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

By

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A thesis submitted to the School of Pharmacy in partial fulfillment of the requirements for the degree of
Bachelor of Pharmacy (Hons.)

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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.

**Student's Full Name & Signature:**

**Md Osama Bin Rofiq**
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# Approval

The thesis titled "*In Vitro* Biological Screening of Ethanol Extract of *Pterocarpus Indicus* Willd. (Fabaceae) Leaves" submitted by Md Osama Bin Rofiq (19146093) of Spring 2019 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy on March 29, 2023.

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## Ethics Statement

No animal, human organ, or tissue was used during this study. Human blood was collected with full consent in compliance with ethical principles and guidelines.

## Abstract

This research was carried out to assess the biological properties of the *Pterocarpus Indicus* Willd. leaves of the Fabaceae family. To study the leaves, antioxidant activity was examined using DPPH free radical scavenging method along with total phenolic content and thrombolytic activity by clot lysis. The results were observed after performing the experiments where *Pterocarpus Indicus* Willd. leaves manifested poor antioxidant activity. The $IC_{50}$ value of the scavenging activity of the plant was 135.211 and for the standard ascorbic acid was 67.842 μg/ml. Additionally, the total phenolic count was 82.693. However, a promising thrombolytic effect was observed from the outcome. The plant extract showed nearly equal clot lysis percentage as the standard Clopidogrel. The in-vitro biological screening indicated the presence of antioxidant and thrombolytic activities. The study exhibits that the leaf of the *Pterocarpus Indicus* Willd. has bioactive chemical compounds which may provide a source of antioxidant and thrombolytic agents.



**Keywords:** *Pterocarpus Indicus* Willd., antioxidant, clot lysis, DPPH assay, Total phenolic content, phytochemistry.

# Dedication

*This work is dedicated to my loving parents as a token of my appreciation and gratitude for their unwavering love, patience, and encouragement throughout my life.*

## Acknowledgment

In the name of Allah, the Most Merciful, the Most Compassionate, I express my gratitude to the Almighty for granting me the strength and perseverance to complete this project.

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## List of Acronyms

- gm= gram
- ml= Milliliter
- mg= Milligram
- µg= Microgram
- IC50= Median Inhibitory Concentration
- DPPH= 2,2-diphenyl-1-picrylhydrazyl
- TPC= Total Phenolic Content
- DMSO= Dimethyl Sulfoxide
- HCL= Hydrochloric Acid
- Conc.= Concentration
- Ascorbic acid= ASA
- NHB= National Herbarium Bangladesh
- TCM= Traditional Chinese Medicine
- TIM= Traditional Indian Medicine
- ROS= Reactive oxygen species
- ALS= Amyotrophic lateral sclerosis
- SK= Streptokinase
- ATOD= Acute thrombotic occlusive disorders
- CVST= Cerebral venous sinus thrombosis
- MI= Myocardial infarction
- DVT= Deep venous thrombosis
- PE= Pulmonary embolism
- APAO= Acute peripheral arterial occlusions
- CVA= Cerebrovascular accident

- GA= Gallic Acid
- F-C reagent= Folin-Ciocalteu reagent
- ASA= Ascorbic Acid

# Chapter 1

## 1. Introduction

Plants are the fundamental basis of medicine. Since prehistoric times, people from numerous cultures have considered plants to heal themselves based on the affluent experience and knowledge acquired from their ancestors(Khan, 2014). With the passage of time, interactions among diverse cultures have eventuated which enriched the expertise of a culture in plants by virtue of the acceptance of others(Houghton, 1995). Medicinal Plants which include all the types of plants having therapeutic properties and utilised in herbalism, are considered as the backbone of the traditional medicines(Rasool Hassan, 2012; Singh R, 2015). For centuries, different cultures have utilized plants for preparing traditional medicines such as Unani, Ayurveda, Traditional Chinese and Korean medicine(Yuan et al., 2016).

At the present time, Medicinal plants are regarded as the major source of elements for the discovery and development of drugs(Singh R, 2015). In many developing countries, the use of medicinal plants is widespread and around 80% of the population relies heavily on them to fulfil their primary healthcare needs(Rakotoarivelo et al., 2015). Besides, medicinal plants have been widely used in the synthesis of drugs like antibiotics, anticoagulants, antimalarial agents and laxatives as raw materials(Singh R, 2015).

As per findings, there are over 400,000 secondary metabolites on earth and most of the plants and their medical activities are not investigated and explored yet(Aslam & Ahmad, 2016; Mohammed, 2019). Moreover, In the Indian subcontinent approximately 8000 species out of 17000 higher plants are explored by the local tribal colonies and utilized as medicinal plants(Singh R, 2015). Hence, investigation and identification of the unexplored plants and their activities can be significant for new drugs findings and treatment in future. The earth is filled with wide a variety of plant species having distinctive chemical diversity which leads to

heterogeneity in biological activities(Yuan et al., 2016). Eventually, utilizing these plants as ingredients or raw materials can lead to prominent drug discovery and development for treating more critical diseases(Yuan et al., 2016).

## 1.1 History of medicinal plants

Since ancient times, humans have been intimately involved with plants as a source of foods, habitats, fire and healings to survive. In searching for food, people sometimes acquired knowledge about the plants and its activity by swallowing, or often through using it to get protection from insects, mammals and disease(Rehman, 2021; Yuan et al., 2016). Back then, for preventing food spoilage different types of spices were used(Billing & Sherman, 1998; Rehman, 2021). There is no precise data to validate exactly from when people started to utilize plants for healing and treating diseases. For instance, farina of the seven species of existing medicinal plants were discovered in the Neanderthal burial site which is 60,000 years old and located in the northern Iraq(Rehman, 2021). Further, a species of mushroom which demonstrates activities against whipworm was explored closer to a 5000 years old frozen corpse also known as Otzi the Iceman in Otztal Alps(Rehman, 2021).

However, every culture had their own adaptive medication systems, materials, experts and practitioners for treating illness.

Greek culture has provided significant contributions in phytotherapy. The Greek medical system is also known as Unani(Yuan et al., 2016). Hippocrates(460-337BC), founder of allopathic medicine hypothesized that clinical disorders occur due to abnormality in the human physiology systems. He also developed the medication of treating the patient by eliminating the cause of the disease so that body systems could regulate properly. Besides, Aristotle, an ancient Greek philosopher and scientist discovered and identified almost 500 crude drugs. His

student named Theophrastus, wrote a book where he introduced another 500 crude drugs for the treatment of pathological conditions(Khan, 2014).

Traditional Chinese Medicine (TCM) is considered as one of the ancient medication therapies which is progressively getting acceptance at this time in the western countries as alternative medicine due to its distinctive theories, treatment and evidence-based methodology(Khan, 2014; Yuan et al., 2016). TCM involves the use of conventional Tai chi, acupuncture, minerals, animals, parts of the medicinal herbs (roots, seeds, leaves, flowers) and combined formulas(Facchinetti et al., 2015). At first, TCM was introduced by Fu His (2953 BC) which was eventually modified by Shen Nung, an emperor to make the medication more precise. Later, Cao-Yuan-Fang (550-630) gathered data from ancestors and surroundings about the medical problems and composed a book about the disease, symptoms and the cause of the pathogenic conditions. Following that, Wang-Tao (702-772) wrote a book along with 600 prescriptions. Ben Ca Gang Mu (1596), a comprehensive pharmacopeia was introduced by Li-Shizen with 1894 prescriptions regarding past diseases, symptoms and treatments which started to be used as reference books for academic and research training in the 1950s(Khan, 2014).

Traditional Indian Medicine (TIM) is also an ancient medical system that has been practiced in the name of Ayurveda for thousands of years in India. It was categorized into psychotherapy, rational and divine therapy(Subbarayappa, 2001). The medicinal system was documented in some of the ancient literatures named Atharva-Veda and Rig-Veda around 5000 BC(Khan, 2014). Moreover, numerous plants and spices were explored from literature which has been used as ayurvedic medicine for their distinctive effects from a long time such as *Curcuma longa*, (Rehman, 2021).

Most Islamic countries still maintain their use of Greco-Arabic medicine which is also recognized as Traditional Arab medicine as well as Islamic medicine. The Arab medicine

period is separated into 3 phases. These are i) Greco-Arab ii) Arab iii) Arab into Latin(Saad, 2013). Greco-Arabic phase initiated in the Islamic golden age when Islamic scholars started learning Greece and Rome literatures as well as composing in their own language(Khan, 2014; Saad, 2013). At the same time, physicians from the Islamic countries started to implement distinctive medical methods such as diet control, therapies and exercise(Khan, 2014). Subsequently in the second phase, evolution of medicinal science took place. For instance, they initiated pharmacy practices, built stores for treatment and buying medicine, divided pharmacological science and medicine which further led to extraction of plants and formulation of drugs and drug ingredients. Following that, Jaber Bin Hayan commenced studying methods for extracting materials from plants and ended up extracting royal acid, sulfuric acid, alcohol and nitric acid. Besides, another physician named Abu Bakr Rhazes in the middle of $8^{th}$ century AC conducted animal trials to test toxicity for ensuring safety and efficacy whereas Shahpur Ibn Sahl composed first ever pharmacopeia and assembled different types of drugs and drug materials for treating health conditions. Moreover, Al-Zahrawi introduced the method of formulating simple and complex drugs(Khan, 2014; Saad, 2013). However, in the evolution of Arab medicine, Islam religion also contributed significantly as many verses of Holy Quran portrayed hygienic lifestyle, aspects of poisons, different species of plants and fruits which later utilized as medicine(Aslam & Ahmad, 2016). Finally, the third phase of Arab into Latin started when European scholars acknowledged them for their inventions and started to learn from them by translating their books into Latin. At that time, there was an extraordinary writer, philosopher and physician in Arab (980-932 AD) named Ibn Sina who wrote the “Canon of medicine” which became the fundamental guideline of medicine for Europeans till the end of sixteenth century(Khan, 2014; Saad, 2013). In that period, Islamic scholars, physicians and philosophers placed remarkable contributions in different sectors of medical science such as

phytotherapy, anatomy, rational medicine, pharmaceutics through their research and writings which gradually wide spread all over the world(Subbarayappa, 2001).

## 1.2 Indigenous Medicinal Plants of Bangladesh

For having Geographical diversity, climate variability, and rich soils, The Indian sub-continent possesses a wide range of diverse forest ecosystems. As a part of the Indian sub-continent and subtropical regions, Bangladesh also achieves favorable environmental conditions for plant growth and great diversity in plant species which is a massive asset for drug finding and development. The Chattogram Hill Tracts, Sylhet, Khulna (Sundarbans), Bandarban are remarkably recognized for their diverse forest ecosystems. It is estimated that out of 5000 species only 400-500 species are yet recorded in Bangladesh from which many of them have been utilized for treating several diseases by Kavirajes, folk medicinal practitioners (Bardhan et al., 2018; Kaisar et al., 2011). Being a part of the Indian subcontinent, Ayurveda and Unani medicine are accessible and well accepted to the people of Bangladesh. Further, folk people and tribal communities are usually looked up to the medicinal plants considering that it is readily available, cost effective and has less side effects. However, herbs are getting acknowledged gradually and considered as resources for drug development.

Table 1.1: Common medicinal herbs in Bangladesh

| Scientific Name | Local Name | Part Used | Use |
|---|---|---|---|
| *Bridelia retusa L.* (Euphorbiaceae) | kantakhasi, Kamkui, Shukujja ghas (Chakma), bobipui (khumi tribe) | Bark, leaf paste, Ripe fruits, | Used to treat skin infections such as impetigo, folliculitis, minor cellulitis, erysipelas, fungal |

| | | | |
|---|---|---|---|
| | | | infections(Hossan et al., 2009). |
| *Ocimum sanctum L.* (Labiatae family) | Tulsi. | Leaves | reduce cold. Act against viral encephalitis and viral hepatitis(Bardhan et al., 2018). |
| *Rauvolfia serpentina* (Apocynaceae) | Sarpagandha | Flower | Reduce hypertension. |
| *Hiptage benghalensis L* (Malphighiaceae) | Madhobi Lata | Root, flower | Act against leprosy and ski infection(Mehedi Hasan et al., 2010). |
| *Amaranthus spinosus L* (Amaranthaceae) | Khuira kanta | Stem, leaf, | To treat jaundice(Rahmatullah et al., 2010). |
| *Kalanchoe pinnata (Lam.) Pers.* (Crassulaceae) | Pathorkuchi | Whole plant | Eliminate stone from stomach(Rahmatullah et al., 2010). |

## 1.3 Botanicals as Leads for Drug Development

Medicinal plants possess a diverse range of biologically active chemical compounds, which offer therapeutic value. This enormous diversity of plant species allows huge resources of chemical compounds which can be used for finding lead compounds. Medicinal plants have been used for centuries for treating several diseases as traditional medicine. Following that,

Traditional knowledge has been utilized to identify therapeutic compounds to use as lead compounds for drug development. However, it is estimated that only 5-15% of the higher plants are tested methodically for finding bioactive compounds(Cragg & Newman, 2001). Another statistic denotes that 119 extensively used chemical components that are utilized in drug formulation are obtained from only 91 plants and covers almost 62 therapeutic classes. Further, nearly 25% of prescribed drugs in the USA are acquired by extracting medicinal plants(Aslam & Ahmad, 2016; Farnsworth,' Olayiwola Akerele et al., 1985). In other words, maximum medicinal plants are still not investigated and identified yet which can generate enormous opportunities of finding peculiar components for drug discovery and development. Ingredients that are extracted from plants have a significant role for treating diseases since they operate as preventive, supportive or as synergic medicines(Mohammed, 2019).

Table 1.2: Therapeutic applications of medicinal plants

| Scientific Name | General Name | Parts of the plants | Functions |
|---|---|---|---|
| *Aloe vera L.* (Asphodelaceae) | Aloe vera | Leaf | Wound healing, anti-oxidation, anti-viral, anti-inflammation, anti-ulcer (Liang et al., 2021). |
| *Ginkgo biloba L.* (Ginkgoaceae) | Maidenhair Tree | Leaf | Used to treat dementia and Alzheimer's disease(Mohanta et al., 2014). |

| | | | |
|---|---|---|---|
| *Withania somnifera* (Solanaceae) | Ashwagandha | Seed, leaf, flower, fruit | Leaves are used to treat fever, seeds for enhancing sperm count, fruits for treating skin disease and flowers provide astringent and diuretic properties(Mandlik & Namdeo, 2021). |
| *Zingiber officinale* (Zingiberaceae) | Ginger | Rhizome | It manifests ani-oxidant, anti-cancer, anti-inflammatory, antipyretic and anti-viral activity. Also, used for increasing appetite and treating throat and tongue infections(Dissanayake et al., 2020). |
| *Cinnamomum zeylanicum* (Lauraceae) | Cinnamon | Bark | It shows antiproliferative, hypoglycemic and antioxidant activity(Shang et al., 2021). |

Table 1.3: Widespread drugs of plant origin

| Plant Origin | Drug | Chemical Class | Indication |
|---|---|---|---|

| | | | |
|---|---|---|---|
| *Taxus brevifolia* | Taxol | Taxoids. | Antitumor and anti-cancer(Yuan et al., 2016). |
| *Penicillium niger* | Penicillin | Beta-lactam antibiotic | Antibiotic |
| *Digitalis lanata* | Digoxin | Alkaloid | Cardiotonic(Aslam & Ahmad, 2016). |
| *Aspergillus terreus* | Lovastin | Statins | Hyperlipidemia(Yuan et al., 2016). |
| *Cinchona officinalis,* | Quinine | Alkaloid | Antimalarial, antipyretic, Analgesic(Aslam & Ahmad, 2016). |
| *Ranunculus ternatus* hunb. | Ternatolide | Terpenoids | Anti-tuberculosis(Yuan et al., 2016). |

## 1.4 Selection of *Pterocarpus Indicus* Willd.

*Pterocarpus Indicus* Willd. is selected due as there is very limited data about *Pterocarpus Indicus* Willd. globally in common journals and publication sites. As a consequence, the plant is chosen for identifying and evaluating antioxidant and of thrombolytic properties. So, determining the properties of *Pterocarpus Indicus* Willd. is the intention of this project.

### 1.4.1 Introduction to *Pterocarpus Indicus* Willd.

*Pterocarpus Indicus* Willd., locally known as Padauk in Bangladesh belongs to the family *Fabaceae*. It is usually found across the South Asia, particularly in India, Sri Lanka and

Bangladesh(Al-Hiari et al., 2021). In Bangladesh, the plant is available in National Botanical Garden, Dhaka, Mymensingh and Chattogram Hill Tracts and Sundarbans.

Fabaceae, also known as legume family, is considered as one of the largest families for having around 19000 species and 751 genera. Fabaceae is recognized as flowering plants. Plant species of this family have numerous utilizations in formulating natural products as well as serving as medicinal herbs, timber, fertilizers and crops. Among all the species of Fabaceae family, *Calpurnia aurea, Millettia ferruginea***,** *Pterocarpus marsupium, Pterocarpus santalinus* are notable medicinal plants in which 19.7% leaves and 17.3% of roots are used. Moreover, maximum species of Fabaceae family plants are shrubs, tree and herbs and the utmost plants are not investigated and identified yet(Asfaw & Abebe, 2021).

### 1.4.2 Morphology of *Pterocarpus Indicus* Willd. leaves

When grown in the open, it can attain a height of 25-35 meters (82-115 feet) with a wide canopy. Under favorable conditions, the tree can grow up to 2 meters per year during the initial 3-4 years, but the growth rate may decline to around 1 meter per year afterwards. Leaves are bright green in colour and size is around 6-12 cm long and 3-7 cm wide. Disc shaped fruits and size is around 5-6 cm. Flowering is commonly induced before the emergence of new leaves but extends beyond the leafing stage. The bright-hued flowers, resembling peas, emit a pleasant fragrance and appear in intermittent short bursts lasting 1-2 days. They are arranged in branched axillary racemes and have a length of approximately 1.5 cm (J Thomson, 2006).

### 1.4.3 Taxonomy of *Pterocarpus Indicus* Willd.

Table 1.4: Taxonomy hierarchy:

| Rank | Name |
|---|---|
| Kingdom | Plantae |
| Division | Magnoliophyta |
| Class | Magnoliopsida |
| Order | Fabales |
| Family | Fabaceae |
| Sub family | Faboideae |
| Genus | *Pterocarpus* |
| Species | *Pterocarpus indicus* Willd. |

### 1.4.4 Pharmacological characteristics of various genera and species of Fabaceae family

*Pterocarpus Indicus* Willd. is belonged to the Fabaceae family and Pterocarpus genus that possess thrived therapeutic value. From the family of Fabaceae, *Acacia catechu*, *Cicerarietinum L.*, *Delonixregia*, *CajanusCajan*, *Delonixregia*, *Pisumsativum* and *Sutherlandia frutescens* have anti-infammatroy, antioxidant, antidiabetics, antipyretic, and analgesic activites(Ahmad et al., 2016a). Moreover, *Pterocarpus marsupium* and *Pterocarpus santalinus Linn* are one of the widely species from the genus of *Pterocarpus.* Among them, *Pterocarpus santalinus Linn* exhibits antidiabetic activities(Arunakumara et al., 2011) and *Pterocarpus marsupium* also demonstrate anti-diabetic properties(Senthilkumar et al., 2020).

### 1.4.5 Related literature

There is very limited data published worldwide about the chemical properties and therapeutic effects of the barks of the plants.

## 1.5 Project Justification

The project to identify the chemical properties of the selected plant *Pterocarpus Indicus* Willd. to acquire deeper understanding regarding their potential use as it is observed that there is very limited data about the chemical components of the plants. However, different species of the same family exhibit significant therapeutic properties such as antimicrobial, antioxidant, anti-inflammation, antiplatelet, antihypertensive, insecticidal, hepatoprotective, and cytotoxic pharmacological activities(Ahmad et al., 2016b). Following that, the target of this study is to explore the pharmacological properties of the *Pterocarpus Indicus* Willd. plant leaves. So, the experiment will be conducted to identify the unknown properties of the selected plant expecting its potential to contribute in the development of drugs.

## 1.6 Aim of the project

This project's aim is to identify the potential medicinal properties of *Pterocarpus Indicus* Willd. leaves through an in-depth study of its chemical composition and biological activity.

## 1.7 Objective of the project

This project will focus on identifying the biological activity of the plant *Pterocarpus Indicus* Willd. To carry out the experiment ethanol extract of the *Pterocarpus Indicus* Willd. will be used to perform the following steps:

1. Evaluation of antioxidant property of *Pterocarpus Indicus* Willd. leaves extract through DPPH free radical scavenging method and (F–C) assay for TPC

2. Determination of thrombolytic property of *Pterocarpus Indicus* Willd. leaves extract.

## 1.8 Evaluation of *In vitro* antioxidant property

Unstable and highly reactive chemical species, called free radicals, are associated with several life-threatening disorders such as cancer, Alzheimer's disease, hypertension, Parkinson's

disease, cardiomyopathy, ALS, and complications of diabetes mellitus(Jamshidi-Kia et al., 2020). ROS such as alkoxyl, peroxyl, hydroxyl and superoxide which is a special type of free radicals, damages the body's defense mechanism to create oxidative stress. To prevent that, human body utilizes the defense mechanism by preventing the damage or repairing the damaged cells with the help of other antioxidant such as ascorbate (Vitamin C), lipoic acid, uric acid, tocopherols (Vitamin E) and ergothioneine. When the activity of the antioxidant defense system is not sufficient to prevent the interaction of ROS, an imbalance occurs, resulting in a state of oxidative stress. Moreover, acute oxidative stress continuously damages DNA, cell structures, lipids proteins which causes mutations, cell destructions and mitochondrial functions and leading to several diseases(Škrovánková et al., 2012).

Hence, Suppression of free radical activity is necessary to prevent damage to cells (Chowdhury et al., 2011a). As a remedy, antioxidant drugs were introduced which acts by donating electron (e-) or atom (H) against free radicals or neutralizing through phenol (-OH) (convert R, RO, ROO into RH, ROH, ROOH) to maintain cellular health and protecting against oxidative stress(Jamshidi-Kia et al., 2020). There are many identified spices and herbs that are utilizing as effective antioxidant sources. Tocopherols, flavonoids, ascorbic acid, beta-carotene, terpenes, and phenolic acid components manifest powerful antioxidant activity, either independently or synergistically in combinations(Škrovánková et al., 2012). "There are several approved methods that can be applied to determine whether an unidentified plant is a potential antioxidant source. These are: DPPH (2,2-diphenyl-1-picrylhydrazyl) radical assay, Phycoerythrin assay, ABTS assay, Total radical-trapping antioxidant parameter and FRAP assay(Antolovich et al., 2002)."

The antioxidant property analysis of *Pterocarpus Indicus* Willd. leaf extract was conducted to evaluate potential sources of antioxidant property. To identify a potential source of

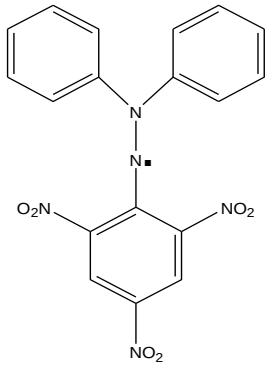
antioxidants, analysis of the antioxidant properties of *Pterocarpus Indicus* Willd. leaf extract was conducted using

i) DPPH radical assay(Antolovich et al., 2002).

ii) Total phenolic content assay(Ainsworth & Gillespie, 2007)

### 1.8.1 Evaluation of DPPH-based antioxidant assay

Various methods available to evaluate the ability of components to scavenge free radicals. Of these, DPPH is a highly considered and frequently used method for plant extracts due to its reliability(Sanna et al., 2011a). This method works by exploiting the ability of antioxidants to scavenge DPPH, causing a decrease in the initial concentration of DPPH (Brand-Williams et al., 1995; Sanna et al., 2011a). Phenolic compounds are assessed for their antioxidant properties by evaluating their ability to donate electrons and hydrogen atoms to free radicals. In this method, DPPH is used as a stable free radical which has unpaired electrons in the outermost shells. After that, phenolic compounds interact with the DPPH to measure the reduction of DPPH through a UV-VIS spectrophotometer. When DDPPH is exposed to a free radical, it gets oxidized and changes color providing an absorbance peak in the UV-Vis spectrophotometer. However, when an antioxidant comes close to the free radical, the free radicals get scavenged which inhibits oxidation. When oxidation is no longer occurred, it also decreases in absorbance. The ability of antioxidants to scavenge free radicals is reflected in the reduction of absorbance, with a lower absorbance indicating a higher scavenging activity(Celiz et al., 2020; Sanna et al., 2011b). The $EC_{50}$ index is used to measure the capacity of donating hydrogen atom of the phenolic compounds. The EC50 value provides an indication of the potency of phenolic compounds as antioxidants, with lower values indicating higher potency(Villaño et al., 2007).




*DPPH (oxidized form)

## 1.8.2 Evaluation of Total Phenolic Contents (TPC)

TPC, using F-C procedure, is one of most commonly and frequently used methods to assess the amount of phenolic content(Lamuela-Raventós, 2018). Phenolic compounds (PCs) are very useful and potent to neutralize the free radicals for having lower electron reduction potential. Further, PCs are able to stop and break the chain reaction of oxidative damages. This method is regarded on electron transfer reactions between the Folin-Ciocalteu(F-C) reagent and the phenolic compounds present in the sample. The F-C reagent oxidizes the phenolic compounds and forms a blue complex that can be measured using UV-VIS spectrophotometer. The intensity of the blue color is proportional to the TPC of the sample(Ainsworth & Gillespie, 2007; Sánchez-Rangel et al., 2013).

## 1.9 Evaluation of *in vitro* thrombolytic activity

Acute thrombotic occlusive disorders (ATOD) refer to a group of pathologic conditions characterized by formation of blood clot that blocks usual blood flow in the circulatory system such as Cerebral venous sinus thrombosis (CVST), acute peripheral arterial occlusions (APAO), myocardial infarction (MI), cerebrovascular accident (CVA), deep venous

thrombosis (DVT) and pulmonary embolism (PE),)(Perler, 2005). The blood clot formation (thrombus) develops in the blood vessels because of the haemostasis dysfunction and morbidity and mortality rates associated with this illness are high(Chowdhury et al., 2011b; Tabassum et al., 2017). According to recent findings, cardiovascular disease is holding the top cause of fatality in the United States and acute coronary thrombosis is responsible for most of the cases(Perler, 2005). To provide a remedy for this situation, Thrombolytic drugs were introduced to dissolve the thrombus and eliminated from the blood vessels as a result of restoring the blood flow(Verstraete & Collen, 1986). Mainly, Heparin, anti-coagulating agent, urokinase, SK and tissue plasminogen activator (tPA) are used to treat ATOD(Chowdhury et al., 2011b; Perler, 2005; Prasad et al., 2006; Tabassum et al., 2017; Verstraete & Collen, 1986). Streptokinase (SK), the first thrombolytic drug was known to be effective in thrombolysis therapy but additional activity to cause hemorrhagic complications pushed further investigation for alternative drugs. Thrombolytic drugs operate primarily by converting plasminogen into plasmin which is responsible for breaking down fibrin along with other haemostatic materials leading to dissolve the blood clots. Finally, dissolving the blood clots lead to reperfusion and diminish the risks and consequences. However, current evidence suggests that treating with thrombolytic drugs reduces the risk of fatality up to 50%(Perler, 2005).

As thrombolytic drugs are essential for treating thrombotic occlusive disorders, *Pterocarpus Indicus* Willd. leaf extract was examined using the following procedure explained by (Prasad et al., 2006) to identify potential thrombolytic compounds from this novel source.

# Chapter 2

# 2. Methodology

## 2.1. Plant Collection

*Pterocarpus Indicus* Willd. was chosen as a plant to conduct this research due to inadequate data on its biological properties. Proceeding from the bibliographic study and availability, the leaf part of the plant was selected for biological analysis. The leaves of the plants were collected in November 2022 from the University of Chittagong, Chattogram, Bangladesh.

Figure 2.1: *Pterocarpus Indicus* Willd. plant (Padauk)

Table 2.1: Identify selected part of the plant

| Name of the Plant | Scientific Name | Family | Part |
|---|---|---|---|
| Padauk | *Pterocarpus Indicus* Willd. | Fabaceae | Leaves |

Figure 2.2: Collection of *Pterocarpus Indicus* Willd. leaves.

## 2.2 Plant Authentication

After gathering the leaves of the *Pterocarpus Indicus* Willd. plant, samples of the leaves were submitted to the NHB, Mirpur, for authentication and verification. The specimen was certified and authenticated by National Herbarium and assigned accession number (DACB87496) within two weeks.


Figure 2.3: Verification of the plant and accession number collection from NHB

## 2.3. Extraction procedure

The medicinal herb was extracted following steps:

The entire procedure of the extraction can be allocated into two portions: 1. Preparation and Drying and 2. Extraction of the plant leaves.

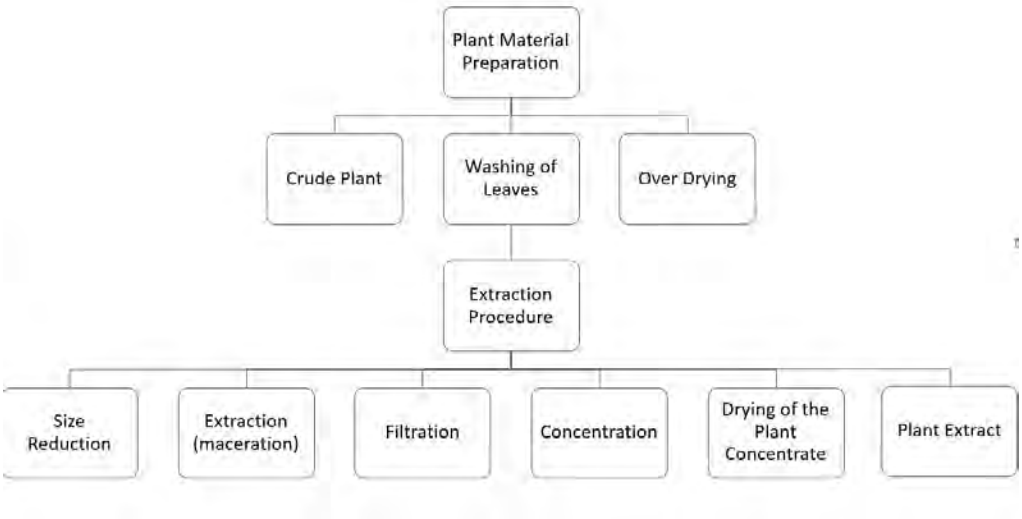



Figure 2.4: Flowchart for preparing plant extract from crude plants

## 2.3.1: Preparation of plant material

The plant's leaves were removed from the stem and rinsed in the clean water to eliminate plant debris and dust. The cleaned leaf was then left to dry for some days under the sun after that they were ready for the extraction phase.

## 2.3.2. Procedure for plant extraction

❖ **Size minimizing and weighing:**

The dry leaves were pulverized into powder particles using a high-performance grinding machine, resulting in approximately 50g of powder. The powder was then placed in an airtight container with proper labeling and stored in a dark, cool, and dry environment to prevent cross-contamination until further investigation.

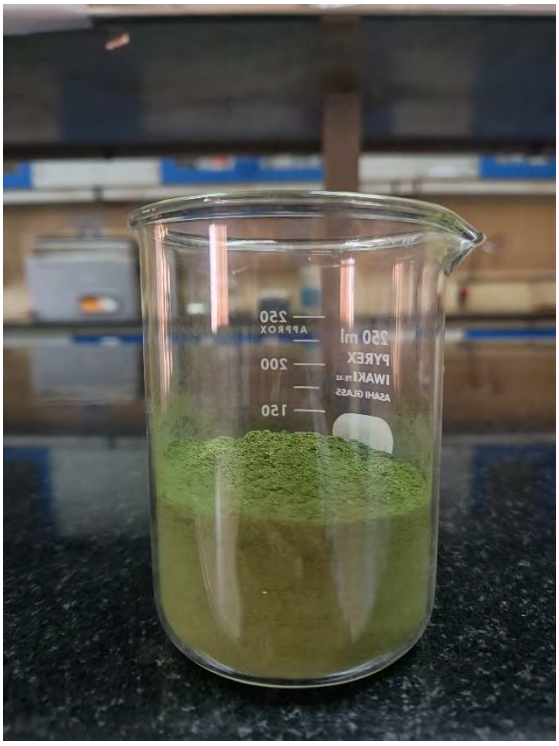



Figure 2.5: Grinded powder of *Pterocarpus Indicus* Willd. leaves.

❖ **Maceration of plant by solvent:**

To begin with, a maceration process was followed for the extraction and ethanol was used as organic solvent. Afterward, the plant material was soaked for the period of 7 days into 500 ml of ethanol in a glass container at room temperature with fitful stirring.


Figure 2.6: Solvent for maceration (Ethanol 2.5L)

❖ **Filtration:**

After the completion of maceration, white fabric, cotton and Whatman filter paper were applied sequentially to separate the extracted solvents.

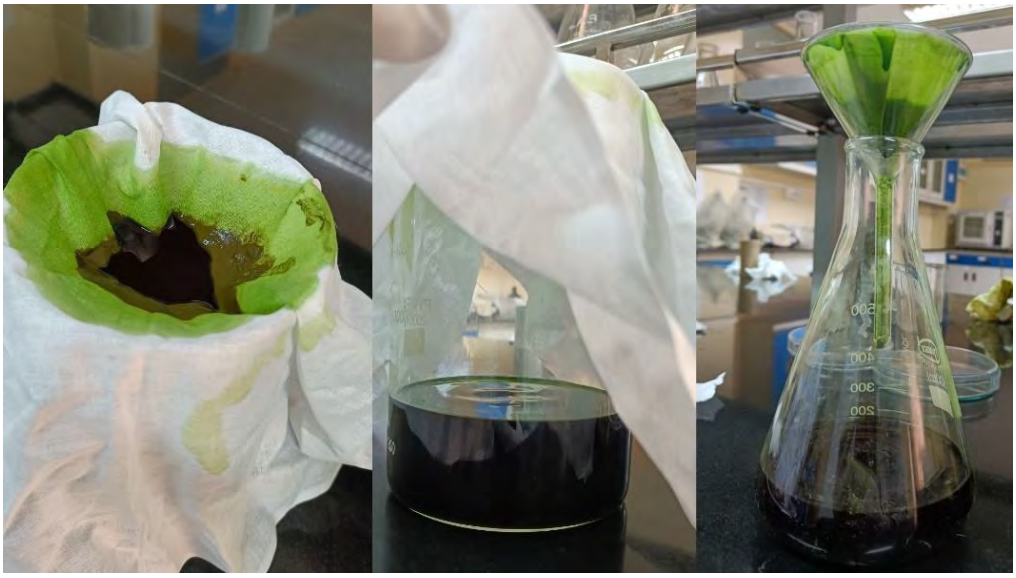


Figure 2.7: Filtration process using cloth (Pic 1 & 2) and filter paper (Pic 3)

❖ **Concentration:**

The acquired filtrate was needed to heat for producing ethanol concentrated plant extract. With this in mind, a water bath is used at 53° C to carry away the solvents from the sample by evaporating. Under the water bath, when thick concentrated extract produced, it was taken to the petri-dish for conducting the following tests.


Figure 2.8: Evaporating solvents using a water bath.

❖ **Drying:**

Eventually, the petri dish was kept under the laminar airflow to ensure as if no contamination occurs as well as avoiding possibility of microbial growth at the time of vaporization.

Figure 2.9: Dried ethanol extract of *Pterocarpus Indicus* Willd. leaves in the petri-dish.

Table 2.2: Obtained weight of the leaves extract after completing extraction.

| | |
|---|---|
| Weight of the petri dish | 149.23g |

| | |
|---|---|
| Weight of the petri dish and extract | 141.23g |
| Weight of the extract | 8.03g |

Total weight of the plant extract was about 6.35g.

## 2.4 *In vitro* antioxidant activity analysis

For the assessment of the antioxidant property of the plant sample, two of the following methods were conducted;

### 2.4.1. DPPH-based free radical scavenging assay

#### ❖ Materials and Reagents:

Table 2.3: Components required for DPPH assay

| Materials | | Reagents | |
|---|---|---|---|
| 1. | UV spectrophotometer | 1. | 2,2-Diphenyl-1-Pycrylhydrazyl (DPPH) |
| 2. | Light-proof box | 2. | Ascorbic Acid (ASA) |
| 3. | Test tubes | 3. | Ethanol |
| 4. | Volumetric Flasks | 4. | Plant extracts |
| 5. | Pipette | 5. | Distilled water |

#### ❖ Control Preparation:

ASA was chosen as the positive control for the evaluation of DPPH free radical screening assay. To prepare the standard solution, 2mg of ascorbic acid *was* dissolved in ethanol and made a concentration of 500 µg/ml. Afterwards, serial dilution process was performed for getting different concentrations solutions ranging from 500 µg/ml to 0.977 µg/ml.

Table 2.4: Components used in control preparation

| Chemicals Name | Amount |
|---|---|
| ASA | 2 mg |
| Ethanol | 4 ml |

## Preparation of the test sample:

In order to prepare the test sample, 2mg of *Pterocarpus Indicus* Willd. leaves extract and 4ml of ethanol were taken and made a solution having concentrations of 500 µg/ml. Then, multiple concentrations of solution were prepared from 500 µg/ml to 0.977 µg/ml through conducting the serial dilution process.

Table 2.5: Components used in sample preparation

| Name of Chemicals | Amount |
|---|---|
| *Pterocarpus Indicus* Willd. leaves extract | 2 mg |
| Ethanol | 4 ml |

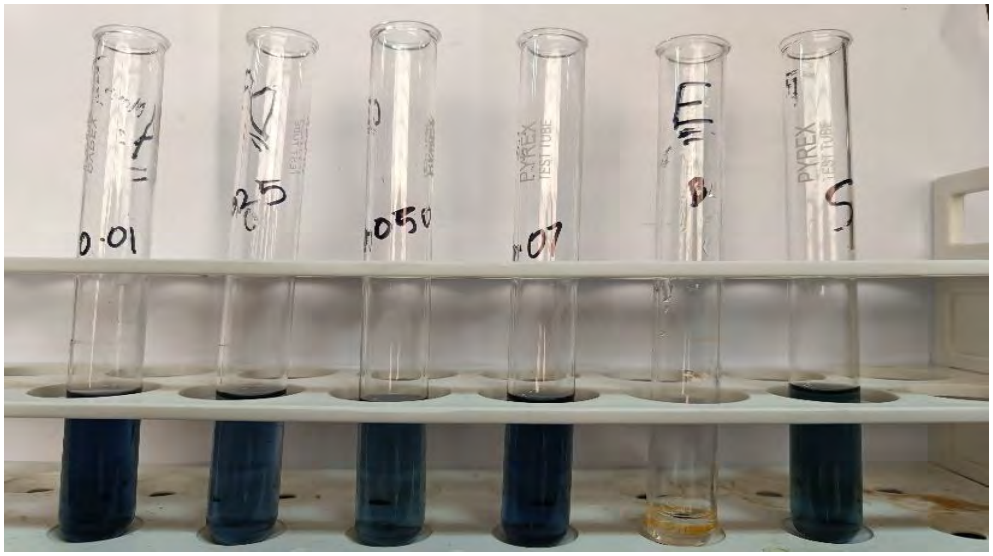



Figure 2.10: Serial dilution for preparing sample solutions of multiple concentrations

## ❖ Preparation of DPPH solution for evaluation:

To prepare DPPH solution, DPPH solution was prepared by dissolving 1 mg of DPPH powder in a 50 ml of ethanol solvent for acquiring 20 µg/ml concentration. Then, the solution was enveloped with aluminum foil paper and placed in a dark place away from sunlight.

Table 2.6: Components used in the preparation of DPPH solution

| Chemicals Name | Amount |
|---|---|
| DPPH<br>(2,2-Diphenyl-1-Pycrylhydrazyl) | 1 mg |
| Ethanol | 50 ml |

## ❖ DPPH-based scavenging activity:

Eventually, 3.0 ml of DPPH ethanol solution was mixed with each of the test sample solutions having multiple concentrations ranging from 500 µg/ml to 0.977 µg/ml and stored in the dark place for 30 minutes to conduct reactions. After that, absorbance of all the standard solutions

and sample solutions were taken using UV spectrophotometer. Ethanol was used as blank for the measurement.

### ❖ Calculation:

Inhibition of free radical DPPH in percent *(I%)* was calculated using this equation:

$$(I\%) = (1 - A_{sample}/A_{blank}) \times 100$$

Here,

$A_{sample}$ = Absorbance of the test samples.

$A_{blank}$ = Absorbance of the control reaction. (Comprising with all the reagents aside from the test material)

Finally, 50% inhibition (IC50) of the extract concentration was calculated using the graph of percentage of inhibition against the plant extract concentration (µg/ml).

## 2.4.2 Determination of TPC

## Materials and Reagents:

Table 2.7: Components required in the determination of total phenolic content

| Materials | Reagents: |
|---|---|
| UV spectrophotometer | Gallic acid (GA) |
| Test tubes | Folin-Ciocalteu reagent(F-C) (10-fold diluted) |
| Vials | $Na_2CO_3$ (7.5%) |
| Micropipette (50-200µl) | Distilled water |

## ❖ Test sample preparation for evaluation:

For preparing the solution, 2 mg of *Pterocarpus Indicus* Willd. leaves extract and distilled water were dissolved in a test tube and obtained concentration of 2mg/ml.

Table 2.8: Components used sample preparation

| Name of sample | Amount |
|---|---|
| *Pterocarpus Indicus* Willd. leaves extract | 2 mg |

After that 2 ml of $Na_2CO_3$ and 2.5 ml of F-C reagent with 0.5 ml of sample solution were added to prepare 5 ml of mixture and kept in incubation for 20 minutes at room temperature.

## ❖ Standard solution Preparation for evaluation:

Gallic acid is widely utilized as standard for TPC. Following that, Different concentrations of gallic acid solutions were arranged from 100 µg/ml to 0 µg/ml using serial dilution process. Then, 5 ml of standard solution was prepared for each of the concentrations through adding 2 ml of Na2CO3 and 2.5 ml of F-C reagent in each 0.5 ml of gallic acid solution. After that, all the standard solutions were placed in the incubation at room temperature for 20 minutes.

## ❖ Analysis of TPC:

In the end of the incubation process, absorbance of the sample solution and standard solutions were taken using a UV spectrophotometer at 760nm. For standard solutions, a liner relationship was acquired in the standard curve after plotting the absorbances in ordinate against the

concentrations. By utilizing the standard curve of the standard solution total phenolic content was measured.

## 2.5 *In vitro* thrombolytic activity analysis.

Thrombolytic property analysis was performed followed by the method of(Prasad et al., 2006).

### ❖ Materials and reagents:

Table 2.9: Components required in thrombolytic activity analysis

| Materials | Reagents |
|---|---|
| Micropipette | Clopidogrel<br>(anti-platelet agent) |
| Eppendorf/<br>Microcentrifuge tube | Blood |
| Syringe | Distilled water |

### ❖ Test sample preparation:

100 mg of *Pterocarpus Indicus* Willd. leaves extract and 10 ml distilled water were taken to make the sample solution and kept for 24 hours. The next day, the supernatant was decanted and filtered properly.

### ❖ Preparation of standard solution:

An antiplatelet agent was required in this experiment for using as a standard. Following that, Clopidogrel was chosen. To prepare the standard solution, 100 mg of Clopidogrel and 10 ml of distilled water were taken and dissolved in a test tube properly. It was prepared to use as the stock solution in the experiment.


Figure 2.11: Preparation of standard solution using Clopidogrel

## ❖ Blood sample preparation:

For collecting blood, 5 healthy volunteers were managed without any history of anticoagulant therapy. With the help of a nurse, 3 ml of blood sample was collected from each of the 5 volunteers maintaining aseptic condition. 15 ml blood was collected from 5 volunteers.

## ❖ Thrombolytic property test process:

For the blood sample of 1 volunteer: 3 ml of blood was taken and poured into 3 different pre-weighed Eppendorf tubes marked as 1E (Volunteer-1, Ethanol extract), 1C (Volunteer-1, Clopidogrel), 1B (Volunteer-1, Blank). Then, the tubes were incubated for 45 minutes at 37$^{o}$ C. After 45 minutes, the formation of clot was observed and weighed the tube after completely discharging all the fluids surrounding the clotting. Afterward, the clot weight was measured by using the tube weight taken before and after the clot formation.

$$\text{Weight of the clot} = \frac{weight\ of\ the\ eppendorf\ tube}{weight\ of\ the\ eppendorf\ tube\ after\ eliminating\ surrouding\ fluids\ from\ the\ clot\ formation}$$

In this experiment, Clopidogrel and distilled water were taken as positive control and non-thrombolytic negative control. Sequentially, 100µl clopidogrel, 100 µl distilled water and 100 µl test sample were added to their designated tubes and incubated at 37° C for another 90 minutes. After the incubation process, the released liquids were eliminated from the tube and weighed again to observe the weight difference after the clot disruption. Moreover, this process was followed for all the blood samples of other volunteers and performed together side by side. Finally, the percentage of clot lysis were calculated using this equation:

$$\text{Percentage (\%) of clot lysis} = \frac{(weight\ of\ released\ clot)}{(clot\ weight)} \times 100$$

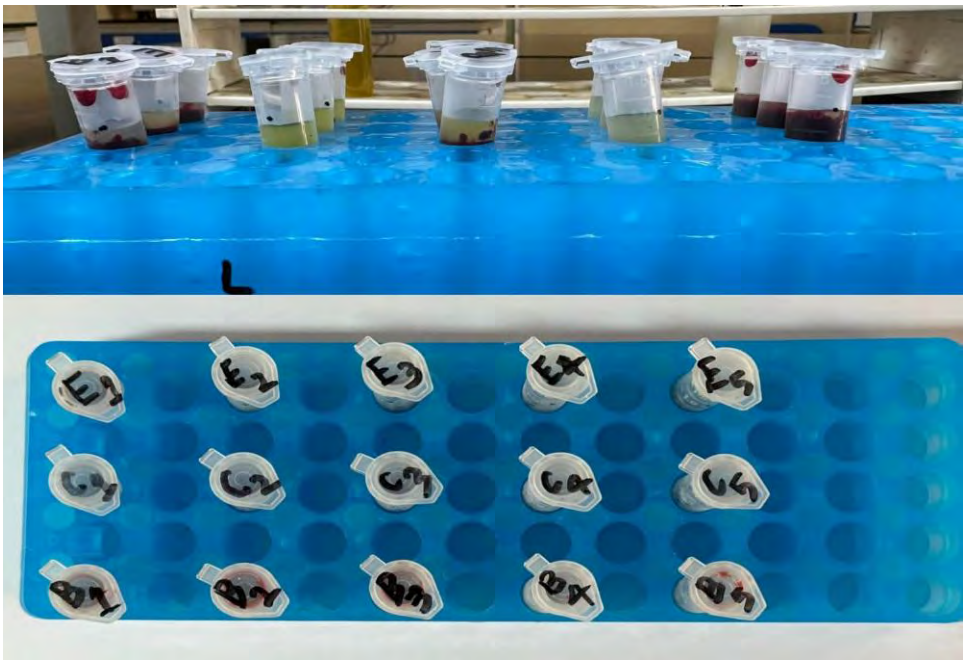


Figure 2.12: Preparation of standard, sample and blank solution.

# Chapter 3

## 3. Observation and results

### 3.1. Determination of Antioxidant activity analysis

#### 3.1.1. DPPH-based scavenging assay of *Pterocarpus Indicus* Willd.

Table 3.1: percentage of inhibition and $IC_{50}$ value of ASA

| Standard | Absorbance of Standard (ASA) | % of inhibition | IC50 µg/ml |
|---|---|---|---|
| 500 | 0.031 | 95.008 | 67.842 |
| 250 | 0.032 | 94.847 | |
| 125 | 0.048 | 92.271 | |
| 62.5 | 0.168 | 72.947 | |
| 31.25 | 0.297 | 52.174 | |
| 15.625 | 0.385 | 38.003 | |
| 7.813 | 0.418 | 32.689 | |
| 3.906 | 0.464 | 25.282 | |
| 1.953 | 0.481 | 22.544 | |
| 0.977 | 0.481 | 22.544 | |
| Blank | 0.621 | | |

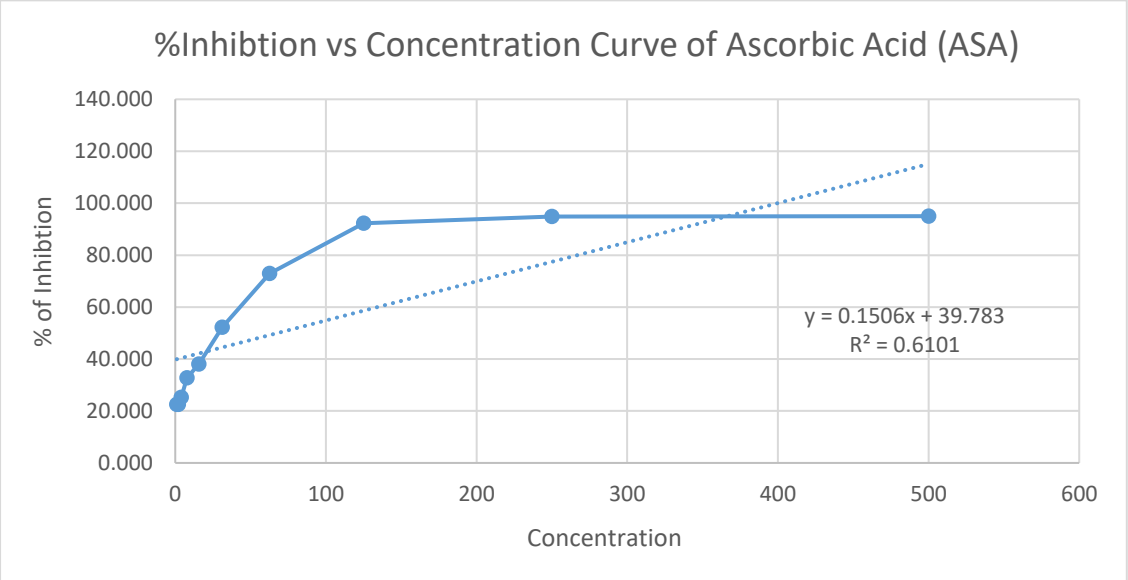


Figure 3.1: Standard curve of Ascorbic acid.

Table 3.2: percentage of inhibition and $IC_{50}$ value of plant extract

| Standard | Absorbance of plant extract | % of inhibition | IC50 µg/ml |
|---|---|---|---|
| 500 | 0.087 | 85.99034 | 135.211 |
| 250 | 0.115 | 81.48148 | |
| 125 | 0.153 | 75.36232 | |
| 62.5 | 0.292 | 52.97907 | |
| 31.25 | 0.405 | 34.78261 | |
| 15.625 | 0.414 | 33.33333 | |
| 7.813 | 0.451 | 27.3752 | |
| 3.906 | 0.491 | 20.93398 | |
| 1.953 | 0.487 | 21.5781 | |
| 0.977 | 0.521 | 16.10306 | |
| Blank | 0.621 | | |

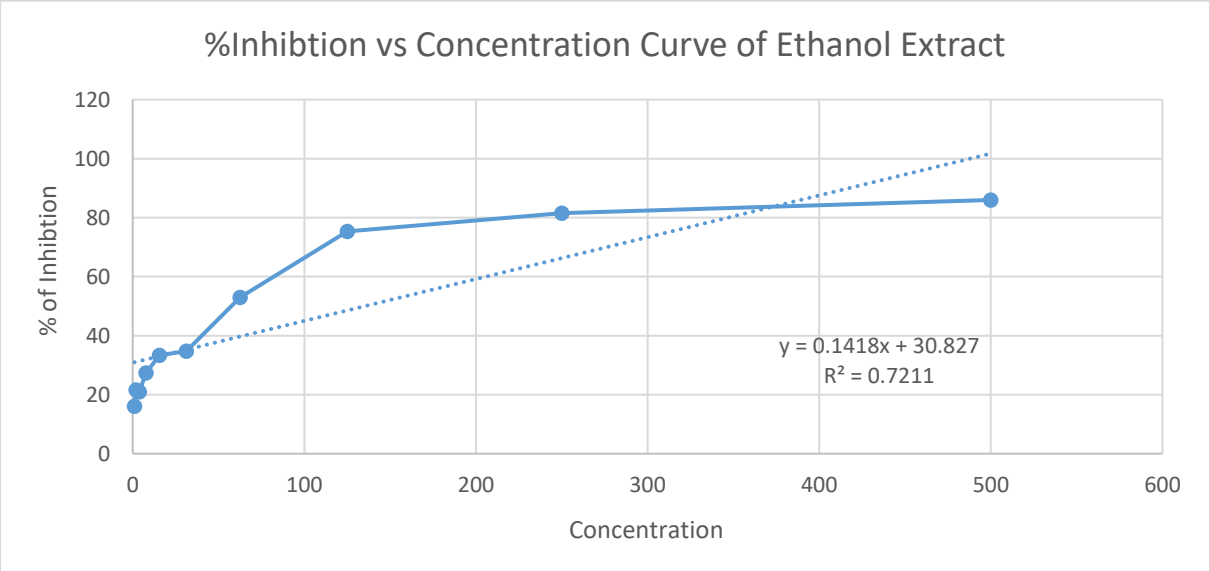



Figure 3.2: Standard curve of plant extract

## ❖ Explanation:

After completion of the test, it was observed that the % of inhibition of ascorbic acid is higher than the plant extract which indicated that ascorbic acid is more effective in neutralizing or scavenging the free radicals than the plant extract and the plant extract had the potential to act

as an antioxidant with poor potency. Further, $IC_{50}$ of the ascorbic acid was found 67.842 whereas plant extract was 135.211 which suggested that ascorbic acid was more effective and almost half the amount of ascorbic acid was needed to achieve the same level of inhibition as a larger amount of the ethanol extract.

### 3.1.2. Determination of TPC:

Table 3.3: Absorbance and regression line of Gallic acid:

| Concentration (µg/ml) | Absorbance | Regression line | R |
|---|---|---|---|
| 100 | 0.741 | y=0.0075x+0.0308 | 0.9891 |
| 75 | 0.617 | | |
| 50 | 0.429 | | |
| 25 | 0.233 | | |
| 0 | 0 | | |

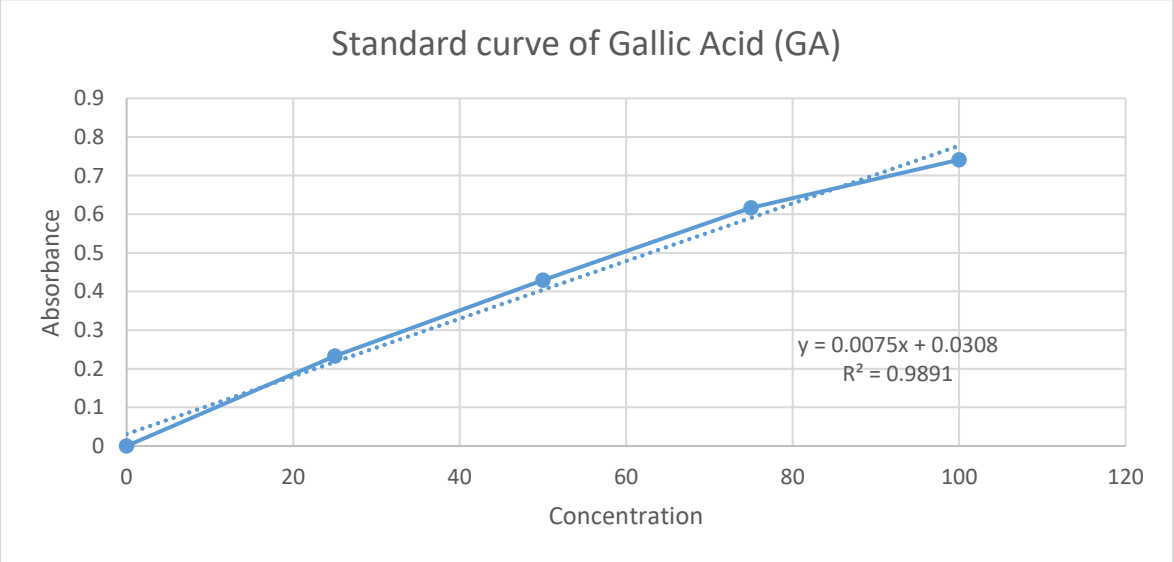



Figure: 3.3: Standard curve of GA

Table 3.4: Calculated TPC from the standard curve.

| Extract Name | Part of the plant | Absorbance | TPC (mg of GAE/ gm of extract) |
|---|---|---|---|
| Ethanol | *Pterocarpus Indicus* Willd. leaves | 0.651 | 82.693 |

So, the total phenolic content of the *Pterocarpus Indicus* Willd. leaves extract was 82.693.

## 3.2. Thrombolytic activity analysis

Table 3.5: Thrombolytic activity evaluation (Volunteer 1):

| Sample (Plant extract) | W1 | W2 | W3 | W4 (W3-W1) | W5 (W2-W3) | Clot lysis percentage |
|---|---|---|---|---|---|---|
| Plant Sample | 0.839 | 1.517 | 1.28 | 0.441 | 0.237 | 53.74% |
| Clopidogrel | 0.871 | 1.498 | 1.28 | 0.409 | 0.218 | 53.30% |
| Blank | 0.859 | 1.506 | 1.389 | 0.53 | 0.117 | 22.08% |

Table 3.6: Thrombolytic activity evaluation (Volunteer 2):

| Volunteer 2: | W1 | W2 | W3 | W4 (W3-W1) | W5 (W2-W3) | Clot lysis percentage |
|---|---|---|---|---|---|---|
| Sample (Plant extract) | 0.847 | 1.251 | 1.106 | 0.259 | 0.145 | 55.98% |
| Clopidogrel | 0.859 | 1.428 | 1.273 | 0.414 | 0.155 | 37.44% |
| Blank | 0.859 | 1.474 | 1.314 | 0.455 | 0.16 | 35.16% |

Table 3.7: Thrombolytic activity evaluation (Volunteer 3):

| Volunteer 3: | W1 | W2 | W3 | W4 (W3-W1) | W5 (W2-W3) | Clot lysis percentage |
|---|---|---|---|---|---|---|
| Sample (Plant extract) | 0.884 | 1.604 | 1.492 | 0.608 | 0.112 | 18.42% |
| Clopidogrel | 0.88 | 1.605 | 1.499 | 0.619 | 0.106 | 17.12% |
| Blank | 0.88 | 1.578 | 1.527 | 0.647 | 0.051 | 7.88% |

Table 3.8: Thrombolytic activity evaluation (Volunteer 4):

| Volunteer 4: | W1 | W2 | W3 | W4 (W3-W1) | W5 (W2-W3) | Clot lysis percentage |
|---|---|---|---|---|---|---|
| Sample (Plant extract) | 0.854 | 1.414 | 1.285 | 0.431 | 0.129 | 29.93% |
| Clopidogrel | 0.849 | 1.429 | 1.284 | 0.435 | 0.145 | 33.33% |
| Blank | 0.847 | 1.378 | 1.347 | 0.5 | 0.031 | 6.20% |

Table 3.9: Thrombolytic activity evaluation (Volunteer 5):

| Volunteer 5: | W1 | W2 | W3 | W4 (W3-W1) | W5 (W2-W3) | Clot lysis percentage |
|---|---|---|---|---|---|---|
| Sample (Plant extract) | 0.88 | 1.553 | 1.372 | 0.492 | 0.181 | 36.79% |
| Clopidogrel | 0.857 | 1.549 | 1.358 | 0.501 | 0.191 | 38.12% |
| Blank | 0.849 | 1.529 | 1.423 | 0.574 | 0.106 | 18.47% |

Here,

$W_1$= weight of the Eppendorf/microtube without blood sample.

$W_2$= sum of the clot weight and Eppendorf tube

$W_3$= sum of the clot weight and Eppendorf tube after clot disruptions

$W_4$= weight of the blood clot only after the clot disruptions

$W_5$= weight of the released clot.

## ❖ Explanation:

In this experiment, bloods were managed from the 5 different healthy volunteers and it was observed that ethanol extract of *Pterocarpus Indicus* Willd. leaves showed similar effect as the antiplatelet agent Clopidogrel and comparatively higher on some points. On the blood sample of volunteer 1,3 and 5 the clot lysis percentage was identical and plant sample showed higher clot lysis percentage on the blood sample of volunteer 2. So, the result suggested that ethanol extract of *Pterocarpus Indicus* Willd. leaves exhibited a persistent thrombolytic effect.

# Chapter 4

## 4. Discussion

Due to having diverse chemical structures, biological activity and fewer side effects, natural products such as plants have been researched widely for their potential application in the drug development. Following this, *Pterocarpus Indicus* Willd., a species of the Fabaceae family was selected to assess the biological properties. In the assessment, antioxidant activity using DPPH assay, TPC and thrombolytic activity test was performed and evaluated the potential of the plant leaves. Before starting the activity test, the plant leaves were dried and extracted using ethanol. Then the ethanol extracted *Pterocarpus Indicus* Willd. leaf was used as sample preparation in the analysis. Antioxidant activity assay was conducted to assess the antioxidant activity of the plant to scavenge or neutralize the free radicals. DPPH was used as the free radicals and percentage of inhibition and $IC_{50}$ value was measured. However, ascorbic acid was also used as a standard antioxidant to compare the outcome. In the experiment, the $IC_{50}$ value of ASA was 67.842 and 135.211 was for the plant sample. Further, in the TPC analysis the value for the plant sample was 82.693. According to the TPC analysis and antioxidant assay, the data established that leaves of *Pterocarpus Indicus* Willd. might contain poor antioxidant activity and further investigation necessary to establish the data.

Thrombolytic activity test was also performed to assess the clot disruption rate by the leaves of *Pterocarpus Indicus* Willd. species. Clopidogrel was tested as a standard anticoagulant and antiplatelet along with the plant sample to compare the obtained data. After the test was conducted, the plant sample demonstrated promising and effective thrombolytic activity. Blood was taken from a total of 5 volunteers and the plant sample exhibited almost similar effect as the standard. So, it suggested that leaves of *Pterocarpus Indicus* Willd. might be used as a thrombolytic agent and to assure that more research is necessary.

## 4.1 Conclusion

The identification of novel plant species with potential therapeutic characteristics is of tremendous interest, particularly given the growing demand for natural products and the need for new treatments for a wide variety of diseases. Following that *Pterocarpus Indicus* Willd. was investigated to identify its biological potentials. This study provided some important insights about its potential applications in medicine. Based on the finding of the research, leaf of the *Pterocarpus Indicus* Willd. is a limited source of natural antioxidants as the leaf demonstrated poor presences of antioxidant. However, it manifested effective thrombolytic activity.

In conclusion, this biological screening assays give some valuable data about the antioxidant and thrombolytic properties of *Pterocarpus Indicus* Willd. leaves. Although the plant showed poor antioxidant activity, it exhibited promising thrombolytic activity, making it a potentially valuable source of new compounds for drug development. Further detailed investigations are required to ensure the availability of antioxidant properties and to isolate and identify the chemical components obligated for the detected thrombolytic activity which can be useful in managing and treating thrombolytic complications as a thrombolytic agent.

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