Phytochemical and Biological Screening of Methanol Extract of *Maesa montana* (Myrsinaceae) Leave

A project submitted

by

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

Certification Statement

This is to certify that this project titled "Phytochemical and Biological Screening of Methanol Extract of *Maesa montana* (Mysinaceae) Leaves" submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (Hons.) from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Dr. Hasina Yasmin, Associate Professor, 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 research was aimed to evaluate the potential biological properties of the plant *Maesa Montana* for the first time. The plant belongs to the family Myrsinaceae. *Maesa Montana* was found to be locally used in arthritis. The methanolic extract of the leaves of the plant was partitioned with petroleum ether, carbon tetrachloride, dichloromethane and the fractionates were subjected to preliminary phytochemical screening. The phytochemical analysis showed that *Maesa montana* contains a mixture of phytochemicals such as alkaloids, glycosides, phenols, tannins, flavonoids, steroids and saponins. The crude methanolic extract of leaves of *Maesa montana* was used to evaluate the antioxidant, cytotoxic and thrombolytic activities. Antioxidant property of the extract was determined by DPPH test and the free radical scavenging activity of the crude methanolic extract was found with IC_{50} value of 62.27 µg/ml. The total phenolic content of the extract was found 295.395 (mg of GAE/gm of extract) suggesting a good source of natural antioxidant. The brine shrimp lethality bioassay was performed to determine the cytotoxicity of the extract. The assay showed LC_{50} value of 9.449 µg/ml which is comparable to the positive control (LC_{50} 0.517 µg/ml.). In the thrombolytic study, the plant showed 19.911 % clot lysis which is also comparable to the standard clopidogrel which showed 40.436 % clot lysis. Thus our study demonstrated that the methanolic extract of leaves of *Maesa montana* possesses antioxidant, cytotoxic and thrombolytic activities.

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List of abbreviations:

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

Chapter One

INTRODUCTION

1. Introduction

1.1 Medicinal Plants and Phytotherapy

Since traditional preparations or pure active principles, plants are most of the cases considered a common basis of medicaments. There are also various other purposes for which plants are randomly used which includes preservation of food, substitute medicine and natural therapies for over several centuries. Generally it is reflected that compounds which are prepared naturally instead of unnaturally are tend to be biodegraded comparatively without difficulty and consequently being more ecologically satisfactory. As a result, natural antioxidants, antibacterial, cytotoxic, antiviral and fungicidal agents have achieved admiration in current years. This ultimately led to the creation of optimistic image among consumers which is also scattering as day passes. A traditional or folk medicine practice focuses on using of different plants of medicinal value (Acharya & Shrivastava, 2008).

A medicinal plant can possibly be defined as one which contain substance that can be utilized for remedial reason or which may act as a forerunner for production of helpful medications. Plants employing favorable pharmacological impacts on physical body are usually titled as "Medicinal plants." It can now be reached to the conclusion that plants which normally integrate and possess some secondary metabolites, similar to tannins, alkaloids, glycosides and comprise minerals as well as vitamins, have therapeutic properties (Ghani, 2005).

Phytochemistry is the branch of study which is concerned with plant constituents**.** These constituents are chemical substances that are present in plant (Ghani, 2005). Phytotherapy is the study which uses medications that are derived from plants for the purpose of treatment and prevention of several diseases**.** It can also be regarded as a medical practice which has a science based foundation and can also be distinguished from several other traditional tactics, for example- medical herbalism (Heinrich, 2012).

The conception of phytotherapy originated from a French physician named Henri Leclerc, who first used the word in the year 1913 and published of the Précis de phytothérapie "Handbook of Phytotherapy", the first in the year 1922. Phytotherapy *first* moved in the English language through its common explanation in 1934, being introduced by Eric Frederick William Powell, an English practitioner who focused his practice on herbalism and homeopathy (Heinrich, 2012).

Although herbal medicines are considered with a distinct custom profile (one based on scientific and medical evidence) as phytotherapeutic goods, similar products in some cases can be considered used as food complements by others. The latter point towards that medicines which are on the basis of herbal substances are considered as undocumented therapies and in several countries they are treated so. The custom of phytotherapy fluctuates extensively all through the world. Countries like South Korea and Japan, established phytotherapy goods incorporated into health insurance reportage. Whereas countries like China, India and Nepal provides extensive health care reportage for herbal medications, which descent upon traditional medication services (Heinrich, 2012).

Table 1.1: **List of some common medicinal plants and their usages**

Plant	Common Name	Botanical Name	Parts Used	Medicinal Use
	Amla	*Emblica officinalis*	Fruit	Vitamin - C, diabetes, laxatives, cold, hyper acidity
	Ashok	*Saraca Asoca*	Bark Flower	Menstrual pain, uterine, disorder, diabetes
	Aswagandha	*Withania Somnifera*	Root, Leafs	Stress, nerves disorder
	Bael	*Aegle marmelous*	Fruit, Bark	Diarrhea, dysentery, constipation
	Bhumi Amla	*Phyllanthous amarus*	Whole Plant	Anemic, jaundice, dropsy
	Brahmi	*Bacopa,Monnieri*	Whole plant	Nervous, memory enhancer, mental disorder
	Chiraita	*Swertia Chiraita*	Whole Plant	Skin disease, burning, cessation, fever
	Madhunasini	*Gymnema Sylvestre*	Leaves	Diabetes, hydrosol, asthma
	Guggul	*Commiphora Wightii*	Gum rasine	Rheumatoid arthritis, paralysis

1.2 History of Traditional Medicine:

For several centuries, plants with medicinal value are being used in various nations as a harmless therapeutic vision. Generally, the task of plants with medicinal value is constructed on the rich familiarities of infinite healers throughout the eras, inherited from descendants, healer-to-healer transference otherwise established through individual familiarities through the period of stint. These old-style methods of treatment include Allopathic, Ayurveda, and Homeopathic etc. Maximum educated nation-states have established their identifiable Materia Medica, accumulating specifics regarding the innumerable plants used for therapeutic resolves.

The archaeological knowledge provide sensible confirmations about the remedial possessions of plants which were acknowledged to inhabit in primitive time (Ahmad, Kamran, & Mobasher, 2014; Halberstein, 2005; Khan, H., Saeed, M., & Muhammad, 2012). So the therapeutic custom of using plants is as prehistoric as humanoid civilization. Particular substantial references that are accessible includes the Artharvaveda, based on the customary Indian medicine called ayurvedic medicine. The use of clay tablets were described by the Mesopotamians (1700 BCE) (Glodsby et al., 2001). Documented evidence also proves the practice of Papyrus Ebers by Egyptians (Ramawat & Merillon, 2008). Several standard data's that expose the remedial convention of plants consist of- De Materia Medica, inscribed thru Dioscorides (Glodsby et al., 2001).

The Greeks provided a praiseworthy contribution towards pharmaceutical sciences, chiefly in phytopharmaceuticals (Helberstein, 2005). Aristotle during his lifespan has described about 500 crude drugs that can be used in the cure of various pathological disorders (Chatard, 1908). Theophrastus, apprentice of the great Aristotle, stated 500 or more crude medications in the manuscript written by him (Scarborough, 1978).

Customary Chinese Medicine signifies as a major primogenital methods of treatment. This system is inimitable in treatment, philosophies and remedies. This effective scheme has been proven of remarkable significance of the antiquity of remedy and at present has gained worldwide acknowledgement owing for confirming the root scheme and also being free of

external influence (Patwardhan et al., 2005). Fu His (2953 BC) is regarded as the discoverer of this scheme. The exogenous aspects that are well-thought-out to be involved in the paleopathology are stated in the Chinese Medicine system. Later on, emperors like Shen Nung and Hong Ti payed contribution towards the advancement of this system. Chinese pharmacopoeia also known as 'Pen Tsao', contains extended number of therapies for several health difficulties. The peak of engraved Chinese medicine goes to Shen Nong Ben Cao Jin. A manuscript 'Zhu Bing Yuan Ji Lun' was written by famous Cao Yuan Fang (550-630), describes the etiology and symptoms of several diseases. Chinese medical students consider this book as a standard reference for their study. Wang Tao (702-772) provided significant support towards the old-style Chinese medicine through his published works like, 'Waitai Miyao'. This describes roughly 600 preparations in total (Kopp et al., 2003).

Customary Indian Medicine or commonly recognized as Ayurveda (acknowledged as the mother of all treatments) has always been regarded by means of primogenital healthiness scheme on globe. Explanations of this scheme can be accessed through prehistoric texts like, 'Rig-Veda' then 'Atharva-Veda'. The term Ayurveda a type of Sanskrit word which implies acquaintance of lifecycle (Hsu & Barrett, 2008; Mukherjee & Wahile, 2006).

The religious conviction of Islam fixed a new scale to maintain discipline of medication in the state of Arabia. Al Tabri was a legendary Muslim genius, whose written manuscript 'Firdous Al Hikmat', comprises generally 7 separate parts, amongst where an individual one is specifically dedicated to medications and toxins (Chatard, 1908). Abu Ali Al Hussan Ibn Sina, is worldwide considered originator of Greco-Arabic institute of medication whose written manuscript 'Canon' can be regarded as per an exemplary on medication in Europe, that pronounces exceeding 1000 medications (Guerra, 1979).

1.3 Plants with Medicinal Value in Bangladesh

Bangladesh is a subtropical nation that is surrounded with more than 5,000 angiosperms 200 families. Table 1.2 includes some medicinal plants along with their uses. The atmosphere of the country is very favorable for the growth and nourishment of medicinal plants. Areas like- Chittagong, Dhaka, Rajshahi, Sylhet are supplemented with approximately 5000 diverse types of plants with medicinal value, as cited in the "'Materia Medica' ". Since the early times, customary medications have been distinguished in the country.

Table 1.2: **Plants with medicinal value available in Bangladesh used for the preparation of traditional medicine**

Plant Name	Purpose of Using
Alstonia scholaris	Antipyretic and anthelmintic
Dioscorea alata	To cure constipation
Heliotropium indicum	To treat cough, fever, abdominal pain
Limonia acidissima	To treat typhoid fever and diarrhea
Terminalia catappa	To treat bronchitis and skin diseases

1.4 Literature Review: *Maesa montana*

Maesa belongs to a genus of flowering plants. At present it is placed in the Primulaceae family. But previously it was placed in Myrsinaceae or Maesaceae family. There are about 150 species, among which the majority are found in Malaysia, New Guinea, Western Asia and the Pacific Islands. Considering the country of Bangladesh normally it exists in the forests of Chittagong and its hill tracks, Sylhet. These plants are climbers, bushes and trees which can grow up to the height of 12 meters.

1.4.1 Plant Description of *Maesa montana*

Maesa montana are big shrubs or minor tree, having a height of (2-3) meters. Branchlets are terete in shape and white velvet-hairy to hairless, pith solid. Leaf-stalks are intensely lined to (1-1.5) cm. Leaves are elliptic, rectangle or spear shaped and in rare cases can be roughly ellipsoid which ranges from (7-14 x 3-7) cm. The leaves are also membranous, having a wedge-shaped or blunt base, border coarsely notched or curly with punctate teeth and maybe of tip pointed or long-pointed. Lateral veins are (8-12) on respective sideways of midrib which finishes in teeth. Flowers are borne in racemes or panicles ranging from (2-7) cm, in leaf axils. Bracteoles are minute, spear shaped or ellipsoid. Flowers are normally near 2 mm. Flower-stalks size ranges from (1-2) mm. Sepals are ellipsoid or rectangular, mostly hairless or sometimes lacey, complete, tip rounded. Flowers are generally of white color being of bell-shaped, ellipsoid petals, can be long or longer than tube, border entire or toothed, rounded tip. Stamens persist in the gullet of the flower. Fruit is of white in color, circular or ovoid berry, size ranging from (3-4) mm in diameter, orange spotted, having persistent sepals that covers (2/3-3/4) of fruit. Flowering time: february-april.

1.4.2 Taxonomy

Kingdom: Plantae

Subkingdom: Tracheophyta

Phylum: Magnoliophyta

Class: Magnoliopsida

Order: Ericales

Family: Myrsinaceae

Genus: *Maesa*

Maesa montana

1.4.3 Pharmacological Properties of Different Species of *Myrsinaceae*

Myrsinaceae family provides several medicinal properties they specially used for arthritis, swelling, anthelmintic, insecticidal, cancer, inflammation, membrane ailments etc. Some important species are listed in Table 1.3.

Table 1.3: **Activities found in various species of *Myrsinaceae***

Species	Explored Activities
Maesa Argentea	Cytotoxicity
Maesa Lanceolata	Cytotoxicity, Antioxidant
Maesa Chisia	Antiviral
Maesa Indica	Thrombolytic
Maesa Japonica	Antioxidant
Maesa Ramentacea	Cytotoxicity (Possible Anticancer Effect)
Maesa Acuminata	Cytotoxicity, Antioxidant, Thrombolytic
Maesa Perlaria	Antioxidant

Using the extract prepared with solvent ethanol and performing the initial level phytochemical screening of the root portion of *Maesa perlaria* showed the presence of major bioactive constituent's like- 2,4-Di-tert-butylphenol, sigmasterol and campesterol (Yang et al., 2016). Several reports provides plant extract containing 2,4-Di-tert-butylphenol possess significant antioxidant and neuronal protective effects (Chen & Dai, 2015; Choi et al., 2013; Yoon et al., 2006).

Using the fruit portion for the preparation of extract prepared with solvent ethanol of *Maesa lanceolata* exhibited ample cytotoxic action counter to Human leuikemia (Mossa, Muhammad, Ramadan, & Al, 1999; Muhammad, Mossa, & El-Feraly, 1993).

An established preliminary study on the methanolic extract of the leaves of *Maesa indica* provided comparative less thrombolytic activity (Jainul et al., 2014).

1.5 Preliminary Screening of Biological Activities of Plants

1.5.1 Antioxidant Property by Total Phenolic Content

Compounds which are phenolic in nature yield antioxidant properties through oxidation-reduction reactions that plays a vital role for absorbing and counterbalancing of unrestricted radicals, solo mastic in addition triad oxygen otherwise disintegrated peroxides (Osawa, 1994). Phenolic components which are responsible for yielding the antioxidant effects includes- flavonoids, phenolic nature acids as well as phenolic nature diterpenes (Shahidi, Janitha and Wanasundara, 1992). Numerous chemicals produced by florae having noteworthy antioxidant properties, could be linked to minor frequency and causing death to quite a lot of mortal inhabitants (Velioglu, Mazza, Gao, & Oomah, 1998). Phenols are entirely ionized in alkaline condition. The principally used reagent in the specified ionic phenolic solution would be Folin-Ciocalteu. This reagent allows to oxidize effortlessly the phenols. The reagent Folin-Ciocalteu generally is of yellow in color but soon completion of oxidation progression, the solution alters to blue color. This color alteration strength is measured at the specific wavelength of 760 nm in a UV-spectrophotometer. The assessed absorbance denotes the components total phenolic content (Harbertson & Spayd, 2006).

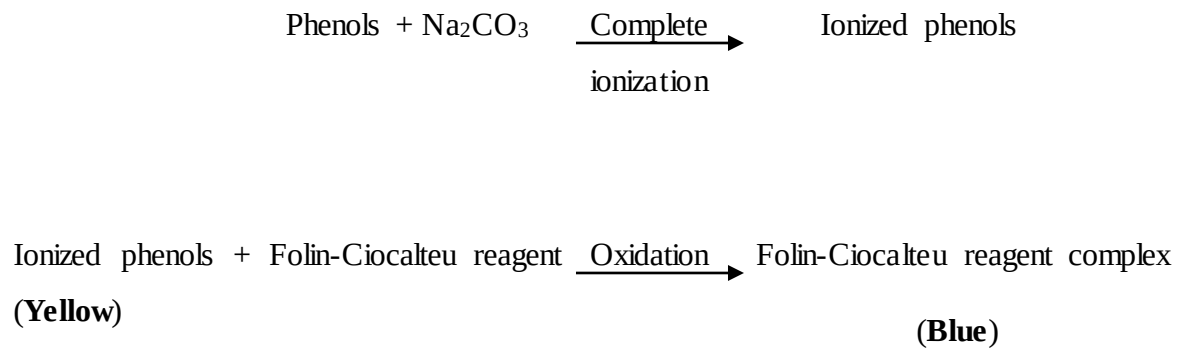

Fig. 1.1 Schematic Representation of Phenolic Content

1.5.2 Antioxidant Property by DPPH Assay

Plant extracts unrestricted radical hunting actions (antioxidant activity) were measured on firm 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH) (Brand-Williams, Cuvelier, & Berset, 1995). A solution of 2 milliliters of methanol of that particular extract at varied concentrations were combined with 3 milliliters of a DPPH methanol mixture (20 µg/ml). Thus the antioxidant action was evaluated through bleaching of the DPPH stained methanol mixture with the vegetable extract equated to tert-butyl-1-hydroxytoluene (BHT) and ascorbic acid (ASA) by UV spectrophotometer.

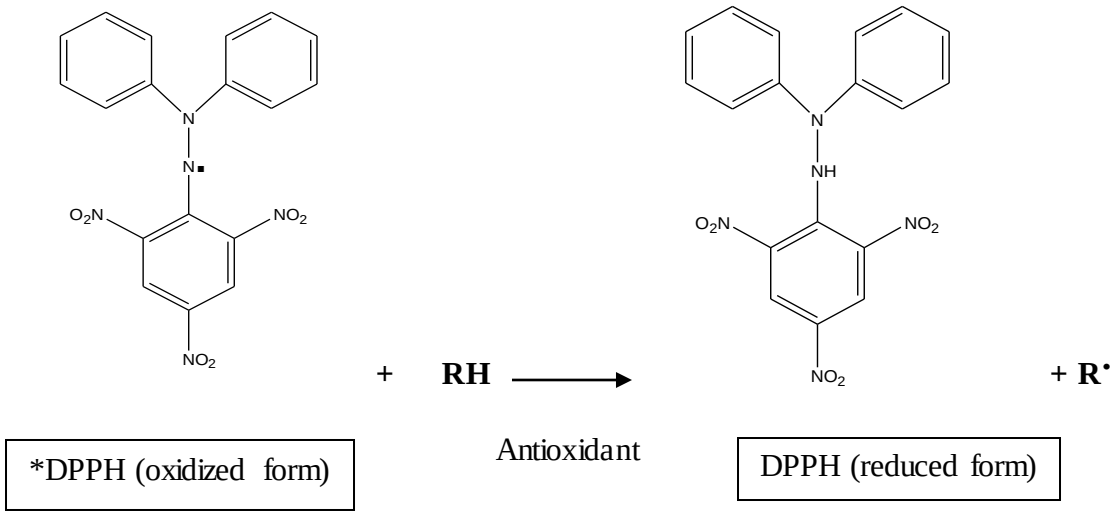

Fig. 1.2 Chemical Structure of DPPH

* DPPH = 1, 1-diphenyl-2-picrylhydrazyl

1.5.3 Cytotoxic Activity

The term cytotoxicity denotes to things which are lethal to cells. Cells being visible to a cytotoxic compound might react in several means, for example- necrosis, which causes the loss of membrane solidity and rapidly death occurs because of destruction of cells, might conclude to propagate and split; or could trigger a hereditarily controlled programmed cell death, a term which is named apoptosis (Cytotoxicity, 2016).

1.5.3.1 Cytotoxic Activity by Brine Shrimp Lethality Bioassay

Biologically active components at all times are noxious to the breathing body at extensive doses. This also validates the statement that "pharmacology is simply the highest dose toxicology and toxicology is simply lower dose pharmacology". A swift and comprehensive biological test for biologically active components or natural and synthetic origin is the lumbar lamb shrimp test (McLaughlin, Rogers, & Anderson, 1998). This method, verifies the bioactivity of natural product fields, fractions along with pure compounds.

Incubation of the salted shrimp eggs required to perform in replicated brine for the growth of nauplii. The projected concentration for the experimented testers is arranged through the totaling of the considered dimethylsulfoxide (DMSO) quantity. Counting of nauplii are performed via optical checkup by further taking these in ampoules which contains 5 milliliters of replicated brine. Afterwards, testers of several concentrations introduced to the pre-selected bottles beforehand branded thru micropipette. These bottles held in reserve approximately 24 hours for finally calculating the stayers (Meyer et al., 1982).

1.5.4 Thrombolytic Property

A severe syndrome like cerebral venous sinus thrombosis (CVST) is closely linked with severe illness and mortality (Watson, Stimpson, Topping, & Porock, 2002). A first line of treatment for CVST can be heparin which is an anticoagulant and also an antidepressant because of its efficiency, being safe and potentials (Biousse and Newman, 2004). Particular

thrombolytic medications such as plasminogen activator (t-PA), urokinase, streptokinase, etc. plays vital title part in administrating sufferers associated via Cerebral Venous Sinus Thrombosis (Baruah, 2006). For that reason, main objective for the study was examining the thrombolytic effectiveness of the extract prepared with solvent methanol of *Maesa Montana* leaves.

Thrombolytic medications mainly considered to prominently improve blood flow and aid in the reduction or lessening the symptoms of several patients without the requirement for supplementary physicians. But then again for several reasons it is not commended to every person. Thrombolytic medications are used for the treatment of myocardial complications. Amid them, streptokinase is the most extensively used. A patient with aforementioned use of blood products, herbal medications or complementary foodstuffs or those who are in menace of hemorrhage thrombolytic medications are not commended to them. The circumstances may include- increased blood pressure, vigorous or severe bleeding and cerebral hemorrhage (Shapira-Rootman, Beckerman, Soimu, Nachtigal, & Zeina, 2015).

1.6 Rationale of the Study

Maesa montana's literature review reveals that the plant was studied by a very limited number of researches. However, studies of the other species of the same genus have stated anthelmintic, antioxidant, analgesic, anti-inflammations, thrombolytic and cytotoxic actions. Thus the core purpose of the study is to identify and discover the several pharmacological properties of the methanolic leaf extract of the plant.

1.7 Aim of the Study

This study aims to investigate and identify the unknown biological potential of the selected plant, *Maesa montana*.

1.8 Objectives of the Study

The objectives of the study includes evaluation of following:

- **A.** Preliminary phytochemical screening of methanolic extract of *Maesa montana* leaves
- **B.** Biological activities of the methanolic extract of *Maesa montana* leaves
    1. Determination of the antioxidant property of the plant extract using *in-vitro* DPPH free radical scavenging technique as well as the total phenolic content
    2. Determination of Cytotoxic activity
    3. Determination of Thrombolytic activity

Chapter Two

Methodology

2. METHODOLOGY

2.1. Plant Assembly

The leaves of *Maesa montana* plant was assembled in the month of May 2017 from the division of Sylhet, Bangladesh. After collection the plant was identifies by the National Herbarium Bangladesh (NHB), Mirpur, Dhaka (accession digit DACB- 44999).

2.2 Development of Plant Substantial

Portion of leaves were detached using bare hands from stalks of the collected plant which was washed away by means of uncontaminated water for eradicating of dirt and filth elements. The fresh leaves were then permitted to dehydrate under shade for quite about a few days. Finally the leaves were dehydrated for 24 hours at a temperature of (30-40) °C in an oven.

2.3 Plant Extraction Procedure

2.3.1 Grinding

The dried leaves were grounded to powder using a grinder and packed in vacuum sealed plastic container with the required label. Finally the container was kept in a cool and dry place until further investigation. About 500 gm of the powder was soaked in a cleaned amber color reagent bottle (5L) using 1.6 liters of methanol. The bottle was airtight and kept for 2 days with occasional shaking.

2.3.2 Filtration

The extract was filtered through a clean cloth, followed by means of cotton and Whatman filter (pore size: 110 mm).

2.3.3 Evaporation

The filtrate was concentrated through the use of a rotational evaporator (Heidolph, Germany) at a speed of 100 rpm and at a temperature of 30°C. The bulk of the crude extract obtained was 44 gm. The extract was poured in a large sized petri-dish and was positioned under laminar airflow in order to evaporate the residual solvent of the extract. The particular

reason for the extract to be kept under laminar airflow was to prevent any sort of microbes growth in the extract while the evaporation was continuing.

2.4 Preliminary Phytochemical Screening of the leaves of *Maesa montana*

2.4.1 Solvent-Solvent Partition of Crude Extract by Modified Kupchan Partition Method

Following the conventions established by Kupchan then developed by Van Wagenen *et al.*, (1993) solvent-solvent partitioning was performed. The whole partitioning process is schematically shown in Figure 2.1. First of all 5 gm of crude extract was taken in a 500 mL beaker. In another beaker 90 milliliters of methanol was combined with 10 milliliters of water. Then slowly this blend was added to the extract to make an aqueous methanol solution. The mixture was then separated with petroleum ether, dichloromethane and carbon tetrachloride.

2.4.1.1 Partitioning with Petroleum Ether

The prime mixture was transported to an unscrambling funnel with addition of 100 milliliters of the petroleum ether. The funnel was kept shaking for near about 5 minutes leaving the mixture uninterrupted for further 15-20 minutes. The organic portion was collected through the repetition of the procedure thrice (100 milliliters x 3). Finally, the fractions were collected and kept for evaporation together while keeping the aqueous portion was taken in a separate beaker.

2.4.1.2 Partitioning with Carbon Tetrachloride

12.5 milliliters of distilled water additionally was included to the prime mixture that remained subsequently partitioning using petroleum ether and was properly mixed. The prime solution was transported to a separating funnel with the addition of 100 milliliters of the carbon tetrachloride (CCl_4). The funnel was kept shaking for near about 5 minutes leaving the mixture uninterrupted for additional 15-20 minutes. The organic portion was

collected through the repetition of the procedure thrice (100 milliliters x 3). Finally the fractions were collected and kept for evaporation together while keeping the aqueous portion was taken in a separate beaker for the upcoming step.

2.4.1.3 Partitioning with Dichloromethane

16 milliliters of distilled water was additionally included to the prime solution that remained subsequently partitioning using petroleum ether and carbon tetrachloride (CCl_4) and was correctly mixed. The prime solution was transported to a separating funnel with the addition of 100 milliliters of the dichloromethane (CH_2CL_2). The funnel was kept shaking for near about 5 minutes leaving the mixture uninterrupted for additional 15-20 minutes. The organic portion was collected through the repetition of the procedure thrice (100 milliliters x 3). Finally the fractions were collected and kept for evaporation together while keeping the aqueous portion was taken in a separate beaker for the upcoming step.

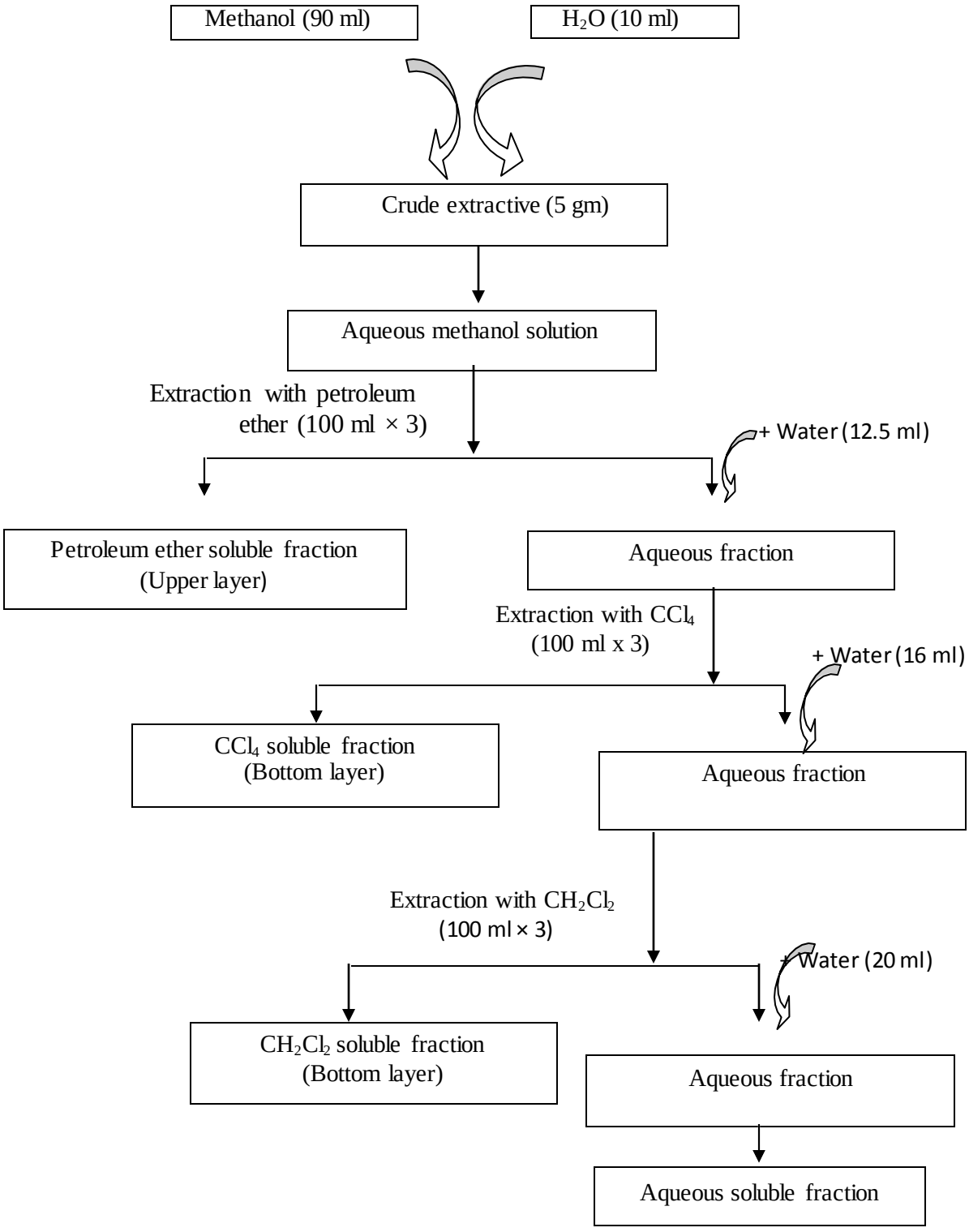

Figure 2.1: Schematic representation of the modified Kupchan Partitioning of methanolic crude extract of *Maesa montana* leaves

2.4.2 Phytochemical Tests Performed:

2.4.2.1 Test for Alkaloids:

a. **Mayer's test**: 1 milliliter from each particular fraction was taken in separate test tubes with the addition of Mayer's reagent (Potassium Mercuric Iodide Solution) drop by drop. The deposition of a white color precipitation designates the existence of alkaloids.

b. **Wagner's test:** 1 milliliter from each particular fraction was taken in separate test tubes and in each fraction 1 milliliter of Wagner's reagent were introduced. Development of a brown or reddish brown color precipitation confirms existence of alkaloids.

c. Hager's test: 1 milliliter from each particular fraction was taken in separate test tubes and in each fraction few drops of Hager's (Saturated picric acid solution) reagent which results in bright yellow shaded precipitate and designates the existence of alkaloids.

2.4.2.2 Test for Glycosides:

a. **Molish's test**: To particular fraction about 2-3 droplets of molish reagent was added further and the mixtures were mixed properly. Now to these mixtures few drops of conc. H_2SO_4 was introduced cautiously. Reddish-purple shaded ring development at the intersection of two layers displays existence of glycosides.

b. **Conc. H_2SO_4 test**: 1 milliliter of conc. H_2SO_4 was added with 1 milliliter from each fraction, and permitted to standstill for 2 minute which gives a reddish color precipitate specifies the existence of glycosides.

c. **Keller Killiani test**: 1 milliliter of each particular fraction was dissolved in 1 milliliter of glacial acetic acid and further cooled for few minutes. After cooling 2-3 droplets of ferric chloride accompanying with 2 milliliters of concentrated H_2SO_4 was added. A reddish brown shaded disc development intersecting the two covers confirms existence of glycosides.

2.4.2.3 Test for Tannins:

a. **Ferric chloride test:** Partitioned each fractions were used to treat separately with couple of drops of $FeCl_3$ solution. Construction of black color precipitation designates existence of tannins.
b. **Lead acetate test:** 1-2 milliliters of each partitioned fractions were treated with basic lead acetate. Construction of bulky red color precipitation designates the existence of tannins.
c. **Alkaline Reagent test:** A mixture of Sodium hydroxide used for treating 1-2 milliliters of each partitioned fractions. Construction of yellow to red color designates existence of tannins.

2.4.2.4 Test for Flavonoids:

a. **Zinc-HCl reduction test:** In each partitioned fraction, a nip of zinc powder accompanying with few droplets of conc. HCl was added. Construction of deep red color designates existence of Flavonoids.
b. **Lead-acetate test:** 1-2 milliliters of each partitioned fractions were treated with basic lead acetate. Construction of reddish brown color precipitation designates existence of flavonoids.
c. **$FeCl_3$ test:** 1-2 milliliters of each partitioned fractions were treated with neutral ferric chloride solution. Construction of blackish red color precipitation designates existence of flavonoids.

2.4.2.5 Test for Sterols:

a. **Liebermann-Burchard test:** In 1-2 milliliters of each partitioned fractions, a few droplets of solution of acetic anhydride was introduced. Further addition of few droplets of conc. H_2SO_4 along the walls of the test tube was done cautiously. Construction of reddish brown color ring at the intersection of two layers designates existence of steroids.
b. **Salkowski test:** In 1-2 milliliters of each partitioned fractions, 5 milliliters of chloroform accompanying with 1 milliliter of conc. H_2SO_4 was introduced

cautiously sideways of the tube with mixing. Construction of reddish color in the bottom layer designates existence of steroids.

2.4.2.6 Test for Courmarin: 1-2 milliliters of each partitioned fractions were taken in separate tubes to be enclosed with a piece of paper soaked in NaOH and further heated. After heating, these tubes yielding a yellow fluorescence under UV light designates existence of coumarins.

2.4.2.7 Test for Resins: In each partitioned fractions 3-4 milliliters of solution of $CuSO_4$ was introduced with vigorous shaking for 1-2 minutes. The mixture was then kept until separation. Construction of green color precipitation designates existence of resins.

2.4.2.8 Test for Phenols:

a. **Ellagic acid test:** Couple of droplets of 5% (w/v) glacial acetic acid accompanied with 5% (w/v) mixture of $NaNO_2$ were used to treat each partitioned fractions. Construction of muddy brown color designates existence of phenols.
b. **Phenol test:** 2 milliliters of each partitioned fractions were individually treated with 1 milliliter solution of $FeCl_3$. Construction of an intense color designates existence of phenols.

2.4.2.9 Test for Saponins: 5 milliliters of each partitioned fractions were taken in separate test tubes and shaken vigorously for obtaining stable froth. 5-6 droplets of olive oil was introduced to these frothy solution. Construction of an emulsion designates existence of saponins.

2.5 Antioxidant Property Analysis

2.5.1. DPPH Free Radical Scavenging Assay

DPPH can generally be consumed in estimation of free radicals effect (antioxidant antidepressants) on several components and plants with medicinal value (Choi et al., 2013; Desmarchelier et al., 1997).

2.5.1.1 Materials and Reagents

The reagents and materials necessary for the assay are listed in Table 2.1.

Table 2.1: Materials and Reagents

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

2.5.1.2 Procedure

Standard Preparation

For positive control ascorbic acid (ASA) was selected and the total required quantity of ascorbic acid were calculated. For acquiring a prime mixture maintaining a concentration to 500 µg/ml, the required quantity was dissolved. Sequential dilution was performed on the prime mixture towards attainment of several concentrations which ranged starting 500.0 to 0.977 µg/ml.

Test Sample Preparation

For the preparation of sample, 2mg of extract of the selected plant *Maesa Montana* and 4 ml of methanol were taken in a test tube and mixed. The concentration of the sample was 500 µg/ml. Now sequential dilution was performed on this tester mixture to attain several concentrations which ranged from 500.0 to 0.977 µg/ml.

Assay of Free Radical Scavenging Activity

Test tubes having concentration which ranged starting 500 µg/ml upto 0.977 µg/ml were lined up serially and in each one 3.0 milliliters of DPPH methanol mixture (20 µg/ml) were added. Mix remained set aside back in a black room for near about 30 minutes for the reaction occurrence. After passing of 30 minutes the absorbance of respective mix was

measured thru UV spectrophotometer at a wavelength of 517 nm keeping methanol as blank.

2.5.1.3 Calculation

Calculation of free radical DPPH inhibition in percentage *(I%)*:

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

A_{blank} = absorbance of the control reaction (which contains all of the reagents excluding the assessed substantial)

Finally the (IC_{50}) or the concentration provided by the extract for 50% was acquired through plotting percentage of inhibition (I %) against the extract concentration (µg/ml) using graphical representation.

2.5.2 Evaluation of Total Phenolic Content

A process denoted by Skerget *et al*., (2005) was used for the assessment of the total phenolic contents of leave extractives of *Maesa Montana*. In this process Folin-Ciocalteu reagent was used as an oxidative agent besides gallic acid was taken as standard (Majhenič, Škerget, & Knez, 2007).

2.5.2.1 Materials and Reagents

The reagents and materials necessary for the determination of total phenolic content in the extract are listed in Table 2.2.

Table 2.2: **Materials and Reagents for Phenolic Content Measurement**

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

2.5.2.2 Procedure

Standard Solution Preparation

Gallic acid is generally considered as standard. Several concentration of gallic acid solutions which range starting 100 µg/ml upto 0 µg/ml. An amount of 2.5 milliliters of Folin-Ciocalteu reagent (10x dilution done using water) as well as 2.0 milliliters of Na_2CO_3 solution (7.5% w / v) were added to 0.5 milliliter of gallic acid of each concentration. This mix was further incubated at normal room temperature for near about 20 minutes. After the incubation the absorbance was measured at a wavelength of 760 nm. Finally, the absorbance was plotted against the concentration in a graphical representation thus achieving a linear relationship.

Sample Preparation

For the desired sample concentration of 2mg/ml, 2 mg of the leaves extractives were dissolved in required amount of distilled water.

Table 2.3: **Sample Amount Used for Phenolic Content Test**

Sample	Concentration
Methanol Extract of *Maesa montana* leaf	2mg/ml

2.5.2.3 Calculation

2.5 milliliters of Folin-Ciocalteu reagent (10x dilution done using water) in addition to 2.0 milliliters of Na_2CO_3 solution (7.5% w/v) were added to 0.5 milliliter of plant extract (2 mg/ml). This mix was further incubated at room temperature for 20 minutes. After the incubation the absorbance was measured at a wavelength of 760 nm. The standard curve attained from gallic acid was used for the determination total phenolic content in the sample. The pehonolic content of the sample was expressed in milligram of GAE (gallic acid equivalent)/gram of extract.

2.6 Cytotoxicity Analysis

2.6.1 Reagents and Materials

The reagents and materials necessary for the analysis are listed in Table 2.4. 4 mg of Methanol extract of *Maesa montana* leaf was used for the study.

Table 2.4: **List of Materials Required for Brine Shrimp Lethality Assay**

Sl. No.	Name of materials
1	*Artemia salina* leach (brine shrimp egg)
2	Sea salt (NaCl)
3	Small tank with perforated dividing dam to hatch the shrimp
4	Lamp to attract shrimps
5	Pipette, Micropipette
6	Glass vials
7	Test tubes
8	Magnifying glass
9	Dimethyl sulfoxide (DMSO)
10	Plant extract

2.6.2 Procedure

Preparation of Seawater for Test

In 1 liter of distilled water 38 grams of salt previously weighed was dissolved and finally filtered for obtaining a clear mixture.

Hatching of Brine Shrimps for Test

Artemia salina, a species of brine shrimp was used for the study. The shrimp eggs added into a small tank earlier filled with seawater. For obtaining mature nauplii, continuous supply of oxygen and lamp light was provided throughout the period of hatch. For the collection of shrimp nauplii a special pipette called Pasteur pipette was used. Not more than

10±2 living shrimps were introduced in each particular test tube consisting of 5 milliliters of seawater.

Preparation of Test Solution

The test sample was dissolved with dimethyl sulfoxide (DMSO) in test tube followed by performing serial dilution for obtaining several concentrations ranging from 0.78125 µg/ml to 400 µg/ml. Now 50 µl of sample having a concentration of 400 µg/ml was introduced in a test tube which already contained not more than 10±2 nauplii in 5 ml seawater. Next, 50 µl other samples of different concentrations was introduced in each of the test tube.

Table 2.5: **Plant sample with different concentration after serial dilution**

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

Control Group Preparation

a) Preparation of Positive Control

Commonly used positive control in Brine Shrimp Lethality assay is vincristine. Stock solution of 20 µg/ml vincristine was prepared using DMSO. After that sequential dilution is performed to attain concentration range starting 10 µg/ml up to 0.0390 µg/ml. Finally the prepared positive control was introduced to test tube which contained 5 ml of seawater in the company of 10±2 nauplii.

b) Preparation of Negative Control

Negative control preparation includes addition of 100 µl of DMSO in 3 test tubes containing 5 ml of seawater and 10±2 nauplii. The speedy death rate of the nauplii will denote the test being invalid as the death of these nauplii occurred for some other reasons excluding the cytotoxicity of the components.

2.6.3 Calculation

24 hours later the results were checked. Through the use of magnifying glass and by visual inspection the total number of persisted nauplii was calculated. After this the (%) of mortality was considered. LC_{50} was calculated using vincristine standard.

2.7 Thrombolytic Property Analysis

The analysis of thrombolytic property determination was performed using clopidogrel (anti-platelet agent) as positive standard, the plant extractive as sample besides water as negative standard.

2.7.1 Materials and Reagents

The reagents and materials necessary for the study are listed in Table 2.6.

Table 2.6: **Used Materials in Thrombolytic Test**

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

2.7.2 Procedure

Test Sample Preparation

Sample preparation involved suspending 100 mg of plant extractives in 10 milliliters of distilled water. This mix was kept for overnight. Afterwards the supernatant was removed by decantation.

Standard Solution Preparation

For standard preparation clopidogrel an anti-platelet agent was considered. In 10 milliliters of distilled water 100 mg of clopidogrel was dissolved through mixing it appropriately.

Blood Sample Preparation

5 human volunteers were chosen for blood sample collection. Aseptic conditions were ensured during the blood sample collection. 1 ml of blood taken from each volunteer were introduced in 5 separate previously weighed micro centrifuge tube. The tubes were then kept aside for incubation at a temperature of 37 °C for approximately 45 minutes for clot formation.

2.7.3 Calculation

Once the construction of the clot, fluid portion was entirely cleared from every micro-tubes and the clot weight was found by the weight of the tube taken beforehand the clot is formed. For this test, 100 µl of clopidogrel was considered as positive standard and 100 µl of distilled water was used as a non-thrombolytic negative control. 100 µl of respective sample was introduced into respective test tubes. Each micro tubes was incubated at a temperature of 37 °C for approximately 90 minutes. Completion of incubation, released liquid was removed. The weight of the tubes were measured to observe the variance in weight after clot disruption. Finally, percentage of clot lysis is calculated by the following formula:

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

Chapter Three

Results

3. Results

3.1 Phytochemical Analysis of *Maesa montana*

Methanolic extract of *Maesa montana* was successively partitioned with petroleum ether, dichloromethane and carbon tetrachloride according to modified Kupchan Partitioning method (VanWagenen et al., 1993). Preliminary phytochemical analysis was performed for all the above fractionates and showed the presence of alkaloids, glycosides, tannins, flavonoids, saponins and steroids which are listed in the following table. Among the fractionates pet ether fraction was found to contain most of the phytochemicals.

Table 3.1: Preliminary phytochemical screening of methanolic extract of *Maesa montana* leaf

Phytochemical tests		**Petroleum ether fraction**	**Dichloro methane fraction**	**Carbon tetrachloride fraction**
Alkaloids	Mayer's test	++	-	-
	Wagner's test	++	+	+
	Hager's test	++	+	-
Glycosides	Keller killani test	++	-	-
	Conc. H_2SO_4 test	+	+	-
	Molish's test	++	+	-
Phenols	Ellagic acid test	+	-	+
	Phenol test	_	+	-
Tannins	Ferric chloride test	++	-	-
	Alkaline reagent test	++	+	+
	Lead acetate Test	-	-	-
Flavonoids	Zinc-HCl reduction test	_	_	-
	Lead acetate Test	+	-	+
	Ferric Chloride Test	++	-	-

Coumarins	Test for coumarins	-	-	+
Resins	Test for Resins	++	-	-
Saponins	Test for Saponins	+	+	-
Steroids	Liebermann-Burchard test	++	++	+
	Salkowski test	++	+	+

++ = Strongly present; + = present; - = absent

3.2. Antioxidant Property Analysis

3.2.1. DPPH Free Radical Scavenging Assay

The extracts prepared with the leaves of *Maesa montana* using methanol as solvent were subjected to unrestricted radical scavenging action by the scheme of Brand-Williams et al., 1995. Here Ascorbic Acid (ASA) was used as reference standard. Table 3.2 and 3.3 include the % inhibition values ASA and samples, respectively. IC_{50} value For ASA and sample were 73.358 µg/ml and 62.27 µg/ml.

Table 3.2: IC_{50} values (µg/ml) of Ascorbic Acid (ASA)

Conc. (µg/ml)	Absorbance of Standard (ASA)	% of inhibition	IC_{50} µg/ml
500	0.036	94.07894737	73.358
250	0.043	92.92763158	
125	0.072	88.15789474	
62.5	0.178	70.72368421	
31.25	0.284	53.28947368	
15.625	0.373	41.11842105	
7.813	0.409	34.04605263	
3.906	0.456	26.48026316	
1.953	0.479	21.21710526	
0.977	0.595	17.26973684	
Blank	0.608		

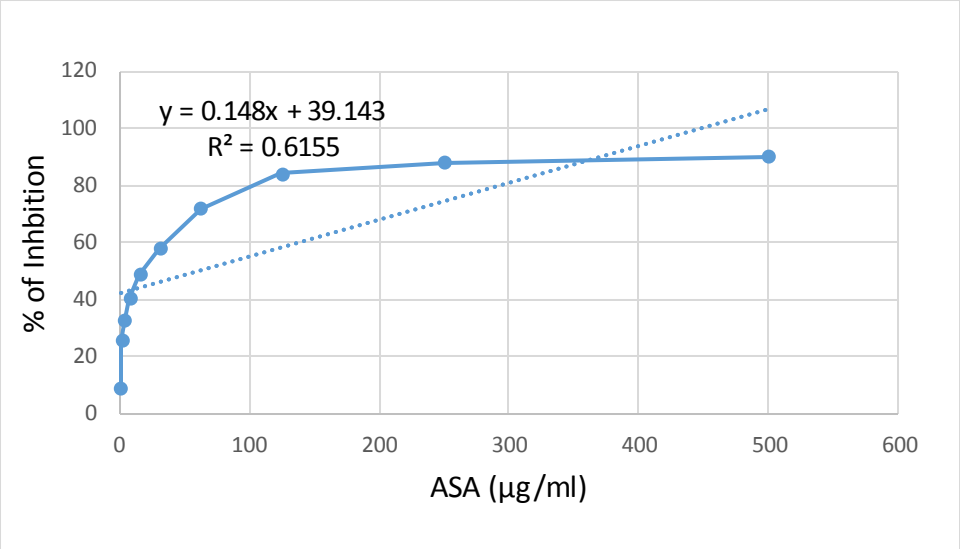

Fig 3.1: Concentration vs % inhibition curve of ASA

Table 3.3: IC_{50} value (µg/ml) of methanol extract of *Maesa montana* leaf

Conc. (µg/ml)	Absorbance of methanol extract	% of inhibition	IC_{50} µg/ml
500	0.061	89.96710526	62.27
250	0.074	87.82894737	
125	0.096	84.21052632	
62.5	0.171	71.875	
31.25	0.257	57.73026316	
15.625	0.311	48.84868421	
7.813	0.362	40.46052632	
3.906	0.407	33.05921053	
1.953	0.453	25.49342105	
0.977	0.552	9.210526316	
Blank	0.061		

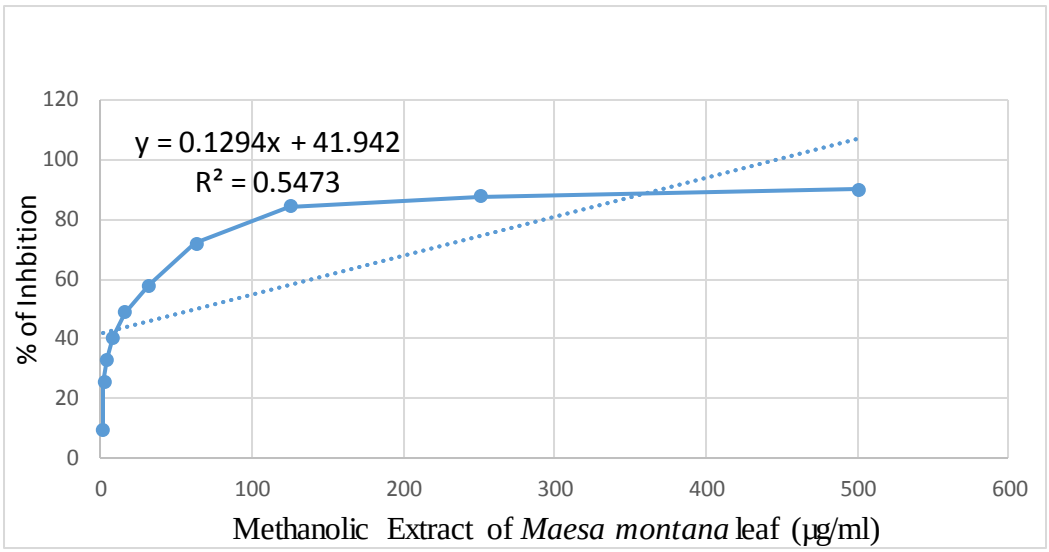

Fig 3.2: Concentration vs % inhibition of methanolic extract of *Maesa montana* leaf

3.2.2. Determination of Total Phenolic Content

Total phenolic content of Maesa Montana was determined by UV-Visible spectroscopic method using gallic acid as the standard drug (Table 3.4). The total phenolic content (mg of GAE/gm of extract) of the methanolic extract of *Maesa montana* leaf was found 295.395 mg of GAE/gm of extract as shown in table 3.5.

Table 3.4: **Absorbance of gallic acid**

Conc. (µg/ml)	Absorbance	Regression line	R^2
100	0.800	y = 0.0081x - 0.0007	0.9975
50	0.423		
25	0.215		
12.5	0.123		
6.25	0.047		
3.125	0.007		
1.5625	0.003		
0.78125	0.000		
0.3906	0.000		
0	0.000		

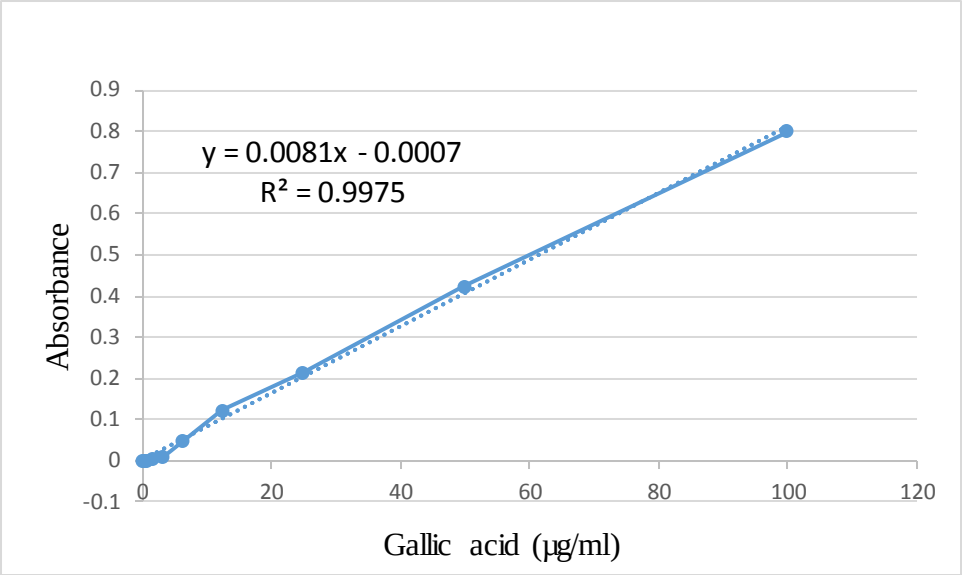

Fig 3.3: Standard curve for determination of total phenolic content

Table 3.5: Total phenolic content of methanolic extract of *Maesa montana* leaf

Sample	Absorbance	Total phenolic content (mg of GAE/gm of extract)
Methanol extract of leaves of *Maesa montana*	2.392	295.395

3.3 Cytotoxicity Analysis: Brine Shrimp Lethality Assay

Methanolic extract of *Maesa montana* was tested for its toxicity against brine shrimp lethality assay (Meyer et al., 1982). Vincristine sulphate was used as positive control. Results of the toxicity of the standard vincristine and crude extract against brine shrimp (LC_{50} values) are shown in Table 3.6 and 3.7. LC_{50} for vincristine sulphate was found to be 0.517 µg/ml (Table 3.5). LC_{50} of the methanolic extract of *Maesa montana* was found 9.449 µg/ml (Table 3.6).

Table 3.6: Effect of vincristine sulphate on shrimp nauplii

Conc. (µg/ml)	Log Conc.	No. of Nauplii taken	No. of Nauplii Dead	No. of Nauplii alive	% of Mortality	Log LC_{50} (µg/ml)	LC_{50} (µg/ml)
0.039	-1.408	10	2	8	20	-0.287	0.517
0.078	-1.107	10	3	7	30		
0.15625	-0.806	10	3	7	30		
0.3125	-0.505	10	4	6	40		
0.625	-0.204	10	5	5	50		
1.25	0.097	10	6	4	60		
2.5	0.398	10	7	3	70		
5	0.699	10	8	2	80		
10	1	10	9	1	90		
20	1.301	10	10	0	100		

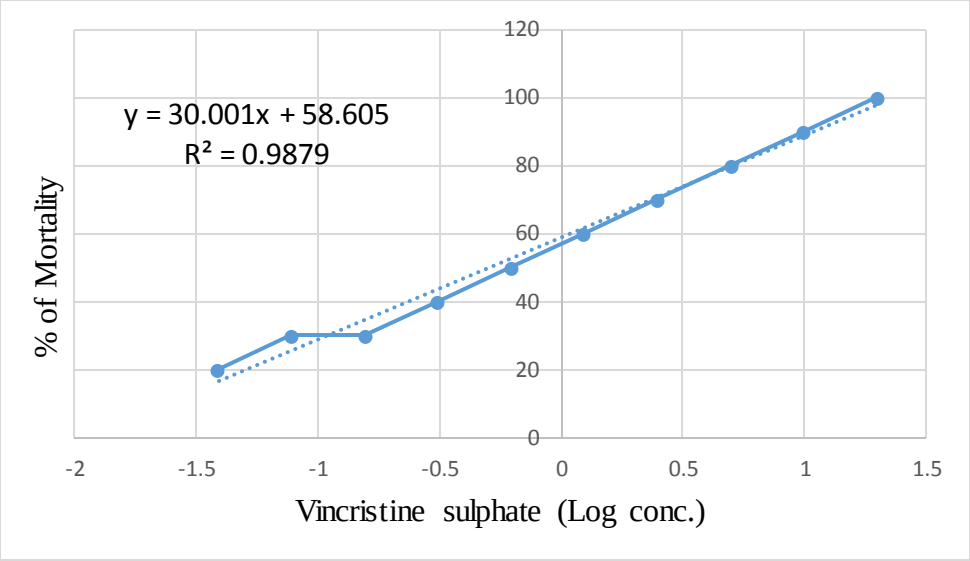

Fig 3.4: % mortality of vincristine sulfate

Table 3.7: **Effects of methanol extract of *Maesa montana* leaf on shrimp nauplii**

Conc. (µg/ml)	Log Conc.	No. of Nauplii taken	No. of Nauplii Dead	No. of Nauplii alive	% of Mortality	Log LC_{50} (µg/ml)	LC_{50} (µg/ml)
0.781	-0.107	10	2	8	20	0.975	9.449
1.562	0.194	10	3	7	30		
3.125	0.495	10	3	7	30		
6.25	0.796	10	5	5	50		
12.5	1.097	10	5	5	50		
25	1.398	10	6	4	60		
50	1.699	10	7	3	70		
100	2	10	8	2	80		
200	2.301	10	9	1	90		
400	2.602	10	10	0	100		

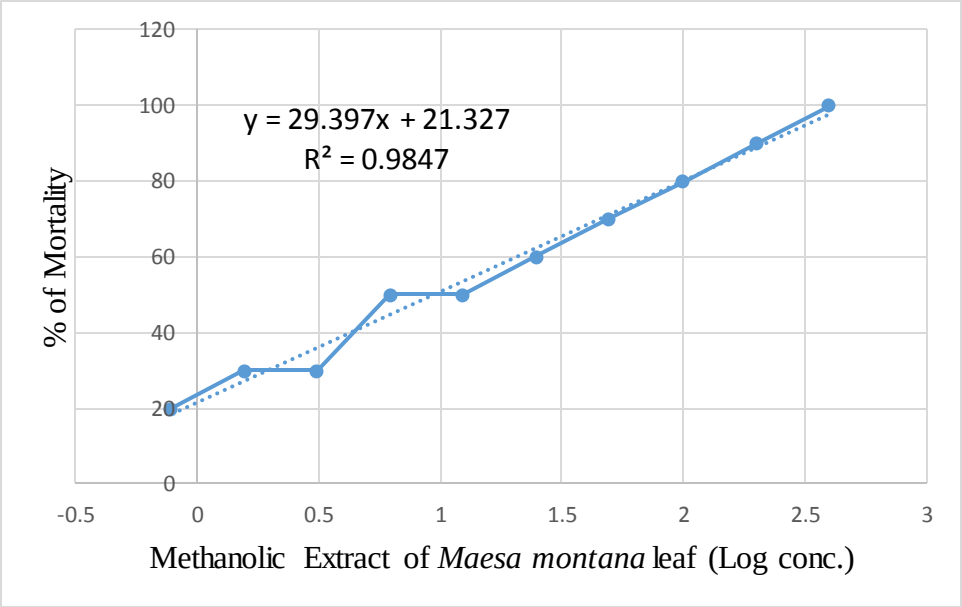

Figure 3.5: **% of mortality and predicted regression line of Methanol extract of *Maesa montana* leaf**

3.4. Thrombolytic Property Analysis

Methanolic extract of *Maesa montana* was assessed for thrombolytic action using clopidogrel as positive control. Subsequent incubation for 90 minutes at 37°C showed 40.436% lysis of clot. On the other hand, distilled water was treated as negative control which exhibited negligible percentage of lysis of the clot (3.27%). In this study methanolic extract of *Maesa montana* exhibited 19.911% clot lysis suggesting moderate thrombolytic activity.

Table 3.8: Evaluation of Thrombolytic Activity (in terms of % of clot lysis)

Samples	W1	W2	W3	W4= W3-W1	W5	% of clot lysis
Methanol extract of *Maesa montana* (10mg/ml)	0.795	1.608	1.473	0.678	0.135	19.911
Clopidogrel (10mg/ml)	0.788	1.625	1.384	0.596	0.241	40.436
Blank	0.778	1.599	1.573	0.795	0.026	3.27

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

Chapter Four

DISCUSSION

Discussion

This is the first report on phytochemical screening and determination of antioxidant, cytotoxic and thrombolytic activities of the methaolic extract of *Maesa montana* leaf**.** The phytochemical analysis of the methaolic extract of *Maesa montana* leaf showed the presence of mixture of phytochemicals such as alkaloids, glycosides, phenols, tannins, steroids, saponins and flavonoids. However, the petroleum ether fraction was found to contain the compounds in greater extent.

Many studies have shown that oxidative stress may potentiate several diseases such as Alzheimer's disease, Parkinson's disease, Down's syndrome, anxiety, schizophrenia, depression etc. (Ganguly et al., 2017). Natural antioxidants are effective free radical scavengers (Brewer, 2011) and thus received great attention. The present study was designed to determine the antioxidant potential of the methaolic extract of *Maesa montana* leaf by using DPPH radical scavenging assay and total phenolic content. The IC_{50} value for the methanolic extract of *Maesa montana* leaf was found to be 62.27 µg/ml which was comparable to the standard ascorbic acid (IC_{50}:73.358 µg/ml). Phenolic compounds act as antioxidants since they have the redox properties (Soobrattee et al., 2005). Free radical scavenging activity is enhanced by the presence of hydroxyl groups in phenolic compounds. For example, the antioxidant activity of flavonoids including flavones, flavonols and tannins depends on the presence of free OH groups. Thus determination of total phenolic content could be a basis for rapid screening of antioxidant activity in crude extracts. The total phenolic content of the methanolic extract of Maesa montana leaf was determined as 295.395 mg of GAE/g of extract suggesting that the extract has a great potential as an antioxidant and can be further subjected to vigorous study to find out the active phenolic and flavonoid compounds.

The methaolic extract of *Maesa montana* leaf presented moderate cytotoxicity against the brine shrimp. Brine shrimp lethality assay is usually performed for the initial screening of the cytotoxic activity of plant and therefore, further studies are required to establish the cytotoxic potential of plant extract against human cancer cell line.

The present study showed that the methaolic extract of *Maesa montana* leaf has thrombolytic effect as it reduces clot weight. Cardiovascular disorders are one of the main causes of morbidity and mortality in the world (Roth et al., 2015) as well as in Bangladesh (Islam & Majumder, 2013). Thrombolytics lyse the clot through plasminogen activation and is a natural thrombolytic agent (Bhaargavi, 2016; Califf et al., 1988). Currently available thrombolytic agents still harm significant shortcoming including large dose, limited specificity, bleeding tendency, allergic reactions etc.(Katzung, 2007). Therefore, to find more effective alternatives for CVS diseases, researchers are working on natural resources. The methanolic extract of *Maesa montana* leaf at 10 mg/ml concentration showed 19.911% clot lysis which is highly significant in comparison to its negative control and further studies maybe conducted to isolate the thrombolytic active chemical constituents for more precise results.

Chapter Five

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