Phytochemical and Biological Investigation of Methanolic Leaf Extract of *Atalantia roxburghiana*

A project submitted

by

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ID: 13146005

Session: Spring 2013

to

The Department of Pharmacy

In partial fulfillment of the requirements for the degree of

Bachelor of Pharmacy (Hons.)

Dhaka, Bangladesh

December 2017

This work is dedicated to my parents and my sibling to whom I owe my achievements.

Certification statement

This is to certify that, this project titled 'Phytochemical and Biological Investigation of Methanolic Leaf Extract of *Atalantia roxburghiana*' 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 Shejuti Rahman Brishty, Lecturer, Department of Pharmacy, BRAC University and this project is the result of the author's original research and has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the project contains no material previously published or written by another person except where due reference is made in the project paper itself.

Signed,

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Countersigned by the Supervisor

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Acknowledgement

I would like to begin my gratitude to Allah for the help in the completion of this research and preparation of this paper.

I would like to thank my supervisor Shejuti Rahman Brishty, Lecturer, Department of Pharmacy, BRAC University for her continuous provision, guidance and patience since the first day of the project work. As a person, she has continuously inspired and motivated me with her skilled knowledge which made me more passionate about the project when it began. I am really indebted to Professor Dr. Eva Rahman Kabir, Honorable Chairperson, Department of Pharmacy, BRAC University, for her support, encouragement and kind cooperation all through the project.

Finally, I would like to thank the laboratory officers and assistants for their continuous guidance and cooperative behavior.

Armin Tania

Abstract

The present study investigated the phytochemical and biological properties of *Atalantia roxburghiana* leaf methanol extract for the first time. The objective was to establish this plant as a potential therapeutic agent in traditional practice of medicine in Bangladesh. The preliminary phytochemical screening of the crude methanolic extract exhibited the presence of alkaloids, carbohydrates, flavonoids, phenols, phytosterols, tannins, glycosides and saponin. DPPH radical scavenging effect, total flavonoid and phenolic contents and antioxidant capacities were studied through established protocols and the leaf extract showed significant antioxidant potential through these experimentations. Moderate cytotoxic effect was observed in brine shrimp lethality bioassay. The antimicrobial activity was evaluated by disc diffusion method where the plant extract showed maximum activity against the bacteria *Escherichia coli* & *Vibrio cholera*. Evaluation of thrombolytic activity using Streptokinase further indicated very significant thrombolytic properties of the methanolic extract. The present data constitutes a preliminary investigation of the plant which might be valuable for performing research and developing new drugs from natural sources in order to treat different kinds of illnesses in our country as well as in other parts of the world.

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Abbreviations

AAE - Ascorbic acid equivalent

ASA - Ascorbic acid

BHT - *tert*-butyl-1-hydroxytoluene

DMSO – Dimethyl sulphoxide

DPPH -1,1-diphenyl-2-picrylhydrazyl

GAE - Gallic acid equivalent

LAF - Laminar Air Flow

ME - Methanolic extract

QE - Quercetin equivalent

ROS - Reactive oxygen species

SK - Streptokinase

TAC - Total Antioxidant Capacity

TFC - Total Flavonoid Content

TPC - Total Phenolic Content

VS – Vincristine sulphate

WHO - World Health Organization

Chapter-1: Introduction

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1.1 General information

Since the beginning of existence of human being on earth, illnesses have assumed a basic part in the occasions of each time and it is hard to exaggerate the effect of diseases on history. To get mitigated from the impacts of a portion of the most exceedingly awful illnesses, distinctive solution immunizations have been imagined for the diagnosis, treatment and prevention. However, there are still some diseases for the treatment of which, no pharmaceutical products have been found and researchers are working to find out the best possible medication for these diseases. In the present time most of the people take antibiotics and other medicines for different disease purposes, but they may sometimes cause harmful effect in the long-term use (Neill et al., 2013). For this reason, researchers are searching for a new way of medications having no side effect. Medicinal plants can be a better choice in this case as plants contain substances which have therapeutic effects with little or no side effects. Plants have been utilized for restorative purposes from the ancient period of time. There are different chemical compounds which are isolated from plants, showing natural capacities or biological activities. Some plants like green tea, ginger, aloe, pepper, walnuts and turmeric and so on are supposed to be nutritious and furthermore, have helpful therapeutic activity. Other than this, a few plants and their subsidiaries are additionally used as active ingredients in the preparation of various medications (Singh, 2015). World Health Organization (WHO) evaluated that 80% of people are presently utilizing natural medications as their essential health care needs. As per WHO, there are more than 21,000 plant species which have the efficiency to cure diseases and are being used as therapeutic plants (Rafieian-Kopaei, 2012). Treatment with medicinal plants is seen as to a great extent ensured as there is no or negligible side effects. Though over the period of time, the utilization of herbal medicines is expanding, there is still some lacking in the site of research action.

1.2 Relationship of the phytotherapy with the medicinal plants

Phytotherapy is the oldest medical practice in the world. It can be defined as the way of treatment using medicinal plants. Phytotherapy is a synergistic way to deal with health-care, consolidating the best of science, medicine and nature into a customized, holistic natural medicine solution for human being.

According to Ghani (2003a), medicinal plants can be defined as "a group of plants that possess some special properties or virtues that qualify them as items of drugs and therapeutic agents, and are useful as articles of medical management and treatment of diseases," and the World Health Organization (WHO) defines medicinal plant as "Any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes or which is a precursor for synthesis of useful drugs" (Sofowora, 1982).

Different types of plant parts are used in the modern treatment system. The different parts of plants can be used as a direct source of active pharmaceutical agents – either as single purified drug or in advanced extract form often in admixtures with other ingredients. Medicinal plants are used in a variety of dosage forms, such as powders, pastes, juices, infusions, decoctions medicinal preparations etc. Isolation of other important plant-derived drugs of modern medicine rapidly followed and since then, many useful drugs have been discovered and introduced into modern medicine. Drugs like caffeine from *Thea sinensis,* quinine from *Cinchona* spp. and colchicines from *Colchicum autumnale* constitute some examples of such early drugs. About 100 such drugs of defined structures are in common use today throughout the world and about half of them are accepted as useful drugs in the industrialized countries. These include drugs like atropine, colchicine, digitoxin, L-Dopa, emetine, ephedrine, ergotamine, hyoscine, hyoscyamine, morphine, quinidine, quinine, rescinnamine, reserpine, sennosides, vinblastine, vincristine etc. In addition to these, there are other plant-derived chemical substances of known structures that are used as drugs or necessary components of many modern medicinal preparations. These include camphor, capsaicin, eucalyptol, menthol, minor cardiac glycosides, various volatile oils etc. These are only a few examples of vast number of drugs that are derived from plants (Burbage, 1981).

1.3 Research of traditional drug in Bangladesh

In Bangladesh, ayurvedic, homeopathic, unani/kabirajis are very common and famous in rural areas. Different parts of plants with medicinal properties are being used in these traditional medicines. People of rural areas are mostly depended on medicinal plants for their primary health care and remedy of diseases. Because of unavailability of drugs and commodities, lack of trained providers, imposition of unofficial fees, a rural-urban

imbalance in health provider's distribution, unfavorable opening hours and weak referral mechanisms; poor people have a little access to health-related services. Thus, these are contributing to low utilization of public facilities in Bangladesh (Nawaz et al., 2009). Bangladesh contains about 5000 plant species because of its productive soils, atmosphere and occasional assortment (Kadir 1990; Yusuf et al., 1994). Around 500 of these species are proposed to have therapeutic properties (Ghani, 2004; Hossain, 2005). Around 85% of the population lives in rustic regions (Halim et al., 2007) and almost 80% are dependent on restorative plants for essential human services (Hossain, 2005). It was expressed that many individuals frequently utilize such treatment, since herbal medication is sometimes considered comparatively low cost. Be that as it may, its popularity originates from the viability of the treatment most of the time and comparatively safe, with few or no side effects (Mukulet al., 2007). Likewise, home grown solutions, in view of their scattered nature, are generally effortlessly and quickly accessible (Elliot and Brimacombe, 1986).

The use of remedial plants in essential health frameworks is imperative, especially in remote commonplace gatherings and insufficiently accessible extents. The course of action of Unani and Ayurvedic remedy was furthermore brought under the National Drug Policy of Bangladesh in 1982 to ensure availability, business collecting and advancing of significant value of Unani and Ayurvedic Pharmaceutical and Medications (Ahsan et al., 1997).

Table 1.1: Some crude drugs used as medicine in Bangladesh (Ghani, 2003a)

Bangali Name	Scientific Name	Part(s) Used	Uses
Nayantara	*Catharanthus roseus*	Flowers	Cancer, insomnia, blood pressure and diabetes.
Sarpagandha	*Rauvolfia serpentina*	Root	Blood pressure and dysentery.
Ghritokumari	*Aloe indica*	Leaves	Constipation, anthelmintics,fistula, piles,leucorrhoea, burns and jaundice.
Lajjabati	*Mimosa pudica*	Whole plant	Blood purification, toothache, convulsion fistula and piles.
Assamlata	*Mikania cordata*	Leaves	Dysentery.
Ulatkambal	*Abroma augusta*	Bark, leaves	Gonorrhea, diarrhea, urethritis and irregular menstruation.
Jogyadumur	*Ficus hispida*	Bark, root	Insects bites, boils, asthma, piles, cough, bronchitis, and diarrhea.
Shatamuli	*Asparagus racemosus*	Roots	Cancer, bacterial and fungal disease, tonic, appetizer, jaundice and diabetes.
Anatamul	*Tylophora indica*	Leaves	Asthma, cough, bronchitis, diarrhea, dysentery and stimulant.
Mahedi	*Lawsonia inermis*	Leaves, flower	Skin disease, pox, burns, dandruff and insomnia.

Bohera	*Terminalia Billerica*	Fruit, bark	Constipation, diarrhea, dysentery, leprosy, rheumatisms and piles.
Bherenda	*Ricinus communis*	Roots, seeds	Constipation and rheumatisms.
Ghandabadal	*Paederia foetida*	Leaves	Diarrhea, urticaria, paralysis, piles and toothache.
Haritaki	*Terminalia chebula*	Fruit, Bark	Indigestions, jaundice, piles, skin disease and ulceration of gum.
Thankuni	*Cliotoria ternatea*	Whole plant	Weakness, dermatitis, jaundice and stomach disorder.
Neem	*Azadirachta indica*	Leaves	Anthelmintic, fever dermatitis, stomach disorder, jaundice, nausea, and ruminates.
Tulshi	*Ocimum sanctum*	Leaves, flower, seeds	Stomach disorder, malaria, common cold, and hypertension.
Nishinda	*Vitex negundo*	Leaves, barks	Weakness, cough, headache, malaria, and kalazar.
Basak	*Adhatoda vasica*	Root, leaves, flowers	Cough asthma, arthritis, dysentery, and malaria.
Arahar	*Cajanus cajan*	Leaves, seeds	Jaundice, mouth sore and leprosy.
Arjun	*Terminalia arjuna*	Bark	Heart disease.
Kalojira	*Nigella sativa*	Seeds	Common cold, rheumatisms, galactagogue and carminative.

Hatishur	*Heliotropium indicum*	Root, leaves	Fever, rheumatisms, wound.
Amloki	*Phyllanthus emblica*	Bark flower, fruit	Hair tonic, cough, diuretic, stomach ache, dysentery, jaundice, dermatitis.
Halud	*Curcuma longa*	Rhizomes	Blood purification, skin disease, eye disease, tonic, and stomachache.
Methi	*Trigonella foenum-graceum*	Seeds	Hypertension and diabetes.

1.4 Name of the Plant

Scientific name: *Atalantia roxburghiana* Hook. f.

Scientific synonym: *Sclerostylis amyridoides* M. Roem

Local name:

Malaysia: Limau hutan or malyan atalantia or limau Pagar

Vietnam: Quyet forest, Green tangerine

China: *Atalantia* Corrêa

1.5 Information about plant

1.5.1 General information about *Atalantia roxburghiana*

Atalantia roxburghiana is a plant belonging to the Rutaceae family. *Atalantia roxburghiana* is a treelet that grows to a height of 6m in open country and on the limestone hills of Cambodia, Laos, Vietnam, Malaysia and Thailand. The hole is crooked, smooth and the bark is dark green. The stems are spiny and leaves are simple, spiral and estipulate. The crushed leaves have a string citrus order. The blade is dotted with oil cells, acute, sharp or minutely blunt-tipped. The flowers are white and minute, with a 4-lobed calyx and stamens which are free. The secondary nerves are inconspicuous and the fruits are pale yellow. In Cambodia, Laos, Vietnam, Malaysia, the leaves are used to treat lung disorders, probably on account of their essential oils. It has not yet been explored for pharmacology. One can reasonably expect the isolation of cytotoxic and/or anti-HIV acridone alkaloids from it. Flowering occurs in April while the edible fruits are produced from June to August. The

leaves used in medicine to treat respiratory diseases. Decoctions of leaves are used to lung disorders (Wiart, 2006). The chloroform extract of *Atalantia roxburghiana* was reported to exert muscle relaxant through inhibition of calcium influx through the calcium channel of the cell membrane (Rashid et al., 1995). Extracts of the plant has been used as antidote for snake venoms (Suthari & Raju, 2016).

The essential oil obtained from the branches and leaves of *Atalania roxburghiana* Hook f. (Rutaceae) has been analyzed by GC and GC/MS. Forty-three components of the essential oil, representing 98.6% of the total amount of the oil were identified. The main constituents were monoterpene hydrocarbon: y-terpinene (38.3%), p-cymene (15.7%), β-pinene (5.2%), and α-pinene (4.7%)(Minh et al., 2010).The plant contains an alkaloid N-methyl flindersine (Baxter et al., 1998).

Figure 1.2: Leaves of *Atalantia roxburghiana*

Figure 1.1: Whole plant *Atalantia roxburghiana*

1.5.2 The plant Family: Rutaceae

Rutaceae is a family usually known as the rue or citrus family. The family is commonly called orange family. This consists of about 160 Genera and about 2070 species. It contains woody shrubs and trees, which is distributed throughout the world, especially in the place having warm temperate and tropical region. The largest numbers of this family are found in Africa and Australia. Most of the plants of this family have medicinal importance. Their fruits are rich in vitamins and minerals (Hùng et al., 2015).

1.5.3 The plant Genus: *Atalantia*

Atalantia is a genus belonging to family the Rutaceae which are considering as flowering plants. This genus is included in the subfamily Aurantioideae, which also includes the genus *Citrus*. It is in the tribe Aurantieae and subtribe Citrinae, which are known technicallyas the citrus fruit trees. *Atalantia* and the genus *Citropsis* are also called near-citrus fruit trees (Yahata et al., 2006).

1.5.4 Some reported species (Yahata et al., 2006)

- *Atalantia acuminata*
- *Atalantia ceylanica*
- *Atalantia citroides*
- *Atalantia dasycarpa*
- *Atalantia fongkaica*
- *Atalantia guillauminii*
- *Atalantia hainanensis*
- *Atalantia henryi*
- *Atalantia kwangtungensis*
- *Atalantia macrophylla*
- *Atalantia monophylla*
- *Atalantia racemosa*
- *Atalantia rotundifolia*
- *Atalantia roxburghiana*
- *Atalantia simplicifolia*

- *Atalantia wightii*

1.5.5 Taxonomic hierarchy

Domain: Eukaryota

Kingdom: Plantae

Phylum: Angiosperm

Division: Magnoliophyta

Class: Magnoliopsida

Order: Sapindales

Family: Rutaceae

Subfamily: Aurantioideae

Genus: *Atalantia*

Species: *Atalantia roxburghiana*

Table 1.2: Description of different plant parts of *Atalantia roxburghiana*

	Description
Roots	*Atalantia roxburghiana* is a small tree which grows up to 10 m high and with little or no spines.
Stem	The stems are spiny.
Leaves	The leaves are in oval shape up to long 9-15 cm long with bulging veins and tendons on both sides. They are simple, spiral and estipulate.
Fruit	The fruits are pale yellow.
Flowers	The flowers are white and minute, with a 4-lobed calyx and stamens which are free.

1.5.6 Chemical constituents of *Atalantia roxburghiana*

- γ-terpinene
- p-cymene
- β-pinene

- α-pinene
- N-methyl flindersine

1.5.7 Edibility of *Atalantia roxburghiana*

- Fruit and leaves treat respiratory disease
- Decoction of root is good for women after child birth.

Chapter-2: Methodology

2.1 Chemical investigation of the experimental plants

There are several species belong to the Rutaceae family, and *Atalantia roxburghiana* is one of them. It was tested for its chemical constituents and biological investigation.

2.2 Collection and preparation of the plant material

Atalantia roxburghiana was selected as the plant for this investigation as there was no conduction of any previous study on it except the chemical constituents of the essential oil of *Atalantia roxburghiana* (Minh et al., 2010). With the help of all comprehensive literature study of this plant and its availability, it was chosen for the investigation. The plant *Atalantia roxburghiana* was collected from Sylhet, Bangladesh in July, 2017. After that, the plant was submitted to the National Herbarium of Bangladesh (NHB), Mirpur (Dhaka) for the authentication. A few days later the plant identified (ACCESSION NO.45300) and authenticated by the taxonomist of National Herbarium of Bangladesh, Mirpur, Dhaka.

The leaves were plucked off from the plant stem and then washed with clean water to remove the dust particles. After proper washing, the leaves were sun dried for several days. The dried leaves of the plant were blended to a rough powder by using grinding machine. The powder was then stored in an air tight container and kept in dry and cool place.

2.3 Extraction of the plant material

385 gm of the coarse powder material was taken in a fresh, round bottomed flask (5 liters) and soaked in 1 liter of methanol. Then the container was sealed by foil and kept for a period of 15 days accompanying random stirring and shaking. Afterwards the mixture was filtered by a fresh cotton plug and finally with a Whatman No.1 filters paper.

The total volume of the filtrate mixture was then reduced by using a Rotary evaporator (Heidolph) at 100 rpm at 30º C until concentrated methanolic extract is produced. Thereafter the mixture was transferred into Petri dishes for drying.

The Petri dishes containing methanolic extract were then kept under the Laminar Air Flow (LAF). This was done for evaporating the solvent from the extract that was leaving behind

dry and semisolid extract and the Laminar Air Flow was used as a preventive way to avoid any microbial growth to the plant extract. After successful drying of the extract, Petri dishes were covered with Aluminium foil and refrigerated for further use. The weight of the crude extract was 35 gm.

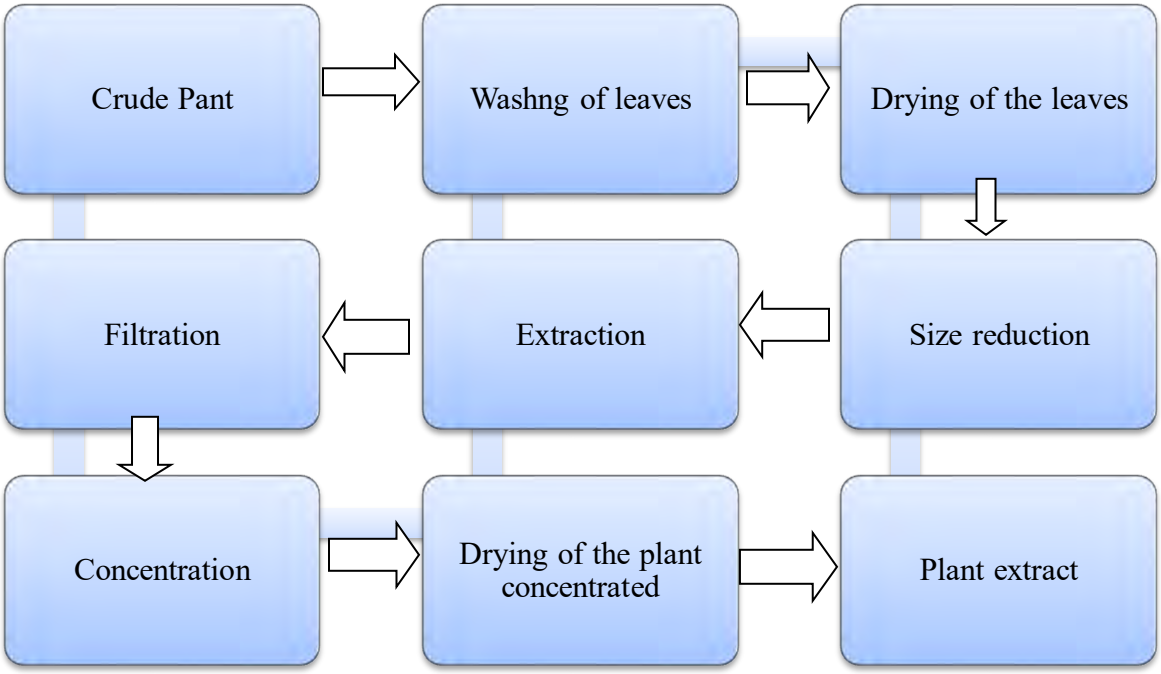

Figure 2.1: Flowchart showing the step by step procedure for the extraction of ingredients from the crude medicinal plants

2.4 Phytochemical screening

Phytochemical screening was performed on the crude extract of *Atalantia roxburghiana* in order to access its qualitative chemical composition such as alkaloids, carbohydrates, tannin, resin, flavonoids etc.

The following qualitative tests were performed:

2.4.1 Detection of alkaloid

Three tests were performed for the qualitative determination of the alkaloids. 0.5 gm of methanolic extract of *Atalantia roxburghiana* was dissolved in 5 ml of 1% Hydrochloric acid, boiled in a water bath followed by filtration. Using the filtrate obtained the following tests were performed:

2.4.1.1 Hager's test

To 2 ml of the filtrate, a few drops of Hager's reagent (1% Picric acid solution) were added and the presence of alkaloids was confirmed by the formation of yellow precipitate (Waldi, 1965).

2.4.1.2 Mayer's test

According to Trease and Evans (1997), 10 ml of Mayer's reagent was prepared by dissolving 0.1358 gm of Mercuric (II) chloride and 0.5 gm of Potassium iodide in 10 ml of distilled water.

Then, to 2 ml of the filtrate, a few drops of Mayer's reagent were added along the sides of the test tube. The formation of a white or creamy precipitate indicated the presence of alkaloids.

2.4.1.3 Wagner's test

According to Wagner (1993), 10 ml Wagner's reagent was prepared by dissolving 0.2 gm of Iodine crystal and 0.6 gm of Potassium iodide in 10 ml distilled water.

To 2 ml of the filtrate, a few drops of Wagner's reagent were added. Formation of a brownish-black precipitate confirmed the presence of alkaloids in the sample.

2.4.2 Detection of carbohydrates

According to Ramkrishnan et al., (1994), carbohydrate can be qualitatively detected by weighing out 0.5 gm of methanolic extract of *Atalantia roxburghiana* and dissolving it in 5 ml of distilled water and then filtering the mixture.

To the filtrate obtained, the following two tests were performed:

2.4.2.1 Molisch'stest

2 ml of filtrate obtained was treated with 2 drops of Molisch's reagent i.e. alcoholic solution of α-napthol to which 2 ml of concentrated Sulfuric acid was pipette along with the sides of the test tube and was allowed to stand for a while. The formation of violate ring indicated the presence of carbohydrates.

2.4.2.2 Fehling's test

To 2 ml of the filtrate, 1 ml of each of the Fehling's solution A and B were added in a 1:1 ratio and then boiled for a few minutes. Formation of break-red precipitate indicated the presence of reducing sugar.

2.4.3 Detection of flavonoids

2.4.3.1 Lead acetate test

The methanolic extract was treated with a few drops of lead acetate solution and the formation of yellow colored precipitate signified the presence of flavonoids.

2.4.3.2 Zinc ribbon test

According to Sindhu and Uma (2013) and Ghani (2003), the presence of flavonoids can be confirmed by another method. To a test tube containing 0.5 ml of methanolic extract, 5-10 drops of concentrated Hydrochloric acid and a small piece of zinc were added. The solution was then boiled for a few minutes and then left to stand. The formation of red to crimson color solution indicated the presence of flavonoids.

2.4.4 Detection of phenols or phenolic compound

Ferric chloride test

According to Soni and Sosa (2013), this test was performed by measuring 2 ml of extract in a test tube followed by adding 3-4 drops of 15% (w/v) Ferric chloride solution. The formation of bluish-black precipitation signified the presence of phenols.

2.4.5 Detection of phytosterols

Libermann Burchard's test

To a small amount of extract, 1 ml of chloroform was added and filtered. The filtrate was then treated with a 2 ml of Acetic anhydride, boiled and cooled. Finally, 1 ml of concentrated Sulfuric acid was added to the solution. Formation of brown ring at the junction indicated the presence of phytosterols (Soni and Sosa, 2013).

2.4.6 Detection of steroids

Salkowski test

To 1 ml of extract, 2 ml of chloroform and 1 ml of Sulfuric acid were added. The appearance of red color indicated the presence of steroids (Ghani, 2003).

2.4.7 Detection of tannin

2.4.7.1 Lead acetate test

To 1 ml of the extract, a few drops of 1% Lead acetate solution were added and the formation of yellow colored precipitate indicated the presence of tannins (Tiwari and Bimlesh, 2011).

2.4.7.2 Potassium dichromate test

10% Potassium dichromate solution was prepared by dissolving 1 gm of Potassium dichromate in 10 ml distilled water. After that, 5 ml aqueous solution of crude extract was dissolved in 1 ml of 5% Ferric chloride solution. The formation of yellow precipitation indicated the presence of tannin (Ghani, 2003).

2.4.8 Detection of resin

According to Soni and Sosa (2013), the presence of resin can be identified by adding 5-10 drops of acetic anhydride to 2 ml of the extract and heating the solution gently. This was then followed by addition of 0.5 ml of Sulfuric acid to the solution. The presence of resin was confirmed by the formation of a bright purple color.

2.4.9 Detection of glycosides

The methanolic extract of *Atalantia roxburghiana* was hydrolyzed with dilute Hydrochloric acid before subjecting it to Brontrager's Test (Kumar, 2013).

Borntrager's test

To the 5 ml of filtrate, 5 ml of 5% Ferric (III) chloride and 5 ml of dilute hydrochloric acid were added. This was followed by heating the mixture for 5 minutes in a boiling water-bath and cooling it down. Then 5 ml of benzene was added to the mixture and shaken thoroughly. The organic layer was then separated by using a separating funnel and an equivalent volume of dilute ammonia solution was added. The formation of pinkish-red color in the ammonical layer signified the presence of glycosides (Kamalakar et al., 2014).

2.5 Evaluation of antioxidant activity

The current development in the information of free radicals and reactive oxygen species (ROS) in science is delivering a therapeutic revolution that guarantees a new period of health and disease management. The vast majority of the possibly harmful impacts of oxygen are because of the arrangement and movement of a number of chemical compounds, known as ROS, which have a tendency to provide oxygen to different substances. Free radicals and antioxidants have turned out to be commonly used terms in modern discussions of disease mechanisms (Lobo et al., 2010). The free radicals are chemical species which are highly reactive and produced in the body and have the potential to damage cells, organelles, DNA, and other biomolecules, resulting in diseases such as cancer, cardiovascular and neurodegenerative ailments. The treatment of those diseases has serious viability and safety issues. In addition, it is so expensive that many people cannot afford it. This situation creates the necessity of discovering safe and effective remedies that will be readily available for common people (Ahmed et al., 2015).

The purpose of this study was to evaluate the different extracts of *Atalantia roxburghiana* as new potential sources of natural antioxidants and phenolic compounds. The therapeutic properties of plants have been researched in the recent scientific developments throughout the whole world, because of their intense antioxidant activity exercises, no side effects and economic feasibility. The antioxidant activity is analyzed in the following ways-

- Determination of Total Phenolic Content (TPC)
- Determination of antioxidant properties: DPPH assay
- Determination of Total Flavonoid Content (TFC)
- Determination of Total Antioxidant Capacity (TAC)

2.5.1 Determination of Total Phenolic Content (TPC)

Phenolic compounds can be considered as an essential part in protecting body cells from damage by hydrogen peroxide and from the harm carried out by unsaturated fats and lipid peroxides, absorbing and neutralizing free radicals (Sroka & Cisowski, 2003).

The phenolic compounds employ their antioxidant properties by redox properties, which enable them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Proestos et al., 2006).

Antioxidants can be found in various foods and medicinal plants and they play an important role in the prevention and treatment of chronic diseases which are caused by oxidative pressure. They frequently have strong antioxidant and free radical scavenging activity, and also anti-inflammatory action, which are similarly the basis of other bioactivities and health benefits, e.g. anti-aging, anticancer, defensive activity for cardiovascular diseases, diabetes mellitus, obesity and neurodegenerative diseases (Li et al., 2014).

2.5.1.1 Principle

Folin-Ciocalteu reagent is used in the phenolic solution as the reagent will easily oxidize in the phenols which will be ionized in alkaline solution. The color of Folin-Ciocalteu reagent is yellow and the solution becomes blue after the oxidation process. The concentration of the color change is measured in a spectrophotometer at 760 nm. The absorbance value will show the total phenolic content of the compound (Harbertson and Spayd, 2006).

$$\text{Phenols} + Na_2CO_3 \xrightarrow{\text{Complete ionization}} \text{Ionized phenols}$$

$$\text{Ionized reagents} + \text{phenols} + \text{Folin-Ciocalteu complex} \xrightarrow{\text{Oxidation}} \text{Folin-Ciocalteu reagent}$$

(Yellow) **(Blue)**

2.5.1.2 Materials and methods

Total phenolic content of leaves of *Atalantia roxburghiana* extract was measured employing the method as described by Skergetet al.,(2005) using Folin-Ciocalteu reagent as oxidizing agent and Gallic acid as standard (Majhenik et al., 2007).

Materials

- Folin-Ciocalteu reagent (10 fold diluted)
- Na_2CO_3 solution (7.5 %)
- Distilled water
- Pet ether
- Methanol

- Chloroform
- Ethyl acetate
- UV-spectrophotometer
- Vial
- Beaker (100 and 200 ml)
- Gallic acid
- Pipette (1ml and 5 ml)
- Micropipette (50-200 µl)

Table 2.1: Composition of Folin-Ciocalteu reagent

Serial No.	Component	Percent
1	Water	57.5
2	Lithium Sulfate	15.0
3	Sodium Tungstate Dihydrate	10.0
4	Hydrochloric Acid>=25%	10.0
5	Phosphoric Acid 85% solution in water	5.0
6	Molybdic Acid Sodium Dihydrate	2.5

2.5.1.3 Standard curve preparation

In the preparation of standard curve Gallic acid was used as standard. Gallic acid solutions were prepared which have different concentrations ranging from 0 µg/ml to 100 µg/ml. 2.5 ml of Folin-Ciocalteu reagent that was diluted 10 times with distilled water and 2.0 ml of Na_2CO_3 (7.5% w/v) solution was added to 0.5 ml of Gallic acid solution. The mixture was incubated for 20 minutes at room temperature. The absorbance was measured at 760 nm after 20 minutes.

After plotting the absorbance against the concentration, a linear relationship between them was obtained which was used as a standard curve for the determination of the total phenolic content of the test samples.

2.5.1.4 Sample preparation

2 mg of the extracts of the leaves was taken and dissolved in the distilled water to get a sample which had a concentration of 2 mg/ml. The sample along with its concentration for the total phenolic content was measured.

2.5.1.5 Total phenolic compound analysis

To 0.5 ml of extract solution (conc. 2 mg/ml), 2.5 ml of Folin-Ciocalteu reagent (diluted 10 times with distilled water) and 2.0 ml of Na_2CO_3 (7.5% w/v) solution were added. The mixture was incubated for 20 minutes at room temperature. After 20 minutes the absorbance was measured at 760 nm by UV-spectrophotometer. Using the standard curve prepared from Gallic acid solution with different concentrations, the total phenolic content of the sample was measured. The total phenolic content of the sample was expressed as mg of GAE (Gallic acid equivalent)/gm of the extract.

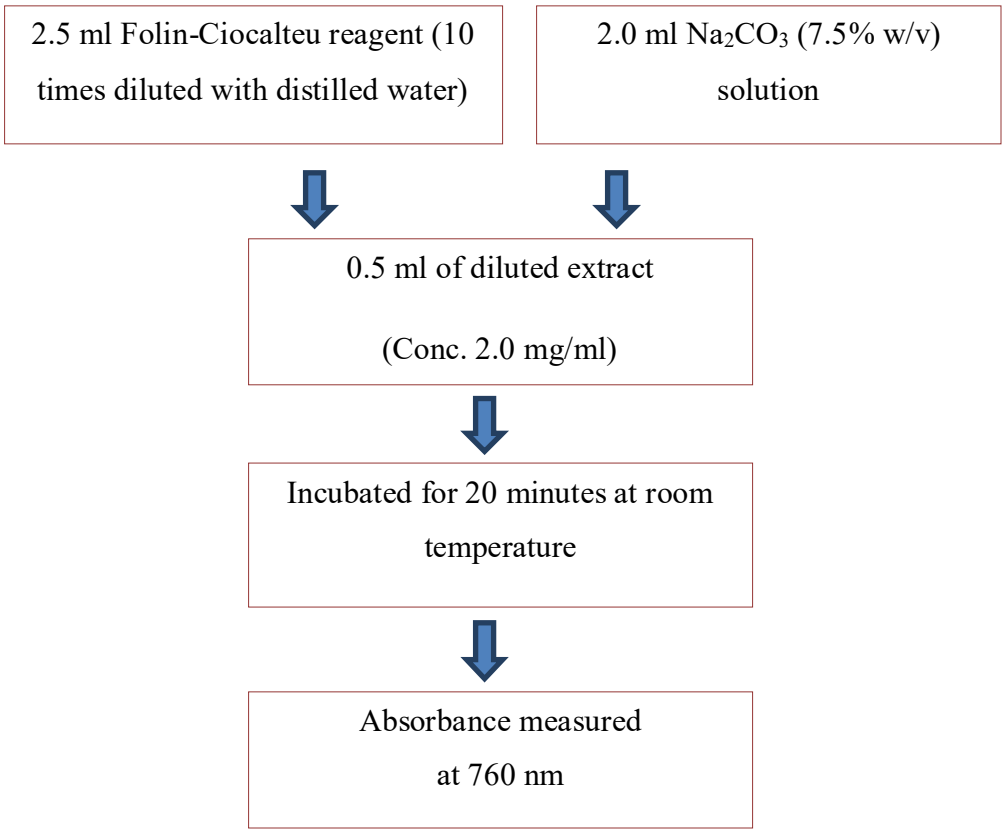

Figure 2.2: Schematic representation of the total phenolic content determination

2.5.2 Antioxidant activity: DPPH assay

2.5.2.1 Principle

According to the method described by Brand-Williams et al., (1995), the free radical scavenging activities (antioxidant capacity) of the plant extract (*Atalantia roxburghiana*) on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) can be measured.

Here, 2 ml of a methanol solution of the sample extract at different concentrations (500 µg/ml to 0.977µg/ml) were mixed with 3 ml of a DPPH methanol solution (20µg/ml). The bleaching of purple colored DPPH methanol solution by the plant extract was compared with two standards, named Ascorbic acid and *tert*-butyl-1-hydroxytoluene (BHT).

2.5.2.2 Materials and methods

DPPH was used to evaluate the free radical scavenging activity (antioxidant potential) of various compounds and medicinal plants (Choi et al., 2000; Desmarchelier et al., 1997).

- 1,1-diphenyl-2-picrylhydrazyl (DPPH)
- Micropipette (50-200 µl)
- Ascorbic acid
- *tert*-butyl-1-hydroxytoluene (BHT)
- Distilled water
- Methanol
- Chloroform
- Ethyl acetate
- UV-spectrophotometer
- Beaker (100 and 200ml)
- Amber reagent bottle
- Test tube
- Light-proof box
- Pipette (5ml)

2.5.2.3 Control preparation for antioxidant activity measurement

Calculated amounts of Ascorbic acid and *tert*-butyl-1-hydroxytoluene (BHT) were dissolved in methanol to get a mother solution having a concentration 1000 µg/ml for each. Serial dilution was made using the mother solution to get different concentrations ranging from 500 to 0.977 µg/ml. Here, both Ascorbic acid and BHT were used as positive control.

2.5.2.4 Test sample preparation

Calculated amount of different extracts was measured and dissolved in methanol to get the mother solution (conc. 1000 µg/ml). Serial dilution of the mother solution gave different concentrations, ranging from 500 to 0.977 µg/ml and was kept in the marked flasks.

2.5.2.5 DPPH solution preparation

20 mg DPPH powder was weighed and dissolved in methanol to get a DPPH solution having a concentration 20 µg/ml. The solution was prepared in the amber reagent bottle and kept in the light proof box.

2.5.2.6 Assay of free radical scavenging activity

2 ml of a methanol solution of the sample extracts at different concentrations (500 µg/ml to 0.977µg/ml) were mixed with 3 ml of a DPPH methanol solution (20 µg/ml). After 30 min reaction period at room temperature in dark place the absorbance was measured at 517 nm against blank by UV spetrophotometer. Here, methanol was used as blank solution.
Inhibition of free radical DPPH in percent (I%) was calculated as follows:

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

Where, A_{blank} is the absorbance of the control reaction (containing reagents except the test material).

Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted percentage of inhibition against extract concentration.

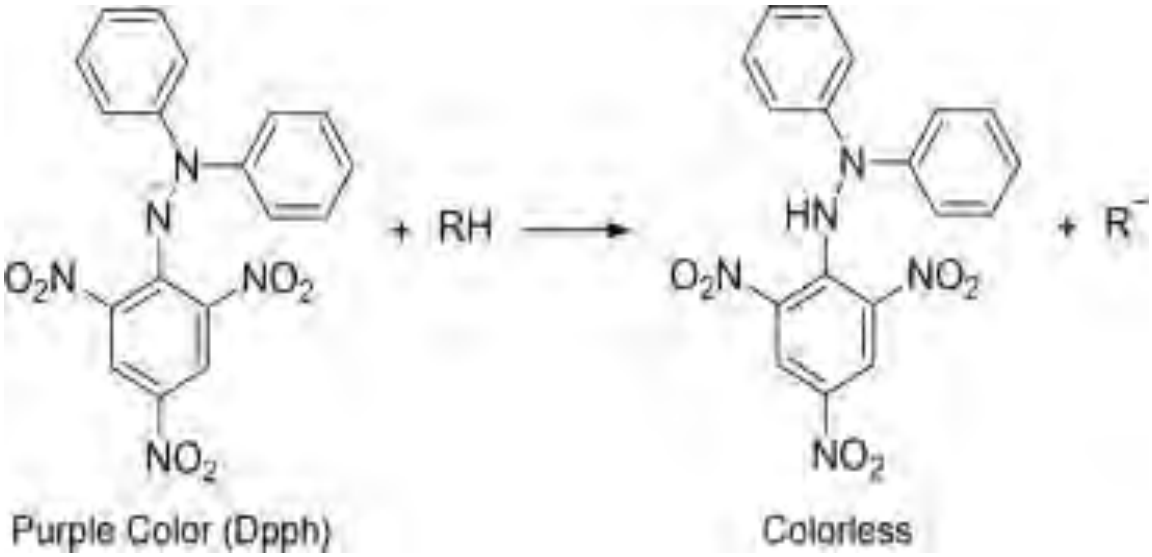

Figure 2.3: Structural change of DPPH

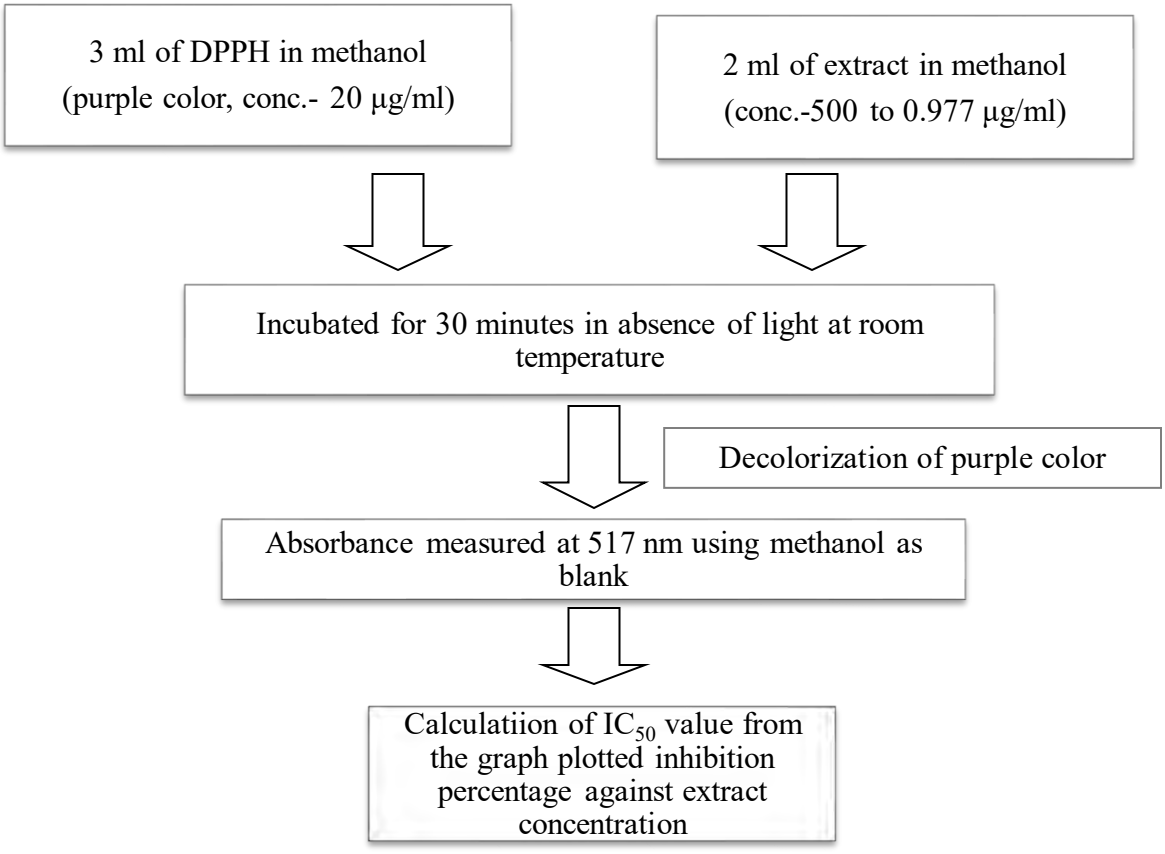

Figure 2.4: Schematic representation of the method of assaying free radical scavenging activity

2.5.3 Determination of Total Flavonoid Content (TFC)

2.5.3.1 Principle

The total flavonoid content of the *Atalantia roxburghiana* was determined according to the method as described by Kumaran and Karunakaran (2007). The total flavonoid content of *Atalantia roxburghiana* extract was determined by the Aluminium chloride colorimetric method.

2.5.3.2 Chemicals and reagents required

- Methanol
- Quercetin (standard)
- Potassium acetate
- Aluminium Chloride

2.5.3.3 Reagent preparation

100 ml of 10% Aluminium chloride solution was prepared by measuring 10 gm of Aluminium chloride in a 100 ml volumetric flask and diluting it with distilled water to100 ml mark.
100 ml of 1M Potassium acetate solution was prepared by measuring 9.815 gm of Potassium acetate in a100 ml volumetric flask and diluting it with distilled water up to100 ml mark.

2.5.3.4 Sample and standard preparation

120 mg of *Atalantia roxburghiana* extract was measured and dissolved in 10 ml of methanol to produce a concentration of 12 mg/ml. This became the sample stock solution. The sample concentrations were prepared by serial dilution of the sample stock solution to derive four serially diluted concentrations: 1200, 800, 400 and 200 µg/ml.

Quercetin was used as the standard and the stock solution was prepared in the same manner as the extract resulting in seven serially diluted concentrations, rangingfrom1200, 800, 400, 200, 100, 50 and 25 µg/ml.

2.5.3.5 Preparation of the blank

The blank solution contained 200 µl of10% Aluminium chloride solution, 200 µl of 1M Potassium acetate solution, 5.6 ml of distilled water and 4 ml of methanol, such that the final volume of the solution was10 ml.

2.5.3.6 Experimental procedure

1ml of each of the fraction of sample and standard (Quercetin) concentrations was taken in test tubes, to which 3 ml of methanol was added. Afterwards, 200 µl of 10% Aluminium chloride solution and 200 µl of 1M Potassium acetate solution were added to each of the test tubes using 1000 µl micropipette. Finally, 5.6 ml of distilled water was added to the test to make the final volume of the solution 10 ml. The test tubes were then incubated in room temperature for 30 min. Afterwards, the absorbance of the each of the sample and standard were measured at 415 nm by using spectrophotometer. The total flavonoid content of each of the fractions was expressed as Quercetin equivalent (QE) using the following equation:

C= (c*V)/m

Where,

C=Total content of flavonoid compounds, mg of Quercetin per gram of dried plant extract, expressed as Quercetin equivalent (QE)

c= Concentration of Quercetin obtained from calibration curve (mg/ml)

V=Volume of sample solution (ml)

m= Weight of the sample (gm)

2.5.4 Determination of Total Antioxidant Capacity (TAC)

The method described by Prieto et al, (1999) was used to determine the total antioxidant capacity of *Atalantia roxburghiana*.

2.5.4.1 Chemical and reagent

- Ammonium molybdate
- L-Ascorbic acid (Standard)

- Sodium Tri phosphate ($Na_3PO_4.12H_2O$)
- Concentrated Sulfuric acid (98%)
- Methanol

2.5.4.2 Reagent preparation

100 ml of 0.6M Sulfuric acid was prepared by measuring 3.28 ml of 98% concentrated Sulfuric acid in a 100 ml volumetric flask and diluting it with distilled water to100 ml mark. 100 ml of 0.004M Ammonium molybdate solution was prepared by measuring 4.494gmofPotassium acetate in a 100 ml volumetric flask and diluting it with distilled water up to 100 ml mark.100 ml of 0.028M Sodium Phosphate solution was prepared by measuring 1.0645 gm of Aluminium chloride in a 100mlvolumetric flask and diluting it with distilled water to 100ml mark.

2.5.4.3 Sample and standard preparation

120 mg of *Atalantia roxburghiana* extract was measured and dissolved in 10 ml of methanol to produce a concentration of 12 mg/ml. This became the sample stock solution. The sample concentrations were prepared by serial dilution of the sample stock solution to derive four serially diluted concentrations: 1200, 800, 400 and 200 µg/ml.

Ascorbic acid was used as the standard and the stock solution was prepared in the same manner as the extract resulting in seven serially diluted concentrations, ranging from 1200, 800, 400, 200, 100, 50 and 25 µg/ml.

2.5.4.4 Preparation of the blank

3 ml of reagent solution and 300 µl of methanol were used for preparing the blank solution.

2.5.4.5 Experimental procedure

300 µl of each of the fraction of sample and standard (L-Ascorbic acid) concentrations were taken in test tubes. After that, 3ml of Reagent solutions (0.6M Sulfuric acid, 0.028M Sodium phosphate and 0.004M Ammonium molybdate) was added in to the test tubes. The test tubes (sample, standard and blank) were then incubated at 95°C in a water bath for 90 min.

Finally, the absorbance of the sample and standard solutions was measured against blank at 695 nm using spectrophotometer (U-2910 UV-Vis Spectrophotometer).The total antioxidant capacities of each of the fractions were expressed as Ascorbic acid equivalents (AAE) using the equation as follows:

$$A = (c*V)/m$$

Where,

A=Total antioxidant capacity, mg of Ascorbic acid per gram of dried plant extract, expressed as Ascorbic acid equivalent (AAE)

c=Concentration of Ascorbic acid obtained from calibration curve (mg/ml)

V=Volume of sample solution (ml)

m=Weight of the sample (g)

2.6 Antimicrobial activity study by disc diffusion method

Several infectious diseases are caused by microscopic organisms and parasites. The expansion of the clinical implications of drug resistant, parasite and bacterial pathogens have allowed further implication to antimicrobial drug studies. Around the world, infectious disease is one of the fundamental reasons of death which represents approximately 50% of all deaths. It may not be shocking to see these statistics in developing countries, but the surprising fact is that mortality rates due to infectious disease are very high in developing countries like the United States. This is shocking specified that it was once thought that we would reduce infectious disease by the end of the era. These increases are attributed the increase in HIV/AIDS and respiratory tract infections. Other contributing aspects include proliferation of antibiotic resistance in nosocomial and community acquired infections. Moreover, the most intensive increases are taking place in between 25–44 years old age people (Pinner et al., 1996).

Antimicrobial screening is the major phase of antimicrobial drug research which is implemented to determine the susceptibility of many fungi and bacteria to any agent. This experiment measures the capability of each test sample to inhibit the *in vitro* bacterial and

fungal growth. This ability may be estimated by any of the following three methods (Ayafor, 1972):

- ✓ Bioautographic method
- ✓ Disc diffusion method
- ✓ Serial dilution method

Among the above stated methods, the disc diffusion method (Bayer et al., 1966) is the most popular *in vitro* study for antimicrobial activity. It is basically a quantitative test representing the sensitivity or resistance of the microorganisms to the test materials. However, no dissimilarity between bacteriostatic and bactericidal activity can be completed by this method (Roland, 1982).

2.6.1Apparatus and reagents

- ➢ Filter paper discs
- ➢ Nutrient agar medium
- ➢ Petri dishes
- ➢ Sterile cotton
- ➢ Micropipette
- ➢ Inoculating loop
- ➢ Sterile forceps
- ➢ Screw cap test tubes
- ➢ Autoclave
- ➢ Laminar Air Flow (LAF) hood
- ➢ Spirit burner
- ➢ Refrigerator
- ➢ Incubator
- ➢ Dichloromethane
- ➢ Ethanol
- ➢ Nose mask and hand gloves

2.6.2 Test organisms

Both gram positive and gram-negative organisms were taken for the test and they are listed in the table below:

Table 2.2: List of bacteria used in the study

Gram positive bacteria	Gram negative bacteria
Staphylococcus aureus	*Escherichia coli*
Bacillus subtilis	*Vibrio cholerae*

2.6.3 Preparation of medium

Medium was prepared by adding 3.8 mg Mueller Hinton agar in 100 ml distilled water. Then it was mixed thoroughly until the agar was completely dissolved. After autoclaving for 20 min at 121^0C, the mixture was cooled up to 45-50^0C and poured into 4 sterile Petri dishes equally to form uniform depth in each plate. Each 120 mm Petri dish got 25 ml of agar solution approximately. Then it was allowed to cool and solidify at room temperature.

2.6.4 Culturing of bacterial strains

Firstly, Nutrient broth medium was prepared by dissolving 0.25 gm Nutrient broth in 10 ml distilled water. Strains were collected from long term preserved medium containing skimmed milk, trypsin, glucose and glycerin which were preserved in Ultra Low Temperature. The bacterial strains were taken from there by a loop and then transferred into Nutrient broth medium and incubated 24 hr to revive those bacteria. After 24 hr of incubation, new bacterial colony was formed and ready to use.

2.6.5 Preparation of disc

The disc was made of Whatman paper with 6mm diameter and autoclaved in a test tube. Then solutions of plant extract were added to the disc. Then it was allowed to soak all the plant extracts for 10-15 min.

2.6.6 Procedure

Firstly, a cotton swab was dipped into the suspension of bacteria. To get rid of excess fluid, the swab was gently squeezed against the tube. Then, the swab was used to streak the bacterial suspension to the nutrient agar plate in one direction and after that in was streaked diagonally. After that, the agar plates were allowed to get dry for 5 min. Then, the discs containing extract of peptides and proteins were placed individually by using forceps, on the surface of the place. Kanamycin 30, Amoxicillin 10, Streptomycin 10 were used as reference standard and also placed on the surface of the plate. The forceps were sterilized by flame after using every time and gently pressed the disc on the plate. Lastly, the Petri dishes were incubated 24 hr at 37^0C to get the lawn growth of bacteria.

2.7 Brine shrimp lethality bioassay

Bioactive compounds always show some toxicity to the human body in higher doses. For the bioactive compounds of natural and synthetic sources, brine shrimp lethality bioassay (Meyer et al., 1982; Mc Laughlin et al., 1998) is a rapid and trustworthy one by which the crude extracts; fractions as well as the pure compounds can be verified for their bioactivity. *In vivo* lethality investigation of a simple zoological organism (brine shrimp nauplii) is a satisfactory monitor for screening and fractionation. Brine shrimp lethality bioassay specifies cytotoxicity and wide spread variety of pharmacological activities such as antiviral, pesticidal, antimicrobial and anti-tumor etc. of the plant extracts (Meyer et al., 1982; Mc Lughilin et al., 1998).

Brine shrimp lethality bioassay system stands better than other cytotoxicity testing methodology since it is quick in process, cheap and requires no exceptional equipment or aseptic procedure. This method is use for huge number of organisms and there need just statistical validation and small amount of sample. It does not require animal serum just like other methods.

2.7.1 Principle

Brine shrimp eggs are hatched in simulated sea water to get nauplii. The desired concentration of the test sample is prepared with the calculated amount of Dimethyl sulphoxide (DMSO). By visual inspection, the nauplii are calculated and are taken in vials containing 5 ml of simulated sea water. Then samples of different concentrations are added to the premarked vials through micropipette. The vials are left for 24 hours and survivors are calculated after 24 hours.

2.7.2 Materials

- Sea salt (NaCl)
- *Artemia salina* leach (brine shrimp egg)
- Small tank with perforated dividing dam to hatch the shrimp
- Lamp to attract shrimps

- Pipettes
- Micropipette
- Glass vials
- Magnifying glass
- Test tubes
- Test samples of experimental plants

2.7.3 Experimental procedure

2.7.3.1 Preparation of seawater

38 gm of sea salt (pure NaCl) was weighed, dissolved in one liter of distilled water and then filtered off to get clear solution.

2.7.3.2 Hatching of brine shrimps

Artemia salina leach (brine shrimp eggs) was collected from pet shops and it was used as the test organism. Seawater was taken in the small chamber and brine shrimp eggs were added to the chamber. Two days were permitted to hatch the shrimp and to be matured as nauplii. Constant oxygen supply and light were carried out through the hatching time and they were taken for assay. Then ten living shrimps were added by the Pasteur pipette to the test tubes containing 5 ml sea water.

2.7.3.3 Preparation of test samples of the experimental plant

The test sample was taken in vial and dissolved in 100 µl of pure Dimethyl sulfoxide (DMSO) to get stock solutions. Then 50 µl of solution was taken in the first test tube containing 5 ml of simulated seawater and 10 shrimp nauplii. Thus, final concentration of the prepared solution in the first test tube was 400µg/ml. Then a series of solutions of varying concentrations were prepared from the stock solution by serial dilution method. 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 2.3).

Table 2.3: 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 .0
5	25 .0
6	12.5
7	6.25
8	3.125
9	1.5625
10	0.78125

2.7.3.4 Preparation of control group

Control groups are used to validate the test method and ensure that the results obtained are only due to the activity of the test agent and the effects of the other possible factors are nullified. Usually two types of control groups are used:

i) Positive control

ii) Negative control

2.7.3.5 Preparation of the positive control group

Positive control in a cytotoxicity study is a generally recognized cytotoxic representative and the consequence of the test is compared with the result got for the positive control. In the present experiment Vincristine sulfate was used as the positive control. Measured amount of the Vincristine sulfate was dissolved in DMSO to get a primary concentration of 20 µg/ml from which serial dilutions are made by using DMSO to get 10 µg/ml, 5 µg/ml, 2.5µg/ml, 1.25 µg/ml, 0.625 µg/ml, 0.3125 µg/ml, 0.15625 µg/ml, 0.078125 µg/ml, 0.0390 µg/ml. The positive control solutions were added to the premarked vials containing ten living brine shrimp nauplii in 5 ml simulated sea water to gain the positive control groups.

2.7.3.6 Preparation of the negative control group

100 µl of DMSO was added to each of three premarked glass vials containing 5 ml of simulated sea water and 10 shrimp nauplii to use as control groups. If the brine shrimps in these vials show a rapid mortality rate, then the test is considered as invalid as the nauplii died due to some reason other than the cytotoxicity of the compounds.

100 µl of DMSO was added to each of three premarked glass vials containing 5 ml of recreated ocean water and 10 shrimp nauplii to use as control gatherings. In the event that the salt water shrimps in these vials demonstrate a quick death rate, the test is considered as invalid as the nauplii kicked the bucket because of some reason other than the cytotoxicity of the mixes.

2.7.3.7. Counting of nauplii

After 24 hours, the vials were inspected using a magnifying glass and the number of survivors were counted. The percent (%) mortality was calculated for each dilution. The concentration-mortality data were analyzed statistically by using linear regression using a simple IBM-PC program. The effectiveness or the concentration-mortality relationship of plant product is usually expressed as a median lethal concentration (LC_{50}) value.

This represents the concentration of the chemical that produces death in half of the test subjects after a certain exposure period.

2.8 Thrombolytic activity test

Thrombolytic drugs are used to breakdown blood clot. Thrombolysis may cause disruption in normal blood flow. For this reason, it is called 'clot busting'. Thrombolysis works by stimulating fibrinolysis by plasmin through infusion of analogs of tissue plasminogen activator (tPA), the protein that normally activates plasmin. Plasmin is a proteolytic enzyme that is fit for breaking cross-links between fibrin particles, from which blood clusters take structural integrity. *In vitro* thrombolytic activity of crude extract was enumerated and was compared with streptokinase, which is a well-known anticoagulant used in myocardial infarction (Sikri and Bardia, 2007).

2.8.1 Collection of blood sample

For the collection of blood sample, two healthy volunteers were taken who have no previous history of oral contraceptives and anticoagulant drugs. Microcentrifuge tubes were weighed and from the donors 6ml of blood was taken into that microcentrifuge tubes. They were assigned by the numbers 1, 2, 3 etc. Each participant provided written consent for the donation of blood sample.

2.8.2 Streptokinase

Commercially available streptokinase (S-Kinase, 1500000IU, Popular Pharmaceuticals Ltd.) was collected and 5ml of sodium chloride (0.9%) was added into the vial to make the concentration 30000 IU. From this, 100 µl suspensions was collected and used as *in vitro* thrombolysis. (Fahad Hussain, 2014)

2.8.3 Sample preparation

100mg of the extracted sample was dissolved into 10ml of the 0.9% sodium chloride solution. The solution was then filtered. (Fahad Hussain, 2014)

2.8.4 Procedure

The blood samples were permitted to incubate for 45 minutes at 37°C. After clot development, serum was totally evacuated (clot ought not to be irritated) and each tube having clot was again weighed to decide the coagulation weight (clot weight = weight of clot containing tube − weight of tube alone). Each microcentrifuge tube containing clot was legitimately marked and 100 µl of plant extract, 100 µl of sodium chloride (0.9%) (as a negative control), 100 µl of 30,000 IU reference streptokinase (as a positive control) were added to tubes with clot. Every one of the tubes was incubated at 37°C for 90 minutes. The liquid left was then precisely removed and the tubes were weighed once more. The distinction in weight prior and then afterward clot lysis was communicated as level of cluster lysis (Biozid, 2015).

Percentage clot lysis = (weight of the clot after lysis by sample and removal of serum/weight ofthe clot before lysis by sample) ×100

Chapter-3: Results and Discussion

3.1 Determination of the percentage yield of the plant extract, *Atalantia roxburghiana*

Table 3.1: Total weight of the powder plant before maceration

Initial weight (beaker)	306 gm
Final weight (powder + beaker)	691 gm
Total weight of the powder plant	385 gm

Table 3.2: Net weight of the plant extract after maceration

Initial weight (beaker)	309 gm
Final weight (extract+ beaker)	344 gm
Total weight of the extract	35 gm

Interpretation: A total weight of 35 gm of extract was produced in the maceration process and after drying of methanolic extract of *Atalantia roxburghiana,* which was carried out in the whole experiment.

3.1.1 Calculation of percentage yield of the extract

Extract yield percentage (%) = $(W_1 \times 100)/W_2$

Where,

W_1= Net weight of extract after maceration

W_2=Total weight of powder taken for maceration

So, percentage yield in Methanol = $(35 \times 100)/385$

= 9.09%

Interpretation: The total weight of the extract after maceration was found 35gmwhereas before maceration was 385gm; therefore, the % yield of *Atalantia roxburghiana was* calculated to be 9.09%.

3.2 Phytochemical screening of *Atalantia roxburghiana*

Table 3.3: Phytochemical analysis of methanolic extract of *Atalantia roxburghiana* leaves

Class of compound	**Result**
Alkaloid	++
Carbohydrate	++
Flavonoid	++
Phenol	+
Phytosterol	+
Steroid	-
Tannin	+
Glycoside	+
Resin	-
Saponin	+

(+ means present in single test method, ++ means present in two experimental method,
- means absent single test method, -- means absent in two experimental method)

Interpretation: The phytochemical screening of methanolic extract of leaves of *Atalantia roxburghiana* was successfully carried out. The screening showed the presence of alkaloid, carbohydrate, flavonoid, phenol, phytosterol, tannin, glycoside and saponin, while there was absence of steroid and resin.

3.3 Determination of Total Phenolic Content (TPC)

The methanolic extract (ME) of the leaves of *Atalantia roxburghiana* was subjected for total phenolic content determination. Folin-Ciocalteu reagent was used for this test. Depending on the absorbance values of the extract solution, the value of total phenolic content of the extract was determined and differentiated with the standard solutions of Gallic acid (Table 3.4) equivalents. The total phenolic content of the sample is expressed as mg of GAE (Gallic acid equivalent)/gm of extract, and is given in Table 3.5.

After investigation, the total phenolic content in ME was found to be 39.25 mg of GAE/gm of extract.

Table 3.4: Standard curve prepration by using Gallic acid

Sl. No.	Conc. of the Standard (µg/ml)	Absorbance	Regression Line	Regression coefficient (R^2)
1	100	0.800	y = 0.0081x - 0.0007	0.9975
2	50	0.423		
3	25	0.215		
4	12.5	0.123		
5	6.25	0.047		
6	3.125	0.007		
7	1.5625	0.003		
8	0.78125	0.000		
9	0.3906	0.000		
10	0	0.000		

Interpretation: It is observed that, as the concentration of *Atalantia roxburghiana* was increased from 0-100 µg/ml, the total phenolic content also increased from 0 to 0.8 mg of Gallic acid per gram of dried extract. Therefore, it indicates that with increase in total phenolic content, the antioxidant activity of plant extract also increased.

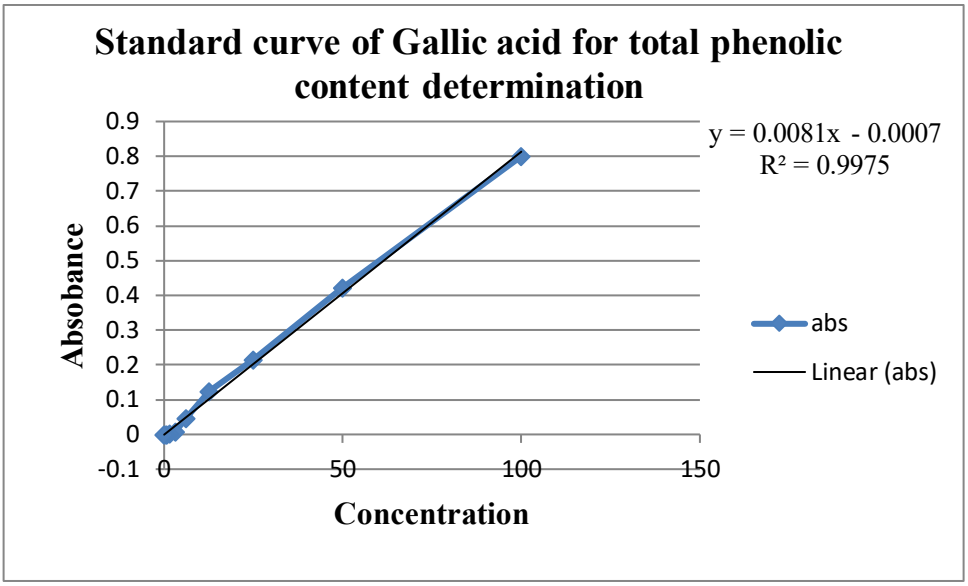

Figure 3.1: Standard curve of Gallic acid for total phenolic content determination

Interpretation: The equation of the calibration curve of Gallic acid was found to be y = 0.0081x-0.0007 which would be used to determine the total phenolic content of methanolic leaf extract of *Atalantia roxburghiana*. A regression coefficient (R^2) of 0.9975 was also obtained indicating a good relationship between the concentration and the absorbance.

Table 3.5: Total phenolic content of *Atalantia roxburghiana* (represented as mg of GAE/gm of extract)

Plant part	Sample code	Test Sample	Total phenolic content (mg of GAE/gm of extract)
Leaves of *Atalantia roxburghiana*	ME	Methanolic extract	39.25

3.4 DPPH free radical scavenging assay

The methanolic extract of the leaves of *Atalantia roxburghiana* (ME) was tested for free radical scavenging activity by using the method of Brand-Williams et al., 1995. Reference standards were Ascorbic acid (ASA) and *tert*-butyl-1-hydroxytoluene (BHT).

In this research, methanolic extract solution (ME) presented notable free radical scavenging activity with IC_{50} value of 554.47 µg/ml for leaves of *Atalantia roxburghiana* (Table 3.6). The free radical scavenging activity of methanolic extract of leaves of *Atalantia roxburghiana* was found to be much better than the standards.

Table 3.6: IC_{50} values of the standards and methanolic extract of leaves of *Atalantia roxburghiana*

Plant part	**Sample code**	**Test Sample**	**IC_{50} (µg/ml)**
Leaves of *Atalantia roxburghiana*	ME	Methanolic extract	554.47
ASA (Ascorbic acid) (standard)			115.98
BHT (*tert*-butyl-1-hydroxytoluene) (standard)			96.74

Interpretation: The IC_{50} value of dried extract of *Atalantia roxburghiana* signifies that a much higher concentration of 554.47 µg/ml was required for inhibiting 50% of all DPPH free radical scavengers, whilst a small concentration of 115.98 µg/ml was required to achieve the same inhibitory effect for Ascorbic acid and 96.74 µg/ml was required for BHT (*tert*-butyl-1-hydroxytoluene).

Table 3.7: IC_{50} value of Ascorbic acid (ASA)

Absorbance of the blank	Conc. (µg/ml)	Absorbance of the extract	% inhibition	IC_{50} (µg/ml)
0.325	500	0.005	98.46	115.98
	250	0.006	98.15	
	125	0.015	95.38	
	62.5	0.024	92.61	
	31.25	0.068	79.07	
	15.625	0.098	69.84	
	7.813	0.139	57.23	
	3.906	0.186	42.76	
	1.953	0.175	46.15	
	0.977	0.193	40.61	

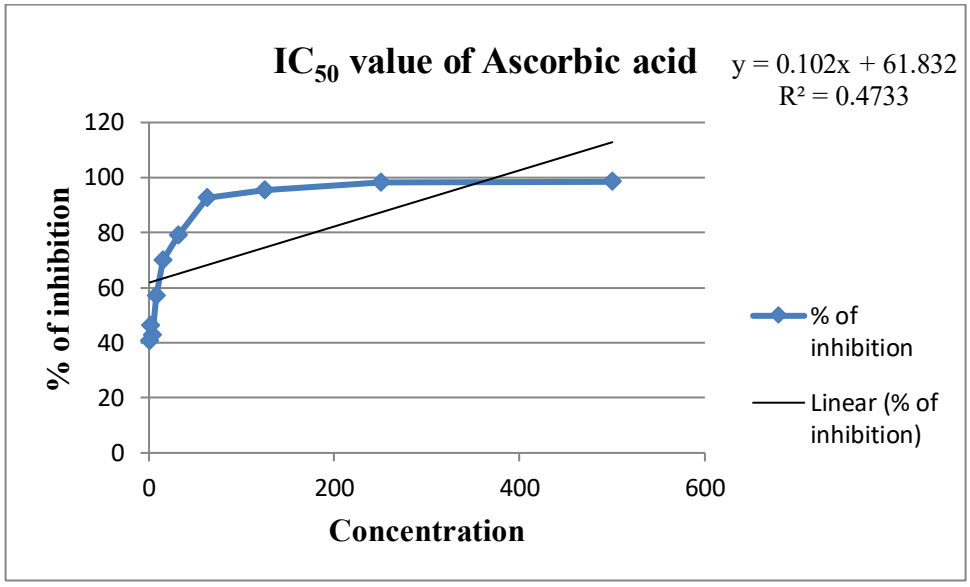

Figure 3.2: IC_{50} value of Ascorbic acid

Table 3.8: IC_{50} value of *tert*-butyl-1-hydroxytoluene (BHT)

Absorbance of the blank	Conc. (µg/ml)	Absorbance of the extract	% inhibition	IC_{50} (µg/ml)
0.325	500	0.018	94.46	96.74
	250	0.068	79.07	
	125	0.097	70.15	
	62.5	0.135	58.46	
	31.25	0.159	51.07	
	15.625	0.175	46.15	
	7.813	0.206	36.61	
	3.906	0.225	30.76	
	1.953	0.238	26.76	
	0.977	0.287	11.69	

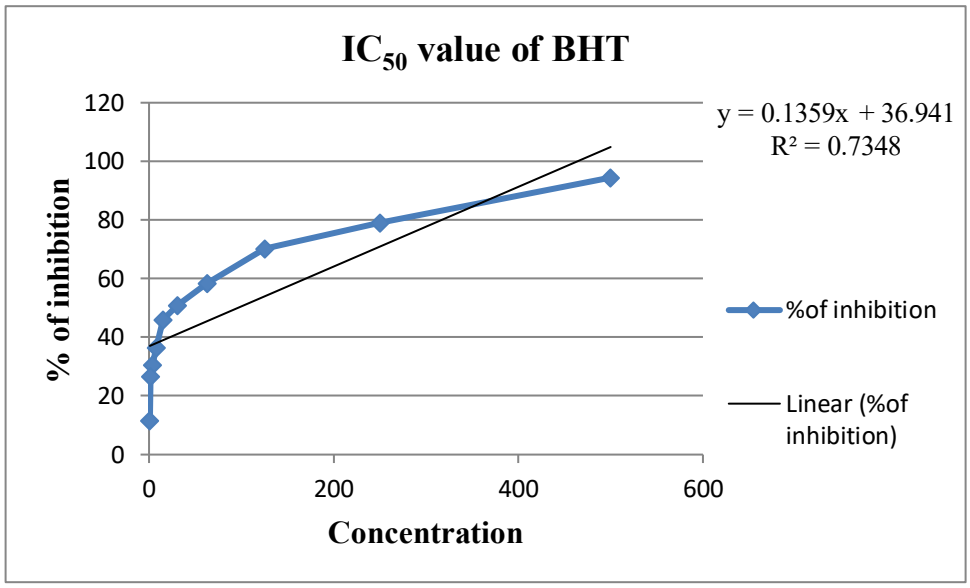

Figure 3.3: IC_{50} value of *tert*-butyl-1-hydroxytoluene (BHT)

Table 3.9: IC_{50} value of methanolic extract (ME) of *Atalantia roxburghiana*

Absorbance of the blank	Conc. (µgm/ml)	Absorbance of the extract	% Inhibition	IC_{50} (µg/ml)
0.325	500	0.182	44.85	554.47
	250	0.193	41.52	
	125	0.199	39.70	
	62.5	0.217	34.24	
	31.25	0.226	31.51	
	15.625	0.229	30.61	
	7.813	0.235	28.79	
	3.906	0.239	27.58	
	1.953	0.243	26.36	
	0.977	0.255	22.73	

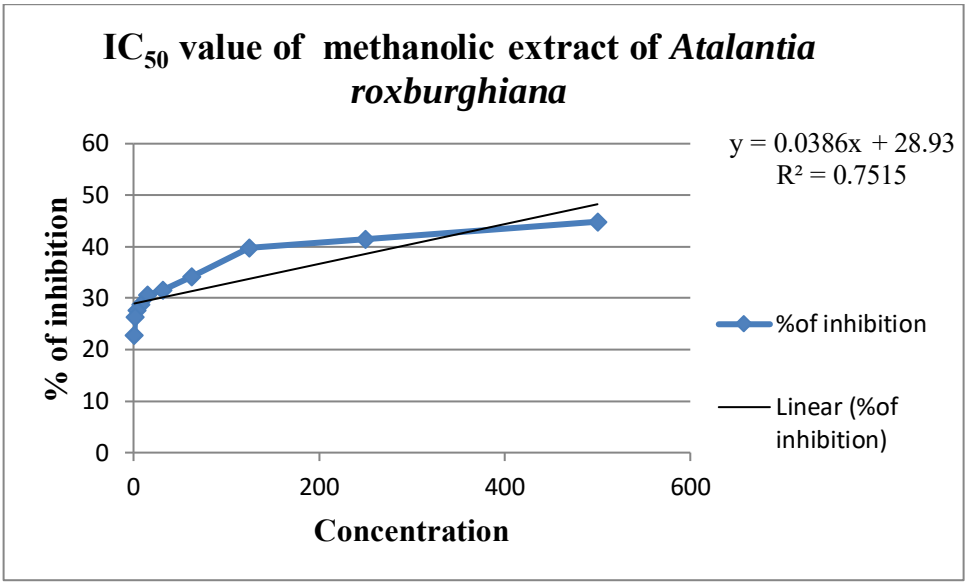

Figure 3.4: IC_{50} value of ME of leaves of *Atalantia roxburghiana*.

3.5 Determination of Total Flavonoid Content (TFC)

The methanolic extract (ME) of the leaves of *Atalantia roxburghiana* was subjected to total flavonoid content determination. Quercetin was used as standard for this test. Depending on the absorbance values of the extract solution, the value of total flavonoid content of the extract was investigated and differentiated with the standard solutions of Quercetin (Table 3.10) equivalents. The total flavonoid content of the sample is expressed as mg of QE (Quercetin equivalent)/gm of extract, and is given in Table 3.11.

The total flavonoid content in the methanolic leaf extract of our plant of interest was found to be 160.85 mg of QE/gm of extract.

Table 3.10: Standard curve prepration by using Quercetin

Sl. No.	Conc. of the Standard (µg/ml)	Absorbance	Regression Line	Regression coefficient (R^2)
1	25	0.021	y = 0.0012x - 0.0127	0.9982
2	50	0.046		
3	100	0.108		
4	200	0.219		
5	400	0.422		
6	800	0.959		
7	1200	1.369		

Interpretation: It is observed that, as the concentration of *Atalantia roxburghiana* was increased from 25–1200 µg/ml, the total flavonoid content also increased from 0.021 to 1.369 mg of Quercetin per gram of dried extract. Therefore, it indicates that there is as well, a positive correlation between total flavonoid content of *Atalantia roxburghiana* and its antioxidant activity.

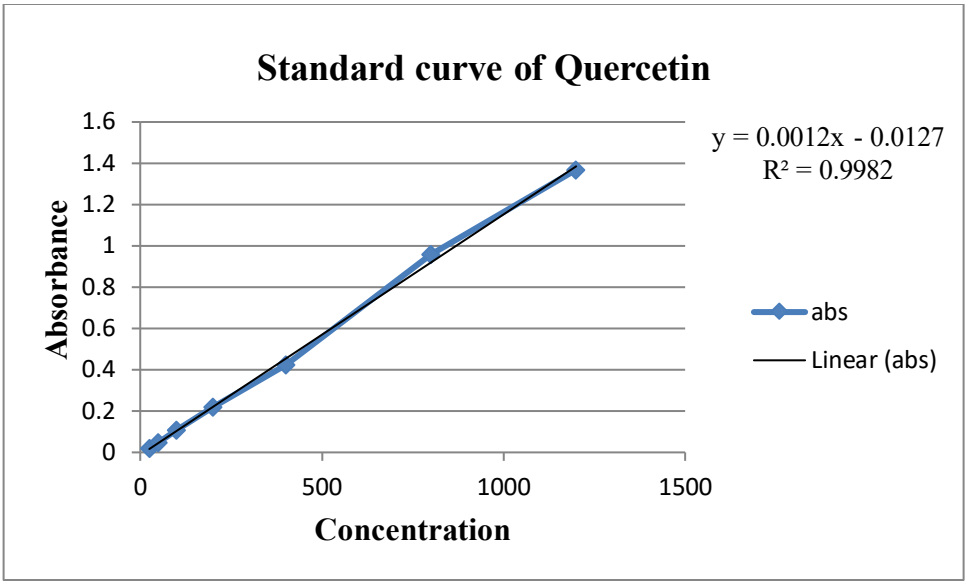

Figure 3.5: Calibration curve of standard Quercetin (at 415nm) for determining TFC in ME of *Atalantia roxburghiana* leaves

Interpretation: The equation of the calibration curve of Quercetin was found to be y = 0.0012x-0.0127 which would be used to determine the total flavonoid content of *Atalantia roxburghiana* leaf extract. A regression coefficient (R^2) of 0.9982 was also obtained pointing towards a good relationship between the concentration and the absorbance.

Table 3.11: Total flavonoid content of *Atalantia roxburghiana* (represented as mg of QE/gm of extract)

Plant part	Sample code	Test Sample	Total flavonoid content (mg of QE/gm of extract)
Leaves of *Atalantia roxburghiana*	ME	Methanolic extract	160.85

3.6 Determination of Total Antioxidant capacity (TAC)

The methanolic extract (ME) of the leaves of *Atalantia roxburghiana* was subjected to total antioxidant capacity determination. Ammonium molybdate reagent was used for this test. Depending on the absorbance values of the extract solutions, the value of total antioxidant capacity of the extract was investigated and differentiated with the standard solutions of Ascorbic acid (Table 3.12) equivalents. The total antioxidant capacity of the sample is expressed as mg of AAE (Ascorbic acid equivalent)/gm of extract, and is provided in Table 3.13.

The total antioxidant capacity investigated in the methanolic extract was 18.73 mg of AAE/gm of extract.

Table 3.12: Standard curve prepration by using Ascorbic acid

Sl. No.	Conc. Ofthe Standard (µg/ml)	Absorbance	Regression Line	Regression coefficient (R^2)
1	200	1.080	y = 0.0031x - 0.7592	0.9212
2	400	2.101		
3	800	3.741		
4	1200	4.122		

Interpretation: It is observed that, as the concentration of *Atalantia roxburghiana* was increased from 200 µg/ml to 1200 µg/ml, the total antioxidant capacity also increased from 1.080-4.122 mg of Ascorbic acid per gram of dried extract. This is indicative of a positive correlation existing between total antioxidant capacity of methanolic extract of *Atalantia roxburghiana* leaves and its antioxidant property.

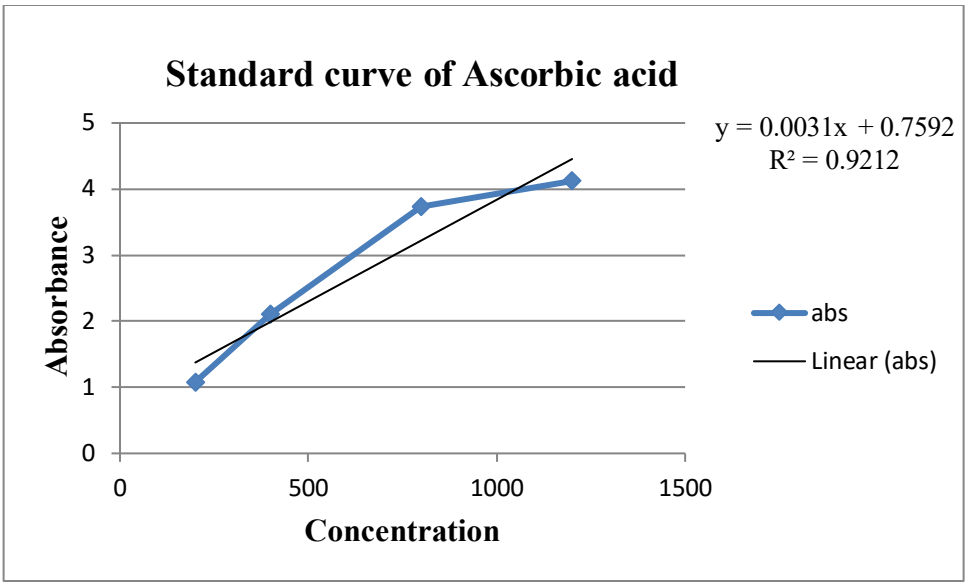

Figure 3.6: Calibration curve of Ascorbic acid (AA) at 695nm for determining TAC in ME of *Atalantia roxburghiana* leaves

Interpretation: The equation of the calibration curve of Ascorbic acid was found to be y = 0.0031x - 0.7592 which would be used to determine the total antioxidant capacity of *Atalantia roxburghiana* methanolic leaf extract. A regression coefficient (R^2) of 0.9212 was also obtained which signified a good relationship between the concentration and the absorbance.

Table 3.13: Total antioxidant capacity of *Atalantia roxburghiana* (represented as mg of AAE/gm of extract)

Plant part	Sample code	Test Sample	Total antioxidant capacity (mg of AAE/gmof extract)
Leaves of *Atalantia roxburghiana*	ME	Methanolic extract	18.73

3.7 Antimicrobial screening:

This study focused on the determination of the antimicrobial activity of the methanolic extract (ME) of leaves of *Atalantia roxburghiana* using two gram positive bacterial strains, *Staphylococcus aureus* & *Bacillus subtilis* and two gram negative bacterial strains,

Escherichia coli & *Vibrio cholera* by disc diffusion method. The methanolic extract was investigated for antimicrobial activity with a concentration of 20, 30, 40 µg/disc. It showed moderate antimicrobial activity against some of the tested microorganisms.

The maximum zone of inhibition exhibited by ME was found to be 20 mm against *Escherichia coli* for which Cefixime was used as standard. The moderate antimicrobial activities were found against *Bacillus subtilis* (having zone of inhibition of 15 mm, standard was Kanamycin) and *Vibrio cholera* (zone of inhibition of 16 mm, standard: Amoxicillin).

Table 3.14: Antimicrobial activity of methanolic extract of leaves of *Atalantia roxburghiana*

Test microorganism	**Diameter of zone of inhibition (mm)**	
	Gram negative bacteria	
Escherichia coli	20	27.5 (Standard: Cefixime)
Vibrio cholera	16	26.5 (Standard: Amoxicillin)
	Gram positive bacteria	
Bacillus subtilis	15	29 (Standard: Kanamycin)
Staphylococcus aureus	10	26.5 (Standard: Cefixime)

3.8 Brine Shrimp Lethality Bioassay

The methanolic extract (ME) of leaves of *Atalantia roxburghiana* was examined for brine shrimp lethality bioassay. The cytotoxicity of the extract to brine shrimp was observed and the results are given in Table 3.17.

The lethal concentrations (LC_{50}) of the test samples were determined by plotting the percentage of mortality rate of shrimps against the logarithm of concentration. The curve of regression analysis helps to provide the best-fit line. Vincristine sulfate (VS) was used as positive control and the LC_{50} was found to be 0.43 µg/ml. The LC_{50} of the methanolic extract of leaves of *Atalantia roxburghiana* was 9.13 µg/ml which is much higher than that of Vincristine sulfate.

Table 3.15: Effect of Vincristine sulfate (positive control) on shrimp nauplii

Conc.(µg/mL)	Log_{10}Conc.	%Mortality	LC_{50}(µg/ml)
0	-	0	0.43
0.039	-1.4089	20	
0.078125	-1.1072	30	
0.15625	-0.8061	30	
0.3125	-0.5051	40	
0.625	-0.2014	60	
1.25	0.09691	80	
2.5	0.39794	80	
5	0.6989	90	
10	1.00	90	
20	1.30102	100	

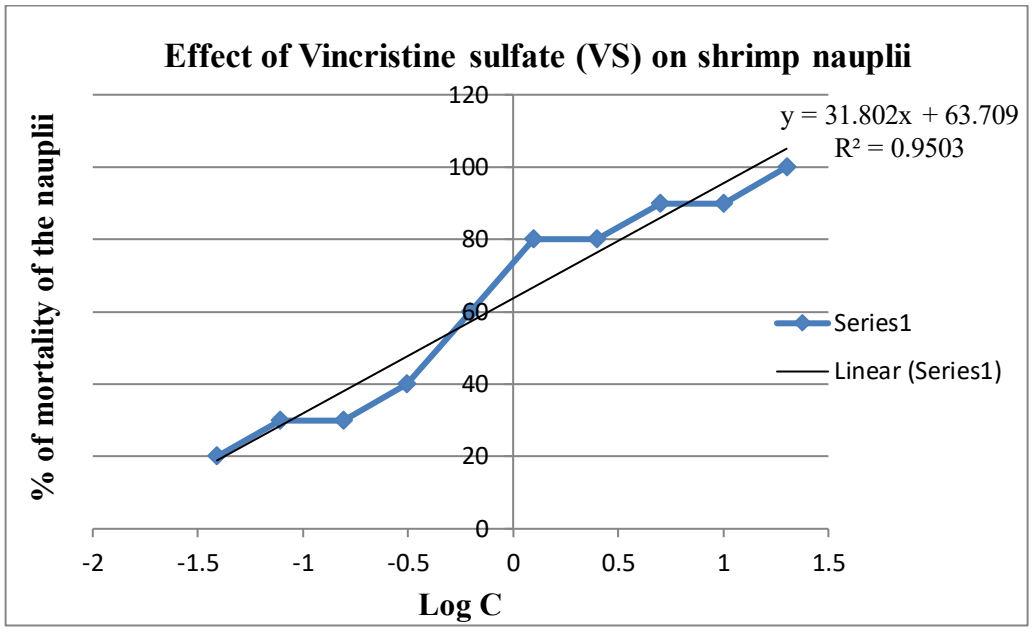

Figure 3.7: Plot of % mortality and predicted regression line of VS

Table 3.16: Effect of the methanolic extract (ME) of leaves of *Atalantia roxburghiana* on shrimp nauplii

Conc. (µg/ml)	Log_{10} conc.	% of mortality	LC_{50}
0	-	0	9.13
0.78125	-1.1072	30	
1.5625	0.19382	40	
3.125	0.49485	40	
6.25	0.79588	50	
12.5	1.09691	50	
25	1.39794	60	
50	1.69897	70	
100	2	80	
200	2.30103	80	
400	2.60206	90	

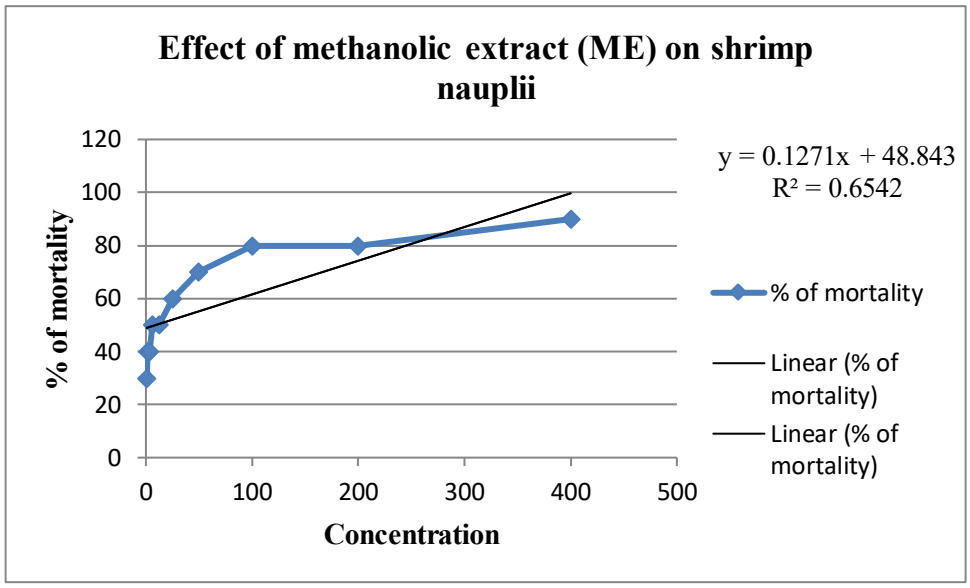

Figure 3.8: Plot of % mortality and predicted regression line of ME of *Atalantia roxburghiana* leaves

Interpretation: It is observed that with the increase in concentration of *Atalantia roxburghiana* from 0 µg/ml to 400 µg/ml, mortality also increased from 30-90% of nuplii. This also indicates a positive correlation existing between concentration and % of mortality.

Table 3.17: LC_{50} values of the test samples (VS and ME)

Test samples	Regression line	Regression coefficient (R^2)	LC_{50} (µg/ml)
Vincristine sulfate (VS)	y = 31.802x + 63.709	0.9503	0.43
Methanolic extract (ME) of *Atalantia roxburghiana* leaves	y = 0.1271x + 48.843	0.6542	9.13

3.9 Thrombolytic activity test

Addition of 100 µl Streptokinase (SK), a positive control (15,00,000 I.U.) to the clots along with 90 minutes of incubation at 37°C, showed 10% clot lysis. Clots when treated with 100 µl sodium chloride (negative control or blank solution) showed only negligible clot lysis (6%). The *in vitro* thrombolytic activity study revealed that the methanolic extract of *Atalantia roxburghiana* leaves lysed around 11% of blood clot. The effective clot lysis percentage by methanolic extract of *Atalantia roxburghiana*, positive thrombolytic control (Streptokinase) and negative control (sodium chloride) is presented below:

Table 3.18: Thrombolytic activity of *Atalantia roxburghiana* leaves

Serial No.	Weight of the tube (W_1)	Weight of the tube with clot (Before lysis) (W_2)	Weight of the clot (W_3)	Weight of tube with clot (After lysis) (W_4)	$W_5 = (W_4 - W_1)$	Percent of unlysed clot	Percent of clot lysis
1 (Methanolic extract)	0.830	1.589	0.759	1.509	0.679	0.895	11%
2 (Streptokinase)	0.797	1.671	0.874	1.584	0.787	0.900	10%
3 (Sodium chloride/blank)	0.784	1.611	0.827	1.565	0.781	0.944	6%

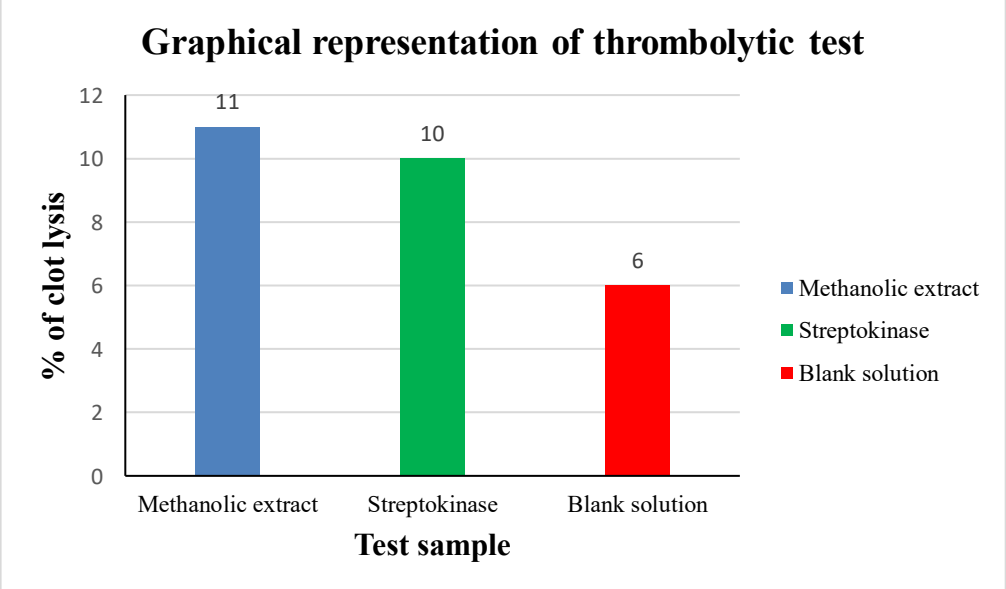

Figure 3.9: Comparison of percent of clot lysis of Streptokinase, blankand methanolic extract of *Atalantia roxburghiana* leaves

Interpretation: Positive control Streptokinase showed about 10% of clot lysis, whereas the methanolic extract of *Atalantia roxburghiana* leaves lysed about 11% of the blood clot. This indicates that the methanolic extract of our plant of interest has very significant thrombolytic potential.

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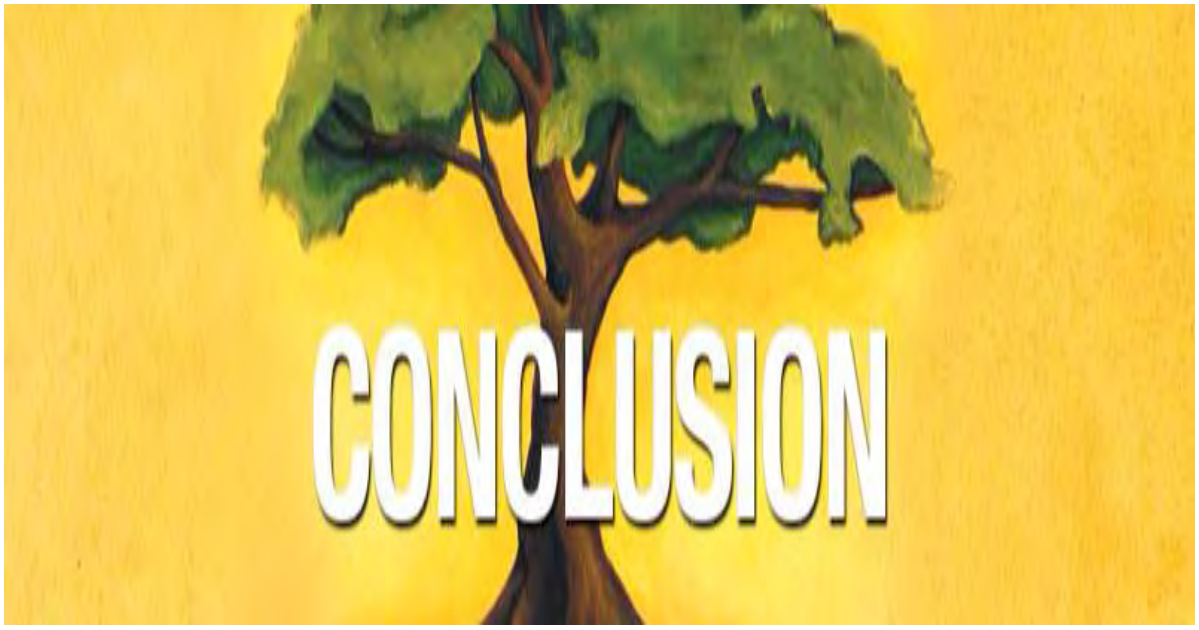
Chapter-4: Conclusion



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Conclusion

The crude extracts of the leaves of *Atalantia roxburghiana* can be a source of herbal medicine to cure a wide variety of human diseases. The phytochemical studies of the plant determined the presence of different compounds bearing the potential for detailed investigation. The plant extract exhibited moderate to high antioxidant properties that can be valuable to treat oxidative stress in the biological system. The antimicrobial test showed that *Atalantia roxburghiana* plant extract has effect against *Escherichia coli* and *Vibrio cholera*. Its antimicrobial potential can be further evaluated by means of determining minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the plant extract.

The thrombolytic test using Streptokinase presented very promising result for the plant extract which indicates its future use as an anticoagulant agent if incorporated in the proper concentration.

Therefore, the study specifies that the extracts of leaves of *Atalantia roxburghiana* might be a significant contributor to define drug development by exhibiting a number of potential biological properties. Further investigations such as partitioning of the plant extract using different solvents and determination of *in vivo* pharmacological properties should also be carried out. Animal models can be valuable to evaluate the therapeutic efficacy of the plant in comparison to synthetic drugs available in the market, as well as to observe the extent to which a preparation from natural sources can affect a biological system.

Chapter-5: References

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