IN VITRO PHARMACOLOGICAL & PHYTOCHEMICAL INVESTIGATION ON N-HEXANE FRACTION OF *FICUS HISPIDA* LEAVES EXTRACT

A Dissertation submitted to the Department of Pharmacy, East West University, in partial Fulfillment of the requirements for the degree of Bachelor of Pharmacy.

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DECLARATION BY THE CANDIDATE

I, Shahminajada, hereby declare that this dissertation, entitled "*In Vitro* Pharmacological & Phytochemical Investigation On n-Hexane Fraction Of *Ficus Hispida* Leaves Extract" Submitted to the Department of Pharmacy, East West University, in the partial fulfillment of the requirement for the degree of Bachelor of Pharmacy (Honors) is a genuine & authentic research work carried out by me under the guidance of Abdullah-Al-Faysal, Senior Lecturer, Department of Pharmacy, East West University, Dhaka. The contents of this dissertation, in full or in parts, have not been submitted to any other Institute or University for the award of any Degree or Diploma of Fellowship.

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CERTIFICATION BY THE SUPERVISOR

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DEDICATION

This Research paper is dedicated to

My beloved Parents,

Who are my Biggest Inspiration…

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ABSTRACT

The study was designed for pharmacological & phytochemical investigation of n-Hexane fraction of methanol extract of the leaves of *Ficus hispida* (Family: Moraceae). The powdered leaves of *Ficus hispida* were extracted with methanol and then partitioned with n-hexane, dichloromethane and ethyl acetate consecutively. The n-Hexane fraction remaining was investigated for total flavonoid content, total phenol content, brine shrimp lethality test. The fraction contained 43.125 mg AAE/gm of dried extract in total phenolic content assay and 29.39623 mg quercetin/gm of dried extract in total flavaniod content assay. Screening for cytotoxic properties using brine shrimp lethality bioassay with tamoxifen (LC_{50} value of 0.78726 µg/ml) as positive control showed that the fraction have considerable cytotoxic potency exhibiting LC_{50} value 1.13675 µg/ml. The n-Hexane fraction showed weak cytotoxic activity, low antioxidant activity. Further investigations are needed for the proper identification and isolation of these bioactive compounds to produce safer drugs for treatment of harmful diseases.

Key words: *Ficus hispida*, Brine shrimp lethality bio-assay, phenolic content, flavonoid content.

CHAPTER-ONE

INTRODUCTION: HISTORY, DEVELOPMENT & REVOLUTION OF DRUGS

Introduction: History, Development & Revolution of drugs

1.1. Role of Traditional medicine in Human society

The traditional medicines have been playing a significant role in the human society for centuries. Traditional medical practice illustrates the medical knowledge practices, which developed centuries ago within a variety of societies before the era of modern allopathic or homeopathic medication begins. Ayurvedic medicine, traditional Chinese medicine, Unani, herbal, African Yoruba Ifa, Muti as well as many other ancient medical practices from all over the world included in these medicines.

Historically, at the end of the twentieth century, a number of traditions came to dominate the practice of traditional medicine. Among all, the herbal medicine system of ancient Greek and ancient Roman sources, the Ayurvedic medicine system from India, traditional Chinese medicine, Unani-Tibb medicine and Shamanic Herbalism were the most dominant at the end of the twentieth century (Medical Reference 2012).

1.2. History of Drug discovery and Development

1.2.1. Early history of medicine

Drug discovery and development has a long history and dates back to the early days of human civilization. In those ancient times, drugs were not just used for physical remedies but were also associated with religious and spiritual healing. Sages or religious leaders were often the administrators of drugs. The early drugs or folk medicines were derived mainly from plant products and supple-mented by animal materials and minerals. These drugs were most probably discovered through a combination of trial and error experimentation and observation of human and animal reactions as a result of ingesting such products.

Although these folk medicines probably originated independently in different civilizations, there are a number of similarities. For example, in the use of the same herbs for treating similar

diseases. This is likely to be a contribution by ancient traders, who in their travels might have assisted the spread of medical knowledge.

Folk medicines were the only available treatments until recent times. Drug discovery and development started to follow scientific techniques in the late 1800s. From then on, more and more drugs were discovered, tested, and synthesized in large - scale manufacturing plants, as opposed to the extraction of drug products from natural sources in relatively small batch quantities.

After World War I, the modern pharmaceutical industry came into being, and drug discovery and development following scientific principles was firmly established.

Although pharmaceutical drugs are now widely used worldwide, many ethnic cultures have retained their own folk medicines. In certain instances, these folk medicines exist side by side and are complemented by pharmaceutical drugs.

The following are some snapshot examples of how drugs were discovered from the early human civilizations.

1.2.1.1. Chinese Medicine

Traditional Chinese medicine (TCM) is believed to have originated in the times of the legendary emperor Sheng Nong in 3500 BC. Some important medical writings are Shang Han Lun (Discussion of Fevers), and Sheng Nong Ben Cao Jing (The Pharmacopoeia of Sheng Nong — a legendary emperor).

The Chinese pharmacopoeia is extensive. Some of the active ingredients from Chinese herbs have been used in Western drugs. For example, reserpine from Rauwouofia for antihypertensive and emotional and mental control, and the alkaloid ephedrine from Mahuang for the treatment of asthma.

Figure 1.1: Chinese Medicine (Anticancer) (Easy Health Options 2017)

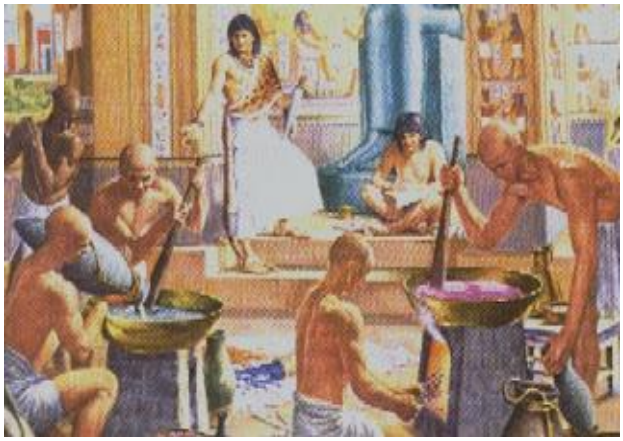


Figure 1.2: Egyptian Medicine (Ancient Egypt 2011)

1.2.1.2. Egyptian Medicine

Ancient papyrus provided written records of early Egyptian medical knowledge. The Ebers papyrus (from around 3000 BC) provided 877 prescriptions and recipes for internal medicine, eye and skin problems, and gynecology.

1.2.1.3. Indian Medicine

The Indian folk medicine, called Ayurvedic medicine can be traced back 3000 – 5000 years and was practiced by the Brahmin sages of ancient times. The treatments were set out in sacred writings called Vedas. The material medica are extensive and most are based on herbal formulations. Some of the herbs have appeared in Western medicines, such as cardamom and cinnamon. Susruta, a physician in the fourth century ad, described the use of henbane as antivenom for snakebites.

Figure 1.3: Indian Medicine (MINERVAH 2013)

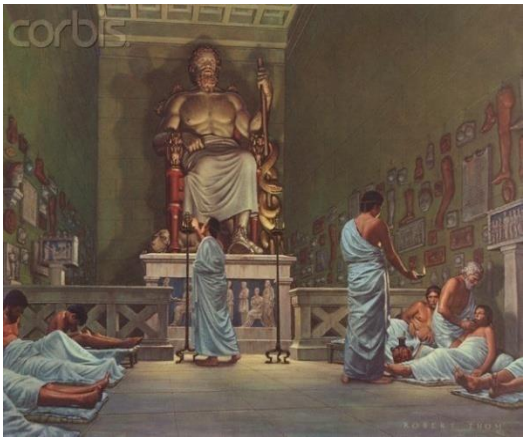


Figure 1.4: Greek Medicine (Forbidden Histories 2015)

1.2.1.4. Greek Medicine

Some of the Greek medical ideas were derived from the Egyptians, Babylonians, and even the Chinese and Indians. Castor oil was prescribed as a laxative; linseed or flaxseed was used as a soothing emollient, laxative and antitussive. The Greeks established that diseases result from natural causes. Hippocrates, the father of medicine, at about 400 bc is credited with laying down the ethics for physicians.

1.2.1.5. Roman Medicine

As great administrators, the Romans instituted hospitals, although these were used mainly to cater to the needs of the military. Through this work, organized medical care was made available. The Romans also extended the pharmacy practice of the Greeks. Dioscorides and Galen were two noted physicians in Roman days. Dioscorides's Materia Medica contains descriptions of treatments based on 80% plant, 10% animal, and 10% mineral products.

Figure 1.5: Roman Medicine (Pinegreenwoods 2015)

1.3. Drug discovery and development in the middle ages

The Middle Ages, from around ad 400 to 1500, witnessed the decline of the Roman influence. This was also the time when plagues scourged many parts of Europe. Diseases such as bubonic plague, leprosy, smallpox, tuberculosis, and scabies were rampant. Many millions of people succumbed to these diseases.

1.3.1. The Early Church

There are some references to herbs in the Bible. However, the Church's main contribution to medicines is the preservation and transcription of Greek medical manuscripts and treatises. This enabled the knowledge developed in the ancient times to be continued and later used in the Renaissance period.

1.3.2. Arabian Medicine

Through trade with many regions, the Arabians learned and extended medical knowledge. Their major contribution is perhaps the knowledge of medical preparations and distillation methods, although the techniques were probably derived from the practices of alchemists. Avicenna, around ad 900 – 1000, recorded a vast encyclopedia of medical description and treatment. Another noted physician was Rhazes, who accurately described measles and smallpox.

Figure 1.6: The Early Church (CHRISTIAN ART & GIFTS 2006)

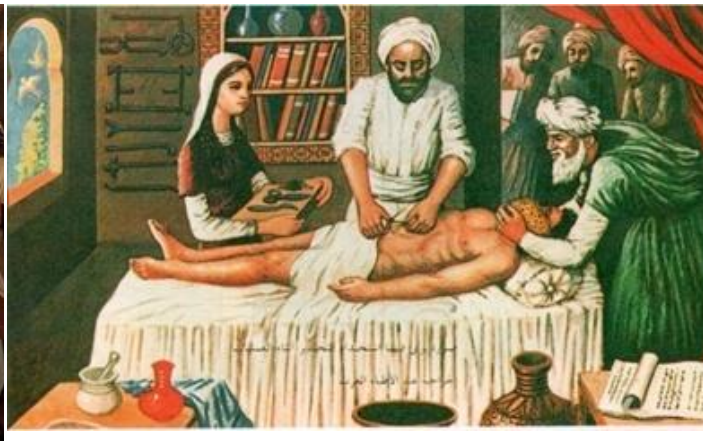


Figure 1.7: Arabian Medicine (Takrouri M S 2005)

1.4. Foundation of current drug discovery and development:

The Renaissance period laid the foundation for scientific thoughts in medicinal preparations and medical treatments. There were many advances made in anatomy, physiology, surgery, and medical treatments, including public health care, hygiene, and sanitation.

1.4.1. Smallpox

In 1796, Edward Jenner successfully experimented with smallpox inoculations. This paved the way for the use of vaccination against some infectious diseases.

1.4.2. Digitalis

In the late 1700s, William Withering introduced digitalis, an extract from the plant foxglove, for treatment of cardiac problems.

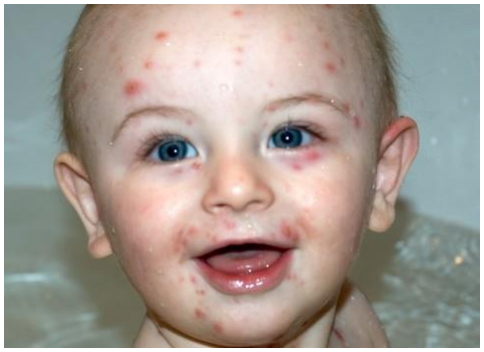


Figure 1.8: Small pox (VANITHA 2013)

Figure 1.9: Digitalis (HOW IT WORKS)

1.4.3. Scurvy

John Hunter (1768) noted that scurvy was caused by the lack of vitamin C. He prescribed the consumption of lemon juice to treat scurvy.

1.4.4. Rabies

Louis Pasteur (1864) discovered that microorganisms cause diseases, and he devised vaccination against rabies. This was achieved through the use of attenuated rabies virus.

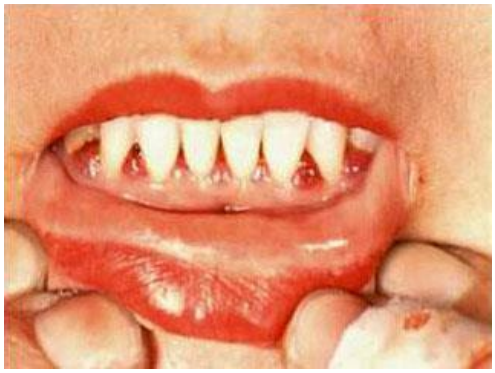


Figure 1.10: Scurvy (WOMEN HEALTH ZONE)

Figure 1.11: Rabies (Australian Government, 2016)

1.5. Beginnings of modern pharmaceutical industry

Despite the advances made in the 1800s, there were only a few drugs available for treating diseases at the beginning of the 1900s:

1.5.1. Digitalis:

Extracted from a plant called foxglove, digitalis stimulates the cardiac muscles and was used to treat cardiac conditions.

1.5.2. Quinine:

Derived from the bark of the Cinchona tree, quinine was used to treat malaria.

1.5.3. Ipecacuanha:

Extracted from the bark or root of the Cephaelis plant, ipecacuanha was used to treat dysentery.

1.5.4. Aspirin:

Extracted from the bark of willow tree, aspirin was used for the treatment of fever.

1.5.5. Mercury:

This was used to treat syphilis.

More systematic research was being performed to discover new drugs from the early 1900s.

Figure 1.12: Cinchona tree (Quinine) **(**Wildscreen Arkive,2017)

Figure 1.13: *Cephaelis ipecacuanha* (Dysentry) (PharmaWiki,2007)

Figure 1.14: Willow Tree (Aspirin) (Anna's Art Blog 2012**)**

Figure 1.15: Penicillin (WIKIPEDIA)

1.5.6. Penicillin

In 1928, Alexander Fleming discovered that Penicillium mold was active against staphylococcus bacteria. Ernst Chain rediscovered this fact some 10 years later, when he collaborated with Howard Florey. By 1944, large - scale production of penicillin was available through the work of Howard Florey and Ernst Chain. This work foreshadowed the commencement of biotechnology, where microorganisms were used to produce drug products.

1.6. Evolution of drug products

In the early days, until the late 1800s, most drugs were based on herbs or extraction of ingredients from botanical sources. The synthetic drugs using chemical methods were heralded at the beginning of the 1900s, and the pharmaceutical industry was founded. Many drugs were researched and manufactured, but mostly they were used for therapeutic purposes rather than completely curing the diseases. From the early 1930s, drug discovery concentrated on screening natural products and isolating the active ingredients for treating diseases. The active ingredients are normally the synthetic version of the natural products. These synthetic versions, called new chemical entities (NCEs), have to go through many iterations and tests to ensure they are safe, potent, and effective.

In the late 1970s, development of recombinant DNA products utilizing knowledge of cellular and molecular biology commenced. The biotechnology industry became a reality. The pharmaceutical industry, together with the advances in gene therapy and understanding of mechanisms of causes of diseases, and the research results from the Human Genome Project have opened up a plethora of opportunities and made possible the development and use of drugs specifically targeting the sites where diseases are caused (Smith CG, O' Donnell JT,eds).

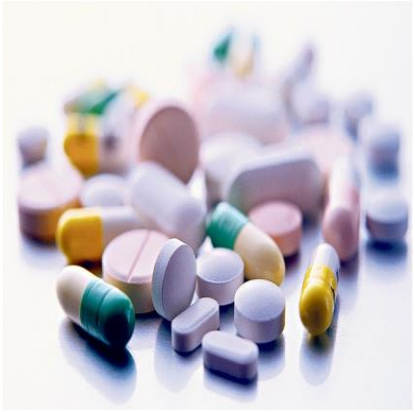


Figure 1.16: Depressant drugs (Imgarcade)

Figure 1.17: Health care (Supercarehealth)

1.7. Sources for Medications and Therapeutic Drugs

1.7.1. Medical Drug Sources

Therapeutic and Pharmaceutical drugs are commonly called medications, medicine, pills, or just "drugs". There are many sources of drugs and many resources are readily available to help identify, dispense, and administer medications competently, safely and in accordance with the law. Whenever healthcare professionals join the workforce in a medical office, free standing clinic, or hospital, they are faced with the fact that part of the everyday job routine is to deal with drugs.

1.7.1.1. Pharmacology

The subject of pharmacology was known as Materia Medica until 1890s when the current term began to come into use. Pharmacology is defined as the study of drugs and their actions. The sub-sciences of pharmacology and their specific fields of study are as follows:

1.7.1.2. Pharmacognosy

The recognition, quality, purity and identifications of drugs.

1.7.1.3. Pharmacy

The preparation, stability, preservation, and storage of pharmaceutical preparations.

1.7.1.4. Posology

Dosage or amount of drugs to be administered.

1.7.1.5. Pharmacodynamics

The response of living tissue to chemical stimulation in the absence of disease. This almost exclusively deals with research and development.

1.7.1.6. Pharmacotherapeutics

The action of drug on living tissue in the presence of disease, treatment of the sick.

1.7.1.7. Toxicology

The study of toxic and poisonous effects of substances.

1.7.2. Sources

There are several sources from which medications derived. Drugs are derived from the following main sources

1.7.2.1. Plant sources

Some Obtained from plant parts or products. Seeds, Stem, Roots, Leaves, Resin and other parts yield these drugs. Example includes digitalis and opium.

1.7.2.2. Animal sources

Glandular products from animals are used, such as insulin and thyroid.

Figure 1.18: Plant (Flower Pictures 2010)

Figure 1.19: Frog (WIKIMEDIA COMMONS)

1.7.2.3. Mineral sources

Some drugs are prepared from minerals. For example, potassium chloride and lithium carbonate (An antipsychotic).

1.7.2.4. Synthetic sources

Laboratories duplicate natural processes. Frequently this can eliminate side effects and increase the potency of the drug. Example includes Barbiturates, Sulfonamides, and aspirin (MA Pharm.com).

Figure 1.20: Minerals (Publish Your Article 2015)

Figure 1.21: Synthesis (COPERNICUS SCIENCE CENTRE)

1.8. Plant-based Drugs and Medicine

Today there are at least 120 distinct chemical substances derived from plants that are considered as important drugs currently in use in one or more countries in the world. Several of the drugs sold today are simple synthetic modifications or copies of the naturally obtained substances. The original plant substance/chemical name is shown under the "Drug" column rather than the finished patented drug name.

1.8.1. Anti-inflammatory agents

Drugs and Physical therapies used to reduce inflammation, e.g. non-steroidal anti-inflammatory drugs (NSAIDs), low-level laser therapy, therapeutic ultrasound, contrast foot baths, icing and complementary therapies. For example: **Aescin** is used as anti-inflammatory which is derived from *Aesculus hippocastanum* (The Free Dictionary).

Figure 1.22: *Aesculus hippocastanum*(Hlasek)

Figure 1.23: *Agrimonia eupatoria* (WIKIMEDIA COMMONS)

1.8.2. Anthelmintics agents

Anthelmintics or antihelminthics are a group of antiparasitic drugs that expel parasitic worms (helminths) and other internal parasites from the body by either stunning or killing them and without causing significant damage to the host. For example, **Agrimophol** is used as Anthelmintics which is derived from *Agrimonia eupatoria* (Wikipedia).

1.8.3. Anti-cholinergic agents

An anticholinergic agent is a substance that blocks the neurotransmitter acetylcholine in the central and the peripheral nervous system. These agents inhibit parasympathetic nerve impulses by selectively blocking the binding of the neurotransmitter acetylcholine to its receptor in nerve cells. For example, **Anisodine** is used as anti-cholinergic agent which is derived from *Anisodus tanguticus* (Wikipedia).

Figure 1.24: *Anisodus tanguticus* (Wikipedia)

Figure 1.25: *Ardisia japonica* (Wordpress, 2013)

1.8.4. Antitussive agents

A large group of opioid and nonopioid drugs that act on the central and peripheral nervous systems to suppress the cough reflex. Because the cough reflex is necessary for clearing the upper respiratory tract of obstructive secretions, antitussives should not be used with a productive cough. For example, **Bergenin** is used as antitussive agents which is derived from *Ardisia japonica* (The Free Dictionary).

1.8.5. Antitumor agent

A drug that inhibits DNA and/or RNA synthesis by intercalation (does not involve covalent bonding) through changing conformation of DNA mostly based on natural products which is used to treat/prevent infections from other cancer treatments. For example, **Colchiceine** amide is used as antitumor agent which is derived from *Colchicum autumnale* (Quizlet).

Figure 1.26: *Colchicum autumnale* (Panoramio)

Figure 1.27: *Mucuna spp* (Seabean)

1.8.6. Antiparkinson agents:

Antiparkinson drugs are medicines that relieve the symptoms of Parkinson's disease and other forms of Parkinsonism. Antiparkinson drugs are used to treat symptoms of Parkinsonism, a group of disorders that share four main symptoms: tremor or trembling in the hands, arms, legs, jaw, and face; stiffness or rigidity of the arms, legs, and trunk; slowness of movement (bradykinesia) and poor balance and coordination. For example**, L-Dopa** is used as antiparkinson agents which is derived from *Mucuna spp* (The Free Dictionary).

1.8.7. Bronchodilator:

A bronchodilator is a substance that dilates the bronchi and bronchioles, decreasing resistance in the respiratory airway and increasing airflow to the lungs. Bronchodilators may be endogenous (originating naturally within the body), or they may be medications administered for the treatment of breathing difficulties. They are most useful in obstructive lung diseases, of which asthma and chronic obstructive pulmonary disease are the most common conditions. For example, **Kheltin** is used as bronchodilator which is derived from *Ammi visage* (Wikipedia).

Figure 1.28: *Ammi visage* (Mullerseeds)

Figure 1.29: *Rauvolfia sepentina* (ECVV 2003)

1.8.8. Circulatory Disorders

It can be any disorder or condition that affects the circulatory system. The circulatory system refers the blood transport system. Circulatory disorders can arise from problems with the heart, blood vessels or the blood itself. For example, **Ajmalicine** is used for treatment of circulatory disorder which is derived from *Rauvolfia sepentina* (Yahoo).

1.8.9. Cardiotonic

A pharmacological agent that increases the force of myocardial contractions. Cardiac glycosides, derived from certain plant alkaloids, exert a tonic effect by altering the transport of electrolytes across the myocardial membrane, causing a decreased efflux of sodium and calcium and a decreased influx of potassium. Digitoxin and digoxin, widely used cardiac glycosides obtained from leaves of a species of foxglove, increase the force of myocardial contractions, extend the refractory period of the atrioventricular node, and, to a lesser degree, affect the sinoatrial node and the heart's conduction system. For example, **Convallatoxin** is used as cardiotonic which is derived from *Convallaria majalis* (The Free Dictionary).

Figure 1.30: *Convallaria majalis* (TRIM TREE NURSERY)

Figure 1.31: *Camellia sinensis* (Camellias R Us)

1.8.10. CNS stimulanting agents

Central nervous system (CNS) stimulants are medicines that speed up physical and mental processes.

Central nervous system stimulants are used to treat conditions characterized by lack of adrenergic stimulation, including narcolepsy and neonatal apnea. Additionally, methylphenidate (Ritalin) and dextroamphetamine sulfate (Dexedrine) are used for their paradoxical effect in attention—deficit hyperactivity disorder (ADHD).

The anerexiants, benzphetamine (Didrex), diethylpropion (Tenuate), phendimetrazine (Bontril, Plegine), phentermine (Fastin, Ionamine), and sibutramine (Meridia) are CNS stimulants used for appetite reduction in severe obesity. For example, Caffeine is used as CNS stimulant which is derived from *Camellia sinensis* (The Free Dictionary).

1.8.11. Local anaesthetic

A local anesthetic (LA) is a medication that causes reversible absence of pain sensation, although other senses are often affected, as well. Also, when it is used on specific nerve pathways (local anesthetic nerve block), paralysis (loss of muscle power) also can be achieved.

Clinical LAs belong to one of two classes: aminoamide and aminoester local anesthetics. Synthetic LAs are structurally related to cocaine. They differ from cocaine mainly in that they have a very low abuse potential and do not produce hypertension or (with few exceptions) vasoconstriction. For example, Cocaine is used as local anesthetic which is derived from *Erythroxylum coca* (Wikipedia).

Figure 1.32: *Erythroxylum coca* (Culturevie)

Figure 1.33: *Cissampelos pareira* (Medicinal Plants 2014)

1.8.12. Skeletal muscle relaxant

A **muscle relaxant** is a drug that affects skeletal muscle function and decreases the muscle tone. It may be used to alleviate symptoms such as muscle spasms, pain, and hyperreflexia. The term "muscle relaxant" is used to refer to two major therapeutic groups: neuromuscular blockers and spasmolytics. Neuromuscular blockers act by interfering with transmission at the neuromuscular end plate and have no central nervous system (CNS) activity. They are often used during surgical procedures and in intensive care and emergency medicine to cause temporary paralysis. Spasmolytics, also known as "centrally acting" muscle relaxants, are used to alleviate musculoskeletal pain and spasms and to reduce spasticity in a variety of neurological conditions. While both neuromuscular blockers and spasmolytics are often grouped together as muscle

relaxants, the term is commonly used to refer to spasmolytics only. For example, **Cissampeline** is used as skeletal muscle relaxant which is derived from *Cissampelos pareira* (Wikipedia).

1.8.13. Mucolytic

Medicine that is able to break down mucus or to reduce mucus viscosity. For example**, Papain** is used as mucolytic which is derived from *Carica papaya* (The Free Dictionary).

Figure 1.34: *Carica papaya* (FloraFinder.org)

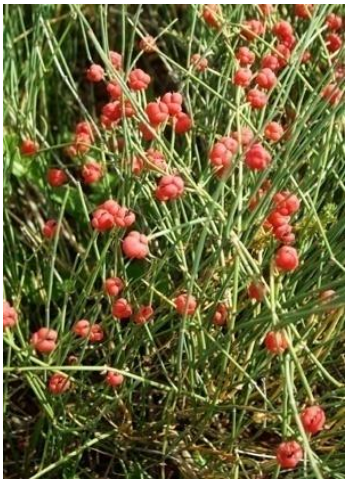


Figure 1.35: *Ephedra sinica* (THE POISION DIARIES)

1.8.14. Sympathomimetic

A pharmacological agent that mimics the effects of stimulation of organs and structures by the sympathetic nervous system. It functions by occupying adrenergic receptor sites and acting as an agonist or by increasing the release of the neurotransmitter norepinephrine at postganglionic nerve endings. Various sympathomimetic agents are used as decongestants of nasal and ocular mucosa, such as bronchodilators in the treatment of asthma and vasopressors and cardiac stimulants in the treatment of acute hypotension and shock; they are also used for maintaining normal blood pressure during operations using spinal anesthesia. Drugs in this group include cyclopentamine, dobutamine, dopamine, ephedrine, isoproterenol, metaproterenol, metaraminol, mephentermine, methoxamine, methoxyphenamine, naphazoline, norepinephrine, phenylephrine, propylhexedrine, protokylol, pseudoephedrine, terbutaline sulfate, tetrahydrozoline, tuaminoheptane, xylometazoline, and epinephrine, a synthetic isomer of the hormone secreted by

the adrenal medulla. Adverse effects of sympathomimetic drugs may be nervousness, severe headache, anxiety, vertigo, nausea, vomiting, dilated pupils, glycosuria, and dysuria. Also called adrenergic drug.

For example, **Pseudoephredrine** is used as sympathomimetic which is derived from *Ephedra sinica* (The Free Dictionary).

1.8.15. Haemostatic agents

Hemostasis or haemostasis is a process which causes bleeding to stop, meaning to keep blood within a damaged blood vessel (the opposite of hemostasis is hemorrhage). It is the first stage of wound healing. This involves coagulation, blood changing from a liquid to a gel. Intact blood vessels are central to moderating blood's tendency to form clots. The endothelial cells of intact vessels prevent blood clotting with a heparin-like molecule and thrombomodulin and prevent platelet aggregation with nitric oxide and prostacyclin. When endothelial injury occurs, the endothelial cells stop secretion of coagulation and aggregation inhibitors and instead secrete von Willebrand factor which initiate the maintenance of hemostasis after injury. Hemostasis has three major steps: 1) vasoconstriction, 2) temporary blockage of a break by a platelet plug, and 3) blood coagulation, or formation of a fibrin clot. These processes seal the hole until tissues are repaired.

Topical hemostatic agents are used when surgical hemostasis is inadequate or impractical. The majority of routine, elective operations are performed in patients with normal hemostasis and with minimal blood loss. The two main categories of topical hemostatic agents are physical agents, which promote hemostasis using a passive substrate, and biologically active agents, which enhance coagulation at the bleeding site. For example, **(+)-Catechin** is used as hemostatic agents which is derived from *Potentilla fragarioides* (UpToDate 2017).

Figure 1.36: *Potentilla fragarioides* (Wild plants in and around Shimane)

1.9. *The Plant Fiscus hispida and its role in medicinal sector*

Ficus hispida is a small but well distributed species of tropical fig tree. It occurs in many parts of Asia and as far south east as Australia. There is a large variety of local common names. Like a number of ficus, the leaves are sandpapery to touch. An unusual feature is the figs which hang on long stems. In Australia the fruit are eaten by cassowaries and double-eyed fig parrots.

Figure 1.37: *Ficus hispida* plant (Medicinal Plants)

Taxonomy

Kingdom: Plantae

(unranked): Angiosperms

(unranked): Eudicots

(unranked): Rosids

Order: Rosales

Family: Moraceae

Genus: *Ficus*

Species: *Ficus hispida*

1.9.1. Local Name

Bengali/vernacular name

Kak dumur, Khoksha-dumur, Dumur, Dhungri, Thoska.

Tribal name

Tammang gaas (Tanchangya), Dhumur gula (Chakma), Fah shai ba (Marma), Thainjang (Tripura), Luhuk (Murong).

1.9.2. Chemical constituents

- Bark contains tannins and saponin glycosides.
- Leaves contain bergapten, psoralen, β-amyrin and β-sitosterol.
- Fruits contain protein, ascorbic acid, carbohydrates and minerals.

The plant also contains 10-keto-tetracosylarachidate. Hydrocarbons present on the leaf surface have also been studied (Medicinal Plants).

1.9.3. Description of plant

A large shrub or small tree, up to 10 (-15) m tall, all parts hispid, hairs pale brown to white. Trunk with lax branches, bark grey, smooth, flaky, young twigs hollow. Leaves opposite, with 105-405 (-10) cm long petiole with a gland near the node; lamina ovate-oblong to ovate-elliptic or ± obovate-oblong, (8-) 10-30 (-35) cm long, 2.5-20 (-25 cm broad, 35-costate at the cuneate to truncate-cordate base, crenate-serrate to ± entire, acute to shortly acuminate, scabrid on both sides, lateral nerves 5-9-pairs, intercostals curved-ascending; stipules lateral, ovate-lanceolate, 10-20 (-25) mm long, hairy beneath, caduceus; cystoliths present only on the lower side. Hypanthodia on 5-10 mm long peduncles, borne in paired clusters on leafless hanging or often trailing branchlets from the trunk or branches (cauliflorous), obovoid or turbinate, 10-15 mm in diameter, thinly hispid, subtended by 3, ± triangular, 1-1.5 mm long basal bracts, apical orifice closed by 5-6 bracts, longitudinally faintly 7-9-ribbed, with a few appressed lateral bracts, internal bristles absent. Male flowers: numerous in 1-2 whorls, ostiolar; sepals 3, concave; stamen single, fllament short. Gall flowers pedicellate or sessile in the male hypanthodium, with sepals enclosing the ovary. Female flowers: sessile or pedicellate, sepals united into a tube round the globose ovary; style subterminal, hairy. Figs depressed-globose to ± pyriform, 2-3 cm in diam, pale-green or greenish-yellow, brown pubescent.

Shrubs or small trees, coarsely hairy; dioecious. Stipules usually 4 and decussate on leafless fruiting branchlets, ovate-lanceolate. Leaves opposite; petiole 1-4 cm, with short thick hairs; leaf blade ovate, oblong, or obovate-oblong, 10-25 × 5-10 cm, thickly papery, abaxially with coarse gray hairs, adaxially rough and with short thick hairs, base rounded to ± cuneate, margin entire or bluntly toothed, apex acute to mucronate; secondary veins 6-9 on each side of midvein. Figs axillary on normal leafy shoots, sometimes on leafless branchlets or branchlets from main branches, solitary or paired, yellow or red when mature, top-shaped, 1.2-3 cm in diam., with short scattered hairs, pedunculate; involucre bracts present; lateral bracts sometimes present. Male flowers: many, near apical pore; calyx lobes 3, thinly membranous; stamen 1. Gall flowers: calyx absent; style subapical, short, thick. Female flowers: calyx lobes absent; style lateral, with hairs. Fl. Jun-Jul (Encyclopedia of Life).

Figure 1.38: *Ficus hispida* (Herbal plants Sri Lanka 2014)

1.9.4. Distribution

It is Very common throughout Bangladesh in homestead and village thickets.

Global Distribution: Indo-Malesia to Australia

Indian distribution: State - Kerala, District/s: All Districts (Encyclopedia of Life).

1.9.5. Uses of *Ficus hispida*

Fig is a fruit that we are familiar to as well as everyone else. The name of a fruit growing in ignorance and neglect is fig. Often found in the biodiversity of the bush As a result, the circulation of figs is as high as it is, there are many different types of unknown nutrition. But the fact that we know the quality is not in the way of his admiration.

1.9.5.1. High Blood Pressure Control

Potassium helps control high blood pressure. There is no pair of figs to supply regularly potassium. Moreover, with the increase of age, it is difficult to find people who do not have high blood pressure problems. To solve this problem, you can take it out of your list now.

1.9.5.2. Keeps the heart healthy

There is an element on the fig and fig leaves that helps control the body's triglyceride level. If this level is controlled, the heart is healthy.

1.9.5.3. Breast cancer prevention

One study found that with the regular consumption of diet rich in fiber, 34% of women had less chances of breast cancer. It is possible to reduce the probability of regular figs up to 50%.

1.9.5.4. Bone strengthens

There may be a shortage of calcium in the body. At this time, hands-on knee pain, bone loss, tooth decay etc. Calcium needs to be saved and prevention from this. And the name of a reliable source of calcium in abundance is known as the best known fig.

1.9.5.5. Regulates diabetes

In order to control diabetes, the patient has to take insulin injections. Studies have shown that there are antioxidants in the fig leaf which helps in reducing the amount of insulin in the patient. With the advice of the doctor, the juice of figs can eat breakfast in the morning.

1.9.5.6. Body weight reduction

Pacatine present in dumour control the blood cholesterol levels. Those who are confused about their weight, they can eat regular figs.

1.9.5.7. Problems with bowel problems

Fig works very well to overcome the stomach problem. Fig works as an aid to prevent constipation and piles. It is also quite useful to cool the bile.

Various uses of figs are also common. The use of figs in the stomach or insect bites will be relieved if used in pomegranate. Acne is also very effective in curing (Amitrabazar).

1.9.6. Morphological of plant

Fruits: A lenticular achene, keeled with prominent hilum. Fruiting throughout the year.

Flower: Figs on special shoots and on the branches. Male, female and gall flowers enclosed in depressed-globose figs; ripening yellow; faintly ribbed. Flowering throughout the year.

Leaf: The shape of leaf is Oblong to elliptic-lanceolate. It is simple type of leaf. Arrangement of leaf looks like Opposite-decussate. The apex of leaf is abruptly acute and the margin is likely entire-minutely toothed.

Field tips: Leaves opposite-decussate, 3-nerved from base. Figs on special shoots. (Encyclopedia of Life).

1.9.7. Leaf morphology of *Ficus hispida*

Leaves simple, opposite, decussate; stipules to 2.5 x 1 cm, caducous, leaving annular scar; petiole 1-10 cm long, canaliculate, hispid; lamina 7-35 x3-16 (40 x 18 cm in saplings), elliptic-oblong, narrow ovate, narrow obovate, apex caudate-acuminate, base rounded subcordate or truncate-subcordate, margin entire or dentate sometimes irregularly toothed, scabrid on both surface, hispid beneath; midrib slightly raised above; 3-nerved at base; secondary nerves 4-9 pairs, often branched, ascending; tertiary nerves broadly reticulo-percurrent.

Figure 1.39: *Ficus hispida* leaf (Flora Fauna World 2011)

1.9.7.1. Behavior of leaf powder with different chemical/reagents

Behavior of leaf powder with different chemical reagents was studied to detect the presence of phytoconstituents with color changes under daylight (RAVICHANDRA V D, et al, 2011).

Regents	Color/ppt	Constituents
Picric acid	Slight ppt.	Alkaloids present
Conc. H_2SO_4	Reddish brown	Steroids/triterpenoids present
Aq. $Fecl_3$	Bluish black ppt	Tannins present
Iodine solution	No change	Starch absent
Ammonia present	No change	Anthroquinone glycosides absent
5% Aq. KOH	No change	Anthroquinone glycosides absent
Mayer's reagent	Slight ppt	Alkaloids present
Spot test	Stains observed	Fixed oils present
Aq. $AgNo_3$	No precipitation	Proteins absent
Aq. NaoH	Yellow	Flavonoids present
Mg – Hcl	Magenta	Flavonoids present
Dragendroff's reagent	No ppt	Alkaloids absent
Aq. Lead acetate	White ppt	Tannins present
Liberman Burchardt's test	Reddish green	Steroids and tannins are present

Figure 1.40: Behavior of leaf powder with different chemical/reagents

CHAPTER-TWO

LITERATURE REVIEW

Literature review

2.1. Hypoglycemic activity of Ficus hispida (bark) in normal and diabetic albino rats:

The main purpose is to find out hypoglycemic activity of *Ficus hispida*(bark) for normal and diabetic activity of albino rats and to find out the propable mechanism of hypoglycemic activity, if there is any action.

Albino rats were divided into 6 groups and they were receiving treatments containing of vehicle, water-soluble portion of the ethanol extract of *Ficus hispida* bark (FH) (1.25 g/kg) and standard antidiabetic drugs, glibenclamide (0.5 mg/kg) and 0.24 units of insulin (0.62 ml of 0.40 units/ml). Before and after 2h of drug administration, blood glucose level was determined by the glucose oxidase method in both normal and alloxan-induced diabetic rats. The probable mechanism of action of FH as a hypoglycemic agent was determined by i) the glycogen content of the liver, skeletal muscle and cardiac muscle, and ii) glucose uptake by isolated rat hemi-diaphragm were estimated.

Through the experiments, the reduction of blood glucose level was determined by FH both in the normal ($P<0.01$) and diabetic ($P<0.001$) rats. However, the reduction in the blood glucose level was less than that of the standard drug, glibenclamide. Through the uptake of of glucose by rat hemi-diaphragm significantly ($P<0.001$), FH is increased. In the glycogen content of the liver ($P<0.05$), skeletal muscle ($P<0.01$) and cardiac muscle ($P<0.001$), there was a significant increase. The amount of glycogen present in the cardiac muscle was more than the glycogen present in the skeletal muscle and liver.

FH has significant hypoglycemic activity. Glycogenesis and peripheral uptake of glucose are increased in the probable mechanisms, which is involved in its hypoglycemic activity. (Ghosh, R. et al, 2004)

2.2. Studies on anti-diarrhoeal activity of Ficus hispida. Leaf extract in rats:

The methanol extract of *Ficus hispida* L. is showed that there having a significant inhibitory action against castor oil-induced diarrhoea and PGE_2-induced enteropooling in rats. A significant reduction in gastro-intestinal motility on charcoal meal test in rats has been found in this experiment. The results is obtained through establish of the *F. hispida* leaf extract as an anti-diarrhoeal agent. (Mandal S C, et al, 2002)

2.3. Protective effect of leaf extract of Ficus hispida Linn. against paracetamol-induced hepatotoxicity in rats:

The hepato-protective activity in rats which is induced an acute liver damage by paracetamol (750 mg/kg, p.o.) was evaluated through the methanol extract of the leaves of *Ficus hispida* Linn. (Moraceae). A significant protective effect by lowering the serum levels of transaminase (SGOT and SGPT), bilirubin and alkaline phosphatase (ALP) is exhibited in the extract at an oral dose of 400 mg/kg. A histo-pathological examination of liver sections is supplemented from these biochemical observations. The activity of extract was also compared to that of Liv-52 a known hepatoprotective formulation. (Mandal S C,et al, 2000)

2.4. Anti-hyperglycemic Activities of Leaves of Three Edible Fruit Plants (Averrhoa carambola, Ficus hispida and Syzygium samarangense) of Bangladesh:

There are three common plants in Bangladesh which fruits having edible use and they are *Averrhoa carambola* L. (Oxalidaceae), *Ficus hispida* L.f. (Moraceae), and *Syzygium samarangense* (Blume) Merr. & L.M. Perry (Myrtaceae). The leaves and fruits of *A. carambola* and *F. hispida* are used by folk medicinal practitioners for treatment of diabetes, while the leaves of *S. samarangense* are used for treatment of cold, itches, and waist pain. It was the objective of the present study to evaluate the anti-hyperglycemic potential of methanolic extract of leaves of the plants in oral glucose tolerance tests carried out with glucose-loaded mice, in where scientific studies are absent on the anti-hyperglycemic effects of the leaves of the three plants. Through the

glucose oxidase method, the extracts at different doses were administered one hour prior to glucose administration and blood glucose level was measured after two hours of glucose administration (p.o.). Significant oral hypoglycemic activity was found with the extracts of leaves of all three plants tested. The fall in serum glucose levels were dose-dependent for every individual plant which having highest at the highest dose tested of 400 mg extract per kg body weight. When comparing with the control animal at this dose, the extracts of A. carambola, *F. hispida*, and *S. samarangense*caused, respectively, 34.1, 22.7, and 59.3% reductions in serum glucose levels. The standard anti-hyperglycemic drug, glibenclamide, caused a 57.3% reduction in serum glucose levels versus control. The methanolic extract of leaves of *S. samarangense* proved to be the most potent in demonstrating antihyperglycemic effects, among three plants. The folk medicinal uses of *A. carambola* and *F. hispida* in the treatment of diabetes, and indicates that the leaves of *S. samarangense* can also possibly be used for amelioration of diabetes-induced hyperglycemia which is shown as a result of this experiment. (Shahreen S, et al 2012)

2.5. Volatiles from Ficus hispida and Their Attractiveness to Fig Wasps:

Receptive (ready to be pollinated), post pollinated, and post parasitized figs, and leaves of *Ficus hispida* having some volatile components and they were analyzed in this experiment. There are having some differences among them which is examined and the specificity of fig wasp attractiveness was investigated. The major constituent of steam-distilled oil of either male or female receptive figs was Linalool, while the major compound of the oils of post parasitized and post pollinated figs was dibutyl phthalate. Male and female receptive figs contains some specific components like as palmitic oil, and 9, 12-octadecadienoic acid which is located in petroleum ether extracts, while hexadecanoic acid ethyl ester was the major compound of post parasitized and post pollinated figs. In dichloromethane extracts, the major constituent of male and female receptive figs was linalool, the major component of male post parasitized figs was 1-hydroxylinalool, and the major constituents of female post pollinated figs were 1-hydroxylinalool and benzyl alcohol. Through bioassays of sticky traps, it is showed that *Ceratosolen solmsimarchal* was attracted to dichloromethane extracts of male and female receptive figs, and also to petroleum ether extracts of female receptive figs, but was not attracted to dichloromethane and petroleum ether extracts of male post parasitized and female

post pollinated figs. Figs were attractive to pollinating wasps only at the receptive stage. The volatile constituents of receptive figs were different from those of post pollinated or post parasitized figs. From a receptive to a post pollinated state, figs changed in their volatile composition. Some compounds disappeared or decreased in amount. Linalool, linalool oxide, α-terpeneol, and 2,6-dimethyl-1,7-octadiene-3,6-diol are those compounds which having an attraction on repellents of the wasps. The dichloromethane extracts of male and female receptive figs having similar activities in attracting fig wasps which indicates the receptive figs of both sexes are similarly attractive to fig wasps, which is further supported by their similar volatile composition. Leaf extract was not attractive to the wasps (Song Q, et al 2001).

2.6. Chemical Ecology of Fruit Bat Foraging Behavior in Relation to the Fruit Odors of Two Species of Paleotropical Bat-Dispersed Figs (Ficus hispida and Ficus scortechinii):

The fruit odors of two bat-dispersed fig species in the Paleotropics, having a relation to the foraging behavior of fruit bats, a test is done for this following hypothesis:

1. For detecting and selecting of ripe figs by fruit bats, fruit odor has a specific role.

2. Bat-dispersed fig species which are characterized by the same or similar, chemical compounds and

3. Total scent production increases, in bat-dispersed fig when the fruit has been ripen.

A bioassays test has been performed for testing the effect of both natural and synthetic fig fruit odors on the foraging behavior of the short-nosed fruit bat (*Cynopterus brachyotis*). *Cynopterus brachyotis* is an important disperser of figs within the study area. When foraging for figs, Fruit bats responded to both visual and chemical (olfactory) cues. However, the strongest foraging reaction that resulted in a landing or feeding attempt was almost exclusively associated with the presence of a ripe fruit odor—either in combination with visual cues or when presented alone. Fruit bats also used fruit odors to distinguish between ripe and unripe fruits. By using gas chromatography (GC) and GC/mass spectrometry (MS), a total of 16 main compounds were identified in the ripe fruit odor of *Ficus hispida* and 13 in the ripe fruit odor of *Ficus scortechinii*—including alcohols, ketones, esters, and two terpenes. Additional compounds were

also recorded in *F. hispida*, but not identified—four of which also occurred in *F. scortechinii*. Total scent production increased in both species when fruits ripened. Both natural and synthetic fruit odors resulted in feeding attempts by bats, with no feeding attempts elicited by unscented controls. Reaction rates to natural fruit odors were higher than those to synthetic blends (Hodgkison R, et al, 2007).

2.7. Responses of the Pollinating Wasp Ceratosolen solmsi marchali *to Odor Variation between Two Floral Stages of Ficus hispida*:

During development of figs on *Ficus hispida*, only the female floral stage is receptive to its pollinator *Ceratosolen solmsi marchali*. After this stage, the quantity of fig odor decreases. The effects of *F. hispida* volatiles from receptive figs (figs at the female floral stage, when they are pollinated) and interfloral figs (between the female floral and male floral stages) on their pollinator were studied, together with responses to compounds present in the odor. Odors emitted by both receptive and interfloral figs were attractive to the pollinator. However, wasps preferred the odor of receptive figs to that of interfloral figs even though the quantity of interfloral volatiles increased. Three monoterpenes that included linalool (major constitutent) and two minor compounds limonene and β-pinene from the receptive fig volatiles were used to test the pollinator responses. The levoisomer and racemic mixtures of linalool were attractive to the pollinator at high doses, but the dextroisomer was neutral. (±)-Limonene and (−)-β-pinene at high doses were even less attractive to the pollinator than clean air and were neutral at low doses, while (*R*)-(+)-, (*S*)-(−)-limonene were neutral at all doses. In blend tests, all four mixtures of (±)-linalool or (*S*)-(−)-linalool combined with (±)-limonene or (−)-β-pinene attracted *C. solmsi marchali* when administered at high doses. (*R*)-(+)-linalool and (−)-β-pinene enhanced the attractiveness of (*S*)-(−)-linalool to the pollinator, while enantiomers of limonene did not. These results suggest that both quality and quantity of fig volatiles regulate *C. solmsi marchali* response and that quality is the main host-finding and floral stage-distinguishing cue for the pollinator. Synergistic effects of some compounds may play a role in enhancing attractiveness of the active compounds (Chen C, et al, 2008).

2.8. Components of reproductive success in two dioecious fig species, Ficus exasperata and Ficus hispida:

We studied components of reproductive success in two dioecious fig species, *Ficus exasperata* and *F. hispida,* at a deciduous and an evergreen site in south India, over a two-year period. Unlike monoecious figs, which produce a mixture of wasps, seeds, and pollen in every fruit, dioecious fig species have hermaphrodite (functionally male) trees, which produce wasps and pollen only, and female trees, which produce seeds only. Dioecious fig species are free of the seed–wasp trade-off involved within each monoecious fig fruit; wasp and seed production in dioecious species reflect selective pressures acting on each sex to maximize offspring production. We studied specific predictions of the reproductive behavior of dioecious fig species, predictions that were made based on studies of monoecious figs.

Pollinator (and associated parasitic wasp) arrivals at trees bearing receptive inflorescences were closely linked to the seasonal variation in receptive fig numbers. More wasps arrived at female trees of *F. exasperata* than at "male" trees, apparently because of synchronized wasp release by male trees during the time of synchronous female tree receptivity. Wasp numbers arriving at male and female trees of *F. hispida* did not differ significantly, apparently because inflorescence production on male and female trees was more evenly spread over time. Female inflorescences had more pollinators than male inflorescences in *F. hispida*; female and male inflorescences had approximately equal numbers of pollinators in *F. exasperata.* Hand-pollination experiments introducing one, three, or eight pollinators into figs of *F. hispida* showed that foundress number was a significant predictor of resultant wasp or seed number. In both fig species, female inflorescences initiated more flowers than male inflorescences, and produced more seeds than male inflorescences produced wasps. Female fruit were not larger than male fruit at maturity in both fig species. Only in *F. exasperata* were male fruit significantly smaller in the dry than in the wet site. Both species had a relatively high average level of pollination, greater than 70%. The mean percentage of fruit matured was 56% in *F. hispida,* and a surprisingly high 78% in *F. exasperata.*

Both fig species exhibited strong sex differences in foundress numbers, numbers of flowers initiated, and numbers of wasps or seeds matured, suggesting strong selection on components of

reproductive success within each sex after dioecy evolved. Our quantitative analyses elucidate how some of the many factors in plant–pollinator relationships affect plant reproductive success in the fig–wasp mutualism (Patel A, et al, 2000).

2.9. Signalling receptivity: Comparison of the emission of volatile compounds by figs of Ficus hispida before, during and after the phase of receptivity to pollinators:

Figs and pollinating fig wasps are involved in highly specific mutualisms. Because associations between figs and their pollinating wasps are horizontally transmitted, partner encounter is a crucial step, and is mediated by the emission by receptive figs of the volatile compounds that are detected by the pollinator. However, pollinator attraction is probably not the only function of the volatile compounds produced by figs. Other likely functions include signalling to wasps that a fig has already been pollinated, and deterring or defending against visitors with negative effects on developing figs or the pollinators they contain. The functions of volatile compounds will also change over the course of fig development, and the composition of the odour bouquet is thus also likely to vary. However, this variation and its likely functional importance have rarely been studied. To address these questions, we investigated changes in the composition of the bouquet of volatile compounds, and in the rate of emission of odour components, before, during and after the phase of receptivity to pollinators in Ficushispida, in Yunnan (China). This first study of the dynamics of variation in signal composition throughout the cycle of fig development, provides support for the hypothesis of chemical mimicry in dioecious (Proffit M, et al, 2008).

2.10. Antinociceptive Activity Studies with Methanol Extracts of Ficus hispida L.f. Leaves and Fruits in Swiss Albino Mice:

The methanol extracts of *Ficus hispida* L.f. leaves and fruits were evaluated for antinociceptive activity in acetic acid-induced gastric pain writhings in Swiss albino mice. The methanol extract of leaves demonstrated dose-dependent and significant antinociceptive activity when administered at doses of 50, 100, 200, and 400 mg per kg body weight. At these doses, the

percent inhibitions of writhing versus control animals (i.e. animals without any extract administration) were, respectively, 65.71, 67.14, 70.19, and 85.05. In comparison, the standard antinociceptive drug, aspirin, when administered at a dose of 400 mg per kg body weight reduced writhings by 88.09%. The methanol extract of fruits also exhibited significant dose-dependent writhings in mice, when administered at doses from 50 to 400 mg per kg body weight. However, in the case of fruit extract, the percent inhibition of writhings was lower than corresponding doses of leaf extract. Writhings were reduced by 49.24, 61.24, 62.67, and 65.19%, respectively, when mice were administered fruit extract at doses of 50, 100, 200, and 400 mg per kg body weight. The results suggest that both leaf and fruit extract of Ficushispida contain strong antinociceptive components, which warrant further studies to be made on these extracts towards discovery of efficacious pain relieving agents (Jahan T, et al, 2011).

2.11. Anti-ulcerogenic evaluation of root extract of Ficus hispida Linn. in aspirin ulcerated rats:

The present study was designed to investigate the anti-ulcer efficacy of methanolic root extract of the *Ficus hispida* Linn. (FH), which was known to possess various therapeutic properties. The reason for the study was that the known non-steroidal anti-inflammatory drugs (NSAIDs) were full of side effects especially ulceration causes Gastric ulceration an economic loss and a source of welfare concern worldwide. There are 350,000 to 500,000 new cases per year and more than one million are ulcer-related hospitalizations. We found that FH decreased the incidence of ulcers and also enhanced the healing of ulcers. Methanolic extract of FH at doses 200 and 400 mg/kg was found to be effective by 63.8 and 68.44% respectively in aspirin (ASP) induced ulcer model and significantly reduced free and total acidity. It was observed that anti-ulcer effect of FH might be due to its cytoprotective effect rather than antisecretory activity. Conclusively, FH was found to possess potent anti-ulcerogenic as well as ulcer-healing properties and could act as a potent therapeutic agent against peptic ulcer disease (Sivaraman D, et al, 2010).

CHAPTER-THREE

METHODS & MATERIALS

Methods and Materials

3.1 Collection and Preparation of Plant Material:

Plant sample of *Ficus hispida* was collected from Dhaka in March, 2017. Then proper identification of plant sample was done by an expert taxonomist. The plant was sun dried for several days. The plant materials were then oven dried for 24 hours at considerably low temperature for better grinding. The dried plant was then ground in coarse powder using high capacity grinding machine in the Phytochemical Research Laboratory, Department of Pharmacy, East West University.

3.2 Extraction of the Plant Material:

About 900 gm of the powdered material was taken in separate clean, round bottomed flask (5liters) and soaked in 3.5 liter of methanol. The container with its content was sealed by cotton plug and aluminum foil and kept for a period of 15 days accompanying occasional shaking and stirring. The whole mixture was then filtered through cotton followed by Whatman No.1 filterpaper and the filtrate thus obtained was concentrated at 390°C with a Heidolph rotary evaporation.

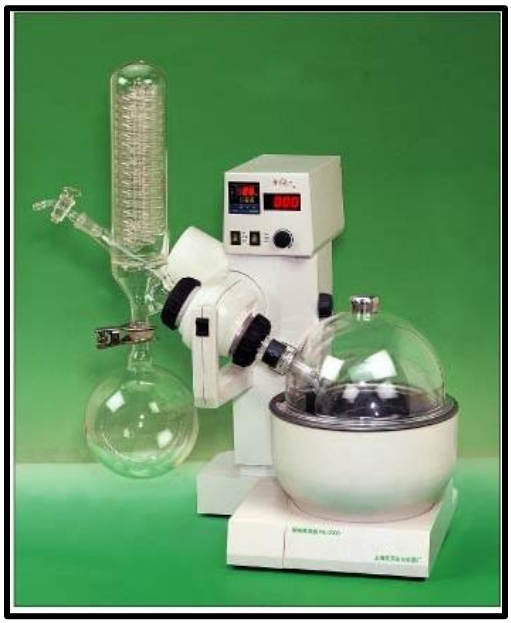


Figure 3.1: Drying of extract using rotary evaporator (NINGSHING 2010)

The concentrated extract was then air dried to solid residue. The weight of the crude methanol extract obtained from the powdered whole plant was 25.18 gm respectively.

3.3 Preparation of Mother Solution

5 gm of methanol extract was triturated with 90 ml of methanol containing 10 ml of distilled water. The crude extract was dissolved completely. This is the mother solution.

3.4 Partition of Mother Solution

The mother solution was then partitioned off successively by four solvents of different polarity.

3.4.1 Partition with n-hexane

The mother solution was taken in a separating funnel. 100 ml of the n-hexane was added to it and the funnel was shaken and then kept undisturbed. The organic portion was collected. The process was repeated thrice (100 ml×3). The n-hexane fraction was then air dried for solid residue.

3.4.2 Partition with Dichloromethane

To the mother solution left after partitioning with n-hexane, 12.5 ml of distilled water was added and mixed. The mother solution was then taken in a separating funnel and extracted with Dichloromethane (DCM). The process was repeated thrice (100 ml × 3). The DCM fraction was then air dried for solid residue.

3.4.3 Partition with Ethyl Acetate

To the mother solution that left after washing with n-hexane, and Dichloromethane, 16 ml of distilled water was added and mixed. The mother solution was then taken in a separating funnel and extracted with ethyl acetate. The process was repeated thrice (100 ml ×3). The ethyl acetate fraction was then air dried for solid residue.

3.4.4 Partition with Aqueous Fraction

After partitioning the mother solution with n-hexane, Dichloromethane and Ethyl acetate, 20 ml of distilled water was added and mixed. The mother solution was then taken in a separating funnel and extracted with aqueous fraction. The process was repeated thrice (100 ml × 3). The aqueous fraction was then air dried for solid residue.

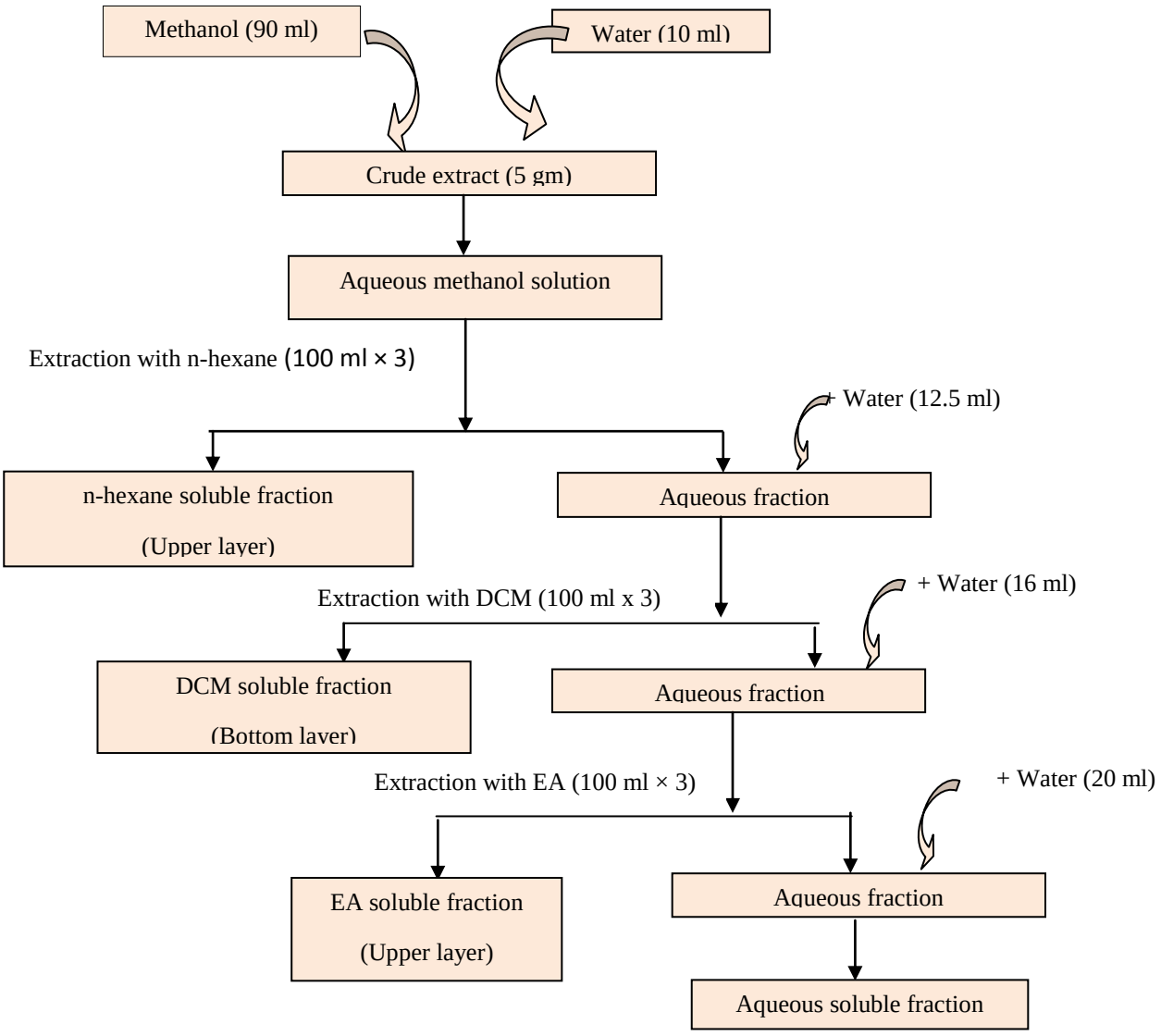


Figure 3.2: Schematic representation of the partitioning of methanolic crude extract of *Ficus hispida.*

3.4.5 Collection of n-Hexane Fraction

After partitioning the mother solution with the four different solvents the n-Hexane fraction of them were collected and air dried. This n-Hexane was further investigated for different pharmacological properties such as Antioxidant and Cytotoxic (Beckett AH and Stenlake JB, 1986).

3.5 Brine Shrimp Lethality Bioassay

3.5.1 Principle

Brine shrimp lethality bioassay is a recent development in the assay procedure for the bioactive compounds and natural product extracts, which indicates cytotoxicity as well as a wide range of pharmacological activities e.g. anticancer, antiviral, and pharmacological activities of natural products etc. Bioactive compounds are almost always toxic in high doses. Pharmacology is simply toxicology at a lower dose or toxicology is simply pharmacology at a higher dose. Thus (*in-vivo*) lethality, a simple zoological organism, (Brine shrimp napulii- *Artemia salina*) can be used as a convenient monitoring for screening and fractionation in the discovery of new bioactive natural products. Natural product extracts, fractions or pure compounds can be tested or their bioactivity by this method. This bioassay is indicative of cytotoxicity and a wide range of pharmacological activity of natural products. Brine shrimp is the English name of the genus *Artemia* of aquatic crustaceans. *Artemia* is the only genus in the family Artemiidae (Olowa *et al.*, 2013).

3.5.2 Apparatus and Reagents

Table 3.1: Apparatus and reagents for Brine shrimp lethality bioassay

Artemia salina leach (brine shrimp eggs)	Pipettes & Micropipette
Sea salt (NaCl)	Glass vials
Small tank with perforated dividing dam to hatch the shrimp	Magnifying glass
Lamp to attract shrimps	Test samples

3.5.3 Procedure

3.5.3.1 Preparation of Sea Water

To hatch the brine shrimp nauplii for the assay, sea water representing brine should be prepared at first. To prepare sea water 38 gm of pure NaCl was dissolved in distilled water and then the volume made up to 1000 ml by distilled water in a 1000 ml beaker for *Artemia salina* hatching. 1-2 drops of 1 N NaOH or 1 N HCl solution was added with a dropper for obtaining the pH 8.4 as sea water.

3.5.3.2 Hatching of Brine Shrimp

A rectangular tank was divided in to two unequal compartments by a porous separator. The larger compartment was darkened while the smaller one was kept illuminated. Then a dry preserved egg of *Artemia salina* Leach was added in the artificial sea water. Oxygen was supplied through an air pump and a table lamp was placed near the beaker. The eggs of *Artemia salina* were hatched at room temperature (25-30ºC) for 18-24 hours. The larvae (nauplii) were attracted by the light and moved to the smaller compartment through the holes. 10 living shrimps were then collected by a pipette and then added to each of the test tubes containing 5 ml of seawater. Those freshly hatched free-swimming nauplii were used for the bioassay (Niazi J. *et al*., 2009).

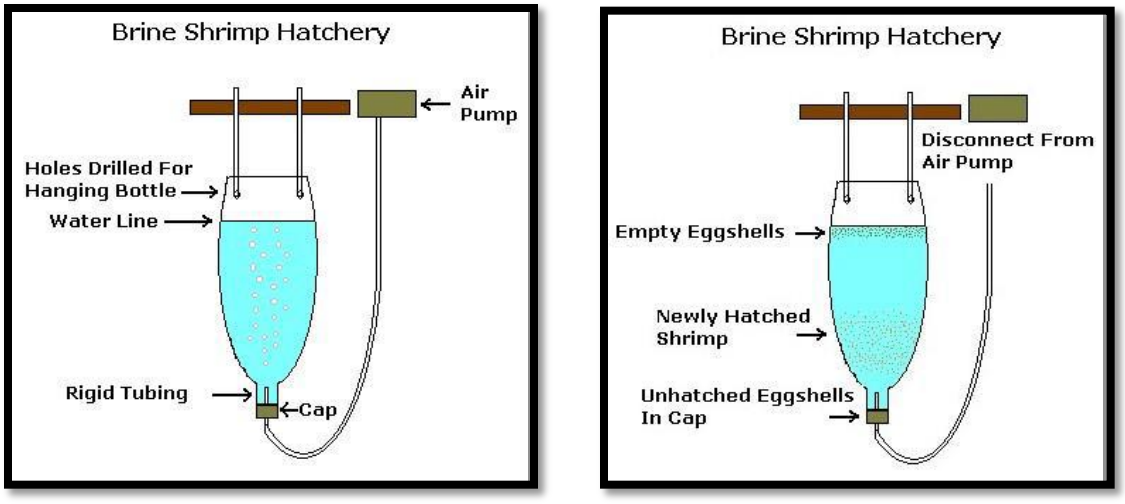


Figure 3.3: Brine shrimp Hatchery

3.5.3.3 Preparation of Test Solutions

Clean test tubes were taken. These test tubes were used for ten different concentrations (one test tube for each concentration) of test samples and ten test tubes were taken for standard drug tamoxifen for ten concentrations of it and another one test tube for control test.

3.5.3.4 Preparation of the Test Samples of Experimental Plant

All the test samples of 4 mg were taken and dissolved in 200 µl of pure dimethyl sulfoxide (DMSO) in vials to get stock solutions. Then 100 µl of solution was taken in test tube each containing 5 ml of simulated seawater and 10 shrimp nauplii. Thus, final concentration of the prepared solution in the first test tube was 400µg/ml. Then a series of solutions of varying concentrations were prepared from the stock solution by serial dilution method. In each case 100 µl sample was added to test tube and fresh 100 µl DMSO was added to vial. Thus the concentrations of the obtained solution in each test tube were 400 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.125 µg/ml, 1.5625 µg/ml and 0.78125 µg/ml for 10 dilutions.

3.5.3.5 Preparation of the Positive Control Group

In the present study tamoxifen is used as the positive control. Measured amount of the tamoxifen is dissolved in DMSO to get an initial concentration of 2000 µg/ml. From that stock solution serial dilutions are made using DMSO to get 400 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.125 µg/ml, 1.5625 µg/ml and 0.78125 µg/ml. Then ten living brine shrimp nauplii in 5 ml simulated seawater are added to the positive control solutions in the pre-marked test-tubes to get the positive control groups.

3.5.3.6 Preparation of the Negative Control Group

100 µl of DMSO was added to the pre-marked test tube containing 5 ml of simulated seawater and 10 shrimp nauplii to use as control groups. If the brine shrimps in these vials show a rapid mortality rate, then the test is considered as invalid as the nauplii died due to some reason other than the cytotoxicity of the compounds (Goldstein *et al.*, 1974).

3.5.3.7 Counting of Nauplii

After 24 hours, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial was counted. From this data, the percent (%) of lethality of the brine shrimp nauplii was calculated for each concentration (Sleet RB and Brendel K, 1983).

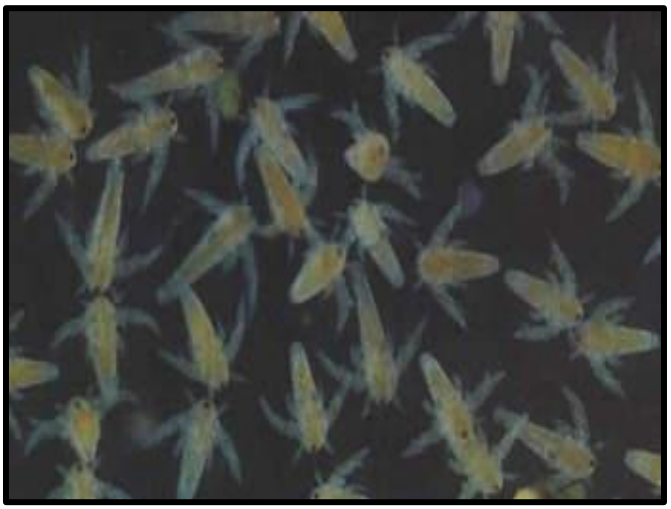


Figure 3.4: Counting of nauplii (Aqua guide 2007)

3.6 Antioxidant Activity

3.6.1 Total Phenolic Content

The antioxidative effect is mainly due to phenolic components, such as flavonoids, phenolic acids, and phenolic diterpenes. The antioxidant activity of phenolic compounds is mainly due to their redox properties, it has been reported that there is an inverse relationship between the antioxidative status occurrences of human diseases. In addition, antioxidant compounds which are responsible For Such antioxidants activity could be isolated and then used as antioxidants for the prevention and treatment of free radical-related disorders. Therefore, research to identify antioxidative compounds is an important issue. Although it remains unclear which of the compounds, of medical plants are the active ones, polyphenols recently have received increasing attention because of some interesting new findings regarding their biological activities. From pharmacological and therapeutic points of view, the antioxidant properties of polyphenols, such

as free radical scavenging and inhibition of lipid per oxidation, are the most crucial. Even though a variety of herbs are known to be sources of phenolic compounds, studies isolating polyphenols and evaluating their antioxidative effects have rarely been carried out. The purpose of this study was to evaluate extractives of *Ficus hispida* new potential sources of natural antioxidants and phenolic compounds. This study also demonstrates a possible relationship between phenolic content and antioxidant activity.

3.6.1.1 Principle

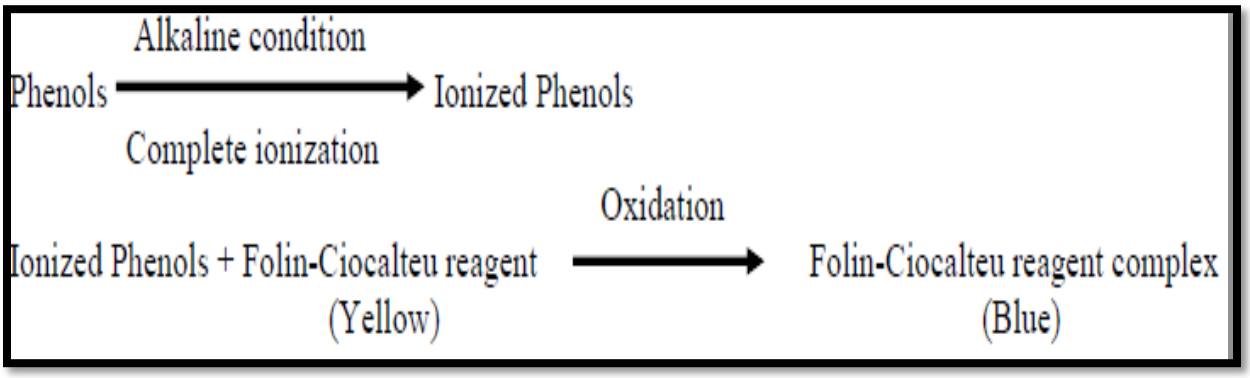
The content of total phenolic compounds in plant methanolic extracts was determined by Folin–Ciocalteu Reagent (FCR). The FCR actually measures a sample's reducing capacity. In the alkaline condition phenols ionize completely.

Table 3.2: Composition of 100 mg Folin-Ciocalteu Reagent

i.	Water	57.5 ml
ii.	Lithium Sulfate	15.0 mg
iii.	Sodium Tungstate Dihydrate	10.0 mg
iv.	Hydrochloric Acid (25%)	10.0 mg
v.	Phosphoric Acid 85% solution in water	5.0 mg
vi.	Molybdic Acid Sodium Dihydrate	2.5 mg

When Folin-Ciocalteu reagent is used in this ionized phenolic solution the reagent will readily oxidize the phenols. Usual color of Folin-Ciocalteu reagent is yellow and after the oxidation process the solution become blue. 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 $(PMoW_{11}O_{40})^{4-}$.

The intensity of the color change is measured in a spectrophotometer at 765 nm. The absorbance value will reflect the total phenolic content of the compound (Singleton et al., 1999).



3.6.1.2 Apparatus and Reagents

Table 3.3: Apparatus and reagents used for total phenolic content

Folin-Ciocalteu reagent (10 fold diluted)	UV-spectrophotometer
Ascorbic acid	Beaker (100 & 200 ml)
Na_2CO_3 solution (7.5%)	Test tube
Methanol	Micropipette (50-200 µl)
Distilled water	Cuvette

3.6.1.3 Procedure

3.6.1.3.1 Standard Curve Preparation

Ascorbic acid was used here as standard. Different ascorbic acid solutions were prepared having a concentration ranging from 120 µg/ml to 80 µg/ml. 5 ml of FCR (diluted 10 times with water) and 4 ml of Na_2CO_3 (7.5% w/v) solution was added to ascorbic acid solution. The mixture was incubated for 20 minutes at room temperature. After 20 minutes the absorbance was measured at 765 nm. After plotting the absorbance in ordinate against the concentration in abscissa a linear relationship was obtained which was used as a standard curve for the determination of the total phenolic content of the test samples.

3.6.1.3.2 Sample Preparation

2 mg of the *Ficus hispida* n-Hexane fraction was taken and dissolved in 1 ml methanol to get a sample concentration of 2 mg/ml.

3.6.1.3.3 Determination of Total Phenol Content

- 1.0 ml plant extract of different concentrations (120 µg/ml, 110 µg/ml, 100 µg/ml, 90 µg/ml and 80 µg/ml) was taken in test tubes.
- 5 ml of Folin–ciocalteu (Diluted 10 fold) reagent solution was added into the test tube.
- 4 ml of Sodium carbonate solution was added into the test tube.
- The test tubes containing the samples were incubated for 1 hour at the room temperature to complete the reaction.
- Absorbance of solution was measured at 765 nm using a spectrophotometer against blank.
- A typical blank solution containing methanol was taken.

3.6.2 Total Flavonoid Content

3.6.2.1 Principle

Aluminium chloride ($AlCl_3$) colorimetric method is incorporated to determine the total flavonoid contents of the crude plant extract. The basic principle of the assay method is that aluminium chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols of the crude extract. In addition aluminium chloride also forms acid labile complexes with the ortho-dihydroxyl groups in the A or B-ring of flavonoids. The formed flavonoid-aluminium complex between flavonoid of the crude extract and aluminium chloride has an absorbance maximum at 510 nm. Therefore, the amount of flavonoid in the crude extract can be quantified by measuring the absorbance of reaction mixture at 510 nm using a UV-visible spectrophotometer against a blank containing all reagents except the extracts. Quercetin at various concentrations was used as standard (Chang C et al., 2002).

Flavonoid (Extract) + $AlCl_3$ (reagent) = Formation of flavonoid-aluminium complex (λmax = 510 nm)

3.6.2.2 Apparatus & Reagents

Table 3.4: Apparatus and reagents used for total flavonoid content

Aluminium chloride	Spatula
Methanol	Analytical balance
Quercetin	Pipette and pumper
Sodium hydroxide	Aqueous fraction
Sodium nitrite	Test tubes and beaker

3.6.2.3 Procedure

3.6.2.3.1 Preparation of 10% Aluminium Chloride ($AlCl_3$) Solution: 1gm of $AlCl_3$ was taken into a 10 ml of a volumetric flask and the volume was adjusted by distilled water.

3.6.2.3.2 Preparation of 4% NaOH Solution: 4 gm of NaOH was taken into a 100 ml volumetric flask and the volume was adjusted by distilled water.

3.6.2.3.3 Preparation of 5% (W/V) $NaNO_2$ Solution: 0.5 gm of $NaNO_2$ was taken into a 10 ml of a volumetric flask and the volume was adjusted by distilled water.

3.6.2.3.4 Preparation of Standard Solution: The stock solution was prepared by taking 10 mg of quercetin and dissolved into 50 ml of methanol. Concentration of this solution was 200 µg/ml of quercetin. The experimental concentrations (0, 4, 8, 12, and 16 µg/ml) were prepared from this stock solution.

Table 3.5: Preparation of standard solution

Concentration (µg/ml)	Solution taken from stock solution (ml)	Volume adjusted by methanol (ml)	Final volume (ml)
0	0	5	5
4	0.1	4.9	5
8	0.2	4.8	5
12	0.3	4.7	5
16	0.4	4.6	5

3.6.2.3.5 Preparation of Extract Solution: 5 mg of each plant extracts were taken and dissolved into 5 ml of methanol. The concentration of the solution was 1 mg/ml of plant extracts. Then the following steps were carried out.

2 ml extract was taken in a test tube and then 6 ml of distilled water was added. Then 5% of $NaNO_2$was added and incubated for 6 minutes. 10% $AlCl_3$ was added and incubated for 6 minutes. 4% NaOH and 0.6 ml distilled water was added. Then it was incubated for 15 minutes. For blank solution 2 ml methanol was taken and same procedure was repeated.

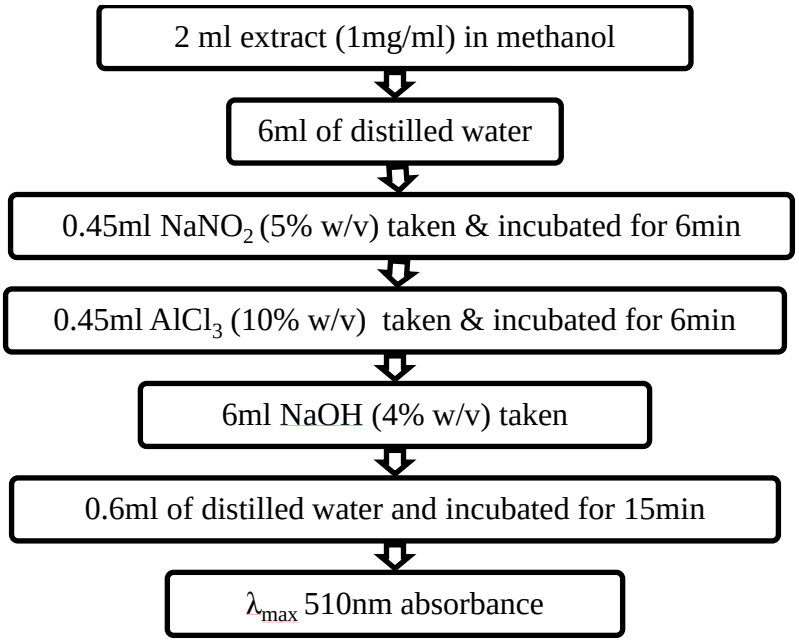


Figure 3.5: Schematic diagram of preparation of extract solution

Preparation of blank solution:

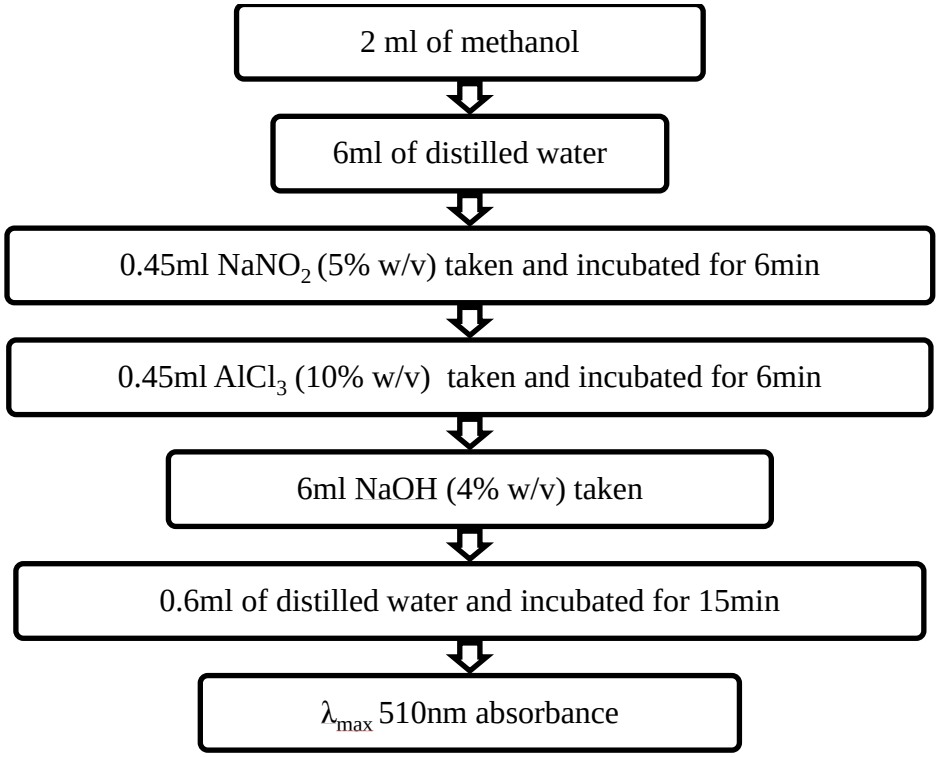


Figure 3.6: Schematic diagram of preparation of blank solution

CHAPTER-FOUR

RESULT & DISCUSSION

Result & Discussion

4.1 Result of Brine Shrimp Lethality Bio-Assay

The n-Hexane fraction of the *Ficus hispida* inspected using a magnifying glass and the number of survivors counted. The effectiveness of the concentration and % mortality relationship of plant product was expressed as a Median Lethal Concentration (LC_{50}) value. LC_{50} represents the concentration of the standard and n-Hexane extract that produces death in half of the test subjects after a certain period. The percentage mortality at each concentration was determined using the following formula:

$$\% \text{ Mortality} = \frac{(\text{Number of dead nauplii}) \times 100}{\text{Total number of nauplii}}$$

The LC_{50} of the test samples was obtained by a plot of percentage of the shrimps died (% Mortality) against the logarithm of the sample concentration (Log C) and the best-fit line was obtained from the curve data by means of regression analysis.

4.1.1 Preparation of Curve for Standard

Here, Tamoxifen was used as reference standard.

Table 4.1: Results of the bioassay of Tamoxifen (standard)

Test tube no.	**Concentration (C) (µg/ml)**	**Log C**	**Number of Nauplii alive**	**Number of Nauplii dead**	**% Mortality**	**LC_{50} (µg/ml)**
1	400	2.602	6	4	40	0.78726
2	200	2.301	0	10	100	
3	100	2.000	7	3	30	
4	50	1.699	4	6	60	
5	25	1.398	5	5	50	
6	12.5	1.097	5	5	50	
7	6.25	0.796	6	4	40	

8	3.125	0.495	3	7	70	
9	1.5625	0.194	10	0	0	
10	.078125	-0.107	1	9	90	

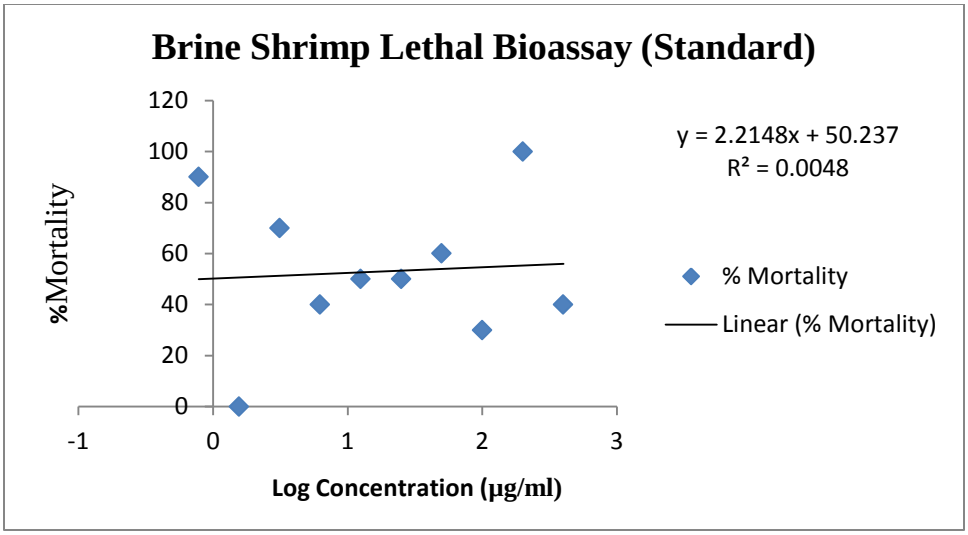


Figure 4.1: % Mortality and predicted regression line of Tamoxifen (standard)

4.1.2 Preparation of n-Hexane Fraction Curve

Table 4.2: Results of the bioassay of n-Hexane fraction (extract)

Test tube no.	**Concentration (C) (µg/ml)**	**Log C**	**Number of nauplii alive**	**Number of Nauplii dead**	**% Mortality**	**LC_{50} (µg/ml)**
1	400	2.602	1	9	90	1.136753
2	200	2.301	2	8	80	
3	100	2.000	4	6	60	
4	50	1.699	5	5	50	
5	25	1.398	5	5	50	
6	12.5	1.097	0	10	100	
7	6.25	0.796	7	3	30	

8	3.125	0.495	7	3	30	
9	1.5625	0.194	3	7	70	
10	.078125	-0.107	4	6	60	

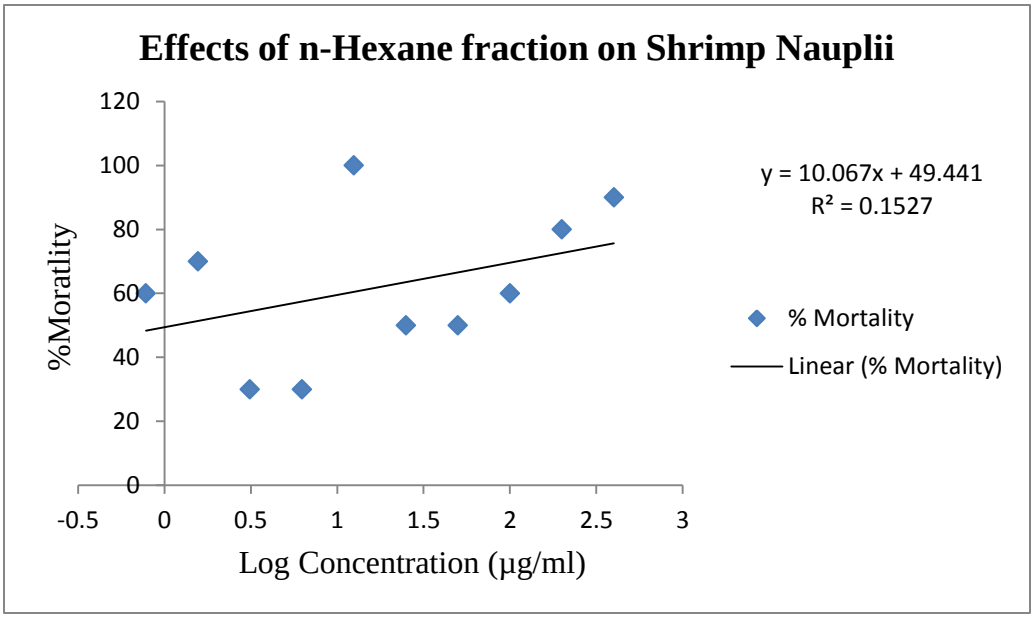


Figure 4.2: % Mortality and predicted regression line of n-Hexane fraction (extract)

4.1.3 Discussion

In Brine Shrimp Lethality bioassay, varying degree of lethality was observed with exposure to different concentrations of the test samples. The degree of lethality was not found to be directly proportional to the concentration ranging from the lowest concentration to the highest concentration in both standard and n-Hexane fraction samples. Mortality is not increased gradually with an increase in concentration of the test samples. Maximum mortalities took place at the highest concentration of 200µg/ml, whereas the least mortalities at lowest concentration 6.25µg/ml as shown in Table 4.1 and Table 4.2.

Table 4.3: Cytotoxic activity of Tamoxifen and n-Hexane fraction of *Ficus hispida* leaves

Sample	Linear regression equation	R^2 value	LC_{50} (µg/ml, 24hr)
Standard (Tamoxifen)	y = 2.214x + 50.23	0.004	0.78726
Extract (n-Hexane fraction)	y = 10.06x+49.44	0.152	1.13675

In this investigation, standard and n-Hexane fraction exhibited cytotoxic activities with the LC_{50} values 0.78726µg/ml and 1.13675µg/ml respectively as shown in Table 4.3. For n-Hexane fraction, LC_{50} value is more than the standard which indicates that the extract has less potent activity than standard against brine shrimp nauplii.

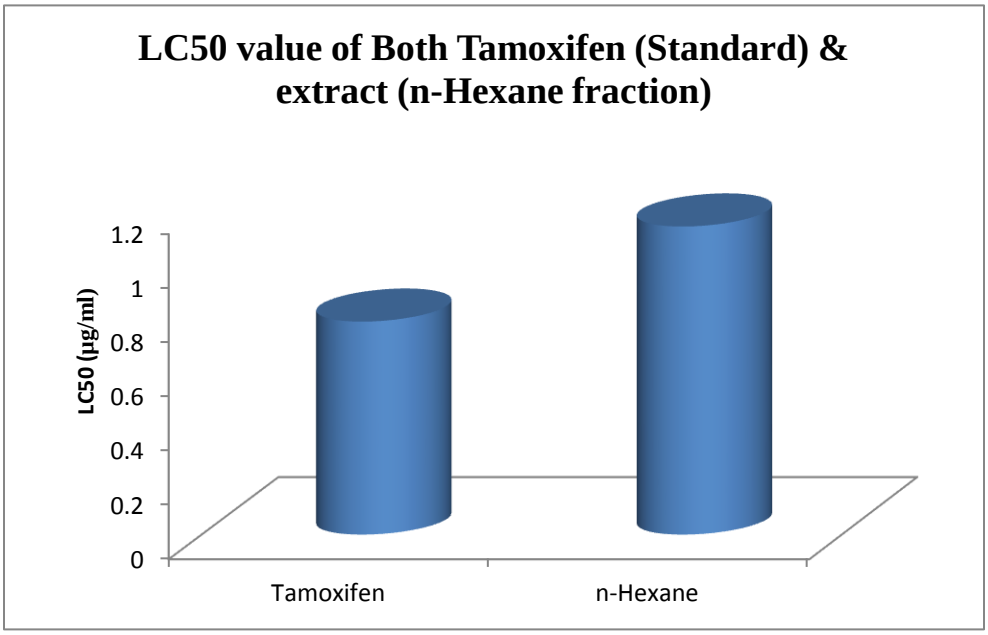


Figure 4.3: Comparison between LC_{50} values of standard and extract

From the above figure it can be concluded that for aqueous fraction the lethal concentration required to kill 50% of the sample population is higher than the standard. So the extract is less potent than Tamoxifen (Standard) at lower concentration.

4.2 Result of Antioxidant Tests

Antioxidant tests are classified by various methods. Samples were subjected to various standard methods to determine various scavenging capacity and amount that is equivalent to the standard like ascorbic acids. Antioxidant property of the n-Hexane fraction of *Ficus hispida* extract was determined by following methods:

- Determination of total phenolic content.
- Determination of total flavonoids content.

4.2.1 Result of Total Phenolic Content

The n-Hexane extract of *Ficus hispida* and the n-Hexane fractions of the methanol extract of *Ficus hispida* were subjected to determine total phenolic content. Ascorbic acid was used as reference standard (Singleton et al., 1999).

4.2.1.1 Preparation of Standard Curve

Table 4.4: Total Phenolic content of ascorbic acid

Concentration (µg/ml)	**Absorbance (at 765 nm)**	**Regression line**	**R^2value**
80	2.642	y = 0.024x+0.660	0.811
90	3.003		
100	2.962		
110	3.121		
120	3.806		

A linear relationship was observed when the absorbances were plotted against concentrations, as shown in Figure 4.4. This linear curve was considered as a standard curve.

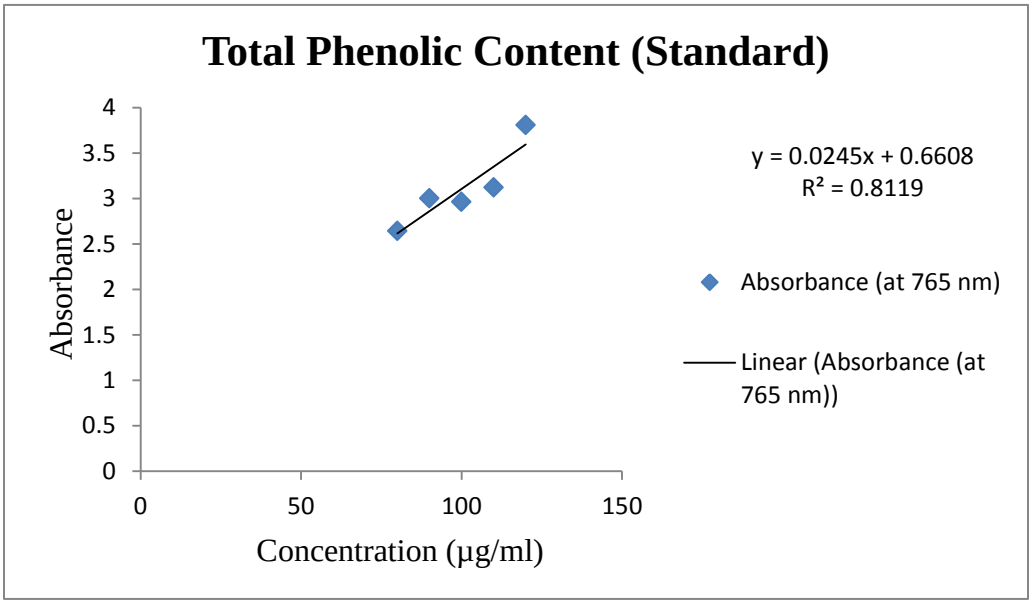


Figure 4.4: Graphical representation of Phenolic content of ascorbic acid

4.2.1.2 Total Phenolic content present in n-Hexane extract of *Ficus hispida*

Based on the absorbance values of the extract solution, reacted with Folin-Ciocalteu reagent and compared with the standard solutions of ascorbic acid equivalents (AAE), the total phenolic content present in the extract is calculated and given in the table below.

Table 4.5: Total Phenolic content in n-Hexane fraction of *Ficus hispida*

Concentration (mg/ml)	Absorbance	mg AAE/g
2	1.695	43.125

4.2.1.3 Discussion

The absorbance was found to be directly proportional to the concentration. Absorbance increased with the increase in concentration indicating increase in phenolic content. Absorbance of the n-Hexane fraction is less than the absorbance of standard. Based on the absorbance values of extract solution and using the regression line equation of the standard curve, 43.125 mg of AAE/gm of dried extract of phenol content was found in the n-Hexane fraction of *Ficus hispida*.

4.2.2 Result of Total Flavonoid Content

The n-Hexane fractions of *Ficus hispida* leaves were subjected to determine total flavonoid content. Quercetin was used as reference standard.

4.2.2.1 Preparation of Standard Curve

Table 4.6: Total flavonoid content of Quercetin.

Concentration (µg/ml)	Absorbance (At 420 nm)	Regression line	R^2 value
0	0	y = 0.053x-0.013	0.999
4	0.193		
8	0.422		

12	0.618		
16	0.834		

After absorbances were taken of different concentrations of quercetin ranging from 0µg/ml to 16µg/ml, a linear relationship was observed when the absorbances were plotted against concentrations, as shown in Figure 4.5. This linear curve was considered as a standard curve.

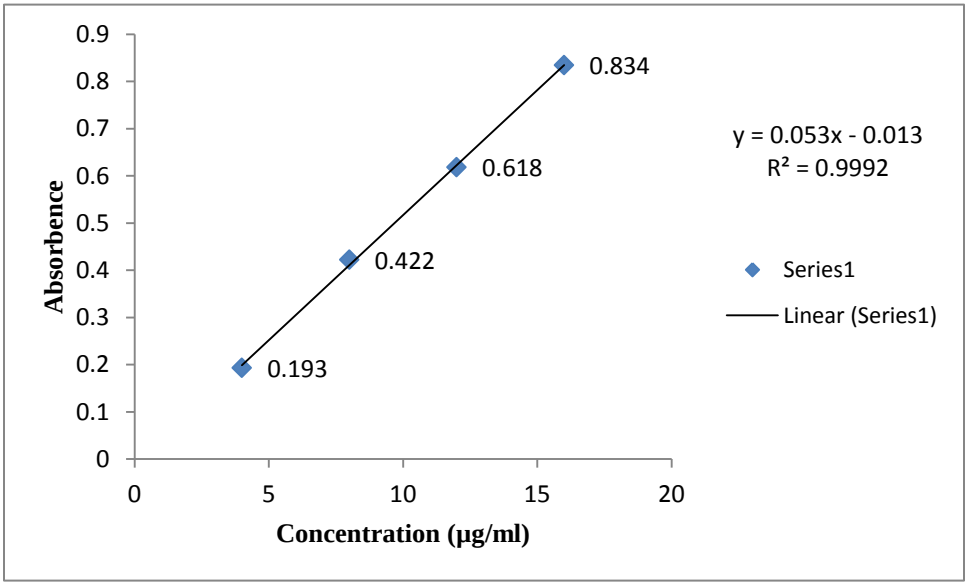


Figure 4.5: Graphical representation of assay of flavonoid content of quercetin

4.2.2.2 Total Flavonoid Content Present in n-Hexane Extract

Based on the absorbance value of extract solution and using the regression line equation of the standard curve, the total flavonoid present in the extract is calculated and is given in Table 4.8.

Table 4.7: Total flavonoid content of n-Hexane fraction of *Ficus hispida* leaves extract.

Sample	**Concentration (mg/ml)**	**Absorbance**	**Total flavonoid content (mg of quercetin/g of dried extract)**
n-Hexane fraction of *Ficus hispida*	1	1.545	29.39623

4.2.2.3 Discussion

To determine the total flavonoid content of the test samples the standard curve was used. For 1mg/ml concentration of n-Hexane fraction of *Ficus hispida* (leaves), 29.39623 mg of quercetin/gm of dried extract of flavonoid content was found. So it can be said that, the extract contains very low antioxidative compounds.

CHAPTER-FIVE

CONCLUSION

Conclusion

As the literature review suggests, the presence of several phytochemical compounds in n-Hexane fraction of *Ficus hispida* (leaves), makes the plant pharmacologically inactive.

LC_{50} value of *Ficus hispida* (leaves)in n-Hexane fraction showed less cytotoxic activity than Tamoxifen. Since n-Hexane fraction of *Ficus hispida* (leaves) exhibited less potent cytotoxic activity, so it can be investigated for anticancer, pesticidal and antitumor properties in future.

Antioxidant property in n-Hexane extract of *Ficus hispida* (leaves) was determined by Phenolic content assay and Flavonoid content assay. Phenolic content was 43.125 mg/gm and Flavonoid content was 29.39623 mg/gm in n-Hexane extract of *Ficus hispida (leaves).* So, n-Hexane extract of *Ficus hispida* (leaves) have poor antioxidant property. Mixture of compounds can lower antioxidant property in n-Hexane fraction of *Ficus hispida* (leaves), if any counteracting compounds were present in mixture. So pure compound isolation should be done in future to confirm antioxidant property of n-Hexane fraction of *Ficus hispida*(leaves)

However, studies are required on higher animal model and subsequently on human subjects to prove efficacy as an antioxidant and cytotoxic agent. It will help in the development of new novel and safe drugs for the treatment of various diseases.

CHAPTER-SIX

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