IN-VITRO BIOLOGICAL SCREENING OF ETHANOL EXTRACT OF *PTEROCARPUS INDICUS* WILLD. (FABACEAE) LEAVES

By

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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

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Approval

The thesis titled "*In-vitro* Biological Screening of Ethanol Extract of ***Pterocarpus Indicus* Willd** (Fabaceae) Leaves" submitted by Anika Tabassum (19146085), of Summer, 2022 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy on 29th March, 2023

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Ethics Statement

I obey the laws, rules, and regulations of my university and country. I work with integrity, fidelity, and honesty. I will take responsibility for my actions.

Abstract

This research was carried out to determine the biological properties (cytotoxic, antimicrobial) of a medicinal plant, named *Pterocarpus Indicus* Willd, which belongs to Fabaceae family. For evaluating cytotoxic property of ethanol extract of *Pterocarpus Indicus* Willd leaves, BrineShrimp Lethality Assay has been done. The obtained LC50 value was 1260.99µg/mL obtained for ethanol extract and for the standard Vincristin Sulfate it was 4.09 µg/mL. Additionally, In high concentration the percentage of mortality was very low and in low concentration the percentage of mortality rate was zero. So, Pterocarpus Indicus Willd do not have cytotoxic effect. For Anti-microbial property evaluation, Disk diffusion method has been done. There were high range of bacterial growth in every concentration of *Bacillus subtilis, Escherichia coli, and Salmonella typhi* bacteria. Only in the bacterial stain of *Staphylococcus aureus*, the range of bacterial growth is low in every concentration so, this plant have low level of antimicrobial effect. This research is essential to the discovery of pharmacological properties of this plant.

Keywrods: *Pterocarpus Indicus* Willd, Fabaceae, Cytotoxic, Antimicrobial property

Dedication

This work is dedicated to my family for their unconditional love and support

Acknowledgement

Alhamdulillah, all praise is due to Allah (SWT), from whom I receive my health and stamina to do this project task. I want to express my thanks to Almighty Allah for giving me the courage, endurance, support, and help I needed to finish the requirements for a bachelor's degree in pharmacy.

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Anika Tabassum

February, 2023

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List of abbreviations:

- mg = Milligram
- mL = Milliliter
- µg = Microgram
- LC50 =Median Lethal Concentration
- UV = Ultraviolet
- DMSO = Dimethyl Sulfoxide
- MDR = Multidrug Resistance
- HCl = Hydrochloric Acid
- R2= Regression Coefficient
- WHO= World Health Organization
- m= Meter
- cm= Centimeter
- gm= Gram
- Conc.= Concentration

Chapter 1

1. Introduction

Plants have been used to treat or reduce sickness for thousands of years. It is the source of unique chemical compounds with the potential uses in medicine and in other various fields. Plants contain various active substances, including alkaloids, volatile oil, steroids, tannins, resins, glycosides and phenols, which are accumulated in various sections of plant, including flowers, leaves, roots, fruits, bark and seeds. The combination of these secondary metabolites usually results in the favorable therapeutic effects of plant components. According to Farnsworth et al. (1985) discovered 119 secondary plants metabolites that utilized as medicines. The World Health Organization considers plants as the source of 11% of the 255 medications classified basic and necessary, and also a number of synthetic pharmaceuticals are derived from the natural precursors. It is because of their various properties like antioxidant, antimicrobial, antidiabetic, anti-inflammatory and radio protective, phytochemicals are widely employed in medicine. The emergence of drug's resistance and also the unwanted effects of many antibiotics which prompt the research for the novel antimicrobial medicines. Researchers have worked hard to develop effective extraction technologies for achieving high efficiency & the efficacy. The product of the extraction is referred as efficiency, but the potentials (magnitude of bioactivity / ability to generate an impact) of extract is referred to as effectiveness. Extraction from plants is one of the most environmentally friendly methods for isolating biological components. To acquire improved quality and high efficiency from herb extraction, procedures must be optimized for efficiency. Analytical techniques include a number of crucial steps such as sampling, sample preparation, quantification, statistical assessments, and so on. The requirement for the selection of the most appropriate process of extraction is clear in the fact that the extraction efficiency can depend greatly when other processes

are used on the same plant substances with same solvents (GUPTA et al., 2012).

Because there are few negative effects, medical plant treatments are regarded as safe. The best benefit is that natural medicines work best. The most important observation is that herbal treatments are safe for use by people of all sexes and ages.

1.1. History of Medicinal Plants

Plants are continuously provided us oxygen, shelter, food and most importantly medicines that gives us better life from the beginning of the earth's history, which is where humans and other animals first developed a link with them. Humans eventually developed the ability to identify and classify plant resources suitable for use in supplying their basic needs, along with the emergence of civilizations. Usage of the herbs and the extracts of herbal for therapeutic effects may be identified to the oldest stories, traditions & literature that were helped to define those plant which can relieve pain and treat illness. The renowned medicine systems such as Ayurvedic & Unani of Indian subcontinent, Native American of the North America, the Chinese & Tibetan of another parts of Asia, Amazonian of South America, Native American of the North America, all originated by the evolution of these plant-based medicine systems, which were primarily based on plants in the local area.

According to WHO, 35,000 - 70,000 species are used in medicines, which equates 14-28% of 250,000 species believed to exist worldwide and 35-70% of all species utilized globally. Over 70 percent of population in this world varies on the various plants for the care of our health. Today, about 50% of the most popular drug on market came from the plants. About 17% of the 250,000 species of plants in world studied for their potentials as medicines. For the purpose of creating novel medications, the chemical & biological variety of the plants represents an endless supply.

The flowering plants in the floras of the China and the North America is practically identical at 35,000. Native Indians employed 2564 medicinal plants, while traditional Chinese medicines are used 5000 of them. Herbal medicine from North America is a vast, untapped source of potential phytopharmaceuticals. Native Indians employed 9 percent of the flower for the therapeutic causes, according to American ethnobotanist Daniel Moerman. Yet, very few screens of medicinal plants in North America have ever been conducted, and the great majority of species are still unknown.

This editorial's goals include evaluating the key factors used in recent studies on medicinal plants and offering suggestions for a beneficial strategy for such research (Mamedov, 2012).

1.2. Medicinal Plants in Bangladesh

In Bangladesh, there are 5000 different plant species. Of them, 1000 are thought to have medical properties, and 250 are commonly utilized in medicines (Kadir, 1990). Researchers are currently interested in looking into the allelopathic/phytotoxic qualities of medicinal plants since they are a significant source of many pharmacological and toxicological property. Islam and Kato-Noguchi (2014) cited 2 factors for these growing interests: (i) simplicity of the separating phytotoxic plants from the therapeutic plants (ii) potential for medicinal plants to have higher concentrations of bioactive chemicals than other plants. Allelopathic property of Bangladeshi medicinal plant species are little recognized. As 20% of Bangladesh's total plant species are thought to be medicinal, they might be used as possible study subjects for allelopathic studies. The discovery of those previously undiscovered allelopathic medicinal herbs in Bangladesh may serve as the basis for the creation of fresh natural herbicides (Mominul Islam et al., 2018).

Table 1.1: Some available medicinal plants are listed below:

Scientific name	**Family Name**	**Plant category**
Acacia auriculiformis A. Cunn. ex Benth.	Fabaceae	Tree
Adhatoda vasica L.	Acanthaceae	Shrub
Bauhinia purpurea L.	Fabaceae	Tree
Calotropis gigantean (L.) W. T. Aiton	Apocynaceae	Shrub
Camellia sinensis (L.) Kuntze	Theaceae	Shrub
Diospyros peregrina (Gaertn.) Gürke	Ebenaceae	Tree
Erythrina variegata L.	Fabaceae	Tree
Eucalyptus camaldulensis Dehnh.	Myrtaceae	Tree
Ficus racemose L.	Moraceae	Tree
Garcinia mangostana L.	Clusiaceae	Tree
Gmelina philippensis L.	Lamiaceae	Tree
Holarrhena antidysenterica (Linn.) Wall.	Apocynaceae	Shrub
Justicia gendarussa Burm. f.	Acanthaceae	Herb
Lagerstroemia Indicus Willd (L.) Pers.	Lythraceae	Tree

(Mominul Islam et al., 2018)

1.3. Medicinal Plants in Drug

Plants are the backbone of many therapy in various rural & tribal parts in Bangladesh. From ancient times, people all across the world have extensively recognized as nature & natural drugs. In past, identification of the diverse microorganisms, the roots, stems, barks & seeds of plants utilized for treating diseases or illnesses caused by bacteria. Hence, it seems that certain plant has significant therapeutic properties and specific pharmacological effects. The rural, folklore and tribal area appreciate traditional process of the plants extraction or easy preparation like infusion, powder & decoction because of the great abundance of plants. Treatment of the infectious illnesses is one of the main uses of medicinal plant. Diseases brought on by harmful microorganisms including bacteria, viruses, and fungus are known as infectious illnesses. Many plants are used to treat STDs including gonorrhea and syphilis as well as pneumonia, meningitis, food-borne infections, ear infections, urinary tract infections, and syphilis. The most typical uses still involve the treatment of skin diseases, sinus infections, and common colds. Furthermore, as science has advanced, humans have learned that the readily available plants contain bioactive substances that work in tiny doses, such as antioxidants, alkaloids, glycosides, gums, flavonoids, terpenes, resins, gums. In general, people still use these treatments for ailments that aren't considered to be life-threatening.

Table 1.2: The oldest and most common methods of treating any diseases is use medicinal plants. Medicinal plants in Bangladesh play the following roles:

Scientific Name	**Family**	**Part used**	**Traditional Disease**
Abutilon indicum L.	Malvaceae	Stem, leaf paste bark, cooked leaves, infusion of leaves, root and seeds	bladder infections, gonorrhoea,vaginal infections,
Adina sessilifolia L.	Rubiaceae	Leaf paste	Impetigo, minor cellulitis,fungal infections
Bridelia retusa L	Euphorbiaceae	Ripe fruits, leaf paste,bark	impetigo, folliculitis, minorcellulitis, fungal infections
Caesalpinia bonduc L	Fabaceae	leaves & roots, dried leaves powder, leafpaste, seed, pod	Urinary tract infection,helminthiasis
Ixora nigricans L	Rubiaceae	Root and leaves extract, pastes	food poisoning, otitis media,diarrhea
Ocimum sanctum L	Labiatae	Leaves, aqueous decoction of leaves	Cold, viral hepatitis and viral encephalitis

(Bardhan et al., 2018)

1.4. Drugs obtained from medicinal plants

Several blockbuster pharmaceuticals generate directly / indirectly from the plants, which are the key source of new pharmacologically active chemicals. Despite the present focus on synthetic chemistry as the means to discover as well as produce medicine, plants continually make important contribution in treatment and in the prevention of illness. At the beginning of the 21st century, 11% of 252 medicines are originated from flowering plants.

Naturally, natural products are remain important as sources of therapeutic compounds. Several more natural items can be used as chemical things or the format for design, synthesis of new chemicals to treat human's illness and to the natural products which found direct therapeutic application as drug entities. Most of the core structures for synthetic chemicals are based upon the natural products, as there are some new ways to discovery of drugs like combinatorial chemistry as well as molecular modeling design based on computer, and various drugs are made up by synthetic chemistry, no ways can completely replace the importance of natural products in discovery and development of drug. Natural products can still be used as sources of new structures. Almost to 50% of medications that have been authorized in the last thirty years have been derived by directly / indirectly from natural products. Among the 175 small molecules that have been used to treat cancer since the 1940s, 85 are either natural products themselves or directly derived from them (Veeresham, 2012).

Table 1.3: Some drugs derived from medicinal plants are given below:

Drug Name	Plant Name	Use
Atropine	*Solanaceae*	Anticholinergic
Morphine	*Papaveraceae*	Opioid analgesic

Vinblastine	*Catharanthus roseus (L.)*	Antitumour
Acetylsalicylic acid	*Salix sp (Salicaceae)*	Anti-inflammatory
Digoxin	*Digitalis sp (Scrophulariaceae)*	Cardiotonic
Rivastigmine	*Physostigma venenosum Balf. (Fabaceaea)*	Cholinesterase inhibitor

(Lopez, 2011)

1.5. Selection of *Pterocarpus Indicus* Willd for this project

After examining several journals and publications on *Pterocarpus Indicus* Willd, it was determined that there was some articles available on this plant. So the main intention of this research is to detect new property about this plant if any or identify which property is not showing in this plant in Bangladesh but which is actually available in this plant in other country. As a result, the plant was chosen to study some properties, including cytotoxicity, antimicrobial. So, the goal of this present effort is to identify these properties of *Pterocarpus Indicus* Willd.

Figure 1.1: *Pterocarpus Indicus* Willd Tree

1.5.1. *Pterocarpus Indicus* Willd

Pterocarpus Indicus Willd is belongs to the family Fabaceae. The family Fabaceae includes the pantropical genus of trees known as Pterocarpus. It is a member of the subfamily Faboideae. And the name of the species of Pterocarpus indicus is Pterocarpus that is indigenous in Southeast Asia, western Pacific Ocean islands, Northern Australasia, East Timor, Indonesia, Malaysia, the Ryukyu Islands, Thailand and the Solomon Islands. This plant also found in Cambodia, Southernmost China, and the Philippines. Pterocarpus indicus is one of two species which is utilized as the source of the traditional diuretic lignum nephriticum, which was used from the 16th to the 18th centuries. Pterocarpus indicus populations are under severe threat from several directions.

1.5.2. Morphology of plant *Pterocarpus Indicus Willd*

This plant is now extensively dispersed across the tropics and is native to southern and eastern Asia as well as the northern and southwestern Pacific. When grown outdoors, this plant normally

grow to a height of 25 - 35 meter and this plant has a wide canopy. With annual rainfall ranging from 1300 to 4000 mm, it grows at elevations between 1 and 1300 m. Over the first three to four years, there may be a 2 m/year height increase until it slows to 1 m/year after that.

Local name: Padauk

1.5.3. Taxonomy (*Pterocarpus Indicus Willd*)

Table 1.4:

Kingdom	*Plantae*
Class	*Magnoliopsida*
Order	*Fabales*
Family	*Fabaceae*
Genus	*Pterocarpus*
Species	*Pterocarpus Indicus* Willd

1.5.4. Pharmacological properties of other genera & species

P. soyauxii is very brilliant red / orange while it first cut but gradually becomes warm brown when exposed to sunshine over time. Woodworkers love it because of its color. The Andaman padauk is *P. dalbergioides*, but the Burmese padauk is *P. macrocarpus*. While padauks and real rosewoods,to which they are partly related, can be mistaken, padauks are often rougher and less ornamental in figure. Like rosewood, padauk is occasionally used to produce guitars, xylophones, organ, and marimba keys. It is a crucial component of conventional Chinese furniture.

1.5.5. Related Publication on *Pterocarpus Indicus* Willd

There are some article on Pterocarpus Indicus Willd plant which is also published.

Some articles are:

- Antiviral Effect of *Pterocarpus indicus Willd* Leaves Extract against Replication of Dengue Virus (DENV) In Vitro (Ernawati Dewi et al., 2018).
- Pre-clinical investigation of analgesic, anti-diarrheal and CNS depressant effect of *Pterocarpus indicus* in Swiss albino mice (Hossen Rajib et al., 2021).

Thus, it is still necessary to identify several useful characteristics and applications of medicinal plants.

1.6. Project justification / rationale

According to an examination of the article on the chosen species, *Pterocarpus Indicus Willd,* some substantial research has been done on it. Yet, some research on this species has revealed potent properties. So, for this study, the primary goal is to identify the pharmacological properties of the plant's leaf extract which is not determined so that making further medicine, research will also look into this plant's undiscovered qualities.

1.7. Aim of Project

Aim of the research is to determine pharmacological properties of selected plant, ***Pterocarpus Indicus* Willd** (Family: Fabaceae)

1.8. Objective of the project

Objective of the project is to-

1) Evaluate cytotoxic property of selected plant.
2) And evaluate the antimicrobial property.

1.9. Evaluation of cytotoxic property of *Pterocarpus Indicus* Willd leaves extract

Cytotoxicity, an in-vivo test, used to detect if a chemical would kill cells directly or by the leaching of poisonous compounds (Medical Devices, 2015). Cytotoxicity, the toxicity carried on by an interaction chemotherapeutic agent with live cells. The identification of the usage of nanoparticles is assisted by the assays of cytotoxicity and it is necessary (Mukherjee, 2019). When cells are exposed to cytotoxic substances, they may react in a variety of ways, for example necrosis in where they miss out integrity of their membrane as well as quickly died due to destruction of cell, they might be stop growing & dividing or else they may be activated a program which can causes controlled cell death genetically, known as apoptosis (cytotoxicity, 2016).

1.9.1. BRINE SHRIMP LETHALITY BIOASSAY

A basic cytotoxicity test for the bioactive compounds is brine shrimp lethality bioassay. It is rely on test chemicals' capacity of killing brine shrimp, a simplistic organism brine shrimp (Artemia salina) (Harwig & Scott, 1971). Nauplii are 22 mm in their length, and they are as large as they can examine without any magnifying glass & also they are perfectly small for hatching in the big

amount and do not need for a large space in lab. Michael et al. were the ones who initially suggested this test, and numerous other groups afterwards improved it. This test is frequently used for assessing toxicity of the substances, for example heavy metals, drugs, herbicides notably plant extracts. It mainly serves as preliminary toxicity check before additional research using mammalian animal models. The solvent employed in this assay can be provided false results because of its toxic activity so the solvent is an essential component of the assay. Some organic solvents and detergents have been reported to exhibit significant cytotoxicities in vivo. It is necessary to conduct a thorough investigation into how high solvent concentrations impact brine shrimp mortality bioassay findings and to provide recommendations for the maximum working concentration of solvents. The authors of this paper conducted an experimental evaluation of the solvent's toxicity on brine shrimp. DMSO was proved as a safer solvent in brine shrimp lethality test, while ethanol was recommended as the solvent with the highest working concentration (Wu, n.d., 2014). The brine shrimp lethality bioassay is a quick (24 hour), easy to learn, doesn't need aseptic methods, is cheap, and test material needs in small amounts might be 2-20 mg or less (Apu et al., 2010).

1.10. Evaluation of antimicrobial activity of *Pterocarpus Indicus* Willd Leaves extract

The global health community is extremely concerned about the emergence and distributed the resistance of antibiotic and also evaluation of various type of disease which are caused by organisms. In general, antimicrobial drugs have a critical role in lowering the burden of infectious illnesses worldwide. Yet, due to the fact that there are fewer, or often no, effective antimicrobial treatments available for the infection caused by pathogenic bacteria, the rise and distribution of MDR variants in bacteria grown to be a serious concern to the public's health. Finding novel antimicrobial drugs is therefore of utmost relevance in view of the evidence of the fast global spread of resistant clinical isolates. The fast, widespread establishment of resistance to recently introduced antimicrobial drugs in the past, however, suggests that even new families of antimicrobial medicines will have a limited shelf life. Most of the medicinal plants are identified as the important sources of naturally occurred antimicrobial chemicals as a strong therapy option for these troublesome bacterial illnesses. WHO claims the greatest place to get a range of medications is medicinal plants. Due to their antibacterial properties, which are brought on by phytochemicals produced during the plant's secondary metabolism, several plants have been employed. Plants carried a high range of metabolites, including flavonoids, phenolic compounds and tannins, which are showed to have antibacterial activities in vitro. Many phytotherapy books have described several medicinal plants to treat various infectious diseases like skin infections, respiratory ailments and urinary tract infections. This study sought to examine the in-vitro antibacterial property from a few medicinal plants against prevalent microbial infections, taking into account the enormous potential of plants as sources for antimicrobial medicines (Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Salmonella typhi) (Sarita Manandhar

et al., 2019). In the research, the antibacterial property of Ethanolic extracts of Pterocarpus Indicus Willd leaves against a few spoilage microorganisms is examined. The minimum inhibitory concentrations of extracts of plant against the Gram-positive bacteria (Staphylococcus aureus, Bacillus subtilis) and also the Gram-negative bacteria (Escherichia coli, Salmonella typhi) both have been determined using the agar well diffusion technique.

1.10.1. Disc diffusion method (principle)

In order to determine if plant extracts have any antibacterial properties, Bauer et al. (1966) used the disc diffusion technique for antimicrobial susceptibility testing. A sterile brush used for uniformly lawn Muller Hinton agar plate with bacterium culture (modified to 0.5 McFarland standard). The sensitivity test was conducted after the plates had been dried for 15 minutes. The Mueller Hinton agar surface was covered with discs that was impregnate with variety of plant extracts. Six discs constitute each test plate. One is positive control and another one is negative control, and four of treated discs. Besides of the controls, every plate has the four treated discs which is place in the equal distance to each other. Then the plates incubated at the temperature of 37°C for one day means twenty-four hours. After the incubation, plates had been examined for the zone of inhibition. Then the inhibition zone was measured by the use of calipers & recorded (Zaidan et al., 2006).

Chapter 2: METHODOLOGY

2.1. Collection of the Plant

Pterocarpus Indicus Willd was collected for this study for confirming the previous research result on its biological characteristics has been done and also trying to identify some new biological characteristics. After a thorough review of the available literature on this species, it was decided to conduct an analysis. On November 5th, 2022, *Pterocarpus Indicus* Willd leaves were taken from the National Botanical Garden in Mirpur, Dhaka, Bangladesh.

Table 2.1: Research of ***Pterocarpus Indicus* Willd**

Plant Name	Scientific Name of Plant	Family of Plant	Part of Plant
Baro-padak	***Pterocarpus Indicus* Willd**	Fabaceae	Leaves

Figure 2.1: *Pterocarpus Indicus* Willd leaves

2.2 Verification of the plant:

After the selection of the leave, it was submitted for the verification of the plant to the National Herbarium Bangladesh in Mirpur, Dhaka. Then after a week, a token was received, and accession number of the plant is DACB- 87496. This plant is verified by Bangladesh National Herbarium in Mirpur, Dhaka had verified as genuine.

Figure 2.2: Collection of the accession number

2.3. Extraction process

Firstly, medicinal plant is extracted then processed for the direct ingestion for the herbal / for the traditional medicines / for using in any research. Concept of processing of medicinal plants in researchable use needs time & appropriate collection of plant, needs suitable process of drying, and lastly suitable grinding procedure. The process of extraction of medicinal plants involves separating active plant substance including flavonoids, alkaloids, steroids, terpenes and glycosides

and also the standard techniques of extraction. Maceration, decoction, infusion, digestion, percolation & Soxhlet extraction, microwave-assisted extraction, and ultrasound-assisted extraction, all of these are utilized in the extraction process. Moreover, secondary metabolites are separated and purified by using thin-layer chromatography, paper chromatography, HPLC, gas chromatography. The suitable process of extraction is determined by the material type of plant, Solvent, solvent pH and temperature. It is also varies on how final items will used (Abubakar &Haque, 2020).

The extraction process is divided into two phases. First is the preparation & drying of the plant materials which have 2 steps, and second is the extraction process which have 5 steps.

Plant Material:

I. Crude Plant

II. Washing

III. Oven Drying

Extraction Process:

I. Reduction of the Size

II. Extraction

III. Filteration

IV. Concentration

V. Drying

2.3.1. Plant material preparation

Firstly, Leave are ripped from stem of the plant and then leaves are washed by the clean water to eliminate scrap and also the dust. After that, cleaned leave were going to dry under the sun for twenty four hours and then in a hot air oven, the leaves are dried for one hour at the temperature of 30-40°c and for the next step, dry leaves was arranged.

2.3.2. Extraction procedure

- **Reduction of the size and weigh**

Dried & crusted leaves was ground in a large capacity grinding machine with coarse dust. Aiming to prevent cross-contamination, about 44.77g of powder were collected, stored in an air tight plastic vessel with appropriate label and then placed in a cold, dry, and dark place pending further examination.

Figure 2.3: Grinded powder

- **Extraction of the plant by solvent**

Two methods of the extraction techniques are classified depended on types the of solvents used:

• Extraction of the plant by the aqueous solvents

• Extraction of the plant by the organic solvents

For this research, plant materials were extracted using the extraction maceration procedure with ethanol serving as the organic solvent. A beaker containing powdered *Pterocarpus Indicus* Willd plant material was immersed in 2.5L of ethanol for one weak at room temperature with periodic shaking.

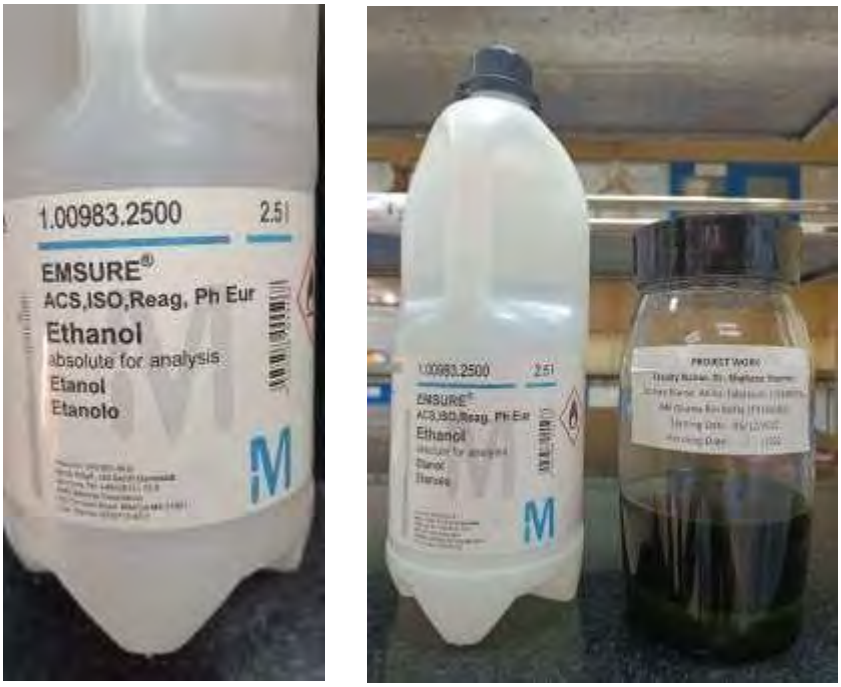

Figure 2.4: Ethanol 2.5L

➢ Filtration

The contents of beaker were filtered by clothes after two days of maceration using cotton & theWhatman filter, pore size was 110 mm.

Figure 2.5: Filtration by cloth

➢ Concentration

In order to create the ethanol concentrate extract, the filtrate was concentrated by the use of a rotary evaporator at 100 rpm with the temperature of 30 °C. After that, a petri dish was filled with a thick, concentrated mixture.

➢ Drying

Finally, laminar airflow (LAF) was applied to the petri dish in order to evaporate the extract's solvent. In order to reduce the likelihood of microbial development in the extract during drying, LAF was added as a preventative step. Following the extract's effective drying, it was stored on a petri plate.

Figure 2.6: Ethanol extract of *Pterocarpus Indicus* Willd leaves in dried form in petri-dish.

Table 2.2: Weight of *Pterocarpus Indicus* Willd ethanol leave extract gained by the complete extraction procedure

Initial weight: Petri-dish	142. 33g
Final weight: Petridish + extract	148. 58g
Weight of extract	6.25g

2.4. Cytotoxicity property Evaluation

2. 4.1. Experimental Procedure of Brine shrimp lethality assay

➢ Materials for the Brine shrimp lethality test

Table 2.3: List of the materials

Number	Materials
1	*Artemia salina* leach
2	Sea salt (NaCl)
3	Dimethyl sulfoxide
4	Lamp
5	Micropipette
6	Plant extract
7	Testtubes
8	Glass Vial
9	Small tank with perforated dividing dam to hatch the shrimp
10	Pipettte

➢ Preparation of seawater

For preparing sea-water, 76g pure NaCl had to weigh, after that this was dissolved in 2L distilled water. Then this was filtered.

➢ Hatching of brine shrimps

Test organism as brine shrimp eggs was Artemia salina leach which was purchased from the pet stores. For hatching, first of all, shrimp eggs was placed in the tiny tank together with seawater, and then exposed side of tank was covered. It was took two days for hatching and for develop into nauplii. Throughout the hatching period, a constant oxygen supply was maintained. As shrimp that have just hatched are drawn to light (phototaxis), nauplii that were free of egg shell were gathered from the tank's lit area. The nauplii were carefully removed from the fish aquarium using a micropipette after being diluted with fresh, clear sea water to improve visibility (Asaduzzaman et al., 2015).

Figure 2.7: Hatching of brine shrimps

➢ Preparation of test solutions with samples of experimental plants

Every test samples were diluted in 200µl of dimethyl sulfoxide (DMSO) using 32 mg of the sample, and then the volume was reduced to twenty mililitre by using the sea water. As a result, stock solution's concentration was 1600 µg/ml. Next, using sea water, the solution was

successively diluted to 800, 400, 200, 100, 50, 25, 12.5 & 6.25 µg/ml.

Table 2.4: 2.5 ml of the plant extract solution introduced to 2.5 ml of the sea-water containing 10 nauplii.

Concentration (µg/ml)	Extract Solution	Sea water containing 10 nauplii	Final volume
800	2.5 ml (1600µg/ml)	2.5 ml	5 ml
400	2.5 ml (800µg/ml)	2.5 ml	5 ml
200	2.5 ml (400µg/ml)	2.5 ml	5 ml
100	2.5 ml (200µg/ml)	2.5 ml	5 ml
50	2.5 ml (100µg/ml)	2.5 ml	5 ml
25	2.5 ml (50µg/ml)	2.5 ml	5 ml
12.5	2.5 ml (25µg/ml)	2.5 ml	5 ml
6.25	2.5 ml (12.5µg/ml)	2.5 ml	5 ml

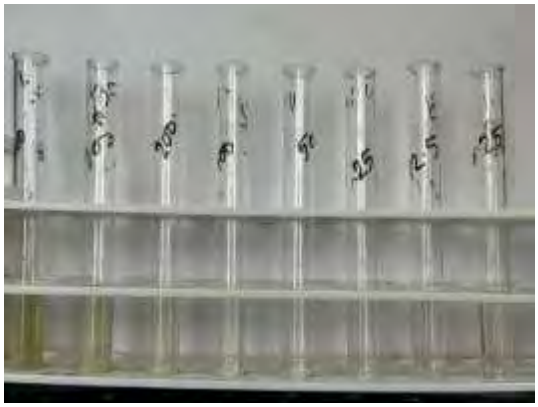

Figure 2.8: Test solutions with samples of the experimental plant

- **Control group preparation**

In the cytotoxicity research, control groups were utilized to confirm the test methodology and guarantee that the results were only activity of the test agent & that effects the other potential variables was eliminated. There were two types of the control groups which used in this test:

- Positive control
- Negative control

The Positive control group preparation

A widely recognized cytotoxic agent works as positive control in a cytotoxicity research, and the test agent's results are compared to positive control. Vincristine sulfate utilized in this research. Vincristine was tested at extremely low concentrations (20, 10, 5, 2.5, 1.25, 0.625, 0. 3125 and 0. 156 µg/ml) since it is a very cytotoxic alkaloid.

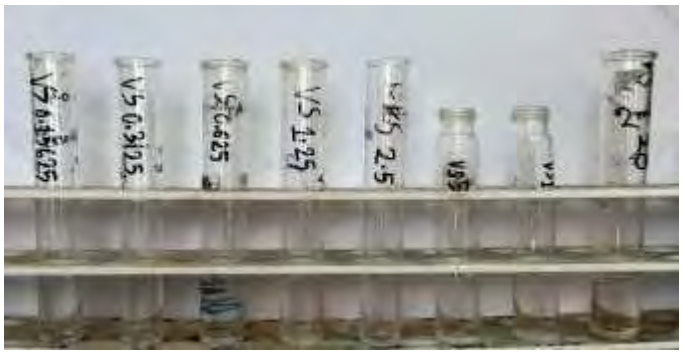

Figure 2.9: The Positive control group

The Negative control group preparation

3 test tubes was carried 4.95 ml simulated sea water and 10 nauplii, was used as control groups,

and then 50µl DMSO added to each of them. Test is deemed invalid if brine shrimps in these vials exhibit a high incidence of mortality because nauplii perished for reasons other than the compounds cytotoxicity.

Figure 2.10: Negative control group

- **Counting of nauplii**

Using a magnifying lens and a dark backdrop, the test tubes were examined after twenty four hours for counting the number of nauplii was survived. This information used to compute the percentage (%) of the lethality of brine shrimp in each concentration. The Abott formula was used to adjust the mortality (*Abott W. S., 1925*).

$P_t = [(P_o - P_c)/ (100 - P_c)] \times 100$

Here,

P_o = Observerved mortality

P_c = Controlled mortality

Often, a plant product's efficacy or concentration-mortality connection is stated as a LC50. It is the concentration of the chemical that, after a specific exposure period, results in the death of half of the test participants and was established using the linear regression approach by graphing the

mortality rate against the corresponding log of concentration (Md. Asaduzzaman et al., 2015).

2.5 : Antimicrobial property Evaluation

As test sample, ethanol extract of *Pterocarpus Indicus Willd* leaves was used.

Table 2.5: Materials used for the test

Number	Material
1	Nutrient Agar Medium
2	M.H. Agar
3	Paper discs
4	Petri dish
5	Micropipettes
6	Forceps
7	Test tubes
8	Autoclave
9	Spirit burner
10	Refrigerator
11	Incubator
12	Laminar air flow hood
13	Nose Masks
14	Hand gloves

- **Microorganism used in test**

Bacterial Strains obtained in pure culture.

Table 2.6: List of microorganism used

Gram-positive Bacteria	Gram-negative Bacteria
Staphylococcus aureus	*Salmonella typhi*
Bacillus subtilis	*Escherichia coli*

- **Sterilization process**

Conical flasks, beakers, cotton swabs, petri dishes and forceps was sanitized and stored in an aseptic environment before the test was conducted. This sterilization is carried out to prevent any microbiological or cross contamination throughout the procedure. Before working in Laminar Air flow Hood, UV lights were turned on for one hour to maintain control of the working environment. UV radiation was also used to disinfect micropipette tips, cotton, forceps, blank discs, and other items. All the equipment were autoclave sterilized for twenty minutes at 121°c and 15 lbs/sq. in of pressure. After the experiment, all the equipment was sterile and the bacterial strains were destroyed to prevent contaminating the environment.

- **Procedure of antimicrobial test**

At first, nutrient broth about 2.5g had to dissolve in 100ml of the distilled water for the production of the culture. Afterwards, 4 conical flasks were obtained, each conical flask was contained 10 ml of broth mixture with 4 distinct strains of bacteria was added to it. After that, conical flasks was placed in shaking incubator at temperature of 37° C for twenty four hours. All flasks was takenout

from incubator after 24 hours after that it was store in controlled atmosphere. Afterwards, 7.6g M.H. Agar had to be dissolved in 200 ml of distilled water for creating agar medium. Then, M.H. Agar was prepared and put into a petri dishes right away. The petri dishes was kept at the room temperature to cool. Meanwhile, the test samples of plant extracts with the concentrations of 500, 250, 125, 61.5, 31.25 and 15.625 mg/ml was made and then placed onto the paper discs. Bacterial strains were speared into the solidified M.H. Agar in patri-dishes using cotton bars. Plant extract test discs and regular streptomycin discs were put in patri-dishes. Then the petri dishes was maintained in the incubator for 24 hours at 37^{o}c to encourage bacterial growth. Once more, 24 hours later, standard and test sample discs were collected, along with patri-dishes harboring various bacterial strains. The inhibitory zone formed by the standards & test sample discs was examined.

- **Determination of the Inhibition Zone for test**

Antimicrobial activity of agent is determined by the capacity of inhibiting bacteria growing around the disc in petri dishes. The growth of microorganism suppression by the disc can provide a distinct zone for the inhibition. Antibacterial properties of test samples was tested after incubation by measured diameter of zone of inhibition with the transparent scale.

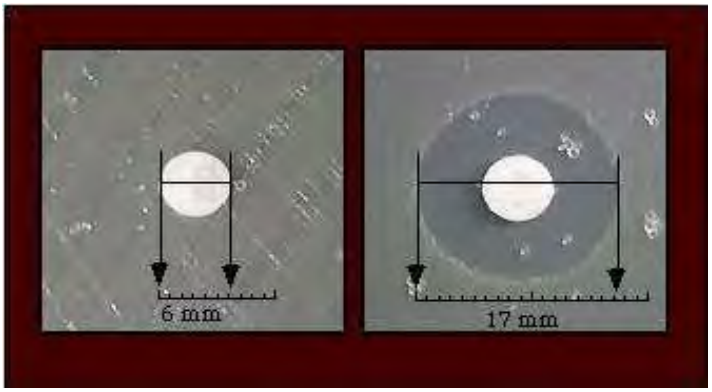

Figure 2.11: Determination of clear inhibition zone

Chapter Three: Observation & Results

3.1 : Cytotoxicity property Evaluation

3.1.1 : Brine shrimp lethality assay Evaluation

Table 3.1.: Positive control (vincristine) effect on shrimp nauplii

Concentration (µg/mL)	Log Concentration	Number of nauplii were taken	Noumber of nauplii were dead	Number of nauplii were alive	% of Mortality	LC50
0. 156	-0.8069	10	1	9	10	
0. 3125	-0.505	10	2	8	20	
0.625	-0. 204	10	3	7	30	
1. 25	0.0969	10	4	6	40	4.09 µg/mL
2.5	0. 3979	10	5	5	50	
5	0.69897	10	9	1	90	
10	1	10	10	0	100	
20	1. 301	10	10	0	100	

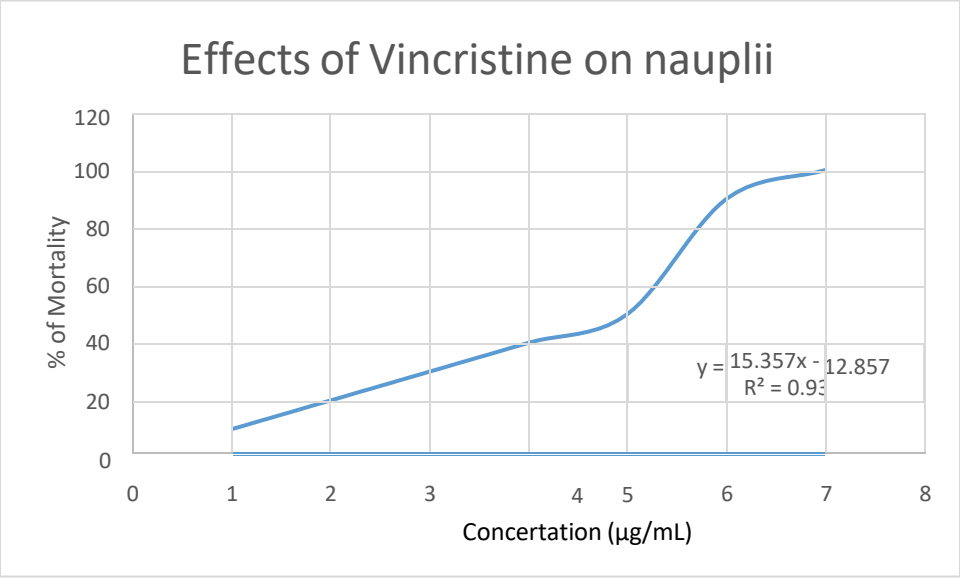

Figure 3.1: Percentage of mortality & predicted regression line for vincristine sulfate

Table 3.2: Effect of ethanol extract of *Pterocarpus Indicus* Willd on the nauplii

Concentration (µg/mL)	**Log Concentration**	**Number of Nauplii were taken**	**Number of Nauplii were Dead**	**Number of Nauplii were alive**	**Mortality %**	**LC50 (µg/mL)**
6.25	0.796	10	0	10	0	1260.99 µg/mL
12.5	1.097	10	0	10	0	
25	1.398	10	0	10	0	
50	1.699	10	1	9	10	
100	2	10	1	9	10	
200	2.301	10	2	8	20	
400	2.602	10	2	8	20	
800	2.903	10	3	7	30	

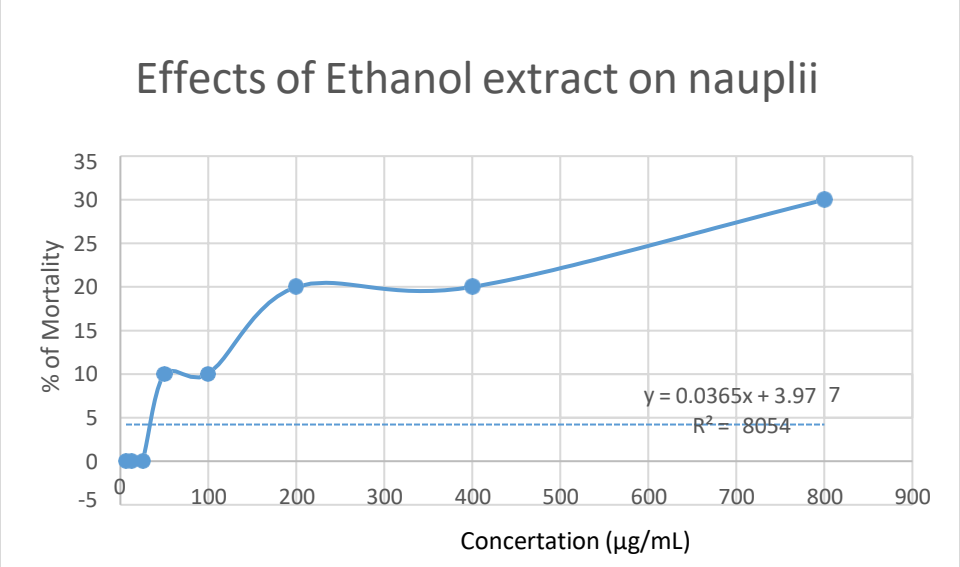

Figure 3.2: Percentage of mortality & predicted regression line for Ethanol extract of *PterocarpusIndicus* Willd

Explanation: From table (3.1 & 3.2), LC50 is 4.09 µg/mL obtained for vincristine and1260.99µg/mL obtained for ethanol extract. In higher concentration, percentage of dying of nauplii very low.

3.2. Anti-microbial property Evaluation

3.2.1. *Pterocarpus Indicus* Willd leaves antimicrobial activity evaluation

In anti-microbial property test of ethanol extract of *Pterocarpus Indicus* Willd leaves, some concentrations ranging 500 mg/ml to 15.625 mg/ml was generated. These concentrations was usedto test each bacterial strain. In this Evaluation, moderate results were identified. There is a small chance that some concentrations will have a very modest impact, but the majority of concentrations will not have any antibacterial function as we can see that in the petri dish of *Bacillus subtilis, Escherichia coli, Salmonella typhi* have bacterial growth in every concentration.Moreover, in the petri dish of *Staphylococcus aureus*, the bacterial growth is low and in every concentration there is a space of not growing bacteria so it can be said that the plant has antimicrobial effect on *Staphylococcus aureus*.

Fig: *Bacillus subtilis*

Fig: *E.coli*

Fig: *Salmonella typhi*

Fig*: Staphylococcus aureus*

Figure 3.3: The antimicrobial property evaluation on petri dishes.

Chapter Four: Discussion

4. Discussion

The Biological Screening Techniques of the ethanol leaf extraction of selected plant named *Pterocarpus Indicus* Willd, give significant information that may be used in the medical field. For evaluating cytotoxicity property of ethanol extract of *Pterocarpus Indicus* Willd leaves, BrineShrimp Lethality Assay was done. LC50 value of tested samples was established by plotting the mortality percentage of nauplii against sample concentrations on a graph. The regression analysiswas performed for obtaining the perfectly fitted line of the data curve. LC50 is 4.09 µg/mL obtained for vincristine and 1260.99µg/mL obtained for ethanol extract. We can see that, in higher concentration very low percentage of mortality happened and in low concentration there was no mortality happened. So, it can be said that, Pterocarpus Indicus Willd do not have cytotoxicity effect but additional study is needed to make sure its activity for benefit of the world healthcare. Antimicrobial property evaluation was done to identify the ethanol extract of the collected plant, *Pterocarpus Indicus* Willd leaves. This experiment could not bring significant result. We can see that from the figure 3.3, in the petri dish of *Bacillus subtilis, Escherichia coli, Salmonella typhi* there is high range of bacterial growth in every concentration. On the other hand, in the petri dishof *Staphylococcus aureus*, the range of bacterial growth is low and in every concentration there isa space of not growing bacteria so it can be said that the plant has antimicrobial effect on *Staphylococcus aureus*. So, the result is, this plant can show moderate level of antimicrobial effect.But further investigation is required for confirming about the presence of plant's antimicrobial property.

4.1. Conclusion

To assess biological activity, the ethanol extract of *Pterocarpus Indicus* Willd leaf was studied. This study's findings is the plant has a low degree of antibacterial activity and no cytotoxicity, but more research on this plant is necessary. Also, recent studies recommend a more thorough analysis of plants to discover unexplained biological qualities that will aid in the advancement of global healthcare and may bring any new useful properties in the health sector.

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