Phytochemical Screening and Biological Activity Evaluation of *Hygrophila schulli*

A project submitted
by
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Dedicated to my family

Certification Statement

This is to certify that this project titled ‗Phytochemical Screening and Biological Activity Evaluation of *Hygrophila schulli*' submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Monica Sharfin Rahman, 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

_____

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Abstract

Hygrophila schulli (Family: Acanthaceae) is a crucial medicinal plant which is commonly habitat in the sub continental zone. The plant is frequently used in the Indian Ayurveda system for the treatment of urogenital tract diseases such as in dysuria, urinary calculi and cystitis due to its diuretic property. Moreover, the plant has also established its significant potentiality in treating kidney stone in the folk medicine system. In spite of its strong medicinal value, only sporadic attempts have been made for the scientific and methodical validation of these traditional uses. The current investigation aimed to determine the phytochemical constituents as well as to evaluate antioxidant, antimicrobial and cytotoxic potentials of the methanolic extract of this plant. The investigation revealed the presence of several phytochemical compounds namely, alkaloids, flavonoids, phenols, glycosides, tannins and steroids. The antioxidant potential of the plant was evaluated by means of DPPH assay and total phenolic content assay. Both the assay showed significant antioxidant potential of this plant. It draws a fine line to use *H. schulli* in cardiovascular and anti-inflammatory diseases. Brine shrimp lethality bioassay was also performed on this plant, which showed a dose dependent relationship of concentration and % mortality of shrimp nauplii. In this study, the LC_{50} values of the standard, vincristine sulphate and methanolic extract of *H. schulli* were obtained 1.115 μg/mL and 294.182 μg/mL respectively. However, the crude methanolic extract as well as the pet ether fraction, chloroform fraction, ethyl acetate fraction and aqueous fraction of *H. schulli* showed no sensitivity in antimicrobial assay. Based on these investigations, it may be suggest that the plant *H. schulli* can be used as a potential medicinal plant.

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List of abbreviation

AA	Ascorbic Acid
GAE	Gallic Acid Equivalent
RSD	Relative Standard Deviation
H. schulli	*Hygrophila schulli*
mg	Milligram
mm	Millimeter
mL	Milliliter
µg	Microgram
BHA	Butyl Hydroxy Anisole
BHT	Butyl Hydroxy Toluene
IC_{50}	Median Inhibitory Concentration
LC_{50}	Median Lethal Concentration
DPPH	2, 2-Diphenyl-1-Picrylhydrazyl
UV	Ultraviolet
DMSO	Dimethyl Sulfoxide
GI	Gastrointestine
HCl	Hydrochloric Acid
FRS	Free Radical Scavengers/Scavenging
R^2	Regression Coefficient
WHO	World Health Organization
m	Meter
Cm	Centimeter

gm	Gram
LAF	Laminar Air Flow
Conc.	Concentration

Chapter 1

Introduction

1 Introduction

1.1 Phytotherapy and medicinal plants

Phytotherapy, an oldest form of medicinal therapy has been used in the medicinal treatment from the very beginning of our existence in this world, initially unintentionally and later on intentionally. Back in ancient age, men‘s eccentric behavior of searching new sources of food and ways to mitigate their health challenges introduced them to the vast ranges of medicinal plants containing potential therapeutically active ingredients. About 200 years ago the first pharmacological compound, morphine, was produced from opium which was extracted from the seed pods of the poppy flower. From that time period to till now, scientists have been acquiring knowledge about plants to create pharmaceutical products that we know today. Today, herbal medicine constitutes about 25% of the prescribed medicine. It is also observed that among the 252 essential drugs that are listed by the World Health Organization, 11% are exclusively of plant origin (Sofowora, 1982).

However, phytomedicine, which is also known as herbal medicine or botanical medicine, refers to the usage of plant‘s seeds, berries, roots, leaves, bark, or flowers for medicinal purposes. These medicines are plant-derived, complex mixtures of organic substances that may come from any crude or processed part of a plant which are used to treat illnesses within local or regional healing practices (Bent, 2008).

Nevertheless, herbalism has a long traditional application outside the conventional medicine. Along with the advances of clinical research, improvements in analysis and quality control has led its more common usage in our today‘s regular life, which clearly indicates its potentiality in mitigating and preventing disease. (Ehrlich, 2015)

Additionally, different researches have also indicated its cost-effectiveness and efficacy in the treatment of different diseases. In fact, among the 177 drugs approved worldwide for the treatment of cancer, more than 70 percent are based on natural products or synthetics that mimic the natural products. Different plants are used as herbal solutions for many diseases. (Ehrlich, 2015)These were also used in the past even before the existence of modern drugs. Some of them are mentioned below:

- **Garlic**

Garlic is an herb that in addition of containing high amount of sulfur, also contains flavonoids, oligosaccharides, selenium and allicin. It is observed to be effective in treating diabetes and inflammation, enhancing immunity, maintaining blood pressure, controlling cardiovascular disease, resisting fungal, viral infection as well as resolving hair loss problem. A moderate success rate is also observed in controlling cholesterol level after increased intake of garlic. A research published in an American journal of nutrition, also indicated a correlation between garlic intake and antioxidant activity increment (Rahman & Lower, 2006).

- **Onion**

Onion is also a very renowned medicinal herb. It is found to be useful as an appetizer, flatulent, antiseptic, antipyretic, aphrodisiac, tissue building and as well as cardiac tonic agent. Chewing onion is also proved to be efficacious for spongy and bleeding gums and inflamed sore throats. Apart from these functions, onion acts as a good tonic and medicine for menstrual disorders in females. It is also found to be useful in strengthening heart muscles and promoting circulation within the heart, which may ultimately prevent and cure ischaemic conditions (Medicinal Herbs & Spices, 2011). Alopecia areata, a hair losing condition, can also be treated with onion juice. Satisfactory outcomes from different researches have also indicated its effectiveness on affected skin (Sharquie & Al-Obaidi, 2002).

- **Ginger**

Ginger is a member of Zingiberaceae family. It is usually used as a spice and medicinal herb in the South-East Asia region. It has a strong phytochemistry which includes ingredients that are useful to treat cancer, such as vallinoids, 6-gingerol and 6-paradol etc. However, presence of other constituents such as shogaols, zingerone etc. is also observed from different investigations (Shukla & Singh, 2007).

In general, ginger is recommended for preventing arthritis, rheumatism, atherosclerosis, hypertension and ulcer. Many resources have also indicated is potentiality in remedying stomach upset and motion sickness. It stops vomiting by acting as a barrier for the release of serotonin. Its role as an antioxidant might also help to control aging (Mashhadi et al., 2013).

- **Cinnamon**

Cinnamon helps in reducing blood glucose level in type 2 diabetic patients. It is also found to be effective in reducing heart risks as well as in maintaining cholesterol level in human body. Therefore, daily consumption of cinnamon is usually recommended for maintaining cholesterol level (―10 Best Healing Herbs", 2015).

- **Turmeric**

Calcium, Phosphorus, Iron, Carotene, Thiamine and Niacin are different ingredients that are found to be present in turmeric. Turmeric is recommended as a powerful stimulating agent for the treatment of gastro intestinal tract diseases as well as for the enhancement of intestine activities. It is regularly used by the Ayurveda, siddha and unani systems of medicine as one of the best blood purifiers and as an antiseptic (―Medicinal Herbs & Spices", 2011).

- **Aloe**

Aloe plant gel is a good source of skin care products ingredients. A thick gel obtained from the leaves of Aloe is used to treat skin burn, sun burn, and skin inflammation etc. (Barros, 2011-2016).

- **Valerian root (*Valerian officinalis*)**

Since the ancient times, the valerian root is used to get relief from insomnia and stress. It produces GABA (Gama Amino Butyric Acid), which is needed by the brain to produce sound and faster sleep (Barros, 2011-2016).

Plants those contain remedial activities or show pharmacological effects on human are generally designated as medicinal plants. From ancient ages, people are relying on such plants for healing several diseases by virtue of their medicinal value. Due to their easy availability and fewer incidents of adverse effects, now a day, it is more popular than the synthetic drugs. As a result, today, about 80% of the world population rely on medicinal plants, sometimes partially or even in some cases, entirely (Batugal, 2004).

1.2 Medicinal plants-history & drug discovery

Since the primitive ages, men have the tendency to look for new natural sources either for the food requirement or for their mitigation of health challenges. Although they had little knowledge about those plants in past, they implemented their experiences in curing diseases. Archaic written evidences for medicinal plant drug preparation has shown us the vast

popularity of medicinal plants throughout the long journey of human in this earth. As observed from different researches, it is predicted that crude plant material was used for medicinal purposes until 16th century. The usages of medicinal plants and process of preparing drugs from those plants has also been indicated in a 5000 years old Sumerian clay slab, which was found from Nagpur, India. The slab contains about 12 recipes which subjects about 250 plants of that time period. The Chinese book on roots and grasses –Pen T'Sao," written by Emperor Shen Nung circa at 2500 BC, also shows us the usage of dried parts of medicinal plants in about 365 drugs. Rhei rhisoma, camphor, Theae folium, Podophyllum, the great yellow gentian, ginseng, jimson weed, cinnamon bark, and ephedra are some of the examples of plants listed in that Chinese book, which are in use till today. The Indian holy book Vedas has also mention the application of plants that are abundant in India in the treatment of diseases. It should also be mention that numerous spice plants such as nutmeg, pepper, clove etc. that are used even today were actually originated from India (Petrovska, 2012).

However, the ancient Greeks and Romans were the renowned herbalists of all times. Physicians traveling with the Roman army spread their herbal knowledge throughout the Roman Empire as well as in the Spain, Germany, France and England. Dioscorides (c. 40-c. 90) and Galen (131-200 A.D.) were the two Greek physicians of the Roman army, who compiled their herbal knowledge in a book, which remained the definitive materia medica text for about 1500 years (–A Brief History of Herbalism", 2007). Nevertheless, Greek civilization's history has indicated the application of about 500 crude drugs in the treatment of diseases. It is observed that the first archetype medical treatment was also formulated during the Greek period. Hippocrates, a Greek physician had also written about 300 books on plants during that period (H. K. Ph.D., 2014).

Considering the traditional Chinese medicine therapy, they are very much enriched with logistic evidence and unique techniques. The official compendium of drugs in China, which is known as the Chinese pharmacopoeia has indicated the usage of a large number of traditional Chinese drugs and their therapeutic indications for different pathological conditions. However, in terms of more effective treatment, the Arab took a revolutionary step by introducing the plant extract in formulating medicine during that time period. This

initiative was later followed by the others as it was found to be more precise and effective than the crude treatment in the later period. The Arab also explained the toxic properties of many plants and prepared antidotes from those plants (–Medicinal plants", 2017)

In the present world, the medicinal values of the plant based products have been proved scientifically. A very good number of strong molecules have been derived and launched as a product from natural sources. Moreover, now a day, the value of natural products is regularly combined with synthetic and biotech drugs for the development of modern medicine (–Medicinal plants definition", 2017). The popularity of plant derived medicine and healthcare products is also growing rapidly in the current world. It is predicted that plant derived products are occupying about 60% of the whole market globally. Moreover, in India, it is seen that about 70% of the natural products are combined with modern medicine for the treatment of diseases (Tariq & Reyaz, 2013).

1.3 Phytochemical screening and biological assay of medicinal plants

Phytochemical screening is a very important procedure to get bioactive constituents, more specifically secondary metabolites from plant materials. Medicinal plants consist of several phytochemicals which are the powerful solution to many diseases and pathological conditions. Phytochemicals derived from medicinal plants can also play a significant role in medicinal science to cure or inhibit various critical health conditions. Today, isolating phytochemicals and conducting study on them has created a new bridge between conventional and modern drug compounds. Identifying natural compounds and formulating drugs from them are regularly helping modern science to meet our day to day challenges. Globally, a huge number of laboratories are working continuously to discover different natural compounds with strong biological activity from distinct parts of medicinal plants (Tariq & Reyaz, 2013)

In our present world, a vast number of therapeutic effects like anti-tumor, anti-viral, anti-inflammatory, anti-malarial, analgesic etc. can be achieved from biologically active compounds. Although plant based cancer therapy has been reported since 1950s, a large number of studies are still conducted on medicinal plants to combat cancer. Statistical review also stated 16 plants which are in the trial period for the treatment of diseases.

Therefore, screening of medicinal plants can play a major role in the development of modern medicine. However, phytochemical screening is usually carried out on whole plant or sometimes on a particular part of plant to get the desired bioactive compounds (Sulaiman & Balachandran, 2012). Some of the bioactive components that may be obtained phytochemical screening are given below:

- **Flavonoids**

Considering the chemical property, flavonoid has a 15 carbon polyphenolic skeleton, which contains two phenyl rings and a heterocyclic ring. It is composed of 6 major subgroups: chalcone, flavone, flavonol, flavanone, anthocyanins and isoflavonoids (–Phytochemicals". n.d.).

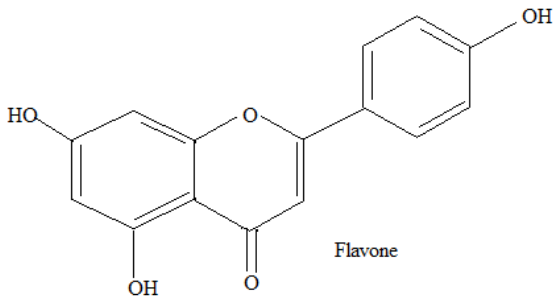

Figure 1.1: Flavonoid.

However, flavonoids are usually known for their pigmentation function in plant. Besides their pigmentation role, in higher plants they also act in the UV filtration, symbiotic nitrogen fixation procedures. Chemical messenger, physiological regulators and cell cycle inhibitors are also some of the roles that are performed by flavonoids (Galeotti et al., 2008). Different studies have also indicated its antioxidant, coronary heart disease prevention, and hepatoprotective, anti-inflammatory, anti-cancer and antiviral activities in physiological systems (Szalay, 2015).

- **Alkaloids**

Alkaloids are complex organic nitrogenous compounds, which contain pyrrole, pyrrolidine, tropane, pyridine, quinolone and isoquinoline in their structures. They are soluble in water,

alcohol, ether and chloroform. They have a prominent physiological function in human body. However, they are also known for their toxic properties and include many poison like atropine, strychnine etc. From the perspective of pharmacology, the alkaloids are found to give pharmacological activity at a minimal dose. The biological function of many alkaloids usually depends on the amine functional group which is transformed into a quaternary system by protonation at physiological pH (Kumar & Pandey, 2013).

Alkaloids which are rich in quaternary amines are usually found naturally (Dr. Mazen & El-Sakka, 2010). A list of naturally occurring alkaloids is given in table 1.1.

Table 1.1: List of naturally occurring alkaloids (Edward J. Big, E., 1944)

Alkaloids	Sources	Uses
Morphine	Poppy	Relief of pain
Codeine	Poppy	Cough control
Cocaine	Coca leaf	Local anesthesia
Apomorphine	Morphine	Cause vomiting
Arecoline	Betal nuts	Worm expeller
Atropine	Belladonna	Mydriatic
Caffeine	Coffee	Diuretics, stimulant
Ephedrine	Ephedra	Vasoconstrictor
Physostigmine	Calabar bean	Apthalonology
Quinine	Cinchona bark	Malaria

- **Tannins**

Tannins are naturally occurring polyphenols. They have a tendency to bind and precipitate proteins (King & Young, 1999). Therefore, it is predicted that they have a significant impact on animal nutrition as well as on inhibition of growth rate digestive enzymes (Bennick, 2002). However, tannins are also found to form complex with starch, cellulose,

minerals etc. (Bennick, 2002). Tannins could be spotted in various parts of plant tissues which may include:

Bud tissues – Tannins are present in the outer part of the bud tissues, which help them to protect the plants from freezing (King & Young, 1999).

Leaf tissues – In evergreen plants, tannins are evenly scattered in all leaf tissues. Here, they serve to reduce the palatability of plants, thereby help plants to be protected from predators (King & Young, 1999).

Root tissues – In root tissues, tannins are usually found in the hypodermis. They act as a chemical barrier of penetration and colonization of roots by plant pathogens in root tissues (King & Young, 1999).

Seed tissues – Tannins are also found to be present in a layer in between the outer integument and the aleurone layer. In seed tissues, the tannins are usually involved with the maintenance of plant dormancy. However, evidence of allopathic and bactericidal properties due to tannins is also prevalent in seed tissues (King & Young, 1999).

Stem tissues – Tannins are often found in the active growth areas of the trees for example, secondary phloem, and xylem and also in the layer in between epidermis and cortex. It is predicted that tannins might have a role in the growth regulation of these tissues (King & Young, 1999).

However, tannins can be broadly classified into two categories as shown in figure 1.2 (Metwaly, 2015).

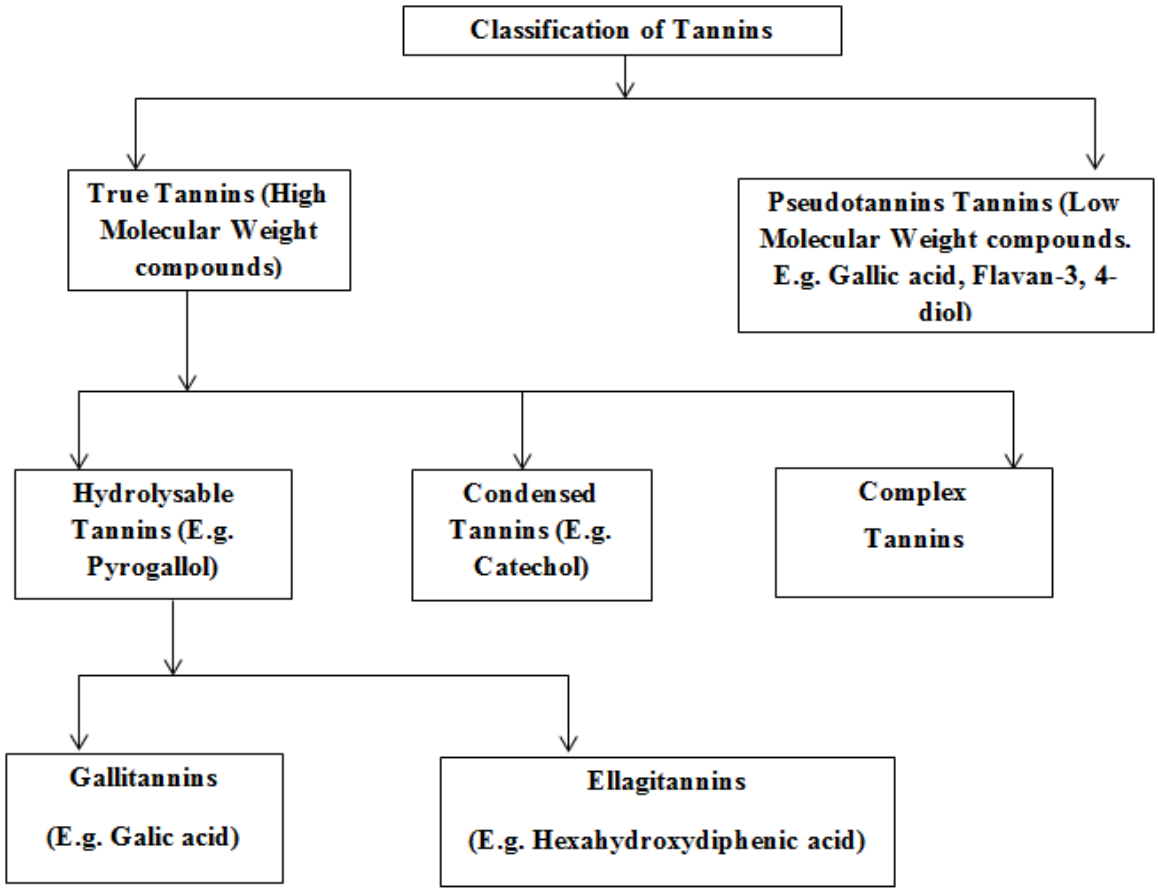

Figure 1.2: Classification of tannins.

Medicinal plants are the prime source for tannins, though the range of pharmacological effects of tannins varies with the type of the plants (Ukoha et al., 2011). However, in general, tannins exhibit potential antimicrobial activities against fungi, yeast, bacteria and viruses (Chung et al, 1998). Beside their antimicrobial property, herbal tannins also found their potentiality in the formulation of mouthwashes, eyewashes, snuff, vaginal douches due to their hemostatic properties. Additionally, tannins can also be used to impede bare swelling as well as to control hemorrhage (Shekhar, 2009). Moreover, if it is used as proanthocyanidins, it can be used to improve vascular health. It is also proposed that, topical use of tannins can remove skin irritation. Apart from these functions, anti-inflammatory, antiseptic, anti-viral, anti-oxidant anti-helminthic and anticancer agents are also some of roles that might be performed by tannins (Chung et al., 1998).

- **Saponins**

Saponins are usually known for their foaming features. Chemically, saponins are glucosides that have a polycyclic aglycone structure attached with one or more sugar side chains. It has a bitter taste. It works as an antioxidant, immunity booster and anti-tumor agents. It is also found to reduce the risk of cancer in human (Shekhar, 2009). Several researches have also established its anti-depressive feature recently. Moreover, adequate evidences constructed on scientific basis showed enhanced neurogenesis, neurotrophic factors and restore monoaminergic tone due to saponins. By obstructing apoptosis and intraneuronal calcium dynamics, saponins can also exhibit neuroprotective effect as indicated from scientific data. As current medication system is continuously failing to provide better treatment to depression, it might be proposed to conduct more study on the anti-depressive property of plant saponins by arranging pre-clinical and clinical trials (Khan et al., 2016) (Abbas et al., 2015).

- **Antioxidants**

Antioxidants are emerging as prophylactic and therapeutic agents. These are the agents, which scavenge free radicals like reactive oxygen species and prevent the damage caused by them (Hamid et al., 2010). Free radicals play a common and probably the most important role in the damage of our body cell which ultimately causes aging problem. They have the potential to damage healthy cells of body by destroying their structure and functions. They are mainly produced from the highly reactive oxygen of our body. Antioxidants are believed to be the body‘s first line of defense against aging. They have the capability to neutralize these free radical activities (Flora, 2009). However, the antioxidants can be classified broadly into two categories which can be classified further. The classification of antioxidants is given in figure 1.3.

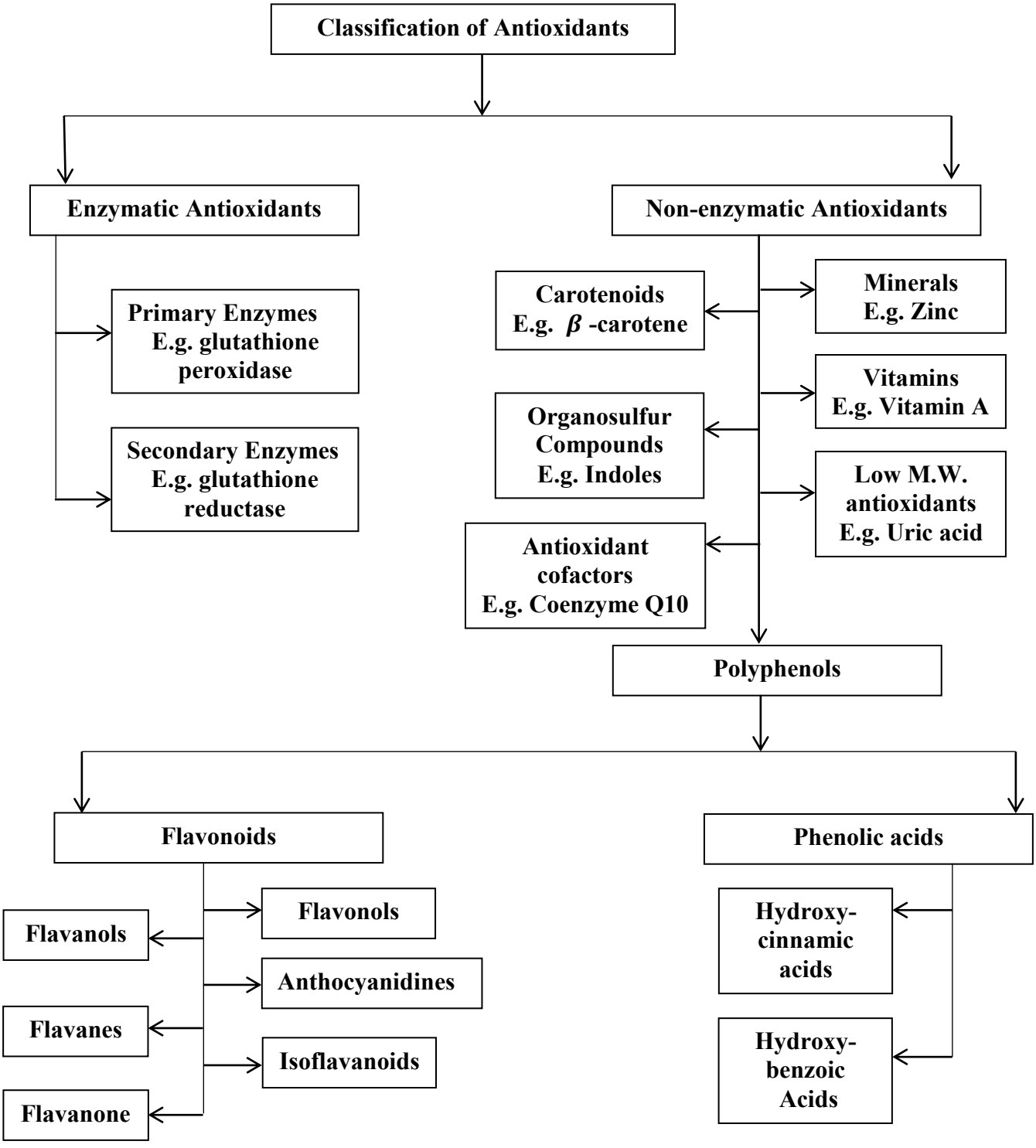

Figure 1.3: Classification of antioxidants (Bunaciu, 2015).

Biological assay is performed for medicinal plants to produce novel bioactive compounds or lead compounds that can contribute to the modern medicine. In this present dissertation, anti-oxidant, anti-bacterial and cytotoxic potential is evaluated for the selected plant.

1.4 Importance of phytochemical, antioxidant, anti-microbial and cytotoxicity evaluation

As observed from different observations, the need for naturally derived drug products is increasing day by day. A number of studies have been carried out for this purpose, which has eventually resulted in the discovery of different potential phytochemical constituents from different crude plant extract (Masih & Singh, 2012). Successful experiments and discovery of high amount of phytochemicals has disclosed the path to obtain drugs that are both cost-effective and safe. Hence, the search for natural products is being still continued. Studies and literature reviews highlighted the fact that, other than allopath, today, around 90% drugs used in the several practicing methods in Asian sub-continent are directly related to plants (Savithramma et al., 2011).

The human body has a complex system of natural enzymatic and non-enzymatic antioxidant defense systems which counteract the harmful effects of free radicals and other oxidants. Free radicals are responsible for causing a large number of diseases which includes cancer (Kinnula & Crapo, 2004), cardiovascular diseases (Singh and Jialal, 2006), neural disorders (Sas et al., 2007), Alzheimer‘s disease (Smith et al., 2000), mild cognitive impairment (Guidi et al., 2006), Parkinson‘s disease (Bolton et al., 2000), alcohol induced liver disease (Arteel, 2003), ulcerative colitis (Ramakrishna et al., 1997), aging (Hyun et al., 2006) and atherosclerosis (Upston et al., 2003) etc. However, protection against free radicals can be enhanced by sufficient intake of dietary antioxidants. Substantial evidences have indicated that foods containing antioxidants and particularly the antioxidant nutrients might play a significant role in disease prevention. There is, however, a growing harmony among the scientists that for a long term therapy, a combination of antioxidants is more effective than single antioxidant therapy (Chanda & Dave, 2009).

Antioxidants may be of great value in improving the quality of life by preventing or postponing the onset of degenerative diseases. In addition, they have the potential to reduce substantial amount of cost in the health care delivery. However, plants have an innate ability to biosynthesize a wide range of non-enzymatic antioxidants that are capable of mitigating ROS- induced oxidative damage. Several in vitro methods have been used to screen antioxidant potential of plants and most of these investigations have revealed potent antioxidant activity of different plants (Chanda & Dave, 2009). Plant antioxidants such as ascorbic acid and flavonoids have been proved to be the best exogenous antioxidants for human body. Indeed, these compounds not only restrain ROS production by scavenging free radicals, but also help in boosting the endogenous antioxidant defense system of body (Halliwell, 2006). Thus, scientists are putting their interest on different natural sources such as plant extracts to obtain natural potential antioxidants (Chanda & Dave, 2009).

However, it is observed that, although a large number of new antibiotics have been produced in the pharmacological areas in the last three decades, resistance to these drugs by microorganisms have also been increased proportionately. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs. Such a fact is cause for concern due to the number of patients who have suppressed immunity, and also due to the new bacterial strains which are multi-resistant of a number of drugs (Cohen, 1992). Nevertheless, the growing microbial resistance is making the use of antimicrobial drugs in the near future uncertain. Therefore, actions must be taken to reduce this problem, for example, different studies can be carried out to develop new drugs of both natural and synthetic origin. However, ultimate goal of all these approaches is to deliver appropriate and efficient antimicrobial drugs to the patients. In this regard, plants have always been a valuable source of natural origin for maintaining human health, especially in the last decade. It is proposed that the most interesting area of application for medicinal plant extracts is the growth inhibition of different serious pathogens (Okolo et al., 1995).

Plant derived components have attracted particular attention as an alternative source to battle several diseases including cancer. Over 3000 species of plants have been reported to have anticancer properties (Graham et al., 2000). Despite such advancement in the cancer therapies which include chemotherapy and radiation therapy, the mortality rate associated

with cancer remained high. Thus, the present scenario and the toxic side effects associated with the available treatments calls for alternative methods, with higher efficacy and lesser side effects, to deal with this disease. Plants have been looked upon as the best substitute in this situation and several have been evaluated in an effort to discover novel, potential anticancer compounds with no toxic effects (Gullet NP et al., 2010). In fact, several studies have elucidated the potentiality of a number of naturally derived phytochemicals as therapeutic agents for cancer. Some of the plants that are advanced towards the clinical trials for cancer treatments include Madagascar periwinkle, Catharanthus roseus L. etc. (Cragg & Newmann, 2005).

Above discussions and literature reviews implicate that, plant source are equally popular like chemical drug components. In fact in some cases, plant sources are prioritized for their less toxic side effects compared to the synthetic agents. In this scenario, sit is obligatory to design more and more research plans on different potential plant extracts (*in vitro* & *in vivo*) to find out new possible drug solutions.

1.5 Rationale of the study

Medicinal plants are the largest sources of attaining new compounds which can contribute to the modern medicines. Although several medicinal plants are being used in Ayurveda and homeopathy, only few research information about those plants are available to establish them as therapeutic agents. Hence, to intensify the medicinal use and rectify the natural constituents as medicine instead of synthetic drugs, it is necessary to organize such kind of research studies. Thus, more attention should be provided to develop better medication from them by using advanced techniques.

The plant under current investigation, *Hygrophila schulli* is a thorny sub-shrub plant which is native to India. The plant has a long traditional history to be used as/in diuretic, antibacterial, jaundice, urogenital tract diseases etc. Till date, *Hygrophila schulli* is subjected to only a limited number of research works to evaluate its potentiality in different pathological conditions (Mazumdar et al., 1997). Diuretic test (Hussain et al., 2009), nephrotoxicity test (Bibu et al., 2010), anti-inflammatory test (Patra et al., 2009) are some of the examples of research works that have been carried out on this particular plant. However,

different studies carried out on similar other species of this plant indicated potential medicinal value of those plants, from which it might be predicted that the selected plant might have some medicinal value in it (Mazumdar et al., 1997). Thus, the rationale of this study is to identify natural constituents and evaluate some biological properties like antioxidant, anti-bacterial and cytotoxic potential from the crude extract of *H. schulli.* In addition, for the goodness of planetary healthcare, the study will look for into the other unknown properties of this plant.

1.6 Aim of the study

The aim of the present study is to identify phytochemical constituents as well as to evaluate antioxidant, cytotoxic and antimicrobial potential of the crude methanolic extract of *H. schulli*.

1.7 Objectives of the study

The study protocol is based on the following objectives:

1. Phytochemical screening of the crude methanolic extract of the powdered leaf material for the identification of the major groups of phytochemicals by using different protocols established by Ghani A, 2003.
2. Evaluation of antioxidant activity by determining IC_{50} value of the crude plant extract using *in-vitro* DPPH free radical scavenging method.
3. Quantifying antioxidant potential by determining total phenolic content of the crude extract using folin-ciocalteu method.
4. Evaluation of cytotoxic activity by determining LC_{50} of the crude methanolic extract of the plant using brine shrimp lethality assay.
5. Evaluation of anti- microbial activity of *H. schulli* by the method described in Bayer et al., 1966.

1.8 Description of plant

Hygrophila schulli is a medicinal plant which belongs to the Acanthaceae family. According to Chandran et al, it is a thorny sub-shrub plant which is generally found in India, Srilanka, Myanmar, Indonesia, China, Africa and Malaya. However, *Hygrophila schulli* (Family: Acanthaceae) is also known as Ikshura, Ikshugandha and Kokilasha (having eyes like Kokila

or Indian cuckoo) in the Ayurvedic literature (Chandran, et al., 2013). Nevertheless, the plant is widely used as medicine in India. The medicinal value of root, leaves and stems of *H. schulli* is also mentioned in the Ayurveda (~~–~~*Hygrophila schulli*", .n.d.).

H. schulli is usually found to grow in wetlands and soggy places (Chandran, et al., 2013). Commonly, it has the tendency to spread prolifically in polluted clogged area, drainage etc. which is not harmful (~~–~~*Hygrophila schulli*", .n.d.). In general, the plant contains eight leaves and six spines at each node. The leaves of *H. schulli* are whorled and contain undulating margins. The plant has purple flowers at the top of leafy bracts and bracteoles which contain about 1 cm long orbicular seeds (Chandran, et al., 2013).

Figure 1.4: *Hygrophila schulli* plant.

1.8.1 Taxonomy

Taxonomy of *H. schulli* is given below: ***Hygrophila schulli* — Overview Long Leaved Barleria**

Kingdom- Plantae

Phylum- Tracheophyta

Class- Magnoliopsida

Order- Scrophulariales

Family- Acanthaceae

Genus- Hygrophila

Species- hygrophila schulli

1.8.2 Synonyms of the plant (Rahman, Hossain, & Islam, 2014)

Asteracantha longifolia (L.) Nees

Bahel schulli Buch.- Ham

Barleria auriculata Schumach

Barleria longifolia L

Hygrophila longifolia (L.) Kurz

Hygrophila spinosa T.Anderson

Hygrophila spinosa T. Anders

1.8.3 The plant family Acanthaceae

The members of Acanthaceae family are usually annual or perennial herbs and shrubs. The family comprises of about 250 genera and 2,500 species. The plants of this family mostly include tropical herbs, shrubs or twining vines. Some epiphytes are also found in this family. However, the plants of this family are found with simple, opposite leaves and tubular, 4 or 5-lobed flowers which have 2 to 4 stamens and 1 to 2 stigmas in a long style. The fruit is capsular with two chambers which often open explosively when it is matured so that the seeds can be scattered at distance from the parent plant (–Acanthaceae", 2017).

However, some species of this family are also observed to be distributed in the temperate region. Indonesia and Malaysia, Africa, Brazil and Central America are the regions where the plants of Acanthaceae are usually found to be distributed. Nevertheless, the representatives of the family can be found approximately in every habitat, including dense or open forests, scrublands, wet fields and valleys, sea coast and marine areas, swamps and even in mangrove woods (Scotland, 1992).

It is proposed that, the Acanthaceae family has low structural diversity than any other family. On the other hand, in terms of number and type of apertures and ultra-sculpturing, the pollen grains of Acanthaceae are as diverse as any other family-level lineage regardless of their size. Their diverse character is also prominent in their chromosome numbers with documented haploid numbers ranging from 7 to 68. This clearly indicates the evolutionary transitions of this family via both dysploidy and polyploidy (M, 2011).

Figure 1.5: Different plants of Acanthaceae family.

1.8.4 Uses

Traditionally, the leaves of *H. schulli* are utilized for the following conditions: (Chandranet al., 2013)

-Gastric disorder	-Urinary calculi	-Anemia
-Jaundice	-Urinary discharge	-Anuria
-Bacterial infection	-Inflammation	-Gleet
-Dropsy	-Joint Pain	-Cough
-Rheumatism	-Biliousness	-Stomachache
-Anasaraca	-Eye disease	-Lumbago
-Urinogenital tract diseases	-Ascites	-Arthritis
-Diarrhea	-Abdominal troubles	-Leucorrhoea

Apart from these conditions, the plant is also used as diuretic, tonic, aphrodisiac, hypnotic as well as demulcent (Chandran, et al., 2013).

1.8.5 Components isolated from *H. schulli*

Previous phytochemical investigations has revealed the presence of numerous constituents such as lupeol, stigmasterol, isoflavone glycoside, an alkaloid and small quantities of uncharacterized bases in *H. schulli* (Nikam, Mundada & Mishra, 2012). However, the phytochemicals that are identified from *H. schulli* mainly include phytosterols, fatty acids, minerals, polyphenols, proanthocyanins, mucilage, alkaloids, enzymes, amino acids, carbohydrates, hydrocarbons, flavonoids, terpenoids, vitamins and glycosides. Phytoconstituents such as lupeol, lupenone, 25-oxo-hentriacontanyl acetate, stigmasterol, betulin, β- carotene, hentriacontane, apigenin-7-O-glucuronide, apigenin-7-O-glucoside, 3-methylnonacosane, 23-ethylcholesta-11(12), 23(24)-dien-3β-ol, luteolin, asteracanthine, asteracanthicine, luteolin-7-rutinoside, methyl-8-n-hexyltetracosanoate, β--sitosterol, histidine, phenylalanine, lysine, ascorbic acid, nicotinic acid, n-triacontane, glucose, mannose, rhamnose, arabinose, xylose, maltose, myristic acid, oleic acid, palmitic acid, stearic acid, linoleic acid etc. are also reported in this plant (Patra, Jha & Murthy, 2009).

1.8.6 Literature review

After going through several articles and literatures, some of the research works conducted on *H. schulli* is found. A study has been carried out with petroleum ether root extract of *H. schulli* on *Ehrlich ascites* carcinoma and sarcoma-180 bearing mice. The study showed positive result of the extract against the standard drug tamoxofen (Mazumdar et al., 1997). In addition of it, antimotility test wih alocoholic leaves (Patra et al., 2008), diuretic activity assay (Hussain et al., 2009), nephrotoxicity test with the ethanolic extract (Bibu et al., 2010), anti-inflammatory test (Patra et al., 2009) have been carried out on this plant previously. But still, the plant has potential to contribute in the development of modern medicine. More research should be conducted to establish the prospects of *H. schulli.*

Chapter 2

Methodology

2 Methodology

The following scheme was followed for the entire investigation:

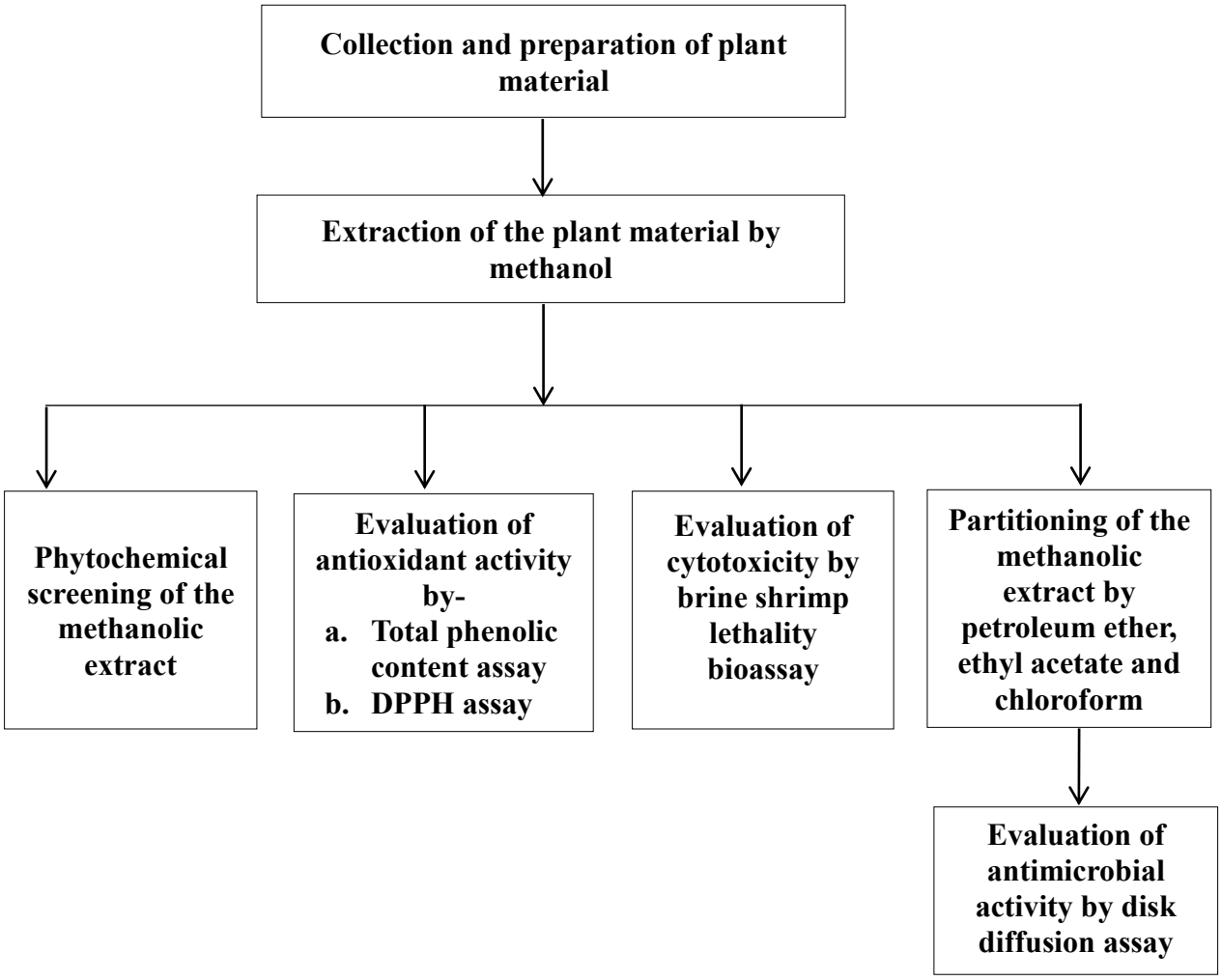

Figure 2.1: Research design.

2.1 Preparation of plant extract

2.1.1 Collection and identification of the plant

The plant *Hygrophila schulli*, was selected for the current study to identify phytochemical constituents as well as to evaluate pharmacological activities of this plant. Plant leaves were collected from the Avoynogor, Jessore, Bangladesh in December, 2016.

Figure 2.2: One of the collected plants of *Hygrophila schulli* before drying.

Later, the plant leaves were identified by the Bangladesh National Herbarium, Mirpur, Dhaka. After identification, an accession number, 43835 was provided by them for future reference.

Figure 2.3*: H. schulli* after drying and identification.

Table 2.1: Chemical investigation of *Hygrophila schulli*

Name of the plant	Family	Plant part
Hygrophila schulli	Acanthaceae	Leave

2.1.2 Plant material preparation and drying

After collecting the leaves of *H. schulli*, the leaves were subjected for water wash to remove plant debris as well as dust. Later, the leaves were separated from the branch and left for shed drying for about two weeks. The leaves were shed dried to prevent any kind of denaturation of chemical constituents of *H. schulli*.

Figure 2.4: Dried plants of *H. schulli.*

2.1.3 Extract preparation

- **Size reduction and weighing**

After completion of drying, a high capacity grinding machine was used to crush the leaves into fine powder. Approximately, 170 gm of powder was obtained after grinding, which was later kept in an airtight container to prevent contamination and for further investigation.

Figure 2.5: Grinded powder of the leaves.

- **Extraction**

A clean big amber jar was taken to prepare methanolic extract of the crude dried powder. Then in around 800 mL of methanol, the powder of the leaves was soaked. It was kept in mind during filling the jar with methanol that no spilling of the materials takes place as the

jar is needed to be shaken frequently. Moreover, it was also taken care that no gaseous substance remains inside the jar during shaking and stirring.

- **Filtration**

The content of the jar was subjected for filtration with the help of a cotton filter on the fourteenth day.

- **Concentration**

A rotary evaporator (Heidolph) with a rotation speed of 100 rpm and temperature of 30°C was used to concentrate the filter. A dense concentrated solution was obtained from the rotary evaporator after three hours.

Figure 2.6: Concentrating the filtrate of *H. Schulli* plant using a rotary evaporator.

- **Drying**

After concentrating the crude extract, the dense concentrated solution was transferred into a beaker. The beaker was kept under Laminar Air Flow (LAF) to evaporate the solvent from the extract. After drying for ten days, the extract became fully dried. The dried extract was then stored in vials to avoid any kind of cross contamination, microbial growth and also for further investigation. A schematic representation of the whole procedure is given in following page:

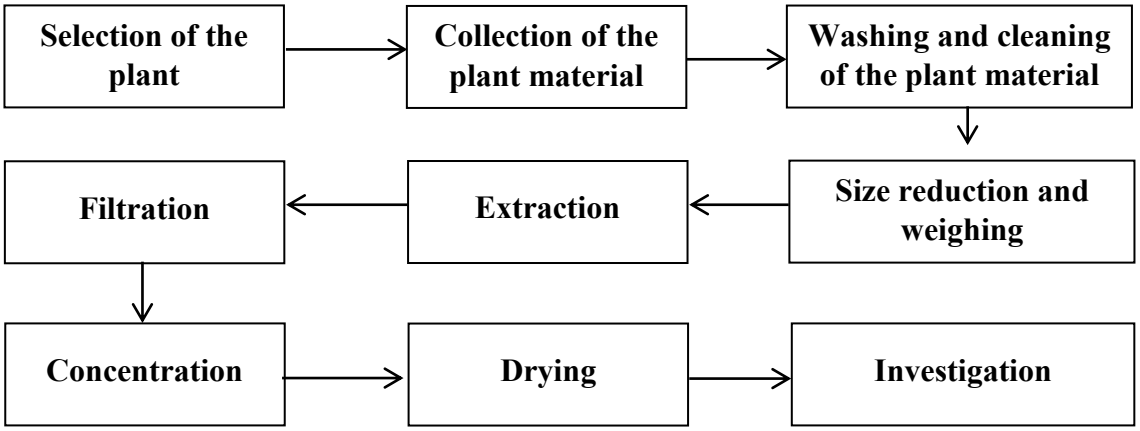

Figure 2.7: Flowchart showing extraction procedure from crude medicinal plant.

2.2 Solvent-solvent partition of the crude extract by modified Kupchan partitioning method

The crude methanolic extract of *Hygrophila schulli* was subjected for fractional separation by using different solvent by following a method developed by Kupchan and modified by Vanwagenen (Vanwagenen, 1993).

At first, mother solution of the test sample was prepared by dissolving 5 gm of the crude extract in 10% methanol. After this, solvent extraction was performed on this mother solution by using three different solvents: Petroleum ether, chloroform and ethyl acetate.

Table 2.2: Solvents used in solvent-solvent partitioning of *H. schulli* crude extract

Name of the solvent	Amount
Petroleum ether	300 mL
Chloroform	300 mL
Ethyl acetate	300 mL

2.2.1 Partitioning with petroleum ether

For partitioning with petroleum ether, initially the mother solution was taken in a separating funnel and then about 100 mL of petroleum ether and 12.5 mL of water were added to it.

After shaking well, the separating funnel was kept for rest without any disturbance for a while. When two different layers occurred in the separating funnels, the stop cork of the separating funnel was opened to collect the organic portion from the funnel. The process was repeated for thrice for better separation. The organic portions were then collected together and evaporated in the Rotary evaporator to get the petroleum ether fraction.

Figure 2.8: Two layers after the first partitioning.

2.2.2 Partitioning with chloroform

After completion of petroleum ether partitioning, about 16 mL of distilled water was added and mixed adequately to the aqueous fraction. This solution was then transferred to a separating funnel, where it was extracted with 100 mL chloroform three times. Later, all the chloroform fractions were collected together and evaporated subsequently. On the other hand, the aqueous fraction was preserved for further fractionation.

2.2.3 Partitioning with ethyl acetate

The aqueous fraction left after chloroform fractionation was mixed with 20 mL of distilled water and uniformly mixed accordingly. This mixture was then extracted with 100 mL of ethyl acetate three times. Then all the ethyl acetate extracted fractions were collected together and evaporated. The residual solution was also then collected to be preserved as aqueous fraction.

Figure 2.9: After partitioning different partitioning solutions.

A scheme of the above mentioned procedure is given in figure 2.10.

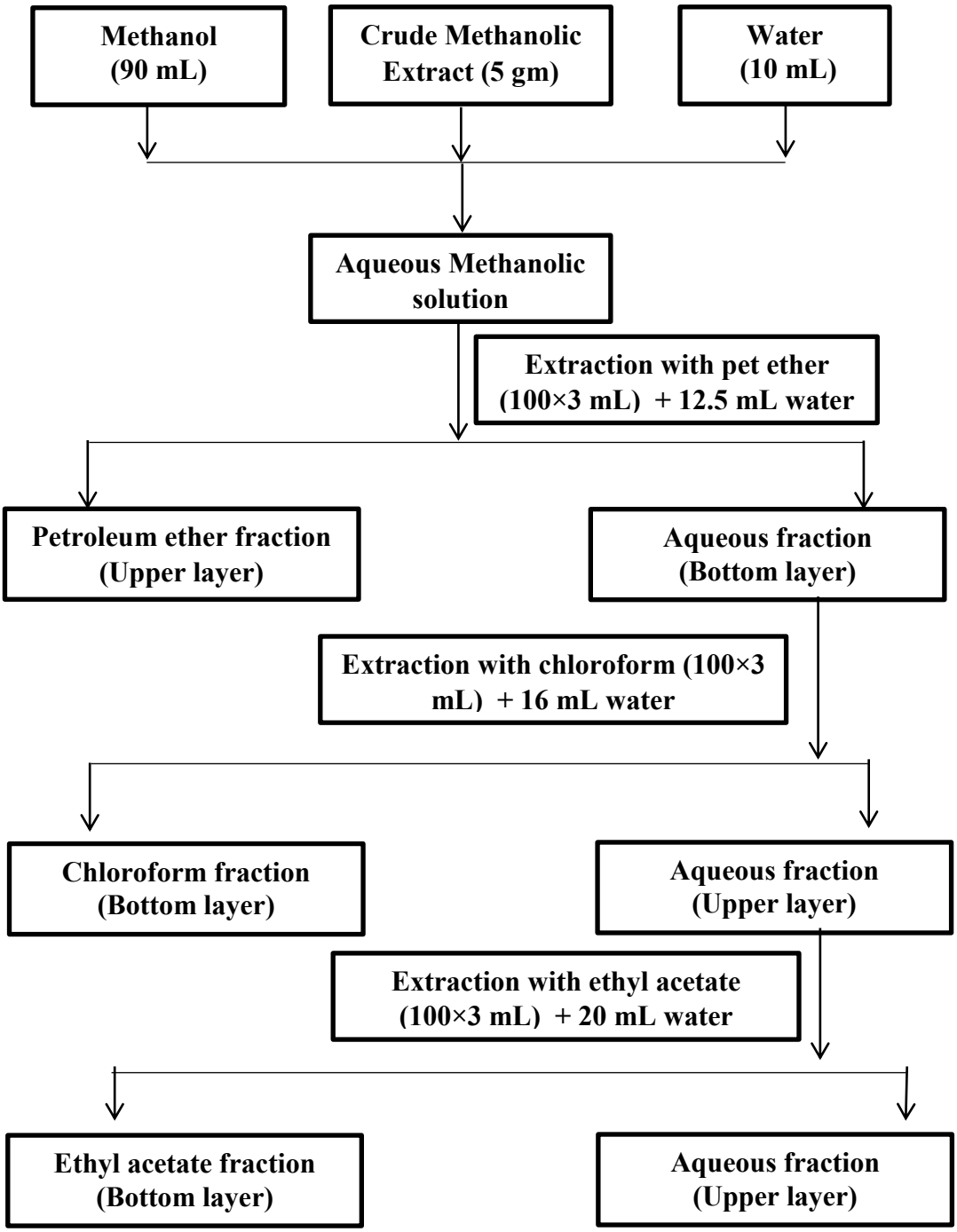

Figure 2.10: Schematic representation of the modified Kupchan Partitioning of methanolic extract of the leaf of *H. schulli.*

Table 2.3: Amount of partitionates obtained from 5 gm of methanolic extract of *H. schulli*

SI. no.	Sample code	Fraction	Weight (gm)
1.	PEF	Petroleum ether fraction	0.83
2.	CLF	Chloroform fraction	0.30
3.	EAF	Ethyl acetate fraction	0.23
4.	AQF	Aqueous fraction	2.00

2.3 Phytochemical screening of the leaf extract

2.3.1 Design of the screening

The phytochemical screening of the crude methanolic leaf extract of *H. Schulli* was performed by using the processes described in Ghani, 2003. The processes described for phytochemical screening in Ghani. A, 2003 are convenient than any other procedures due to the easy availability of the required reagents in the laboratory. Moreover, they are easily understood; thus can be performed without any difficulty (Ghani, 2003). The methanolic extract of *H. schulli* was subjected for phytochemical screening for the following constituents:

- ✓ Carbohydrate
- ✓ Glycosides
- ✓ Flavonoids
- ✓ Tannins
- ✓ Alkaloids
- ✓ Resins
- ✓ Saponins

2.3.2 Chemicals and reagents

The reagents and chemicals used for phytochemical screening were of analytical grade and were obtained from the Department of Pharmacy, BRAC University.

Table 2.4: Chemicals and reagents used during phytochemical screening of *H. schulli*

Chemicals	Reagents
Sodium hydroxide	Dragendorff‘s reagent
Ferric chloride	Molisch‘s reagent
Acetic anhydride	Mayer‘s reagent
Sulfuric acid	Wagner‘s reagent
	Hager‘s reagent

2.3.3 Procedure for screening of phytochemical constituents

To determine the phytochemical constituents from the plant extract, all the processes were done by following standard procedures described in Ghani, 2003. The processes are briefly discussed below:

2.3.3.1 Test for carbohydrates

- **Molisch’s test**

Molisch‘s test is performed to determine the presence of carbohydrate in the sample under investigation. To carry out the test, about 2 mL of aqueous extract and few drops of 10% newly prepared ethanolic solution of alpha-naphthol (prepared by dissolving 10 gm alpha-naphthol in 10 mL ethanol) was mixed together in a test tube. A few milliliter of concentrated sulphuric acid was then poured along the side of the tube to form a layer in the solution. Appearance of red ring between the acid and test layer will confirm the presence of carbohydrate in the sample.

2.3.3.2 Test for glycosides

- **General test for glycosides**

In order to conduct test for glycoside, a very small amount (0.5 gm) of methanolic extract was dissolved in 1 mL of distilled water in a test tube. A 5% sodium hydroxide solution

prepared earlier to this investigation was then added to the test tube and mixed accordingly. If yellow color appears following this, presence of glycoside will be confirmed.

2.3.3.3 Test for saponins

- **Forthing Test**

For conducting the Forthing test, about 0.1 gm of the powdered plant material in 10 mL of distilled water was heated to boil for about 55 minutes. When the solution was boiled, it was allowed to cool for sometimes, after which it was subjected for filtration. Then about 5 mL of distilled water was added to the filtrate to dilute it which was shaken vigorously subsequently. After this, the solution was allowed to stand for about 10 minutes. Presence of saponins will form a dense persistent forth inside the test tube.

2.3.3.4 Test for flavonoids

To screen the presence of flavonoids in the test sample, 1 mL of distilled water and small amount of extract was mixed together in a test tube. Following the addition of few drops of dilute sodium hydroxide, an intense yellow color will appear inside the test tube. Disappearance of this intense color upon addition of acid will indicate the presence of flavonoids in the sample.

2.3.3.5 Test for tannins

- **Lead acetate test**

About 5 mL of aqueous extract and few drops of 1% lead acetate solution were mixed together in a test tube. If a red or yellow colored precipitate is formed following this, result will be considered as positive.

2.3.3.6 Test for resins

A small amount of ethanolic or chloroformic extract was dissolved in about 5-10 mL of acetic anhydride in presence of heat. After cooling for sometimes, about 0.05 mL of sulphuric acid was added to it. Following this, if change of purplish red color of the solution to violet occurs, it will confirm the presence of resins in the sample.

2.3.3.7 Test for alkaloids

- **General laboratory test for alkaloids**

About 0.5 gm of the plant extract and 5 mL of 1% hydrochloric acid were stirred together on a steam bath for sometimes. After this, the solution was subjected for filtration. From the obtained filtrate, 1 mL was taken each time, which was subjected to treatment with each of the following reagents. Presence of alkaloids will be confirmed in the plant extract if the below mentioned colored precipitates are formed following the treatment.

- **Mayer's reagent**

When Mayer's reagent is added to the filtrate, presence of alkaloid will produce white or creamy precipitation in the solution.

- **Hager's reagent**

Addition of Hager's reagent to filtrate will produce yellow color precipitate if alkaloid is present in the test sample.

- **Wagner's reagent**

A brown or deep brown precipitation will appear following addition of Wagner's reagent to the filtrate if alkaloid is present in the sample.

- **Dragendorff's reagent**

Addition of Dragendorff's reagent into the filtrate will produce orange or orange-red precipitation in the solution if the test sample contains alkaloid.

Dragendorff's reagent

Stock solution- For preparing the stock solution, about 4 gm of sodium iodide, 5.2 gm of bismuth carbonate and 50 mL of glacial acetic acid were all boiled together for a short amount of time. Following this, the boiled mixture was allowed to rest for 12 hours, which produced sodium acetate crystal type precipitate in the solution. Then the precipitate was filtered by a sintered glass funnel. Following the filtration procedure, a clear reddish brown filtrate was obtained, from which about 40 mL was taken and added to a solution of 160 mL

of ethyl acetate and 1 mL of water. The solution was then stored in amber colored bottle for later use.

Working solution- The working solution was prepared by mixing 10 mL of stock solution and 20 mL of acetic acid. Later, the volume of the solution was adjusted to 100 mL by adding water to it.

2.3.3.8 Test for steroids

- **Libermann-Burchard's test**

In this test, initially about 1 mL of chloroform was taken to dissolve a small amount of petroleum ether extract of the test sample. This was followed by the addition of about 2 mL of acetic anhydride and 1 mL of concentrated sulphuric acid to it. A greenish colored appearance followed by changing to blue color upon standing will indicate the presence of steroid in the sample.

2.3.3.9 Test for proteins

- **Millon's test**

Followed by dissolution of small amount of aqueous extract in 1 mL distilled water, about 5-6 drops of Millon's reagent was added to the solution. This will form a white precipitate in the solution. This precipitate will turn into red upon heating, if the test sample contains protein in it.

2.4 *In-vitro* antioxidant activity test

2.4.1 Determination of Total Phenolic Content (TPC)

The TPC of the plant extract of *H. schulli* was determined by the modified Folin-Ciocalteu method as mentioned by Wolfe, Wu and Liu (2003).

2.4.1.1 Principle

The crude methanolic extract of *Hygrophila schuli* was subjected to the evaluation of total phenolic content by means of Folin-Ciocalteu Reagent (FCR). In the total phenolic content evaluation, the FCR basically measures the reducing capacity of the sample. Although the chemical property of FCR is not known accurately, it is predicted that it might contain

heteropolyphosphotungstates- molybdates in its structure. In TPC evaluation, a series of reversible one or two –electron reduction reactions produces a blue color, might be $PMoW_{11}O_{40}$. It is proposed that, reduction of molybdenum is easier to carry out in complex form and the electron-transfer reaction usually occurs in between reductants and Mo (VI) (Wolfe, Wu and Liu, 2003).

$$Mo\ (VI) + e^{-} \rightarrow Mo\ (V)$$

2.4.1.2 Reagents and chemicals

Table 2.5: Reagent/chemicals used in Folin-Ciocalteu method

S.I. #	Name of Reagents/Chemicals	Manufacturing Company
1.	Folin-Ciocalteu Reagent (FCR)	LOBA Chemie Pvt. Ltd., India
2.	Gallic acid monohydrate (Standard)	Sigma Aldrich, USA
3.	Sodium carbonate	Merck Specialities Pvt. Ltd., Mumbai
4.	Methanol	Active Fine Chemicals Ltd., Bangladesh

- **Reagent preparation**

About 250 mL of 10% FCR solution was prepared by taking 25 mL of FCR in a 250 mL volumetric flask and diluting it with distilled water to 250 mL mark.

On the other hand, 100 mL of 7.5% (w/v) sodium carbonate was prepared by dissolving 7.5 gm of sodium carbonate in a 100 mL of distilled water in a 100 mL volumetric flask.

- **Sample, standard and blank preparation**

The sample stock solution was prepared by dissolving 120 mg of *H. schulli* extract in 10 mL of methanol which produced a concentration of 12 mg/mL of the sample stock solution. Then serial dilution was performed on this sample stock solution to derive 4 serially diluted concentration: 1200, 800, 400 and 200 ug/mL respectively.

Gallic acid was used as the standard in this experiment. Stock solution of gallic acid was prepared in same manner as it was prepared for the sample stock solution. Serial dilution was also performed for the standard stock solution to produce 4 serially diluted

concentration: 1200, 800, 400 and 200 ug/mL respectively. From these serially diluted solutions, a calibration curve of absorbance vs. concentration was later developed for the investigation.

The blank solution was prepared by mixing 5 mL of FCR solution and 4 mL of sodium carbonate. However, the volume of the blank was finally adjusted to 10 mL by adding 1 mL of methanol to it.

2.4.1.3 Experimental procedure

Initially, 1 mL of the methanolic fraction of sample and standard (gallic acid) in different concentration were taken in different test tubes. Then about 5 mL of FCR solution and 4 mL of sodium carbonate (7.5%) were added to the test tubes. The mixture was then vortex for about 15 seconds and then allowed to stand for 30 minutes at 40°C in a water bath. Finally, the absorbance of standard and sample solutions was measured against blank at 765 nm using spectrophotometer (U-2910 UV-Vis Spectrophotometer). The total phenolic content of the methanolic fractions was then expressed as Gallic Acid Equivalents using the following equation:

$$C = \frac{(c \times V)}{M}$$

Where, C = Total content of phenolic compounds, mg of gallic acid per gram of dried plant extract, expressed as Gallic acid equivalent (GAE)

c = Concentration of gallic acid obtained from calibration curve (mg/mL)

V = Volume of sample solution (mL)

m = weight of the sample (gm)

The experiment was repeated to confirm reproducibility.

2.4.2 DPPH (1, 1-diphenyl-2-picryl hydrazyl) free radical scavenging assay

Antioxidant activity of *H. schulli* was determined by the method described by Brand-Williams et al., (1995). DPPH is considered as the most accepted method in the field of research, to determine the ability of free radical scavenging activity of any new and latent

drug**.** However, this method basically evaluates the free radical scavenging activity of the sample on 2-2-Diphenyl1-1-Picrylhydrazyl (DPPH) free radical (Brand-Williams et al., 1995**)**.

2.4.2.1 Principle

To evaluate the antioxidant activity by DPPH method, 1 mL of methanolic extract solution of the crude plant extract at different concentration was mixed with 3 mL of methanolic solution of DPPH. As a standard, ascorbic acid in a concentration range of 1-100µg/mL was used. Blank sample was also prepared for the investigation. After preparing the sample and blank solution, they were kept in a dark place for 30 minutes. Then the antioxidant activity of the plant extract was measured by analyzing their absorbance under UV-Spectrophotometry at 517 nm wavelength.

To calculate the % of inhibition the following formula was used:

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

Where, A_{sample} is absorbance of the sample and A_{blank} is absorbance of control, which contains all reagents except the test sample.

The extract concentration which will provide 50% inhibition will be then calculated from the graph plotted by % of inhibition vs. extract concentration.

Due to the presence of odd electron in the DPPH free radical, DPPH gives a strong absorption maximum at 517 nm. If the test sample contains antioxidant property, it will reduce the DPPH to DPPH-H, when the odd electron of antioxidant is paired with the hydrogen from the free radical scavenging antioxidant. Moreover, due to the reduction of DPPH, the purple color of DPPH will turn into yellow color. However, DPPH radical scavenging activity is expressed as IC_{50} which is the concentration of samples to produce 50% reduction of the DPPH.

2.4.2.2 Materials

Table 2.6: List of materials used in the DPPH assay

Materials	Reagents \ Solvents
Pipette (1mL and 5mL)	Extracts of the experimental plant
Light proof box	2,2-Diphenyl-1-Picrylhydrazyl (DPPH)
UV-spectrophotometer	Ascorbic Acid
Volumetric flask (25mL)	Methanol UV-spectrophotometer
Test tubes	Distilled water

2.4.2.3 Procedure

- **Preparation of control for the evaluation of antioxidant activity**

Table 2.7: Amount of reagents required for preparing stock solution

Name	Amount
Ascorbic acid	0.0005gm
Methanol	1.000mL

For the current investigation, ascorbic acid was used as the positive control. To prepare the stock solution of positive control, about 0.0005 gm of acid was taken in a volumetric flask. Then 1 mL of methanol was added to it so that the concentration of the final solution becomes 500 µg/mL. After that the stock solution was subjected for serial dilution method to get solutions of different concentration ranging from 500 µg/mL to 0.977 µg/mL.

- **Preparation of test samples**

Table 2.8: Test sample of experimental plant

Sample code	Test sample	Calculated amount (gm)
ME	Methanolic Extract	0.0005

In order to prepare the test sample, at first 0.0005 gm of the methanolic fraction of *H. schulli* was taken in a separate test tube. After that, 1 mL of methanol was added to the test tube to get a concentration of 500 µg/mL. Then this stock solution of each fraction was subjected to serial dilution to get solutions of different concentration ranging from 500 µg/mL to 0.977 µg/mL.

- **Preparation of DPPH Solution**

Table 2.9: Amount of reagents required for preparing DPPH solution

Name	Amount
DPPH	2.00 mg
Methanol	50.00 mL

To prepare the DPPH solution, 2 mg of DPPH was measured accurately which was then dissolved in 50 mL of methanol to get a concentration of 40 µg/mL. Then the solution was kept in a dark box that was covered with aluminum foil paper.

For conducting the experiment, about 2 mL solution was taken from each of the serially diluted solutions (500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.91, 1.95 and 0.977 µg/mL) in separate test tubes. After that, each of the solution was mixed with 3.0 mL of DPPH methanolic solution. Then, all the samples were kept in a dark place for 30 minutes for the reaction to take place. The absorbance of each sample were measured by using UV-spectrophotometry at 517 nm wavelength. During the measurement of absorbance, methanolic solution was taken as blank for the experiment.

Free radical DPPH inhibition was expressed in percent (I %) using the following formula:

$$\text{Inhibition \% (I \%)} = [(\text{Absorbance}_{\text{Blank}} - \text{Absorbance}_{\text{Sample}}) / \text{Absorbance}_{\text{Blank}}] \times 100$$

Finally, the extract concentration which had rendered 50% inhibition (IC_{50}) was determined from the graph of inhibition percentage (I %) against the concentration of extract (µg/mL).

2.5 Brine shrimp lethality bioassay

2.5.1 Principle

In current investigation, cytotoxic activity of the methanolic crude extract of *Hygrophila schulli* was examined using the brine shrimp lethality bioassay. To carry out the experiment, about 4 mg of the methanolic extract was dissolved in 200 μl of dimethylsulfoxide (DMSO) initially. Then serial dilution was performed on this solution using simulated sea water to produce different concentration of the test sample: 400, 200, 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78 μg/mL. These serially diluted solutions were then added to the pre-marked vials previously containing 10 live brine shrimp naupli in 5 mL simulated sea water. After 48 hours, the survived naupli in the vials were counted using a magnifying glass (Mayer et al., 1982).

2.5.2 Materials

Table 2.10: Materials used for the test

Artemia salina leach (brine shrimp egg)	Micropipette
Sea salt (NaCl)	Glass vials
Small tank with perforated dividing dam	Magnifying glass
Lamp	Test tubes
Pipette	Extracts of experimental plant

2.5.3 Procedure

- **Preparation of simulated sea water**

For the experiment, simulated sea water was prepared by dissolving 38 gm of sea salt in 1 liter of distilled water, which was filtered off subsequently to get clear solution for the experiment.

- **Preparation of test organisms**

As test organism, *Artemia salina* leach (brine shrimp) eggs were collected from a pet shop for the experiment. A small tank was taken, in which brine shrimp eggs were added to one

side while being the other side covered. It took two days for the eggs to be hatched as shrimp and matured into nauplii. During all this period of hatching, oxygen was supplied to the tank continuously. A lamp was also arranged at top of the tank to attract the shrimp towards the illuminated part of the tank. After hatching, a pipette was used to transfer the nauplii to a fish tank in which fresh water was added further to improve the visibility of the nauplii. From this tank, 10 nauplii was transferred to different test tubes each time with the help of a micropipette.

- **Test solution preparation with sample of experimental plant**

At first, about 16 mg of the methanolic crude extract of *Hygrophila schulli* was dissolved in 200 μl of pure dimethyl sulfoxide (DMSO). The volume of this solution was then adjusted to 20 mL by adding sea water to it, which made the concentration of final solution 800 μg/mL. This solution was then serially diluted with sea water to give a series of different concentration of solutions: 400, 200, 100, 50, 25, 12.5, 6.25 μg/mL. From this series of solutions of different concentration, about 2.5 mL was taken each time and added to 10 nauplii containing 2.5 mL of sea water. This made the final volume of the solution 5 mL.

Table 2.11: Test samples with concentration values after serial dilution

Test Tube No.	Concentration (µg/mL)
1	400.0
2	200 .0
3	100 .0
4	50 .00
5	25 .00
6	12.50
7	6.250
8	3.120
9	1.560
10	0.780

- **Control groups preparation**

To validate the experimental method as well as to ensure that the results obtained from the experiment are only due to the test agent activity in absence of other possible effects, two control groups were used during the experiment:

i) Positive control: For the current investigation, vincristine sulphate, a widely accepted cytotoxic agent was used as the positive control. The stock solution of vincristine sulphate was prepared by dissolving 2 mg vincristine sulphate in 100 mL of DMSO to make the final concentration 20 µg/mL. Then serial dilution was carried out on this stock solution in order to obtain various concentrations (10, 5, 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078 and 0.0390 µg/mL). From this series of solutions of different concentration, about 2.5 mL was taken each time and added to 10 nauplii containing 2.5 mL of sea water.

ii) Negative control: To prepare negative control, about 5 mL simulated sea water was taken in three different pre-marked vials. After this, 100 µL of DMSO and 10 shrimp nauplii were added to each of these vials. A constant mortality of the nauplii in the three vials will point out the invalidity of the test results as it will indicate that nauplii died because of other reasons rather than the test sample.

- **Nauplii counting:**

After 48 hours, all the vials were examined using a magnifying glass against a black background to count the survived nauplii. This data was then used to calculate the percent (%) of lethality of the brine shrimp for each concentration.

In general, the median lethal concentration (LC_{50}) is used to represent the effectiveness or the concentration-mortality relationship of the experimental plant compound. It is the concentration of the chemical that produces death in 50 % of the test subjects. It is determined by linear regression analysis of plot of % mortality against respective concentration.

2.6 Anti-microbial assay

In current investigation, different fractions of crude methanolic leaf extract of *Hygrophila schulli* were subjected for antibacterial screening against a number of gram positive and gram negative bacteria.

Table 2.12: Gram (+ve) and Gram (-ve) Bacteria

Gram (+ve) Bacteria	Gram (-ve) Bacteria
Staphylococcus aureus	*Salmonella paratyphi*
Bacillus megaterium	*Escherichia coli*
Bacillus subtilii	*Shigella dysenteriae*
Bacillus cereus	*Vibrio mimicus*
Sarcina luteae	*Vibrio parahaemolyticus*

Generally, evaluation of anti-bacterial activity is usually done in two phases which are primary qualitative assay and secondary assay. Primary qualitative assay is performed to determine the presence or absence of anti-bacterial activity in the sample. In general, three methods are available to serve the purposes (Bayer et al., 1966):

i. Diffusion method
ii. Dilution method
iii. Bio autographic method

From the above mentioned three methods, the disc diffusion method is the most accepted method for the primary screening of anti-bacterial activity in the supplied sample. Although, the method cannot distinguish between bactericidal and bacteriostatic effects, it can indicate the sensitivity or resistance of a particular microorganism to the test agent (Bayer et al., 1966).

On the other hand, secondary assay is very useful in developing a new antibacterial agent. The advantage of this method is that it can quantify the relative potency of a pure compound. It is expressed as Minimum Inhibitory Concentration (MIC) (Bayer et al., 1966).

However, the current study evaluated the anti-bacterial potency of the supplied sample by disc diffusion method.

2.6.1 Principle

The disc diffusion method evaluates the antibacterial potency of a particular sample by a observing the ability of that sample in diffusing from a confined source to a nutrient agar media by creating a concentration gradient.

To carry out the investigation, solutions of known concentration of the test samples were initially prepared by dissolving calculated amount of test sample in definite volume of solvents. Then known amounts of the test samples were impregnated on sterile filter papers (5 mm in diameters) which are subsequently dried. After that petridishes (120 mm in diameter) containing a suitable medium seeded with microorganisms were arranged in order to place dried discs in them. To allow maximum diffusion, the disc containing petridishes were then kept at low temperature (4℃) for about 24 hours. During this period, following events took place simultaneously:

i. Absorption of water by the dried discs from the agar medium, which causes the material under test to dissolve.
ii. Diffusion of the test material from the discs to the surrounding agar medium.
iii. A gradual change of concentration of test material surrounding each disc in the agar media.

To allow maximum growth of the organisms, these petridishes were then allowed to incubate at 37℃ for 12-18 hours. Presence of antibacterial activity in the sample will inhibit the growth organisms in the petridishes and give a clear, distinct zone of inhibition called ―zone of inhibition‘‘ in the petridishes. The diameter of the zone of inhibition, expressed in millimeter will then indicate the anti-bacterial activity of the test sample. In general, greater the zone of inhibition, greater the anti-bacterial activity of the test sample is considered. However, the size of the zone of inhibition is usually depends on a number of factors such as intrinsic antimicrobial susceptibility of the test sample, growth rate of test organisms etc. (Gami & Parabia, 2011).

2.6.2 Test organisms

In order to evaluate the antibacterial activity of different fractions of crude extracts of *Hygrophila schulli* (methanolic fraction, petroleum ether fraction, chloroform fraction, ethyl acetate fraction and aqueous fraction), both gram (+ve) and gram (-ve) bacterial strains were used in the current investigation. For this investigation, the organisms were arranged from the Department of Mathematics and Natural Sciences, BRAC University.

2.6.3 Apparatus/Reagents

During antibacterial investigation, the below mentioned reagents and equipment were used:

1. Filter paper discs
2. Petridishes
3. Sterile forceps
4. Test tubes
5. Inoculating loop
6. Sterile cotton
7. Bunsen burners
8. Laminar air-flow unit
9. Micro-pipette
10. Incubator
11. Autoclave
12. Alcohol
13. Methanol
14. Nutrient agar media

Apart from these, different fractions of crude extracts of *Hygrophila schulli* (methanolic fraction, petroleum ether fraction, chloroform fraction, ethyl acetate fraction and aqueous fraction) were as used as the test materials.

2.6.4 Procedure

- **Sterilization procedure**

Sterilization of petridishes and other glass wares were performed by autoclaving at 121℃ and 152 1bs/sq inch pressure for 20 minutes. On the other hand, dry heat sterilization was

performed for the blank discs by keeping them in covered petri-dishes at 180℃ for 1 hour. Followed by the sterilization procedure, the sterilized glass wares and blank discs were then kept in a laminar hood under UV light for 30 minutes. To avoid any kind of accidental contamination, the UV light of the laminar hood was switched on one hour prior working.

- **Culture media**

The growth of bacteria in a culture media depends on the following requirements:

1. Energy source such as carbohydrate, protein and nucleic acid.
2. Essential trace elements for example, Fe, Co, Mg etc.
3. Optimal media pH.
4. Optimal incubation temperature.

However, a number of culture media can be used to evaluate antibacterial activity:

1. Nutrient broth media
2. Nutrient agar media
3. Muller-Hinton agar media
4. Tryptic soy broth (TSB)

For the current investigation, Nutrient agar media was selected, composition of which is given below:

Table 2.13: Composition of Nutrient Agar Media

Ingredient	Amount
Bactopeptone	0.5 g
Sodium chloride	0.5 g
Bacto yeast extract	1.0 g
Bacto agar	2.0 g
Distilled water	100 mL
pH	7.2 at 25℃

- **Preparation of medium**

For preparing culture media, accurately weighed nutrient agar media was taken in a conical flask which was reconstituted with distilled water immediately as indicated in specification (2.8% w/v). To dissolve the agar in the water, the mixture was heated in a water bath until a clear solution was obtained.

Figure 2.11: Freshly prepared liquid agar medium.

When the media was prepared, it was transferred to test tubes for preparing plates and slants. The plates and slants were prepared by taking 20 mL and 5 mL of the media respectively in different test tubes. From the prepared slants, the sub-culture of microorganisms was made subsequently. These test tubes were then cotton plugged and autoclaved at 121℃ and 152 Ibs/sq inch for about 15 minutes.

- **Subculture preparation**

Under a laminar air flow unit, the test organisms were transferred from the pure culture to the previously prepared agar slants with the help of an inoculating loop. To ensure the growth of the test organisms, these inoculated slants were then kept in incubator at 37℃ for about 18-24 hours. After incubation, the culture was used for the antibacterial test.

- **Test plates preparation**

From the subculture, each of the microorganisms were transferred to different test tubes previously containing 20 mL agar media with the help of inoculating loop under a laminar hood. These test tubes were than rotated in order to get a uniform suspension of the organisms. Immediately after rotation, they were taken to different sterile petridishes in an

aseptic area, where they were rotated again both in clockwise and anti-clockwise direction for several times. This had ensured uniform distribution of the micro-organisms into the medium. It was taken into consideration that the depth of media in the petridishes remains within 4 mm. These plates were then cooled to room temperature and kept in refrigerator at 4℃.

- **Disc preparation**

Disc diffusion assay is generally carried out by using two types of discs: sample discs and blank discs.

For preparing sample discs, previously sterilized filter discs, each 5 mm in diameter, were taken in a blank petri-dish. In an aseptic condition, sample solution of desired concentration was applied to each of the discs with the help of a micropipette. Sample solution of desired concentration was prepared by dissolving 10 mg of each of the different fractions of crude extracts of *Hygrophila schulli* (methanolic fraction, petroleum ether fraction, chloroform fraction, ethyl acetate fraction and aqueous fraction) in 200 μl methanol in order to obtain a concentration of 500 μg/10 μl for each concentration. For complete solvent removal, these discs were then kept for few minutes in an aseptic condition.

In the investigation, blank discs were used in order to determine anti-microbial effect of the solvent. These discs were prepared by applying only solvent to it.

Apart from these discs, ciprofloxacin discs were used as the reference standard.

- **Disc placement, diffusion and incubation**

With the help of a sterile forceps, both the sample and standard discs were carefully placed on the solidified agar plates freshly seeded with the test organisms. A complete contact with the medium surface was ensured during this placement. Additionally, the distance between each disc was also kept at least 15 mm in order to prevent overlapping with the zone of inhibition. After this, the plates were inverted and then kept in a refrigerator at 4℃ for about 24 hours to allow diffusion of the material into the surrounding media. At the end, they were kept in an incubator at 37℃ for about 12-18 hours.

- **Evaluation of anti-bacterial activity**

After completion of incubation for about 24 hours, the zone of inhibition was measured for each of the discs in order to evaluate the antibacterial activity of test sample with the help of a transparent scale.

Figure 2.12: Close inspection after 24 hours of the growth of bacteria around the disks.

Chapter 3

Results

3 Results

3.1 Phytochemical screening of methanolic extract of *Hygrophila schulli*

Table 3.1: Result of phytochemical screening of *Hygrophila schulli*

S.I. no.	Class of compound	Result
1.	Alkaloids	+++
2.	Flavonoids	+
3.	Phenols/Phenolic compounds	+
4.	Glycosides	+
5.	Tannins	+
6.	Carbohydrates	-
7.	Steroids	+
8.	Saponins	-

Note: (+) means presence in a single method test, (+++) means presence in three method tests, and (-) means absence.

3.2 Evaluation of antioxidant activity

3.2.1 Total phenolic content (TPC) of methanolic extract of *H. schulli*

Table 3.2: Absorbance of standard (gallic acid) and methanolic extract of *H. schulli* against their respective concentration

Conc. of standard (μg/mL) (gallic acid)	Absorbance of standard (gallic acid)	Concentration of extract	Absorption of extract
200	0.405	200	0.619
400	0.898	400	0.635
800	1.980	800	0.611
1200	3.021	1200	0.541

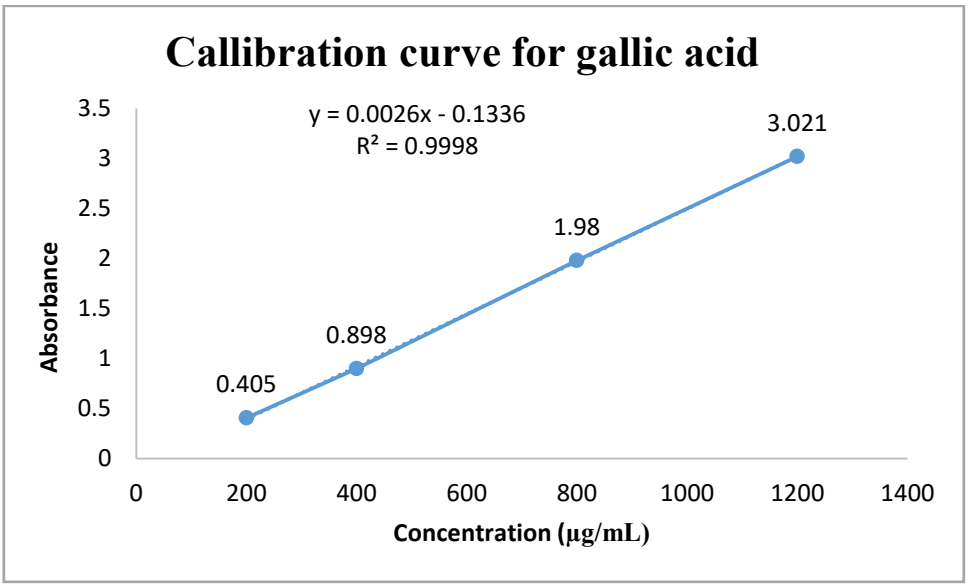

Figure 3.1: Standard calibration curve of gallic acid at 765 nm for determining TPC of *H. schulli*.

Table 3.3: Total phenolic content (TPC) in methanolic extract of *H. schulli*

Sample solution(mg /mL)	Weight of dry extract per mL(gm), m	Absorbance	GAE concentration (mg/mL), c	TPC as GAE, A =(c X V)/m (mg/mL)	Mean value±stdv
12	0.012	0.619	0.289	24.083	23.539±1.34
12	0.012	0.635	0.296	24.66	
12	0.012	0.611	0.286	23.833	
12	0.012	0.541	0.259	21.583	

3.2.2 DPPH free radical scavenging activity of methanolic extract of *H. schulli*

Table 3.4: IC_{50} value (μg/mL) of ascorbic acid

Absorbance of blank	Conc. of Ascorbic acid (μg/mL)	Absorbance of the ascorbic acid	% of inhibition	IC_{50} (μg/mL)
	500.000	0.004	98.87	
	250.000	0.007	98.03	
	125.000	0.016	95.49	
	62.500	0.030	91.55	
0.355	31.250	0.075	78.87	156.313
	15.625	0.094	73.52	
	7.813	0.142	60.00	
	3.906	0.182	48.73	
	1.953	0.174	50.98	
	0.977	0.195	45.07	

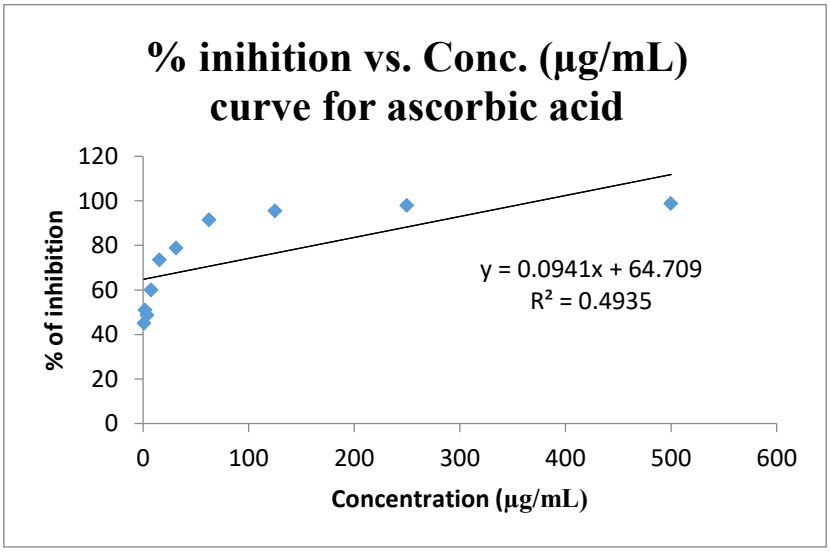

Figure 3.2: % inhibition vs. Conc. (µg/mL) curve for ascorbic acid.

Table 3.5: IC_{50} value (μg/mL) of methanolic extract of *H. schulli*

Absorbance of blank	Conc. of extract (μg/mL)	Absorbance of the extract	% of inhibition	IC_{50} (μg/mL)
	500.000	0.045	94.73	
	250.000	0.056	93.45	
	125.000	0.060	92.98	
	62.500	0.058	93.22	
0.355	31.250	0.062	92.74	978.704
	15.625	0.071	80.00	
	7.813	0.072	79.72	
	3.906	0.074	79.15	
	1.953	0.082	76.90	
	0.977	0.088	75.21	

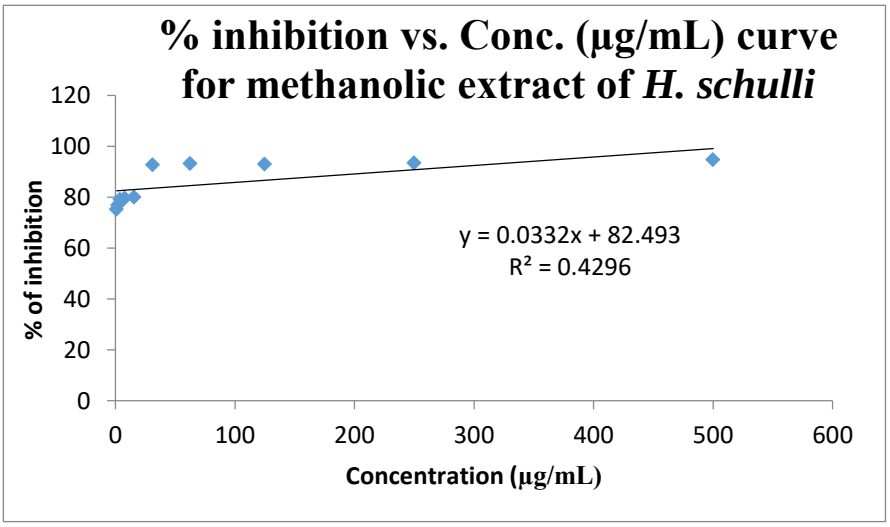

Figure 3.3: % inhibition vs. Conc. (µg/ for mL) curve methanolic extract of *H. schulli.*

3.3 Brine shrimp lethality assay of methanolic extract of *H. schulli*

Table 3.6: Effect of vincristine sulphate (positive control) on the shrimp nauplii

Concentration (μg/mL)	Log_{10} Concentration	% Mortality	LC_{50} (μg/mL)
10.000	1.000	100	1.115
5.000	0.699	90	
2.500	0.398	80	
1.250	0.097	60	
0.625	-0.204	50	
0.312	-0.505	50	
0.156	-0.806	40	
0.078	-1.107	30	
0.039	-1.409	20	

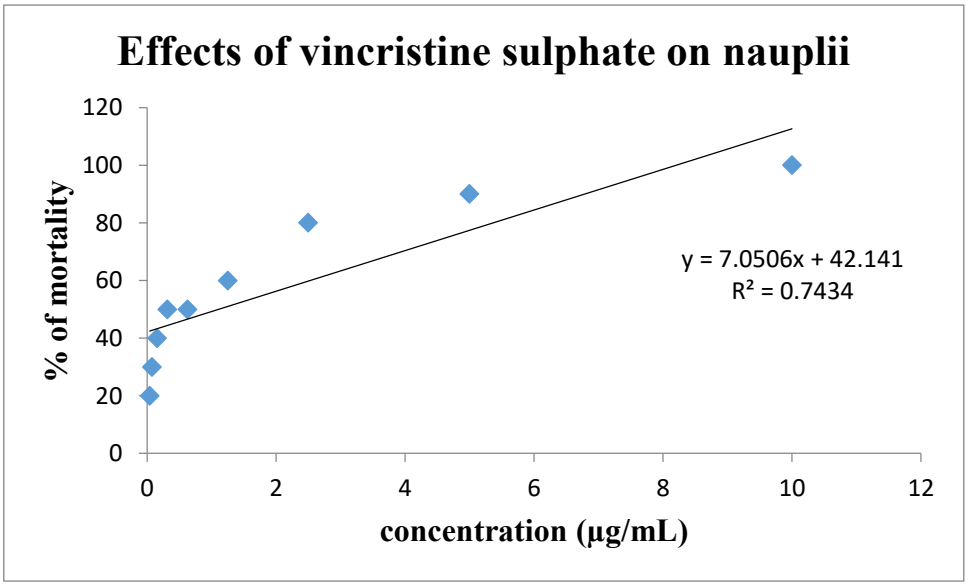

Figure 3.4: % of mortality vs. Conc. (µg/mL) curve for vincristine sulphate.

Table 3.7: Effect of methanolic extract of *H. schulli* on the shrimp nauplii

Concentration (μg/mL)	Log_{10} Concentration	% Mortality	LC_{50} (μg/mL)
400.0	2.602	60	294.182
200.0	2.301	40	
100.0	2.000	30	
50.00	1.699	30	
25.00	1.398	20	
12.50	1.097	20	
6.250	0.796	20	
3.125	0.495	10	
1.562	0.194	10	
0.781	-0.107	10	

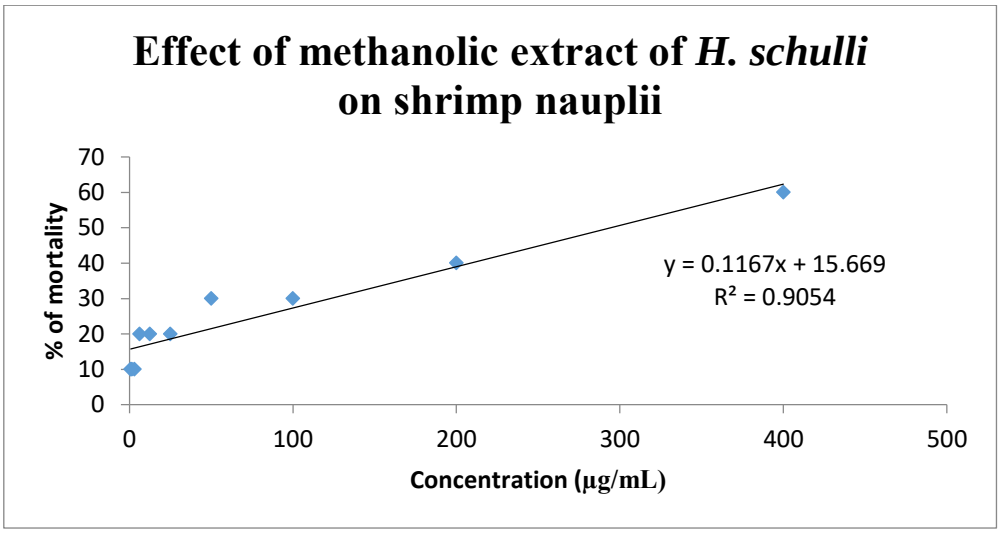

Figure 3.5: % of mortality vs. Conc. (µg/mL) curve for methanolic extract of *H. schulli.*

3.4 Anti-microbial assay of fractional extracts of *H. schulli*

Table 3.8: Anti-microbial assay of fractional extracts of *H. schulli*

Test microorganisms	**Diameter of zone of inhibition(mm)**					
	MEF	**PEF**	**CF**	**EAF**	**AQF**	**Ciprofloxacin**
Gram positive bacteria						
Staphylococcus aureus	-	-	-	-	-	40
Bacillus megaterium	-	-	-	-	-	46
Bacillus subtilii	-	-	-	-	-	34
Bacillus cereus	-	-	-	-	-	41
Sarcina luteae	-	-	-	-	-	42
Gram negative bacteria						
Salmonella paratyphi	-	-	-	-	-	38
Escherichia coli	-	-	-	-	-	37
Shigella dysenteriae	-	-	-	-	-	43
Vibrio mimicus	-	-	-	-	-	35
Vibrio parahaemolyticus	-	-	-	-	-	45
(-) means zone of inhibition is below 6mm						

Here, MEF=Methanolic Extract Fraction, PEF=Pet Ether Fraction, CF=Chloroform Fraction, EAF= Ethyl Acetate Fraction and AQF=Aqueous Fraction.

Chapter 4

Discussion

Discussion

In this present study, different phytochemical screening procedures performed on the methanolic leaf extract of *Hygrophila schulli* revealed the presence of constituents like alkaloids, flavonoids, phenols, glycosides, tannins as well as steroids in this specific plant. Thus, it can be suggested that the plant might have strong medicinal value due to the presence of all these compounds.

The antioxidant potential of methanolic extract of *H. schulli* was also evaluated in this present study by means of DPPH free radical scavenging (FRS) assay and total phenolic content (TPC) assay. In DPPH assay, ascorbic acid was selected as the reference standard, for which fifty percent inhibition was found at 156.313 μg/mL concentration (IC_{50} value). On the other hand, the methanolic extract of *H. schulli* showed fifty percent inhibition at 978.704 μg/mL concentration (IC_{50} value).

The total phenolic content of methanolic extract of *H. schulli* was evaluated by Folin-Ciocalteu method using gallic acid as the reference standard. The total phenolic content of the extract was expressed in terms of gallic acid equivalent (the calibration curve equation: y= 0.0026x-0.1336, R^2=0.9998). In the investigatigation, the total phenolic content of the test sample was obtained in the range of 23.539$\pm$1.34 mg GA/g. Based on the DPPH free radical scavenging (FRS) assay and total phenolic content (TPC) assay of methanolic extract of *H. schulli,* it can be predicted that the selected plant has significant anti-oxidant potential.

In order to study the cytotoxic potential of the plant, brine shrimp lethality assay was performed on the methanolic extract of *H. schulli*, taking vincristine sulphate as the reference potential. However, the study may also indicate anti-fungal, pesticidal, teratogenic effects alongside the cytotoxic activity of the plant (Meyer et al., 1982). The present investigation showed LC_{50} values of methanolic extract of *H. schulli and* vincristine sulphate at 1.115 μg/mL and 294.182 μg/mL concentration respectively. The investigation has also showed a proportionate relationship between concentration and degree of lethality for both reference standard and test sample. As indicated by Meyer, LC_{50} value lower than 1000 μg/mL usually indicate considerable bioactivity (Meyer et al., 1982), based on the value obtained from this study, it can be predicted that the plant might have potential

cytotoxic activity. Moreover, since the presence of constituents like alkaloid, steroids, phenols and flavonoids are determined from the preliminary phytochemical investigation; it can be proposed that, their presence in the plant might be responsible for their cytotoxic activity.

The present dissertation also aimed at the evaluation of anti-microbial potential of different fractions of leaf extract of *H. schulli*: methanolic extract fraction (MEF), pet ether fraction (PEF), chloroform fraction (CF), ethyl acetate fraction (EAF) and aqueous fraction (AQF). In this study, ciprofloxacin was taken as a reference standard for which zone of inhibition was obtained in the range of 35-46 mm against the selected gram (+ve) and gram (-ve) bacteria. On the other hand, all the test samples showed negative response against all the test organisms. Thus, from the observation, it can be proposed that the plant has no antibacterial activity and it cannot be used as antibacterial agent.

Chapter 5

Conclusion

Conclusion

In this present investigation, the methanolic extract of *Hygrophila schulli* was subjected to phytochemical screening as well as to anti-oxidant activity and cytotoxic activity evaluation while fraction extracts have been taken for anti-microbial activity. The phytochemical screening of *H. schulli* indicated the presence of many phytochemical constituents namely, alkaloids, tannin, flavonoids, phenolic compounds, glycosides, steroids and the absence of proteins and saponins in this plant. In total phenolic content assay, a higher value of total phenolic content was obtained for the methanolic extract which clearly indicates its potentiality as antioxidant drug in the modern medicine practice and research. Brine shrimp lethality assay, performed on the methanolic extract of *H. schulli* also showed a positive response towards the shrimp nauplii. In this study, vincristine sulphate was taken as the standard to compare the result. On the other hand, the plant showed no sensitivity in antimicrobial assay, which clearly indicates lack of antimicrobial effect of the test sample. The antimicrobial assay was carried out by taking ciprofloxacin as the reference standard.

Thus the present study has revealed potential bioactivity of *H. schulli* which has encouraged further extensive studies in terms of antioxidant and cytotoxic activity to be carried out in the *in-vivo* model to demonstrate the plant efficiency in terms of pharmacological effects in physiological system.

Chapter 6

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