Investigation of *In-vitro* Biological Potential of Methanol Extract of *Aporosa Wallichii* Stem

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Declaration

It is hereby declared that

1. The thesis submitted is my/our 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/We have acknowledged all main sources of help.

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Approval

The thesis/project titled “Investigation of In-vitro biological potential of methanol extract of Aporosa wallichii stem” submitted by Rezowana Islam (13346004) of Summer, 2019 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of bachelor of pharmacy (hons.) on 22nd August.

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Ethics Statement

1. I have donated the blood for the current project.
2. This work will only be published after ethical permission has been taken.

Abstract

This research was conducted to discover and evaluate the possible biological properties of the medicinal plant stem called *Aporosa wallichii*, which belongs to the family Phyllanthaceae. To comply with the research findings, several experiments were conducted, such as the antioxidant properties test, which includes the DPPH test and the total phenolic content test, the brine shrimp lethality test, the thrombolytic properties test and the antimicrobial properties test. After completing these mentioned experiments from several observations it was found that, this plant stem showed a good level of antioxidant property, a moderate level of thrombolytic property and a significant level of cytotoxicity property. In addition, the antimicrobial properties test did not show the desired result for this plant stem, indicating that it may not contain any antimicrobial properties. However, this research is initial to find the pharmacological property of this plant stem. Finally, based on this research, it can be declared that this *Aporosa wallichii* plant stem can provide a good impact on the world of medicine and the progress of global healthcare.

Keywords: DPPH free radical elimination; Total phenolic content; Brine shrimp lethality bio assay; Thrombolytic properties; Cytitoxicity.

Dedication

This work is dedicated to my parents and husband for their absolute support and motivation

Acknowledgement

All admiration belongs to Allah S.W.T. alhamdulillah, from whom I obtain strength to complete the work of this project. I would like to show my gratitude to Almighty Allah for blessing me with immense patience, strength, corporation and assistance when necessary to complete the Pharmacy degree processes.

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List of Acronyms

mg	Milligram
mL	Milliliter
µg	Microgram
IC50	Median Inhibitory Concentration
LC50	Median Lethal Concentration
ASA	Ascorbic acid
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
UV	Ultraviolet
DMSO	Dimethyl Sulfoxide
HCl	Hydrochloric Acid
FRS	Free Radical Scavengers/Scavenging
R_2	Regression Coefficient
A.wallichii	*Aporosa wallichii*
WHO	World Health Organization
m	Meter
cm	Centimeter
gm	Gram
Conc.	Concentration
ME	Methanol Extract

Chapter 1

Introduction

The term "medicinal plant" comprises some types of plants used in herbology. Plants are used here for therapeutic purposes to treat infections. Nature is the main bright blessing for us, it increases with millions of puzzling components among all the components that the environment favors us. Plants are important part of this environment and exceptionally valuable for the welbing of human health. Previously, various types of diseases have been routinely treated with specialists determined from therapeutic plants. Plant-based medication preparations that had been used to recover diseases at the early stages when the existent of human had created at the beginning for drug therapy. Thus, from here to the present, it is considered one of the main important sources of medication. Therefore, this is essential to distinguish normally reachable herbal medicines to the betterment of humanity that have unbelievable pharmacological effects.

Our soil is full of various types of plants and people designed around 7.5 lac of plant species, of which 5 lac are classified as "upper plants" and 2.5 lac as "lower plants" (Devi, 2015) . Among clinically used medications 60% are invented from natural component part and their subsidiaries and natural herbal component. Almost 87% of all classified medications, such as antimicrobials, operators of anti-tumors and anticoagulants, come through normal products and their supplements. The chemicals which have been accessed recently in the world are more than 28% of the common elements (Akter, 2013). Lately, the World Health Organization (WHO) said that around 80% of people worldwide depend specifically on recuperative plants for basal treatment. In addition, 21,000 species of plants which have potential impacts on pharmacology that can also be used as medicinal plants.

Treatment with medicinal plants is considered safe as there are fewer side impacts. The most prominent advantage is that priests are in tune with nature. The main reality is that people of all ages and sexes can take a home treatment.

1.1. Summary of the history of medicinal plants

Therapeutic plants are used to treat infections initiated by our precursor, but when they started it is still dark. While searching for food they found various plants that appear to have a harmful impact and have distinctive abilities such as the ability to create sweat, decrease torment and inflammation.

The explanatory continuum of therapeutic plants reveals that is our precursors, like the Assyrians, the Babylonians and the Egyptians, were aware well the therapeutic properties of herbs and trees. The Babylonians (around 300 BC) knew a huge sum of vital medicines and expressed that current medicine still uses certain plants in the same way as the Babylonians (Ghani, 2003). For starters, the original copy about the use of therapeutic plants is almost 400 years old. The first copy had been composed by a unit of individuals from the ancient Sumerian culture living in the Euphrates and the Tigris. They were composed in small pieces of clay. The pieces were found at that time by later Iraqi analysts. The Egyptians also composed an original copy in therapeutic plants known as Ebers Papyrus. Above 700 strategies were found, composed almost 1700 BC. An amazing transcribed way of using therapeutic plants from the book entitled "Write Tsao" which contains more than 300 therapeutic plants.

Indian therapeutic framework titled Ayurveda, which alluded to the utility of therapeutic plants, from 800 BC (Cures with conventional plants, 2016).

Among outdated civilizations, India was persistently renowned for its utilized of restorative plants. Throughout the forest of India is the extraordinary amount of restorative and fragrant plants, forming there producers of drugs and fragrance things that collected their raw accessories. The Indian pharmaceutical framework called AYUSH coded about eight thousand homemade cures together for the innate pharmaceutical framework. The tribal pharmaceutical frameworks, Siddha, Unani and Ayurveda are preeminent. Be that as it may, the Indian people deeply used the Ayurveda and Unani framework as a result of which this framework created more.

The Chinese also have incredible information on therapeutic plants that use a conventional split treatment, known as "Chinese herbs." This should be famous that botanical roots support Chinese herbalism. Over 1200 plants are included within the conventional care framework and for treatment design, almost 500 restorative plants are used to remedy some infections. The Chinese used therapeutic herbs in the same way they used them in the past. It ought to be celebrated that one fifth of the Chinese pharmaceutical industry comprises approximately 5,000 conventional therapies (Li, 2000). Old researchers believe that numerous wellness problems and infections have as solutions that are herbs. Old researchers conducted an investigation in this regard, they strive to evaluate with particular discoveries about the viability of the different herbs that give restorative importance to numerous infections. These defined medications do not have or have exceptionally minor difficulties or antagonistic responses. For this type of treatment with home grown plants, is increasingly common worldwide. These herbs that have therapeutic qualities treat many internal diseases that other things are considered difficult to cure. Organizations such as ESCOP (European Organization Cooperative On Phototherapy, 1999), German Commission E (Bluemental et.al, 1989) as well as WHO (World Health Organization)

assumed that plants which are used for diseases remediation is recognized through worldwide and with uses of restorative plants are continually increasing every day.

1.2. Medicinal plants available in Bangladesh

Subtropical nation like Bangladesh, covering more than 5,000 angiosperms 200 families. Bangladesh gives culminating period to evolve and nourish therapeutic plants. Chittagong, Dhaka, Rajshahi, Sylhet, and other ranges throughout Bangladesh are reinforced by around 5000 distinctive types of restorative plants, as stated in the "Medical Matter". From the beginning of their presence, conventional medicines are well known within the nation. The fruitful improvement environment of restorative plants in Bangladesh favors the treatment of 500 classic diseases between 2000. In expansion, later in a long time, the use of remedial plants by giant companies and companies of Bangladesh are expanding impressively. Manifold pharmaceutical impulsive companies in Bangladesh currently use a variety of restorative plants. Maximum tribal and populations of Bangladesh rely heavily on the restorative plant for primary treatment, perhaps it is accepted by them that their well-being will not harm by nature. According to this trust and credence, they use contrastive parts of the plants: bark, steam, natural elements, flowers, etc.

1.3. Importance of medicinal plants in drug finding

Restorative plants provide us with different types of useful specialists that are used to treat various diseases. These restorative operators are inferred from plants and are specifically used as sedatives or synthesized medications or synthesized medications. In the past, medications were

administered by concoctions, extracts or arrangements organized by botanists who seem to be continuously aware of the recovery treatment for most infections. But wound healing was continuously exceptionally critical for the elderly, since for this reason "Sushruta Samhita" dedicates two full chapters in wound healing and realizes more than 100 plants that can be used to heal wounds, as well as the necessary solutions to perform a clean wound (Idris Singh, B. Recuperating and Singh, G., 1994, 37-41, Deshpande, Pathak and Dildo, 1970, p 260-303).

According to different calculations, about 60% of anti-tumor, anti-infective and antimicrobial drugs are currently on the market or in clinical trials (Yue-Zhong Shu, 1998). The largest are still obtained from wild plants or developed plants, but many of them are not synthesized economically (Taux, 2001). Taking after Table 1.1 gives some restorative values that contain plants that are used to recover the common disease.

1.4. Drug as natural product obtained from medicinal plants

Herbal plants remained a basic asset of unused compounds that are pharmacologically dynamic, from these plants viable commercial medications are inferred, either specifically or optionally. About 25% of the suggested medications in the world start from herbal plants, about 121 plants of which the active compounds are currently being used. The World Health Organization (WHO) announced 252 medicines that are included in the list of essential medicines, about 11% of the medicines come from plant sources and from the normal originators, a tremendous quantity of manufactured medicines was obtained. The digoxin of the species Digitalis, vinblastine and vincristine attained from Catharanthus roseus, Atropa belladonna administers atropine together with the morphine jointly codeine of Papaver somniferum (Yue-Zhong Shu, 1998). Lately, use natural products as a rapidly expanding therapeutic agent (Goldfrank et al., 1982; Mentz and

Schenkel, 1989). Liu et al. (2000) detailed as about 50% of all flourished medications were determined through natural products.

During the past ten years, medicinal products derived from plants such as Arteether, sesquiterpene lactone terminal peroxide and natural semi-synthetic products derived from artemisinin used to the medication of malaria, the natural alkaloid Galantamine, has been used in the treatment of the disease Alzheimer's, natural product. Nitisinone derived from leptospermone is used in the treatment of antithrosinemia, apomorphine, a semi-synthetic compound derived from morphine used in the Parkinson's disease treatment, for chronic obstructive pulmonary disease tiotropium, a simulated atropine that is obtained as of Atropa belladonna, Cannabis and capsaicin. Dronabinol and cannabidiol obtained have been used as analgesics (Veeresham C. 2012).

1.5. Selection of Aporosa wallichii (Hook.f.) for this project

Aporosa wallichii (Hook.f.) is one of the plants in a family of Phyllanthaceae. There are several therapeutic plants that contain numerous useful exercises to treat cancer, skin diseases, irritation, loose intestines, diarrhea, jaundice, brain pain, etc. (Rahman and Akter, 2013). Be that as it may, the past considers that the totally different species of the Phyllanthaceae family have strong antioxidant, anthelmintic, antimicrobial, antidiarrheal, antitumor, anti-inflammatory and insecticidal properties (Li, 2000). Aporosa Lindleyana is additionally of the same class and has antioxidant, anti-amylase and lipid properties (Kathirgamanathar et al., 2018). This plant also has antimicrobial, analgesic (Srikrishna et al., 2008) and antidiuretic movement (Ganegamage et al., 2014). Other species such as Baccaurea Parviflora, Antidesma tomentosum, Aporosa aurea and Mallotus Paniculatus have some pharmacological properties that incorporate cytotoxicity and antitipanosomal movement (Mohmod et al., 2015). Croton gratissimus also includes a standard

antiplasmodial rate action. On the other hand, Croton argyratus has an excellent antiprotozoal action (Abdullah et al., 2007). Therefore, there is a high probability of the presence of distinctive types of pharmacological properties in Aporosa wallichii Hook.f. stem. Free radicals are not capable of aging, but also of numerous related infections (Harman, 2009). Testing from some sources recommends that free radicals trigger components of cells that pass inside the body, such as apoptosis and necrosis (Chatterjee et al., 2011). The blockage of the veins can be a thrombosis that influences distinctive organs and this thrombosis. It can cause some pathological conditions (Bekker et al., 2009). Antithrombotic and thrombolytic treatments have an incredible impact on thrombosis and play a vital role in the treatment of thromboembolic disorders (Hirsh et al., 2008). The cytotoxic property is exceptionally critical for annihilating cancer cells. Subsequently, this stem may be a potential source of medicine. For this reason, this consideration focused on finding agents for cancer prevention, cytotoxicity and stem thrombolytic action (Sharmin et al., 2018).

1.5.1. Introduction to the selected stem Aporosa wallichii (Hook.f.)

Aporosa wallichii (Hook.f.) exists for the Phyllanthaceae family. This type of stem is found in the mountainous areas of the Asian subcontinent. In Bangladesh, they are generally available in mountainous areas such as Chittagong, Sylhet.

Aporosa wallichii (Hook.f.) is one of the prevailing mother species of the Kamalachari natural woodland of Chittagong, Bangladesh. In addition, this stem is also available in the mountainous areas of Moulovibazar, Sylhet. (Hossain, Hossain, Alam and Uddin, 2015).

Phyllanthaceae, a family of flowering plants, contains approximately 7,800 species distributed in approximately 300 genera and 5 subfamilies. Many members are important sources of food along with medications. They are also a good source of waxes and oils and are also dangerous for their

toxic fruits, leaves or fresh fruits; Or attractive to your brothers of color. This family grows mainly in tropical and subtropical regions. The family consists of annual and perennial shrubs or woody trees and climbers. There are several plants that belong to this family that have great financial value, for example, the species include Ricinus communis (castor plant), Manihot esculenta (yucca) and Jatropha curcas (Barbados).

1.5.2. Morphology of stem Aporosa wallichii (Hook.f.)

Trees 5 to 9 m high; young pubescent buds; Of glabrous twigs. elliptical spears tightly oblong late, sharp, opaque leaves rounded to the base, joints of grenadine and, sometimes, notches along the edges, or rarely acuminate opaque on top, 8-20 x 2-5 (- 6) cm, often finely grated, glabrous; lateral nerves 5-10 pairs; Petioles 5 to 25 mm long; Very ovate, 5-7 x 2-3 mm, hard wood. Male inflorescence 2 to 5 cm in length; Triangular bracts from 0.7 to 1.5 mm in length; Sepal 3 or 4, oval or oblong-triangular, approximately 1 x 0.5 mm; Stamens 2 or 3, 1.5 to 2 mm long. Inflorescence

female (female); Triangular bracts, 0.8 to 1.2 mm; Sepals 3 or 4, triangular, 1.5-2 x 0.6-1.2 mm; or bottle with ovoid shape c.a. 2 x 1.5 mm ovary, 2 unicellular, glabrous; plug 1.5 - 2 mm in length; Stigma 2, approximately 1 mm wide. Ovoid oblong, 10-15 x 7-8 mm, thin wall, 2 unicellular, glabrous, brown to dry black; Ellipsoidal seeds, 7 -10 x 3-5 mm, black. (Kanjilal et al.,1940).

Origin: Evergreen or deciduous dry forestry of Tripura and Meghalaya in Bangladesh, India, Thailand and Myanmar. (Kanjilal et al., 1940).

Local name: Kukhra in Bangladesh (Hossain et.al, 2015)

1.5.3. stem taxonomy (Aporosa wallichii)

Table 1: Taxonomy hierarchy of Aporosa wallichii

Rank	Scientific name (Common name)
Kingdom	Plantae (stems)
Phylum	Magnoliophyta (Flowering plants)
Class	Magnoliopsida (Dicotyledons)
Order	Malpighiales
Family	Phyllanthaceae
Genus	*Aporosa*
Species	*Aporosa wallichii*

1.5.4. Pharmacological characteristics of other genera and species

Aporosa wallichii is the plant of a family that has a lot of medicinal value. The Phyllanthaceae family provided us with many medicinal properties that they used especially for diseases such as jaundice, asthma, anthelmintics, bronchitis, cancer, diarrhea, eczema, dysentery, headache, leprosy, inflammation, kidney disease, skin diseases, etc.). These are some of the main genera and species in Phyllanthaceae listed below:

Aporusa acuminata (Nirvetti), Actephila albidula , Phyllanthus abnormalis , Leptopus australis etc.

1.6. Project justification / rationale

Review of the literature of the selected plant called Aporosa wallichii (Hook.f.), with observation it is found that no significant study on Aporosa wallichii (Hook.f.) has been performed. However, previous studies in several species of this type have reported potent antimicrobial, antitumor, antidiarrheal, anthelmintic, antioxidant, anti-inflammatory, renal, skin diseases and cytotoxic activities. Therefore, the main objective of this study is to know the different pharmacological characteristics of the raw stem extract of the plant. This research aim is also seek to examine the unknown properties of the selected plant in the improvement of the world of medicine.

1.7. Aim of the project

The final objective of the project is to inspect and discover the unknown biological potential of the selected plant stem, Aporosa wallichii (Hook.f.) (Family: Phyllanthaceae)

1.8. Objective of the project

This project protocol contains the following steps that are carried out with methanol extract from the stem of Aporosa wallichii.

1) Evaluation of the antioxidant property of the methanol extract from the stem of the plant by applying the in vitro DPPH free radical elimination method and determining the total phenolic content of the plant.

2) Evaluation and detection of the antimicrobial activity of the plant.

3) Evaluation of the cytotoxic activity.

4) Determination of thrombolytic activity.

1.9. In-vitro evaluation of antioxidant property of Aporosa wallichii stem extract

Most diseases / disorders are mainly related to oxidative stress induced by free radicals. Substances of Antioxidant have the cognition to affect the oxidation process that participates in the reaction with free radicals, chelating agents, catalytic metals, and also act as oxygen absorbers from any types of source (Buyukokuroglu et al., 2001). Antioxidants effects on plant-derived compounds increase in interest, which may be relevant in terms of nutritional impact and their role in health care and various diseases (Steinmetz and Potter, 1996, Couladis et al., 2003). A large number of isolation reports and tests of antioxidant-derived plants have been defined during the previous period. (Velioglu et al., 1998, Pietta et al., 1998). The assets of medicinal plants are examined through the latest scientific research in medicine worldwide only for their effective antioxidant activity, the minimum amount of side effects and also the financial viability (Auudy et al., 2003).

Another synthetic antioxidant, for instance propyl gallate (PG), tert-butyl-1-hydroxytoluene (BHT), hydroxystyrene butylate (BHA) and tertiary butyl hydroquinone, is used as an additional ingredient to increase potency with effects known, not just toxic. but also human carcinogen (Ito et al., 1986; Wichi, 1988). Therefore, in recent years, the demand for natural antioxidants (plant derivatives) increases considerably (Jayaprakasha J. R., 2000). Plants-derived polyphenols has studied essentially with the probability that those may be the basis of defending effect of the consumption of fruits and vegetables contrary to malignance and some other chronic infections (Elena et al., 2006).

Finally, the purpose of these experiments was to discover and evaluate the stem extract of Aporosa wallichii as possible fresh sources of regular antioxidants and phenolic compounds.

The antioxidant property can be evaluated by

- Evaluation of total phenolic content.
- Evaluation of antioxidant properties: DPPH assay

1.9.1. Evaluation of phenolic content

The plants phenolic compounds mostly exhibit antioxidant assets through redox reactions that play a decisive character in the absorption and neutralization of free radicals, triplet oxygen and decomposed peroxides (Osawa, 1994). The antioxidant effect was revealed mostly owing to phenolic constituents such as phenolic diterpenes, flavonoids and phenolic acids (Shahidi, Janitha and Wanasundara, 1992). Many chemicals obtained from plants have substantial antioxidant properties that are partially linked to a lower mortality rate in various human populations (Velioglu et al., 1998). Into alkaline phase, phenols are ionized entirely. The Folin-Ciocalteu chemist effectively oxidizes the phenols when these chemicals utilized in this phenolic ionic arrangement, the reagent effortlessly oxidizes the phenols. When the oxidation prepare within the arrangement was completed, the yellow color of the chemical Folin-Ciocalteu turned dark blue. This constrain of color alter is measured in a 760 nm spectrophotometer.The absorbance value indicates the total phenolic content of the component. (Harbertson and Spayd, 2006).

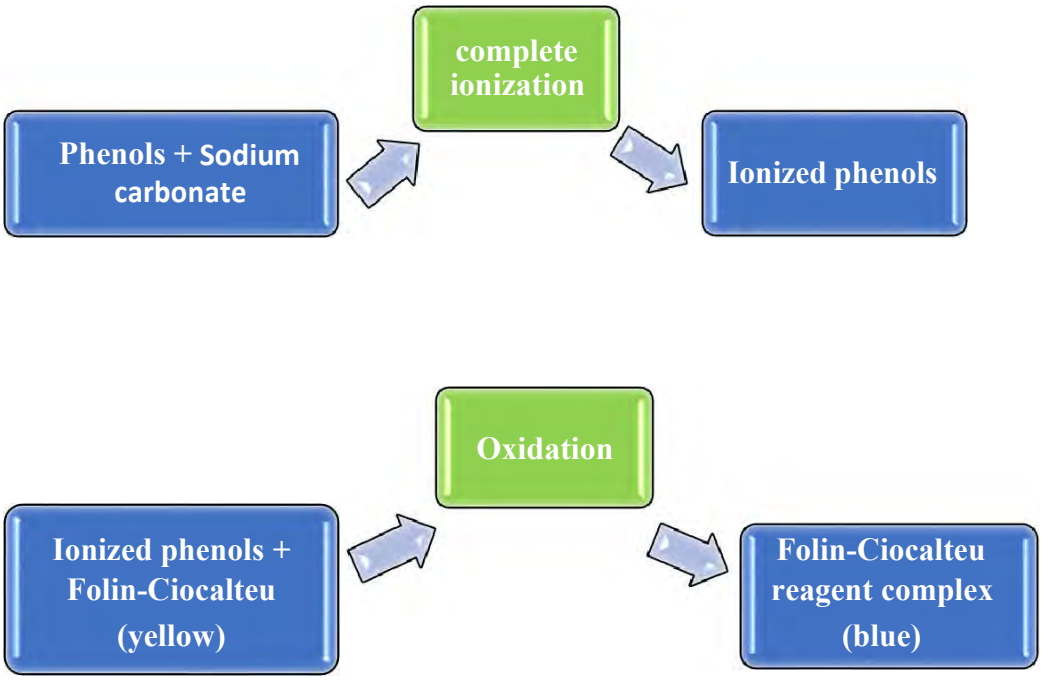
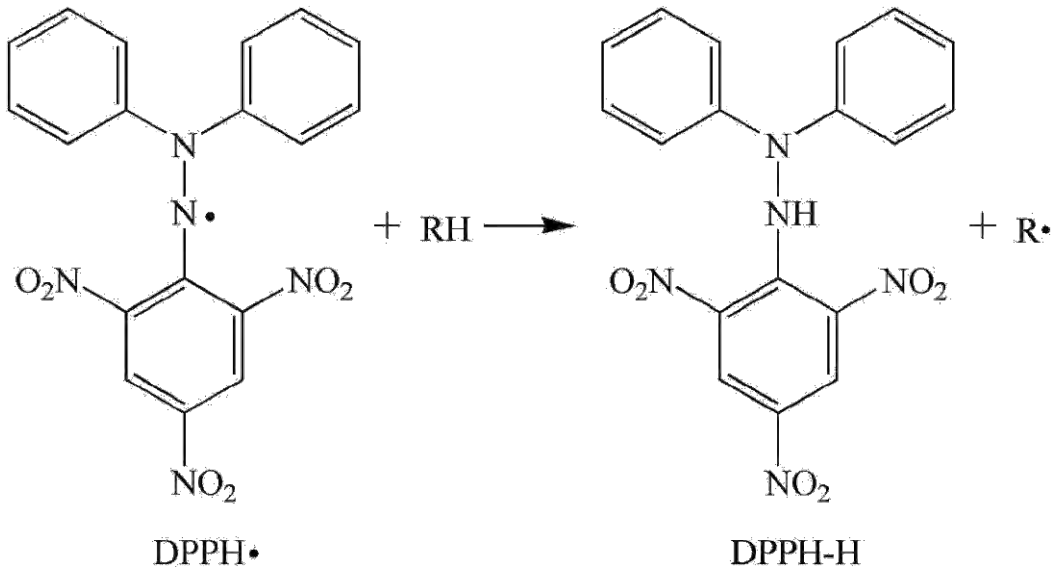


Figure 1: Flow diagram of phenolic content

1.9.2. Evaluation of antioxidant property by DPPH examine

The antioxidant capacity (free radical uptake activity) of methanol plant extracts in stabilized 1, 1-diphenyl-2-picrylhydrazyl radicals (Brand-Williams et al., 1995) was evaluated. With different concentrations, Solution of 2ml of methanol stem extract was integrated with DPPH solution of 3ml (20 µg / ml). Therefore, antioxidant properties were analyzed with bleaching the DPPH color mixture with the methanol stem extract. Therefore, antioxidant properties were analyzed with bleaching the DPPH color mixture with the methanol stem extract. By using UV spectrophotometer the similarities with ascorbic acid (ASA) is observed.



*DPPH (oxidized	**Antioxidant**	DPPH (reduced form)

Here, * DPPH = 1, 1-diphenyl-2-picrylhydrazyl Figure 1.2: Chemical structure of DPPH

1.10. In-vitro evaluation and discover of cytotoxic property of Aporosa wallichii stem extract

Cytotoxicity is the term that refers to a toxicity to cells. When cells come into contact with any cytotoxic substance, the results appear in several ways: first, they lose the strength of the plasma membrane and die quickly due to the destruction of the cells. or you can activate a genetically controlled cell death program, a term called apoptosis (Cytotoxicity, 2016).

1.10.1. Brine shrimp lethality assay

Bioactive substances are every time noxious to the living organism with advanced doses and justify the title that "pharmacology is simply the highest dose toxicology and the lowest dose pharmacology." The test of brine shrimp (McLaughlin, 1998) is the rapid as well as complete 27

biological trial for bio-active components of synthetic, semi-synthetic and natural origin. This method allows the identification of the bioactivity of natural products, fractions of substances and pure substances. In vivo lethality tests in a single animal technical organism (such as shrimp nauplii) are always used for monitoring and detection of new bioactive natural products.

However, the biological test specifies cytotoxicity and an extensive series of pharmacological or biological properties, such as antivirals, antimicrobials, antibodies and pesticides, etc. of compounds (Meyer, 1982; McLaughlin, 1998). The lumbar biological dosing technique designed for Salmon shrimp is superior to some further cytotoxicity analysis procedures because this process is fast, economical and does not involve distinct apparatus or sterile techniques. It uses a enormous amount of organisms because of statistical authentication with a moderately tiny trial size. In addition, contrasting to other procedures, animal serum does not required here.

Offspring of salted shrimp have been incubated in replicated saltwater for growing of nauplii. After addition of the calculated amount of dimethylsulfoxide (DMSO), the test is ready at the specified concentration by dilution. Nauplii are checked through a visual exam and put in vials containing recreated saltwater of approximately 5 ml. In this way, a few concentrations of tests are included to the micropipette tubes that were already labeled.These tubes are left at room temperature for about 24 hours. Finally, survivors are counted later 24 hours. (Meyer et al., 1982).

1.11. In-vitro evaluation of thrombolytic property of Aporosa wallichii stem extract

Since ancient times, plant preparation has been used to treat various diseases. Leaves and / or branches, stems, barks and soil areas are regularly used for traditional medications. Herbal products are often considered nontoxic as they are "natural" (Gesler, 1992). (CVST) Cerebral venous sinus thrombosis is a serious condition associated with severe disease and death (Watson

et al, 2002). Heparin is an antidepressant, anticoagulant, it is the first step of CVST treatment due to its efficacy, safety and possibilities (Biousse and Newman, 2004). Thrombolytic drugs such as urokinase, clopidogrel, streptokinase, etc. They provide important support with the control of patients diagnosed with CVST (Baruah, 2006). Therefore, the precise objective of this experiment was to introduce the thrombolytical efficacy of the methanol extract of the Aporosa wallichii leaves.

Thrombolytic medications are used more efficiently and effectively to improve blood flow and help reduce or decrease the symptoms of many patients without the need for additional surgeons, but it is not recommended for everyone. Thrombolytic agents are utilized to cure myocardial infections. Among them, streptokinase is widely used. Previously patient uses blood products, medicinal herbs or complementary foods or people at risk of bleeding; The thrombolytic agent is not recommended for them. These situations may include (Beckerman, 2015): high blood pressure, active bleeding or severe bleeding and cerebral hemorrhage from cerebral hemorrhage.

1.12. Antimicrobial property evaluation

Infectious diseases are the result of the death of thousands of people worldwide, especially the leading causes of death in tropical regions. However, it is very surprising to see these figures in developing countries, but mortality rates from infectious diseases actually increase in advanced countries for instance the United States. The infective disease mortality rate, which ranks fifth in 1981, became third in 1992, with an increase of about 58%. Infectious diseases are the leading cause of death in the United States of about 8% (Pinner et al., 1996). It is an alarming situation, since we once thought of eliminating infectious diseases by the end of the millennium. Infections of the upper respiratory tract and HIV / AIDS are the result of the increase in infectious diseases. Other causative features are the proliferation of antibiotic resistance in infections attained by our

nosocomial and community infections. In addition, the most affected increases in infectious diseases were observed with the 25-44 age group (Pinner et al., 1996). For this reason, medical and health communities need to renew their interest in infectious diseases, as well as in new treatment and prevention strategies due to these negative health trends. Therefore, the preceding solution point would be the growth of a novel antibiotic (Fauci, 1998).

The antimicrobial test is the major step in the search for antimicrobial drugs to determine the vulnerability of different fungi and bacteria to each mediator. This experiment dealings the capacity of every single test sample inhibits the in vitro growth of fungi and bacteria. Capacity of this can be assessed by one of the subsequent three techniques (Ayafor, 1972). Disc diffusion technique, serial dilution technique and bio-autographic technique.

Nonetheless here is no way to estimate the results of the antimalarial test (Ayafor, 1982). Some researchers use the area of space control and / or minimum production to control product growth. However, many factors, such as factors, production methods and body mass, the traditional mediation process (Bayer et al., 1966) and pH and summer temperatures could affect the consequences.

These techniques are an exhaustive study of the in vitro test for preliminary diagnostic tests of agents that may be involved in antimicrobial movement. However, bacteriostatic and bacterial variations can be performed in this way (Roland R, 1982).

1.12.1. Disc diffusion method (principle)

In this type, antibiotics are separated from sources limited by a nutrient and formulate the target size. Dry and hygienic documents (with an average of 6 mm) that include sampling to determine the known value are placed in a neutral medium called germ. Antibiotics (bleaching) and empty

layers are used for poor control. These instructions are kept at low temperatures (4 ° C) for up to 24 hours to allow most of the detection of the surrounding vehicle. The plates can be transferred and heated at 37 ° C for 24 hours to increase biodegradation. Examples of antimicrobial properties can prevent microbial growth in the media by killing microbes near the discs and, therefore, providing a clear and defined defined space in the exchange zone. Finally, the antimicrobial property of the test sample is identified by evaluating the diameter on the millimeter scale (Barry, 1976, Bayer et al., 1966).

Chapter 2

Methodology

2.1 Plant stem collection

Aporosa wallichii is selected as a plant stem for this research, since no previous study on its biological properties has been carried out. A meticulous bibliographic study of this plant and its availability, the plant was chosen for analysis. The stem portion of the Aporosa wallichii stem was collected in December 2018 in the Moulovibazar district of Sylhet division, Bangladesh.

Table 2: Research of Aporosa wallichii

Name of the Plant	Scientific Name	Family	Part
Kukhra (In Bangladesh)	*Aporosa wallichii*	Phyllanthacea	Stems

2.2 Verification of plant

After collecting the stems, it was lead to the National Herbarium of Bangladesh (NHB), Mirpur, Dhaka for authentication. A week later, its file was collected and the access number of the plant identified for our given specimen is (DACB-44996) and is authenticated by National Herbarium, Mirpur, Dhaka, Bangladesh.

2.3 Process of extraction

The extraction of the medicinal plant involved several steps:

The whole extraction process can be divided into two parts:

- first, preparation and sunbaked of the stem substantial (2 steps),
- second, extraction process (5 steps)

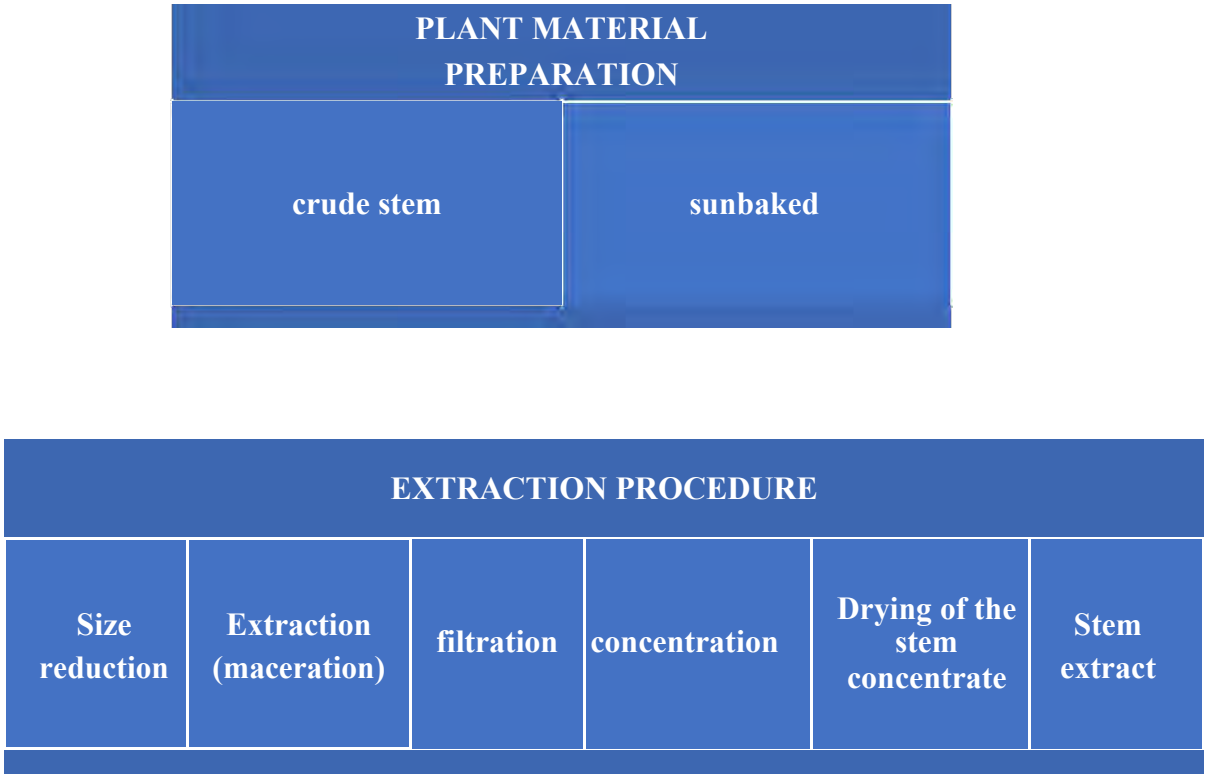


Figure 2: Flowchart showing a flow chart for the stem extraction

2.3.1 Preparation of stem material for crude extract

The leaves were plucked from the stem of the plant and water-washed to remove stem debris and trash particles. The clean sheet was permitted to dry within the sun for one day, after which the stem were dried for 1 hour at 30-40 ° C in a hot air stove. Then dried leaves were placed for the next step.

2.3.2 Plant Extraction procedure

➢ Size reduction and weighing

Dry and crunchy stems were crushed with stiff powder by using a high volume grinding machine. Approximately 483.52 g of dust had collected and then packed in airtight plastic containers with the necessary label that was finally leftward in a cool, dry and shadowy place till next investigation was done, essential measures were taken to avoid cross contamination.

Figure 3: Grinded powder of the Aporosa wallichii stems.

➢ Extraction of plant by solvent

Depending on the types of solvents used, the extraction methods can be divided into two parts:

✓ Extraction with aqueous solvents.

✓ Extraction with organic solvents

For the purpose of this study, the process of maceration by extraction was used for the extraction of stem materials as well as methanol as an organic solvent. The beaker including Aporosa 34

wallichii powder stem material in 2.2 liters of methanol was soaked for a period of 2 days at average room temperature (22-25 ° C) with infrequent stirring.

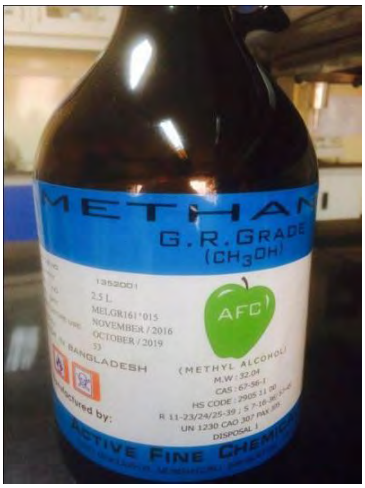


Figure 4: Solvent for extraction (Methanol 2L)

➢ Filtration

After maceration of two days, the content of the jar was filtered with a cloth and then with cotton and a Whatman filter (pore size: 110 mm).

➢ Concentration

Now filtrate collected from the extraction of the stem remained concentrated by via a rotary evaporator (Heidolph) with 100 rpm at 30 ° C, unless the concentrated methanol extract was produced. The concentrated mixture is then thickened in a petri dish.

Figure 5: Concentrate the filtering of the Aporosa wallichii stem using an evaporator rotator

➢ Drying

Finally, the Petri dish was placed under a flow of laminar air (LAF) to vaporize the extract solvent. LAF was used as a preventive measure, a measure to avoid any possibility of microbial development within the extract throughout drying. After successful drying of the extract preserved in the Petri dish.

Figure 6: Dried methanol extract of Aporosa wallichii stems in petri-dish.

Table 3: The weight of the methanol stem extract of Aporosa wallichii obtained as a result of a complete extraction procedure.

Initial weight/g (Petri-dish)	70.126g
Final weight/g (Petri-dish + extract)	90.72g
Weight of extract/g	483.52g

2.4 In-vitro antioxidant property analysis

2.4.1 Free radical scavenging DPPH assay Evaluation

DPPH is always used to discover and evaluate the effects of free radicals (antioxidants) for different composites and remedial plants (Choi et al., 2000; Desmarchelier et al., 1997).

➢ Mechanism of DPPH test

For discovering and estimating the antioxidant activity by DPPH, from different amounts of concentration, 1 ml of methanol was extracted using a solution of 3 ml of DPPH methanol. BHT and ascorbic acid are generally used as a standard to concentrate between 1 and 100 mg / ml. A blank sample was also prepared for this study. After preparation of sample and the blank solution, it was kept in dark for about 30 minutes. Therefore, the antioxidant activity of the extract is dignified by spectrophotometric analysis under UV at a wavelength of 517 nm. The DPPH test is mainly used to detect a decrease in free DPPH with radicals. Because electrons

produce DPPH free radicals, UV spectrophotometry provides high absorbance at 517 nm of nickel. Afterwards the reaction of the stable free radical of DPPH, the antioxidant runs to the production of hydrogen and the reduction of DPPH, which leads to a reduction in absorbance with respect to DPPH. The discoloration is due to the fact that DPP-H produces a yellow color with respect to the total number of electrons. A greater capacity for reduction is shown when the discoloration gradually increases. DPPH is considered the best way to investigate research by determining the potency of new-made and potential medications (Brand-Williams et al., 1995).

➢ Materials and reagents list

Table 4: Materials and reagents

Materials	**Reagents**
UV-spectrophotometer	DPPH (2,2-Diphenyl-1-Picrylhydrazyl)
Test tubes	Ascorbic Acid (ASA)
Volumetric flask	Methanol
Light proof box	Distilled water
Pipette (1mL and 5mL)	Extracts of the experimental plant

➢ Control preparation for evaluation

In this experiment, ascorbic acid (ASA) was used to prepare the standard (positive control). The calculated amount of ascorbic acid remained dissolved into the methanol solvent for obtaining a solution whose concentration was 500 µg / ml. Afterwards , to obtain a different concentration, a serial dilution of 500.0 to 0.977 µg / mL was performed.

Table 5: Amount used in preparation of control

Name of chemicals	**Calculated amount**
Ascorbic acid	2mg
Methanol	4mL

➢ Test sample preparation for evaluation

To prepare the test sample, 2 mg of Aporosa wallichii stem extract was placed in a clean test tube and then methanol of 4 ml was added in the test tube to obtain a concentration of 500 µg / ml. Now, by way of the sequential dilution method, the concentration ranging from 500 µg / ml to 0.977 µg / ml was reached and preserved dry with the label. The calculated amount of methanol extract was 2 mg.

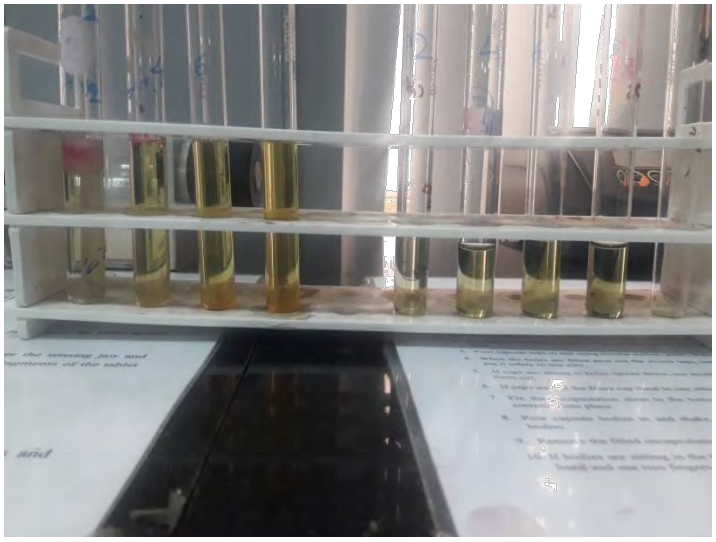


Figure 7: Dilution in series of the stem extract in different test tubes.

➢ Preparation of DPPH solution for evaluation

For the preparation of the DPPH solution, powder containing 1 mg of DPPH was correctly measured and dissolved into 50 ml of methanol to collect the concentration of 20 µg / ml. Then, the solution was stored in a dark box covered with aluminum foil.

Table 6: Amount used in DPPH solution preparation

Name	**Calculated amount**
DPPH (2,2-Diphenyl-1-Picrylhydrazyl)	1mg
Methanol	50ml

➢ Inspect of DPPH free radical scavenging activity

Now, the experiment sample solution contains each test tube having a different concentration ranging from 500 µg / ml to 0.977 µg / ml was mixed with 3.0 ml of a DPPH solution whose concentration is 20 µg / ml . Then, this mixture is kept in a dark place for 30 minutes for the reaction to take place. After 30 minutes, the absorbance of the mixture was measured by a UV spectrophotometer at a wavelength of 517 nm. Here, methanol is used as blank..

➢ Calculation

The percent inhibition (I%) of DPPH from free radicals was calculated as follows:

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

Where X_{blank} represents the absorbance of control reaction

Subsequently, a 50% inhibition (IC 50) was provided through the the methanol stem extract concentration and the value obtained from where the percent inhibition (I%) was plotted in graph contrary to the concentration of the stem extract (µg / mL).

2.4.2 Evaluation of total phenolic content

According method which described by Skerget et al., (2005) which involved the chemical Folin-Ciocalteu as an oxidant and gallic acid by means of standard, the total phenolic content of stem extracts of Aporosa wallichii was easily measured.

➢ Materials and reagents

Table 7: Materials and reagents for the measurement of phenolic content.

Materials	**Reagents**
Test tube	Folin-Ciocalteu reagent (10 fold diluted)
UV-spectrophotometer	Na_2CO_3 solution (7.5 %)
Vial	Gallic acid
Micropipette (50-200 µl)	Distilled water

➢ Examination of test sample grounding for evaluation

2 mg of the stem extract had taken in a test tube and had dissolved within the distilled water, that gave a sample concentration of 2 mg / ml. Show along with your concentration given below.

➢ Preparation of standard solution for gallic acid curve

Gallic acid is generally used throughout the world by means of a standard in the total phenolic content examination. Several solutions of gallic acid were prepared with 100 µg / ml at 0 µg / ml. Concentration via using 2.5 ml of chemical Folin-Ciocalteu (diluted 10 times with water) and 2 ml of (7.5% w / v) Na2CO3 solution was added in gallic acid of 0.5 ml. The solution mixture was placed in a shadowy place on ambient temperature intended for 20 minutes. Afterwards, using UV spectrophotometer, the mixture was measured at 760 nm and the absorbance had

taken. Then, the absorbance had designed contrary to the concentration and, as a result, a linear correlation was obtained that had used to obtain the result of the sample.

➢ Analysis of the total phenolic content

To obtain the mixture, 0.5 ml of stem extract (2 mg / ml), 2.5 ml of Folin-Ciocalteu chemical (10 times mixed with water) and 2.0 ml of (7.5%) were added. p / v) of Na2CO3. Then the mixture is preserved in a dark space at room temperature for 20 minutes. Afterward the specific time elapsed at 760 nm, the absorbance had estimated with a UV spectrophotometer together by using of a standard curve of gallic acid, the total sample had estimated. The phenolic content of the sample was declared by means of GAE mg (gallic acid equivalent)/g extract.

2.5 *In-vitro* analysis of cytotoxicity properties

2.5.1 Experimental Procedure Brine shrimp lethality bioassay

➢ Materials used for examination

Table 8: Materials required for Brine shrimp lethality assay are listed below:

Sl. no.	Name of materials
1	Brine shrimp (*Artemia salina)* egg
2	NaCl
3	Small tank
4	Test tubes
5	Pipette, Micropipette
6	Glass vials

7	Lamp to attract shrimps
8	Magnifying glass
9	Dimethyl sulfoxide (DMSO)
10	Stem extract

➢ Preparation of salt-water for examination

For preparation of the saltwater solution, 38 g of salt (pure NaCl) had weighed accurately, then dissolved in distilled water (1 l) and then the water was filtered to obtain a clear solution.

➢ Hatching of brine shrimps eggs for test

For the test, brine shrimp eggs (Artemia salina) collected in the stores for the test were used. He took a small tank full of sea water and then added shrimp eggs. To obtain mature nauplii two days provided with continuous supply of oxygen along the hatch. Lamp light attracts shrimp through the perforated dam. The Pasteur pipette is used to collect 10 ± 2 live shrimp that are added to each test tube containing 5 ml of seawater.

➢ Preparation of test solution for experiment

The test sample was taken in a test tube and dissolved with dimethylsulfoxide (DMSO). Afterward, by serial dilution, a different concentration was achieved that ranged between 400 µg / ml and 0.78125 µg / ml. First 50 µl of sample whose concentration was 400 µg / ml retained in a test tube comprising 5 ml of DMSO together with 10 ± 2 nauplii. After that, addition of 50 µl

of DMSO to the test tube cause dilution of the solution by this method, obtaining a different concentration.

Table 9: Sample of Stem with different concentration later serial dilution

Test tube no.	Concentration (µg/mL)
1	500
2	250
3	125
4	62.5
5	31.25
6	15.625
7	7.813
8	3.906
9	1.953
10	0.977

➢ Control group preparation for investigation

In cytotoxicity studies toward approve the analysis process and ensure that the results achieved are equivalent to the performance of the test agent and the possible effects of other conceivable stoppage control groups are very essential. In general, there are 2 types of control groups that are practical, are negative and positive control.

➢ Grounding of positive control

Through the cytotoxicity experiment, the positive control is broadly known as a cytotoxic compound, which aids into the test compared to the result of the positive control. At this point, vincristine sulfate and a cytotoxic compound were used as standard (positive control). The dosage of vincristine sulphate was dissolved into DMSO to obtain the first dose about 20 µg / ml at that time by serial dilution, truth concentration of the standard solution obtained, such as 10 µg / ml, 5 µg / ml, 2 , 5 µg / ml, 1.25 µg / ml. 0.625µg / mL, 0.3125µg / mL, 0.15625µg / mL, 0.078125µg / mL and 0.039µg / mL. Finally, a standard (positive control) was added to the test tubes containing 5 ml of sea water together with 10 ± 2 nauplii.

➢ Grounding of negative control

For preparation of the negative control, 3 test tubes were taken and 100 µl of DMSO was added to each of the test tubes containing 5 ml of sea water together with 10 ± 2 nauplii. If the mortality rate of the nauplii is rapid, which indicates that the test is undesirable and the nauplii died as a result of some unwanted reason.

➢ Nauplii counting

The result obtained after 24 hours, by the help of a magnifying glass and the number of stickers, had accurately counted in each of the test tubes. For each dilution, the percentage of mortality by linear regression of the IBM-PC program was calculated, which is used to evaluate the concentration-mortality data (%). In addition, the relationship between concentration and mortality of the stem extract is expressed by a value (LC50) which means the value of the mean

lethal concentration. Therefore, the concentration of the chemical is responsible for death in partial of the experiment nauplii later a specific period of time.

2.6 *In-vitro* thrombolytic property analysis

The thrombolytic property can be evaluated by a simple method in which the stem extract as a sample, clopidogrel (antiplatelet agent) as a positive standard and water as a negative standard.

➢ Materials and reagents

Table 10: Materials used in thrombolytic test are:

Sl. no.	Name of materials
1.	Blood
2.	Clopidogrel (anti-platelet agent)
3.	Micro centrifuge tube
4.	Distilled water
5.	Saline
6.	Plant extract

➢ Experiment Sample preparation

For preparing the test sample, a test tube comprising distilled water (10 ml) was taken and then 100 mg of stem extract was suspended therein, after the test tube had preserved in a dry and shadowy place for whole night and as a result the desired soluble supernatant was transferred in solution and then the solution is filtered adequately.

➢ Standard solution preparation

Clopidogrel, an antiplatelet agent used as a standard for this experiment. 100 mg of clopidogrel were dissolved in distilled water (10 ml) and mixed properly for this moment, this suspension was stored as a standard standard solution from which 500 µl of solution was applied in the thrombolytic activity test.

➢ Blood sample preparation

2 healthy volunteers (n = 2) have no history of anticoagulant treatment selected from them. The blood sample was collected ensuring an aseptic condition. After collecting the blood, 1 ml of blood was changed to previously weighed microcentrifuge tubes. After that the microcentrifuge tubes are stored to form clots.

➢ Thrombolytic property test process

At the beginning of the test, 5 ml of fresh lifeblood was collected as of each volunteer worker. Blood samples had been taken from five altered pre-weighted sterilized microbes and permitted to incubate at 37 ° C intended for 45 minutes. As soon as the clot is formed, the upper fluid had completely discharged from all the microtube pipettes. The weight of the clot was determined by the weight of the tube taken before the clot formed. In this case, 100 µl of clopidogrel used as a positive control and 100 µl of water (distilled) as a non-thrombolytic negative control were used with 100 µl of each sample as of each test tube. Aimed at the observation of clot formation, the microtubes had been incubated at 37 ° C intended for 90 minutes. Then, when the incubation was completed, the fluid that had free from the clot was removed and the pipettes were weighed again for observing the transformation in weight after the distraction of the clot.

Finally, the percentage (%) of clot lysis as shown below:

Percentage (%) of clot lysis = (weight of clot released / weight of clot) × 100

2.7 Antimicrobial property analysis

Methanol extract of *Aporosa wallichii* stems used as test sample.

➢ Apparatus and reagents used for antimicrobial analysis

Table 11: Used apparatus are listed below:

Sl. no.	Apparatus
1.	Filter paper discs
2.	Nutrient Agar Medium
3.	Petri-dishes
4.	Micropipette
5.	Sterile forceps
6.	M.H. Agar
7.	Screw cap test-tube
8.	Autoclave
9.	Spirit burner
10.	Refrigerator
11.	Nose mask and Hand gloves
12.	Incubator
13.	Laminar air flow hood

➢ Micro-organism used in the test

Bacterial strains collected from pure culture and here both gram positive and gram negative organisms were taken for the experiment and the organism list provided below:

Table 12: List of microorganisms used in the antimicrobial analysis

Gram positive Bacteria	Gram negative Bacteria
Enterobacter faecalis *Bacillus cereus*	*Klebsiella pneumoniae* *Salmonella typhi* Enterotoxigenic *Escherichia coli (E.coli)* or (ETEC) Enteropathogenic *Escherichia coli (E.coli)* or (EPEC) *Klebsiella variicola* *Shigella flexneri*

Sterilization procedure of test

Before performing the test, all devices include beakers, conical flasks, cooking plates, cottons and forceps sterilized and kept in an aseptic place. This sterilization is done to avoid any kind of cross contamination or microbial contamination during the process. To maintain the control environment, all work performed under the laminar air flow hood, before starting the experiment, the UV light was lit for 1 hour in the laminar air flow hood. In addition, micropipette tips, cotton, tweezers, blank discs were also treated with UV light. However, by autoclave, Petri dishes and other apparatuses were sterilized at a temperature of 121 ° C and a pressure of 15 lb / m2. inch

for 1 hour. After the experiment, all sterilized and used bacterial strains were destroyed to avoid environmental contamination.

➢ Procedure of antimicrobial test

At the beginning, for the preparation of the culture, 2.5 g of nutrient broth were dissolved in 100 ml of distilled water. Then, 8 conical flasks were taken, each containing 10 ml of broth mixture, 8 different bacterial strains were added and these conical flasks were stored in an incubator with shaking. Temperature at 37 ° C for 24 hours. After 24 hours, these conical flasks were removed from the incubator with stirring and kept in a controlled environment. After that, agar medium was prepared using 7.6 g of M.H. agar that was dissolved in 200 ml of distilled water. Immediately after preparation M.H. The agar was placed on patriotic plates and these patriotic plates were stored for cooling to room temperature. Meanwhile, a test sample of plant extract concentration ranging from 500 mg / ml to 15,625 mg / ml was prepared and left on filter paper discs. When M.H. The agar in the traditional dishes became solid and then the bacterial strains were introduced into it using cotton bars. Streptomycin is used as standard discs and shows discs of test tube extracts placed on patriotic plates. Then, these standards were placed in the incubator at 37 ° C for 24 hours to provide an optimal environment for bacterial growth. Again, after 24 hours, the back plates contain different bacterial strains together with the standard sample and the collected test discs and the observed inhibition zone produced by the standard sample and the test discs.

➢ Determination of inhibition zone for test

Antimicrobial property of the agents is estimated by the ability to stop the microorganism growth around the discs in the patri-dishes. Prevention of microorganism growth indicated when discs gives clear region of inhibition. When the incubation is completed antimicrobial property of the sample were evaluated by calculating the diameter of the inhibition area with a clear scale.

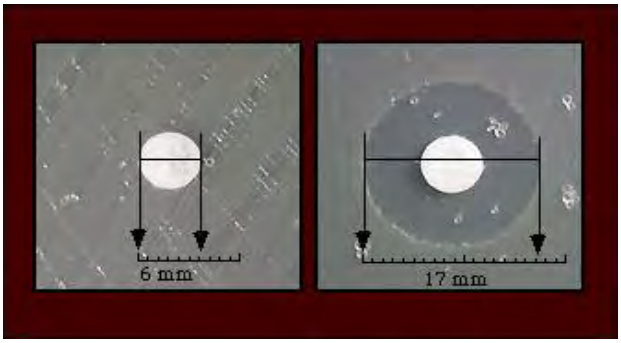


Figure 8: Determination of the clear inhibition zone

Chapter 3

Observation and results of all the experiments

3.1 Antioxidant property analysis

3.1.1 Estimation of DPPH free radical scavenging assay of *Aporosa wallichii*

Table 13: IC_{50} value (µg/mL) of Ascorbic acid (ASA)

Conc. (µg/mL)	**Absorbance of Standard (ASA)**	**% of inhibition**	**IC_{50} µg/mL**
500	0.031	94.97568882	75.688
250	0.043	93.03079417	
125	0.065	89.46515397	
62.5	0.184	70.17828201	
31.25	0.292	52.67423015	
15.625	0.385	37.6012966	
7.813	0.418	32.2528363	
3.906	0.464	24.79740681	
1.953	0.481	22.0421394	
0.977	0.497	19.4489465	
Blank	0.617		

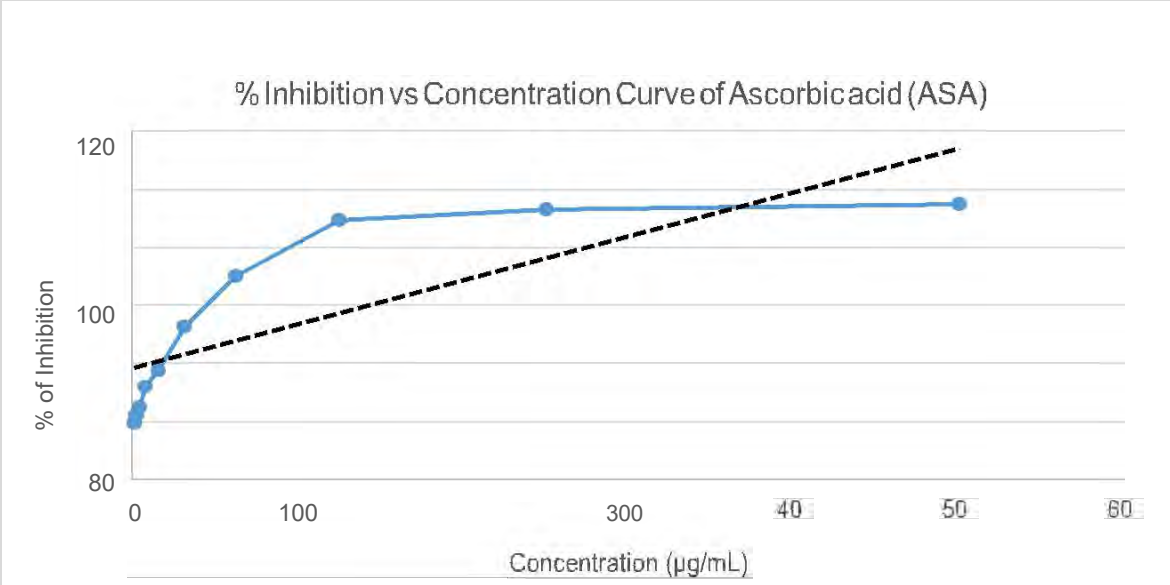


Figure 9: % Inhibition vs. Concentration curve of ASA

Table 14: IC_{50} value (µg/mL) of Methanol stems extract of Aporosa wallichii

Conc. (µg/mL)	**Absorbance of Methanol stem extract**	**(%) of inhibition**	**IC_{50} µg/mL**
500	0.033	94.4256	91.77
250	0.072	87.8378	
125	0.124	79.054	
62.5	0.198	66.554	
31.25	0.263	55.5743	
15.625	0.339	42.7364	
7.813	0.391	33.9527	
3.906	0.464	21.6216	
1.953	0.489	17.3986	
0.977	0.515	13.0067	
Blank	0.592		

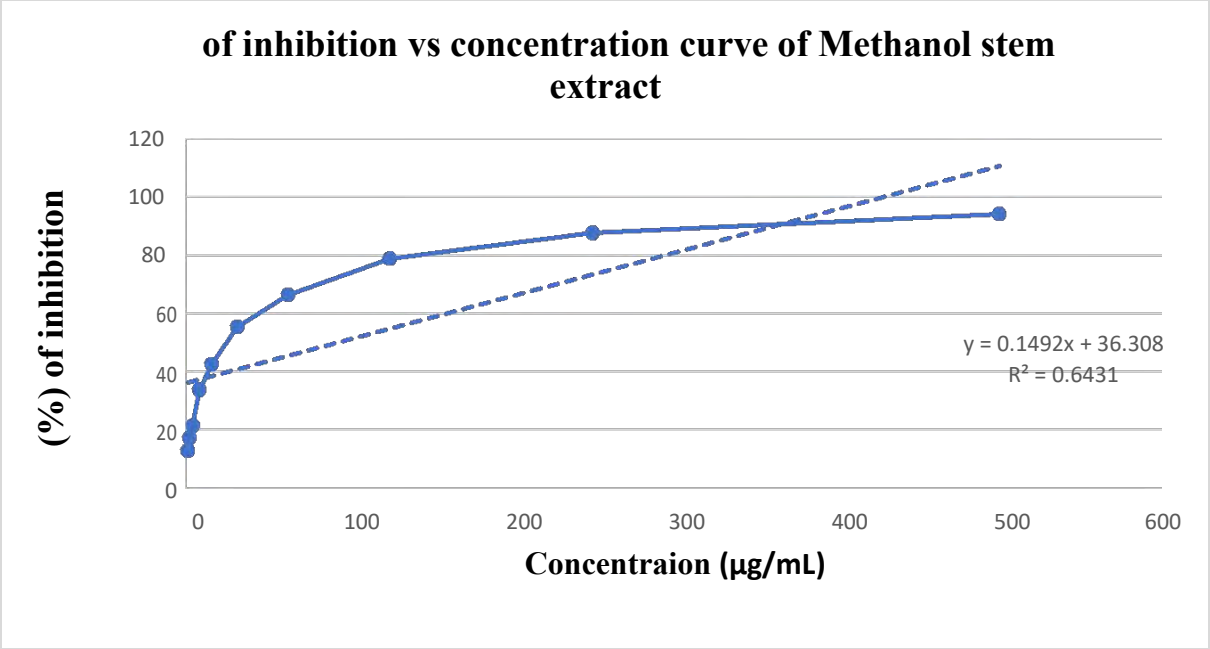


Figure 10: (%) of inhibition vs. Concentration curve of Methanol extract of Aporosa wallichii

Explanation: After observation, the table (3.1 and 3.2) showed that percentage (%) inhibition of free radical DPPH scavenging of the extract of *A. wallichii* was slightly lower at some points but in some points *A. wallichii* have higher % of inhibition than the equivalent concentration of ascorbic acid. And also the value of IC50 µg/mL of methanol extract of *Aporosa wallichii* was higher than ascorbic acid which means 91.77 ug/mL is required to achieve the same inhibitory effect for ascorbic acid.

3.2. Evaluation of total phenolic content

Table 15: Absorbance of Gallic acid

Conc. (µg / mL)	Absorbance	Regression line	R^2
100	0.800	y = 0.0081x - 0.0007	0.9975
50	0.423		
25	0.215		
12.5	0.123		
6.25	0.047		
3.13	0.007		
1.56	0.003		
0.78	0.000		
0.39	0.000		

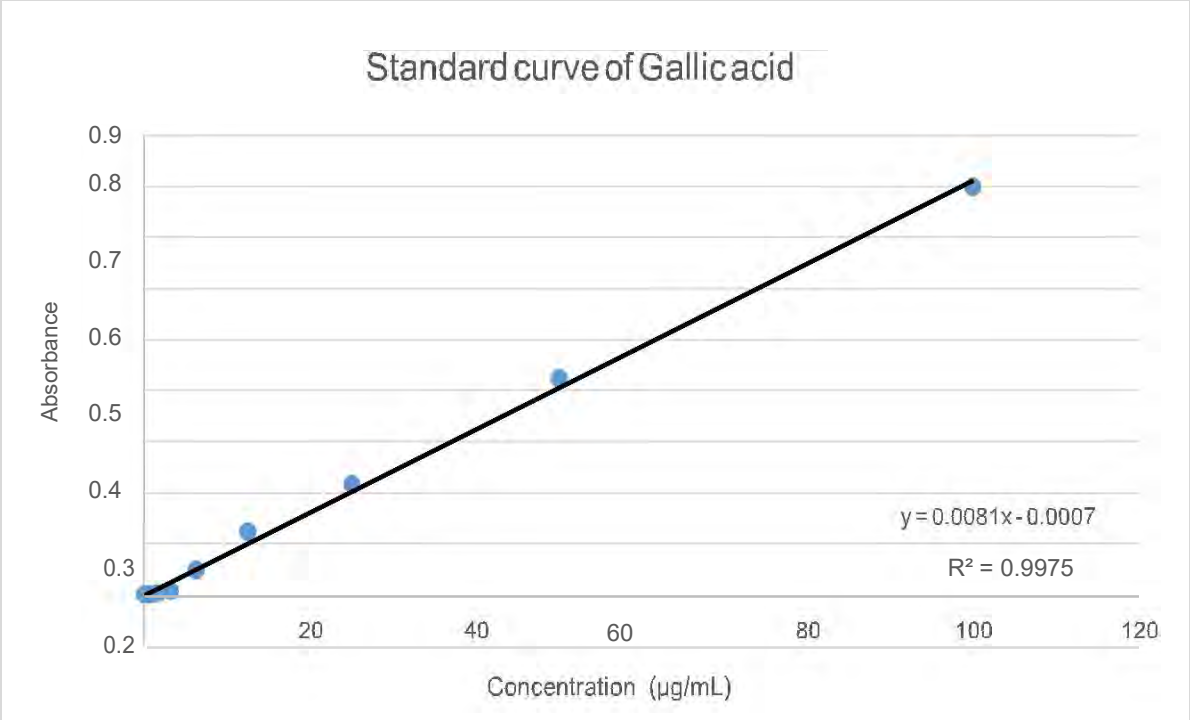


Figure 11: Standard curve of Gallic acid's for total phenolic content investigation

Table 16: Outcome of total phenolic content of experimental sample

Sample code	**Name Of extract**	**Plant part**	**concentration (µg / mL)**	**Absorbance of methanol stem extract**	**Total phenolic content (mg of GAE/gm)**
ME	Methanol extract	Stems of *Aporosa wallichii*	2	0.078	9.71
			4	0.155	19.22
			6	0.212	26.26
			8	0.284	35.15

So, Total phenolic content obtained from the concentration 2(µg/mL), 4(µg/mL), 6(µg/mL) and 8(µg/mL) were 9.71, 19.22, 26.26 and 35.15 (mg of GAE/gm of extract) respectably of the methanol extract of Aporosa wallichii stem.

3.3 *In-vitro* Cytotoxicity property analysis

3.3.1Evaluation of Brine shrimp lethality assay

Table 17: Positive control (vincristine sulphate) effect on shrimp nauplii

Concentration (µg/mL)	Log Concentration	Nauplii withdrawn	Nauplii Dead	Nauplii active	% of Death	LC_{50} (µg/mL)
0.039	-1.408	10	2	8	20	
0.078	-1.107	10	3	7	30	
0.156	-0.806	10	3	7	30	
0.312	-0.505	10	4	6	40	
0.625	-0.204	10	5	5	50	
1.25	0.097	10	6	4	60	2.0203
2.5	0.398	10	7	3	70	
5	0.699	10	8	2	80	
10	1	10	9	1	90	
20	1.301	10	10	0	100	

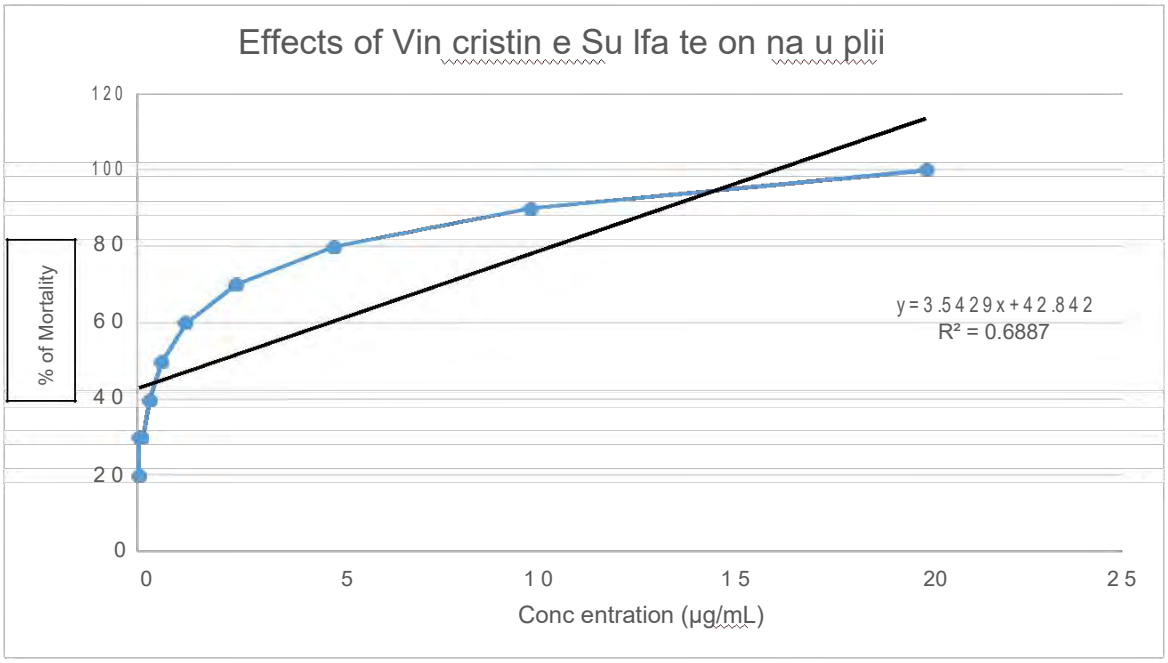


Figure 12: Proportion (%) death and expected deterioration line of vincristine sulfate.

Table 18: Special effects of the methanol extract of A. wallichii over nauplii

Concentration (µg/mL)	**Log Concentration**	**Nauplii withdrawn**	**Nauplii Dead**	**Nauplii active**	**% of Death**	**LC_{50} (µg/mL)**
0.977	-0.010	10	4	6	40	0.484
1.953	0.290	10	5	5	50	
3.906	0.591	10	5	5	50	
7.813	0.892	10	6	4	60	
15.625	1.193	10	6	4	60	
31.25	1.494	10	7	3	70	
62.5	1.795	10	8	2	80	
125	2.096	10	8	2	80	
250	2.397	10	9	1	90	
500	2.698	10	10	0	100	

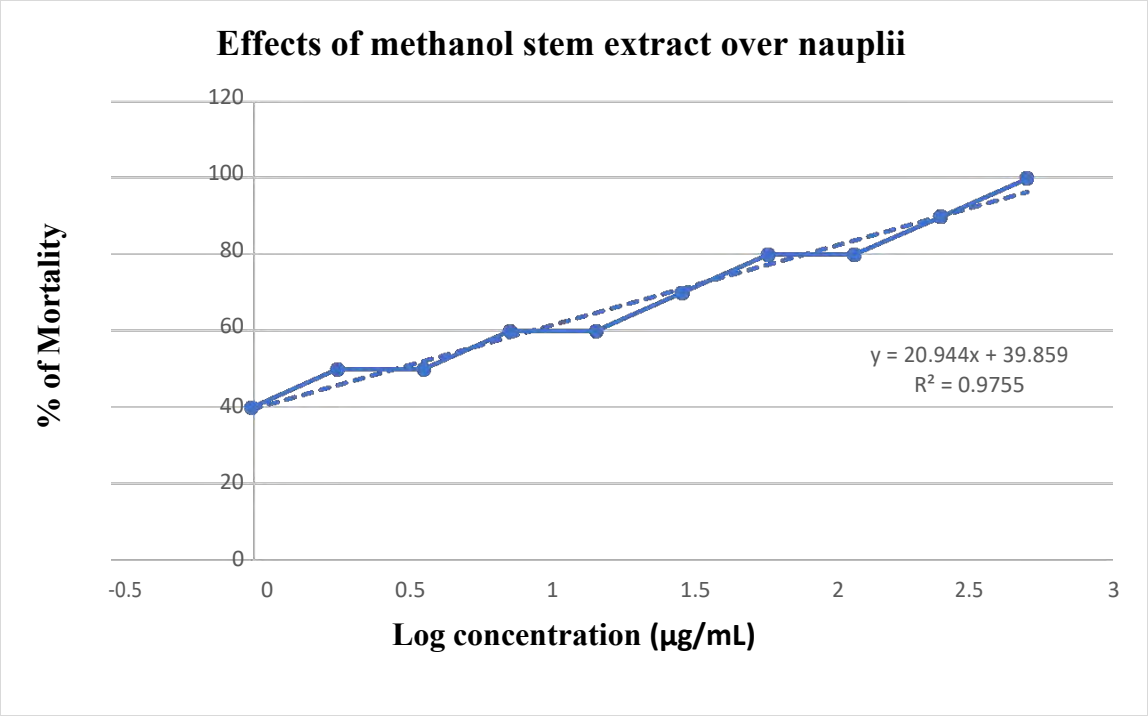


Figure 13: Effects of methanol stem extract over nauplii

3.4 Analysis of Thrombolytic properties

Table 19: Estimation and result of Thrombolytic Action

For female candidate:

Name of Sample taken	A1	A2	A3	A4	A5	% of clot formation
Methanol extract of *A. wallichii*	4.645	7.925	7.220	2.575	0.705	27.38
Clopidogrel (Anti-platelet agent) as standard	4.649	7.992	7.011	2.362	0.981	41.53
Blank	4.654	7.285	7.279	2.625	0.006	0.22

For male candidate:

Name of Sample taken	A1	A2	A3	A4	A5	% of clot formation
Methanol extract of *A. wallichii*	4.629	7.764	7.162	2.533	0.602	23.76
Clopidogrel (Anti-platelet agent) as standard	4.682	7.978	7.056	2.374	0.922	38.84
Blank	4.662	7.358	7.346	2.684	0.012	0.44

Here,

A1= Micro tube weight,

A2= Micro-tube weight with clot,

A3= Micro tube weight with clot after clot disruption,

A4 (A3-A1) = Weight of clot after clot disruption,

A5 (A2-A3) = Weight of released clot.

From this research it can be declared that, Methanol extract of *A. wallichii* stem showed moderate effect on clot lysis but compare to clopidogrel clot lysis rate was lower.

3.5 Anti-microbial property analysis

3.5.1 *Aporosa wallichii* leaves antimicrobial activity evaluation

The methanol extract from the leaves of Aporosa wallichii was taken to perform an analysis of antimicrobial activity, different concentrations were prepared ranging from 500 mg / ml to 15,625 mg / ml. At these concentrations, each of the bacterial strains was examined but no significant results were found. There is little chance of any concentration showing a very mild effect, but most of the concentration showed no antimicrobial property. The reason behind this negative result may be experimental errors and others. But during this experiment streptomycin is used as a standard, it showed antimicrobial activity correctly in both gram positive and negative.

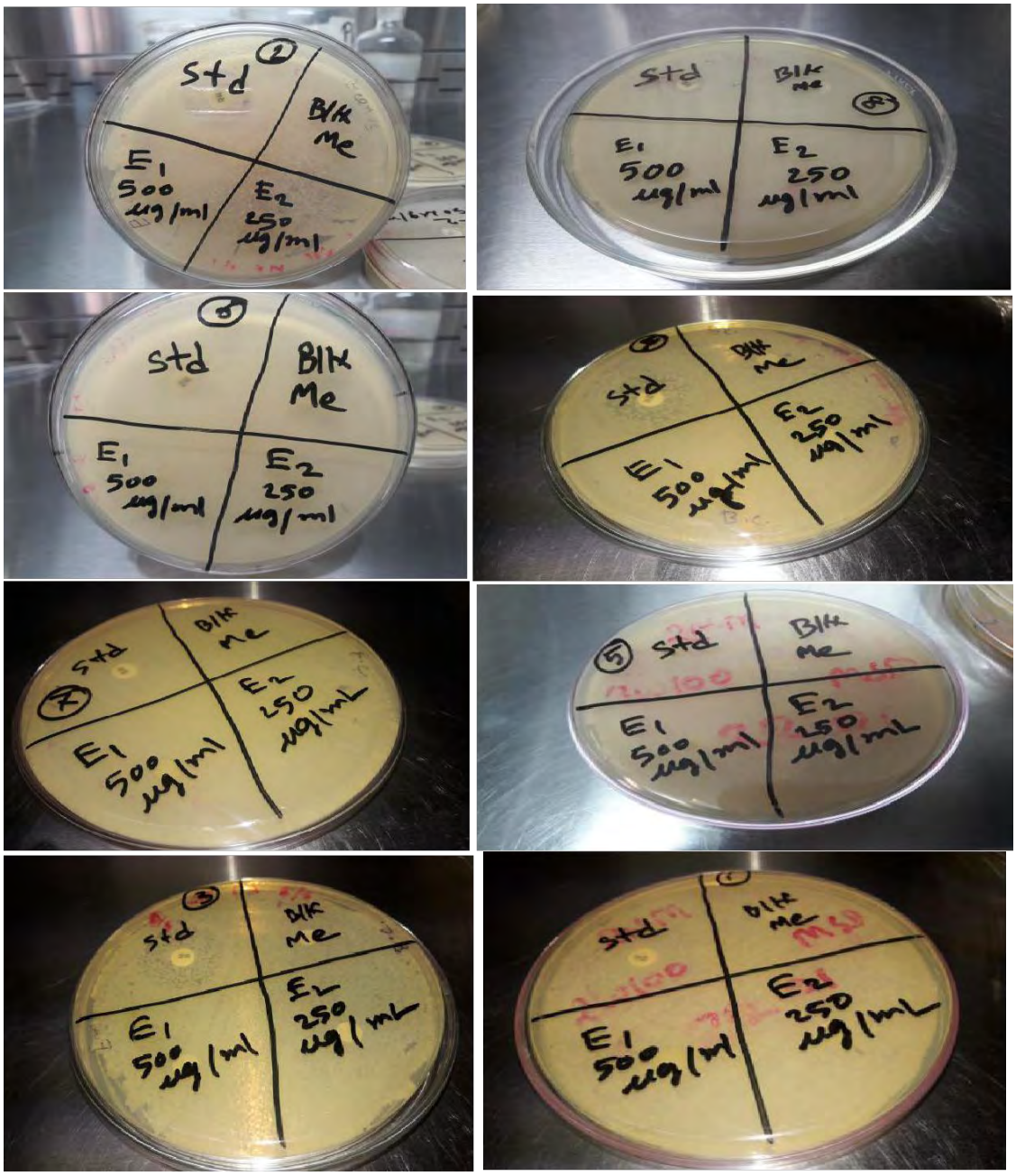


Figure 14: Antimicrobial property of A. wallichii stems evaluation on patri-dishes.

Chapter 4

Discussion

Methods of biological selection of methanol stem extract from the harvested plant, *Aporosa wallichii* has provided a sufficient amount of information about its usefulness in the medical world. The methanol extract from *Aporosa wallichii* stems was correctly verified through the DPPH test to regulate the antioxidant properties of this stem. As a standard reference, ascorbic acid remained used in this research aimed at which an IC50 value of 75,688 μg / ml and the methanol stem extract of *Aporosa Wallichii* had attained, whose IC50 value was obtained of 58,725 μg / ml. In addition, the test of the total phenolic content of this plant showed a satisfactory result. Therefore, the present study might suggest that this plant can be used as an antioxidant agent.

The lethality test of the brine shrimp was obtained to evaluate the property of the cytotoxicity of the methanol extract of the *Aporosa wallichii* stems, the LC50 value of the studied section was resolute after the graph where the percentage of mortality was represented (nauplio) versus the concentration of the test sample. To determine the best fit line of the curve obtained from the data, regression analysis was used. For positive control Vincristine sulfate was taken as standard in this investigation, whose LC50 value had obtained 2.0203μg/mL, with comparison to the standard methanol stem extract of the *Aporosa wallichii*, it provided an LC50 value of about 26.751 μg/mL. Therefore, it can be stated that *Aporosa wallichii* showed the property of cytotoxicity as well as this research could be followed for the improvement of global medical care.

The methanol stem extract of *Aporosa wallichii* showed a significant result in the thrombolytical action check. Here, as a positive control clopidogrel was used for which 59.33% of clot lysis was observed. Distilled water was used as a negative control, which showed a lysis of 2.47% of the blood clot. The methanol stem extract of *Aporosa wallichii* indicated 24.47% of clot formation. By equating the lysis value of the methanol stem extract clot with the value of positive control, the plant stem revealed an effective thrombolytic activity.

An antimicrobial properties test was also performed to evaluate the methanol extract of *Aporosa wallichii* stems. From this experiment a significant result was not obtained, which means that the plant stem did not contain any antimicrobial property, this may be the result of some experimental error. But more research is needed to confirm whether the antimicrobial property of the plant stem is present or not.

Chapter 5

Conclusion

The methanol stem extract of *Aporosa wallichii* was explored to estimate the biological properties. After conducting all investigation, it was clarified that the plant stem showed different biological properties. This research study showed that the plant's stem have a modest level of antioxidantal and thrombolytical properties through a significant level of cytotoxicity properties. However, the plant stem showed no antimicrobial property, but requires more research on the antimicrobial property of this plant. In addition, current research suggests a more detailed investigation of the stem of the *Aporosa wallichii* plant to discover hidden biological properties through phytochmical screening; several in-vivo and in-vitro tests that will benefit in the growth of global medical care also can introduce any novel and active property in the medical arena.

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