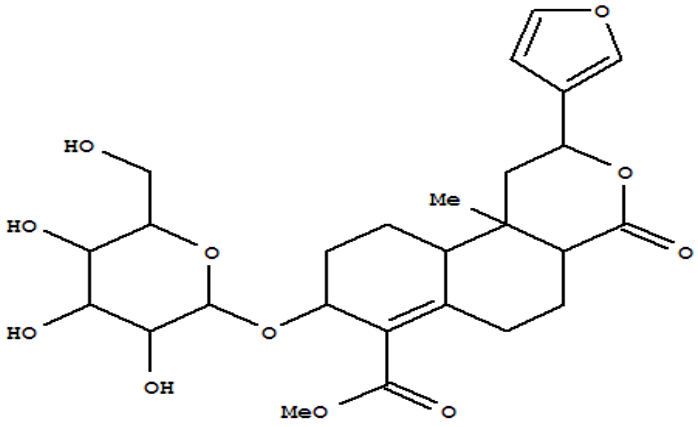
Cordifolioside A



Syringin



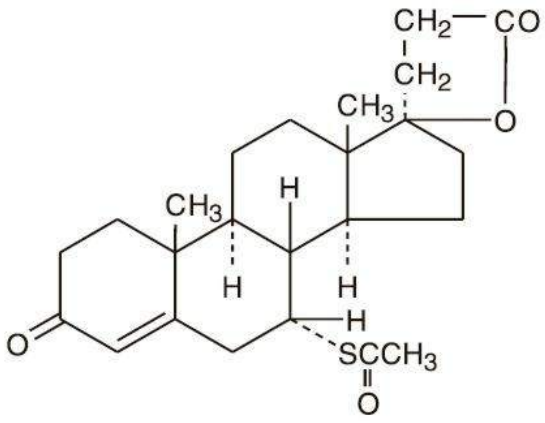
Palmatosides C



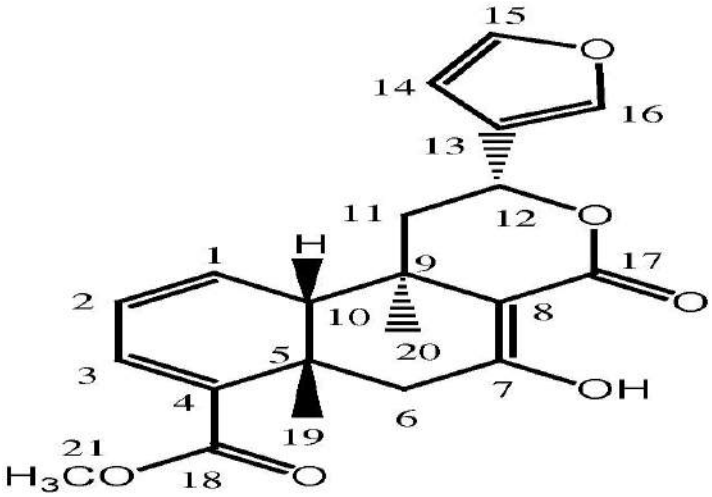
Cordifoliside A

Table-1.6: Different types of Diterpenoid Lactones isolated from various parts of *Tinospora cordifolia*

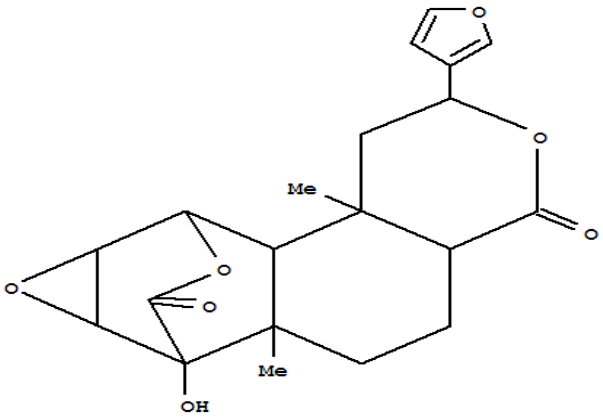
Type of Chemical	Active principle with references	Part in which present
Diterpenoid Lactones	Furanolactone, Clerodane derivatives, 14-dieno-17,12S: 18,1S-dilactone] and Tinosporon, Tinosporides, Jateorine , Columbin	Whole plant



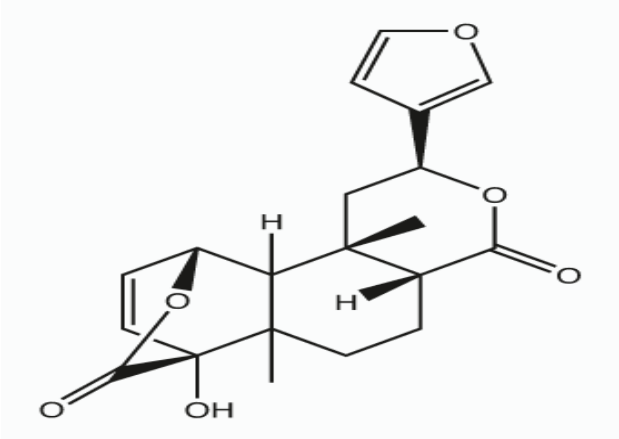
Furanolactone



Clerodane



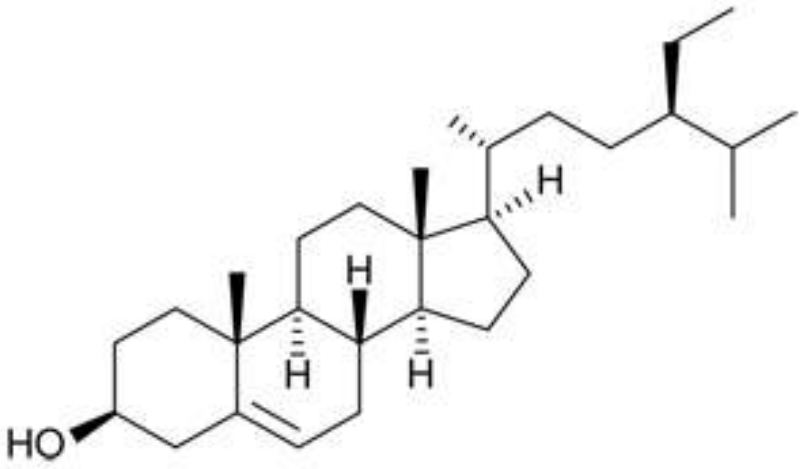
Tinosporides



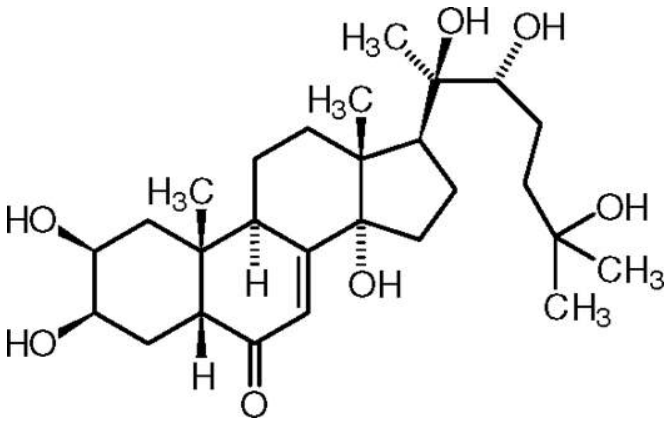
Columbin

Table-1.7: Different types of Steroids isolated from various parts of *Tinospora cordifolia*[114]

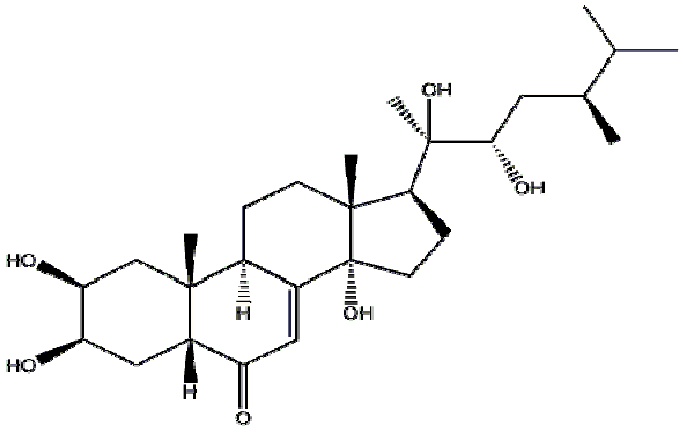
Type of Chemical	Active principle with references	Part in which present
Steroids	β-sitosterol, δ-sitosterol, 20β- hydroxy ecdysone, Ecdysterone, Makisterone A, Giloinsterol.	Aerial part Stem



β-sitosterol



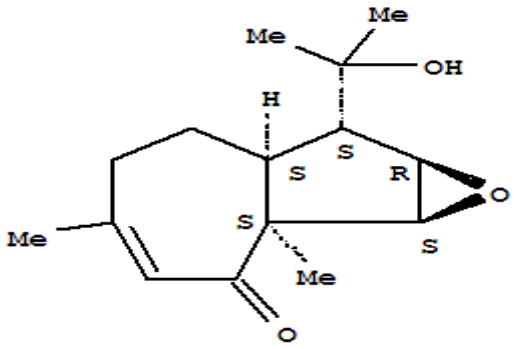
Ecdysterone



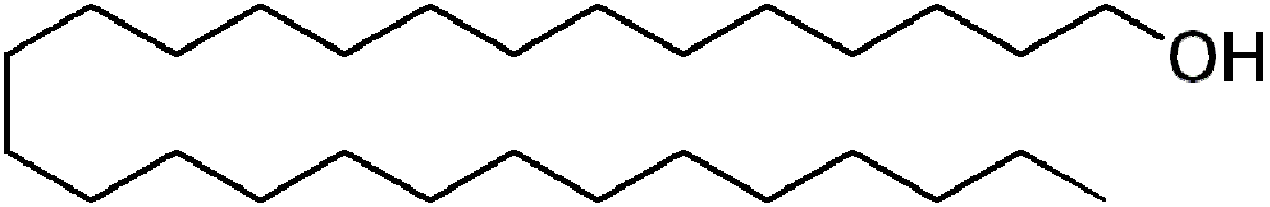
Makisterone A

Table-1.8: Different types of Sesquiterpenoid and Aliphatic compound isolated from various parts of *Tinospora cordifolia*[113]

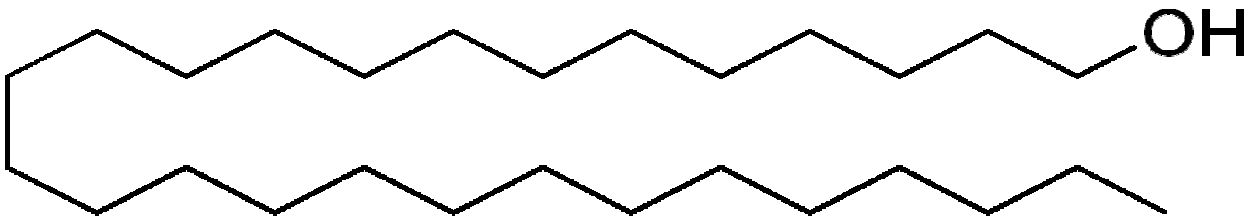
Type of Chemical	Active principle with references	Part in which present
Sesquiterpenoid	Tinocordifolin	Stem
Aliphatic compound	Octacosanol, Heptacosanol, Nonacosan-15-one	Whole plant



Tinocordifolin



Octacosanol

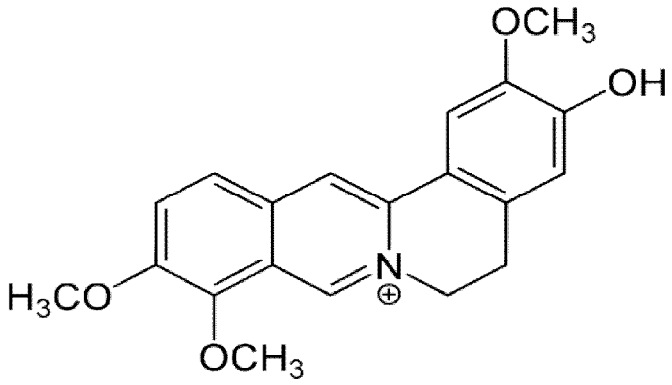


Heptacosanol

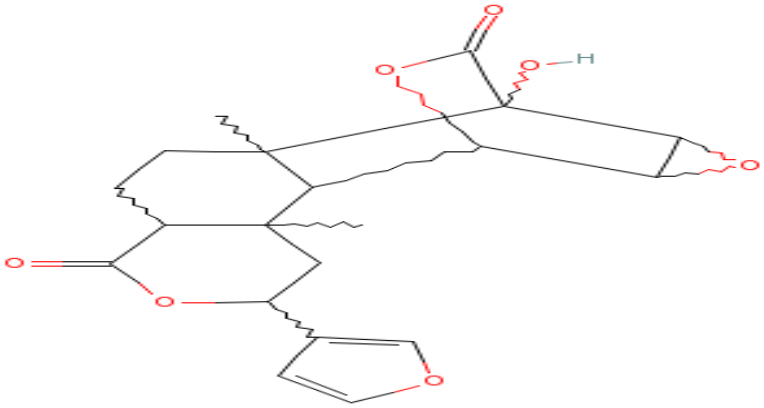
Table-1.9: Different types of Miscellaneous compounds isolated from various parts of *Tinospora cordifolia*[113]

Type of Chemical	Active principle with references	Part in which present
Miscellaneous compounds	3,(α,4-di hydroxy-3-methoxy-benzyl)-4-(4-hydroxy-3-methoxy-benzyl)-tetrahydrofuran,	Whole plant
	Jatrorrhizine,	Root
	Tinosporidine, Cordifol, Cordifelone, N-	Whole plant

	trans-feruloyl tyramine as diacetate, Giloin, Giloinin, Tinosporic acid	



Jatrorrhizine



Tinosporidine

2.2 Pharmacological Review:

Tinospora cordifolia Miers. (Menispermaceae) popularly known as Amrita in Sanskrit, has been used for several centuries in Ayurvedic medicine for the treatment of various ailments. It is an important medicinal plant used in traditional system of medicine. Aphrodisiac potential of this plant has been reported on administration of hydroalcoholic extract of *Tinospora cordifolia* stem

(400 mg/kg body weight) on male wistar albino rats based on the significant increase in number of mounts and mating performance.

The stem of *Tinospora cordifolia* is one of the constituents of several ayurvedic preparations used in general debility, dyspepsia, fever and urinary diseases. The stem is bitter, stomachic, diuretic, stimulates bile secretion, causes constipation, allays thirst, burning sensation, vomiting, enriches the blood and cures jaundice. The extract of its stem is useful in skin diseases. The root and stem of *T. cordifolia* are prescribed in combination with other drugs as

an anti-dote to snake bite and scorpion sting. Dry barks of *T. cordifolia* has anti-spasmodic, antipyretic, anti-allergic, anti-inflammatory and anti-leprotic properties.

The aqueous extract of the stem antagonizes the effect of agonists such as 5-hydroxytryptamine, histamine, bradykinin and prostaglandins E1 and E2 on the rabbit smooth muscle, relaxes the intestinal, uterine smooth muscle and inhibits the constrictor response of histamine and acetylcholine on smooth muscle. Intravenous exposure to aqueous extract of

T. cordifolia in doses of 5.0, 10.0 and 15.0 mg/kg body weight produces a temporary but marked fall in blood pressure and bradycardia in anaesthetized dogs. **[107].**

2.2.1 Anti-hyperglycemic effect:

T. cordifolia is widely used in Indian ayurvedic medicine for treating diabetes mellitus. Oral administration of an aqueous *T. cordifolia* root extract to alloxan diabetic rats caused a significant reduction in blood glucose and brain lipids. Though the aqueous extract at a dose of 400 mg/kg could elicit significant anti-hyperglycemic effect in different animal models, its effect was equivalent to only one unit/kg of insulin.

The anti-hyperglycemic effect of aqueous and alcoholic extracts as well as lyophilized powder of the plant was evaluated in diabetic animals using different doses of diabetogenic agents for

varying duration (21–120 days) so as to assess its effect in mild (plasma sugar>180 mg/dl, duration 21 days), moderate (plasma sugar>280 mg/dl, duration 120 days) and severe (plasma sugar>400 mg/dl, duration 60 days) diabetes mellitus. In the pilot study (mild diabetes), maximum reduction of 70.37% in glucose levels was seen in animals receiving 400 mg/kg per day of aqueous extract after 3 and 15 weeks of treatment. The percent reduction in glucose decreased significantly in the moderate and severe diabetes; 48.81 and 0% at the similar time intervals.

2.2.2 Immuno-modulatory activity:

T. cordifolia is reported to benefit the immune system in a variety of ways. The alcoholic and aqueous extracts of *T. cordifolia* have been tested successfully for immuno-modulatory activity. Pre-treatment with *T. cordifolia* was to impart protection against mortality induced by intra-abdominal sepsis following coecal ligation in rats. It has also significantly reduced the mortality from *E. coli* induced peritonitis in mice. In a clinical study, it has afforded protection in cholestatic patients against *E. coli* infection. These activities are not due to its anti-bacterial activity as shown by the negative in-vitro anti-bacterial activity of the plant extract. It is reported that the treatment in rats had resulted in significant leucocytosis and predominant neutrophilia. It has been also observed that it stimulates the macrophages as evidenced by an increase in the number and % phagocytosis of *S.aureaus* by peritoneal macrophages in rats. Other workers have also supported these observations. The phagocytic and Intra-cellular killing capacity of polymorphs in rats, tested at 3.5 h after *E. coli* infection was significant**[105].**

2.2.3 Anti-stress and Tonic property:

The anti-stress and tonic property of the plant was clinically tested and it was found that it

brought about good response in children with moderate degree of behavior disorders and mental deficit. It has also significantly improved the I.Q. levels.

Tinospora cordifolia Miers. was screened for their putative antistress activity in a battery of experiments. Ethanol extracts of plant at 100 mg/kg exhibited significant antistress activity in all the parameters studied compared with diazepam at 2.5 mg/kg**[102].**

2.2.4 Hepatoprotective activity:

The hepatoprotective action of *T. cordifolia* was reported in one of the experiment in which goats treated with *T. cordifolia* have shown significant clinical and hemato-biochemical improvement in CCl_4 induced hepatopathy. Extract of *T. cordifolia* has also exhibited in vitro inactivating property against Hepatitis B and E surface antigen in 48-72 h.

Effect of *Tinospora cordifolia* extract on modulation of hepatoprotective function in carbon tetrachloride (CCl_4) intoxicated mature rats is reported here. Administration of CCl_4 (0.7 ml/kg body weight for 7 days) produces damage in the liver as evident by estimation of enzymes such as serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transminase (SGPT) and alkaline phosphatase (ALP) as well as serum bilirubin level. However, treatment with *T. cordifolia* extract (100 mg/kg body weight for 15 days) in CCl_4 intoxicated rats was found to protect the liver, as indicated by enzyme level in serum. A significant reduction in serum levels of SGOT, SGPT, ALP, bilirubin were observed following *T. cordifolia* treatment during CCl_4 intoxication[**100].**

2.2.5 Anti-inflammatory activity:

The aqueous extract of *T. cordifolia* exerted a significant anti-inflammatory effect on cotton pellet granuloma and formalin induced arthritis models. Its effect was comparable with Indomethacin and its mode of action appeared to resemble that of a non-steroidal anti-

inflammatory agent. The dried stem of *T. cordifolia* produced significant anti-inflammatory effect in both acute and subacute models of inflammation. *T. cordifolia* was found to be more effective than acetylsalicylic acid in acute inflammation. But in subacute inflammation, the drug was inferior to phenylbutazone. In a clinical evaluation, a compound preparation 'Rumalaya' containing *T. cordifolia* was reported to significantly reduce the pain in patients suffering from rheumatoid arthritis.

Another study shows that it significantly inhibited acute inflammatory response evoked by carrageenin in a doss of 50 mg/100 g given orally and intraperitoneally. In chronic inflammation produced by croton-oil in granuloma pouch technique, 20 mg/100 g of the water extract significantly inhibited granulation tissue response; the reduction in exudative response and increase in the weight of adrenal glands were not significant. A significant inhibition of primary and secondary phases was observed in adjuvant-induced arthritis. A mild analgesic effect of its own as well as potentiation of morphine analgesia were possessed by the extract but it was devoid of antipyretic effect**[107].**

2.2.6 Anti-oxidant action:

The aqueous extract of roots of *T. cordifolia* has shown the anti-oxidant action in alloxan diabetes rats. The administration of the extract of *T. cordifolia* roots (2.5, 50 mg/kg body weight) for 6 weeks resulted in a significant reduction of serum and tissue cholesterol, phospholipids and free fatty acids in alloxan diabetic rats**[104].**

2.2.7 Anti-neoplastic activity:

Administration of *Tinospora cordifolia* stem methanolic extract found to significantly increase humoral immune response, as seen from the increase in plaque-forming cells in the spleen (1575 PFC/10^6 spleen cells) and circulating antibody titre (256), and to produce an enhancement

(129%) in macrophage activation. *Tinospora* extract reduced solid tumour growth and synergistically acted with cyclophosphamide in reducing (83%) the animal tumours.

Jagetia *et al*., have found that guduchi killed the HeLa cells very effectively *in vitro* and thus it indicates that guduchi needs attention as an anti-neoplastic agent. In this study exposure of HeLa cells to 0, 5, 10, 25, 50 and 100 mg/ml of guduchi extract (methanol, aqueous and methylene chloride) resulted in a dose dependent but significant increase in cell killing when compared to non drug treated controls.

2.2.8 In Urinary tract infection:

'Septilin' syrup, a compound preparation containing *T. cordifolia* (7.82% in 5 ml of syrup) was found to elicit good clinical response in children suffering from upper respiratory tract infection and chronic otitis media.

2.2.9 Radioprotective potential of extract:

A preparation of *Tinospora cordifolia* administered i.p. (200 mg/kg b.w.) to a strain male mice 1 h before whole body gamma-irradiation was evaluated for its radioprotective efficacy in terms of whole body survival, spleen colony forming units (CFU), hematological parameters, cell cycle progression, and micronuclei induction. Preirradiation treatment with *Tinospora cordifolia* rendered 76.3% survival (30 days), compared to 100% mortality in irradiated control and prevented radiation induced weight loss. On 10th postirradiation day, the endogenous CFU counts in spleen were decreased with increasing radiation doses 12.0 (5 Gy), 2.16 (7.5 Gy) and 0.33 (10 Gy) but pre-irradiation administration of 200 mg/kg b.w. of RTc increased CFU counts to 31.16, 21.83 and 3.00 respectively. Pre-irradiation *Tinospora cordifolia* treatment could restore total lymphocyte counts (TLC) by the 15th day to normal. It also increased the S-phase cell population that was reduced following 2 Gy irradiation in a time dependent manner. 2 Gy

irradiation-induced micronuclei were also decreased by a pre-irradiation administration of RTc from 2.9 to 0.52%[**103]**

2.2.10 Anti-allergic efficacy:

The efficacy of *Tinospora cordifolia* extract in patients of allergic rhinitis was assessed in a randomized double blind placebo controlled trial. Seventy-five patients were randomly given either *Tinospora cordifolia* or placebo for 8 weeks. At the end of trial baseline investigations were repeated, drug decoded and results analyzed. With *Tinospora cordifolia* treatment 100% relief was reported from sneezing in 83% patients, in 69% from nasal discharge, in 61% from nasal obstruction and in 71% from nasal pruritus. In placebo group, there was no relief in 79% from sneezing, in 84.8% from nasal discharge, in 83% from nasal obstruction, and in 88% from nasal pruritus. The difference between *Tinospora cordifolia* and placebo groups was highly significant. After *Tinospora cordifolia*, eosinophil and neutrophil count decreased and goblet cells were absent in nasal smear. After placebo, decrease in eosinophil and neutrophil count was marginal and goblet cells were present. TC significantly decreased all symptoms of allergic rhinitis. Nasal smear cytology and leukocyte count correlated with clinical findings. *Tinospora cordifolia* was well tolerated[**106].**

2.2.11 Anti-ulcer activity:

The ethanol extracts of the roots of T. cordifolia Miers was observed to induce a marked protective action against an 8 h restraint stress induced ulcerization, the activity being comparable to that of diazepam**[101].**

2.2.12 Cardioprotective activity:

The present study was designed to investigate the effects of pretreatment with alcoholic extract of *Tinospora cordifolia* in an *in vivo* rat model. The model adopted was that of surgically-

induced myocardial ischemia, performed by means of left anterior descending coronary artery occlusion (LAD) for 30 min followed by reperfusion for another 4 h. Infarct size was measured by using the staining agent TTC (2,3,5-triphenyl tetrazolium chloride). Lipid peroxide levels in serum and in heart tissue were estimated spectrophotometrically by the methods developed by Yagi and Ohkawa *et al.* respectively. A lead II electrocardiogram was monitored at various intervals throughout the experiment. A dose dependent reduction in infarct size and in lipid peroxide levels of serum and heart tissue were observed with the prior treatment of *T. cordifolia* with various doses for 7 d compared to control animals. Hence, the present study suggests the cardioprotective activity of *T. cordifolia* in limiting ischemia-reperfusion induced myocardial infarction. **[113].**

2.2.13 Antifertility effect:

Oral administration of 70% methanolic extract of *T. cordifolia* stem to male rats at the dose level of 100 mg/rat/day for 60 days did not cause body weight loss but decreased the weight of testes, epididymis, seminal vesicle and ventral prostate in a significant manner. Sperm motility as well as sperm density were reduced significantly which resulted in reduction of male fertility by 100%. The stem extract brought about an interference with spermatogenesis. The round spermatids were decreased by 73.12%. However, the population of preleptotene and pachytene spermatocytes were decreased by 47.60% and 52.85% respectively, followed by secondary spermatocytes (48.10%). Leydig cell nuclear area and mature Leydig cell numbers were significantly reduced when compared with controls. Serum testosterone levels showed significant reduction after *Tinospora* extract feeding. Seminiferous tubule diameter, Leydig cell nuclear area as well as cross sectional surface area of Sertoli cells were reduced significantly when compared to controls. Biochemical parameters i.e. protein, sialic acid, glycogen contents of

testes decreased significantly. Seminal vesicular fructose also depleted whereas, testicular cholesterol was elevated significantly followed by a reduction in testosterone levels. These results suggested antifertility effects of the stem extract of *T. cordifolia* in male rats[**107].**

2.2.14 In stomach worm:

A number of natural products are commercially available in Bangladesh most of which are used as feed additives, though few have antibacterial and anticoccidial use. Ethanol extracts of Padmagulancha (Tinospora tomentosa) were highly effective against common stomach worm Haemonchus contortus in both in vitro and in vivo studies. This presentation will cover the details of the currently used natural products in Bangladesh and our efforts in revealing the greatness of these natural products.

2.2.15 Anti-parasitic activity:

The *in vivo* antimalarial effect of crude extract of *Tinospora crispa*, a Thai traditional medicine plant. Mice were inoculated with *Plasmodium yoelii* then treated with the crude extract of *Tinospora crispa* at doses of 20, 40 and 80 mg/kg. Mice receiving the dose of 20 mg/kg died on average on Day 8. Mice remained alive longer when treated of the dose of 40 mg/kg or even longer under the treatment of the dose of 80 mg/kg. Surprisingly and interestingly, one mouse from the group in which the dose of 80 mg/kg was administrated is still alive and the parasite was cleared from the blood stream. In conclusion, *T. crispa* has an *in vivo* antimalarial effect in dose dependent manner.

2.2.16 Antiosteoporatic potential of extract:

Present animal studies were conducted to investigate the potential of *Tinospora cordifolia* ethanolic stem extract as an antiosteoporotic agent. Three-month-old female Sprague-Dawley rats were either ovariectomized (ovx) or sham operated and treated with vehicle (benzyl

benzoate:castor oil; 1:4), E(2) (1 microg/day) or *Tinospora cordifolia* (10, 50, 100 mg/kg b.wt) subcutaneously for 4 weeks. At the end of experiment bone mineral density of tibiae was measured by quantitative computer tomography. Serum was analyzed for the activity of alkaline phosphatase and levels of osteocalcin, cross-laps and lipids. Uterus and mammary gland were processed for histological studies. Ovx rats treated with *Tinospora cordifolia* (10 mg/kg b.wt) showed an osteoprotective effect as the bone loss in tibiae was slower than ovx controls. Serum osteocalcin and cross-laps levels were significantly reduced. All the above effects of *Tinospora cordifolia* were much milder than those produced by E(2). Alkaline phosphatase activity was higher in *Tinospora cordifolia* treatment groups. Total cholesterol and LDL levels remained unaltered but HDL levels were significantly lowered with *Tinospora cordifolia* (50 mg/kg b.wt) treatment. Uterus and mammary gland showed no signs of proliferation after treatment with *Tinospora cordifolia* extract. *Tinospora cordifolia* extract showed estrogen like effects in bone but not in reproductive organs like uterus and mammary gland. Thus, this study demonstrates that extract of *T. cordifolia* has the potential for being used as antiosteoporotic agent.

2.2.17 Antileishmanial potential:

The chemotherapeutic interventions against visceral leishmaniasis are limited and facing serious concerns of toxicity, high cost, and emerging drug resistance. There is a greater interest in new drug developments from traditionally used medicinal plants which offers unprecedented diversity in structures and bioactivity. With this rationale, ethanolic extract of *Tinospora sinensis* Linn and its four fractions were tested in vitro against promastigotes and intracellular amastigotes and in vivo in *Leishmania donovani* infected hamsters. Ethanolic extract exhibited an appreciable activity against promastigotes (IC_{50} 37.6 ± 6.2 μg/ml) and intracellular amastigotes (IC_{50} 29.8 ± 3.4 μg/ml). In hamsters, it resulted in $76.2 \pm 9.2\%$ inhibition at 500 mg/kg/day × 5

oral dose level. Among fractions, *n*-butanol imparted highest in vitro and in vivo activities. Ethanolic extract and butanol fraction also enhances reactive oxygen species (ROS) and nitric oxide (NO) release. The results indicate that *T. sinensis* may provide new lead molecules for the development of alternative drugs against visceral leishmaniasis.

2.2.18 Antimicrobial activity:

In this investigation, the antibacterial modes of action of Radix Tinosporae, its major single components, and nine antibiotics with different targets or modes-of-action on Staphylococcus aureus were studied. Metabolic profiles of cultures treated with different medicines were acquired by HPLC/ESI-MS. After HPLC-MS data pretreatment, those profiles acquired were reduced into several MS vectors. Then statistical processing by principal components analysis was carried out upon those vectors, two conclusions could be drawn: (1) the antibacterial mode of action of Radix Tinosporae is similar to that of rifampicin and norfloxacin, which act on nucleic acid; (2) its active components playing main antimicrobial roles on Staphylococcus aureus might be alkaloids, such as palmatine and jatrorrhizine. **[97].**

2.3 Tinospora tomentosa

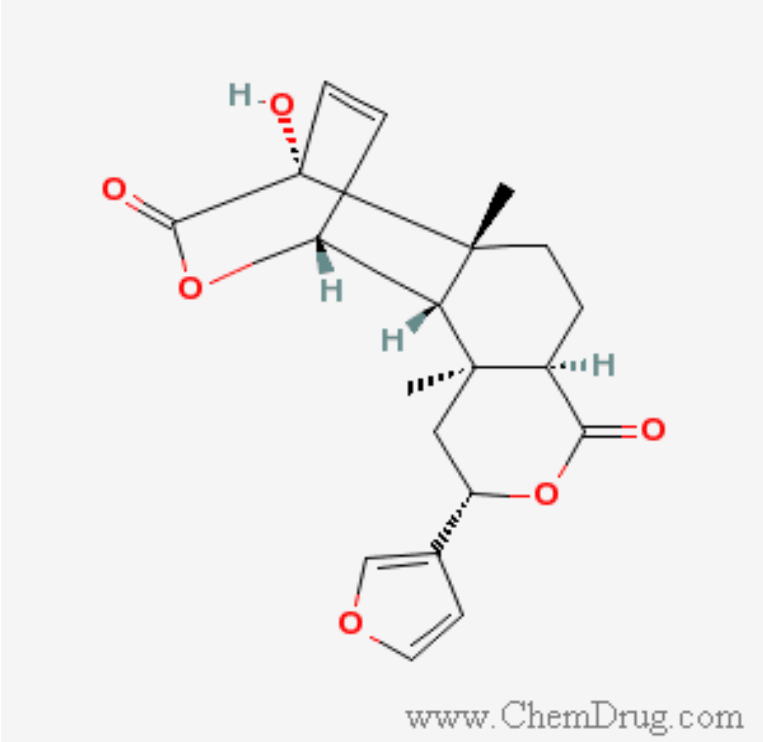
2.3.1 Phychemical Constituents

The main important chemical constituent of the plant are tinosporin, perberillin, palmarin, berberine, tinosporon, hepta consol tinosporic acid adntinosporol. The fresh stem bark yield giloin, giloinin and gilosterol. Hypoglycaemia agent and phenolic lignin have also been isolated from this plant.

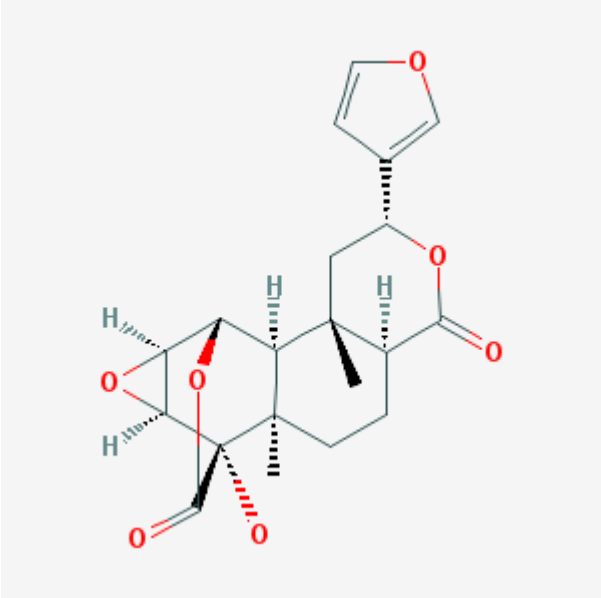
Table 2.1: Chemical constituent of Plant

Chemical Constituents	**Part from which it is isolated**
Tinosporin	Leaf

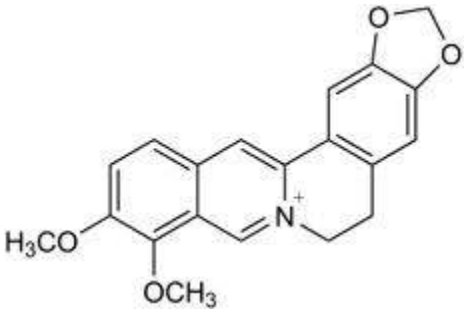
Perberillin	Leaf
Palmarin	Leaf
Berberine	Leaf
Tinosporon	Leaf
Hepta consol tinosporic acid adntinosporol	Leaf
Giloin	Fresh stem bark
Giloinin	Fresh stem bark
Gilosterol	Fresh stem bark
Phenolic lignin	Fresh stem bark



Structure of tinosporin



Structure of palmarin



2.3.3 Medicinal uses:

• Skin diseases: Juice taken with neem, haldi and amla is very effective.

• Piles: Juice of Tinospora with butter milk is useful.

• Breast milk: Decoction of the stem is given to improve the quality of breast milk.

• Toxins: It is considered a best herb for clearing microcirculatory system. Its juice is very effective in removing both exogenous and endogenous toxins. It clears out the brain toxin that

inhibits mental function.

• Asthma: The root and bark with whey is used in the treatment of respiratory troubles particularly in asthma.

• Diabetes: Juice is taken in high quantities.

• Excessive bleeding during menstruation, bleeding after abortion or delivery: Stem, leaves and roots can be used. About 5 g each of leaves and roots are crushed together to extract the juice. Consume 2 cup of this juice for a few days after diluting it with water (2 to 3 ml in half a cup of water) till the condition improve.

• Malaria and other fever : Decoction of the stem with pipli (Piper longum) and honey is taken.

• Indigestion: The juice with honey or the paste of leaves can be given with butter milk.

• Conjuctivitis and cataract : In some parts of India the juice is applied inside the eyes.

2.3.2 Other uses:

It is used in cancer prevention, cancer treatment support, high cholesterol and liver protection. It is used as strong anti-aging factor. Many natives use the fruits of Tinospora in face care. It has been used to treat convalescence from severe illness, arthritis, food allergies and anemia. According to some herbalists, Tinospora has adaptogen effects, a term that indicates it helps the body to adopt to stress. In children it is used in general debility, digestive disturbance, loss of appetite and fever.

Chapter 3:

Preparation of plant extract for experiment

3.1 PLANT SELECTION

There are thousands of medicinal plants in Bangladesh and in this Indian subcontinent. Among these plants it was not easy to select a few plants for the research purpose. The selection of plant greatly affects the research work if there is carelessness takes place. Plant secondary metabolites often accumulate in specific plant parts. Thus, unless it is already known which parts contain the highest level of the compounds of interest, it is important to collect multiple plant parts, or the whole plant to ensure the extracts prepared representative of the range of secondary metabolites. For drug discovery from plants, sample may be selected using a number following criteria by which the research work will run smoothly.

From the literature review it is seen that there are lots of work on the plant *Tinospora cordifolia* about the pharmacological activity of the plant. But there is a least of work has been found about chemical investigation of this plant, especially about the stems of this plant. So I got a chance to select the stems of *Tinospora tomentosa Miers* for my research work to see whether the leaves have antidiabetic and antimicrobial activity or not.

3.2 PLANT COLLECTION

After selection of plant it is must to collect the plant parts for the research purpose. Throughout Bangladesh the plant *Tinospora tomentosa Miers* is available. From the district Brahmanbaria, Chittagong, Bangladesh the plant stems were collected.

3.3 PLANT IDENTIFICATION

It is advisable to attempt field identification of sample collected. To aid taxonomic experts in confirming the field identification and to get a permanent scientific record (accession number) a voucher specimen was prepared on 20th December, 2011. In the voucher specimen the dried leaves of sample plant were attached and some information like local name, medicinal use,

location of the sample plant were also written on that voucher specimen. Finally, from BNH (Bangladesh National Herbarium) I got the identification or accession number of collected sample on 12th May, 2012, and the accession number is 36559 with *Tinospora tomentosa Miers* and minosporaea scientific name and family name of the plant respectively.

3.4 DRYING OF PLANT SAMPLE

After the collection of sample it needs to be dried to make the sample extract. In general the plant material should be dried at temperature below 30^0C to avoid the decomposition of thermolabile compounds. So sun drying can be very effective but drawback is sometimes water molecules are absorbed by the sample and hence microbial growth can affect the phytochemical study. The stems were dried in the sun light thus chemical decomposition can not take place.

3.5 GRINDING OF DRIED SAMPLE

Small amount of plant material can be milled using grinder or blender. But if the sample is in high amount then it is easier to get powdered sample by grinding from a spice mill. Grinding improves the efficiency of extraction by increasing surface area. It also decreases the amount of solvent required for the extraction. The dried samples were ground to coarse powder with a mechanical grinder (Blender) and powdered samples were kept in clean closed glass containers pending extraction. During grinding of sample, the grinder was thoroughly cleaned to avoid contamination with any remnant of previously ground material or other foreign matter deposited on the grinder.

3.6 MACERATION OF DRIED POWDERED SAMPLE

3.6.1 Principle

The choice of extraction procedure depends on the nature of the plant material and the components to be isolated. The principle of solid-liquid extraction is that when a solid material

comes in contact with a solvent, the soluble components in the solid material move to the solvent. Thus, solvent extraction of plant material results in the mass transfer of soluble active principle (medicinal ingredient) to the solvent, and this takes place in a concentration gradient. The rate of mass transfer decreases as the concentration of active principle in the solvent increases, until equilibrium is reached, i.e. the concentrations of active principle in the solid material and the solvent are the same. Thereafter, there will no longer be a mass transfer of the active principle from plant material to the solvent. Since mass transfer of the active principle also depends on its solubility in the solvent, heating the solvent can enhances the mass transfer. Moreover, if the solvent in equilibrium with the plant material is replaced with fresh solvent, the concentration gradient is changed**[108].**

3.6.2 Procedure

After getting the sample as dried powdered, the sample (100 Gram) was then soaked in 1000mL of methanol for five days, the process is known as maceration technique. A glass made jar with plastic cover was taken and washed thoroughly with methanol and dried. Then the dried powder sample was taken in the jar. After that methanol (600mL) was poured into the jar up to 1-inch height above the sample surface as it can sufficiently cover the sample surface. The plastic cover with aluminium foil was closed properly to resist the entrance of air into the jar. This process was performed for five days. The jar was shaked in several times during the process to get better extraction.

3.7 FILTRATION OF THE EXTRACT

After the extraction process the plant extracts was filtered with sterilized cotton filter. The cotton was rinsed with methanol and fitted in a funnel. The filtrate was collected in a beaker. Then again it was filtered and this time What man's filter was used for getting more clear

extract which would be useful making the sample more concentrate in Rotary Evaporation Technique. Then the filtrate was taken into a volumetric flask and covered with alumina foil paper and was prepared for rotary evaporation.

3.8 SAMPLE CONCENTRATION BY ROTARY EVAPORATION TECHNIQUE

3.8.1Principle

A rotary evaporator is a device used in chemical laboratories for the efficient and gentle removal of solvents from samples by evaporation. Rotary evaporators are also used in molecular cooking for the preparation of distillates and extracts. A rotary evaporator consists of following parts-

- A motor unit that rotates the evaporation flask or vial containing the user's sample.
- A vapor duct that is the axis for sample rotation, and is a vacuum-tight conduit for the vapor being drawn off of the sample.
- A vacuum system, to substantially reduce the pressure within the evaporator system.
- A heated fluid bath (generally water) to heat the sample.
- A condenser with either a coil passing coolant, or a "cold finger" into which coolant mixtures such as dry ice and acetone are placed.
- A condensate-collecting flask at the bottom of the condenser, to catch the distilling solvent after it re-condenses.
- A mechanical or motorized mechanism to quickly lift the evaporation flask from the heating bath**[109].**

Figure 3.1: Rotary Evaporator in EWU Laboratory (IKA ®RV05 Basic, Biometra)

3.8.2 Affecting Factors

There are following factors, omission of one of the following factor may interfere the sample concentration procedure and thus which may interfere the phytochemical investigation. Remove the flask from the heat bath.

- Opening the stopcock
- Halting the rotor
- Turning off the vacuum/aspirator
- Disconnecting the flask
- Dropping flask in heat bath.

3.8.3 Procedure

After the filtration process two parts were obtained namely ‘residue part’ and ‘filtered part or filtrate’. The filtered part, which contains the substance soluble in methanol, was putted into a1000ml round bottom flask (BOROSIL), and then the flask was placed in a rotary evaporator. The evaporation was done at 45 degree Celsius temperature. The number of rotation per minute

was selected as 130 RPM. The pressure of the vacuum pumper machine (Biometra) was 6bar. The water flow through the distillation chamber was also provided in a satisfactory flow rate. When the evaporation seemed to be satisfactory, then the methanolic extract was collected in a 50mL beaker. The evaporator flask was rinsed by diethyl ether. Then the beaker was covered with aluminum foil paper and kept on the water bath for 60 minutes and the temperature of water bath maintained as 50^0C. Finally the concentrated methanolic plant extract was found and stored in the laboratory refrigerator from which the extract was used for many chemical investigations.

3.9 Ethylacetate extract:

3.9.1 Procedure

Now we can use the remaining dried plant part. After that we took 200 gm sample and 800 ml ethylacetate which was then putted into a1000ml round bottom flask (BOROSIL), and then the flask was placed in a rotary evaporator. The evaporation was done at 45 degree Celsius temperature. The number of rotation per minute was selected as 130 RPM. The pressure of the vacuum pumper machine (Biometra) was 6bar. The water flow through the distillation chamber was also provided in a satisfactory flow rate. When the evaporation seemed to be satisfactory, then the ethyl acetate extract was collected in a 50mL beaker. The evaporator flask was rinsed by diethyl ether. Then the beaker was covered with aluminum foil paper and kept on the water bath for 60 minutes and the temperature of water bath maintained as 50^0C. Finally the concentrated ethyl acetate plant extract was found and stored in the laboratory refrigerator from which the extract was used for further chemical investigation.

Chapter 4:

Method and material

4.1 CHEMICALS AND OTHER REAGENTS

Sulfuric acid, Folin Ciocalteu reagent, prolein amino acid (protein), 1-butanol, glacial acetic acid, Ninhydrine solution, Glucose, Galactose, Maltose, Lactose, Acetone, Phosphate buffer, Anisaldehyde, deionized water, Gallic acid, Sodium nitrite, Normal saline, Wagner's reagent, Hydrochloric acid, Glacial acetic acid, Ammonia, Phoshomolybdic acid, Acetic anhydride, Alcoholic ferric chloride, 5-aqua copper sulphate, Sodium potassium tartrate.

4.2 EQUIPMENTS AND OTHER NECESSARY TOOLS

In case of the extraction procedure and for various phytochemical tests many equipments and materials were used. Some of them are analytical balance, beaker (in various size), pipette, micro-pipette, rotary evaporator, hot air oven, dryer, storage cabinet, spatula, test tube, volumetric flask, conical flask, test tube holder, test tube rack, aluminum foil paper, scotch tape, refrigerator, water bath, electronic shaker, ultra violate lamp, mask, gloves, lab coat, sprayer, reagent bottle, TLC plate, TLC tank, scale, pencil, TLC plate cutter, capillary tube, mortar and pestle, laminar air flow cabinet, loop, burner, micropipette tip, petri dishes, glass rod, cotton, filter paper, funnel, hot plate, centrifugal machine, autoclave, glassware washers, stirrer, UV spectroscopy, knife, ephedrine tube, Whatman’s filter paper, paper disc, incubator, vortex machine, P^H meter.

4.3 SOLVENTS FOR EXPERIMENTS

Dimethylsulfoxide(DMSO), Acetone, Chloroform, Distilled water, Ethanol, Methanol, Diethyl ether, Acetic acid, n-Hexane, Ethyl acetate, Dichloromethane, Benzene, Ammonium hydrooxide, Formic acid.

4.4 THIN LAYER CHROMATOGRAPHY (TLC)

4.4.1 Principle

The extracts were analyzed by performing TLC to determine the composition of each extract. TLC was done under three solvent systems. Successively the polarity of the solvent systems was increased to get a clear graph of all the possible compounds present in the extract. Most organic solvents are nonpolar in nature and so organic compounds such as benzene was used to elute the non polar compounds present in the extract. Again, since Alkaloid and terpenoid type compounds are basic in nature, ammonium base is used in the solvent system to help them run over TLC plate**[44].**

Table 4.1: The compositions of various solvent systems for TLC

Nonpolar Basic solvent	**Intermediate polar Basic Solvent**	**Polar Basic solvent**
Benzene 9Ml Ethanol 1mL Ammonium hydroxide 0.1mL	Chloroform 5Ml Ethyl acetate 4mL Formic acid 1mL	Ethyl acetate 8mL Ethanol 1.2mL Water 0.8mL

4.4.2 Apparatus

17. TLC tank
18. Pencil
19. Scale
20. UV-lamp
21. Spray bottle
22. Heat gun
23. Petri dish
24. Capillary tube

4.4.3 Reagents

41. Benzene
51. DPPH

42. Ethanol
43. Ammonium hydrooxide
44. $CHCl_3$
45. Formic acid
46. Ethyl acetate
47. EtOH
48. Water
49. Nutrient agar
50. H_2SO_4
52. Folin & ciocalteu solution
53. 1-butanol
54. Glacial acetic acid
55. 2% ninhydrine solution
56. Acetone
57. Phosphate Buffer
58. Methanol
59. N-Hexane
60. Standard flavonoid solution

4.4.4 Procedure

- Firstly, above three solvent systems were prepared.
- In the next step, TLC plates were prepared. For this nine TLC plates were prepared, three for each solvent system to run with. The TLC plates were labeled in the following maner:

  1-C, 1-D, 1-F

  2-C, 2-D, 2-F

  3-C, 3-D, 3-F

  Where,

  1 denoted run with nonpolar solvent system, 2 denoted run with intermediate polar solvent system, 3 denoted run with polar solvent system and C denoted treatment with 10% Sulphuric acid, D denoted treatment with 0.04%DPPH solution, F denoted treatment with 10% Folin ciocalteu solution**[44].**

- On each plate four spots were spotted. The spotting patterns were as follows:

  1st spot was spotted with Solvent; in this case methanol was used.

  2nd & 3rd spots were spotted with the extracts of shefali leaf.

  4th spot was the control, spotted with standard flavonoid solution

- After spotting the respective TLC plate was exposed to the respective solvent system. Like all plates that were marked 1 were run with nonpolar solvent system, those that were marked 2 were run with intermediate polar solvent system and the 3 marked plates run with polar solvent system.
- Upon completion of TLC, the plates were exposed to reagent for compound detection and identification. For this the C marked plates were exposed to 10% sulphuric acid solution, dried and then heated to 80-90^0 C for charring purpose. This helped in the fixation of spot and allowed it to be prominently visible.
- Likewise, the D marked plates were dipped into 0.04% DPPH solution and dried while keeping in a dark place.
- The F marked plates were washed with Folin ciocalteu reagent and dried**[44].**

4.5 Total Phenolic Assay

4.5.1 Principle

The content of total phenolic compounds in plant methanolic extracts was determined by Folin–Ciocalteu Reagent (FCR). The FCR actually measures a sample's reducing capacity. The exact chemical nature of the FC reagent is not known, but it is believed to contain heteropolyphosphotunstates - molybdates. Sequences of reversible one- or two-electron reduction reactions lead to blue species, possibly $(PMoW_{11}O_{40})^{4-}$. In essence, it is believed that

$$Mo\ (VI) + e \rightarrow Mo\ ()$$

the molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reductants and Mo (VI):

4.5.2 Apparatus

Test tube

Spatula

Vortex

Pipette

Analytical balance

Uv-spectrophotometer

4.5.3 Reagents

Folin-Ciocalteu

deionized water

Na_2CO_3

Ethanol or Methanol

Galic acid (Analytical or Reagent grade)

4.5.4 Procedure

- 0.3 ml of plant extract 20mg/ml or standard of different concentration solution was taken in a test tube.
- 0.6 ml of Folin – ciocalteu reagent solution was added into the test tube.
- 6ml water & 4 ml of Sodium carbonate(15%) solution was added into the test tube.
- The test tube was incubated for 120 minutes at 25^0C to complete the reaction in a dark place.
- Then the absorbance of the solution was measured at 765 nm using a spectrophotometer against blank.
- The Total content of phenolic compounds in plant methanolic extracts in gallic acid equivalents (GAE) was calculated by the following formula equation

$$C = (c \times V)/m$$

Where:

C = total content of phenolic compounds, mg/g plant extract, in GAE;

c = the concentration of gallic acid established from the calibration curve, mg/ml;

V = the volume of extract, ml;

m = the weight of pure plant methanolic extract, g.

$y = 0.0162x+0.0215$, $R^2=0.9972$.

PROTEIN ISOLATION & DETECTION

4.6.1 Protein (lectin) Isolation

Dried padmaguruj powder was soaked in 0.15N NaCl solution in the ratio 1:10 at 4°C for 72 hours. Then the resulting mixture was filtered & the filtrate was centrifuged at 4°C temp by 9000 rpm for 30 min. Then the upper fraction was carefully collected. The supernatant was treated with ammonium sulphate (100% saturation) to recover total protein. The protein precipitate was collected by filtration method which was then preserved at -80°C temp.

4.6.2 Protein Detection by TLC

4.6.2.1 Apparatus

TLC plate

TLC tank

Pencil

UV lamp

Capillary tube

4.6.2.2 Reagents

Prolein amino acids	Water
1-butanol	Ethanol
Glacial acetic acid	2% ninhydrine

4.6.2.3 Procedure

- A TLC plate was taken and to both of its ends lines were drawn using pencil at 1cm from the edge. The bottom line was for spotting the samples, while the upper edge line was solvent line.
- To the bottom line two sample spots were made using capillary tube. One was the positive control having prolein amino acid and the other another was sample extracts. All the spots were labeled using pencil and they equidistant apart from each other.
- The TLC tank was saturated with mobile phase having the composition of 1-butanol, glacial acetic acid and water in the ratio of 4:1:1 respectively.
- To the saturated tank the previously spotted plate was placed and the mobile phase was allowed to run through the spots until the solvent line was reached.
- The plate was then taken out of the tank, dried and then visualized under UV light in dark room.
- After marking the florescent compounds, the plate was sprayed with 2% ninhydrine in ethanol solution, dried and then heated using heat gun to make the protein or amino acid component spots visible**[45].**

4.6.3 Protein Quantification by Lowry Method

4.6.3.1 Principle

A standard curve of absorbance as a function of initial protein concentration was made by preparing solution of varying protein concentration using a stock solution of standard protein (e.g., bovine serum albumin fraction V). The stock solution had a concentration of 200mcg/ml & 500mcg/ml protein in distilled water, stored frozen at -80^{0}C. Using it different concentration of standard protein was made by diluting it with distilled water as follows**[46].**

Table 4.2: Different concentration of standard protein in Lowry method

Stock Solution(µL)	0	2.5	5	12.5	25	50	125	250	500
Water(µL)	500	498	495	488	475	450	375	250	0
Protein conc(µg/ml)	0	10	20	50	100	200	500	1000	2000

4.6.3.2 Apparatus

Refrigerator

Conical flask

Beaker

Pipette

Glass rod

Vortex mixer

Test tube

Water bath

UV spectrometer

4.6.3.3 Reagents

2% (w/v) Na_2CO_3

1 % (w/v) $CuSO_4.5H_2O$

Distilled water.

2% (w/v) sodium potassium tartrate

Bovine serum albumin fraction V

2N NaOH

1N Folin reagent

4.6.3.4 Procedure

- In this process complex forming reagent used was prepared just before using. Complex forming reagent consisted of 3 solutions (Solution A, Solution B, Solution C) in the ratio of 100: 1:1 respectively and those having following composition:

  Solution A had 2% (w/v) Na_2Co_3 in distilled water.

  Solution B had 1 % (w/v) CuSO4.5H2O in distilled water.

  Solution C had 2% (w/v) sodium potassium tartrate in distilled water.

- 0.1ml of each standard protein conc. solution was taken in a test tube.
- To it 0.1ml of 2N NaOH solution was added and the resulting solution as let to hydrolyse at 100^0C for 10min in boiling water bath.
- The resulting solution or hydrolysate was cooled to room temperature and to it 1ml of freshly mixed complex forming reagent was added and the solution was left to stand for 10mins in room temperature.
- After 10 minutes to the solution 0.1 ml of 1N conc. Folin reagent was added and using a vortex mixer it was thoroughly mixed. The mixture was left to stand for 30-40minutes at room temperature.
- Then the absorbance reading of all the resulting mixture was taken at 750nm**[46].**

4.7 Haemagglutination Assay (HA)

4.7.1 Principle

Many viruses attach to molecules present on the surface of RBCs. A consequence of this is that

at certain concentrations, a viral suspension may bind together (agglutinate) the RBCs, thus preventing them from settling out of suspension. Since agglutination is rarely linked to

infectivity, attenuated viruses can therefore be used in assays. By serially diluting a virus suspension into an assay tray (a series of wells of uniform volume) and adding a standard amount of blood cells, an estimation of the number of virus particles can be made. While less accurate than a plaque assay, it is cheaper and quicker (taking just 30 minutes). This assay may be modified to include the addition of an antiserum. By using a standard amount of virus, a standard amount of blood cells, and serially diluting the antiserum, one can identify the concentration of the antiserum (the greatest dilution which inhibits hemagglutination). **[42].**

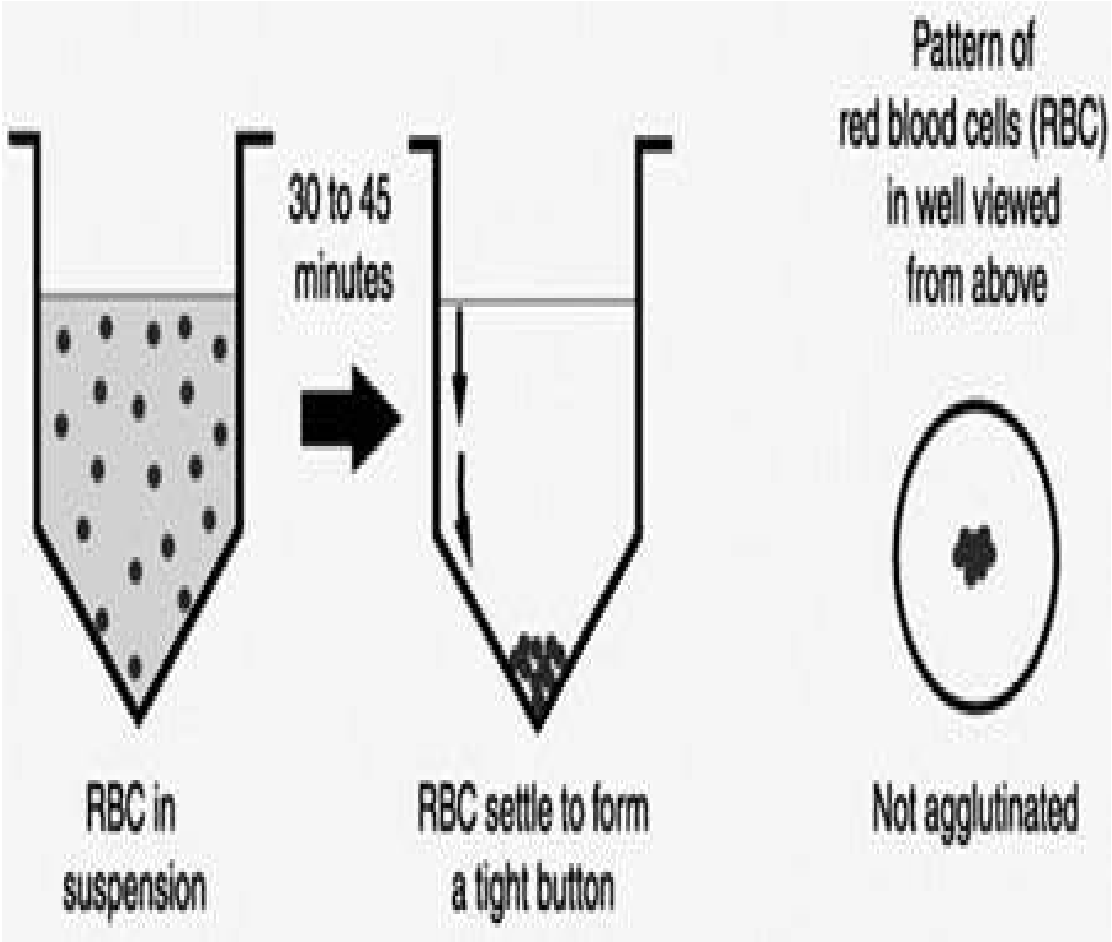


Figure 4.1: Formation of tight button with no agglutination

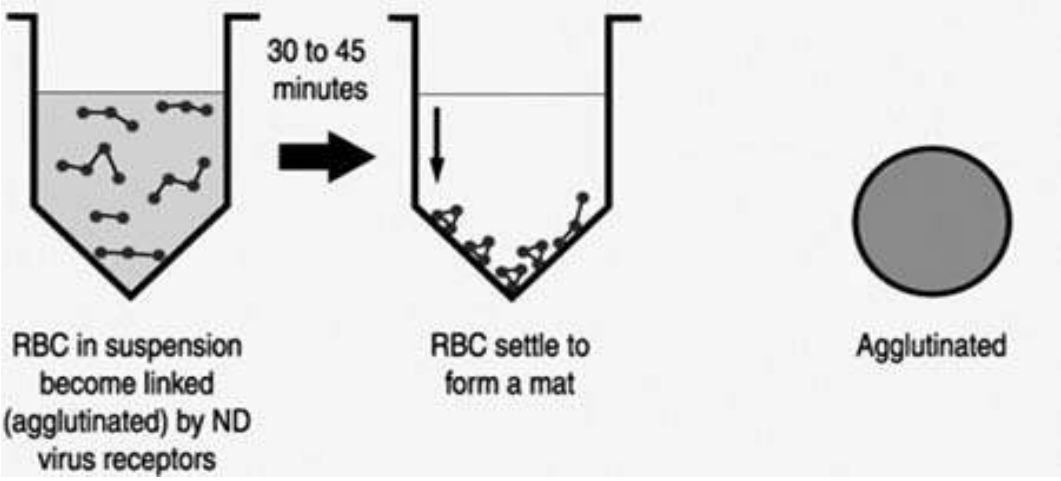


Figure 4.2: Formation of agglutination

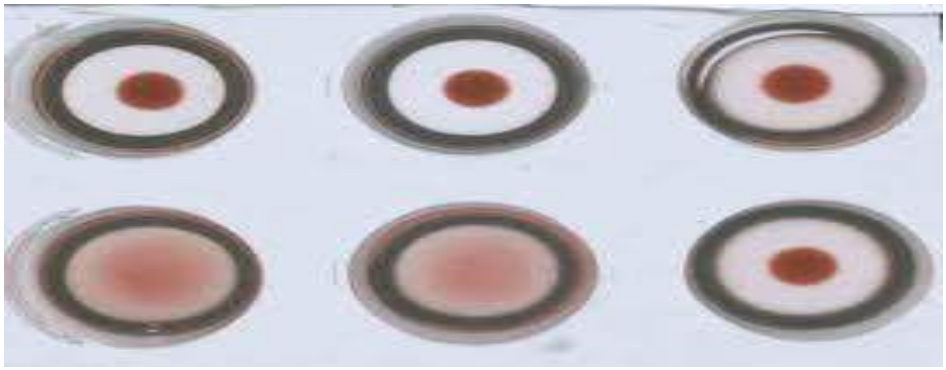


Figure 4.3: Overview of HA and not HA

4.7.2 Apparatus

Centrifuge machine	Refrigerator
Ephindorf tube	Test tube
Micro pipette	Eppendorf tube box
Syringe	CD marker
Cotton	

4.7.3 Reagents

Isotonic phosphate buffer Blood

4.7.4 Procedure

- Stock solution of the test sample was prepared at concentration of 5 mg/ml and each solution was serially diluted.
- Fresh blood from healthy person was collected only for the test of Haemagglutination Assay (HA). The blood group A^+, B^+, AB^+, O^+ were collected from healthy person for this test of East West University students.
- Then the all bloods were centrifuged and the erythrocytes were separated.
- 4% erythrocyte suspension was prepared in phosphate buffer (pH 7.4) of all blood groups.
- 1 ml of the test sample dilution was taken with 1 ml of 4% erythrocyte and incubated at 4°C.
- After incubation, the results were noted. Smooth button formation in bottom indicated negative activity, while a rough granular deposition at bottom showed positive activity.
- The intensity of haemagglutination was determined from the extent of deposition**[30].**

4.8 MINIMUM CONCENTRATION REQUIRE FOR HAEMAGGLUTINATION ACTIVITY

The aim of this study is to determine the minimum concentration of padmaguruj that give haemagglutination activity. In the experiment 96 well plate was used where the test sample was added to the 0.5 % A+ Rbc suspension. Concentration of the test sample (padmaguruj extract) was gradually decreased and in this way 10 different concentration samples were prepared. The result in each well was noted as either haemagglutination activity (+) or no haemagglutination

activity (-).The concentration from which agglutinated RBC appears i.e. –ve sign appears is the minimum concentration of padmaguruj to exhibit haemagglutination activity**[30].**

4.9 CARBOHYDRATE DETECTION BY TLC

4.9.1 Apparatus

TLC plate	TLC tank
Pencil	UV lamp
Capillary tube	

4.9.2 Reagents

Glucose	Phosphate buffer
Galactose	Acetone
Maltose	Sulphuric acid
Lactose	1-butanol

4.9.3 Procedure

- A TLC plate was taken and to both of its ends lines were drawn using pencil at 1cm from the edge. The bottom line was for spotting the samples, while the upper edge line was solvent line.
- To the bottom line two sample spots were made using capillary tube. Four were the positive control having Glucose, galactose, maltose & lactose and the sample extracts. All the spots were labeled using pencil and they equidistant apart from each other.
- The TLC tank was saturated with mobile phase having the composition of 1-butanol, acetone & phosphate buffer in the ratio of 4:5:1 respectively

- To the saturated tank the previously spotted plate was placed and the mobile phase was allowed to run through the spots until the solvent line was reached.
- The plate was then taken out of the tank, dried and then visualized under UV light in dark room.

After marking the florescent compounds, the plate was sprayed with anisaldehyde with 0.5% sulphuric acid, dried and then heated using heat gun to make the carbohydrate component spots visible **[112].**

4.10 ANTI-MICROBIAL ASSAY

4.10.1 Determination of Antimicrobial activity by Disc Diffusion Method

Worldwide, infectious disease is one of main causes of death accounting for approximately one-half of all deaths in tropical countries. Perhaps it is not surprising to see these statistics in developing nations, but what may be remarkable is that infectious disease mortality rates are actually increasing in developed countries, such as the United States[**35].**

Death from infectious disease, ranked 5th in 1981, has become the 3rd leading cause of death in 1992; an increase of 58% .It is estimated that infectious disease is the underlying cause of death in 8% of the deaths occurring in the US (Pinner *et al.*, 1996). This is alarming given that it was once believed that we would eliminate infectious disease by the end of the millenium. The increases are attributed to increases in respiratory tract infections and HIV/AIDS. Other contributing factors are an increase in antibiotic resistance in nosicomial and community acquired infections. Furthermore, the most dramatic increases are occurring in the 25–44 year old age group (Pinner *et al.*, 1996) **[30].**

These negative health trends call for a renewed interest in infectious disease in the medical and public health communities and renewed strategies on treatment and prevention. It is this last solution that would encompass the development of new antimicrobials (Fauci, 1998).

The antimicrobial screening which is the first stage of antimicrobial drug research is performed to ascertain the susceptibility of various fungi and bacteria to any agent. This test measures the ability of each test sample to inhibit the *in vitro* fungal and bacterial growth. This ability may be estimated by disc diffusion method.

But there is no standardized method for expressing the results of antimicrobial screening (Ayafor*et al*., 1982). Some investigators use the diameter of zone of inhibition and/or the minimum weight of extract to inhibit the growth of microorganisms. However, a great number of factors viz., the extraction methods, inoculum volume, culture medium composition (Bayer *et al*., 1966), p^H, and incubation temperature can influence the results.

Among the above mentioned techniques the disc diffusion (Bayer *et al*., 1966) is a widely accepted in vitro investigation for preliminary screening of test agents which may possess antimicrobial activity. It is essentially a quantitative or qualitative test indicating the sensitivity or resistance of the microorganisms to the test materials. However, no distinction between bacteriostatic and bactericidal activity can be made by this method (Roland R, 1982). **[40]& [41].**

4.10.2 Principle of Disc Diffusion Method

In this classical method, antibiotics diffuse from a confined source through the nutrient agar gel and create a concentration gradient. Dried and sterilized filter paper discs (6 mm diameter) containing the test samples of known amounts are placed on nutrient agar medium uniformly seeded with the test microorganisms. **[41].**

Standard antibiotic discs and blank discs are used as positive and negative control. These plates are kept at low temperature (4°C) for 24 hours to allow maximum diffusion of the test materials to the surrounding media (Barry, 1976). The plates are then inverted and incubated at 37°C for 24 hours for optimum growth of the organisms. The test materials having antimicrobial property inhibit microbial growth in the media surrounding the discs and thereby yield a clear, distinct area defined as zone of inhibition. The antimicrobial activity of the test agent is then determined by measuring the diameter of zone of inhibition expressed in millimeter (Barry, 1976; Bayer *et al.,* 1966.)In the present study the crude extracts, fractions as well as some pure compounds were tested for antimicrobial activity by disc diffusion method. The experiment is carried out more than once and the mean of the readings is required (Bayer *et al.,* 1966**)[30].**

4.10.3 Apparatus and Reagents

16. Filter paper discs

17. Autoclave

18. Nutrient Agar Medium

19. Laminar air flow hood

20. Petri dishes

21. Spirit burner

22. Sterile cotton

23. Refrigerator

24. Micropipette

25. Incubator

26. Inoculating loop

27. Ethanol

28. Sterile forceps

29. Nosemask and Hand gloves

30. Screw cap test tubes

4.10.4 Test Materials

Methanolic extract of *padmaguruj*,ethyl acetate extract of padmaguruj & isolating lectin form padmaguruj at different concentraion were taken as test sample.

4.10.5 Test Organisms

The bacterial strains used for the experiment were collected as pure cultures from the East West University microbiology laboratory. Both gram positive and gram-negative organisms were taken for the test and they are listed in the following table.

Table 4.3: List of micro-organisms used

Gram positive Bacteria	**Gram negative Bacteria**
Bacillus cereus *Staphylococcus aureus*	*Salmonella typhi* *Shigella dysentery* *Vibrio mimicus*

4.10.6 Culture Medium and Their Composition

The Nutrient Agar medium was used normally to demonstrate the antimicrobial activity and to make subculture of the test organisms. Nutrient Agar medium contains following things

Table 4.4: Composition of nutrient agar medium**[41].**

Ingredients	**Amount**
Bacto peptone	0.5 gm
Sodium chloride	0.5 gm
Bacto yeast extract	1.0 gm
Bacto agar	2.0 gm
Distilled water q.s.	100 ml

Agar medium having this composition was directly brought from the market.

4.10.7 Preparation of the Medium

To prepare required volume of this medium, calculated amount of agar medium was taken in a

bottle with a cap and distilled water was added to it to make the required volume. The contents were then autoclaved to make a clear solution.

4.10.8 Sterilization Procedure

In order to avoid any type of contamination and cross contamination by the test organisms the antimicrobial screening was done in Laminar Hood and all types of precautions were highly maintained. UV light was switched on one hour before working in the Laminar Hood. Petridishes and other glassware were sterilized by autoclaving at a temperature of 121^0C and a pressure of 15-lbs/sq. inch for 20 minutes. Micropipette tips, cotton, forceps, blank discs etc. were also sterilized.

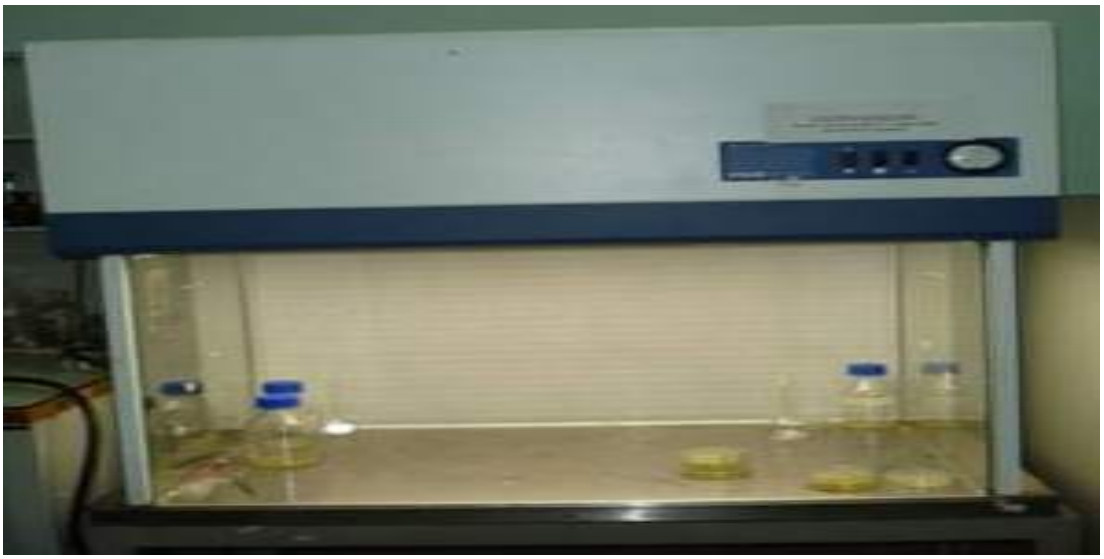


Figure 4.4: Laminar hood

4.10.9 Preparation of Subculture

In an aseptic condition under laminar air cabinet, the test organisms were transferred from the pure cultures to the agar slants with the help of a transfer loop to have fresh pure cultures. The inoculated strains were then incubated for 24 hours at 37^0C for their optimum growth. These fresh cultures were used for the sensitivity test.

Figure 4.5: Incubator

4.10.10 Preparation of the Test Plate

The test organisms were transferred from the subculture to petridish containing about 10 ml of melted and sterilized agar medium.

The bacterial and fungal suspension was taken by a loop a mixed with normal saline with the help of vortex machine. Then a sterilized cotton bud was taken and dipped into the bacterial/ fungal suspension. Then the bacterial/fungal sample is applied to the petridish with the help of this cotton bud.

4.10.11 Preparation of Discs

4.10.11.1 Standard Discs

These were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of the test sample. In this investigation, Azithromycin (30µg/disc) standard disc was used as the reference.

4.10.11.2 Blank Discs

These were used as negative controls which ensure that the residual solvents (left over the discs even after air-drying) and the filter paper were not active themselves.

4.10.11.3 Preparation of Sample Discs with Test Sample

Measured amount of each test sample was dissolved in specific volume of solvent to obtain the desired concentrations in an aseptic condition. Sterilized metrical (BBL, Cocksville, USA) filter paper discs were taken in a blank Petri dish under the laminar hood. Then discs were soaked with solutions of test samples and dried.

4.10.12 Diffusion and Incubation

The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test bacteria and fungi. The plates were then inverted and kept in an incubator at 37^0C for 24 hours.

4.10.13 Determination of Antimicrobial Activity by Measuring the Zone of Inhibition

The antimicrobial potency of the test agents are measured by their activity to prevent the growth of the microorganisms surrounding the discs which gives clear zone of inhibition. After incubation, the Antimicrobial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale.

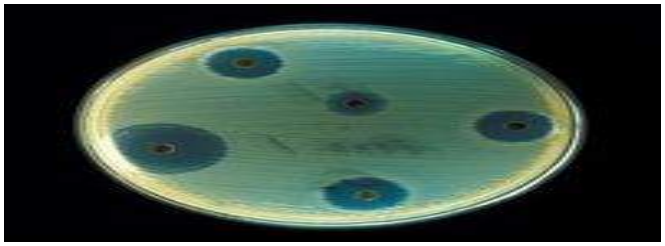


Figure 4.6: Zone of inhibition

4.11 Determination of MIC (Minimum Inhibitory Concentration)

The aim of this study was to determine the minimum inhibitory concentration (MIC) of the crude extract required to kill microorganism. In the experiment, medicaments were added to bacterial species into eppendrof tube, in 5 different concentrations. The MIC was the lowest concentration of the drug at which bacterial growth could not be observed.

4.11.1 Principle of MIC

The disc diffusion method, which is a 'semi-quantitative' method, determination of MIC values provides a quantitative measure for the level of resistance expressed by the test organism. The minimal inhibitory concentration (MIC) values, which represent the lowest extract concentration that completely inhibits the growth of microorganisms. Next to the agar dilution method, the broth dilution method is one of the most frequently used methods to determine MIC's. The method is based on the inoculation of a standardized liquid culture of the test strain in a dilution series of the extract for which the MIC is determined. The first concentration in the dilution series at which no visual growth can be determined is then considered as the MIC **[41]&[35].**

4.11.2 Apparatus and Reagents

Nutrient Agar Medium	Autoclave
Petridishes	Laminar air flow hood
Sterile cotton	Spirit burner
Micropipette	Refrigerator
Inoculating loop	Incubator
Sterile forceps	Ethanol
Nutrient Broth Medium	Screw cap test tubes
Eppendrof tube	Nosemask and Hand glove

4.11.3 Test Materials of *Tinospora tomentosa*

Isolating lectin of padmaguruj was taken as test sample.

4.11.4 Test Organisms

The bacterial strains (*Vibrio mimicus*) used for the experiment were collected as pure cultures

from the East West University microbiology laboratory.

4.11.5 Culture Medium and Their Composition

The Nutrient Agar medium was used normally to demonstrate the antimicrobial activity and to make subculture of the test organisms. Nutrient Agar medium contains following things

Table 4.5: Composition of nutrient agar medium**[35]**

Ingredients	**Amount**
Bacto peptone	0.5 gm
Sodium chloride	0.5 gm
Bacto yeast extract	1.0 gm
Bacto agar	2.0 gm
Distilled water q.s.	100 ml

Table 4.6: Composition of nutrient broth medium**[35]**

Ingredients	**Amount**
Bacto beef extract	0.3 gm
Bacto peptone	0.5 gm
Distilled water q.s.	100 ml

Agar and broth medium having this composition was directly brought from the market and the PH = 7.2 + 0.1 at 25^0C was maintained.

4.11.6 Procedure

- The agar diffusion method was employed for the determination of antibacterial activities of *padmaguruj*.

- The stem extracts were dissolved in 100% water to a final concentration of 100 mg/ml.
- The bacterial strains were cultured in a nutriment broth for 24 hours. **[35]**
- Then, previously prepared 1ml of suspension bacteria (30 X 1012 CFU estimated) was spread on nutrient Broth agar.
- Disks were made by using a sterile filter paper and were loaded with 20 µl of each sample extract.
- 0.15N NaCl PH 7 water was used as negative control and Azithromycin (30 mcg/disk) as positive reference standard.
- All the plates were incubated at 37°C for 24 hours.
- Antibacterial activity was evaluated by measuring the zone of inhibition in millimetres.
- All experiments were done in triplicates. **[35]**

4.12 Sensitivity test

The same disk diffusion method was used to test sensitivity of the crude extract on three classes of organisms (gram +ve, gram –ve and fungi) namely *S.cerevasecis, E.coli & S.dysentry* to determine its spectrum of activity.

4.13 TLC Bioautography

4.13.1 Apparatus

TLC plate	Laminar airflow
Pencil	Incubator
Nutrient Agar	

4.13.2 Reagents

Chloroform	Hexane
Methanol	*Vivrio mimicus* bacterial inoculums

4.13.3 Procedure

- TLC bioautography assay was performed by agar overlay bioautography technique.
- Plant extract samples were applied 1 cm from the base of the silica plate.
- After drying, the plates were developed using solvent chloroform: methanol (8.2: 1.8) and chloroform: hexane (5.4: 6.6).
- Developed TLC plates were carefully dried for complete removal of solvents. Aliquot of 50 ml of nutrient agar was prepared by adding *E.coli* bacterial inoculums.
- Now, the dried TLC plate was overlaid on inoculums containing agar under aseptic condition in laminar airflow. The plates were incubated at 37°C and examined for the zone of inhibition. **[37].**

4.14 IR DATA ANALYSIS

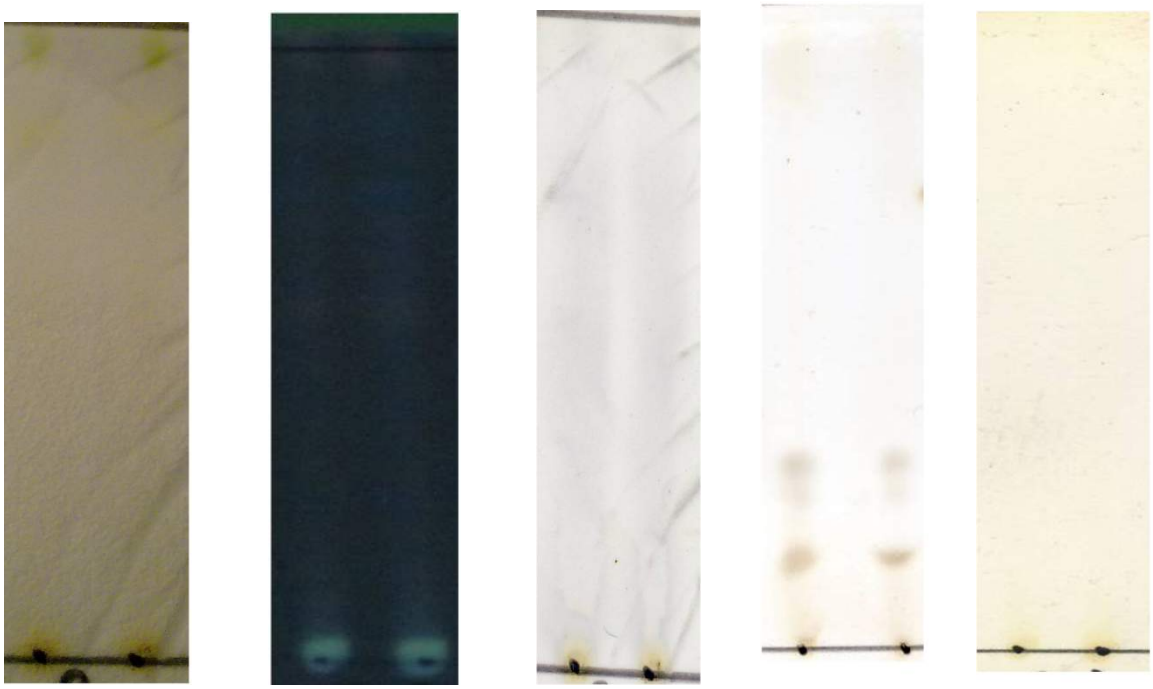
The sample was placed in an IR machine. The automated machine produces a graph showing all the functional groups present in the protein fraction obtained from Tinospora tomentosa.

Chapter 5:

Result and discussion

5.1 Thin Layer Chromatography (TLC)

The results obtained after TLC of the methanolic extract of the *padmaguruj* in solvent system 1 is given below-



1 2 3 4 5

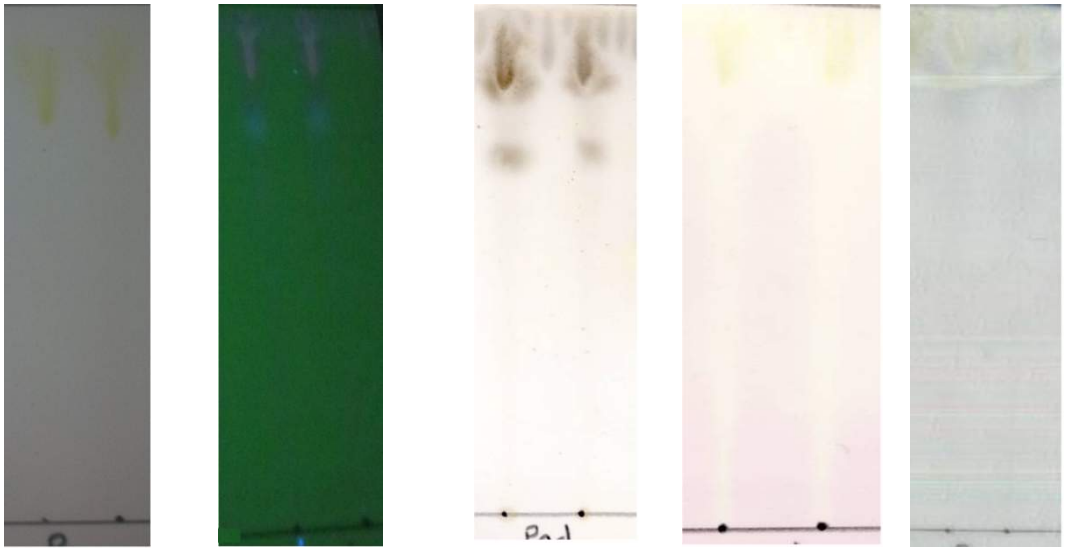
Figure 5.1: Results for TLC in nonpolar basic solvent (1= naked eye view; 2 = UV light view; 3= after application of FC reagent; 4= after charing; 5= after application of DPPH)

The naked eye view of the TLC was mentioned in the plate 1 which did not show two clear spot. Then the plate was observed under UV which is shown in the plate 2 and 3. It showed some spots which indicate the presence of different compounds in that sample. Folin & ciocalteu solution was used to determine the presence of the phenolic compouns in the sample. The formation of intense violet color after application of the Folin & ciocalteu solution in the sample indicates the presence of phenolic compound in the sample. Spraying of the Folin & ciocalteu solution in the TLC plate showed moderate violet color which indicates the presence of phenolic

compound in that fraction (plate 3). After charing of the TLC plate with sulfuric acid has showed (plate 4) two spots at the bottom as well as at the top of the TLC plate.

Spraying of DPPH solution on the TLC plate did not show any significant color changes which indicate the less free radical scavenging property of that fraction.

The results obtained after TLC of the methanolic extract of the *padmaguruj* in solvent system 2 is given below-



1 2 3 4 5

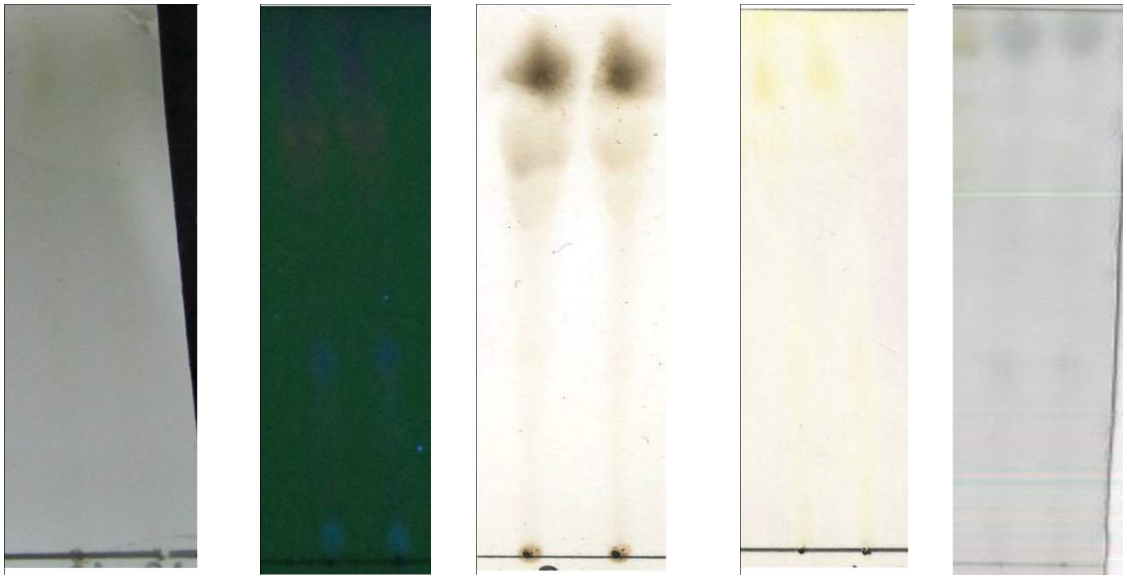
Figure 5.2: Results for TLC in intermediate polar basic solvent (1= naked eye view; 2 = UV light view; 3= after charing; 4= after application of DPPH solution; 5= after application of FC solution)

The naked eye view of the TLC is mentioned in the plate 1 which showed one clear spots at the top of the plate. Then the plate was observed under UV which is shown in the plate 2. It showed some additional spots which indicate the presence of different compounds in that sample. After charing of the TLC plate with sulfuric acid (plate 3) has revealed many spots in the TLC plate.

Spraying of DPPH solution on the TLC plate have shown slightly formation of pale yellow color (plate 4).

Folin & ciocalteu solution was used to determine the presence of the phenolic compouns in the sample. The formation of intense violet color after application of the Folin & ciocalteu solution in the sample indicates the presence of phenolic compound in the sample. Spraying of the Folin ciocalteu solution in the TLC plate shows formation light violet color which indicates the presence of phenolic compound in that fraction very few amount (plate 5).

The results obtained after TLC of the methanolic extract of the *padmaguruj* in solvent system 3 is given below-



1 2 3 4 5

Figure5.3: Results for TLC in polar basic solvent (1= naked eye view; 2 = UV light view; 3= after charing; 4= after application of DPPH solution; 5= after application of FC solution)

The naked eye view of the TLC was mentioned in the plate 1 which showed clear spot upper portion of TLC plate. Then the plate was observed under UV which is shown in the plate 2. It showed some spots which indicate the presence of different compounds in that sample. After charing of the TLC plate with sulfuric acid (plate 3) has revealed many spots in the TLC plate.

Spraying of DPPH solution on the TLC plate did not show significant formation of pale yellow color (plate 4) in the place of the spots which indicates less significant free radical scavenging property of that fraction.

Folin ciocalteu solution was used to determine the presence of the phenolic compounds in the sample. The formation of intense violet color after application of the Folin & ciocalteu solution in the sample indicates the presence of phenolic compound in the sample. Spraying of the Folin ciocalteu solution in the TLC plate shows very light violet color which indicates the presence of trace amount of phenolic compound in that fraction (plate 5).

5.2 Total Phenolic Assay

The crude methanolic extract of *Tinospora tomentosa* was subjected to total phenolic content determination. Based on the absorbance values of the extract solution, reacted with Folin-Ciocalteu reagent and compared with the standard solutions of gallic acid (table 6.6) equivalents, result of the colorimetric analysis of the total phenolics are given in table 6.7. Total phenolic content of the samples are expressed as mg of GAE (gallic acid equivalent) per mg of dried extract. The phenolic content found in the crude methanolic extract of Tinospora tomentosa was 5.266 mg of gallic acid (GAE) per mg of dried extract.

Table 5.1: Standard curve preparation by using gallic acid

SL. No.	Concentration (µg	Absorbance	Regression line	R^2
1	100	1.620	y = 0.0162x + 0.0215	0.9985
2	50	0.866		
3	25	0.450		
4	12.5	0.253		
5	6.25	0.120		
6	3.125	0.059		
7	1.5625	0.034		

8	0.78125	0.022		
9	0.3906	0.020		
10	0	0.011		

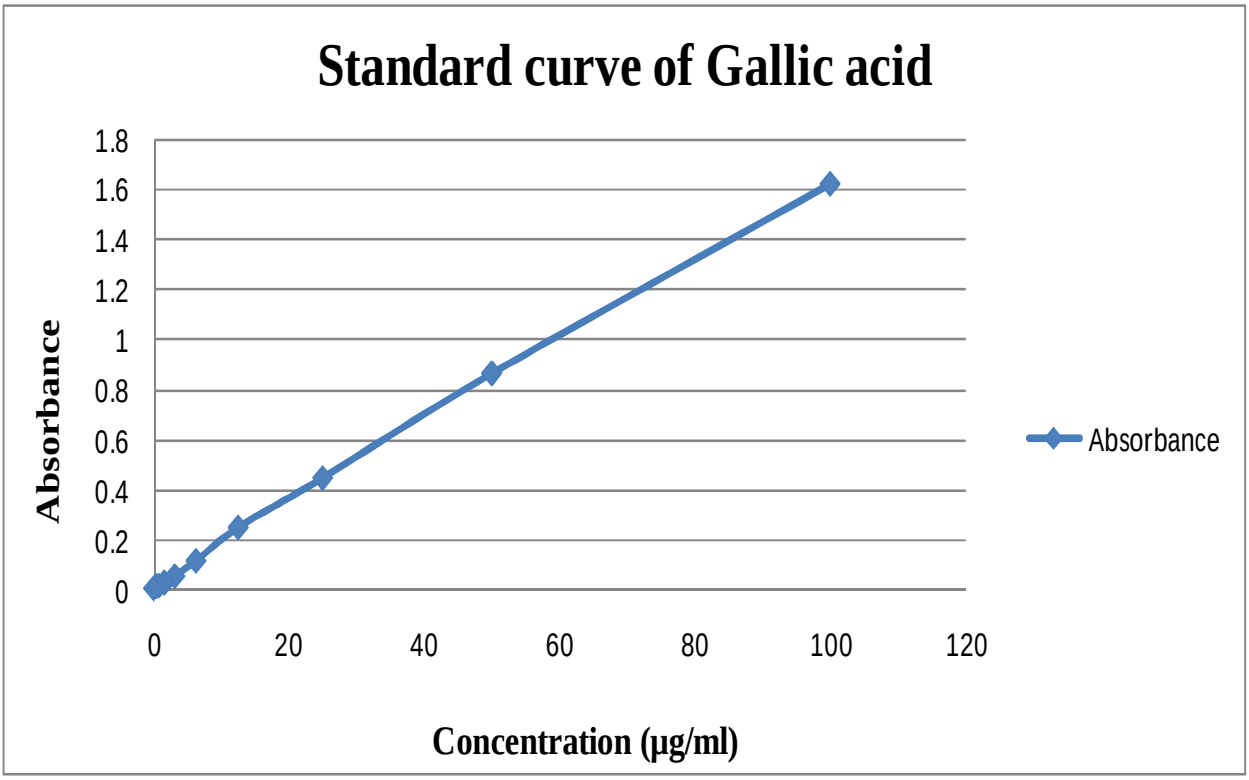


Figure 5.4: Standard curve of gallic acid

Table 5.2: Determination of total phenolic content (mg of GAE/mg of dried extract).

Sample	**Concentration**	**Absorbance**	**Total Phenolic content(mg of GAE/g of dried extract)**
METT	0.674 mg/ml	0.079	5.266

5.3 Protein Detection

5.3.1 Protein Detection by TLC

To detect protein (amino acid) prolein was used as positive control. After TLC, it was found that

the extract made some spots which were very much visible when it was sprayed by Ninhydrin solution under UV light. The brown spots indicate that there may be amino acids present in the extract thus we may go further test for the protein detection.

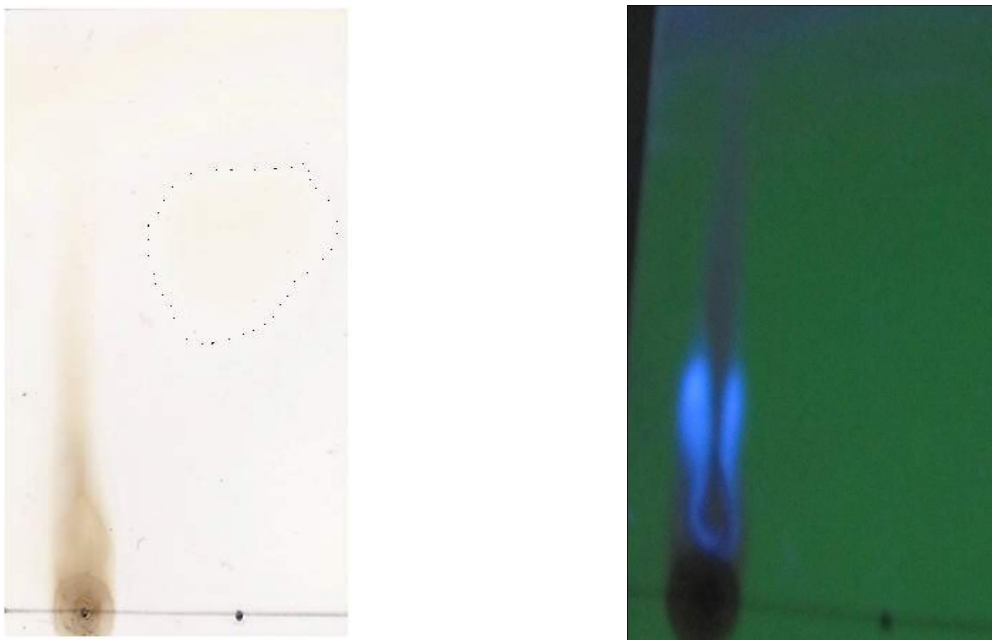


Figure 5.5: Protein detection eye view (left) and UV view (right)

5.3.2 Protein quantification by lowry method:

It was found a curved line from the standard curve of the reference standard bovine serum albumin collected form cow in which absorbance was taken in the Y axis and concentration was taken in the X axis. From the different concentration of the serum albumin different absorbance were found. The standard curve equation for Bovine serum albumin was $Y= 0.083\ln(X) – 0.247$, $R^2 =0.906$.

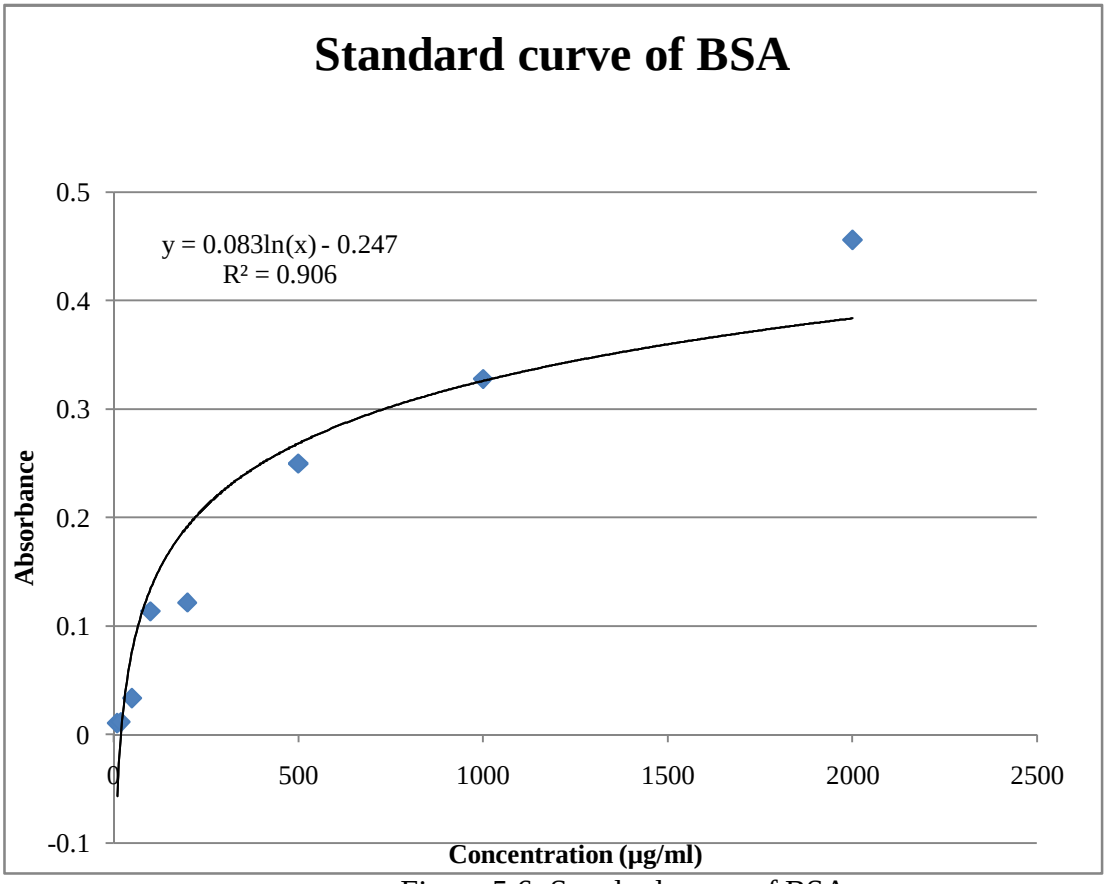


Figure 5.6: Standard curve of BSA

The isolating protein of *Tinospora tomentosa* was compared to the reference standard of bovine serum albumin. From the principle of total protein content by Lowry method, two concentrations of the plant extract was taken, that was 200 µg/ml and 500 µg/ml and their absorbance were found 0.047 and 0.139 respectively. For the comparison of plant extract with the serum albumin, the absorbances of the extract were put in the standard curve equation of the bovine serum albumin, from this two concentrations of serum albumin were found that were equivalent to the concentration of the plant extract respectively. From this relation, total protein content was found in which g of bovine serum albumin per mg of dried extract was measured that were 172.71 for the concentration of 200 µg/ml and 209.3 for the concentration of 500 µg/ml.

Table 5.3: Determination of protein content (mg of BSA/mg of dried extract).

Sample	**Concentration**	**Absorbance**	**Total Protein content(g of BSA/mg of dried extract)**
IPTT	200 µg/ml	0.047	172.71
IPTT	500 µg/ml	0.139	209.3

*IPTT=Isolating Protein of *Tinospora tomentosa*

5.4 Haemagglutination test for the lectin protein identification:

Haemagglutination test is identical test for lectin protein. So to ensure the protein Haemagglutination test was performed.

Sample	Concentration	result
P1	5mg/ml	+++
P2	2.5mg/ml	+++
P3	1.25mg/ml	+

Here from the table it was clear that Tinospora tomentosa contains lectin protein by performing the haemagglutination test that was found from the precipitation of protein by ammonium sulfate. In the test by increasing the concentration of the extract more and more rough granular deposition was occurred at bottom that means the RBC cannot bind each other, lectin protein was bind to the RBC surface. In case of the concentration of extract 5 mg/ml, the more significant result was obtained rather than the 2.5 mg/ml and 1.25 mg/ml.

5.5 Minimum concentration required for Haemagglutination test:

Table 5.4: Determination of minimum concentration required for haemagglutination activity

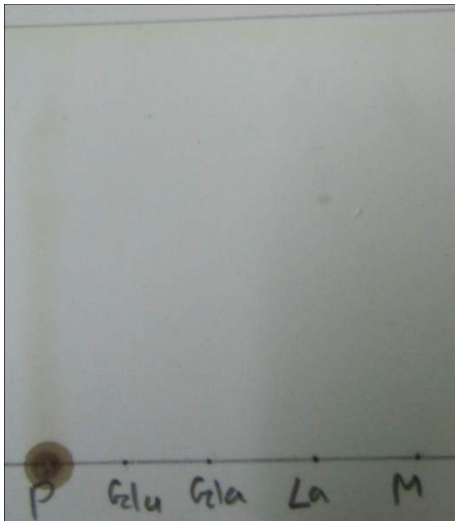
Sample	Concentration	Result
P1	204mg/ml	+
P2	102mg/ml	+
P3	51mg/ml	+
P4	25.5 mg/ml	+
P5	12.75 mg/ml	-
P6	6.375 mg/ml	-
P7	3.1875 mg/ml	-
P8	1.594 mg/ml	-
P9	0.79687 mg/ml	-
P10	0.39483 mg/ml	-

In the following table different concentration was observed for obtaining the minimum concentration in which the haemagglutination test shows the positive result. So this case in the concentration of 25.5 mg/ml was the minimum concentration for the haemagglutination test. The negative result was observed in further decreasing the concentration of the extract that was smooth button formed at the bottom of the surface, RBC was clot each other. In the result 204 mg/ml, 102 mg/ml, 51 mg/ml, 25.5 mg/ml showed positive result.

5.6 Carbohydrate detection:

To detect carbohydrate glucose, galactose, lactose & maltose were used as positive control. After TLC, it was found that the extract made some spots which were very much visible when it was

treated by anisaldehyde with 0.5% sulphuric acid solution. After giving heat there some visible spot shown which indicate the presence of carbohydrate.



1 2

Figure5.7: carbohydrate detection test on TLC plate (1=naked eye view; 2= charing view)

5.7 Antimicrobial test

5.7.1 Antimicrobial sensitivity test of Methanolic extract of *Tinospora tomentosa* by Disc diffusion method:

Table 5.7: Antimicrobial activity of Methanolic extract of *Tinospora tomentosa* by Disc diffusion method

Microorganisms	**ZMETT (1mg/ml)**	**ZMETT (10mg/ml)**	**Positive control Azithromycin (30 mcg/disk)**	**Negative control**
Vibrio mimicus	6	7	22	0

Salmonella typhi	0	7	21	0
Shigella dysentery	6	6	23	0
Staphylococcus aureus	7	8	25	0
Bacillus cereus	0	8	23	0

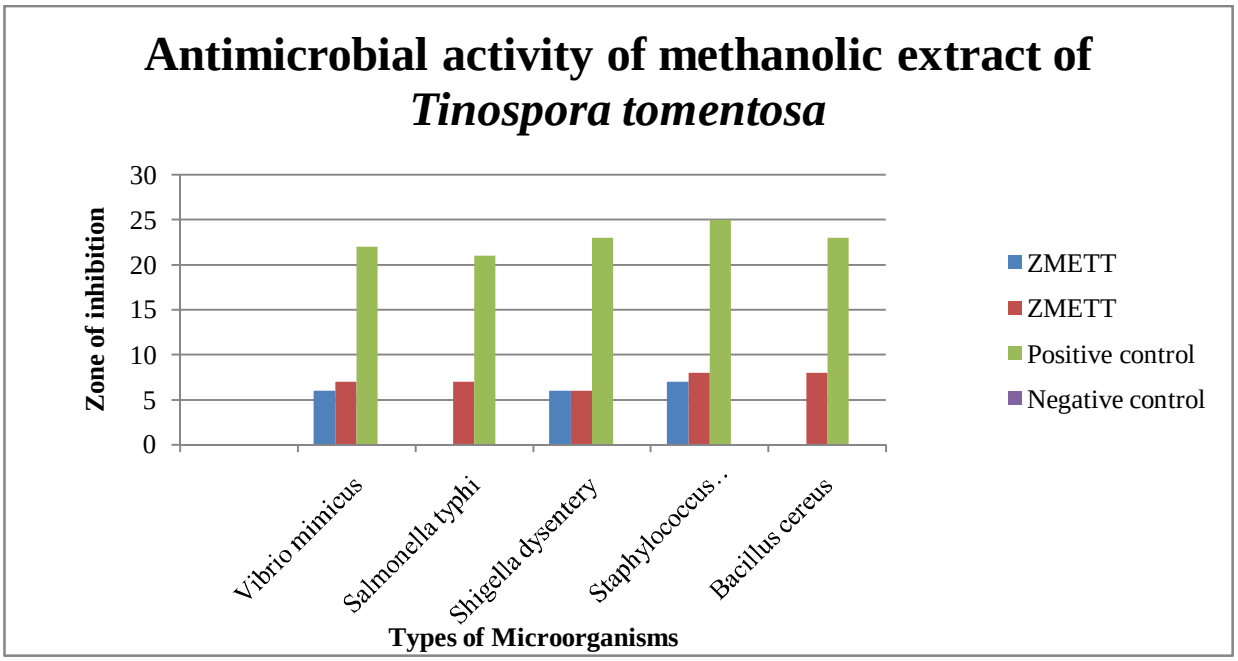


Figure 5.8: Antimicrobial activity of methanolic extract of *Tinospora tomentosa*

Antimicrobial activity of MeOH extract of *Tinospora tomentosa* has been evaluated in vitro against five bacterial species (shown in table) which are known to cause infections in humans. As summarized in the table, extract showed antimicrobial activity at a dose 1 mg/ml and 10 mg/ml disk against tested microorganisms. The highest inhibition zone diameter was 7 mm on *Staphylococcus aureus with the dose of 1 mg/ml and 8mm on Staphylococcus aureus* and

Bacillus sereus respectively in the concentration of 10mg/ml. The lowest inhibition zone diameter was nil on both *Bacillus sereus* and *Salmonella typhi* when working with the 1 mg/ml dose and in case of 10 mg/ml concentration the lowest zone diameter was 6 mm on *Shigella dysenteriae*. The average zone diameter was found in between 6 to 8 mm in case of other bacterial spice, which is *Vibrio mimicus*. The Reference drug azithromycin showed highest zone of inhibition of 25mm on *Staphylococcus aureus* and lowest zone of inhibition of 21 mm on *Salmonella typhi* at 30µg/disk. There were null effect found in the each concentration of extract in case of negative control.

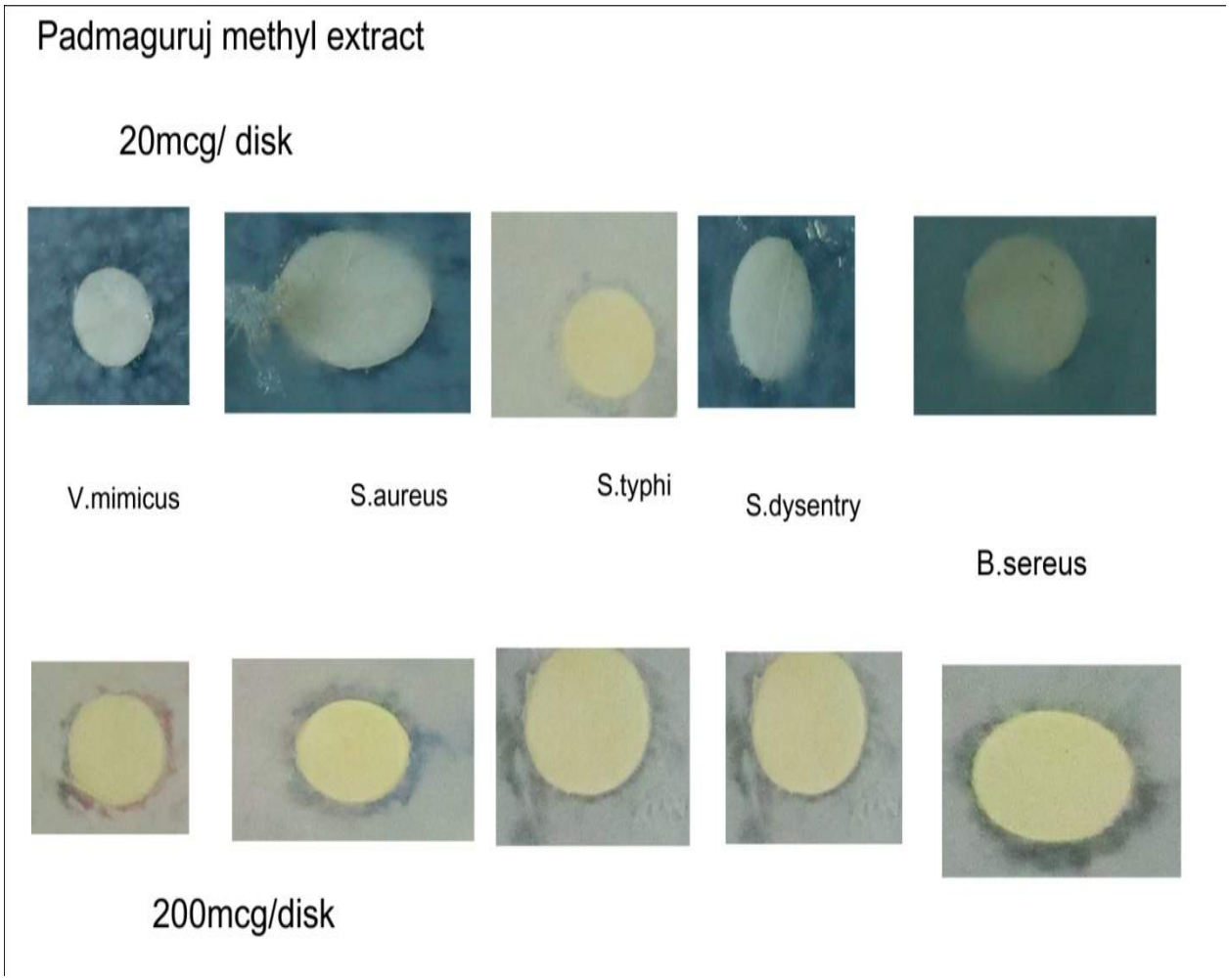
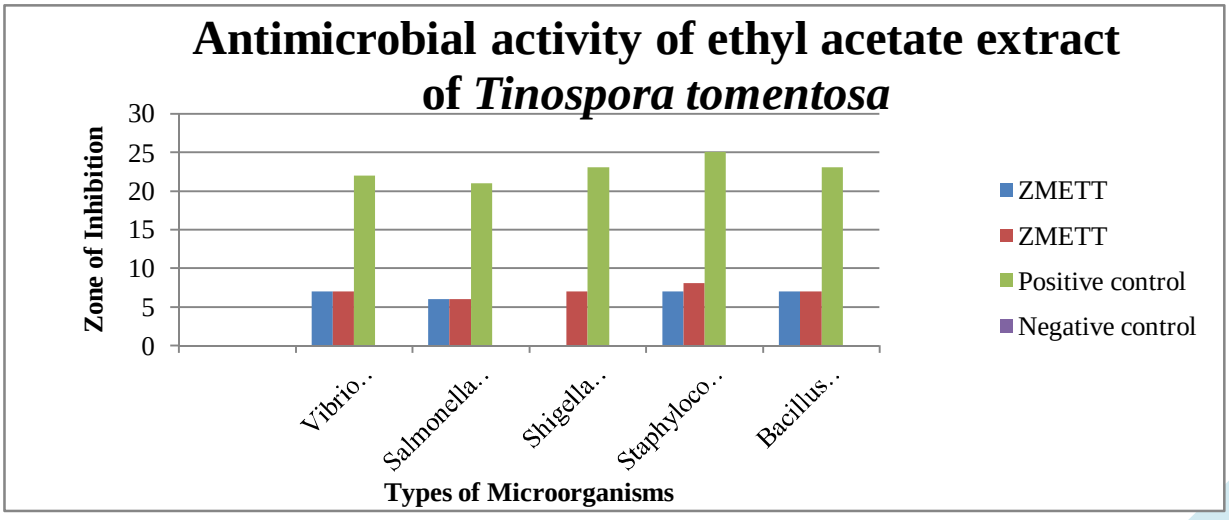


Figure 5.9: Antimicrobial activity of methyl-acetate extract of *Tinospora tomentosa* by Disc diffusion method

5.7.2 Antimicrobial sensitivity test of Ethyl-acetate extract of *Tinospora tomentosa* by Disc diffusion method:

Table 5.8: Antimicrobial activity of Ethyl-acetate extract of *Tinospora tomentosa* by Disc diffusion method

Microorganisms	**ZMETT (1mg/ml)**	**ZMETT (10mg/ml)**	**Positive control Azithromycin (30 mcg/disk)**	**Negative control**
Vibrio mimicus	7	7	22	0
Salmonella typhi	6	6	21	0
Shigella dysentery	0	7	23	0
Staphylococcus aureus	7	8	25	0
Bacillus cereus	7	7	23	0



Antimicrobial activity of ethyl acetate extract of *Tinospora tomentosa* has been evaluated in vitro against five bacterial species (shown in table) which are known to cause infections in humans. As summarized in the table, extract showed antimicrobial activity at a dose 1 mg/ml and 10 mg/ml disk against tested microorganisms. The highest inhibition zone diameter was 7 mm on *Staphylococcus aureus, Bacillus sereus and Vibrio mimicus with the dose of 1 mg/ml and 8 mm on Styphylococcus aureus* in the dose of 10mg/ml. The lowest inhibition zone diameter was nil on *Shigella dysenteriae* when working with the 1 mg/ml dose and in case of 10 mg/ml concentration the lowest zone diameter was 6 mm on *Salmonella typhi*. The average zone diameter was found in between 6 to 8 mm in case of other bacterial spice, which is *Vibrio mimicus*. The Reference drug azithromycin showed highest zone of inhibition of 25mm on *Staphylococcus aureus* and lowest zone of inhibition of 21 mm on *Salmonella typhi* at 30µg/disk. Every concentration of negative control showed no effect.

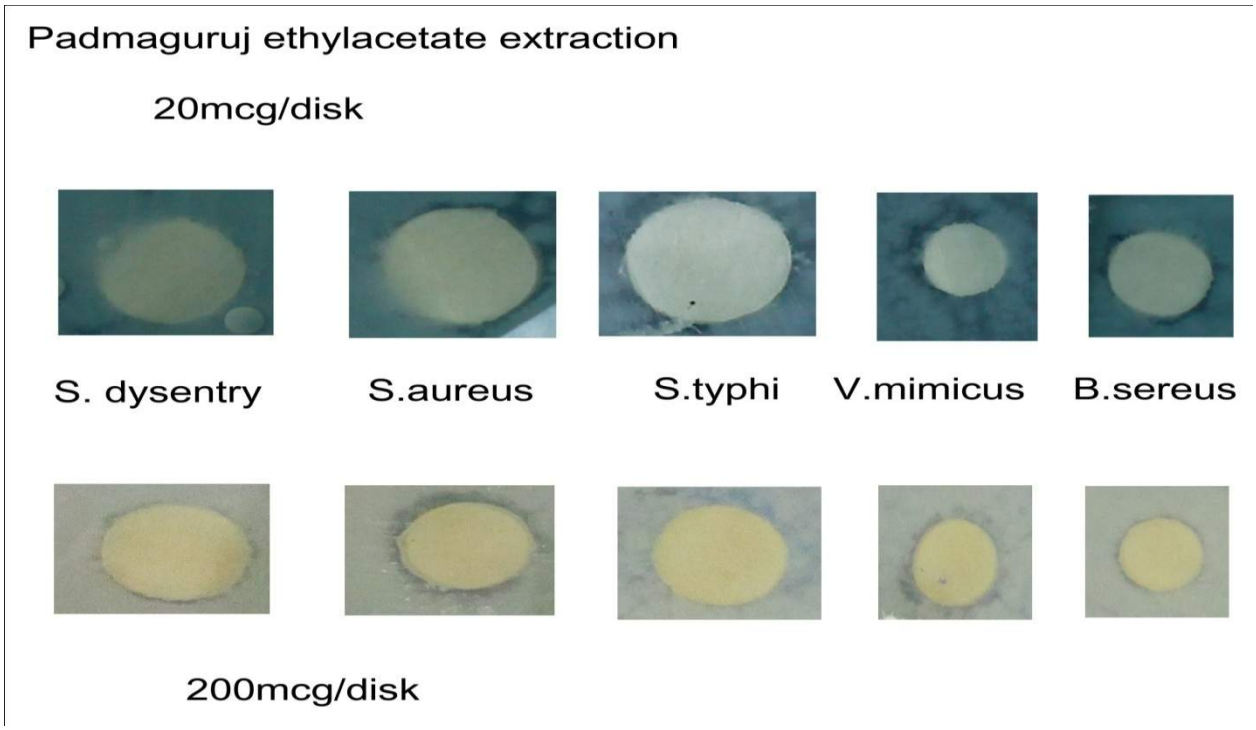


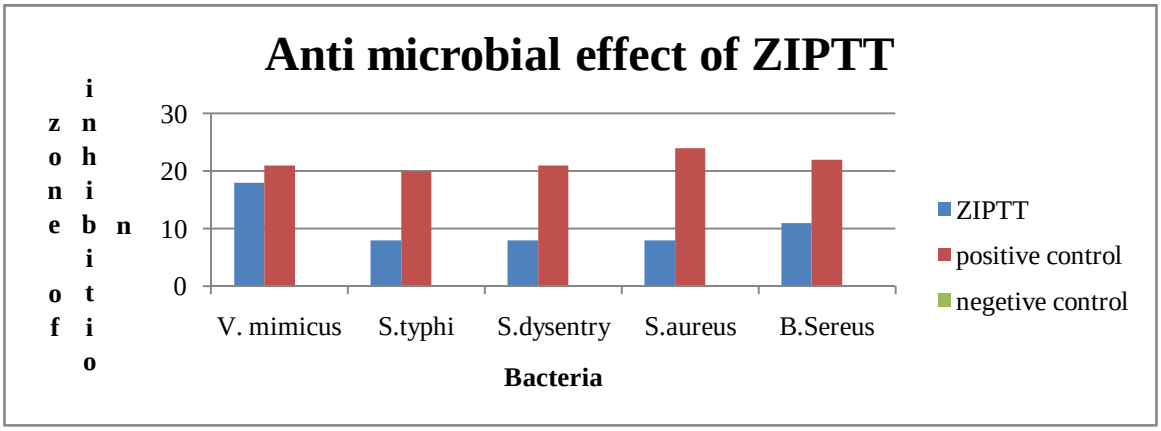
Figure 5.10: Antimicrobial activity of ethyl acetate extract of *Tinospora tomentosa*

5.7.3 Anti Microbial Test of Protein Sample

Table 5.9: Anti microbial activity of Padmaguruj

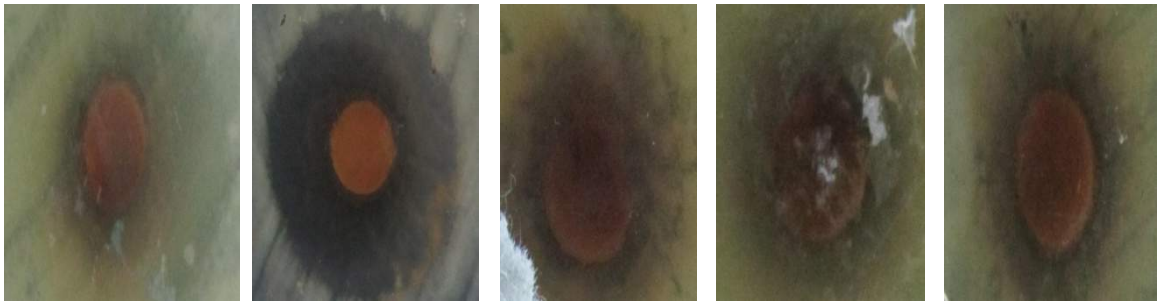
Microorganisms	ZIPTT 4000mcg/disk	Positive control Azithromycin(30 mcg/disk)	Negative control
Vibrio mimicus	18	21	0
Salmonella typhi	8	20	0
Shigella dysentery	8	21	0
Staphylococcus aureus	8	24	0
Bacillus cereus	11	22	0

*ZIPTT- Zone of inhibition of Isolating Protein of *Tinospora tomentosa*



Antimicrobial activity of isolating protein of *Tinospora tomentosa* has been evaluated in vitro against five bacterial species (shown in table) which are known to cause infections in humans.

As summarized in the table, extract showed antimicrobial activity at a dose 4000mcg/disk against tested microorganisms. The highest inhibition zone diameter was 18 mm on *Vibrio mimicus*. The lowest inhibition zone diameter was 6mm on *Shigella dysentery, Salmonella typhi, Staphylococcus aureus*. The Reference drug azithromycin showed highest zone of inhibition of 24mm on *Staphylococcus aureus* and lowest zone of inhibition of 20 mm on *Salmonella typhi* at 30µg/disk. Every concentration of negative control showed no effect. So, maximum effect was observed against *Vibrio mimicus*



S.aureus **V.mimicus** **B.sereus** **S.dysentry** **S.typhi**

Figure5.11: Anti microbial effect of IPTT on different type of bacterial

5.8 MIC

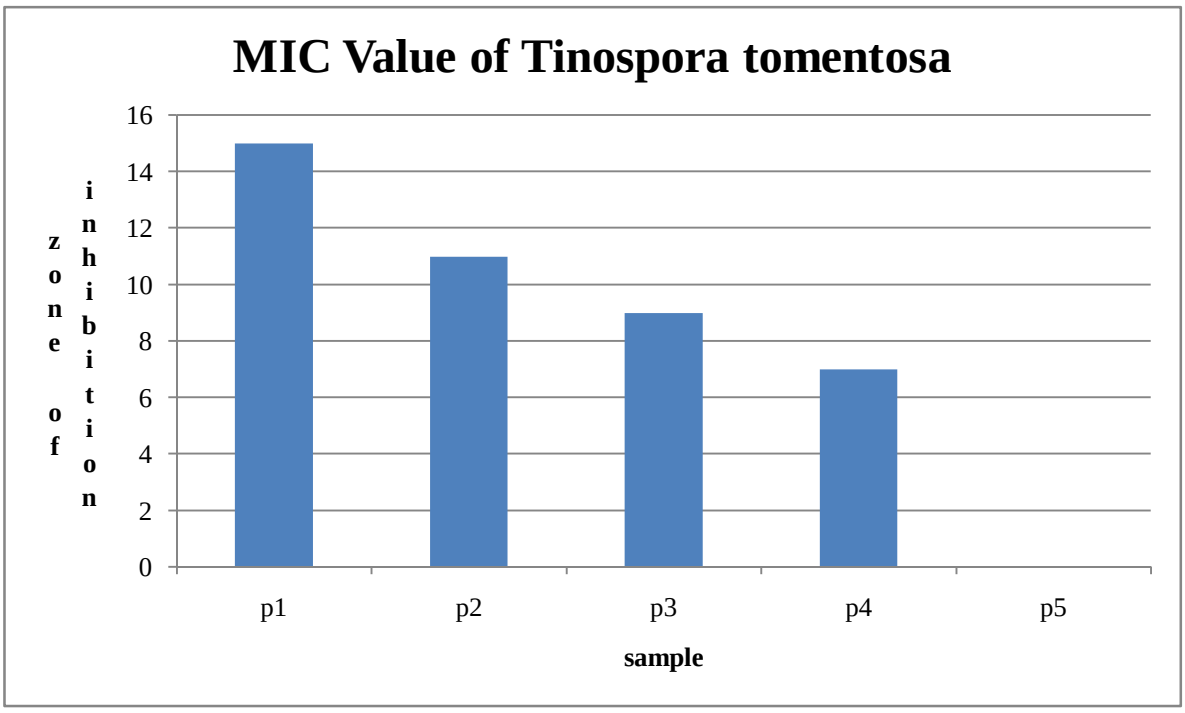
Zone of inhibition by MIC method, the results obtained are shown in the table 6.13 and in the figure 6.19. When the concentration was 2000 and 200 µg/disc, then the zone of inhibition found 8 and 6 mm respectively. In case of 20, 2, 0.2 µg/disc no zone of inhibition was found.

Table 5.10: Zone of inhibition by MIC method

Sample	**Concentration** µg/disc	**Microorganism**	**MIC in Zone of Inhibition Method in mm**
ZIPTT	2000		15
	200		11

ZIPTT	20	*Vibrio mimicus*	9
	2		7
	0.2		0

N.B: Z IPTT= zone of inhibition of isolating protein of *tinospora tomentosa.*



5.9 Sensitivity test:

Zone of inhibition by sensitivity method, the results obtained are shown in the table and in the figure. When the concentration was 2000 µg/disc, then the zone of inhibition found 24, 27 and 20 mm respectively for S.cerevaceae, E.coli & S.dysentry bacteria.

Name	**S.cerevasecis**	**E.coli**	**S.dysentry**
Tinospora tomentosa	**24**	**27**	**20**

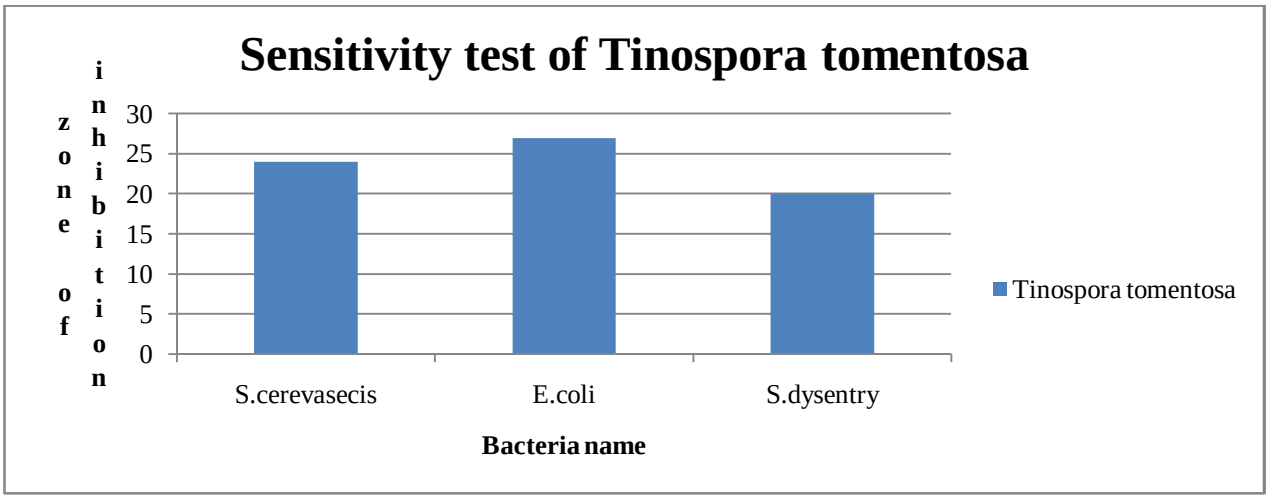
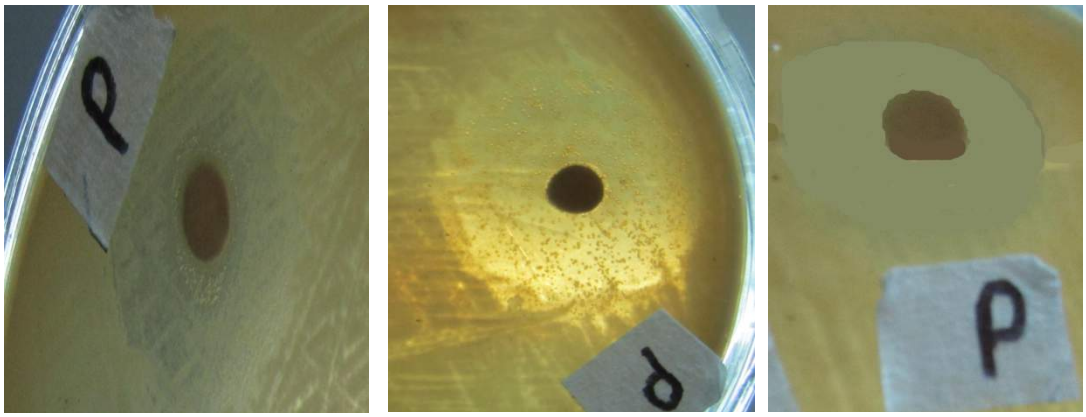


Figure 5.12: Bar diagram of Sensitivity test of Tinospora tomentosa



S. cerevacesis E.coli S. dysentery

Figure5.13: sensitivity test of Tinospora tomentosa

5.10 TLC Bioautography:

When this test was run then a clear zone of inhibition was found against *E.coli.* From the following figure white circle shows that a clear zone of inhibition was found.

Figure5.14: TLC bioautography

5.11 IR data result

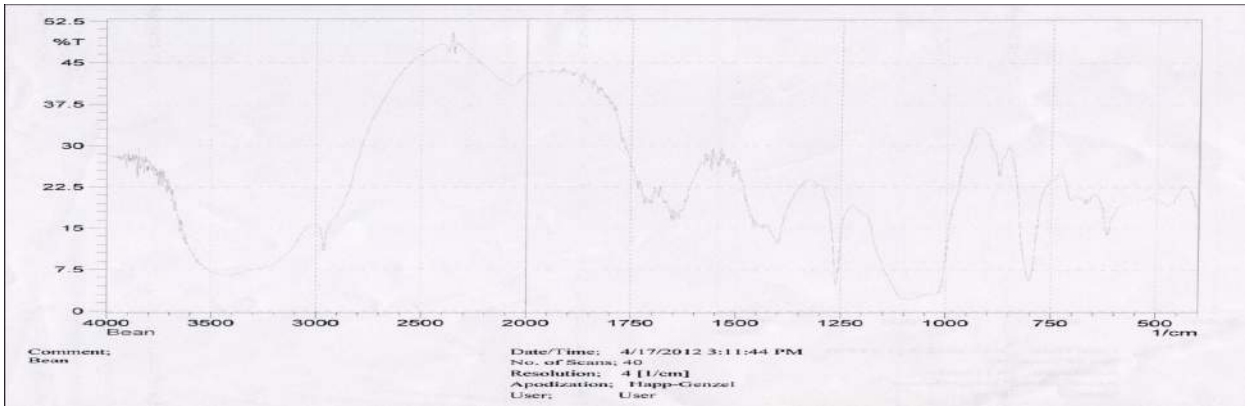


Figure5.15: IR data of padmaguruj protein

The figure above shows the IR spectrum of the isolating protein of the *Tinospora tomentosa.* As we know a plant extract contains several compounds and IR spectrum gives the idea about the functional group present in the compounds. So, we can get idea about the functional groups present in compounds present in the sample.

Table5.11: Prominent peaks observed in the IR spectrum of the extract from padmaguruj with justification.

Peak	Intensity	Probable functional group
1550-1650	Strong	NH_2 Scissoring(1^o amides)
690-750	Medium	NH_2 & N-H wagging
1680-1690	Strong	Unsaturation/aromatic carboxylic acid
1650	Strong	associated amides
1550	Strong	amino acid zwitterions
1695	Medium	Enol

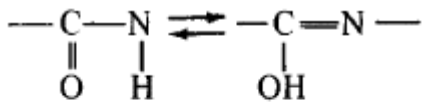
1710-1740	Medium	Aldehyde or keto with saturated aliph./cyclic 6-membered C=O (Saturated aldehyde/ketone)
1685	Medium	aromatic ketones/ α,β-unsaturated aldehyde or ketone
1200	Medium	Ether
1330,1430	Medium	O-H bend(in plane)
660,700,750	Weak	O-H bend(out of plane)
1690	Medium	aryl ketone
1260	Strong	Phosphoramide
2300	strong	Phosphonates

Interpreting the spectrum:

- Amino group: The broad peak in the range 1550-1650 confirms the presence of a primary amine (-NH_2) in the structure of the compound extracted.
- Carboxylic acid group: The peak in the region of 1680-1690 shows the presence of carboxylic acid
- Zwitterions ion: Peak at 1550 confirms the structutre to have zwitterions ion configuration like amino acid **[110 & 111].**
- Amide group: Broad peak at 1650 ensures the presence of amide group.

- Enol group: At 1695 peak observed in due to the presence of an enol group in the structure.

The amino & carboxylic group in the spectrum gives a rough idea of the structure to an amino acid because amino acid has both -NH_2 and –COOH groups. The presence of a peak for amide group shows that the compound is protein in nature as peptide linkage is nothing other than amide linkage. Moreover the presence of enol group also confirms it to be a protein as the —CO—NH— group in proteins exhibits a keto-enol tautomerism.



Keto form (60 percent) Enol form (40 percent)

So it can be confirmed that the protein extract from T. tomentosa actually had protein in it.

Again interpreting the spectrum

- Carbonyl group: Peak observed in the range 1710-1740 confirms the presence of carbonyl group (aldehyde/ ketone) in the structure.
- Hydroxyl group: Both in plane and out-of planr –OH groups are present in the structure as peaks are observed both at 1330,1430 & 660,700,750 respectively.
- Ether group: Peak at 1200 confirms the presence of ether(-C-O) group.

The presence of aldehyde/ ketone and hydroxyl group in the structure confirms the compound to be carbohydrate in nature. Moreover because of the peak of Ether group it is evident that

condensation reaction between aldehyde and hydroxyl group occurred which is characteristic for carbohydrates while forming ring structure from straight chain.

From the above study it can be confirmed that in the sample carbohydrate group & protein group was present. These results ensured the presence of lectin protein, which is mainly a glycoprotein, likely to be found protein extract of the Tinospora tome

Chapter 6:

CONCLUSION

6. CONCLUSION:

The microbiology screening of Tinospora tomentosa Miers gives some sigfificant data on Tinospora tomentosa Miers is used as a medicinal plant in several countries for several purposes. The use of plant extracts and phytochemical, with known antibacterial properties, may be of immense importance in therapeutics treatments. In past few years, a number of studies including phytochemical and pharmacologically have been conducted in different countries to prove such efficiency, but further investigation is needed to identify other activities of this plant.

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