PHYTOCHEMICAL AND BIOLOGICAL INVESTIGATIONS OF *TINOSPORA TOMENTOSA*

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

Supervised By

Dr. Repon Kumer Saha

Assistant Professor, Department of Pharmacy

East West University

Submitted By

Syed Sohidul Haque Shovon

2008-3-70-027

Dedicated

To

My

Parent

Certificate

This is to certify that the thesis In-vitro Biomedical & antimicrobial assay of the plant ***Tinospora tomentosa Miers*** 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 Syed Sohidul Haque Shovon (ID#2008-3-70-027) 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.

Sufia Islam, PhD

Chairperson & Associate Professor

Department of Pharmacy

East West University

Aftabnagar, Dhaka.

Certificate

This is to certify that the thesis In-vitro Biomedical & antimicrobial assay of the plant ***Tinospora tomentosa Miers*** 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 Syed Sohidul Haque Shovon (ID#2008-3-70-027) 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.

Dr. Repon Kumer Saha

Supervisor

Assistant Professor, Department of Pharmacy

East West University

Table of Content

Acknowledgement

All praise is for Allah 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, Assistant 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 Sciences and associate 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 methanolic extracts and buffer extracts derived from the Tinospora tomentosa was screened in vitro for possible phytochemical, antibacterial and biomedical activities. Under phytochemical analysis, Chemical screening was done whereas in biomedical analysis haemolytic activity & haemagglutination test was performed.

The aqueous extracts 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 aqueous extract of Tinospora tomentosa.

Hence the plant species can be a source of antibacterial agent(s) as well as the plant has good property to prevent haemolysis & have high haemagglutination activity.

Keywords: Tinospora tomentosa, Haemagglutination, antimicrobial, IR, MIC.

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 **[1].**

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 **[41].**

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 **[1].**

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.

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 **[2]**.

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. 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 **[2].**

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 **[34].**

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* - Miers

Botanical name: *Tinospora tomentosa* Miers **[37]**

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. ***[34].***

1.8 Medicinal properties:

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.

It has been reported that extract of *Tinospsora cordifolia 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 **[42, 45].**

1.8.1 Hypoglycemic effect

In Indian Ayurvedic medicine, Tinospora cardifolia is widely used for diabetes mellitus. ***[45].***

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.

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 **[39].**

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 **[39].**

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 **[38].**

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 minimum inhibitory concentration (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 **[38]**

1.9.5 Interpetation 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 **[38].**

Chapter 2:

Literature Review

2. Leterature 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 **[43].**

Table-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 **[44].**

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:

Table 2 Qualitative phytochemical evaluation of the *Tinospora tomentosa* Miers. Extracts

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 **[44].**

Table-3 Determination of calcium, potassium and sodium levels of *Tinospora tomentosa* Miers.

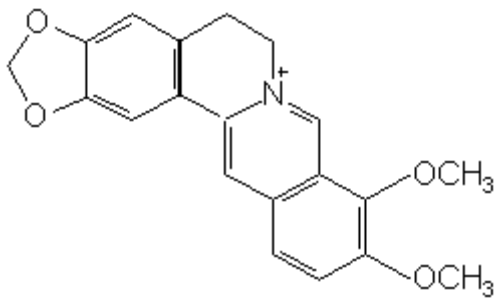
Calcium	Potassium	Sodium
82.65	79.20	UD

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 **[44]**

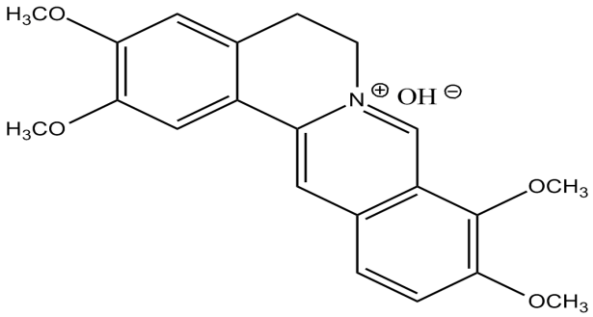
. A variety of constituents have been isolated from *Tinospora cordifolia* plant and their structures were elucidated. They belong to different classes such as alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds and polysaccharides.

Table-4 Different types of alkaloids isolated from various parts of *Tinospora cordifolia*

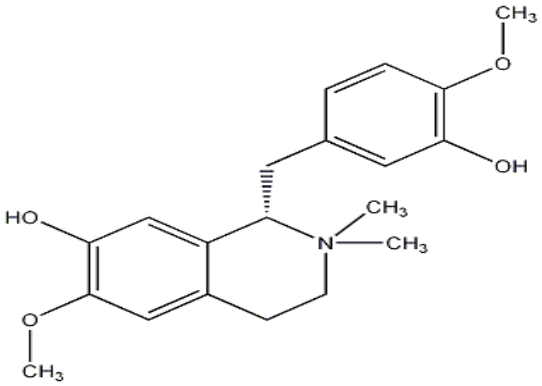
Type of Chemical	**Active principle with references**	**Part in which present**
Alkaloids	Berberine, Palmatine, Tembetarine , Magnoflorine , Choline , Tinosporin, Isocolumbin, Palmatine, Tetrahydropalmatine, Magnoflorine	Stem Root



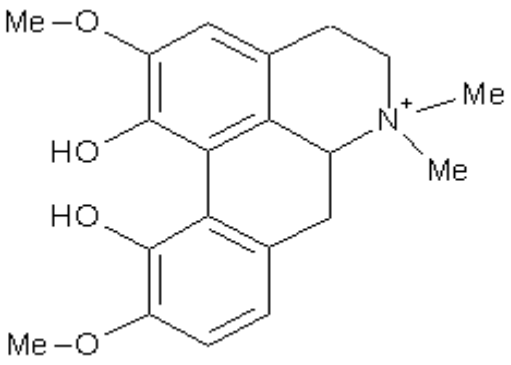
Berberine



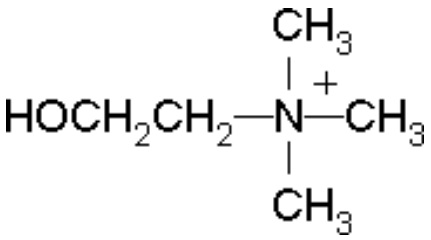
Palmatine



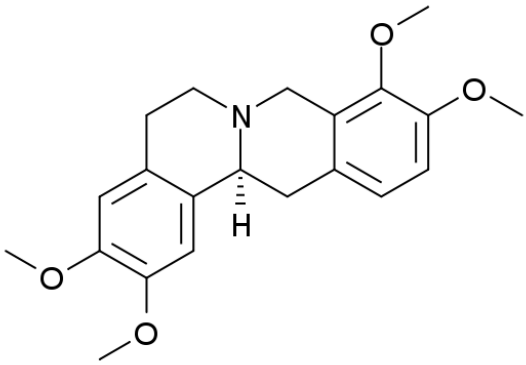
Tembetarine



Magnoflorine



Choline

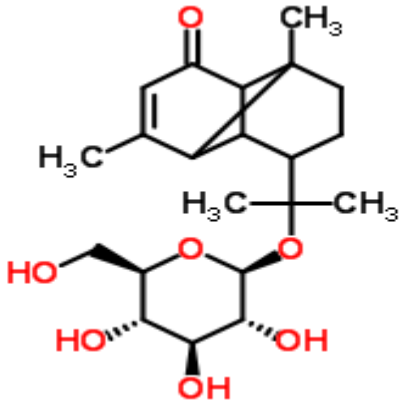


Tetrahydropalmatine

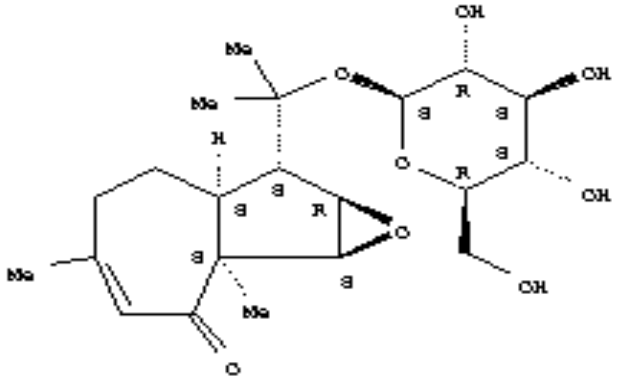
Table-5

Different types of Glycosides isolated from various parts of *Tinospora cordifolia*

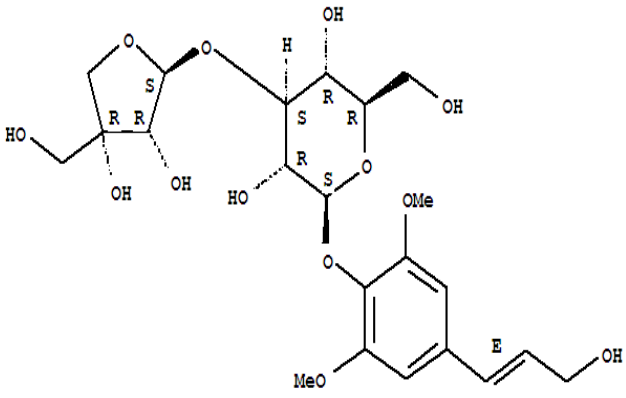
Type of Chemical	Active principle with references	Part in which present
Glycosides	18-norclerodane glucoside, Furanoid diterpene glucoside, Tinocordiside, Tinocordifolioside, Cordioside, Cordifolioside A, Cordifolioside, Syringin, Syringinapiosylglycoside, Palmatosides C, Palmatosides P, Cordifoliside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



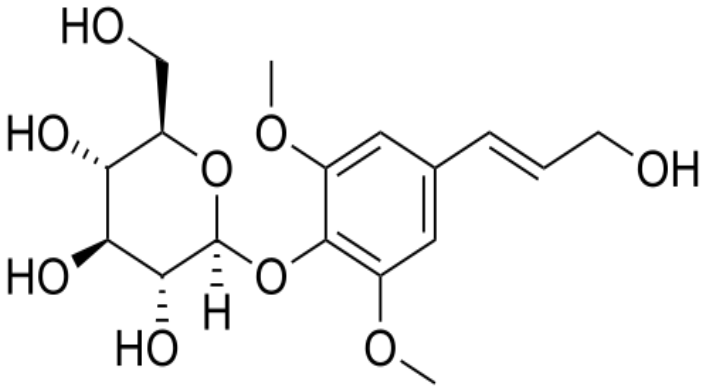
Tinocordiside



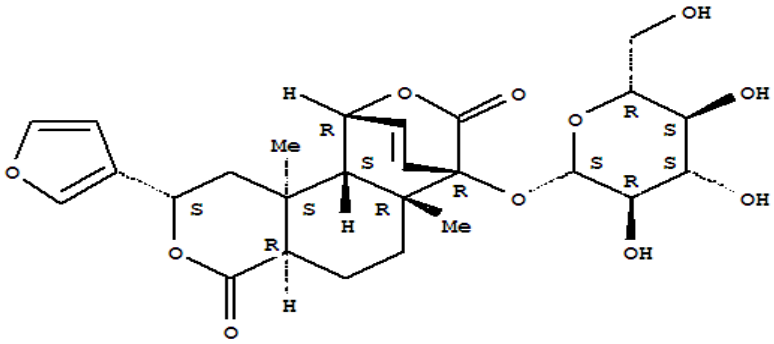
Tinocordifolioside



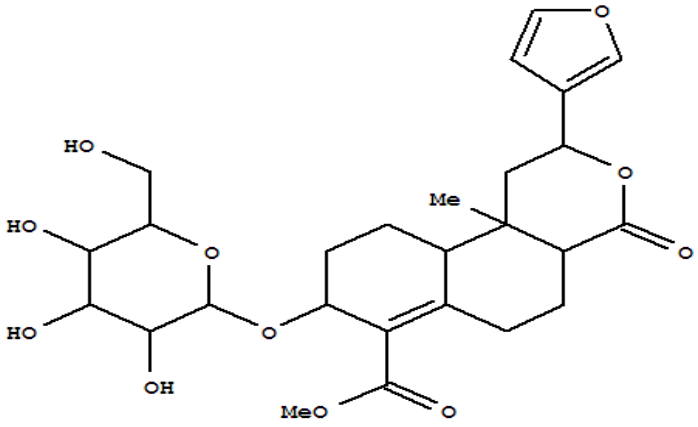
Cordifolioside A



Syringin



Palmatosides C

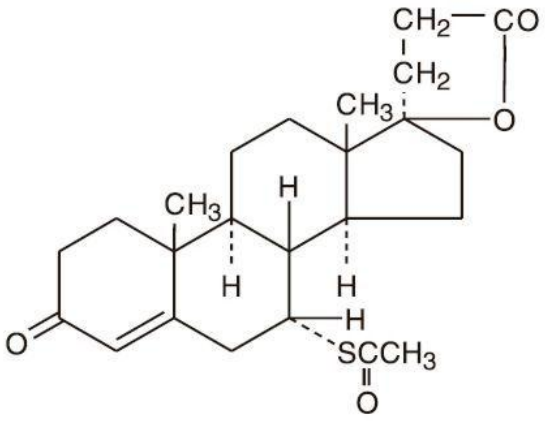


Cordifoliside A

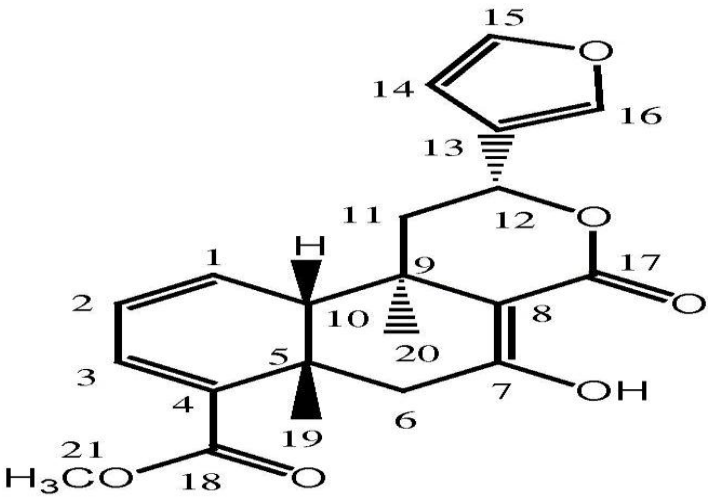
Table-6

Different types of Diterpenoid Lactones isolated from various parts of *Tinospora cordifolia*

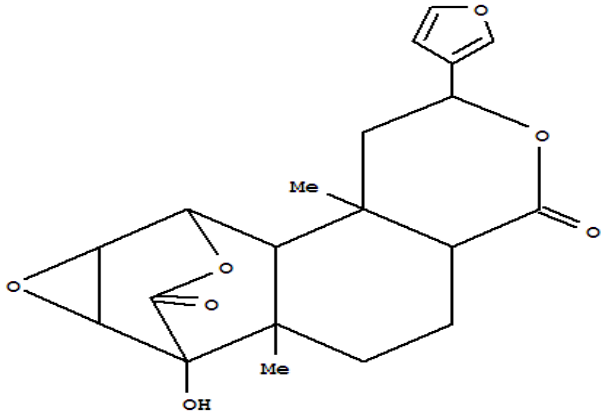
Type of Chemical	Active principle with references	Part in which present
Diterpenoid Lactones	Furanolactone, Clerodane derivatives, 14-dieno-17,12S: 18,1S-dilactone] and Tinosporon, Tinosporides, Jateorine , Columbin	Whole plant



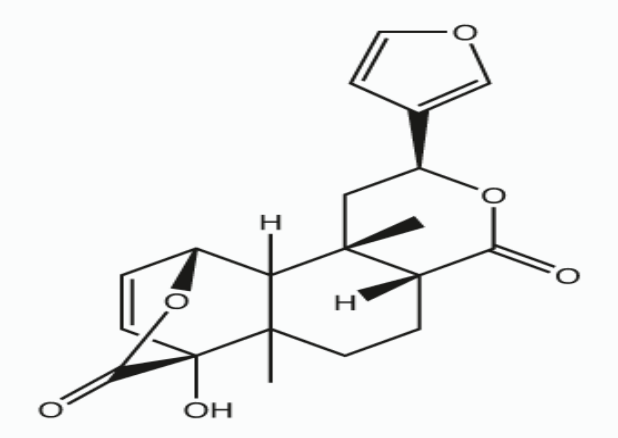
Furanolactone



Clerodane



Tinosporides

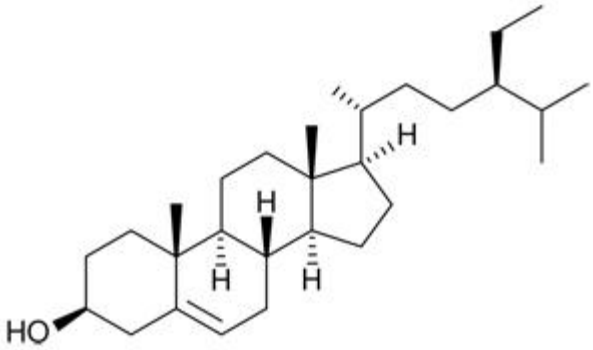


Columbin

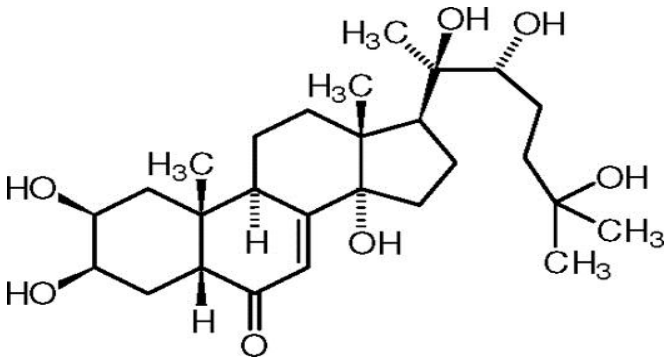
Table-7

Different types of Steroids isolated from various parts of *Tinospora cordifolia*

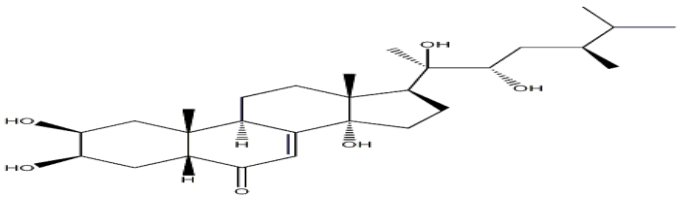
Type of Chemical	Active principle with references	Part in which present
Steroids	β-sitosterol, δ-sitosterol, 20β- hydroxy ecdysone, Ecdysterone, Makisterone A, Giloinsterol.	Aerial part Stem



β-sitosterol



Ecdysterone

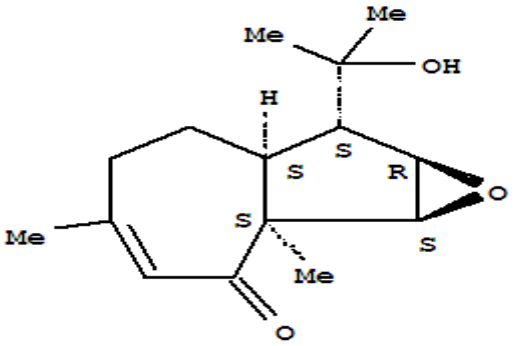


Makisterone A

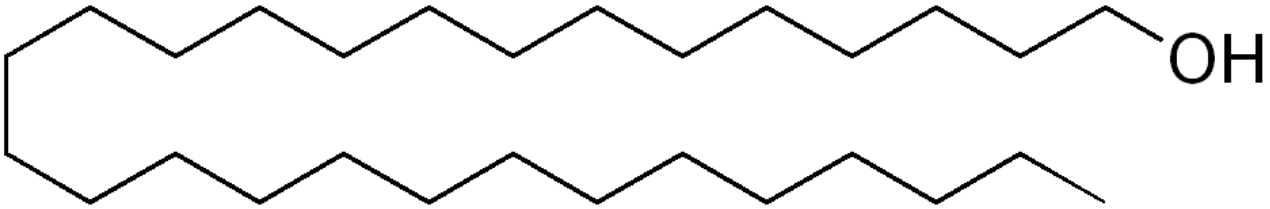
Table-8

Different types of Sesquiterpenoid and Aliphatic compound isolated from various parts of *Tinospora cordifolia*

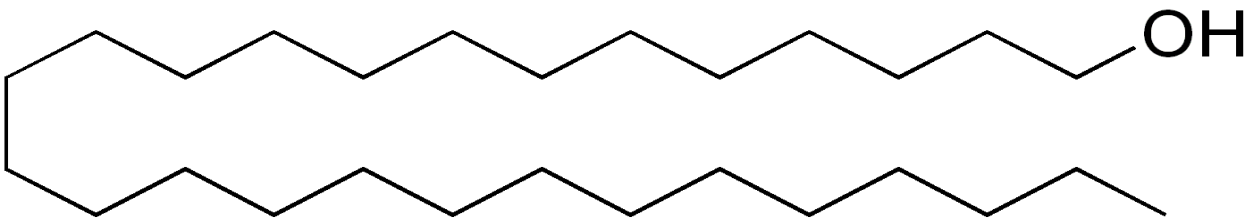
Type of Chemical	Active principle with references	Part in which present
Sesquiterpenoid	Tinocordifolin	Stem
Aliphatic compound	Octacosanol, Heptacosanol, Nonacosan-15-one	Whole plant



Tinocordifolin



Octacosanol

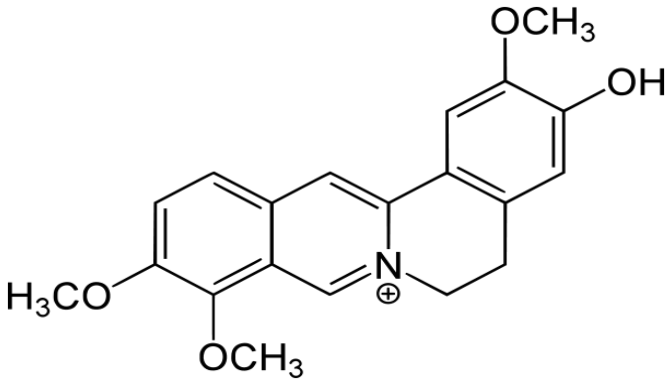


Heptacosanol

Table-9

Different types of Miscellaneous compounds isolated from various parts of *Tinospora cordifolia*

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, Jatrorrhizine, Tinosporidine, Cordifol, Cordifelone, N-trans-feruloyl tyramine as diacetate, Giloin, Giloinin, Tinosporic acid	Whole plant Root Whole plant



Jatrorrhizine

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 **[44].**

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 **[18].**

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 **[5].**

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 **[3].**

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 **[20].**

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 **[11].**

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% **[8].**

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 **[19].**

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 **[4].**

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.

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 **[7].**

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:

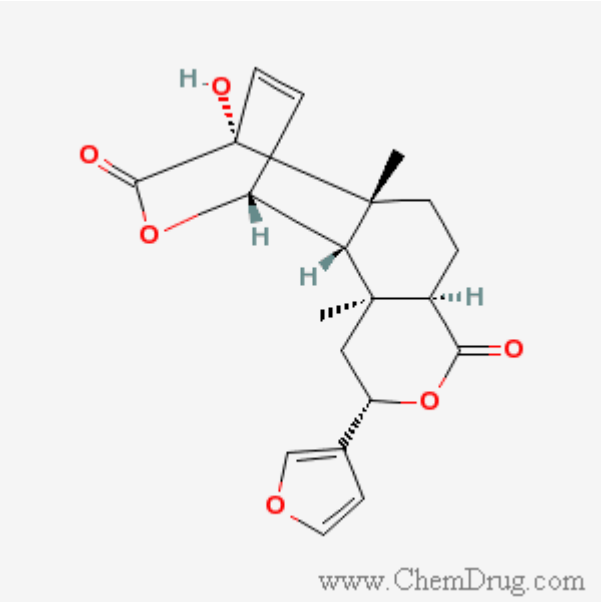
In this investigation, the antibacterial modes of action of Radix Tinosporae, its major single components, and nine antibiotics with different targets or modes-of-action on Staphylococcus aureus were studied. Metabolic profiles of cultures treated with different medicines were acquired by HPLC/ESI-MS. 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.

2.3 Tinospora tomentosa

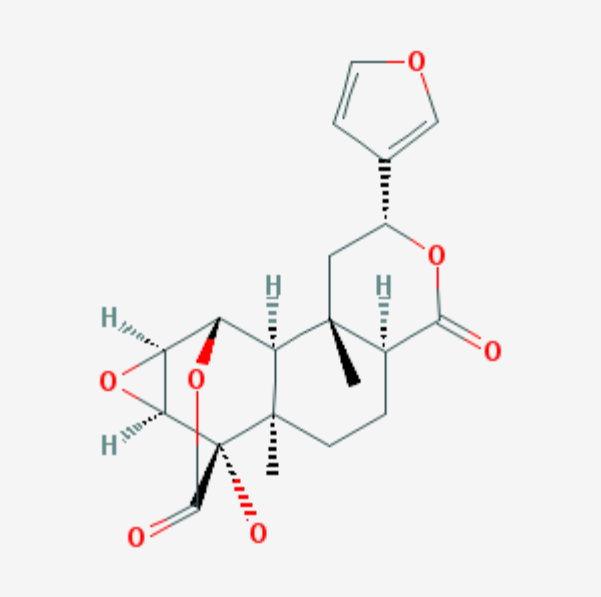
2.3.1 Phychemical Constituents

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 **[34].**

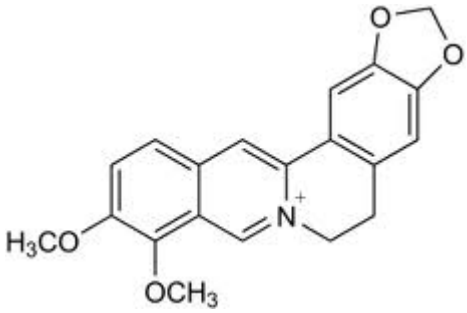
Chemical Constituents	**Part from which it is isolated**
Tinosporin	Leaf
Perberillin	Leaf
Palmarin	Leaf
Berberine	Leaf
Tinosporon	Leaf
Hepta consol tinosporic acid adntinosporol	Leaf
Giloin	Fresh stem bark
Giloinin	Fresh stem bark
Gilosterol	Fresh stem bark
Phenolic lignin	Fresh stem bark



Structure of tinosporin



Structure of palmarin



Structure of Phenolic lignin

2.3.2 Medicinal Properties:

The plant is used almost in the same way as T. cordifolia. It is reported to be used for fumigation in piles and ulcerated wounds, and for the preparation of medicated baths for liver-complaints. Boiled roots are given in fever. Fresh leaves and stems are used in chronic rheumatism. As per traditional use, the different parts like stems, leaves and roots of the plant are used as stomachic, bitter-tonic, anti-periodic, mild diuretic, emetic, anti-purgative, antipyretic, analgesic, antiinflammatory, anti-diabetic, anti-leprotic, anti-gout **[35].**

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 **[36].**

2.3.4 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 **[36].**

Chapter 3:

Preparation of plant extract for experiment

3.1 SELECTION OF PLANTS

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 **[21]**. 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.

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

3.8.1 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 **[22].**

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 **[22].**

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 **[22].**

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 REAGENTS AND SOLVENTS

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.

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.

Table 5.1: 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

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
11. DPPH
12. Folin & ciocalteu solution

3. Ammonium hydrooxide
4. $CHCl_3$
5. Formic acid
6. Ethyl acetate
7. EtOH
8. Water
9. Nutrient agar
10. H_2SO_4
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,

  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.

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

$$\text{Mo (VI)} + e \rightarrow \text{Mo ()}$$

4.5.2 Apparatus

Test tube	Pipette
Spatula	Analytical balance
Vortex	Uv-spectrophotometer

4.5.3 Reagents

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

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

4.6.2.3 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 **[24].**

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:

Table 5.2: Different concentration of standard protein in Lowry method **[25]**

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
Vortex mixer
Conical flask
Test tube
Beaker
Water bath
Pipette
UV spectrometer
Glass rod

4.6.3.3 Reagents

2% (w/v) Na_2CO_3
Bovine serum albumin fraction V
1 % (w/v) $CuSO_4.5H_2O$
2N NaOH

Distilled water. 1N Folin reagent

2% (w/v) sodium potassium tartrate

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) Sodium carbonate in distilled water.

  Solution B had 1 % (w/v) blue vitriol 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 **[25].**

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).

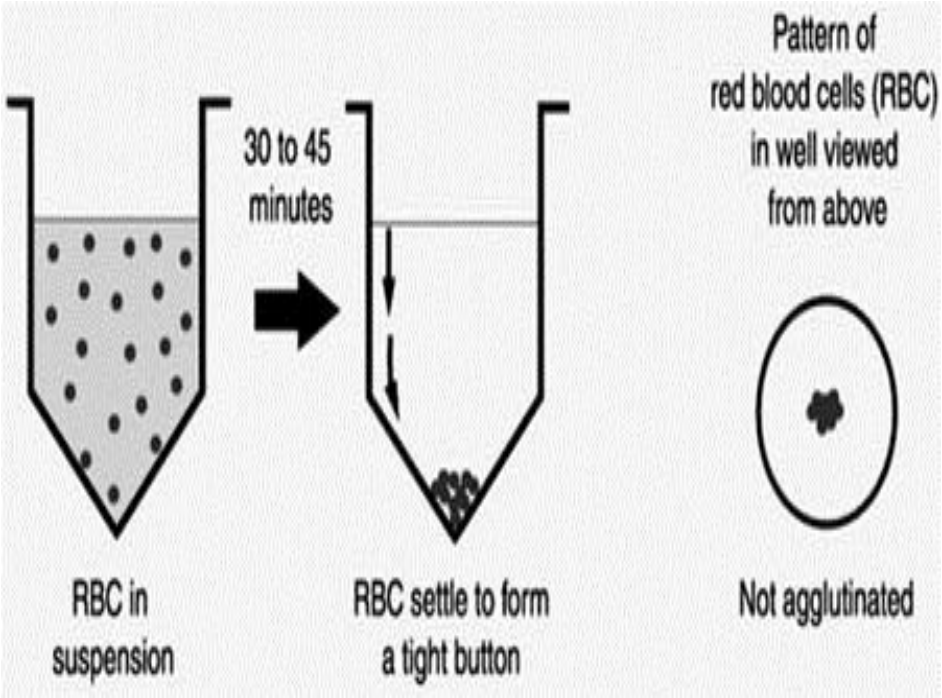


Figure 5.4: Formation of tight button with no agglutination

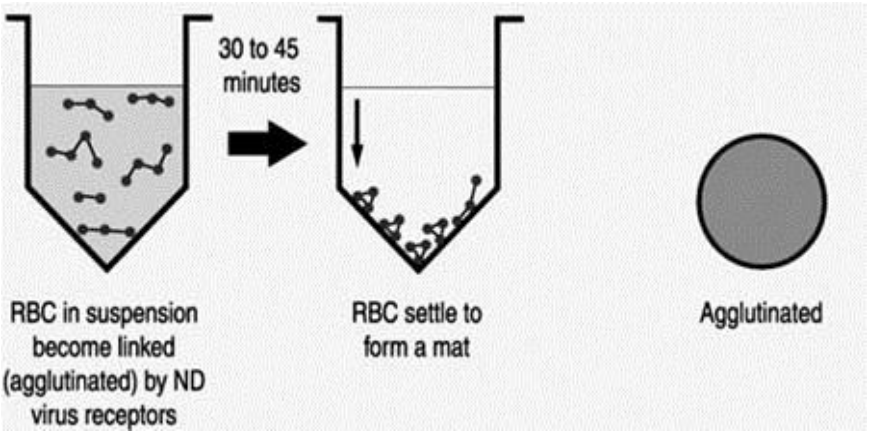


Figure 5.5: Formation of agglutination

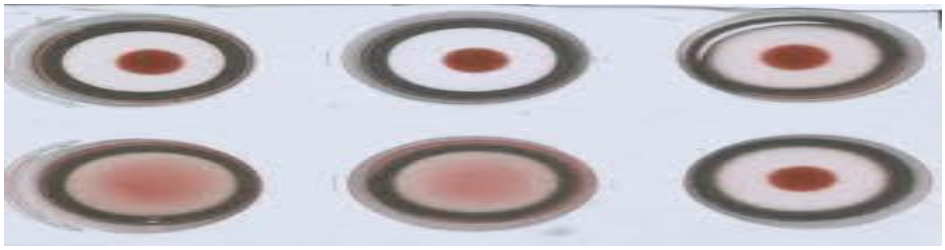


Figure 5.6: Overview of HA and not HA

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.

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.

4.9 CARBOHYDRATE DETECTION BY TLC

4.9.1 Apparatus

TLC plate

TLC tank

Pencil

UV lamp

Capillary tube

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.

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.

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).

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).

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.

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).

4.10.3 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

4.10.4 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 5.5: 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 5.6: Composition of nutrient agar medium

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 5.1: 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 5.2: 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.

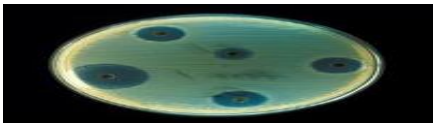


Figure 4.3: Zone of inhibition

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.

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 5.7: Composition of nutrient agar medium

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 5.8: Composition of nutrient broth medium

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.
- 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.

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 **[23].**

4.14 IR DATA ANALYSIS

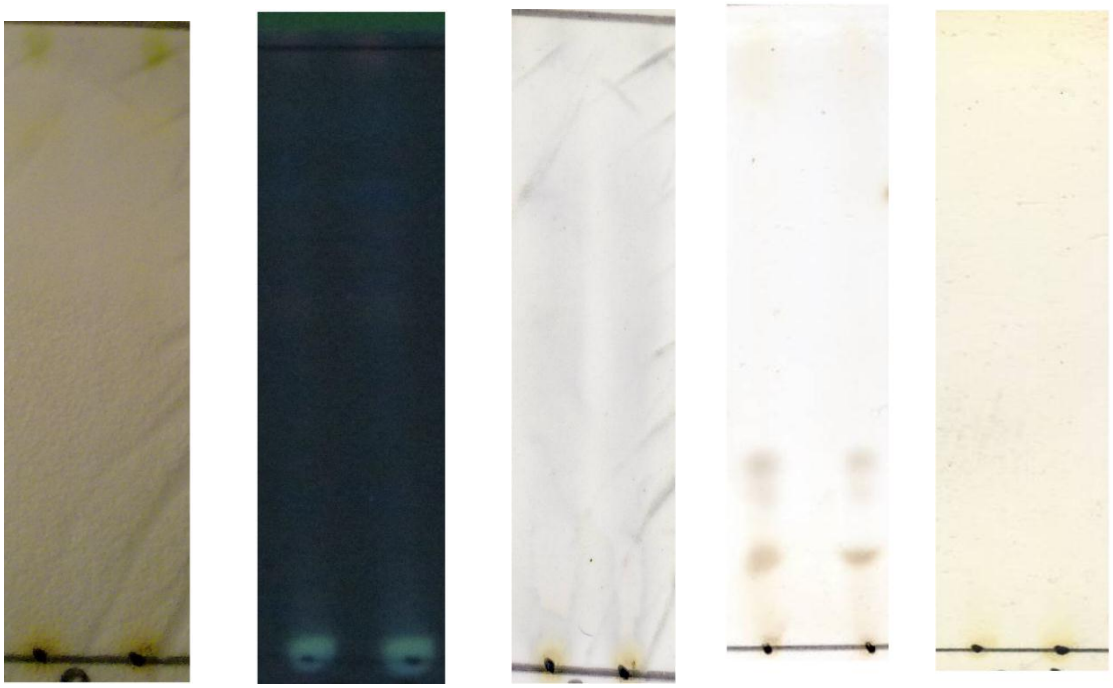
The sample was placed in a IR machine. The automated machine produces a graph showing all the functional groups present in the protein fraction obtained from Tinospora tomentosa.

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

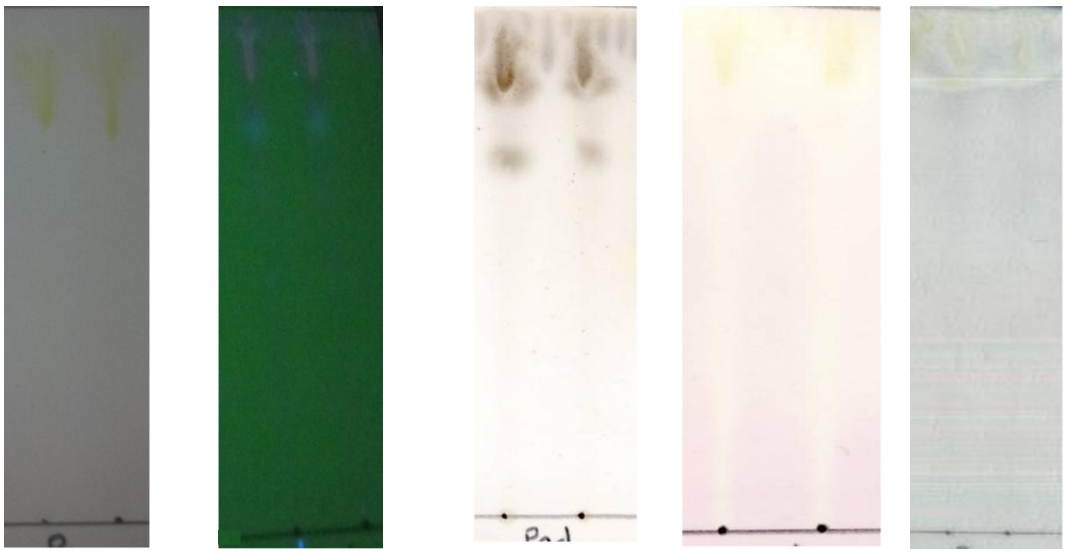
Figure 5.1: Results for TLC in nonpolar basic solvent (1= naked eye view; 2 = UV light view; 3= after application of FC reagent; 4= after charing; 5= after application of DPPH)

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

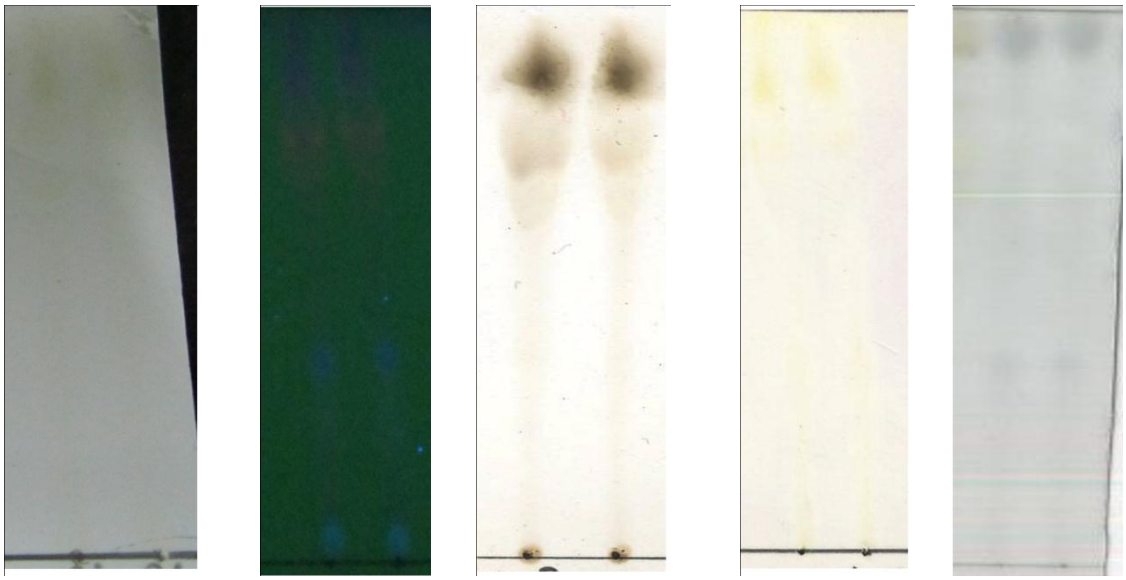
Figure 5.2: Results for TLC in intermediate polar basic solvent (1= naked eye view; 2 = UV light view; 3= after charing; 4= after application of DPPH solution; 5= after application of FC solution)

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 5.6: 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		

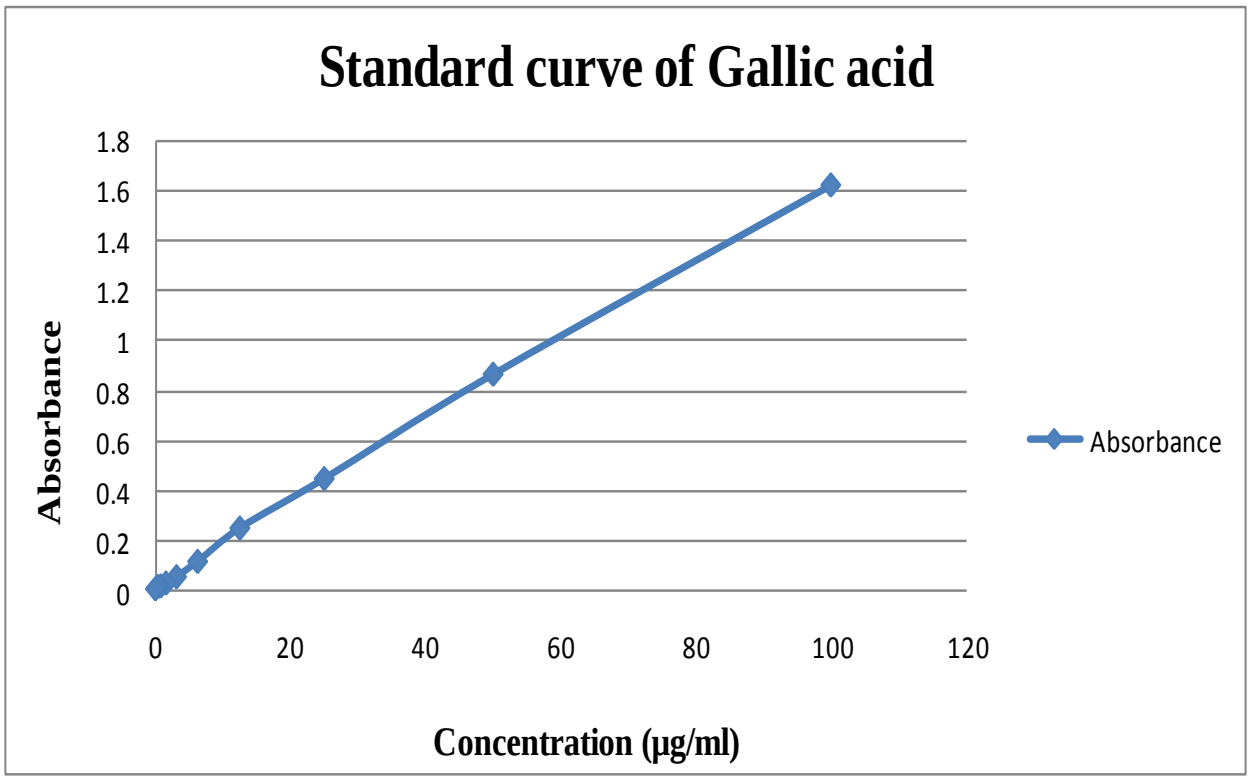


Figure 5.5: Standard curve of gallic acid

Table 6.7: 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.

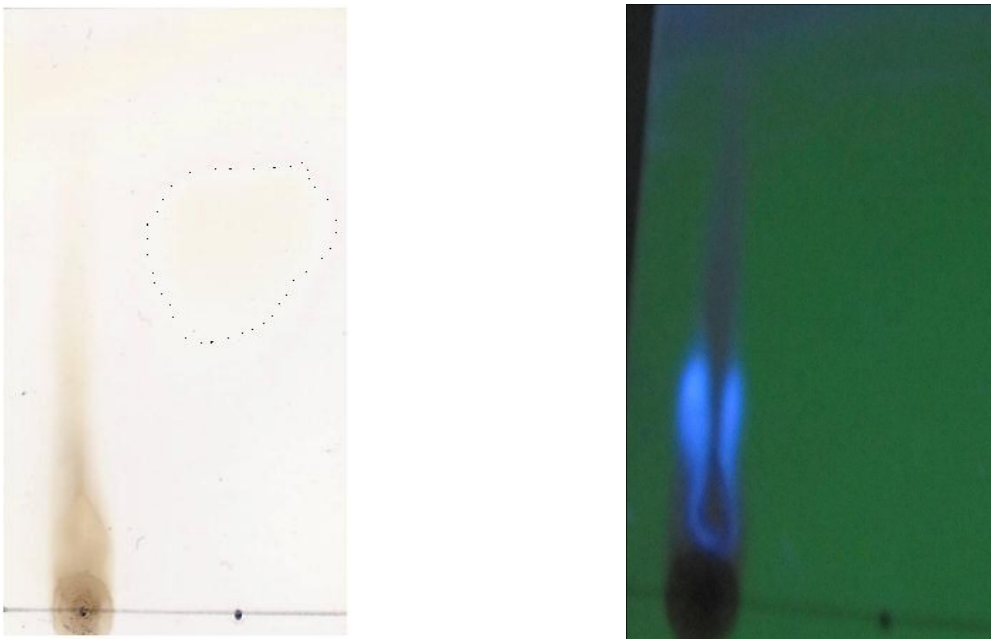
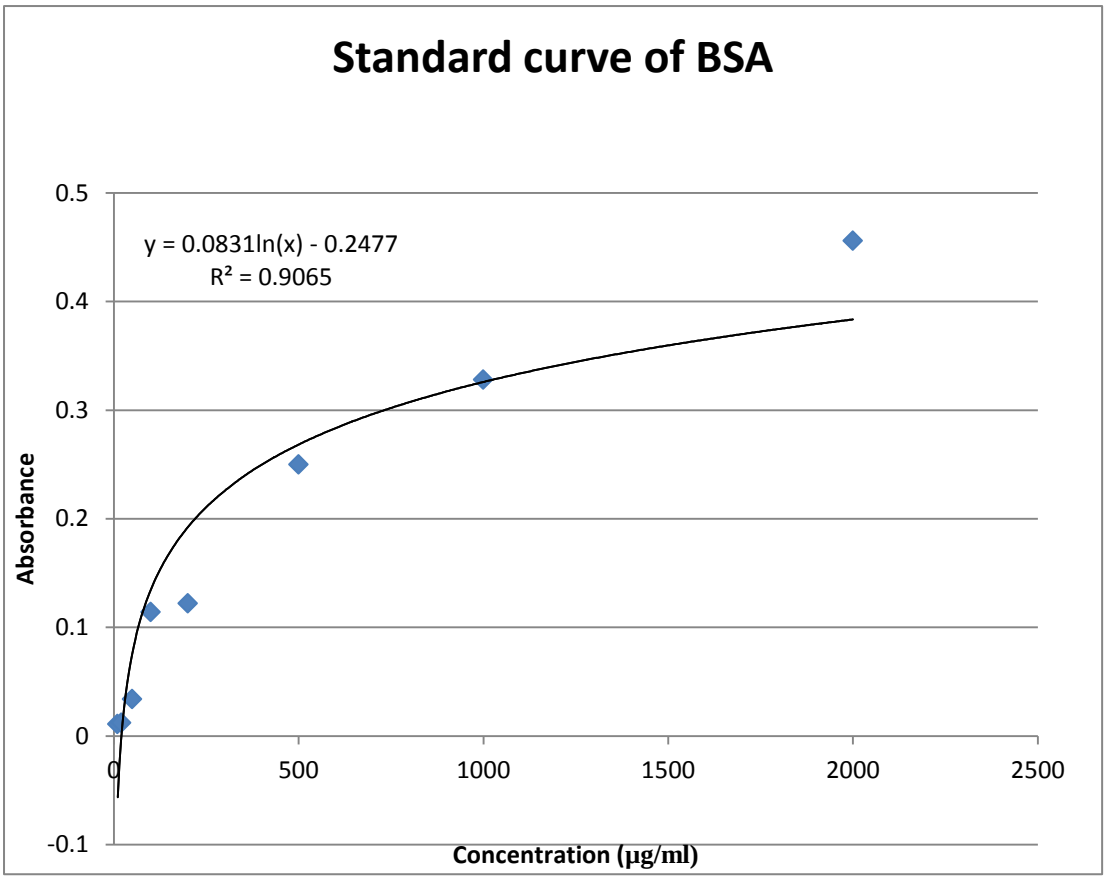


Figure 6.6: Protein detection eye view (left) and UV view (right)

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= $0.083\ln(X) - 0.247$, $R^2 = 0.906$.



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.

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 **[32].**

5.5 Minimum concentration required for Haemagglutination test:

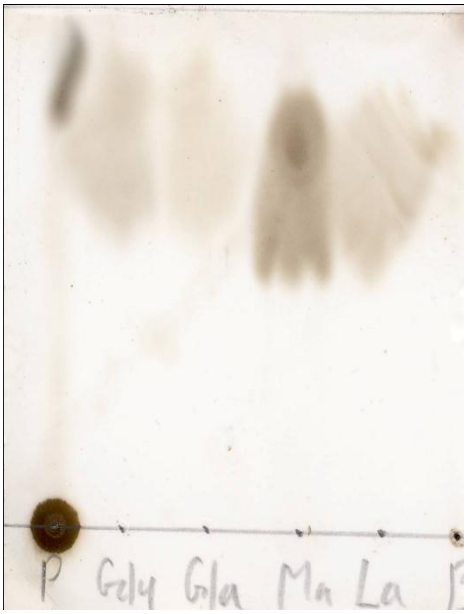
Table 5.6 Minimum concentration require for Haemagglutination test

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 **[32].**

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

Figure: 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:

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

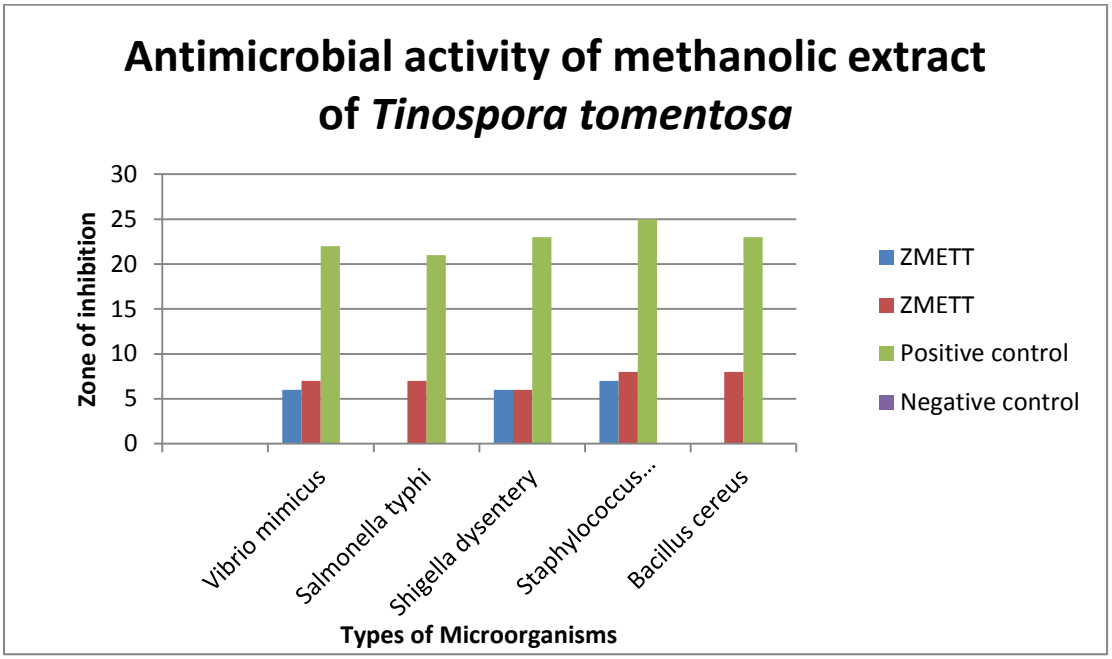
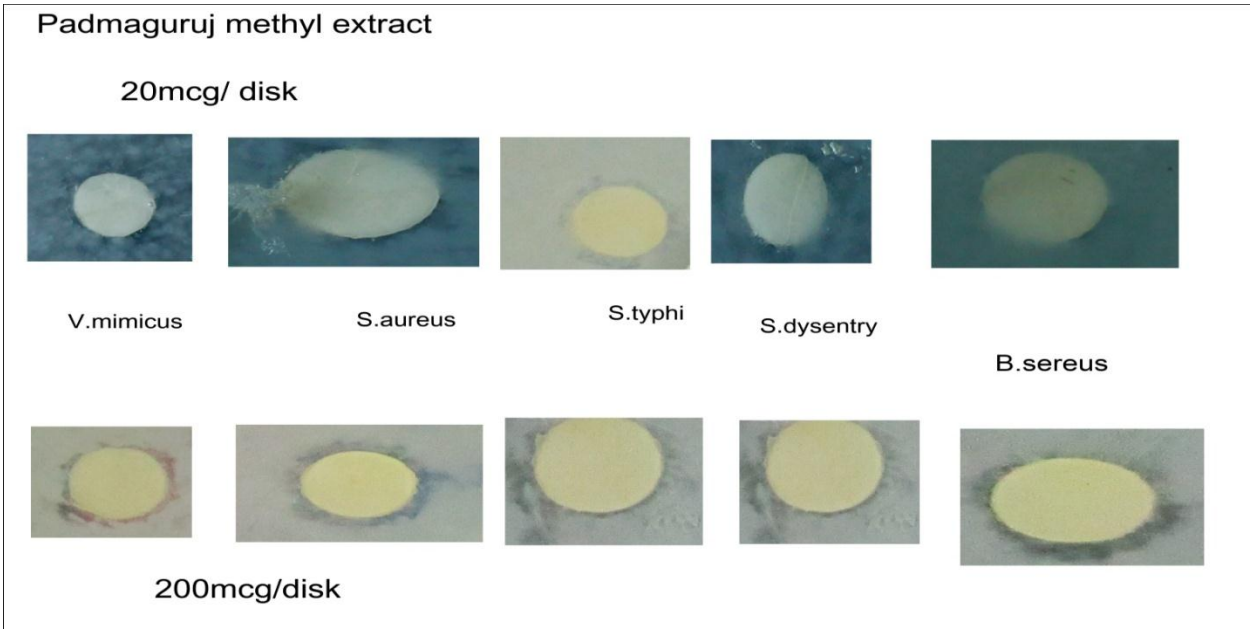


Figure 5.7 : Antimicrobial activity of Methanolic extract of *Tinospora tomentosa*

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 **[30].**



5.7.2 Antimicrobial sensitivity test 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

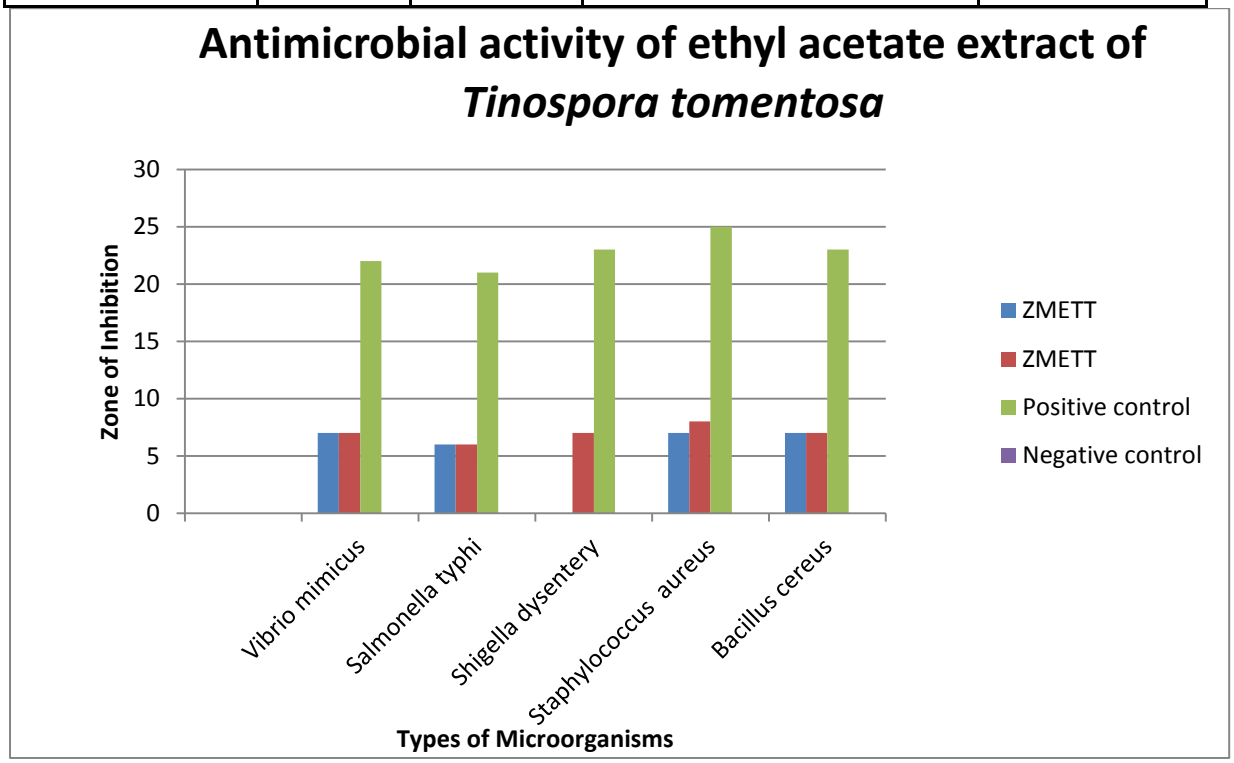
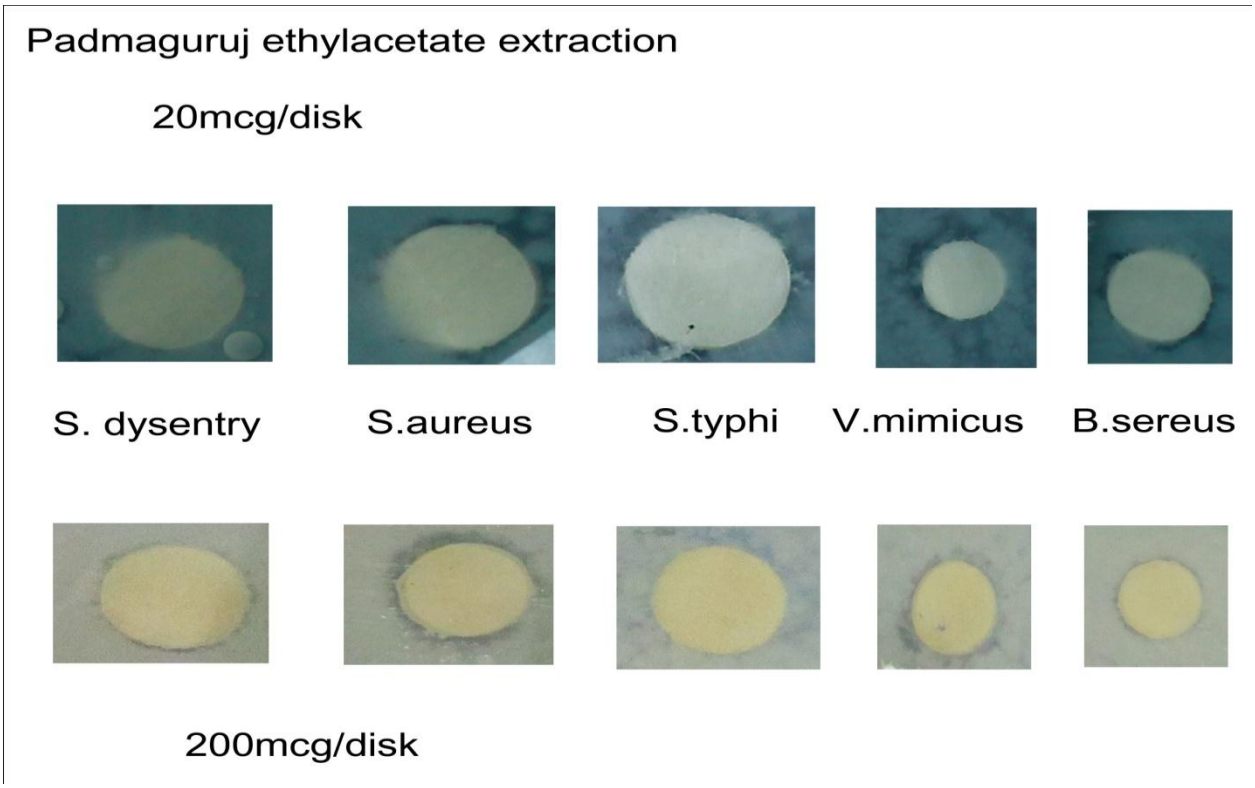


Figure 5.7: Antimicrobial sensitivity test of Ethyl-acetate extract of *Tinospora tomentosa*

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.



5.7.3 Anti Microbial Test of Protein Sample

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

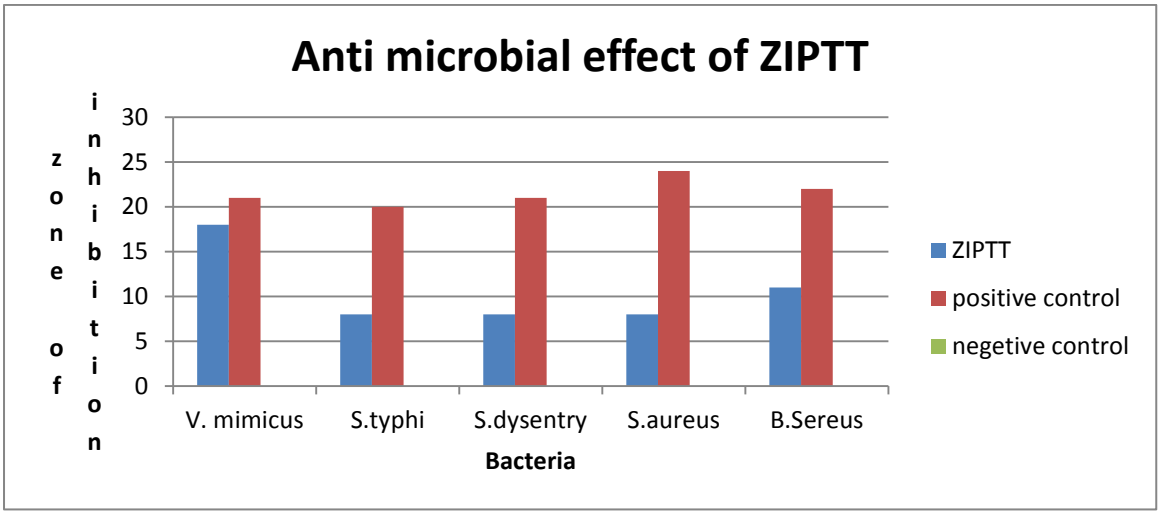
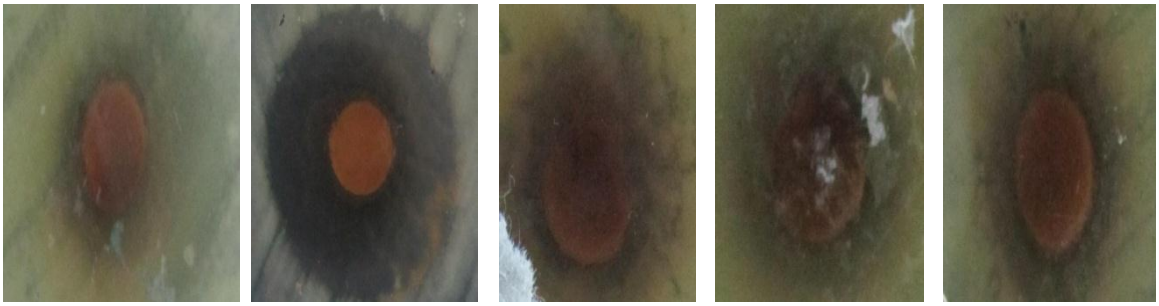


Figure 5.8 : antimicrobial activity of ZIPTT

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**

Figure 5.9: Anti microbial effect of IPTT on different type of bacterial

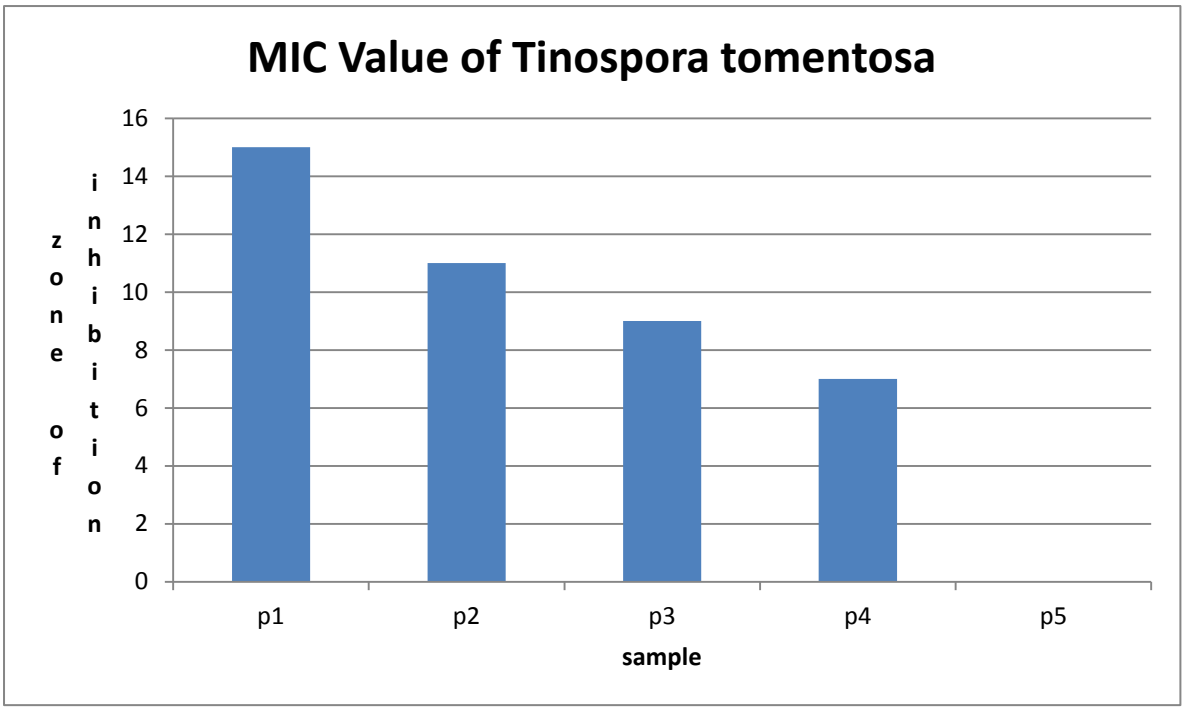
5.8 MIC

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 6.13: Zone of inhibition by MIC method

Sample	**Concentration** µg/disc	**Microorganism**	**MIC in Zone of Inhibition Method in mm**
ZIPTT	2000	*Vibrio mimicus*	15
	200		11
	20		9
	2		7
	0.2		0

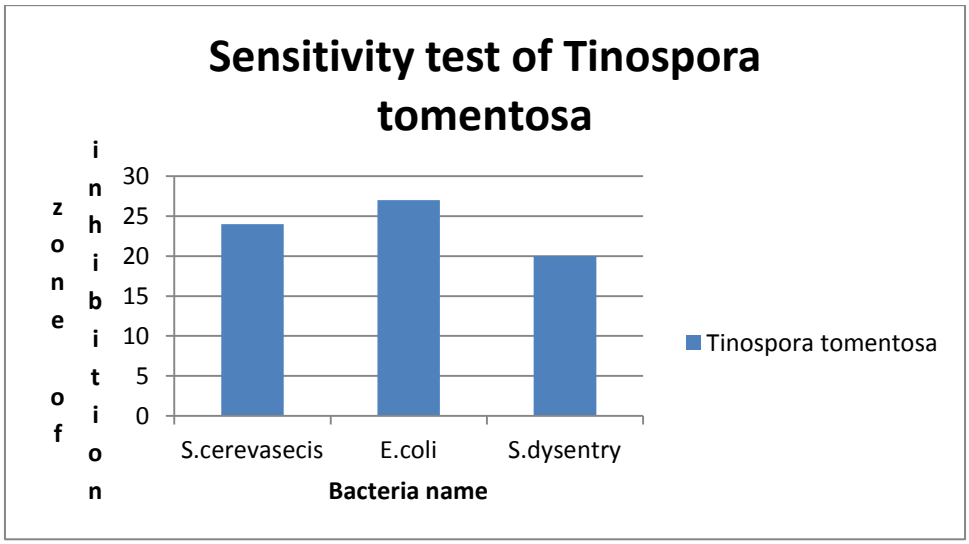
N.B: Z IPTT= zone of inhibition of isolating protein of *tinospora tomentosa.*

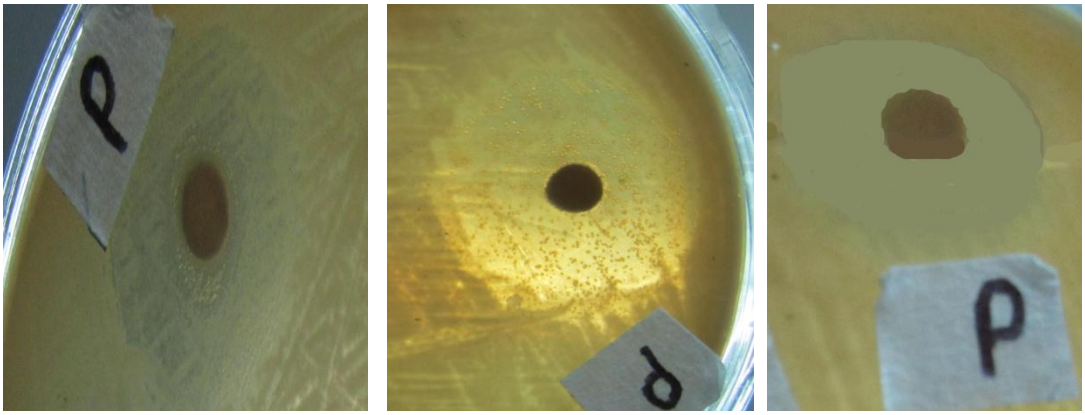


5.9 Sensitivity test:

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





S. cerevacesis E.coli S. dysentery

Figure 5.10: 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.

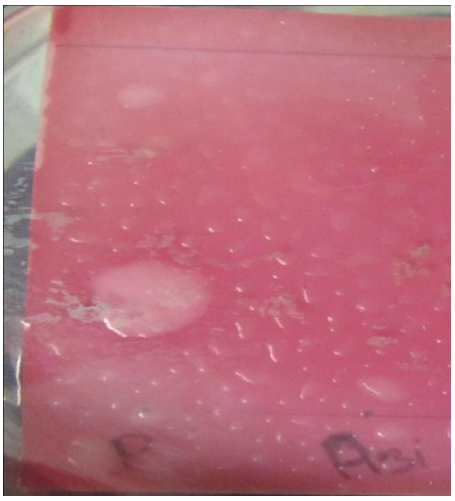


Figure: TLC bioautography

5.11 IR data result

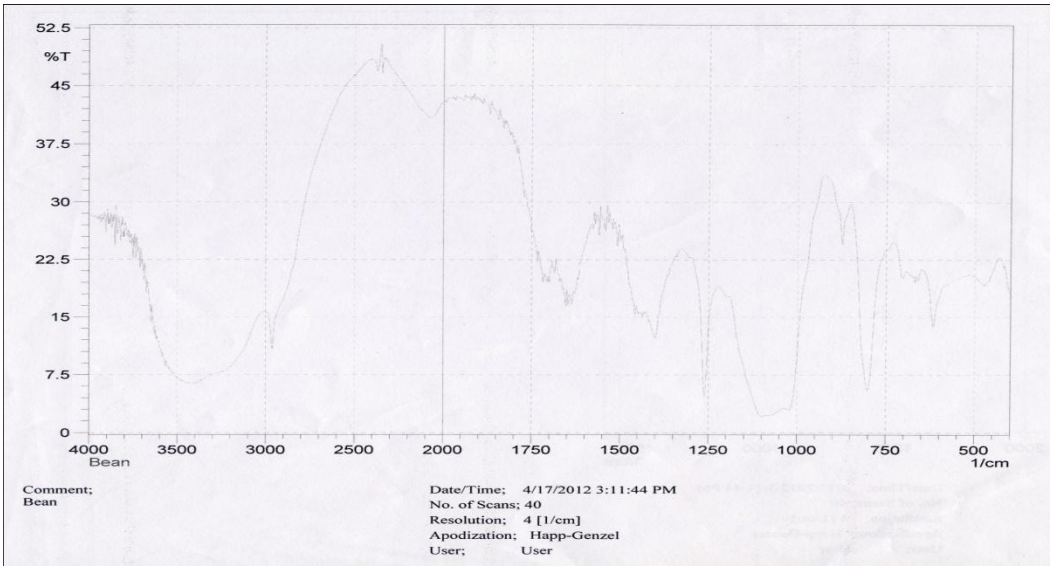


Figure: IR data of padmaguruj protein

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. Table: 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

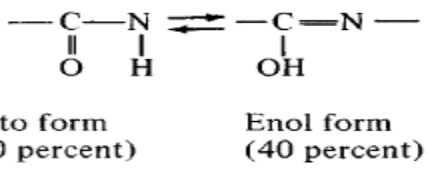
1550	Strong	amino acid zwitterions
1695	Medium	Enol
1710-1740	Medium	Aldehyde or keto with saturated aliph./cyclic 6-membered C=O (Saturated aldehyde/ketone)
1685	Medium	aromatic ketones/ α,β-unsaturated aldehyde or ketone
1200	Medium	Ether
1330,1430	Medium	O-H bend(in plane)
660,700,750	Weak	O-H bend(out 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.
- 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.



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 tomentosa*

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.

REFERENCES

1. Tiwari P., Kumar B., et al (2011). Phytochemical screening and Extraction: A Review. *Journal of Internationale Pharmaceutica Sciencia*, 1: 98-106.

2. Plant life (2012). *Metabolites: Primary vs Secondary*. Retrieved on 5th April from:

http://lifeofplant.blogspot.com/2011/03/metabolites-primary-vs-secondary.html

3. Bishayi B, R. S. (2002). *UKPMC Funders' Group*. Retrieved 06 06, 2012, from Hepatoprotective and immunomodulatory properties of Tinospora cordifolia in CCl4 intoxicated mature albino rats.

4. D. N. K. Sarma, R. L. (1995, 12). *john Wiley & Sons*. Retrieved 06 20, 2012, from Antiulcer activity of Tinospora cordifolia Miers and Centella asiatica linn extracts.

5. D. N. K. Sarma, R. L. (1998, 12 04). *John Wiley & Sons*. Retrieved 06 06, 2012, from Antistress Activity of Tinospora cordifolia and Centella asiatica Extracts:

6.Goel HC, P. K. (2002, 06). *Pubmed*. Retrieved 06 06, 2012, from Free radical scavenging and metal chelation by Tinospora cordifolia, a possible role in radioprotection.

7. Gupta RS, S. A. (2003, 08). *Pubmed*. Retrieved 06 20, 2012, from Antifertility effect of Tinospora cordifolia (Willd.) stem extract in male rats.

8. Harish Chandra GOEL1), *. P. (2004, 05 07). *Journal of Radiation Research*. Retrieved 06 06, 2012, from Radioprotective Potential of an Herbal Extract of Tinospora cordifolia

9. J.K Grovera, V. V. (2000, 06 29). *ScienceDirect*. Retrieved 04 2012, 2, from Journal of Ethnopharmacology

10. Jampani B. Hanuman, R. B. (2001, 03 01). *ScienceDirect*. Retrieved 06 20, 2012, from A diterpenoid furanolactone from Tinospora cordifolia:

11. Mathew S, K. G. (1997, 12). *Pubmed*. Retrieved 05 23, 2012, from Antioxidant activity of Tinospora cordifolia and its usefulness in the amelioration of cyclophosphamide induced toxicity.

12. Nirmala N. Rege, U. M. (1999, 6 18). *John Wiley & Sons*. Retrieved 06 06, 2012, from Phytotherapy Research

13. P.K. Raveendran Naira, S. J. (2006, 08 28). *ScienceDirect*. Retrieved 06 20, 2012, from Mechanism of macrophage activation by (1,4)-α-d-glucan isolated from Tinospora cordifolia

14. P.K. Raveendran Naira, S. R. (2004, 08 25). *ScienceDirect*. Retrieved 06 06, 2012, from International Immunopharmacology

15. P.V. Leyon, G. K. (2004, 08 12). *ScienceDirect*. Retrieved 06 20, 2012, from Effect of Tinospora cordifolia on the cytokine profile of angiogenesis-induced animals

16. Pragada Rajeswara Rao, V. K. (2005, 12 01). *Biological and Pharmaceutical Bulletin*. Retrieved 06 20, 2012, from Cardioprotective Activity of Alcoholic Extract of Tinospora cordifolia in Ischemia-Reperfusion Induced Myocardial Infarction in Rats

17. Rege NN, N. H. (1989, 12). *Pubmed*. Retrieved 06 20, 2012, from Modulation of immunosuppression in obstructive jaundice by Tinospora cordifolia

18. S Mathew, G. K. (2010, 01 10). *ScienceDirect*. Retrieved 06 06, 2012, from Fitoterapia

19. V.A. Badara, V. T. (2004, 11 23). *ScienceDirect*. Retrieved 06 06, 2012, from Efficacy of Tinospora cordifolia in allergic rhinitis

20. VK Pendse, A. D. (1977). *Indian Journal og Pharmacology*. Retrieved 06 20, 2012, from Antiinflammatory, immunosuppressive and some related pharmacological actions of the water extract of Neem Giloe (Tinospora cordifolia): A preliminary report

21. Singh J. (2012). Maceration, Percolation and Infusion Techniques of Extraction of Medicinal and Aromatic Plants. Retrieved on 10th April, 2010.

22. Wikipedia (2012). Sample Concentration By Rotary Evaporation Technique. Retrieved on 12th, 2012 may from: http://en.wikipedia.org/wiki/Rotary_evaporator

23. Yesilada E., Tanaka S. et al (1988). Isolation of an Anti-inflammatory Principle from the Fruit Juice of Ecballium elaterium. *Journal of Natural Products*, 51 (3): 504-508.

24. Holland W. L., Erinn C. S. et al (2003). Quantification of phosphatidic acid and lysophosphatidic acid by HPLC with evaporative light-scattering detection. *Journal of Lipid Research*, 44: 854-858.

25. Bhunia A.K., Jhonson M.C. (1987). Direct detection of an antimicrobial peptide of *Pediococcus acidilactici* in sodium dodecyl sulfate-polyacrylamide gel electrophoresis**.** *Journal of Industrial Microbiology & Biotechnology*, 2: 319-322.

26. Velioglu Y. S., Mazza G., Gao, L., Oomah B. D. (1998). Antioxidant activity and total phenolics in selected fruits, vegetables and grain products. *J Agric Food Chem*, 46:4113-4117.

27. Zarai Z., Kadri A. (2011). The in-vitro evaluation of antibacterial, antifungal and cytotoxicproperties of Marrubium vulgareL. essential oil grown in Tunisia. *Lipids in Health and Disease,* 10: 161-170.

28. Ahmed b., Azam S. et al (2011). Phytotoxic, Antibacterial and Haemagglutination activities of the aerial parts of *Myrsine africana* L.

29. Ayafor, J.F. et al. (1972). *Tetrahedron*, 28**:**9343.

30. Barry A.L. (1976) .Principle & practice of Microbiology. *Lea & Fabager,*

31. Sujogya K.P., Pandhi L.P. (2011). Antibacterial activities and phytochemical analysis of *Cassia fistula* (Linn.) leaf. *Journal of advanced Pharmaceutical Technology and Research,* 2(1): 61-67.

32. Wikipedia (2012). Principle of HA method. Retrieved on 3rd June, 2012 from: http://en.wikipedia.org/wiki/Hemagglutination

33. Bhunia A.K., Jhonson M.C. (1987). Direct detection of an antimicrobial peptide of *Pediococcus acidilactici* in sodium dodecyl sulfate-polyacrylamide gel electrophoresis**.** *Journal of Industrial Microbiology & Biotechnology*, 2: 319-322.

34. T.cordifolia, an important drug plant of traditional medicine . Retrieved from http://www.science20.com/humboldt_fellow_and_science/blog/tinospora_sinensis_lour_merr_syn_t_malabaricamiers_ex_hook_f_t_tomentosa_miers_ex_hook_f_simila

35. Chemical constituent of *Tinospora tomentosa*,. Retrieved from http://www.liveindia.com/herbs/Giloy_Herb.html

36.http://www.science20.com/humboldt_fellow_and_science/blog/tinospora_sinensis_lour_merr_syn_t_malabaricamiers_ex_hook_f_t_tomentosa_miers_ex_hook_f_simila

37. Scientific classification of *Tinospora tomentosa* http://zipcodezoo.com/Plants/T/Tinospora_tomentosa/

38. Sher A. (2009). Antimicrobial activity of natural products from medicinal plants. *Gomal Journal of Medical Sciences*, 7:72-78.

39. Shankhala L.N (2012), Chemical and biological properties of Tinospora cordifolia, *Gomal Journal of Medical Sciences*, page 340-345

40. Samy R.P., Pushparaj P.N., Gopalakrishnakone P. (2008). A compilation of bioactive compounds from ayurveda. *Journal of Bioinformation,* 3(3): 100-110.

41. Faysal M.M. (2008). Justification of use of some Medicinal Plants to Treat Various Diseases in Khulna,Bangladesh. *Journal of Ethnobotanical Leaflets*, 12: 1231-1235.

42. 2003, I. J., & 83-91, 3. (2012). a review on chemical & biological property of tinospora cardifora. *Indian Journal of Pharmacology* , 1-5.

43. Javeed Ahmed Wani, R. N. (2011). Phytochemical Screening and Aphrodisiac Property of Tinospora cordifolia. *International Journal of Pharmaceutical and Clinical Research* , 1-6.

44. S. Mohana Lakshmi*, Y. B. (2012). Aphrodisiac agents from medicinal plants: an ethnopharmacological and phytochemical review. *Journal of Pharmacy* , 1-4.

45. S.S. SINGH, S. P. (2003). CHEMISTRY AND MEDICINAL PROPERTIES OF TINOSPORA CORDIFOLIA. *Indian Journal of Pharmacology* , 1-9.

46. Ahmed b., Azam S. et al (2011). Phytotoxic, Antibacterial and Haemagglutination activities of the aerial parts of *Myrsine africana* L. *African Journal of Biotechnology*, 10(1): 97-102.