Investigation of Phytochemical and *In-vitro* Biological Potential of *Anisoptera scaphula*

A project submitted

by

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Dedicated to my family

Certification Statement

This is to certify that this project titled 'Investigation of Phytochemical and i*n-vitro* Biological Potential of *Anisoptrea scaphula*' 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

_____

Acknowledgement

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Abstract

This study was intended to identify and assess the potential phytochemical as well as biological properties of the medicinal plant *Anisoptrea scaphula* (family: Dipterocarpaceae). To fulfill these purposes, different experiments were carried out, such as phytochemical screening, antioxidant activity test, brine shrimp lethality bioassay and thrombolytic activity test. It is evident from different observations that, this plant is an abundant source of large number of long chain hydrocarbons, beta-setosterol, beta-setosterolpalmitate, beta-setosterol-beta-D-glucosidase, mannitol. In the existing investigation, phytochemical screening has indicated the presence of tannins, saponins, carbohydrates, glycosides and glucosides in this particular plant. Additionally, moderate antioxidant, antimicrobial activity and thrombolytic activity of*Anisoptrea scaphula* were observed from the experiments. However, it showed significant cytotoxicityin brine shrimp lethality bioassay. So, on the basis of the present investigation, it can be proposed that the plant "Anisoptrea scaphula" can be used as a medicinal plant.

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List of abbreviations:

- 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
- *A.scaphula= Anisoptera scaphula.*
- WHO= World Health Organization
- m= Meter
- cm= Centimeter
- gm= Gram
- Conc.= Concentration
- ME= Methanolic Extract
- PEF= Pet Ether Fraction
- CF= Chloroform Fraction
- EAF=Ethyl Acetate Fraction

Chapter 1

Introduction

1. Introduction

Man has always cherished nature for its resources from the very beginning of its existence to make his life comfortable. Man is dependent on nature's elements for different purposes starting from their food to cure diseases. Our universe is enriched with millions of wonderful and mysterious elements and our environment is one of them. Medicinal plants or trees are of a great use for human health. As observed in the past era, a vast number of diseases of different kinds were traditionally treated with agents that are either collected or derived from plants and trees, considered as 'Herbal Medicine'. Hence, from then to till now, it is considered as one of the most powerful source of medicine.

Disease free, safe and sound life is everyone's desires in life and to achieve this inclination, human are always engaged in discovering things to make their life comfortable. In this long search, our ancestor discovered the magical power of plants around them, the power which can help them to get cured from diseases. However, during that period, they were unaware about the 'Pharmacological Properties' of plants.

Nevertheless, people of ancient civilization did know the key uses of some herbal plants, these plants were able to cure simple disease and more complicated live threatening diseases. Different herbs are being used as a source of safe medication from the early period to modern civilization. However at that time, the modern medicines was not even invented (Mercola, 2014). Some of the traditional herbal medication systems are given below:

- Amla (*Phyllanthus emblica*): The plant is mainly known for its antioxidant properties,for which it is used in the Indian Ayurvedic medicine system to treat inflammation of joints, fever and to maintain blood sugar level l (Barros, Healing Herbs and Medicinal Plants List, 2011-2016).
- Aloe (*Aloe vera*): 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, Healing Herbs and Medicinal Plants List, 2011-2016).

- Lemongrass (*Cymbopogon citratus):* It is used to promote sound sleep and to reduce anxiety. Lemongrass tea is also used as a relaxing beverage (Barros, Healing Herbs and Medicinal Plants List, 2011-2016).
- Cinnamon (*Cinnamonum verum):* Cinnamon is most often used in treatingconstipation. The derived oil from Cinnamon has essential antifungal, antibacterial and antispasmodic properties (Barros, Healing Herbs and Medicinal Plants List, 2011-2016).
- Turmeric (*Curcuma longa):* Turmeric has potent antioxidant properties. However, it is also found to function as a natural detoxifier (Barros, Healing Herbs and Medicinal Plants List, 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, Healing Herbs and Medicinal Plants List, 2011-2016).

With these continuous discoveries and development, human gradually came into conclusion that plants are actually the 'Global Medicinal Assets' because they contain such properties which can combat and heal numerous diseases of our body.

1.1 Medicinal plants and herbal medicine

Plants those contain characterized natural constituents, which can be used as medicinal purposes, although are not necessarily a product or available to be marketed, are called 'Medicinal plants'.

Now a day, plant derived materials or products containing raw or processed materials from one or more plants, which can be used for therapeutic or other beneficial purposes as well for human health, are known as 'Herbal Medicine'. For example, *Allium cepa (*Onion*)* contains antibiotic substances. It is also known to have blood cholesterol level and blood pressure lowering properties. Moreover, juice of onion is a popular home remedy of cold and cough. According to the approximation of World Health Organization (WHO), 80% population of few Asian and African countries uses herbal medicines to get relief from superior illness (Farnsworth, 2001).

Traditionally used medicinal plants have been included in a study which is known as 'Ethnobotany'. Now-a-days in modern medicine almost 122 compounds are used which are isolated from the "Ethnomedical" Plants properties (Farnsworth, 2001).

As medicinal plant derived properties are playing a great role in treating various diseases from the ancient period so, more investigational research on medicinal plant will reveal new hope of ray in the Pharmaceutical sector.

1.2 A brief overview on the history of medicinal plants

The exact period of time, from when medicinal plants are used to cure or treat human diseases is still unknown to us. It is predicted that the actual use of medicinal plant was actually started by our ancestor. When they were searching of food, they tried to justify every new substance as food. They found some plants with poisonous effects, some plants to be used as food and some with very different potentialities:

- potentiality of producing sweat.
- help to get rid themselves from joint pain.
- ability to reduce inflammation etc.

There is a myth that, once a Spanish solider caught fever and he drunk water from a pool. In that pool, a bark of Cinchona was fallen. After drinking water from that pool, his fever got cured. From this accidental cure, quinine, the main component of Cinchona was discovered to be used for periodic fever.

The very first writing text on the uses of medicinal plant was almost 400 years old. The text was written by a group of antique people belonging to Sumerian culture who used to live by the river of Euphrates and Tigris. They used to write on small clay plates. The plates were later discovered by the Researchers of Iraq later (Traditional Remidies with Plants, 2016).

The Egyptians also wrote some principle of medicinal plants in the document called Papyrus of Ebers. More than 700 formulas have found, which were written around 1700 A.C (Traditional Remidies with Plants, 2016).

An admirable documented form of medicinal plant use is the book called 'Pen Tsao' which contains use of more than 300 medicinal plants. The Indian medicinal system, called Ayurveda, which has left references of medicinal plant use, from 800 A.C (Traditional Remidies with Plants, 2016).

The analytical history of medicinal plant reveals that our ancestor such as Assyrians, Babylonians, Egyptians were familiar with the medicinal properties of the herbs and trees. A large number of medicinal principal was well known to the Babylonians (about 300 B.C) and it's been said that modern medicine still uses some plants with the same manner like the Babylonians did (Ghani, 2003).

In Egypt and ancient China, the use of plants and animal substances, secretions, minerals are engaged in multiple outline of medicine. These important evidences are the evidences of their tendency in using medicinal plants and their secretion in the past epoch (Dubick, 2015).

However, the Chinese are using a separate orthodox of remedies, named 'Chinese Herbal'. It is observed that botanical origins are in the support of Chinese herbalism. More than 1200 plants are implicated in the traditional treatment system and about 500 plants are routinely used in the treatment of several diseases (Li, 2000).

Chinese people use the herbal medicine in the same way they had used them in previous era. It is observed that one fifth part of the pharmaceutical industry of Chinese market consists of almost 5000 traditional remedies (Li, 2000)

In developed countries, the use of medicinal plants in curing diseases is gradually increasing day by day. Plants usage on the purpose of curing illnesses have been recognized by different well-known organization such as ESCOP (European Organization Cooperative On Phototherapy, 1999), German Commission E (Bluemental et.al, 1989) and WHO (World Health Organization) (ESCOP (European Scientific Cooperative on Phytotherapy, 1999).

1.3 Scenario of medicinal plants in Bangladesh

Bangladesh is a subtropical country which contains more than 5000 angiosperm of 200 families. It has a soothing and perfect weather to grow and nurture medicinal plants.

Chittagong, Dhaka, Rajshahi, Sylhet and other hilly areas of Bangladesh are enriched with about 5000 different species of medicinal plants as mentioned in the 'Materia Medica' (Fakir, 2015).

From the very beginning of its existence, traditional remedies are very famous in the country. Satisfactory growing environment of medicinal plant in Bangladesh has favored it to treat 500 diseases among 2000 diseases by conventional medicines (Fakir, 2015).

Moreover, the use of medicinal plants by the giant industries and companies of Bangladesh has also increased tremendously in the recent years. Many of the leading pharmaceutical companies of Bangladesh are now using a variety of medicinal plants. Digitalis produced digitoxin, vincristine extracted from *Catharanthus roseus,Terminalia arjuna, T. chebula, T. bellerica, Aegle marmelos, Withania somnifera, Cassia angustifolia, Saraca asoca* are some of the most significantly used medicinal plants, that are used in Ayurvedic and Unani medicine from the very beginning in Bangladesh(Fakir, 2015).

The role of wild medicinal plant has put great impact on the life of remote rural part of Bangladesh. This is because of the basic health treatment of the flock people mainly depends on wild medicines. The majority of tribal and flock people of Bangladesh greatly rely on the medicinal plant to have their primary treatment, the reason may be they belief that nature will not do any harm to their health. By depending on this faith and belief, they use various parts of plants: barks, steam, fruit, flower, rhizome etc.(Fakir, 2015).

1.4 Rationale of the study

From the previous literature review, it is observed that, no significant amount of work has been performed on the selected plant called *Anisoptera scaphula (Roxb.)* of this genus. However, previous studies performed on various species of this genus reported potent antimicrobial, anti-tumor, anti-HIV, CNS-depressant, hypotensive, antioxidant, antimicrobial, β-glycosidic and cytotoxic activities. So, the major objective of the study is to discover the natural compound as well as the biological activity of the crude extract of the plant leaves. The investigation will also try to look into the unknown properties and opportunities of the selected plant in the goodness to the planetary healthcare.

1.5 Aim of the study

The aim of the study is to discover unknown natural compounds as well as to evaluate the possible biological profile of the plant, *Anisoptera scaphula (Roxb.)*(Family: Dipterocarpaceae).

1.6 Objectives of the study

The study protocol is consists of the following steps:

1. Phytochemical evaluation of the crude methanolic extract of the powdered leaf material for the identification of the major group of phytochemicals.
2. Evaluation and screening of antioxidant activity and determination of IC_{50} of the crude extract as well as different partitionate of the plant using *in-vitro* DPPH free radical scavenging method.
3. Evaluation of cytotoxic activity and determination of LD_{50} of the crude methanolic extract as well as different partitionate of the plant using brine shrimp.
4. Evaluation of thrombolytic activity.

1.7 Description of the selected plant

The plant under current investigation is *Anisoptera Scaphula (Roxb.),* belongs to the family Dipterocarpacea. It is an evergreen tree, commonly found in the hilly areas of Asian subcontinent. The tree is very tall with a height of 30-45m and perimeter of 3-4.5m (Ecocrop, 2007).

1.7.1 Taxonomical hierarchy

Kingdom	**Plantae**	
Unranked	**Angiosperm**	
Unranked	**Edictos**	
Unranked	**Rosids**	
Order	**Malvales**	
Family	**Dipterocarpaceae**	
Genus	***Anisoptera***	
Species	***Scaphula***	(Ashton K. , 1998)

1.7.2 The plant family Dipterocarpaceae

The family Dipterocarpacea family belongs to the evergreen tropical plant, particularly found in the rainforest of lowland areas. The word Dipterocarpaceae is mainly derived from Greek words (*di* = two, *pteron* = wing and *karpos* = fruit) which actually means 'two winged fruit' (Ashton, 2004).

The family contains 16 genera and more than 695 recognized species. (M. Christenhusz, J.Byng, 2016). Dipterocarpaceae plants are the tallest plant (40-70m); some species of this family have the height of about 80m (The plant List, 2010).

The plants belonging to this family plant are called pantropical due to their large distribution in South America, Africa, India, Indonesia, and Malaysia. However, being a potential source of wood, resin and plywood, several species of this particular family are becoming endangered species, those are fighting for their existence due to illegal and overcutting for logging (Ecocrop, 2007).

Plants under this family are found to possess various biological activities as well as also found to contain several chemical constituents. The stem and leaves of various species belonging to this family show antioxidant, antibacterial, anticancer and antidepressant activities. They are also enriched in various resins and essential oils (Parvez M. , 2012).

- **Genera in Dipterocarpaceae**

About 15 genera belonging to the species of Diptarocarpace have been included in 'The Plant List' (The Plant List, 2010).

Table 1.1: Genera in Diptarocarpaceae(The plant List, 2010)

Anisoptera	Pakaraimaea
Cotylelobium	Parashorea
Dipterocarpus	Pentacme
Dryobalanops	Shorea
Hopea	Vateria
Marquesia	Vateriopsis
Monotes	Vatica
Nebolanocarpus	

1.7.3 A brief description on the *Anisoptera scaphula*

It is a long buttressed, angiosperm tree, found to be widely distributed in the semi evergreen and evergreen dipterocarp forest. Regeneration of *Anisoptera scaphula* is very poor and that's why the plant is considered as 'Critically Endangered plant' (Ashton P. , 1998).

Figure 1.1: *Anisoptera sacphula* tree with leaves (Rambe, 2011)

Synonyms

- ✓ *Anisoptera glabra* Kurz.
- ✓ *Hopea scaphula* Roxb.
- ✓ *Hopeoides scaphula* (Roxb.) Cretz.
- ✓ *Scaphula glabra* Parker.
- ✓ *Vatica scaphula* (Roxb.) Dyer. (R.J. Johns, 2016)

Origin

The plant is mostly found in the tropical Asian countries such as Malaysia, Thailand, Burma, Bangladesh, Southern Indonesia and North of Negri. In Bangladesh, the plant is abundantly found in hilly areas such as, Chittagong, Rajshahi, Shitakundu, Mymensing and Sylhet (R.J. Johns, 2016).

Common name

- **Malaysia**: Mersawa,Gajah, Sanai, Terbak .
- **Burma:** Kaunghmu (general) Ataran, Toungoo.
- **Thailan:** Karbark konao, Chambailek.
- **Bangladesh:** Balishur, Bailam, Shada Bailam.(R.J. Johns, 2016)

1.7.4 Morphological description

The plant *Anisoptera scaphula* grows mainly in the tropical forest areas. It is an ever green plant commonly found in Asia. The plant is well known for its heights, with a height of about 30-45m and perimeter of 3-4.5m (Ecocrop, 2007).

Leaf: Leaves 8-16 cm × 3.5-8 cm, oblong-lanceolate to oblanceolate, greyish-green lepidote beneath, with 13-18 pairs of secondary veins prominent on both surfaces. Leaves alternate, simple, entire, oblong to ovate, base usually obtuse, apex shortly acuminate, lower surface persistently covered with peltate hairs; secondary veins curved and anastomosing at the apex; petiole distinctly geniculate; stipules relatively large, narrow and fugacious (Schulte, 1998).

Flower: Flowers bisexual, regular, 5-merous, distinctly pedicellate in bud; calyx imbricate or rarely valvate, 2 outer lobes obtuse and slightly more thickened than the 3 inner acute ones, united at base in an indistinct tube; corolla with oblong-linear petals, which are shed separately; stamens 15-65, in 3 verticils or irregularly placed, the outer somewhat shorter than the inner, glabrous, filaments rather short, slender, filiform, connate at base, anthers latrorse, the inner 2 pollen sacs shorter than the outer 2, with a long or short appendage; ovary 3-locular, semi-inferior, with enlarged stylebase forming a distinct stylopodium, style long or short, obscurely trifid, stigma minute (Dallwitz, 2016).

Fruit: A globose or subglobose nut, the fruit calyx with an ellipsoid tube almost entirely enclosing the nut and adnate to it, with 2 long, narrowly spatulate, obtuse, untwisted, 3-veined lobes and 3 acuminate short lobes (Dallwitz, 2016).

Seed: Lack endosperm.Seedling with epigeal germination; cotyledons unequal, chlorophyllous; the first leaves paired, with interpetiolar stipules, or in a whorl of 4 without stipules, subsequent leaves arranged spirally (Hossain, 2014).

1.7.5 Plant phytochemistry

Previous phytochemical investigations of *Antisoptera* species led to the isolation of different constituents such ashopeanolin (-) hopeaphenol, balanocaprol, dibalanocaprol, cycloartane triterpenoid, hopeaphenol A, isohopeaphenol A and vaticanol C, stigmasterol, 1,2-dimethoxy-4-allylbenzene,3-4dimethoxycinnamaldehyde and amyrin(beta). Investigations also revealed it's antioxidant as well as glucosidase inhibitory potential (Parvez M. M., 2012).

1.7.6 Pharmacological properties

The genus *Antisoptera* belongs to the Dipterocarpacae family and the plant of this family reported to contain resveratrol oligomers. Because of the presence of these oligomers, the plant shows various pharmacological activities. For example, strong cytotoxic effect has been reported to be shown by this plant due to the presence of a resveratrol tetramer called 'Vaticanol C' (S.Mishima, K.Matsumoto, 2003). *A.scaphula* also might have anticancer, antimicrobial, antioxidant and β-glycosidase inhibitory activity. Various species of this genus are also reported to have antitumor,

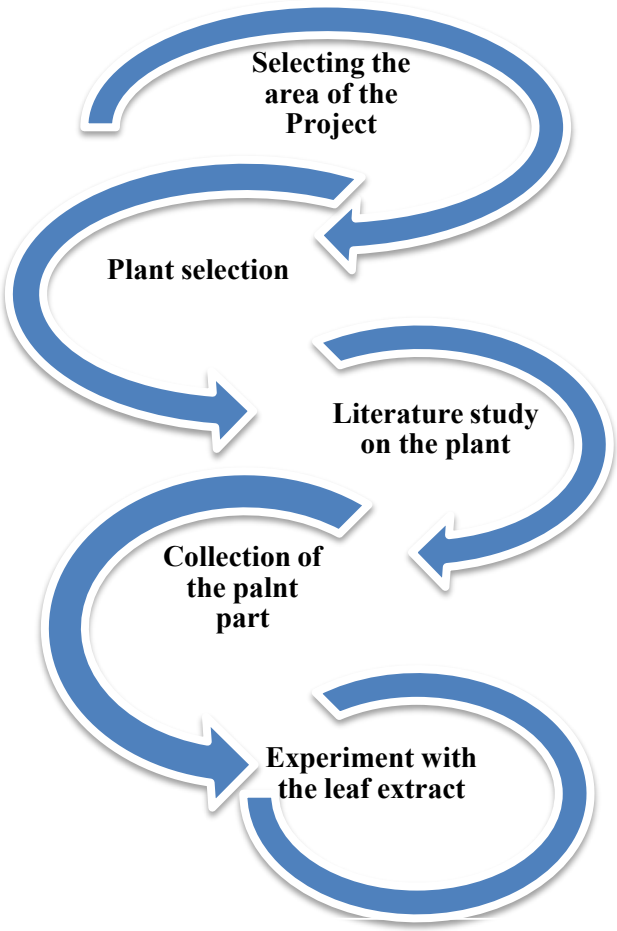
anti-HIV, astringent, CNS depressant, hypotensive and antifungal properties (Parvez M. M., 2012).

1.7.7 Related publication on *Anisoptera scaphula*

A.scaphula is still an unrevealed plant as not much research has been done yet on its chemical properties as well as on pharmacological uses. Therefore, many valuable properties and medicinal uses of the plant are yet to be found. A publication has been found which was mainly carried out to find the chemical properties of *A.scaphula.*(Parvez M. M., 2012).

- Compound Isolation and purification by Chromatographic Method of stem bark of *Anisoptera scaphula (Roxb.)*(Parvez M. M., 2012).

1.7.7 Research design



1.8 Phytochemical screening of crude extract of the plant

According to the pharmaceutical prospect, phytochemical extraction is defined as the process of obtaining or separating medicinally active compound by using various solvent systems of standard procedure (Prashant Tiwari, 2011).

Phytochemicals are considered as the most important health beneficiary compounds derived from plants. Scientists have discovered thousands of phytochemicals from plants with extreme potentiality to prevent different diseases in human body. Thought it is difficult to manipulate the advantage of each phytochemicals separately but researchers believe that phytochemicals might work together to provide health benefit. For example; carotenoids (beta-carotene, lutein, lycopene) has the capability to prevent cell damage and thus considered as a potential antioxidant. But it is observed that, carotenoids can also reduce the risk of heart attack and various cancers (Prashant Tiwari, 2011).

➢ 1.8.1 Classification of bioactive compounds

Bioactive compounds of the plant can be classified according to their clinical and pharmacological activity. Biological activity of plant derived compounds became more complex when their clinical outcome does not match with the chemical nature of that compound.

According to botanical categorization, plants from the same family and genera might possess similar or closely related bioactive compounds as well as similar pharmacological effects.

Few major bioactive compound derived from plant has been described below:

- **Glycosides**

Glycosides consist of various secondary metabolites those are found to bind with mono- or oligosaccharide or to uronic acid. There are mainly two Part in the glycoside one is called ‘Glycon’ (consists of saccharide or uronic acid) and other is called ‘Aglycon’ (consist of the several parts).

Table 1.2: The major groups of glycoside (Aksel, 2008)

Name of the glycoside	Medicinal effects
Cardiac glycosides	▪ Inhibition of Na+/K+-ATPase-pumps in the cell membranes. ▪ Increases heart contractility and reduced rate.
Clucosinolates	▪ Effects cytochrome P450 isoforms in various cells and decrease hepatic bioactivation of environmental procarcinogens. ▪ Produce skin irritation. ▪ Induce hyperthyroidism and goiter.
Anthraquinone glycosides	▪ Induce of water and electrolyte secretion as well as peristalisis in colon.
Saponnins glycosides	▪ Haemolysis of red blood cells. ▪ Cause emulsifying properties and can be used as detergents. ▪ Induce photosensitisation and jaundice.

- **Flavonoids and proanthocyanidins**

Flavonoids consist of a three ring structure. Oligomer of flavonoids is called proanthocyanidins. Both groups of compounds are found to produce general antioxidant effects as they contain phenol groups.

- **Alkaloids**

Alkaloids are organic compounds, consists of hydrogen, oxygen, carbon and nitrogen. There are different types of alkaloids with different activities (Alkaloid, 2016).

Medicinal uses:

- Curarine, found in the deadly extract curare, is a powerful muscle relaxant.
- Atropine is used to dilate the pupils of the eyes.
- Narcotic alkaloids such as morphine and codeine are used in the relief of pain. Another alkaloid called cocaine is used as a local anesthetic (Alkaloid, 2016).

- **Tannins**

There are two different types of tannins:

1. Condensed Tannins: Large polymers of flavonoids.
2. Hydrolysable Tannins: Polymers composed of a monosaccharide core (most often glucose) with several catechin derivatives attached.

Medicinal use of both Tannins:

- Larger tannins are used as astringents in cases of diarrhea, skin bleedings and transudates.

- **Resins**

Resins are mainly mixtures of organic acids and esters. In general, resin exists as a lipid soluble mixture. They are found both in volatile and non-volatile state. They are mainly secreted by woody plants but their presence is also observed in herbaceous plants. When the resins come in contact with air they harden (Resin, 2016).

Medicinal use:

- Antimicrobial and wound healing (Resin, 2016).

- **Diterpenoids**

Diterpenoids are composed of 20 carbons consisting of 4 isoprene units. They are lipophilic in nature with strong odors and have tendency to evaporate readily.

Medicinal uses:

- Less information has been found on the toxicological properties of this component but several of them are found to produce antineoplastic activity (Aksel, 2008).

- **Mono- and sequi-terpenoids, and phenylpropanoids**

The terpenoids are low molecular weight and synthesized by five-carbon building block isoprene. Monoterpenoids consist of two isoprene units and three sesquiterpenoids units.

Medicinal use:

- Antineoplastic.
- Antibacterial.
- Antiviral.
- Gastrointestinal stimulation.

1.9 *In-vitro* screening of antioxidant activity of *A.scaphula* leaf extract:

Free radicals play a common and the most important role in damage for which our body cell faces aging problem. Antioxidants are believed to be the body's first line of defense against aging. Free radicals have the potential to damage healthy cells of body by destroying their structure and functions. Free-radicals are mainly produced from the highly reactive oxygen of our body. Antioxidants have the capability to neutralize these free radical activities (Flora, 2009).

Nature contains a large and good source of antioxidant elements in the form of grain, fruits and vegetables. Plant has great number of constituents called: vitamin C, vitamin E, carotenes, phenolic acids, phytate and phytoestrogens. These are recognized as the most potential compound to reduce the risk of oxidative cell damage by free radicals (Flora, 2009).

1.9.1 Classification of antioxidant

Table 1.3: Classifications of antioxidants by their functions (Butnariu, 2012)

Primary antioxidant(Mainly function as antioxidant)	▪ Ascorbic acid and its derivatives. ▪ Tocopherols, the esters of gallic acid. ▪ Erythorbic acid and its sodium salt. ▪ BHA, BHT and other substances. ▪ THBP and TBHQ.
Secondary antioxidants (substances with antioxidant action but also have other functions as well.)	▪ Sulphur dioxide and ▪ Sulphites.

Table 1.4: Classification of antioxidants by their alphabetic order (Flora, 2009)

Alphabetical name	**Categories of antioxidants**	**Examples**
Antioxidant C	Carotenoids	β-carotene, Lycopene, Lutein.
Antioxidant E	Enzymes	SOD, Catalase, GPx
Antioxidant G	Glutathione	Glutathione
Antioxidant H	Hormones	Melatonin, Oestrogen
Antioxidant L	Lipid associated chemicals	Ubiquinol-10, M-acetyl cysteine, lipoic acid
Antioxidant M	Minerals	Zinc, Selenium, Copper
Antioxidant P	Phenolics	Quercetin, Catechin
Antioxidant S	Saponin, Steroids	Cortisone, Estradiol, Estriol
Antioxidant V	Vitamins	α-tocopherol, Ascorbic Acid

Table 1.5: Kinetic classification of antioxidants along with examples (Flora, 2009)

Class	Examples
• Antioxidants that break chains by reacting with peroxyl radicals having weak O-H or N-H bonds	Phenol, Naphthol
• Antioxidants that break chains by reacting with alkyl radicals	Quinones, nitrones, iminoquinones
• Hydro peroxide decomposing antioxidants	Sulfide, Phosphide, Thiophosphate
• Metal deactivating antioxidants	Diamines, Hydroxyl acids, bifunctional compounds
• Cyclic chain terminating by antioxidants	Aromatic amines, nitroxyl radical, variable valence metal compounds
• Synergism of action of several antioxidants	Phenol sulfide in which the phenolic group reacts with peroxyl radical and sulfide group with hydro peroxide

1.8.2 Chemistry of free radicals

- **Reaction of free radicals**

Free radicals can be defined as the molecules or molecular fragments containing one or more unpaired electrons, involved in oxidative reactions (Flora, 2009).

Antioxidants play a key role in the adsorption and neutralization of free radicals, quenching singlet and triplet oxygen or decompose peroxides in the living systems. Within the living system, reactive

oxygen species (ROS) such as superoxide anion ($O_2^{-\bullet}$), hydroxyl radicals ($OH^{\bullet}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) are produced as a result of normal metabolic processes (Endogenous sources) and from exogenous sources (Flora, 2009).

- **Oxygen radicals**

There are many types of radicals in biological systems which are derived from oxygen which are collectively known as reactive oxygen species. Oxygen has two unpaired electrons in separate orbitals in its outer shell. This electronic structure makes oxygen especially susceptible to radical formation (Antiox B, 1995).

Sequential reduction of molecular oxygen (equivalent to sequential addition of electrons) leads to formation of a group of reactive oxygen species:

- **superoxide anion**
- **peroxide** (hydrogen peroxide)
- **hydroxyl radical**

The structure of these radicals is shown in the figure below, along with the notation used to denote them. The difference between hydroxyl radical and hydroxyl ions can be observed from the following figure:

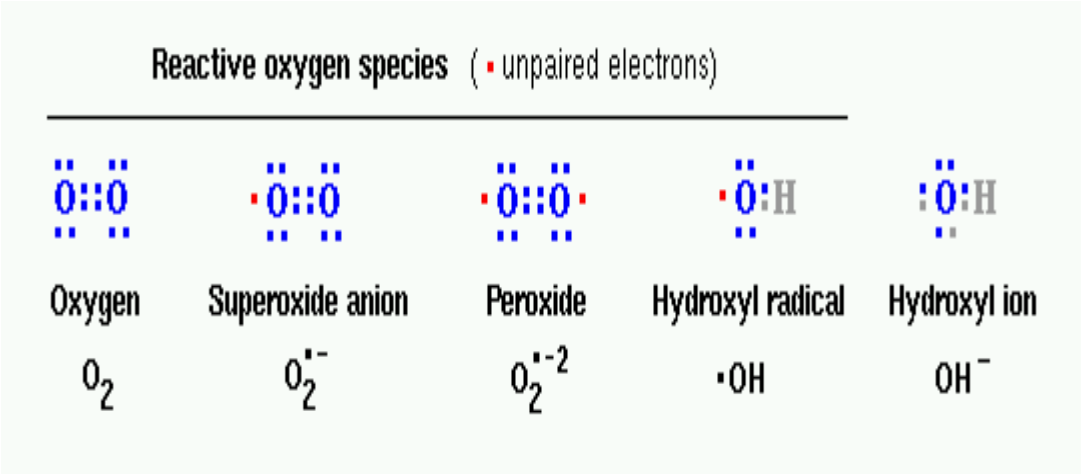


Figure 1.2: reactive oxygen species (Antiox B, 1995).

Table 1.6: Common diseases caused by the ROS and free radical

Alzheimer	Cancer
Parkinson	Dementia
Cellular oxidative stress	Atherosclerosis
Cancer risks	Vascular diseases
Chronic inflammatory diseases	Hypercholesterolemia

1.10 *In-vitro* screening of cytotoxic activity of the plant extract

1.10.1 Cytotoxicity

Cytotoxicity is the quality of being toxic to cells. Cells exposed to a cytotoxic compound can respond in a number of ways. The cells may undergo necrosis, in which they lose membrane integrity and die rapidly as a result of cell lysis, they can stop growing and dividing; or they can activate a genetic program of controlled cell death, all these are termed apoptosis (Cytotoxicity, 2016).

1.10.2 Classification

Basically there are two types of cytotoxicity are present:

1. **Cell-mediated Cytotoxicity:** In this process, target cells are destroyed by using specific lymphocytes. For example: Cytotoxic T lymphocyte is one of them.
2. **Antibody-dependent Cell-mediated Cytotoxicity (ADCC):** In this process, an effecter cell of the immune system spontaneously destroyed a specific cell, whose membrane surface has been bounded by specific antibodies (Pollara, et al., 2011).

1.10.3 Lymphocytes

Three groups of cytotoxic lymphocytes are distinguished as:

- Cytotoxic T cells
- Natural killer cells
- Natural killer T cells

1.11 *In-vitro* screening of thrombolytic activity of the plant extract:

1.11.1 Thrombus

In the regular blood circulation, formation of blood clot has been recognized as a major problem. Thrombus or embolus disturbed the blood flow by creating blockage of the blood vassals and create the deprivation of blood and oxygen to the tissues. This incidence can cause necrosis of the tissue in that particular area of the vessels. Blood clot is formed by the thrombin and lysed by plasmin, which is activated from plasminogen by tissue plasminogen activator (tPA). The reason behind using a fibrinolytic drug is to dissolve thrombin in acutely occluded coronary arteries to restore the blood supply (Ali Ramjan, 2014).

1.11.2 Risks of thrombolysis

Thrombolytic agents are mainly used to effectively and safely improve the blood flow and relief or reduce the symptoms in various patients without the need for more invasive surgery, it is not recommended for everyone (Beckerman, 2015).

Various thrombolytic agents are used for the treatment of myocardial infraction. Among them streptokinase is widely used. However, it is observed that another agent called Tissue-type Plasminogen activator has better effect over streptokinase or urokinase type activators (Ali Ramjan, 2014).

Patients who already use blood thinning agents, herbs, or dietary supplements or people who have the increased risk of bleeding; thrombolytic agent is not recommended for them. These conditions might include (Beckerman, 2015):

- severe high blood pressure

- active bleeding or severe blood loss
- hemorrhagic stroke from bleeding in the brain
- severe kidney disease
- recent surgery

It is noted that all available thrombolytic agents still have significant deficiencies, including the necessity of large doses to be maximally effective, limited fibrin specificity and a significant associated bleeding tendency. Therefore, steps are taken to develop improved recombinant variants of these drugs in order to minimize deficiencies of the available thrombolytic drugs (Ali Ramjan, 2014).

Chapter 2

Methodology

2. Methodology

2.1 Preparation of plant extract

2.1.1 Collection of the plant

The plant under the study is named *Anisoptera scaphula,* was selected as for the current study to identify phytochemical constituents and pharmacological activities of this plant. Plant leaves were collected from the Chittagong district of Bangladesh in March 2016. The plant is found in eastern part of Bangladesh.

Table 2.1: Chemical investigation of *Anisoptera scaphula*

Name of the plant	Family	Plant part
Anisoptera scaphula	Dipterocarpacea	leave

2.1.2 Identification of the plant

To get Taxonomical Identification of the plant, leaves of *A.scaphula*was submitted to Bangladesh National Herbarium, Dhaka. After investigating the leaves, they provided an accession number (DACB- 41235) for our given specimen.

2.1.3 Preparation of the crude extract:

- **Plant material preparation and drying**

Leaves of *A. scaphula* was collected and washed by water to remove plant debris and dust. After that, leaves were separated from the brunch to dry. The leaves were sun dried for only one day then kept under shade to dry rest of the days. This process was followed to prevent the denaturation of the chemical constituent of *A. scaphula.*

- **Extraction process**

➢ ***Size reduction and weighing***

After complete drying, the leaves were crushed to fine powder by using a high capacity grinding machine. Approximately, 400gm of powder were collected which was kept in an airtight container to prevent contamination and for further investigation.

Figure 2.1: Grinded powder of the leaves.

➢ ***Extraction***

To prepare methanolic extract from dried crude, two clean amber jars was taken and powder of the leaves was soaked in it in around 2 liter of methanol. The jars were needed to shake frequently, so at the time of filling the jar with methanol, it was kept in mind that no spilling of the materials should take place. When shaking and stirring the content inside the jars, it was also a matter of concern that the jars were handled carefully to let the gaseous substance move out of the jars.

➢ ***Filtration***

On the seventh day, the content from both of the jars were filtered sincerely with the cotton filter.

➢ ***Concentration***

The filtrate was concentrated by using rotary evaporator (Heidolph) with a rotation speed of 100 rpm and temperature of 30°C. After three hours, a thick concentrated solution was collected.

Figure 2.2: Concentrating the filtrate of *A.scaphula* plant using a rotary evaporator.

- ***Drying***

The thick concentrated solution was poured into several petri dishes. The petri dishes were kept under Laminar Air Flow (LAF) to evaporate the solvent from the extract. After drying for seven days, the extract became fully dried and they were stored in vials to avoid any kind of cross contamination, microbial growth and also for further investigation.

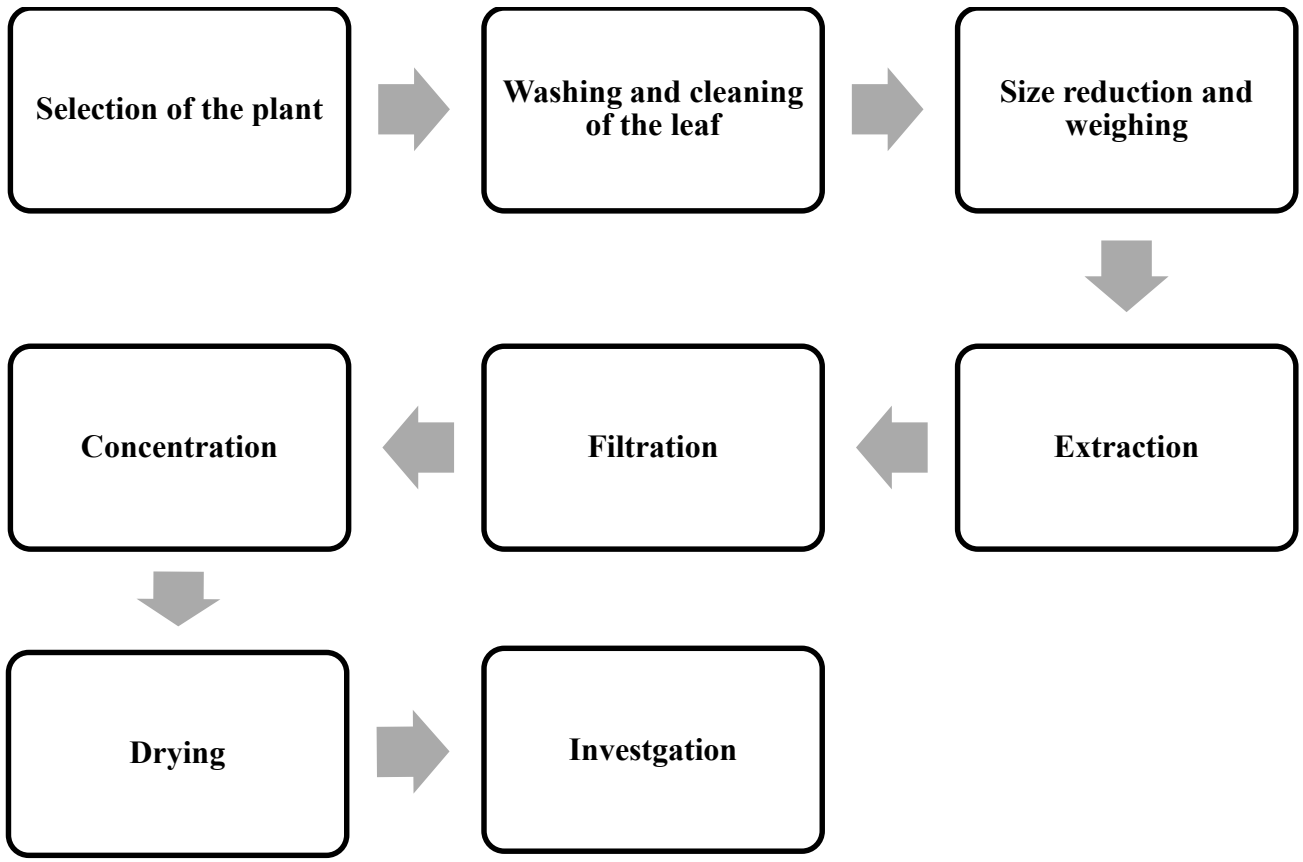


Figure 2.3: Flowchart showing the step by step procedure of extraction from the crude of medicinal plant.

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

Fractional separation by using different solvent was carried out by following Kupchan method which was modified by Vanwagenen (Vanwagenen BC, 1993).

To carry out the test, at first 5 gram of crude extract was dissolved in 10% methanol, which was considered as mother solution. The mother solution was then extracted with three different solvents.

Table 2.2: Solvents used in separation technique

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

2.1.4.1 Partitioning with Petroleum Ether

At first, mother solution was taken in a separating funnel and then 100mL of petroleum ether was added to it. The funnel was shaken very well and kept for rest without any disturbance. Then the organic portion was collected and the process repeated for thrice. All collected portion was evaporated together in the Rotary evaporator.

2.1.4.2 Partitioning with Chloroform

After partitioning with the Petroleum ether, 16mL distilled water was added to the residual solution and mixed uniformly. The mother solution was then taken in a separating funnel and extracted with dichloromethane ($CHCl_3$) (100 mL X 3). The $CHCl_3$ soluble fractions were collected together and evaporated. After that, the aqueous methanolic fraction was preserved as aqueous fraction.

2.1.4.3 Partitioning with ethyl acetate

To the residual solution, left after washing with petroleum ether, carbon tetrachloride and dichloromethane, 20mL of distilled water was added and mixed uniformly. The mother solution was then taken in a separating funnel and extracted with ethyl acetate ($CH_3COOC_2H_5$) (100 mL x3). Then the $CH_3COOC_2H_5$ soluble fractions were collected together and evaporated. Finally the aqueous methanolic fraction was preserved as aqueous fraction.

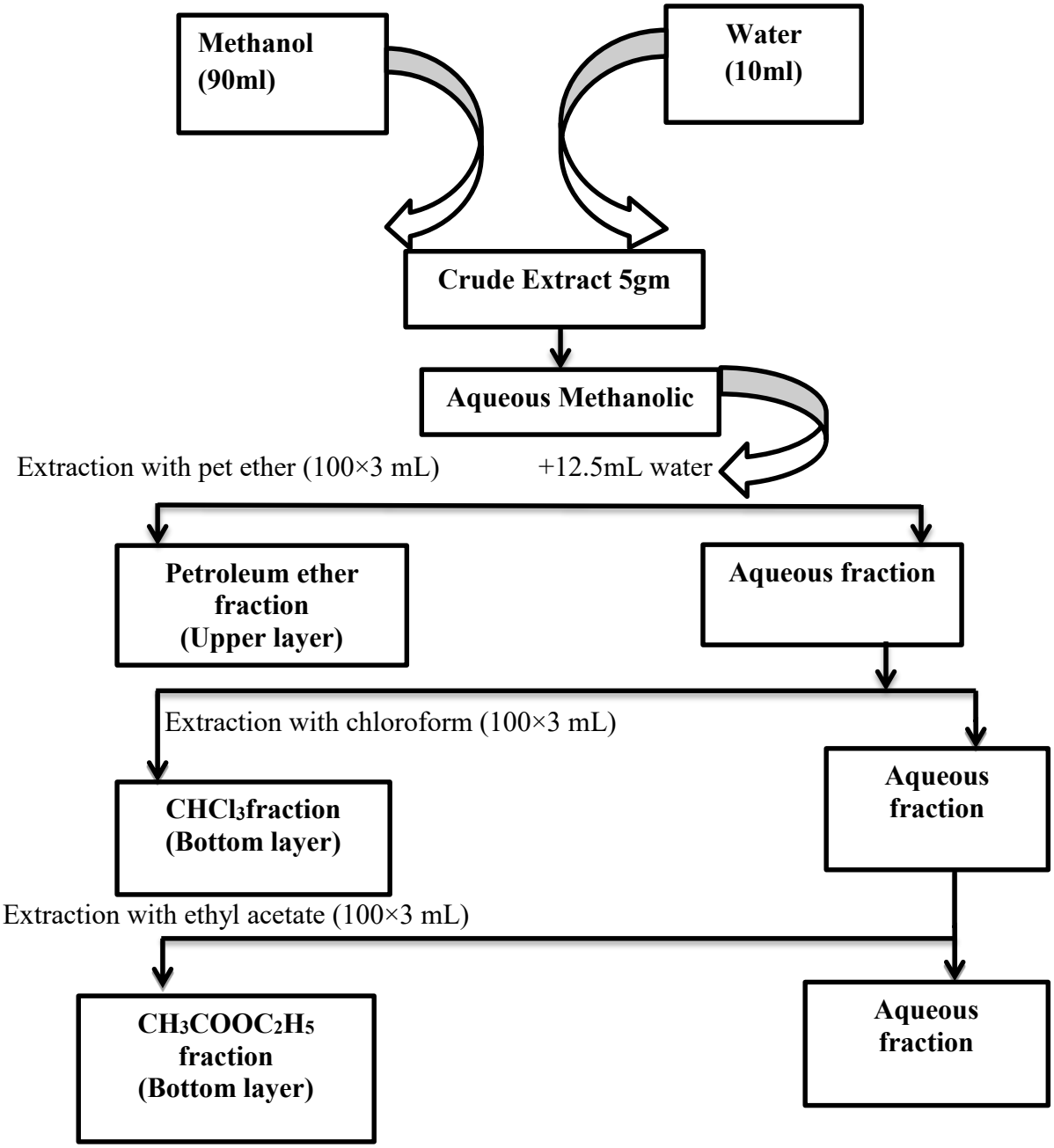


Figure 2.4: Schematic representation of the modified Kupchan Partitioning of methanolic extract of the leaf of *A. scaphula.*

Table 2.3: Amount of partitionates obtained from (5gm) methanolic extract

Sl.no	Sample code	Fraction	Weight (gm)
1.	PEF	Petroleum ether fraction	0.35
2.	DCMF	Dichlomethane fraction	0.20
3.	EAF	Ethyl acetate fraction	0.28
4.	AQF	Aqueous fraction	1.00

2.2 Phytochemical screening of the leaf extract

2.2.1 Design of the screening

The phytochemical tests were analyzed by the process described in (Ghani, 2003).Reasons behind selecting these particular methods were reagents used in the methods were available in the laboratory and also they were easy to understand to find out the chemical compounds.

To determine the phytochemical compound from the methanolic extract of *A. scaphula,* tests were done for:

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

2.2.2 Chemicals and reagents

Table 2.4: Chemicals and reagents were used for the determination of phytochemicals

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

2.3 Procedure for screening of phytochemical compounds

To determine the chemical compounds from the plant extract all the process was done by following a standard procedure. All the process has been briefly discussed below:

2.3.1 Test for Carbohydrates

- **Molisch's Test**

This test is mainly conducted to examine the presence of carbohydrate. Initially, 2mL of aqueous extract was taken in a test tube and few drops of 10% (10gm alpha-naphthol in 10mL ethanol) freshly prepared ethanolic solution of alpha-naphthol was mixed with it. Then (under the fume hood) few mL of concentrated sulphuric acid was poured down the side of the test tube in order to create a layer under the solution. If a red ring appears between the acid and test layers, the result will be considered as positive.

2.3.2 Test for glycosides

- **General test for glycosides**

At first 1mL distilled water was taken in a test tube, a very small amount (0.5gm)of methanolic extract was added to the test tube and dissolved. After that, previously prepared 5% sodium hydroxide solution was added to the test tube solution. Glycoside compound will show yellow coloration.

2.3.3 Test for saponins

- **Forthing Test**

To run this test, a very first small amount (0.1gm) of powdered plant material was heated to boil for 55 minutes with 10mL of distilled water. Then the solution was cooled and filtered. The filtration was diluted with 5mL of distilled water with vigorous shaking and was left to stand for 10 minutes. A dense persistent forth will indicate the presence of saponins.

2.3.4 Test for flavonoids

About 1mLdistilled water and small amount of extract was taken in a test tube to examine the presence of Flavonoids. Then few drops of dilute sodium hydroxide were added to the mixture afterwards intense yellow color will appear. If the intense yellow color disappeared with the addition of acid, the result will be considered as positive.

2.3.5 Test for tannins

- **Lead acetate Test**

At first 5mL of queues extract was taken in a test tube then few drops of 1% solution of lead acetate was added to the solution. If red or yellow precipitation is appeared in the test solution it indicates the presence of Tannin in the test sample.

2.3.6 Test for resins

In order to carry out this test, a small amount of ethanolic or chloroformic extract was taken in a test tube, which was dissolved with 5-10mL of acetic anhydride by the means of heat. Then the solution was cooled. After that 0.05mL of sulphuric acid was added to it the purplish red color changes to violet it will indicate the presence of resins in the sample.

2.3.7 Test for alkaloids

- **General laboratory test for Alkaloids**

At first 0.5gm of the plant extract was stirred with 5mL of 1% hydrochloric acid on a steam bath. Then the solution was filtered. After that, 1mL of the filtration was treated with few drops of each of the following reagents separately. Formation of the following colored precipitation indicates the presence of alkaloids in the plant extract.

Mayer's reagent

With the addition of the Mayer's reagent to the solution white or creamy precipitation appears.

Hager's reagent

With the addition of Hager's reagent yellow crystal type precipitation will appear.

Dragendorff's reagent

With the addition of Dragendorff's reagent orange or orange-red precipitation will occur.

Wagner's reagent

With the addition of Waner's reagent to the solution brown or deep brown precipitation will appear.

Dagendorff's reagent

Stock solution- 4gm of sodium iodide was boiled with 5.2gm of bismuth carbonate with 50mL of glacial acetic acid for a short amount of time. After letting it rest for 12hours, the sodium acetate crystals precipitate are filtered by a sintered glass funnel. A clear reddish brown filtrate was obtained and 40mL of which was added to 160mL of ethyl acetate and 1mL of water. The solution was then stored in amber colored bottle for later use. Working solution- 10mL of stock solution was mixed with 20mL of acetic acid. By adding water it was prepared as 100mL solution.

2.3.8 Test for steroids:

- **Libermann-Burchard's Test**

A small amount of petroleum ether extract was dissolved in 1mL of chloroform and then 2mL of acetic anhydride and 1mL of conc. sulphuric acid was added to the solution. If a steroid is present in the test sample, a greenish color will appear which will turn blue on standing.

2.3.9 Test for proteins

- **Millon's test:**

A small amount of aqueous extract was dissolved in 1mLof distilled water. Then in the solution 5-6 drops of Millon's reagent was added to the extract solution after which a white precipitation will appear. If on heating, the precipitation turns into red, it will confirm the presence of protein.

2.4 *In-vitro* antioxidant activity test

2.4.1 DPPH free radical scavenging assay

- **Principle**

Antioxidant activity of *A.scaphula* was determined by the method described by Brand-Williams et al., (1995). This method basically described the free radical scavenging activity of the plant extract on the free radicals 2-2-Diphenyl1-1-Picrylhydrazyl which is called DPPH.

- **Mechanism**

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

To calculate the % of inhibition the following formula is used here,

% of Inhibition = [(Absorbance Control - Absorbance Sample) / Absorbance Control] ×100.

DPPH assay is mainly used to determine the reduction of free radicals DPPH, which has an odd electron. Because of this odd electron carried by free radical DPPH, we get a highest absorbance at the wavelength of 517nm in UV-Spectrophotometry. After reacting with the stable free radical DPPH, antioxidants get paired off in the hydrogen donor and consequently, get reduced to DPPHH, which results in the decrease of the absorbance from that of DPP-H. Decolorization occurred as a consequence when radicals in DPP-H produces yellow color as a respect to the total number of electrons caught. At the same time decolorization gradually increases which indicates towards the higher reducing ability. 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 (Brand-Williams et al., 1995**).**

- **Materials**

Table 2.5: List of materials used in the method

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

Preparation of Control for the Evaluation of antioxidant activity*:*

Table 2.6: Amount required for preparing stock solution

Name	**Amount**
Ascorbic acid	0.005gm
Methanol	1.000mL

In this method, ascorbic acid has been used to make the positive control. At first, to make the positive control about 0.005gm of acid was taken into a volumetric flask. 1mL of methanol was added to it to make the concentration of the solution 5000µg/mL; it was considered as the stock solution. After that the stock solution was diluted by following serial dilution method to get 1250µg/mL to 78.125µg/mL concentration ranging solutions.

- **Preparation of test samples**

Table 2.7: Test samples of experimental plant

Sample code	Test sample	Calculated amount (gm)
ME	Methanolic Extract	0.005
PEF	Pet ether Fraction	0.005
CLF	Chloroform Fraction	0.005
EAF	Ethyl Acetate Fraction	0.005

In order to prepare the test samples, at first 0.005gm of all the extracts of *A. sacphula* was taken to separate test tubes. After that, in every test tube 1mL of methanol was added to get a concentration of 5000µg/mL. Then this stock solution of each extract was diluted to get concentration ranging from 1200 µg/mL to78 µg/mL by following the serial dilution method.

- **Preparation of DPPH Solution**

Table 2.8: Amount required for preparing DPPH solution

Name	Amount
DPPH	2.00mg
Methanol	50.00mL

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

- **Assay of for free radical scavenging activity**

Now, from the stock solutions (control) with having various concentrations ranging from 1200µg/mL to 78µg/mL, 2mLsolution was taken from each concentration. After that, each of the solution was mixed with 3.0mL of DPPH methanolic solution. Then, all the samples were kept in the dark place for 30 minutes for the reaction to take place. The absorbance of the each sample was measured by using VU-Spectrophotometry at 517nm. Here the methanolic solution was kept as blank.

- **Calculation**

Using the following formula, inhibition of free radical DPPH was calculated in percent (I %) :

Inhibition % (I %) = [(Absorbance$_{Blank}$– Absorbance$_{Sample}$) / Absorbance$_{Blank}$] × 100

Subsequently, extract concentration rendering 50% inhibition (IC_{50}) was determined from the graph, by plotting inhibition percentage (I %) against the concentration of extract (µg/mL).

2.5 Brine shrimp lethality bioassay

- **Principle**

In this method of bioassay by brine shrimp, naupolii is hatched from the shrimp leach called "*Artemia saline"* in simultated sea water. Then a calculated amount of DMSO (Dimethyl Sulphoxide) is added to prepare preferred concentration of the sample under the test. The naupolis were counted by visual inspection in vial containing 5mL of sea water. Samples of different concentration were added to the pre marked vials with the help of micropipette. To count the survivors, the vials were left for 24 hours (Meyer et al., 1982).

- **Materials**

Table 2.9: Materials used for the test

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

Table 2.10: Test samples of experimental plant

Sample code	**Test sample**	**Calculated amount (mg)**
ME	Methanolic extract	4.00
PEF	Pet ether fraction	4.00
CF	Chloroform fraction	4.00
EAF	Ethyl Acetate fraction	4.00

- **Experimental Procedure**
- **Preparation of sea water**

In order to prepare the sea water, at first 38gm of sea salt was dissolved in one liter of distilled water. To get a clear solution the water was then filtered off by using filter paper.

- **Hatching of brine shrimp**

In this test as a test organism brine shrimp eggs (*Artemia salina* leach) were used. The eggs were collected from the pet shop. At first the shrimp egg was taken and added to the small tank containing the sea water. Two days were allowed to hatch shrimp and the nauplii. Constant oxygen was supplied to the tank. A light source was also arranged during the matured period of the nauplii. The hatched shrimp were attracted to the lamp through the perforated dam and which were taken for the test.

A vial was taken containing 5mL of sea water, 10 living shrimp was added to the vial with the help of Pasteur pipette.

- **Preparation of the test sample with the experimental plant**

In order to prepare the stock solution, all the samples (Table 2.7) were taken in separate vials and dissolved with dimethyl sulfoxide (DMSO). After that, 50 µL of solution was taken in the first test tube containing 5 mL of simulated seawater and 10 shrimp nauplii, To make actual concentration of the final solution of first test tube 400 µg/mL. By following the serial dilution method, a series of solution of different concentration was prepared from the stock solution. In every case, 50 µL samples were added to test tube and fresh 50 µL DMSO was added to vial. Thus different concentrations were found in the different test tubes (Table 2.8)

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

- **Preparation of control group**

A control group was also prepared ensure that the result only obtained because of the test reagent and other effects has been diminished. In the cytotoxicity test normally two types of control are used:

a. Positive control
b. Negative control

- **Preparation of the positive control**

In order to compare the results obtained by the test samples of the experimental plant samples, a positive control is used. By using positive control we compare the results against the result obtained by assessing the positive control. In this experiment, Vincristine sulphate has been used as a positive control. To make the stock solution of an accurate amount of vincristine sulphate was measured and dissolved in DMSO to make the concentration of 20 µg/mL. Then, from this stock solution a serial dilution was carried out using DMSO in order to obtain various concentrations ranging from 10 µg/mL, 5 µg/mL, 2.5 µg/mL, 1.25 µg/mL, 0.625 µg/mL, 0.3125 µg/mL, 0.156 µg/mL, 0.078 µg/mL and 0.0390 µg/mL. Then a vial was taken which contained 5mL sea water and 10 living nauplii and positive solution was added to the vial in order to have a positive control group.

- **Preparation of the negative control**

At first, three pre-marked glass vials containing 5mL of simulated sea water was taken. 100µmL of DMSO and 10 shrimp nauplii were added to it to prepare the control group. If the brine shrimp in three vials present constant mortality, then the test will be considered as invalid as the nauplii died due to some other reason than the cytotoxicity of the compounds.

- **Counting of the nauplii**

To obtain the result, the vials were kept for 48 hours. After 48 hours, all the vials were examined by using the magnifying glass to count the survived nauplii. The % of mortility of each dilution was calculated by using the formula mentioned earlier. The effectiveness or the concentration-mortality relationship of the experimental plant compound is normally expressed as median lethal concentration (LC_{50}). This LC_{50} is calculated by using linear regression equation; this represents the concentration of the chemical. This is responsible for the death of half of the test subjects for a certain period of time.

2.6 *In-vitro* thrombolytic activity test

- **Principle**

To evaluate the thrombolytic potential of the plant extracts an easy method was followed where streptokinase and water were used as positive and negative control respectively.

- **Materials**

Table 2.12: Materials used in thrombolytic test

Streptokinase	Blood
Distilled water	Plant extract
Micro centrifuge tube	Saline

- **Procedure**

➢ **Streptokinase**

In this method Streptokinase (15, 00,000 I.U.) used as standard. The brand name of the streptokinase was STK and it was collected from Incepta Pharmaceuticals Ltd, Bangladesh. About 5mLof dextrose saline was added to the streptokinase vial and mixed properly. From the diluted suspension 100µl (30,000 I.U) was used for in-vitro thrombolysis (Sweta Prasad, 2007).

- **Preparation of sample**

At first 100 mf of ME, CF, PEF, EAF, and AQF of the plant was dissolved respectively in 10mL of methanol, chloroform, pet-ether, and ethyl acetate .then the soluble supernatants was decanted and filtered.

➢ **Blood sample**

Blood sample were collected from healthy human (male) volunteers (n=7) by maintaining aseptic condition without a history of anticoagulant therapy. Then1mL of blood was transferred to the previously weighed microcentrifuge tubes to form clots.

➢ **Thrombolytic activity**

To carry out the test at first, 5 mL of venous blood were drawn from each volunteer. Blood samples were taken in five different pre-weighed sterile micro centrifuge tubes and allowed to incubate at 37 °C for 45 minutes. After clot formation, fluid was completely released from each microcentrifuge tubes. The clot weight was determined by subtracting the weight of tube alone from the weight of clot containing tube. Here as a positive control, 100µL of streptokinase and as a negative non thrombolytic control 100µL of distilled water was used along with 100µL of each samples added separately to each test tubes. For observing the clot lysis, all the microcentrifuge tubes were incubated at 37 °C for 90 minutes. After incubation, the released fluid was discarded and tubes were again weighed to observe the difference in weight after clot disruption. Finally percentage of clot lysis was determined by following formula as followings:

% of clot lysis = (wt of released clot /clot wt) × 100

Chapter 3

Results

3. Results

3.1 Phytochemical screening of *Anisopter scaphula*

Table 3.1: Results of phytochemical screening of *Anisoptera scaphula*

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

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

3.2 Evaluation of antioxidant activity

- **DPPH free radical scavenging activity of *A. scaphula.***

Table 3.2: IC_{50}value (µg/mL) of ascorbic acid

Absorbance of blank	Conc. of extract (µg/mL)	Absorbance of the extract (nm)	% of inhibition	IC_{50} µg/mL
	78	0.084	96.11	
0.850	156	0.033	97.70	88.731
	312	0.028	97.17	
	625	0.024	97.52	
	1200	0.020	97.64	

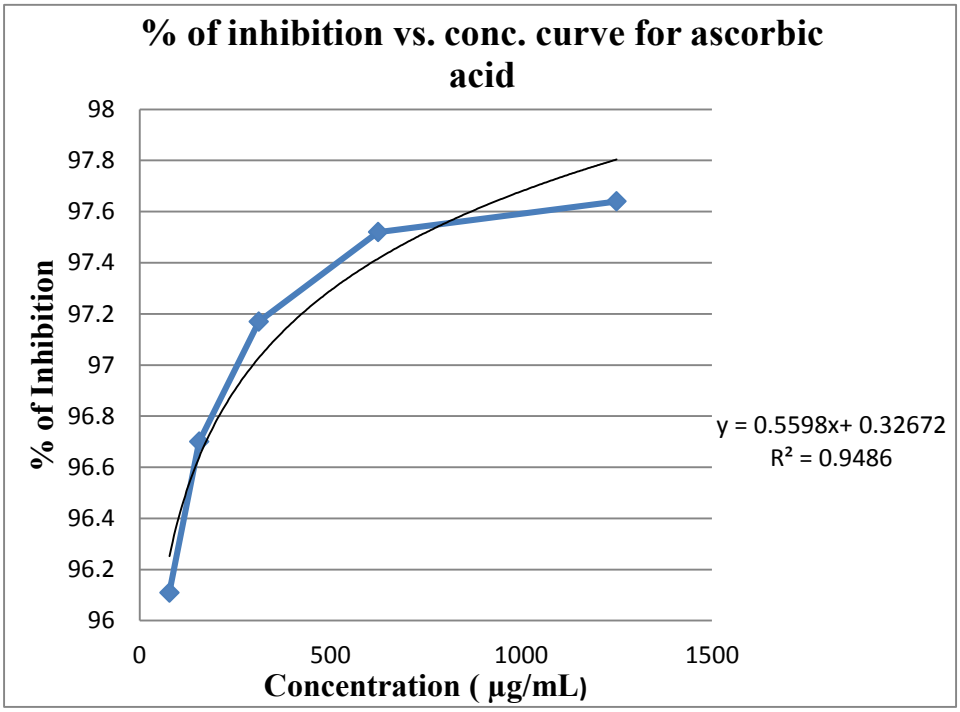


Figure 3.1: % of inhibition vs. conc. curve for ascorbic acid

Table 3.3: IC_{50} value (µg/mL) of methanolic extract of *A. scaphula*

Absorbance of blank	Conc. of extract (µg/mL)	Absorbance of the extract (nm)	% of inhibition	IC_{50} µg/mL
	78	0.084	90.05	
0.850	156	0.065	92.34	50.630
	321	0.021	97.42	
	625	0.019	97.67	
	1200	0.079	90.70	

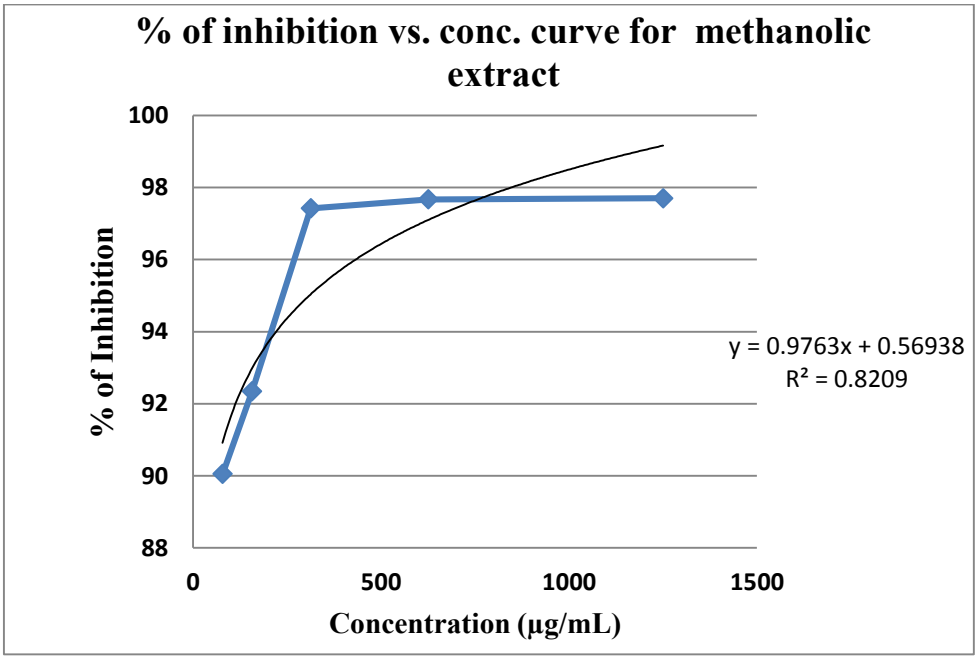


Figure 3.2: % of inhibition vs. conc. curve for methanolic extract.

Table 3.4: IC_{50} value (µg/mL) of pet ether (µg/mL) fraction of *A. scaphula*

Absorbance of blank	Conc. of extract (µg/mL)	Absorbance of the extract (nm)	% of inhibition	IC_{50} µg/mL
	78	0.084	91.00	
0.850	156	0.065	93.00	61.931
	321	0.021	95.00	
	625	0.019	95.28	
	1200	0.079	95.29	

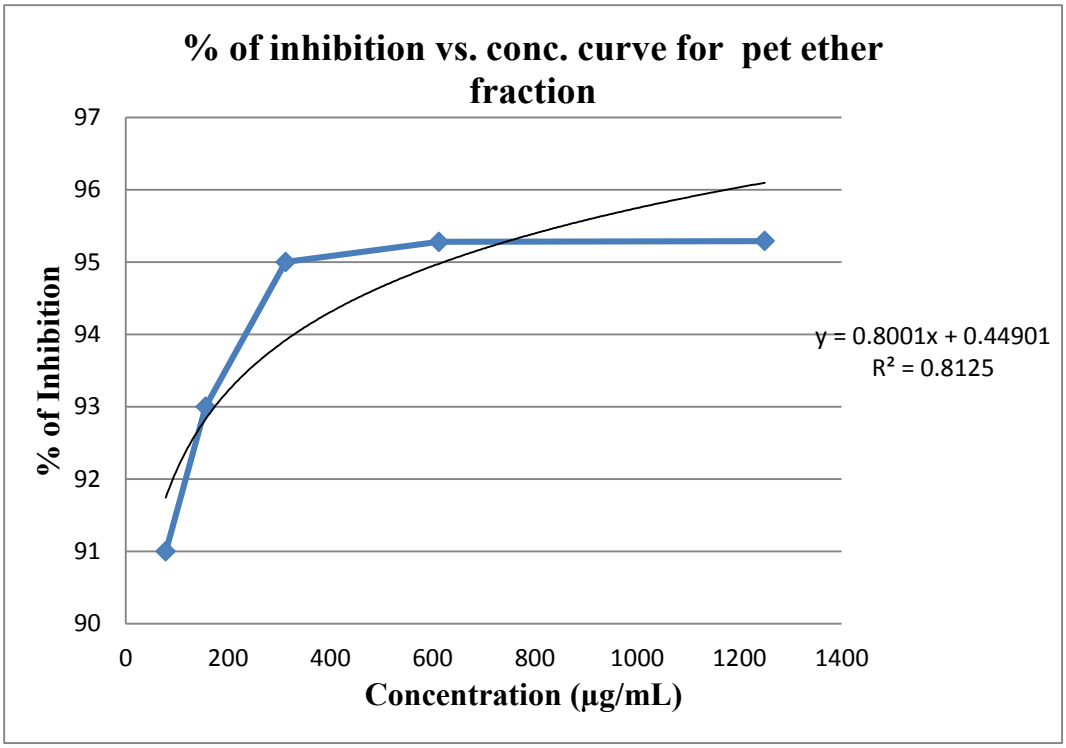


Figure 3.3: % of inhibition vs. conc. curve for pet ether fraction.

Table 3.5: IC_{50}value of chloroform (µg/mL) fraction of *A. scaphula*

Absorbance of blank	Conc. of extract (µg/mL)	Absorbance of the extract (nm)	% of inhibition	IC_{50} µg/mL
	78.12	0.084	91.00	
0.850	156.25	0.065	93.00	53.90
	321.50	0.021	95.00	
	625.00	0.019	95.28	
	1250.00	0.079	95.29	

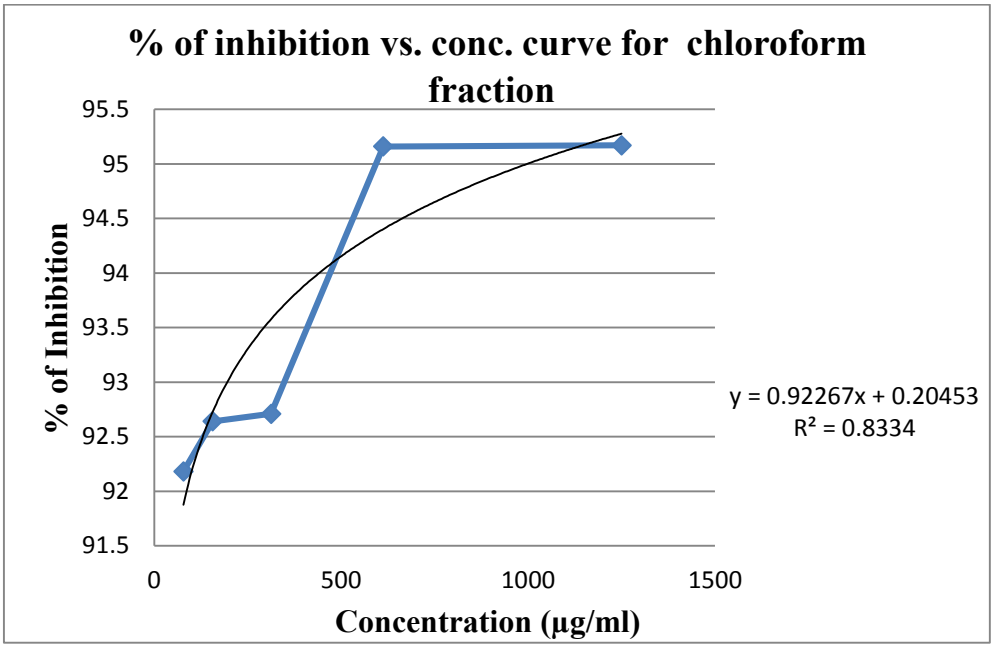


Figure 3.4: % of inhibition vs. conc. curve for chloroform fraction.

Table 3.6:IC_{50} value of ethyl acetate (µg/mL) fraction of *A. scaphula*

Absorbance of blank	Conc. of extract (µg/mL)	Absorbance of the extract (nm)	% of inhibition	IC_{50} µg/mL
	78	0.085	91.00	
0.850	156	0.065	93.00	64.101
	321	0.022	95.00	
	625	0.020	95.28	
	1200	0.079	95.29	

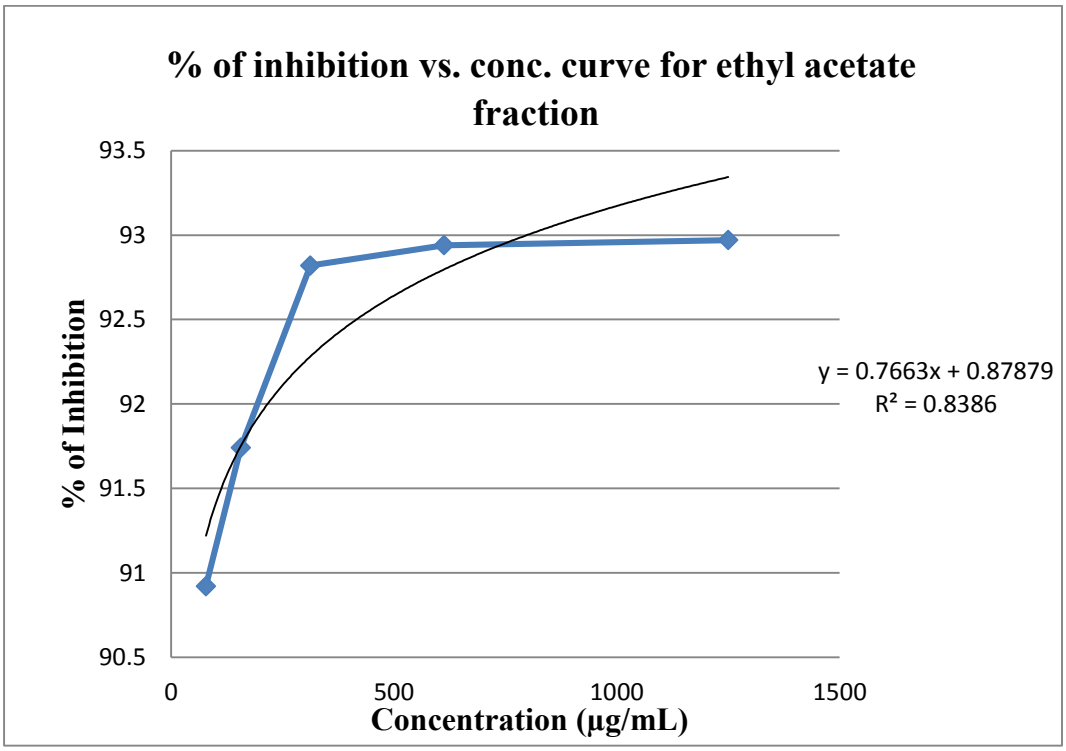


Figure 3.5: % of inhibition vs. conc. curve for ethyl acetate fraction.

Table 3.7: IC_{50} values of the test samples of leaves of *Anisoptera scaphula.*

Test samples	Regression line	R^2	IC_{50} (μg/mL)
ME	y= 0.9763x+0.56938	0.8209	50.630
PEF	y = 0.8001x+0.44901	0.8125	61.931
CF	y = 0.92267x+0.20453	0.8334	53.90
EAF	y = 0.7663x+o.87879	0.8386	64.101

3.3 Evaluation of cytotoxicity:

3.3.1 Brine shrimp lethality bioassay

Table 3.3.1.1: Effect of vincristine sulphate (positive control) on shrimp nauplii

Conc. (µg/mL)	Log_{10} Conc.	% Mortality	LC_{50} (µg/mL)
0.000		0	
0.039	-1.409	20	
0.078	-1.107	30	
0.156	-0.806	30	
0.312	-0.505	40	
0.625	-0.201	50	0.457
1.250	0.097	70	
2.500	0.398	80	
5.000	0.699	80	
10.00	1.000	90	
20.00	1.301	100	

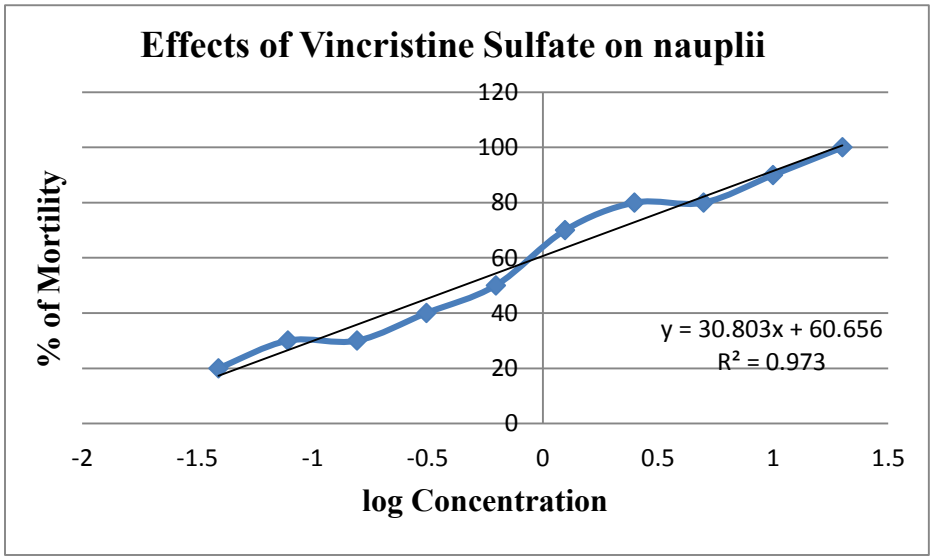


Figure 3.6: Plot of % mortality and predicted regression line of vincristine sulfate.

Table 3.8: Effect of the methanolic extract (ME) of leaves of *Anisoptera sacphula* on shrimp nauplii

Conc. (µg/mL)	Logconc.	% of mortality	LC_{50} (µg/mL)
0.000		0	
0.781	-0.107	10	
1.562	0.194	20	
3.125	0.495	20	
6.250	0.796	20	
12.50	1.097	30	2.680
25.00	1.398	30	
50.00	1.699	40	
100.0	2.000	30	
200.0	2.301	50	
400.0	2.602	50	

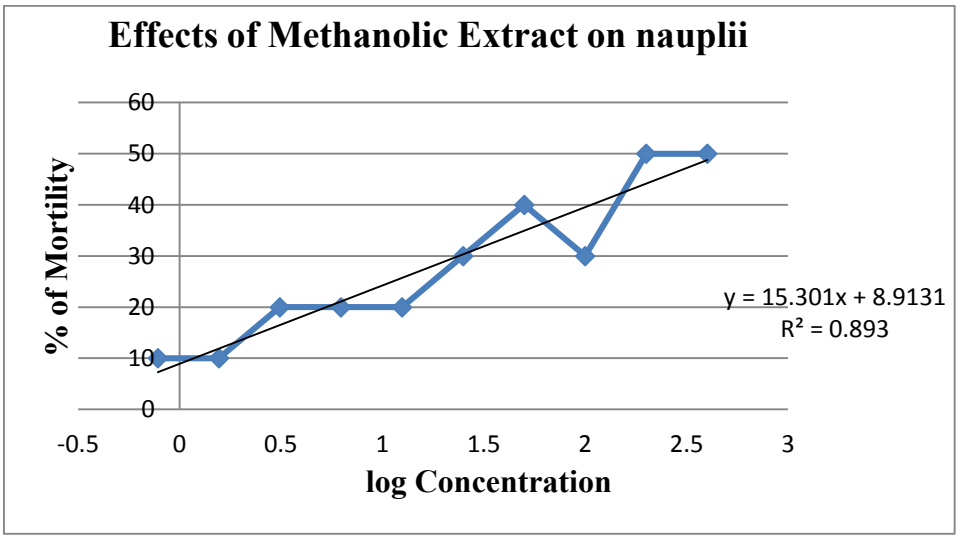


Figure 3.7: Plot of % mortality and predicted regression line of ME

Table 3.9: Effect of petroleum ether fraction (PEF) of the methanolic extract of *Anisoptera scaphula* leaves on shrimp nauplii

Conc. (µg/mL)	Log_{10} conc.	% of mortality	LC_{50} (µg/mL)
0.000		0	
0.781	-0.107	10	
1.562	0.194	10	
3.125	0.495	10	
6.250	0.796	20	
12.50	1.097	20	2.814
25.00	1.398	20	
50.00	1.699	30	
100.0	2.000	30	
200.0	2.301	40	
400.0	2.602	60	

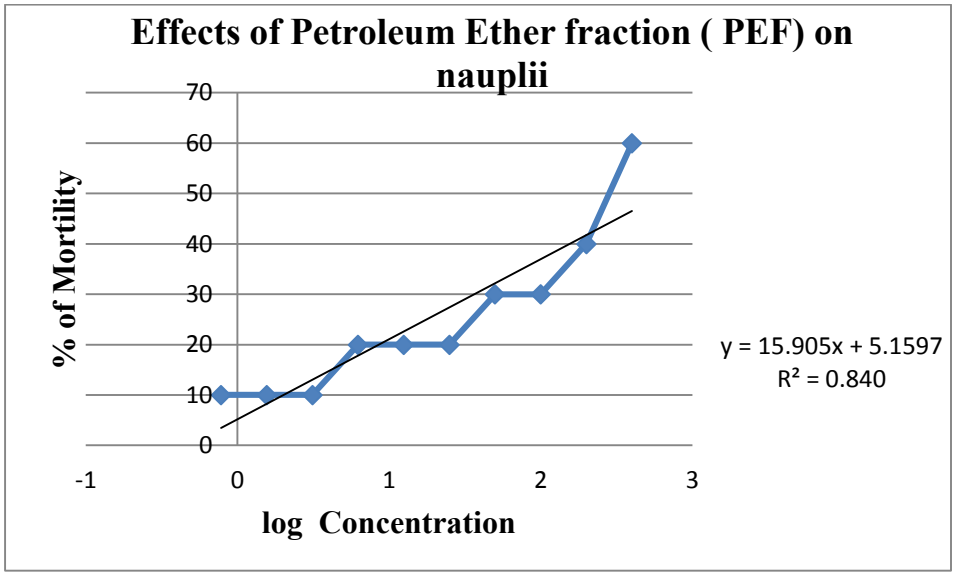


Figure 3.8: Plot of % mortality and predicted regression line of PEF

Table 3.10: Effect of chloroform fraction (CF) of the methanolic extract ofleavesof *Anisoptera scaphula* shrimp nauplii

Conc. (µg/mL)	Log_{10} conc.	% of mortality	LC_{50} (µg/mL)
0.000		0	
0.78125	-0.107	10	
1.5625	0.194	10	
3.125	0.495	20	
6.25	0.796	20	
12.5	1.097	20	3.160
25	1.398	20	
50	1.699	30	
100	2.000	30	
200	2.301	40	
400	2.602	50	

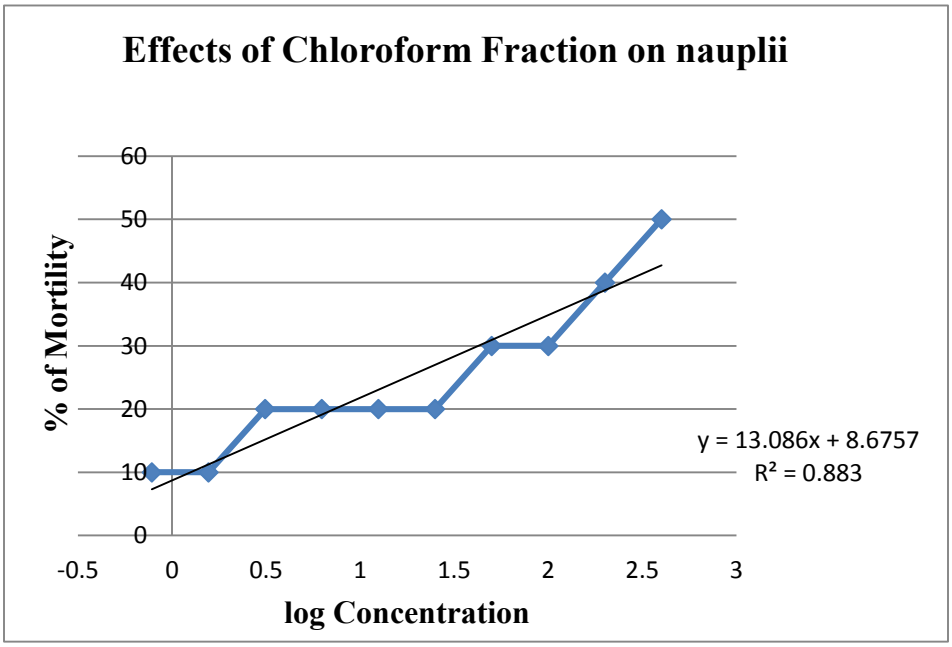


Figure 3.9: Plot of % mortality and predicted regression line of CF

Table 3.11: Effect of carbon tetrachloride soluble fraction (CTCSF) of the methanolic extract of leaves of *A. scaphula* on shrimp nauplii

Conc. (µg/mL)	Log_{10} conc.	% of mortality	LC_{50} (µg/mL)
0.000		0	
0.78125	-0.107	10	
1.5625	0.194	10	
3.125	0.495	10	
6.25	0.796	10	
12.5	1.097	20	3.642
25	1.398	30	
50	1.699	30	
100	2.000	30	
200	2.301	30	
400	2.602	40	

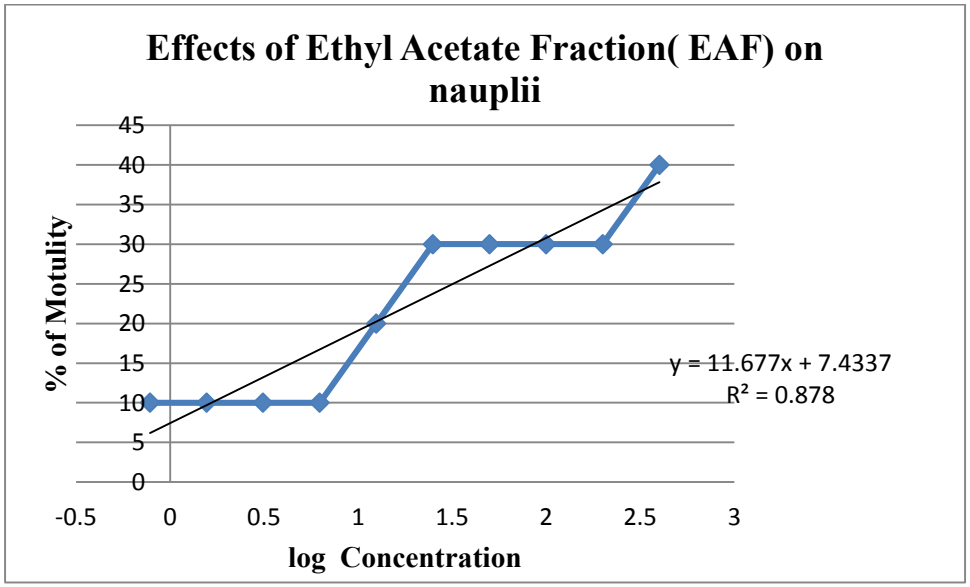


Figure 3.10: Plot of % mortality and predicted regression line of EAF.

Table 3.12: LC_{50} values of the test samples of leaves of *Anisoptera scaphula*

Test samples	Regression line	R^2	LC_{50} (µg/mL)
VS	y = 30.803x+60.656	0.973	0.457
ME	y = 15.301x+4.8131	0.893	2.680
PEF	y = 15.905x+5.1597	0.840	2.814
CF	y = 13.086x+8.6757	0.883	3.160
EAF	y = 11.677x+7.4337	0.878	3.642

3.4. Evaluation of thrombolytic activity:

Table 3.13: Thrombolytic activity (in terms of % of clot lysis) of *A. scaphula*

Test samples	% of clot lysis
Streptokinase	81.99
MEF	30.50
PEF	20.10
CF	21.60
EAF	37.48
AQF	15.00

Chapter 4

Discussion

4. Discussion

In this present dissertation, the methanolic leaf extract of the selected plant, *Anisoptera scaphula* was subjected to different phytochemical screening procedures. The screening procedure revealed the presence of alkaloids, flavonoids, glycosides, resins, steroids and tannins in this particular plant. The presence of these compounds might suggest that the plant contains medicinally active components.

The methanolic leaf extract as well as different solvent fractions of *Anisoptera scaphula,* viz., MEF, PEF, CF and EAF were subjected to DPPH free radical scavenging (FRS) assay in order to determine the antioxidant potential of this plant. In this experiment, ascorbic acid was used as reference standard for which, the IC_{50} value was found to be 88.732µg/mL. The IC_{50}values of MEF, PEF, CF, and EAF were found to be 50.630µg/mL, 61.931µg/mL, 53.90µg/mL, 64.101µg/mL respectively. Thus, the current study might suggest the traditional use of this plant as an antioxidant agent.

The brine shrimp lethality bioassay was performed to evaluate the cytotoxicity of MEF, PEF, CF and EAF of *A. scaphula*. The LC_{50} (lethal concentration) value of the investigated samples was determined after passing 24 hours of shrimp hatching by means of creating a plot of percentage of the died (% of Mortality) brine shrimp (nauplii) against the logarithm of the concentration of the test sample. Regression analysis was used to determine the best fitted line from the curve obtained from the data. In this investigation, an anticancer drug, vincristine sulphate was used as a positive control, which showed the LC_{50} value of 0.451µg/mL. On the other hand, MEF, PEF, CF, EAF of the leaf extract of the plant *A. scaphula* gave LC_{50} values of 2.680 mg/mL, 2.814µg/mL, 3.160 µg/mL and 3.642 µg/mL respectively. Therefore, it can be proposed that this test plant *A. scaphula* may have cytotoxicity. So, more investigation on this plant may be effective to establish it as a new medicine.

Thrombolytic activity of *A. scaphula* was determined by using streptokinase as positive control for which, 81.99% of clot lysis was observed. As a negative control, distilled water was used which showed 15% of lysis of blood clot. The MEF, PEF, CF and EAF of *A. scaphula*, exhibit 30.50%, 5.00%, 20.10%, 21.60%, and 37.48% of clot lysis. Comparing the values of clot lysis

obtained from different fractions of *A. scaphula* with the positive control value, it may be suggested that, the plant may have significant thrombolytic activity.

Conclusion:

The methanolic extract of the leaf and branch of the plant *Anisoptera scaphula*, has been studied for the evaluation of phytochemical as well as its biological profiles. After performing the phytochemical investigation of the plant, it has been found that this plant contains some of the significant bioactive substances such as carbohydrates, tannins, saponins, glycosides and protein, resins. From the biological investigation, it has been found that this plant extract showed a positive response in the brine shrimp lethality bioassay. On the other hand, this plant extract demonstrated a moderate antioxidant and thrombolytic activity test. Accordingly, the current study has demonstrated a potential bioactivity of the plant *Anisotera scaphula* offering further detailed investigations on this plant. The investigation might assist to invent the unfamiliar effectiveness of this plant, as well as may introduce the plant as a new source of medicine.

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