In-vitro Biological Screening of Methanol Extract of *Aporosa wallichii* (Euphorbiaceae) Leaves

A project submitted

by

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

Certification Statement

This is to certify that this project titled "*In-vitro* Biological Screening of Methanol Extract of *Aporosa wallichii* (Euphorbiaceae) Leaves" submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (Hons.) from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Shahana Sharmin , Senior Lecturer, Department of Pharmacy, BRAC University and that appropriate credit is given where I have used the language, ideas or writings of another.

Signed,

Countersigned by the supervisor

Acknowledgement

Alhamdulillah, all the admiration belong to Allah S.W.T. from whom I get strength and health to complete this project work. I would like to show my thankfulness and gratitude to Almighty Allah for blessing me with immense patience, strength, corporation and assistance when necessary to complete the processes of bachelors in Pharmacy.

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Abstract

This research was conducted to find out and evaluate potential biological properties of the medicinal plant called *Aporosa wallichii* which belongs to family Euphorbiaceae. To fulfill the research determinations several experiment were done like antioxidant property test which include DPPH test and total phenolic content test, brine shrimp lethality assay, thrombolytic property test and antimicrobial property test. After completing these experiments evident from various observation that, this plant showed good level of antioxidant property, moderate level of thrombolytic property and significant level of cytotoxicity property. Additionally, antimicrobial property test did not showed desired result for this plant, which indicate it may not contain any anti-microbial property. However, this research is initial to the finding of pharmacological property of this plant. Finally, on the basis of this research it can be claimed that this plant *Aporosa wallichii* able to provide good impact in medicine world and development of global healthcare.

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List of abbreviations:

- ✓ 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 one

INTRODUCTION

1. Introduction

Nature is the most wonderful gift to us it is augmented with millions of mysterious elements among all of the elements environment is the blessing for us. Plants are the part of our environment and they are very useful for human health. As noted in the past, different kind of diseases has traditionally been treated with agents that are derived from medicinal plants. The term "medicinal plant" consists of several types of plants used in herbology. Here plants use for medical purposes to treat diseases. Herbal plant preparations that have been used to treat diseases in the early stages of human life have developed the foundation for drug therapy. Therefore, from here until now, it is considered one of the most valuable sources of medicine. So, it is necessary to detect naturally available herbal medicines for the betterment of mankind which have great pharmacological effects.

Our earth is full of different types of plants and humans invented about 7.5 lakhs of plant species, of which 5 lakh are classified as "higher plants" and 2.5 lakh as "lower plants" (Devi, 2015). About 60% of all clinically used medicines are produced from natural products and their natural herbal derivatives and products. About 87% of all classified medicines, like antibiotics, antitumor agents and anticoagulants, come from natural products and their derivatives. Chemicals that newly available in the world are more than 28% of natural products (Akter, 2013). Recently, World Health Organization (WHO) stated that nearly 80% people around the world directly relying on medicinal plants for primary treatment. Moreover, 21,000 species of plants have potential pharmacological effects which can be used as medicinal plants

Treatment with medicinal plants is considered harmless because there are minimum side effects. Greatest benefit is remedies are in tune with nature. The major fact is that people of all ages and sex can take herbal treatment.

1.1. Summary of the history of medicinal plants

It is known to all that use of medicinal plant to treat diseases started by our ancestor but when they started is still unknown. While looking for food they found different plants which shows poisonous effect and shows different abilities like capacity to produce sweat, reduce pain and inflammation.

Analytical history of medicinal plants discloses that our ancestors, like the Assyrians, the Babylonians, the Egyptians, knew well about the medicinal properties of herbs and trees. A big amount of important medicines were well known to Babylonians (around 300 BC) and stated that modern medicine still uses certain plants in the same way as Babylonians (Ghani, 2003).

The first manuscript on the use of medicinal plants is almost 400 years old. The manuscript was written by a group of people from the ancient Sumerian culture living on the Euphrates River and the Tigris. They wrote on small clay slabs. The slabs were then discovered by later Iraqi researchers. The Egyptians also wrote a manuscript on medicinal plants called Ebers Papyrus. More than 700 methods were discovered, written about 1700 AC. An excellent documented way of using medicinal plants is the book titled "Pen Tsao" holding the use of more than 300 medicinal plants. The Indian medical system called Ayurveda, which referred to the use of medicinal plants, from 800 A.C (Remedies with Traditional Plants, 2016).

Among ancient civilizations, India was always famous for their use of medicinal plants. Throughout the Indian forest the great quantity of medicinal and aromatic plants are located, form there manufacturer of medicines and perfume items collected their raw ingredients. Indian medicine system named AYUSH coded around eight thousand herbal remedies and also for indigenous medicine Tribal, Siddha, Unani and Ayurveda medication systems are foremost. However, Indian people highly used Ayurveda and Unani system as a result these system developed most.

Chinese were also have great knowledge of medicinal plants they use a separate traditional therapy, called "Chinese Herbs". It should be noted that the botanical origins support the Chinese herbalism. More than 1200 plants are involved in the traditional treatment system and for the treatment purposes about 500 medical plants used to cure several diseases. The Chinese used medicinal herbs in the same way they used them in the past. It should be noted that one fifth of the Chinese pharmaceutical industry comprises about 5,000 traditional remedies (Li, 2000)

Ancient scholars trust that many health problems and diseases have only one solution which is herbs. Ancient scholars conducted a research on it, attempt to evaluate with specific findings on the effectiveness of diverse herbs that provide medicinal significance for many diseases. These formulated medications have no or very less side effects or adverse reactions. For this quality treatment with herbal plants is becoming increasingly popular all over the world. These herbs

that have medicinal qualities provide treatment for many internal diseases that otherwise are considered hard to cure.

Organizations like ESCOP (European Organization Cooperative On Phototherapy, 1999), German Commission E (Bluemental et.al, 1989) and WHO (World Health Organization) stated that the use of plants to cure diseases has been recognized by whole world and the use of medicinal plants increases gradually every day.

1.2. Medicinal plants available in Bangladesh

Subtropical country like Bangladesh which surrounds more than 5,000 angiosperms 200 families. Bangladesh provides perfect time to grow and nourish medicinal plants. Chittagong, Dhaka, Rajshahi, Sylhet and other areas of Bangladesh are enriched by about 5000 different types of medicinal plants, as mentioned in the "'Materia Medica' ". From the beginning of its existence, traditional medicines are well known in the country. The environment of successful development of medicinal plants in Bangladesh prefers to treat 500 classical diseases among 2000. In addition, in recent years, the use of medicinal plants by giant industries and companies in Bangladesh has increased considerably. Many leading pharmaceutical companies in Bangladesh now use a variety of medicinal plants. Most tribal and Bangladeshi populations rely heavily on the medicinal plant for primary treatment, perhaps they believe that nature will not harm their health. According to this faith and conviction, they use various parts of plants: barks, steam, fruits, flowers etc.

Table 1.1: List of Medicinal plant used in preparation of traditional medicine

Plant name	**Medicinal uses of plant**
Allium sativum	Reduction of cholesterol in the blood
Centella asiatica	Curing diarrhea and dysentery
Andrographis paniculata	Treating Fever and liver diseases
Coccinea indica	Diabetes management
Rauvolfia serpentina	Cure of insomnia, insanity and hypertension

1.3. Importance of medicinal plants in drug finding

Medicinal plants provide us with various types of therapeutic agents which used for treating different disease. These therapeutic agents are derived from plants and used directly as drug or as semi synthesized drug or synthesized drug.

In the past remedial was produced by concoctions, extracts or preparations prepared by herbalists who seem to always know the corrective treatment for most diseases. But wound healing was always very important for ancient people because of this reason "Sushruta Samhita" dedicates two whole chapters in wound healing and mentions more than 100 plants that can be used to heal wounds, as well as medicines needed to achieve a clean wound (Idris Singh, B. Healing and Singh, G., 1994, 37-41, Deshpande, Pathak and Dildo, 1970, p 260-303).

Various calculation showed that nearly 60% of anti-tumor, anti-infective, anti-microbial natural drugs are now in the market place or in the clinical trials (Yue-Zhong Shu, 1998). Maximum of them are still gotten from wild plants or cultivated plants, but many of them are not yet synthesized economically (Taux, 2001). Following table 1.1 provides some medicinal values containing plants which used for healing general disease.

Table 1.2: Some of the medicinal values containing plants used for healing general diseases

Scientific name	**Part used**	**Traditional disease**	**References**
Aloe vera L.	Leaf gel	Skin diseases like eczema, wounds, sun burns, microbial skin diseases, cosmetic application and hair loss.	Zari, S. T., & Zari, T.A. (2015)
Cleome viscosa L.	Leaves and seeds	Anthelmintic, carminative and diaphoretic.	Bobbarala, Katikala, Naidu, & Penumajji, 2009
Cinnamonum verum L.	Bark	Constipation, antifungal and antibacterial.	Barros, Healing Herbs and Medicinal Plants List, (2011-2016).

Table 1.2: Some of the medicinal values containing plants used for healing general diseases (continue)

Scientific name	Part used	Traditional disease	References
Melia azedarach	Leaf, flower and seed oil	astringent, antiviral antibiotic antidiabitic and insecticide	Bobbarala, Katikala, Naidu, & Penumajji, (2009)
Acacia farnesiana	Bark and roots	Stomachic, poultice, demulcent, and astringent	Bobbarala, Katikala, Naidu, & Penumajji, (2009)
Holarrhena antidysenterica Wall.	Bark	Used in the treatment of fever, dysentery, diarrhea, dropsy and intestinal worm infections	Chopra,R.N., Nayer, & Chopra,I.C. (1992).
Tinospora cordifolia	Stem	Pain killing and anti-inflammatory	Bobbarala, Katikala, Naidu, & Penumajji, (2009)

1.4. Drug as natural product obtained from medicinal plants

The herbal plants remained an essential resource of new compounds which are pharmacologically active, from these plants effective commercial drugs are derived either directly or secondarily. Approximately 25% of the drugs recommended in the world originate from herbal plants, around 121 plants of which active compounds are now in using. World Health Organization (WHO) declared 252 drugs which are registered in essential medicine list, around 11% of drugs are came from plant source and from natural originators a huge quantity of synthetic drugs obtained. Examples of that drugs which obtained from herbal plants they are Digoxin from *Digitalis* species, vincristine and vinblastine gotten from the *Catharanthus roseus*, *Atropa belladonna* provide atropine along with morphine and also codeine from *Papaver somniferum* (Yue-Zhong Shu, 1998). Recently, use of natural product as therapeutic agent increasing rapidly, (Goldfrank et al., 1982; Mentz and Schenkel, 1989). Liu et al. (2000) reported that about 50% of all marketed drugs were derived from natural products.

For the last ten years, medicinal products derived from plants such as Arteether, End peroxide sesquiterpene lactone and natural semi-synthetic products derived from Artemisinin used for the treatment of malaria the natural alkaloid Galantamine have been used in the treatment of Alzhemer's disease, natural product Nitisinone derived from Leptospermone is used in treatment of antityrosinaemia, Apomorphine a semi-synthetic composite which is resulting from morphine used in treatment of Parkinson's disease, for chronic obstructive pulmonary disease tiotropium a imitative atropine used which obtained from *Atropa belladonna,* from Cannabis and Capsaicin plants Dronabinol and Cannabidiol obtained are used as analgesics.(Veeresham C. 2012). Following table 1.2 provides some drugs which derived from natural plant source.

Table 1.3: Some drugs previously isolated from natural plant sources (Meshnick & Dobson, 2001, Serturner, 1805, Hartunf, 1954, Seader, 2005).

Drug	**Chemical class**	**Source**	**Medical use**	**Mechanism of action**
Penicillin	Penicillin	Fungus, *Penicillium niger*	Antibiotic	Inhibition of Peptidoglycan synthesis
Quinine	Alkaloid	Cinchona bark, *Cinchona officinalis*	Anti-malarial agent	Protein synthesis inhibitor
Morphine	Alkaloid	Dried latex of Opium poppy, *Papaver somniferum*	Potent Painkiller/Analgesic	Opioid agonist by binding to opiate receptors
Digoxin	Cardiac glycoside	*Digitalis purpurea*	Atrial fibrillation and Congestive heart failure	Na^+/K^+-ATPase pump inhibition.
Mevastatin[2]	Polyketide	Fungus, *Penicillum citrinum*	Cholesterol lowering drug, (Li, 2009, p. 71-96).	Inhibition of the HMG- coenzyme-A

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

Selected plant is an unrevealed plant after searching different journals and publications on *Aporosa wallichii* (Hook.f.) there's no sufficient amount of information was found, for this reason this plant is selected to identify various properties like anti-oxidant, cytotoxicity, anti-microbial, thrombolytic etc. So, this initiative to determine these properties of *Aporosa wallichii* (Hook.f.) is the target of this current project.

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

Aporosa wallichii (Hook.f.) is belongs to the family Euphorbiaceae also known as Euphorbias. This type of plant is found in the hilly areas of Asian subcontinent. In Bangladesh they usually available in the hilly areas like Chittagong, Sylhet.

Aporosa wallichii (Hook.f.) is one of the dominant plant species of Kamalachari Natural Forest of Chittagong, Bangladesh. Moreover, this plant is also available in the hilly areas of Moulovibazar, Sylhet. (Hossain, Hossain, Alam & Uddin, 2015).

Euphorbiaceae, spurge family of flowering plants, it containing about 7,800 species distributed in approximately 300 genera and 5 subfamilies. Many members are important sources of food along with medicines. They also good source of waxes and oils and also hazardous to their toxic fruits, leaves or fresh fruit; or attractive for their colored brothers. This family grows mostly are in tropical and subtropical regions. The family is made up of annual and perennial shrubs or woody trees and climbers. There are number of plants belongs to this family which have great financial worth, for instance species include *Ricinus communis (*castor plant), *Manihot esculenta* (manioc) and *Jatropha curcas* (Barbados).

1.5.2. Morphology of plant *Aporosa wallichii* (Hook.f.)

Trees 5 to 9 m in height; young pubescent shoots; of glabrous twigs. closely oblong elliptic lance late leaves, acute, dull rounded to the base, together grenadine and sometimes notches along the edges, or seldom acuminate dull at the top, 8-20 x 2-5 (- 6) cm, often finely graceful, glabrous; lateral nerves 5-10 pairs; 5 to 25 mm long petioles; tightly ovate, 5-7 x 2-3 mm, hardwood. Male inflorescence from 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, about 1 x 0.5 mm; stamens 2 or 3, long 1.5 to 2 mm. Inflorescence

feminine (female); triangular bracts, 0.8 to 1.2 mm; Sepals 3 or 4, triangular, 1.5-2 x 0.6-1.2 mm; o ovoid shaped vial c.a. 2 x 1.5 mm ovary, 2 unicellular, glabrous; plug 1.5 - 2 mm in length; stigma 2, approximately 1 mm wide. Oblong ovoid, 10-15 x 7-8 mm, thin wall, 2 unicellular, glabrous, brown to black dry; ellipsoid seeds, 7 -10 x 3-5 mm, black. (Kanjilal et al., 1940).

Origin: Dry evergreen or deciduous forests of Meghalaya and Tripura in India. Bangladesh, Myanmar and Thailand. (Kanjilal et al., 1940).

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

1.5.3. Plant taxonomy (*Aporosa wallichii)*

Table 1.4: Taxonomy hierarchy of *Aporosa wallichii*

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

1.5.4. Pharmacological properties of other genera and species

Aporosa wallichii is the plant of a family which has much medicinal value. Euphorbiaceae family provided us with many medicinal properties they specially used for disease like jaundice, asthma, anthelmintic, bronchitis, cancer, diarrhea, eczema, dysentery, headache, leprosy, inflammation, kidney disease, skin diseases etc. (Rahman & Akter 2012-2013). Here are some major genera and species in Euphorbiaceae given below:

Manihot esculenta (Cassava), *Hippomane mancinella* (manchineel), *Hevea brasiliensis* (rubber tree), *Codiaeum variegatum* (croton), purging croton (*Croton tiglium*), *Euphorbia pulcherrima*

(poinsettia), *Hura crepitans (*sandbox tree), and *Euphorbia tithymaloides* (slipper spurge). (Encyclopedia Britannica, 2015)

1.5.5. Related publication on *Aporosa wallichii*

Aporosa wallichii is still an unpublished plant because no studies have yet been carried out on its chemical properties and pharmacological uses. Therefore, many valuable properties and uses of medicinal plants still need to be identified.

1.6. Project justification / rationale

Literature review of the selected plant called *Aporosa wallichii* (Hook.f.), it is noted that no significant study has been conducted on *Aporosa wallichii* (Hook.f.). Though, earlier studies in several species of this kind have reported powerful antimicrobial, anti-tumor, anti-diarrheal, anthelmintic, antioxidant, anti-inflammations, kidney disease, skin diseases and cytotoxic activities. So, the main purpose of this study is to find out the different pharmacological properties from the raw leaf extract of the plant. Research will also seek to examine the unknown properties of the selected plant in the betterment of medicine world.

1.7. Aim of the project

The ultimate aim of the project is to examine and discover unknown biological potential of the selected plant, *Aporosa wallichii* (Hook.f.) (Family: Euphorbiaceae)

1.8. Objective of the project

This project protocol contain following steps which done with methanol extract of the *Aporosa wallichii* leaves.

1) Evaluation of the antioxidant property of the methanol extract of the plant leaves by applying in-vitro DPPH free radical scavenging method and determination of the total phenolic content of plant.

2) Evaluation and screening of the plant’s antimicrobial activity.

3) Evaluation of cytotoxic activity.

4) Determination of thrombolytic activity.

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

Most illnesses / disorders are mainly related to free radical-induced oxidative stress. Antioxidant substances have capability to affect the oxidation process by involve in reaction with free radicals, catalytic metals, chelating agents, and also act as an oxygen absorbers from any source (Buyukokuroglu et al., 2001). The effects of antioxidants on plant-derived compounds are increasing in interest, which may be relevant in terms of nutritional impact and their role in health care and various disease (Steinmetz and Potter, 1996; Couladis et al., 2003). Huge number of isolation and antioxidant-derived plant test reports have been defined over the previous decade. (Velioglu et al., 1998; Pietta et al., 1998). Properties medicinal plants have been examined in recent medicine science expansions around the world only because of their effective antioxidant activity, minimum amount of side effects and also financial viability (Auudy et al., 2003).

Another synthetic antioxidant such as propyl gallate (PG), tert-butyl-1-hydroxytoluene (BHT), hydroxystyrene butylate (BHA) and tertiary butyl-hydroquinone used as added ingredient to increase potent with the known effects not only toxic and also carcinogenic on human (Ito et al., 1986; Wichi, 1988). Therefore, in recent years natural antioxidant (plant derived) demand increases greatly (Jayaprakasha J. R., 2000). Plant derived polyphenols have been studied largely because of the possibility that they may be the basis for the protective effect of fruit and vegetable consumption against cancer and other chronic diseases (Elena et al., 2006).

Finally, the purpose of this experiments was to discover and evaluate *Aporosa wallichii* leaf extract as potential fresh sources of regular antioxidants and phenolic compounds.

Antioxidant property can be evaluate by

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

1.9.1. Evaluation of phenolic content

Phenolic compounds of the plants usually showed their antioxidant property by redox reactions, which plays very crucial part in the absorption and neutralization of free radicals, triplet oxygen and decomposed peroxides (Osawa, 1994). Antioxidant effect showed up mainly because of phenolic components such as flavonoids, phenolic diterpenes and phenolic acids (Shahidi, Janitha and Wanasundara, 1992). Many chemicals obtained from plants own substantial antioxidant property which partially linked with minor rate of mortality in diverse human populations (Velioglu et al., 1998). In the stage of alkaline the phenols completely ionize. Folin-Ciocalteu chemical easily oxidizes the phenols when these chemicals used in this ionic phenolic solution, the reagent easily oxidizes the phenols. When the oxidation procedure completed in the solution yellow color of Folin-Ciocalteu chemical turned into dark blue. This color change strength is measured in a 760 nm spectrophotometer. Value of the absorbance indicate the total phenolic content of the substance. (Harbertson and Spayd, 2006).

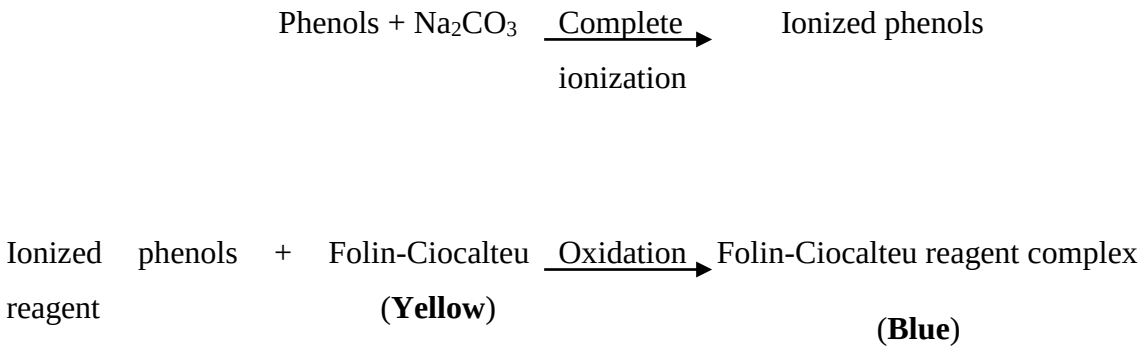
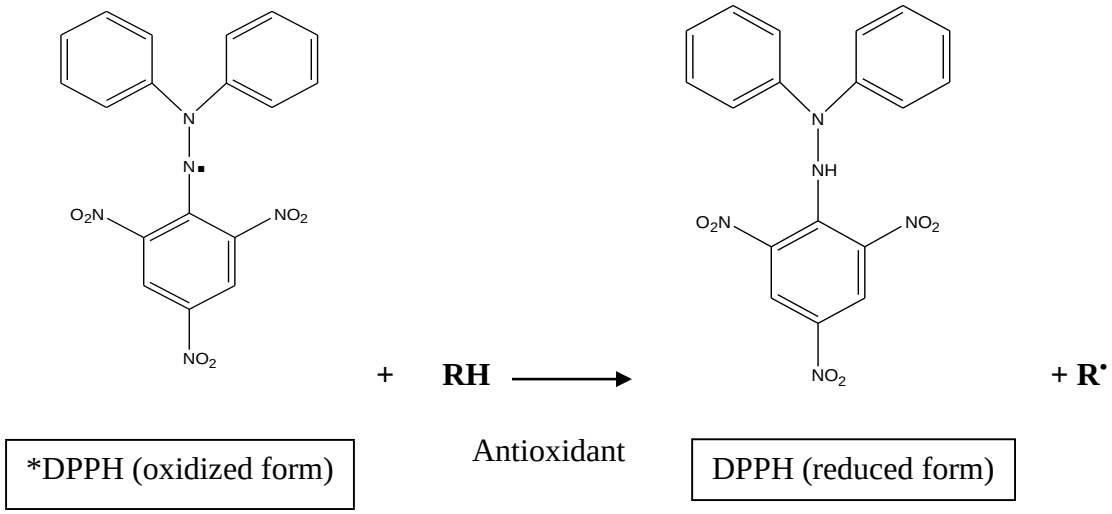


Figure 1.1: Flow diagram of phenolic content

1.9.2. Evaluation of antioxidant property by DPPH examine

Antioxidant capability (free radical scavenging activities) of methanol plant extracts on stable 1,1-diphenyl-2-picrylhydrazyl radicals were assessed (Brand-Williams et al., 1995). At different concentration 2 mL of a methanol solution of the plant extract were mixed with 3 mL of DPPH solution (20µg/mL). Therefore, antioxidant property was analyzed by bleaching the DPPH colored solution with the methanol plant extract note the similarities with ascorbic acid (ASA) by UV spectrophotometer.



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* leaves extract

Cytotoxicity is the term refers to being toxicity or poison for the cells. When cells come contact with any cytotoxic substance results showed up in various ways firstly, they lose plasma membrane strength and die rapidly due to cell destruction this situation called necrosis, may cease to grow and divide; or can activate a genetically controlled cell death program, term called apoptosis (Cytotoxicity, 2016).

1.10.1. Brine shrimp lethality assay

Bioactive substances are always poisonous and toxic for the living body at higher doses and justify the claim that "pharmacology is simply the highest dose toxicology and simply lower dose pharmacology". The brine shrimp test (McLaughlin, 1998) is a quick and complete biological test for the bioactive substances of synthetic, semi synthetic and natural origin. This method allow the identification of bioactivity of natural product, fractions of substances as well as pure substances. In vivo lethality test in a single animal technical organism (like shrimp nauplii) always used for monitoring and screening in the detection of new bioactive natural products.

However, biological test specifies cytotoxicity and an extensive series of pharmacological or biological properties such as antimicrobials, antivirals, pesticides and antibodies, etc. of compounds (Meyer, 1982; McLaughlin, 1998). The biological lumbar dosage technique for salmon shrimp is superior to other cytotoxicity testing procedures because it is fast, inexpensive and does not require special equipment or aseptic techniques. It uses a large number of organisms for statistical validation and a relatively small sample size. Also, unlike other methods, it does not require animal serum.

The salted shrimp offspring are hatched in replicated seawater to grow nauplii. By adding the calculated amount of dimethylsulfoxide (DMSO), the sample is prepared in desired concentration by dilution. The naupliis are counted with visual examination and placed them in vials which contains simulated seawater around 5 mL. Subsequently, various concentration of samples are added to the tubes with micropipette which were previously labeled. These tubes are then left in a room temperature for 24 hours. Finally, survivors are counted after 24 hours. (Meyer et al.,1982)

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

Since ancient times, plant preparation has been used to treat various diseases. Leaves and / or branches, stems, barks and ground areas are often used for traditional medicines. Herbal products are often considered safe because they are "natural" (Gesler, 1992). (CVST) Cerebral venous sinus thrombosis is a serious condition associated with serious illness and death (Watson et al.,

2002). Heparin, an anticoagulant antidepressant, is the first line of CVST treatment due to its efficacy, safety and possibilities (Biousse and Newman, 2004). Thrombolytic medication such as urokinase, clopidogrel, streptokinase, etc. They provide an important support in the controlling of patients with CVST (Baruah, 2006). Hence, precise goal of this experiment was to introduce the thrombolytic efficacy of methanol extract of *Aporosa wallichii* leaves.

Thrombolytic drugs 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 used to treat myocardial infections. Among them, streptokinase is widely used. The previous patient uses blood products, herbal medicines or supplementary foods or people who are at risk of bleeding; Thrombolytic agent is not recommended for them. These situations may include (Beckerman, 2015): high blood pressure, active bleeding or severe bleeding and cerebral hemorrhage of cerebral bleeding.

1.12. Antimicrobial property evaluation

Infectious diseases consequence death of thousands of people across the world especially they are leading cause of death in tropical regions. However, it is very surprising to see these numbers in developing countries, but mortality rates from infectious diseases actually increase in advanced countries like United States. Infectious disease death rate, occupying the fifth place in 1981, became the third in 1992, increase about 58%. Infectious diseases are the top reason of death in the United States around 8% (Pinner et al., 1996). It is an alarming situation, since we once thought of eliminating infectious diseases by the end of the millennium. Higher respiratory infections and HIV / AIDS are the result of infectious diseases increased. Other contributing factors are increased resistance to antibiotics in infections acquired by our nosocomial and communal infections. In addition, the most affected increases of infectious diseases observed in the age group of 25-44 (Pinner et al., 1996). For this reason medical and health communities need to renewed interest in infectious diseases as well as new treatment and prevention strategies due to this negative health trends. Hence, the last point of solution would be the development of new antibiotic (Fauci, 1998).

Antimicrobial screening is the first step in the search for antimicrobial drugs to determine the susceptibility of different fungi and bacteria to each agent. This assay measures the ability of

each test sample to inhibit the in vitro growth of fungi and bacteria. This capacity can be estimated by one of the following three methods (Ayafor, 1972). Disc diffusion method, Serial dilution method and Bio-autographic method.

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

The above techniques is a comprehensive study of the vitro test for preliminary diagnostic tests of agents that may be involved in the antimicrobial movement. However, bacteriostatic and bacterial variations can be made 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 (averaging 6 mm) including sampling to determine the known value are placed in a neutral medium called germs. Antibiotics (bleaching) and empty layers are used for poor control. These instructions are maintained 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 may prevent microbial growth in the media by killing microbes nearby to the discs and thus provide a clear and definite defined space in the exchange zone. Finally, the antimicrobial property of the test sample is then identified by assessing the diameter in the scale of millimeter (Barry, 1976, Bayer et al., 1966).

Chapter Two

Methodology

2. Methodology

2.1. Plant collection

Aporosa wallichii has been selected as a plant for this research as no previous study on its biological properties has been carried out. Thorough bibliographic study of this plant and its availability, the plant was chosen for analysis. The leaf part of *Aporosa wallichii* plant was collected in May 2017 from Moulovibazar district of Sylhet division, Bangladesh.

Table 2.1: Research of *Aporosa wallichii*

Name of the Plant	Scientific Name	Family	Part
Kukhra (In Bangladesh)	*Aporosa wallichii*	Euphorbiaceae	Leaves

Figure 2.1: collection of *Aporosa wallichii* leaves

2.2 Verification of plant

After collecting leaves, it was sent to the National Herbarium Bangladesh (NHB), Mirpur, Dhaka for verification. A week later, its token was collected and the plant identified accession number for our given specimen is (DACB- 44996) and it is authenticated by National Herbarium, Mirpur, Dhaka, Bangladesh

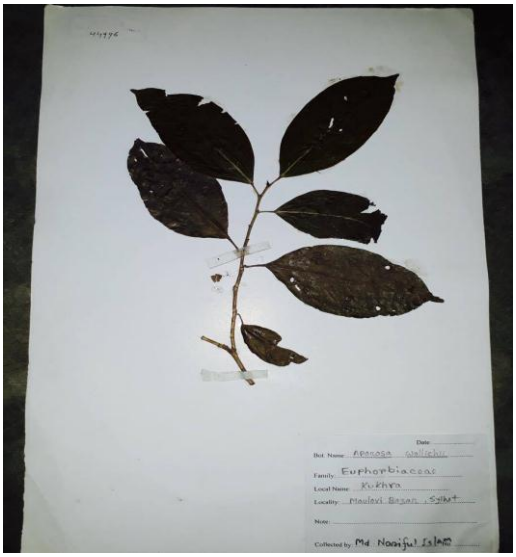


Figure 2.2: *Aporosa wallichii* plant's accession number collected from the National Herbarium of Bangladesh, Mirpur, Dhaka.

2.3. Process of extraction

Extraction of the medicinal plant involved various steps:

The entire extraction procedure can be divided into two parts they are firstly, Preparation and drying of plant material (2 steps), secondly, Extraction process (5 steps).

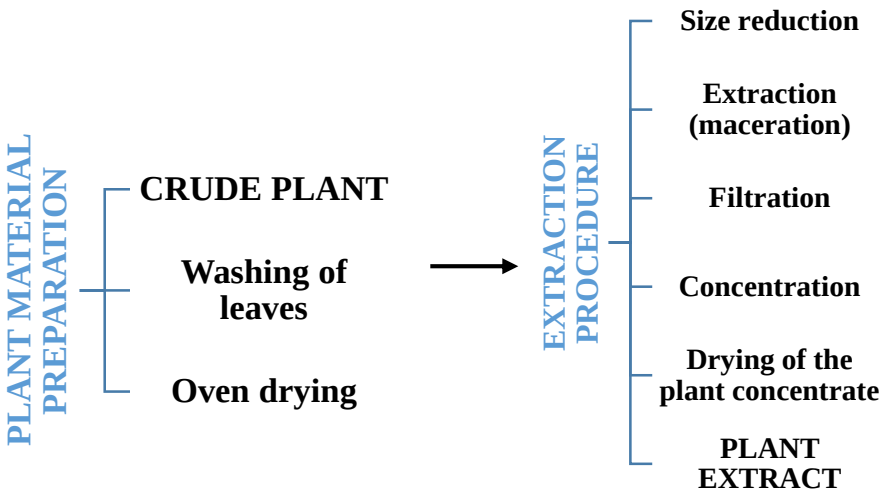


Figure 2.3: Flowchart showing a step-by-step technique for the plant's part extraction

2.3.1. Preparation of plant material for crude extract

The leaves were ripped from the stem of the plant and washed with clean water to remove the plant scrap and dust particles. The clean leaf was then allowed to dry under the sun for a day after which the leaves were dried for 1 hour at 30-40°C in a hot air oven. Dry leaves were then arranged for the next step.

2.3.2. Plant Extraction procedure

- **Size reduction and weighing**

The dry and crusty leaves were then grinded with coarse dust using a high capacity grinding machine. Approximately, 900g of powder collected and this was followed by packing in air-tight plastic containers with the necessary label that was finally left in a cool, dry, and dark place until further investigation, essential steps were taken to avoid cross-contamination.

Figure 2.4: Grinded powder of the *Aporosa wallichii* leaves.

➢ Extraction of plant by solvent

Based 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 aim of this study, the extraction maceration process was used for extraction of Plant materials and methanol as organic solvent. Beaker containing plant material of *Aporosa wallichii* powder was soaked in 2.5L of methanol for a period of 2 days at normal ambient temperature (22-25°C) with occasional stirring.

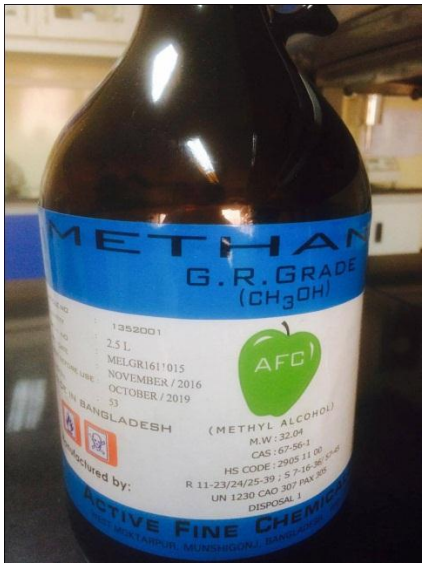


Figure 2.5: Solvent for extraction (Methanol 2.5L)

➢ Filtration

After two days of maceration, the contents of the beaker were filtered using the cloth following by using cotton and Whatman filter (pore size: 110 mm).

➢ Concentration

The collected filtrate of plant extraction was concentrated using a rotary evaporator (Heidolph) at 100 rpm at 30°C, until the methanol concentrate extract is produced. Then thick concentrated mixture collected in a petri-dish.

Figure 2.6: Concentrate the filtrate of the *Aporosa wallichii* plant using an evaporator rotator

- **Drying**

Finally, the petri-dish was placed under laminar airflow (LAF) to vaporize the solvent of the extract. LAF was used as a preventive measure, measured to avoid any possibility of microbial growth in the extract while drying. After the successful drying of the extract kept in the petri dish.

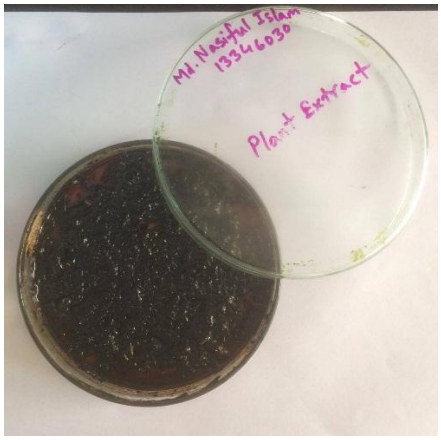


Figure 2.7: Dried methanol extract of *Aporosa wallichii* leaves in petri-dish.

Table 2.2: The weight of *Aporosa wallichii* methanol leaf extract obtained as a result of complete extraction procedure

Initial weight/g (Petri-dish)	63.9
Final weight/g (Petridish + extract)	86.3
Weight of extract/g	22.4

So, Total weight of plant extract after methanol extraction was 22.4g

2.4. In-vitro antioxidant property analysis

2.4.1. Evaluation of free radical scavenging DPPH assay

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

- **Mechanism of DPPH test**

In order to discover or estimate the antioxidant activity by DPPH, 1 mL of extracted methanol extracted from different amounts of concentration was obtained using a 3 mL methanol DPPH solution. BHT and ascorbic acid usually as standard is generally used to concentrate between 1 and 100 mg / mL. Blank sample were also prepared for the study. When sample and the black solution are prepared, it was kept in the dark for 30 minutes. Therefore, the antioxidant activity of the extract is measured by spectrophotometric analysis under UV absorbance at 517 nm wavelength. The DPPH test is mainly used to detect a decrease in free DPPH with radicals. As electron produces DPPH free radicals, UV spectrophotometry gives a high absorbance at 517 nm of nickel. After the reaction of the stable free radical DPPH, the antioxidant leads to the production of hydrogen and to the decrease of the DPPHH, which leads to a reduction in the absorbance with respect to the DPPH. Decolorization results by the fact that DPP-H produces a yellow color with respect to the total number of electrons. Higher reducing ability shows when

decolorization gradually increases. DPPH is considered the best way to investigate research to determine the strength of new drugs and potentials (Brand-Williams et al., 1995).

➢ Materials and reagents

List of the materials and reagents given below

Table 2.3: 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, to prepare standard (positive control) ascorbic acid (ASA) was used. Calculated amount of ascorbic acid were dissolved in the methanol solvent to acquire a solution which concentration was 500 µg/mL. After that to get different concentration ranging from 500.0 to 0.977 µg /mL serial dilution was done.

Table 2.4 Amount used in preparation of control

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

➢ Test sample preparation for evaluation

To prepare test sample, 2mg of *Aporosa wallichii* leaves extract was placed in a cleaned test tube then 4mL of methanol added to the test tube to get concentration 500 µg/mL. Now by serial dilution method desired concentration ranging from 500µg/mL to 0.977µg /mL achieved and kept them dry place with mark. Calculated amount of methanol extract was 2mg.

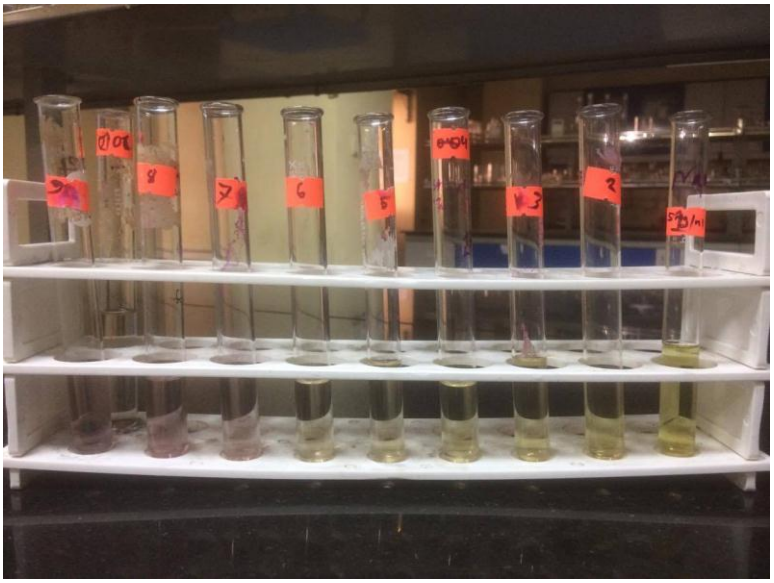


Figure 2.8: Serial dilution of plant extract in different test tube.

➢ DPPH solution preparation for evaluation

In order to prepare the DPPH solution, 1 mg of DPPH powder were correctly measured and dissolved in 50 mL of methanol to collect 20µg/mL concentration. Then the solution was stored in a dark box which is covered by aluminum foil paper.

Table 2.5: Amount used in DPPH solution preparation

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

➢ Assay of DPPH free radical scavenging activity

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

➢ Calculation

Inhibition in percentage *(*I%) of Free radical DPPH was calculated as follows:

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

Where, A_{blank} stands for absorbance of the control reaction

Afterwards, 50% inhibition (IC_{50}) was provided by methanol plant extract concentration and value obtained from the graph where plotted inhibition percentage (I%) against the concentration of plant extract (µg/mL).

2.4.2 Evaluation of total phenolic content

According to the method as described by Skerget *et al*.,(2005) which involved Folin-Ciocalteu chemical as oxidative and Gallic acid as standard the total phenolic content of leaves extractives of *Aporosa wallichii* was measured easily.

➢ Materials and reagents

Table 2.6: Materials and reagents for phenolic content measurement

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

➢ Test sample preparation for evaluation

2 mg of the plant extract was taken in a test tube and dissolved in the distilled water which give a sample concentration of 2 mg/mL. Sample along with its concentration given below

➢ Standard solution preparation for gallic acid curve

Gallic acid is usually used worldwide as a standard in total phenolic content test. Various Gallic acid solution were prepared with 100µg / mL to 0µg / mL. Concentration by using 2.5 mL of Folin-Ciocalteu chemical (10 times diluted with water) and 2mL of (7.5% w / v) Na2CO3 solution were 0.5 mL of Gallic acid added. The solution mixture was placed in a dark place at

room temperature for 20 minutes. After that by using UV spectrophotometer mixture was measured at 760 nm and absorbance was taken. Then the absorbance was plotted against the concentration as a result a linear relationship was obtained which was used get sample result.

- **Analysis of total phenolic content**

To obtain mixture, 0.5mL of plant extract (2 mg / mL), 2.5 mL of Folin-Ciocalteu chemical (10 times mixed with water) and 2.0 mL of (7.5% w / v) Na2CO3 were added. Then the mixture is kept in dark place at room temperature for 20 minutes. After specific time passed at 760 nm absorbance was estimated with UV spectrophotometer along with by using standard curve of Gallic acid, the total sample was estimated. Sample's phenolic content was stated as mg of GAE (Gallic acid equivalent) / g of extract.

2.5. *In-vitro* cytotoxicity property analysis

2.5.1. Experimental Procedure Brine shrimp lethality assay

- **Materials for test**

Table 2.7: List of materials required for Brine shrimp lethality assay

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	Plant extract

➢ Preparation of seawater for test

To prepare seawater solution, 38g of salt (pure NaCl) was weighted accurately then it dissolved in distilled water (1L) and then filtered the water to acquire clear solution.

➢ Hatching of brine shrimps eggs for test

For examination, brine shrimp (*Artemia* salina) eggs collected from stores was used for examination. A small tank taken and filled with seawater then brine shrimp eggs added into it. To get mature nauplii two days provided with continuous supply of oxygen throughout the time of the hatch. Lamp light attracts shrimps through the perforated dam. Pasteur pipette used to collect 10±2 living shrimps which added into each test tube which containing 5mL of seawater.

➢ Preparation of test solution for experiment

Test sample was taken in a test tube and dissolved with dimethyl sulfoxide (DMSO). After that by serial dilution different concentration ranging from 400µg/mL to 0.78125µg/mL achieved. First 50µl of Sample which concentration was 400 µg/mL placed into test tube holding 5mL of DMSO along with 10±2 nauplii. After that fresh 50µl DMSO was added to test tube to dilute the solution by this method different concentration obtained.

Table 2.8: Plant sample with different concentration after serial dilution

Test tube no.	**Concentration (µg/mL)**
1	400.0
2	200 .0
3	100 .0
4	50 .0
5	25 .0
6	12.5
7	6.25
8	3.125
9	1.5625
10	0.78125

➢ Preparation of control group for experiment

In cytotoxicity studies to approve the analysis process and ensure that the results achieved were equivalent to the performance of the test agent and the possible effects of other conceivable stoppages control group is very essential. Usually 2 types of control groups are practice, they are- positive and negative control.

1) Preparation of positive control

In cytotoxicity experiment positive control is widely known as a cytotoxic compound, which help in test compared to the result of positive control. Here, vincristine sulfate a cytotoxic compound was used as standard (positive control). The dosage of vincristine sulfate was dissolved in DMSO to obtain the first dose of 20µg/mL at that moment by serial dilution verity concentration of standard solution obtained such as 10µg/mL, 5µg/mL, 2.5µg/mL, 1.25µg/mL, 0.625µg/mL, 0.3125µg/mL, 0.15625µg/mL, 0.078125µg/mL and 0.0390µg/mL. Finally, standard (positive control) added in the test tubes holding 5mL seawater along with 10±2 nauplii.

2) Preparation of negative control

In order to prepare negative control, 3 test tube were taken and 100 µl of DMSO was added to the each of the test tube which contain 5mL of seawater along with 10±2 nauplii. If the death rate of nauplii is rapid, which indicates the test is unacceptable and the nauplii died due to some unwanted reason.

➢ Nauplii counting

Result obtained after 24 hours, by the help of a magnifying glass and the number of survivors was counted accurately in the each of the test tube. From each dilution, (%) percentage of mortality was calculated by linear regression of IBM-PC program which is used to evaluate the concentration-mortality data. Furthermore, the concentration vs. mortality relationship of plant extract is expressed by (LC_{50}) value which means median lethal concentration value. So, the concentration of the chemical is responsible for the death in half of the test nauplii after a specific period of time.

2.6 *In-vitro* thrombolytic property analysis

Thrombolytic property can be evaluated by an easy method where plant extract as sample, Clopidogrel (anti-platelet agent) as positive standard and water as negative standard.

➢ Materials and reagents

Table 2.9: Used materials in thrombolytic test

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

➢ Test sample preparation

For preparation of test sample, a test tube was taken which containing distilled water (10mL) then 100mg of plant extract was suspended in it, after that test tube was kept in dry, dark place for overnight and as a result desired soluble supernatant was transferred in solution and then solution filtered properly.

➢ Standard solution preparation

Clopidogrel an anti-platelet agent used as a standard for this experiment. 100mg of clopidogrel dissolved in distilled water (10mL) and mixed appropriately by this time this suspension stored as a stock standard solution from which 500µl solution was applied in thrombolytic activity test.

➢ Blood sample preparation

5 healthy volunteers (n=5) have no history of anticoagulant therapy selected from them blood sample were collected by ensuring aseptic condition. After collecting blood 1mL of blood was shifted into pre-weighed micro centrifuge tubes. After that micro centrifuge tubes kept to form clots.

➢ Thrombolytic property test process

At beginning of the test, 5mL of fresh blood was collected from each volunteer worker. Blood samples were taken from five different pre-weighed sterile microbes and allowed to incubate at 37°C for 45minutes. When the clot is formed, the upper fluid was entirely discharged from all micro-tube lines. The weight of the clot was determined by the weight of the tube taken before the clot is formed. In this case, 100µl of clopidogrel used as positive control and 100µl of water (distilled) were used as a non-thrombolytic negative control with 100µl of each sample was added from each test tube. For observation of clot lysis, micro tubes were incubated at 37°C for 90minutes. Afterward when the incubation was completed, the liquid was removed which was released from clot and again weighted the tubes to observe the weight difference after the clot distraction. Finally, percentage (%) of clot lysis as shown underneath:

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

2.7 Antimicrobial property analysis

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

➢ Apparatus and reagents used for antimicrobial analysis

Table 2.10: List of apparatus used

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 tubes
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 list of the organism given below:

Table 2.11: List of micro-organism used in antimicrobial analysis

Gram positive Bacteria	Gram negative Bacteria
Streptococcus pyogene	*Salmonella typhi*
Staphylococcus aureus	*Escherichia coli*
Bacillus subtilis	*Proteus vulgaris*
	Vibrio cholera
	Shigella floxenere

➢ Sterilization procedure of test

Before conducting test all apparatus include beakers, conical flasks, patri-dishes, cottons and forceps sterilized and kept in aseptic place. This sterilization done to avoid any kinds of cross contamination or microbial contamination during the process. To maintain control environment all work done in under Laminar Air flow Hood, before starting experiment UV light was turned on for 1 hour in the Laminar Air flow Hood. In addition, micropipette tips, cotton, forceps, blank discs were also treated by UV light. However, by autoclave machine petri-dishes and other apparatus were sterilized at a temperature of 121ºC and a pressure of 15-lbs/sq. inch for 1 hour. After experiment all the apparatus sterilized and used bacterial strains were destroyed to avoid environmental contamination.

➢ Procedure of antimicrobial test

At first, for culture preparation 2.5g of nutrient broth dissolved in 100mL distilled water. Then 8 conical flask were taken each of the conical flask contains 10mL of broth mixture there 8 different bacterial strains added into it and kept these conical flasks in shaking incubator at

temperature 37 ºC for 24 hours. After 24 hour these conical flask were removed from shaking incubator and kept in controlled environment. After that, agar medium prepared by using 7.6g of M.H. Agar which dissolved in 200mL of distilled water. Immediately after preparation M.H. Agar placed into patri-dishes and kept these patri-dishes for cool down at room temperature. Meanwhile, test sample of plant extract concentration ranging from 500mg/mL to 15.625mg/mL were prepared and let them socked into the filter paper discs. When M.H. Agar in patri-dishes became solid then bacterial strains speared into it by using cotton bars. Streptomycin used as standard discs and test sample plant extract discs placed in the patri-dishes. Then these patri-dishes placed in the incubator at 37ºC for 24 hours to provide optimum environment for bacterial growth. Again after 24hours later patri-dishes containing different bacterial strains along with standard and test sample discs collected and observed inhibition zone produced by standard and test sample discs.

- **Determination of inhibition zone for test**

Antimicrobial property of the agents is estimated by the capability 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.

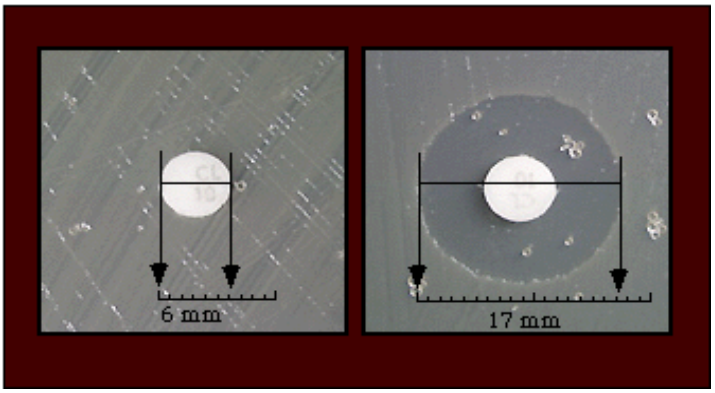


Figure 2.9: Determination of the clear inhibition zone

Chapter Three

Observation and Results

3. Observation and results of all the experiments

3.1. Antioxidant property analysis

3.1.1. Evaluation of DPPH free radical scavenging assay of *Aporosa wallichii*

Table 3.1: 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		

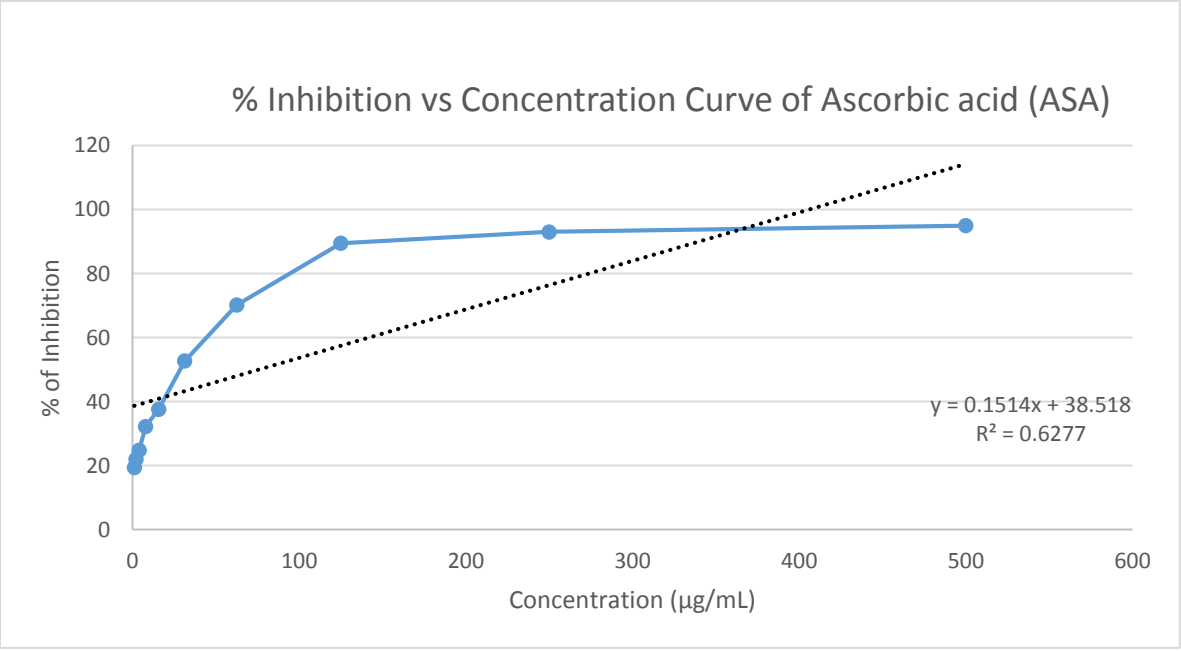


Figure 3.1: % Inhibition vs. Concentration curve of ASA

Table 3.2: IC_{50} value (µg/mL) of Methanol plant extract of *Aporosa wallichii*

Conc. (µg/mL)	**Absorbance of Methanol plant extract**	**(%) of inhibition**	**IC_{50} µg/mL**
500	0.058	90.59968	58.725
250	0.077	87.52026	
125	0.091	85.25122	
62.5	0.113	81.68558	
31.25	0.274	55.59157	
15.625	0.314	49.10859	
7.813	0.368	40.35656	
3.906	0.418	32.25284	
1.953	0.465	24.6353	
0.977	0.574	6.96921	
Blank	0.617		

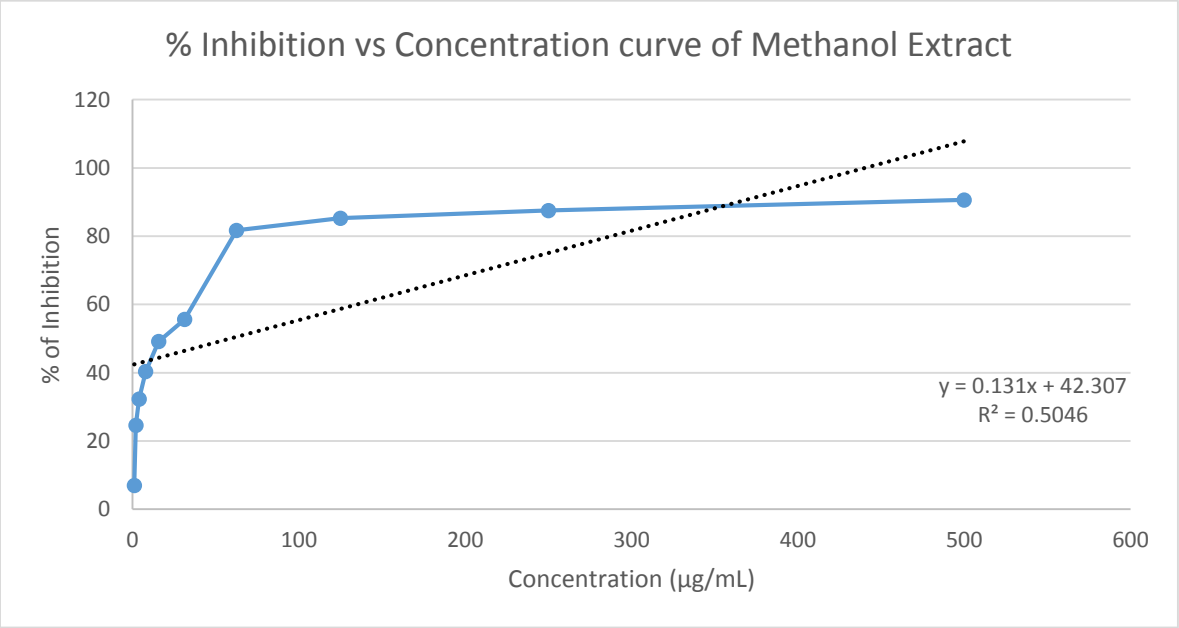


Figure 3.2: % Inhibition vs. Concentration curve of Methanol extract of *A. wallichii*

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 IC50 µg/mL value of methanol extract of *Aporosa wallichii* was lower than ascorbic acid which means 58.725 ug/mL is required to achieve the same inhibitory effect for ascorbic acid.

3.1.2. Evaluation of total phenolic content

Table 3.3: 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		

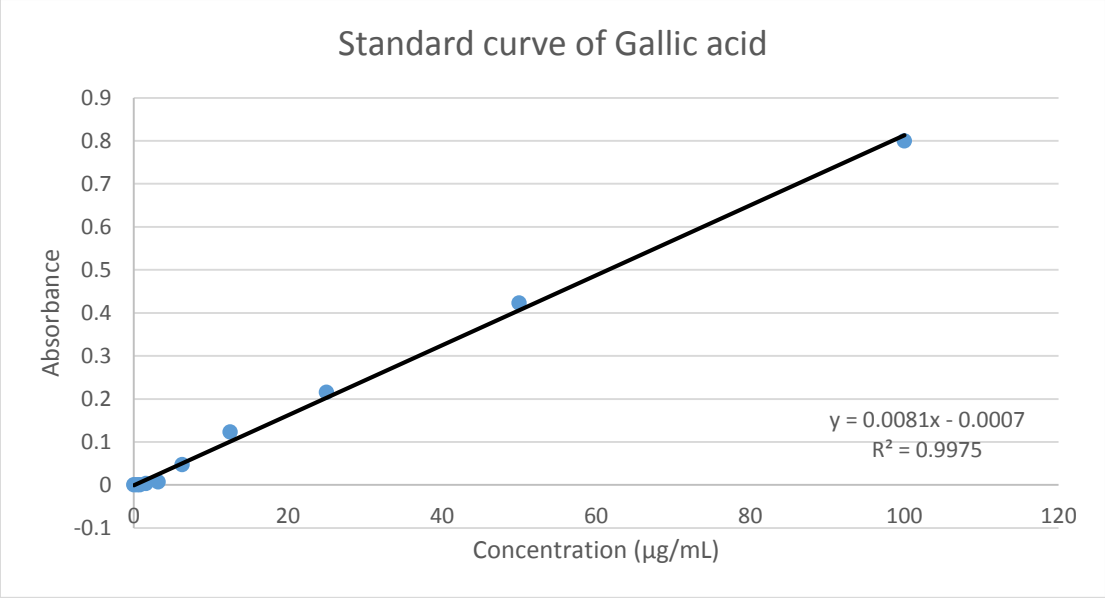


Figure 3.3: Gallic acid's standard curve for total phenolic content test.

Figure 3.4: Dark blue color appeared indicated presence of phenolic content

Table: 3.4: Result of total phenolic content of test sample

Sample code	**Name of extract**	**Plant part**	**Absorbance of methanol plant extract**	**Total phenolic content (mg of GAE / gm of extract)**
ME	Methanol extract	Leaves of *Aporosa wallichii*	2.502	308.97

So, Total phenolic content was obtained 308.97 (mg of GAE / gm of extract) of the methanol extract of Aporosa wallichii leaves.

3.2 *In-vitro* Cytotoxicity property analysis

3.2.1. Evaluation of Brine shrimp lethality assay

Table 3.5: Positive control (vincristine sulphate) effect on shrimp nauplii

Concentration (µg/mL)	Log Concentration	Nauplii taken	Nauplii Dead	Nauplii alive	% of Mortality	LC_{50} (µg/mL)
0.039	-1.408	10	2	8	20	2.0203
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	
2.5	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	

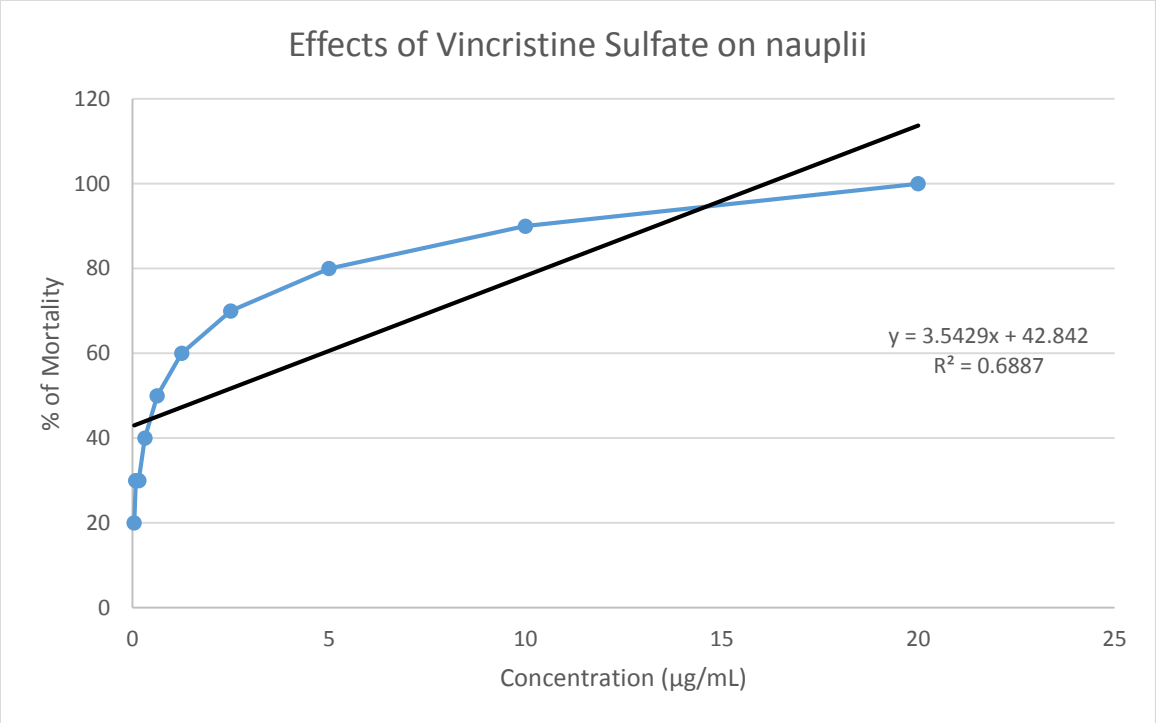


Figure 3.5: Percentage (%) mortality and predicted regression line of vincristine sulfate.

Table 3.6: Effects of the methanol extract of A. wallichii on nauplii

Concentration (µg/mL)	**Log Concentration**	**Nauplii taken**	**Nauplii Dead**	**Nauplii alive**	**% of Mortality**	**LC_{50} (µg/mL)**
0.781	-0.107	10	2	8	20	26.751
1.562	0.194	10	2	8	20	
3.125	0.495	10	3	7	30	
6.25	0.796	10	5	5	50	
12.5	1.097	10	6	4	60	
25	1.398	10	7	3	70	
50	1.699	10	7	3	70	
100	2	10	8	2	80	
200	2.301	10	9	1	90	
400	2.602	10	10	0	100	

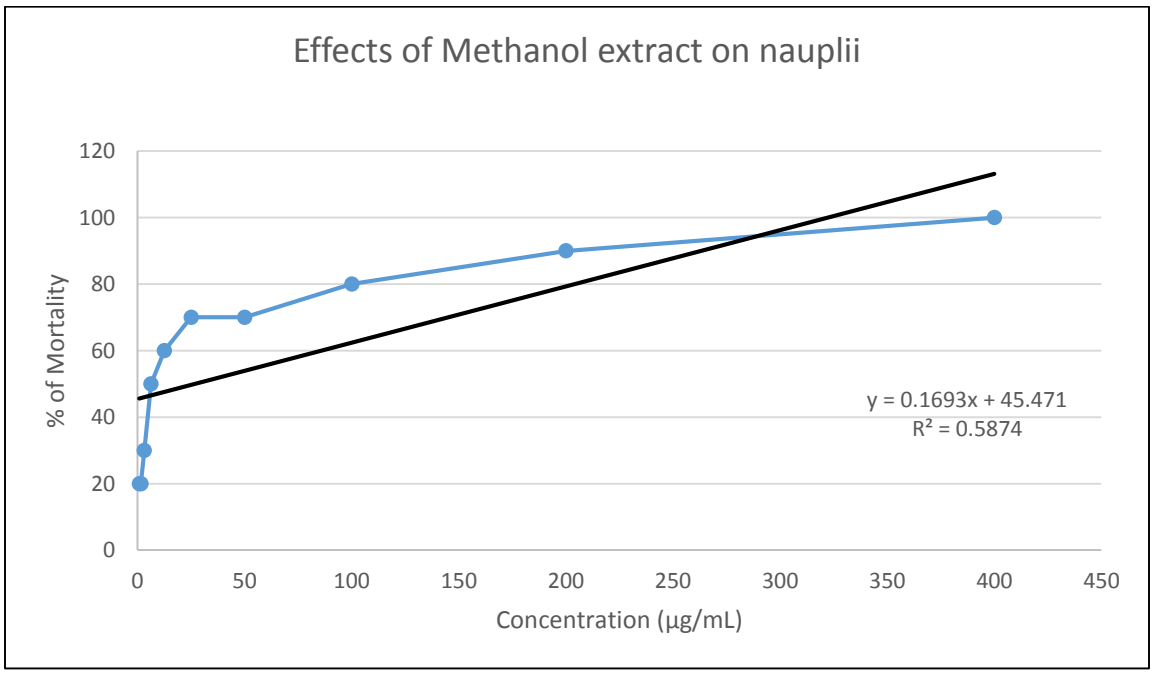


Figure 3.6: Percentage (%) mortality and predicted regression line of Methanol extract of *A. wallichii*

Explanation: From the table (3.5 and 3.6) LC50-26.751 µg/mL obtained for methanol extract and LC50-2.0203 µg/mL obtained for vincristine Sulfate. Which means methanol extract of *A. wallichii* require higher concentration to provide cytotoxicity effects compare to vincristine Sulfate.

3.3. Thrombolytic property analysis

Table 3.7: Evaluation and result of Thrombolytic Activity

Name of Samples	**W1**	**W2**	**W3**	**W4**	**W5**	**% of clot lysis**
Methanol extract of *A. wallichii*	0.789	1.547	1.398	0.609	0.149	24.47
Clopidogrel (Anti-platelet agent) as standard	0.799	1.559	1.276	0.477	0.283	59.33
Blank	0.787	1.533	1.515	0.728	0.018	2.47

Here, W1= Micro-tube weight, W2= Clot with micro-tube weight, W3= Clot with micro-tube weight after clot disruption, W4= Clot weight after clot disruption, W5= Weight of released clot.

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

3.4 Anti-microbial property analysis

3.4.1. *Aporosa wallichii* leaves antimicrobial activity evaluation

Methanol extract of *Aporosa wallichii* leaves was taken to perform anti-microbial activity analysis, different concentration was prepared ranging from 500mg/mL to 15.625mg/mL. With these concentration each of the bacterial strain examined but not any significant result was found. There are little possibilities of some concentration to showed very mild effect but most of the concentration did not showed any antimicrobial property. Reason behind this negative result might be experimental errors and others. But during this experiment streptomycin which is used

as standard showed antimicrobial activity properly on both gram positive and gram negative bacteria.

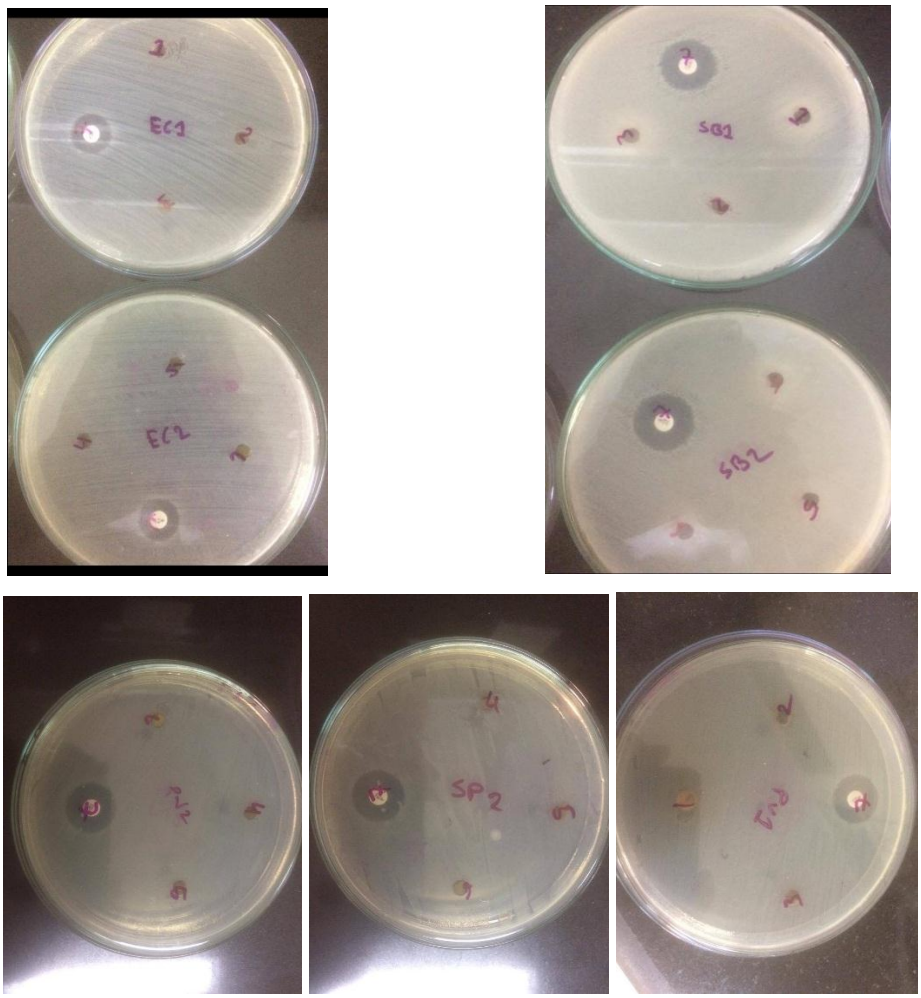


Figure 3.7: Antimicrobial property of plant *A. wallichii* leaves evaluation on patri-dishes.

Chapter Four

Discussion

4. Discussion

Biological screening methods of the methanol leaf extract of the picked plant, *Aporosa wallichii*, and Provides sufficient amount of information which can be utilized in medicine world. The methanol extract of *Aporosa wallichii* leaves was verified properly through DPPH assay to determine the antioxidant property of this plant. As the reference standard, ascorbic acid was used in this experiment for which IC50 value was obtained 75.688µg/mL and the methanol extract of *Aporosa wallichii* which IC50 value of was obtained 58.725µg/mL. Additionally, total phenolic content test of this plant showed satisfactory result, value obtained 308.97 (mg of GAE / gm of extract). Hence, the present study might suggest that this plant can be used as an antioxidant agent.

The brine shrimp lethality assay was attained to assess the cytotoxicity property of methanol extract of *Aporosa wallichii* leaves, LC50 value of the examined sample was determined from the graph where plotting the percentage of mortality (nauplii) against the test sample concentration. To determine the best fitted line from the curve attained from the data, Regression analysis was used. Vincristine sulfate was used in this experiment as a standard (positive control), which LC50 value was obtained 2.0203µg/mL, compared to the standard methanol extract of the plant *Aporosa wallichii*, gave LC50 value 26.751µg/mL. Consequently, it can be raised that this *Aporosa wallichii*, showed the cytotoxicity property more research required to ensure its property for the betterment of the global healthcare.

Methanol extract of *Aporosa wallichii*, showed significant result on thrombolytic activity test. Here, clopidogrel used as a positive control for which 59.33% clot lysis was viewed. Distilled water was used as a negative control, which proved 2.47% lysis of the blood clot. The methanol extract of *Aporosa wallichii*, showed 24.47% clot lysis. Comparing the clots lysis value of methanol extract with the positive control value, plant revealed effective thrombolytic activity.

Antimicrobial property test was also done to evaluate methanol extract of *Aporosa wallichii* leaves. From this experiment there no significant result obtained, which means plant may not contain any antimicrobial property this may be result of some experimental error. But more investigation required confirming about the plant's antimicrobial property present or not.

Conclusion

Methanol extract of the *Aporosa wallichii* leaf was investigated to evaluate the biological properties. After conducting this research it has been cleared that the plant showed different biological properties. This research study showed that plant has moderate level of antioxidant and thrombolytic property along with significant level of cytotoxicity property. However, this plant did not show any antimicrobial property, but it requires further investigation on antimicrobial property of this plant. Furthermore, current research suggest more detailed investigation of this plant *Aporosa wallichii* to find out unidentified biological properties which will help in the development of the world healthcare and may introduce any new effective property in field of medicine.

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