Phytochemical and Biological Evaluation of *Lindenbergia indica*

A Project Submitted
by
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Dedicated to my parents

Certification Statement

This is to certify that this project titled "Phytochemical and Biological Evaluation of *Lindenbergia indica*" submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy, BRAC University constitutes my own work under the supervision of Monica Sharfin Rahman, Lecturer, Department of Pharmacy, BRAC University and that proper credit has been given where I have used the language, ideas or writings of another.

Signed,

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Countersigned by the supervisor

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Acknowledgement

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Abstract

This study was intended to identify and assess the potential phytochemical as well as biological properties of the medicinal plant *Lindenbergia indica* (Scrophulariaceae family). To achieve these purposes, different experiments were performed, such as phytochemical screening, antioxidant activity test, brine shrimp lethality bioassay and antimicrobial activity evaluation. However, the plant *Lindenbergia indica* is locally used for the treatment of chronic bronchitis, dysentery, arthritis, hydrophobia and sepsis. It is also evident from different observations that, this plant is an abundant source of large number of long chain hydrocarbons, beta-setosterol, beta-setosterolpalmitate, beta-setosterol-beta-D-glucosidase, mannitol and apigenin. In the existing investigation, phytochemical screening has indicated the presence of tannins, saponins, carbohydrates, glycosides and glucosides in this particular plant. Additionally, moderate antioxidant as well as antimicrobial activity of *Lindenbergia indica* was observed from the experiments. However, in brine shrimp lethality bioassay, it didn't show any significant cytotoxicity. So, on the basis of the present investigation, it can be proposed that the plant "*Lindenbergia indica"* can be used as a medicinal plant.

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List of Abbreviations

- HDL = High Density Lipoproteins
- mg = Milligram
- mm = Millimeter
- mL = Milliliter
- µg = Microgram
- BHA = Butyl Hydroxy Anisole
- BHT = Butyl Hydroxy Toluene
- IC_{50} = Median Inhibition Concentration
- LC_{50} = Lethal Concentration
- DPPH = 2,2-Diphenyl-1-Picrylhydrazyl
- UV = Ultraviolet
- DMSO = Dimethyl Sulfoxide
- GI = Gastrointestinal
- HCl = Hydrochlorc Acid

Chapter 1

Introduction

1.1 Introduction

From the prehistoric time, human beings are depending on the plant kingdom for their survival as well as for the remedy of various diseases. However, the history and success of herbal medicine in the treatment of different diseases are very effective and affluent. In a publication, a list of 20000 plants have been published which are used medicinally. There was also indication of large number of flowering plants that had been used medicinally (Deans & Svoboda, 1990). Apart from these, primary metabolites such as proteins, fats, carbohydrates are synthesized by plants which have great significance not only for the plants themselves, but also for the animals and humans as well.

1.1.1 Medicinal Plants

According to Fellows (1991), "The term 'medicinal' as applied to a plant indicates that it contains a substance or substances which modulate beneficially the physiology of sick mammals and that it has been used by man for that purpose" (Lewington, 1993). However, in the simplest language, "Medicinal plants are those plants which are used in official and various traditional systems of medicines throughout the world" (Maiti & Geetha, 2007).

1.1.2 History of Medicinal Plants

Shen Nung's "Pen T'Sao" or "Shennong Ben Cao Jing" (c. 3000 B.C.) is known as the oldest list of medicinal herbs (Petrovska, 2012).The ancient Romans and Greeks were known to be famous as herbalists.In that time, the surgeons that traveled with the Roman army gave their herbal knowledge to the whole Roman territory, Germany, Spain, England and France. Furthermore, two Greek surgeons in the Roman army, called Dioscorides (c. 40-c. 90) and Galen (131-200 A.D.) accumulated herbs which were mentioned in the book "*Materia Medica"* (Claude Moore Health Sciences Library, 2007).

The existence of herbalism is evident throughout the middle ages, in Europe and Britain. During the eleventh and twelfth centuries, monasteries were used as medical schools before the foundation of universities. The monks imitated and translated many works of Dioscorides, Hippocrates and Galen. Moreover, their "Physick" gardens used to function as training centers for their future generation (Claude Moore Health Sciences Library, 2007).

During the seventh and eighth centuries, the Arabic scholars gained manyof the Roman and Greek medicals texts. An Iranian physician Ibn Sina (known as Avicenna as well) compiled the herbal traditions of Dioscorides and Galen with the ancient practices of his own country and wrote one of the most valuable medical text books called "The Canon of Medicine" which spread throughout the Europe during the eleventh and twelfth centuries (Claude Moore Health Sciences Library, 2007).

Theophrastus Bombastus von Hohenheim, also known as Paracelsus (1493-1541) emphasized on the significance of experience with patients and wrote against beliefs of the previous physicians. Paracelsus revitalized the first century "Doctrine of Signatures" according to which, every herb had its individual sign (Claude Moore Health Sciences Library, 2007).

A century afterward, Nicholas Culpeper (1616-1654) revived an ancient feature of herbalism called Astrology (Claude Moore Health Sciences Library, 2007).

Some scientists like Francis Bacon (1561-1626) and William Harvey (1578-1657) renovated science from a tentative to an experimental procedure. This new perception however, did not combine well with the revitalization of the "Doctrine of signatures" as well as with "Astrology". In this way, biological as well as medical science started to be detached from traditional herbalism. So, the herbalists who focused on the classification and ignored to admit signatures as well as stars, created the science of "Botany" and the Physicians who granted Harvey's circulation of the blood more constructive than Culpeper's movements of the planets, created the sector of "Scientific Medicine" (Claude Moore Health Sciences Library, 2007).

1.1.3 Medicinal Plants in Bangladesh

In Bangladesh, herbal drugs have bright prospects, as the country is considered rich in medicinal plants genetic resources by virtue of its favorable agroclimatic condition and seasonal diversity. With productive soils, a tropical climate, and seasonal diversity, Bangladesh contains about 6500 plants species including bryophytes, pteridophytes, gymnosperms, and angiosperms; among them, 500 plant species have medicinal values and

they grow in the country's forests, wetlands, homestead forests, and even roadside as indigenous, naturally occurring, or cultivated plants. According to Abdull Ghani (2003), names of 400-500 medicinal plants have been listed either as growing or as prevalent in Bangladesh (Ghani, 2003). About 75% (10 million households in over 85,000 villages) of the country's total population lives in rural areas, and almost 80% are dependent on natural resources (e.g., medicinal plants) for their primary healthcare with herbal medication remaining a popular and accepted form of treatment. Moreover, rural peoples are capable to identify many species of plants from which various products, including food, firewood, medicine, forage, etc. can be obtained, and the customary homestead tree production system also serves as a source of plant products and remedies (Rahman, 2014).

Despite such a high demand of herbal medicine and promising business opportunities of herbal medicine in Bangladesh with the presence of more than 500 companies producing herbal medicines, more than 90% of the plants and products needed to meet domestic demand are imported from other countries, such as India, Nepal, and Pakistan (Rahman, 2014).In addition, potential of higher plants as source for new drugs is still largely unexplored (Mahesh & Satish, 2008). It is estimated that between 25000 and 75000 plant species are used for traditional medicine, of which only 1% are known by scientists and accepted for commercial purposes (Aguilar, 2001). Among the estimated 250,000 - 500,000 plant species, only a small percentage has been investigated phytochemically and the fraction submitted to biological or pharmacological screening is even smaller. Historically pharmacological screening of compounds of natural or synthetic origin has been the source of innumerable therapeutic agents (Mahesh & Satish, 2008). It is estimated that the number of higher plant species (angiosperms and gymnosperms) on this planet is about 250,000 (Ayensu, DeFilipps & R.A., 1978), with a lower level at 215,000 (Cronquist, 1981) and an upper level as high as 500,000 (Tippo & Stern, 1977). Of these, only about 6% have been screened for biological activity and a reported 15% have been evaluated phytochemically (Verpoorte, 2000).

For many of the medicinal plants of current interest, a primary focus of research to date has been in the areas of phytochemistry, pharmacognosy, and horticulture. In the area of phytochemistry, medicinal plants have been characterized for their possible bioactive

compounds, which have been separated and subjected to detailed structural analysis (Briskin, 2000). Chemical constituents in plants are classified into primary and secondary metabolites (Bulock, 1965).

Primary products, such as carbohydrates, lipids, proteins, heme, chlorophyll, and nucleic acids, which are common to all plants are involved in the primary metabolic processes of building and maintaining plant cells. Whereas, the beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant (Briskin, 2000).

Basically, "Phytochemistry is the branch of science that deals with the secondary metabolites of plants" (Harborne & Harborne, 1998).Secondary metabolites are mainly the bioactive natural products, which are used by the host as a means of defensive and self-protective mechanism against their enemies as well as predatory animals. However, screening of these secondary metabolites is called phytochemical screening which is usually a complicated task and requires lots of efforts including botanical identification and collection of the plant, isolation, purification of the extract as well as the identification and assessment of pharmacological and clinical testing (Harborne & Harborne, 1998).

However, in Bangladesh, there might be many other unknown plants with potential medicinal uses, for which further investigation and research are needed to be carried out to identify them.

1.2 Rationale of the Study

Wide-ranging phytochemical investigation as well as isolation of active constituents in the pure form of plants is essential in order to avoid inconvenient effects which will make sure the safe as well as effective use of herbal medicines. The dissertation portrayed for the present study is an effort to identify and characterize the chemical components by means of phytochemical screening and to assess the potential biological outline of the medicinal plant *Lindenbergia indica* (Scrophulariaceae family).Since this plant *Lindenbergia indica* has been traditionally being used in the treatment of chronic bronchitis (Ghani,2003), on skin eruptions (Herbpaht, 2016) and dysentery and a few works have been done on this plant, so

there might be some unexplored medicinal uses of this plant as well. So in this current dissertation, phytochemical screening as well as biological investigation including antioxidant activity test, brine shrimp lethality test and antimicrobial activity test has been performed since on these activities of the plant *Lindenbergia indica*, no work has been done yet; that is why this present study may work as a basis to identify some unfamiliar properties and medicinal uses of this plant. In this current dissertation, methanolic extract of the leaf and branches of the plant *Lindenbergia indica* has been used. The aim of this dissertation was to evaluate the phytochemical constituents and biological activities like antioxidant activity, cytotoxic activity, and antimicrobial activity of the plant *Lindenbergia indica.*

The objective of this study was:

- To see phytochemical constituents like alkaloid, tannins, saponins etc. of the plant *Lindenbergia indica*.
- To evaluate biological activities, like antioxidant activity, cytotoxic activity test and antimicrobial activity test of the plant.

1.3 Plant

1.3.1 The Plant family Scrophulariaceae:

The plant which has studied belongs to the family Scrophulariaceae.

Scrophulariaceae falls under the major groups, named Angiosperms (flowering plants). According to a plant list the species of Scrophulariaceae has 87 genera and 4883 scientific plant names (The Plant List, 2010).

The plants under this Scrophulariaceae family are most commonly herbs or sometimes small shrubs. This family encompasses about 190 genera and 4000 moderate species, together with many other species which are slightly root parasites. However, a small number of them are also found without chlorophyll as well as being entirely parasitic. The leaves of the plants of this family are alternate, opposite, or even sometimes whorled as well as simple to pinnately divide. The stipules are not present (Carr, 2005).

The flowers of the plants under Scrophulariaceae are bisexual as well as zygomorphic and have deeply colored and prominent associated bracts (Carr, 2005).The fruit of the plants under this family is generally a capsule. Flower arrangement of the plants of this family differs with species and is categorized into the following four types (Plant and Soil Sciences eLibrary, 2016):

i) Spike
ii) Raceme
iii) Thyrse
iv) Panicle

Spike **Raceme** **Thyrse**

Figure 1.1: Flower arrangement of Scrophulariaceae family (Plant and Soil Sciences eLibrary, 2016)

1.3.2 Genera under the family Scrophulariaceae:

Amongst the 87 genera of this family, name of some genera are given below (Table 1.1):

Table 1.1: Genera under the family Scrophulariaceae (The Plant List, 2010):

Agathelpis	Hemimeris

Anamaria	Jamesbrittneia
Botia	Limosella
Buddelja	Manulea
Celsia	Nemesia
Colpias	Oftia
Cromidon	Pocilla
Diascia	Reyemia
Dischisma	Sutera
Emorya	Teedia
Fonkia	Verena
Glekia	Walafrida
Gosela	Zaluzianskya

1.3.3 Description of the Plant Genera:

According to the Plant List 2013, under this species rank, 44 names of scientific plants for the genus *Lindenbergia* has been listed amongst these, only 4 species names have been accepted and are given below (The Plant List, 2013):

- *Lindenbergia muraria*
- *Lindenbergia indica* Vatke
- *Lindenbergia grandiflora*
- *Lindenbergia philippensi*

1.3.4 Description of the Plant *Lindenbergia indica*

1.3.4.1 Taxonomic Hierarchy of *Lindenbergia indica* (Widescreen Arkive, 2016)**:**

Kingdom: Plantae

Phylum: Tracheophyta

Class: Magnoliopsida

Order: Scrophulariales

Family: Scrophulariaceae

Genus: *Lindenbergia*

Scientific Name: *Lindenbergia indica* (Linn.)

1.3.4.2 General Botanical Data of the Plant:

Growth Form: It is an annual, small and erect herb of 15-50 cm height (Uddin, 2014).

Habit: *Lindenbergia indica* is an annual, diffusely glandular and pilose herb (Uddin, 2014).

Root: Its root is tap root (Verma & Verma, 2011).

Stem: Its stem is herbaceous, branched from the bottom, hairy and solid (Verma & Verma, 2011).

Leaves: Leaves of this plant are about 1.5-5.5 × 1-3 cm in length, broadly ovate, subacute, crenate-serrate, base rounded or cuneate, glandular pubescent or both sidesand generally glandular-villous (Roy *et al*., 1992).

Flowers: Flowers are yellow in color, pedicillate, subsessile, bracteate as well as complete. Flowers of *Lindenbergia indica* are also bisexual, zygomorphic, hypogynous as well as pentamerous (Verma & Verma, 2011).

Calyx: Calyx of this plant has five sepals and is gamosepalous which is imbricate and pilose on both sides and persistent (Verma & Verma, 2011)

Corolla: Corolla of this plant is gamopetalous which has five petals and is yellow personate, posterior lip of the corolla sharply tapers from wide base to the emarginated apex (Verma & Verma, 2011).

Fruit: Its fruit is capsule (Verma & Verma, 2011).

Figure 1.2A : *Lindenbergia indica* with flowers (Flowers of India, 2016)

Figure 1.2B: *Lindenbergia indica*

Figure1. 2: Plant *Lindenbergia indica*

1.3.4.3 Chemical Constituents:

It is evident from different experiments that the plant contains a large number of long chain hydrocarbons, beta-setosterol, beta-setosterolpalmitate, beta-setosterol-beta-D-glucosidase, mannitol and apigenin (Ghani, 2003).

1.3.4.4 Medicinal Use:

Plant juice: Plant juice is usually given in chronic bronchitis (Ghani, 2003). However, plant juice mixed with Coriander plant, is also applied to skin eruption (Flowers of India, 2016). Additionally, the practice of applying plant juice by rubbing over the body in pestilent fevers is also seen. The plant is also used in combination with cumin, ginger as well as with other aromatics in the treatment of dysentery (Vardhana, 2008).

Herb: Herb is used as antiseptic and is used in the treatment of elephantiasis in combination with coconut oil (Vardhana, 2008).

In addition, this plant is used for the treatment of Arthritis and Hydrophobia as well (Herbpathy, 2016).

1.3.4.5 Distribution/Habitat of the Plant:

Lindenbergia indica is prevalent in Bangladesh, Africa, Malaysia, China and India. Seven species of *Lindenbergia indica* Linn. have also been identified in India (Purohit & Vyas, 2003). This plant is prevalent throughout India ascending to 2100 m in the Himalayas (Khare, 2007).

In Bangladesh, *Lindenbergia indica* is found all over the country on damp and old walls (Flowers of India, 2016).

1.3.4.6 Common Names of the Plant:

The common name of *Lindenbergia indica* is Nettle leaved Lindenbergia. In India, this plant is termed in different names in different states, such as Patthar-chatti (in Gujrat), Dhol (in Maharashtra) and Bheet-chatti (Khare, 2007).

In Bangladesh, this plant is termed as Basanti, Haldi Basanti etc. (Flowers of India, 2016).

1.4 Experimental Design

- **Phytochemical Screening of Plant Extract:**

Phytochemical analysis of plant extracts are performed in order to identify the chemical constituents that are of medicinal use. These chemical constituents of plants having medicinal importance are basically secondary metabolites of plants and the investigation of chemical constituents of plants can only expose those components which have gathered at a particular organ of a certain plant. Different chemicals compounds with potential medicinal use such as flavonoids, alkaloids, glycosides, steroids, phenols, terpenoids, saponins, resins, tannins, coumarins, quinones etc. can be isolated from plant extracts using their specific tests and chromatographic procedures like TLC (Thin Layer Chromatography) (Tiwari *et al.*, 2011).

Phytochemical screening of the plant under study named *Lindenbergia indica* has been carried out in order to identify the chemical constituents of this plant which will lead to have

a precise idea about the chemical components present in this plant and will work as a basis to estimate the possible pharmacological effects as well as medicinal uses of it.

- **Evaluation of Antioxidant Activity:**

According to Halliwell and Gutteridge, antioxidants are compounds, which inhibit or delay the oxidation of the substance, when present in low concentration in relation to the oxidant. Some recent scientific studies have revealed that antioxidants have the ability to decrease the risk of many chronic diseases including cancer as well as heart disease. Plant is a vital source of antioxidants, rich in vitamin C, vitamin E, phenolic acids, carotenes etc. which have a significant potential to reduce the risk of many diseases (Shekhar & Anju, 2014).

In this current exploration, evaluation of antioxidant study of the plant extract *Lindenbergia indica* has been performed in order to identify the antioxidant activity of this plant and this way to identify the prospective of *Lindenbergia indica* to be consumed as a new source of natural antioxidant.

- **Evaluation of Cytotoxicity by Brine Shrimp Lethality Test :**

Brine Shrimp Lethality Test is carried out in order to assay the cytotoxicity level of plants. This bioassay can detect a broad spectrum of bioactivity, which is present in crude plant extracts. The objective of this method is to facilitate a front-line screen which is capable of being backed up by relatively precise as well as comparatively expensive bioassays, when the active components have been separated. Brine Shrimp Lethality Test has various advantages for example it is cheap, easy to perform and requires small amount of test material (Taha & Alsayed, 2000).

In this dissertation, Brine Shrimp Lethality Test has been performed to evaluate the ultimate level of cytotoxicity of the plant *Lindenbergia indica*. This test will function as a basis to measure the level of toxicity, safety, therapeutic index, as well as to assess the potential of the current plant under investigation (*Lindenbergia indica*) to be used as a new drug.

- **Antimicrobial Activity Test:**

Plants have been the most common basis of antimicrobial drugs. Moreover, many aromatic plants have been consumed conventionally in case of folk medicine and also have been used to increase the shelf life of food items by providing inhibition against bacteria, yeast as well as fungi. It has been found that the antimicrobial effectiveness of some plants in treating diseases is unbelievable. In addition, it is anticipated that local people have consumed only about 10% of all flowering flora that are found on earth in order to treat a variety of infections but only about 1% of these has achieved the recognition from modern scientists (Khan *et al.*, 2009). Because of the prominent use plants as a source of antimicrobial agent in different infectious diseases, the investigation for plants having antimicrobial activity have become common these days. For example, many plants have been obtained to be effective in curing respiratory tract infections, gastrointestinal (GI) disorders, urinary tract infections, cutaneous infections etc. That is why, there is a fundamental necessity to find out new antimicrobial agents having various chemical structures with sophisticated mechanism of action (Khan *et al.*, 2009).

The rationale of this dissertation is to evaluate the antimicrobial activity of the methanolic extract of the leaf and branch of the plant *Lindenbergia indica* by Disc Diffusion Method in order to identify the antimicrobial activity in it as well as to ascertain this plant as a new prospective source of naturally found antimicrobial agent.

1.5 Literature Review

i) Antifertility Effect of *Lindenbergia indica* (70% EtOH) Extract:

In this study, 70% of EtOH extract of *Lindenbergia indica* was orally administered to female rats and 1000 mg/kg body weight dose remarkable decrease in serum cholesterol, HDL (High Density Lipoproteins), triglycerides and phospholipids but no considerable decrease in the protein levels. In addition, the fertility test exhibited complete, that means 100% negative results. This negative fertility result also indicated the size of oogenesis and exhaustion of estrogen level. Overall, this study suggested the antiestrogenic nature of *Lindenbergia indica* (Purohit & Vyas, 2003).

ii) Phytochemical characterization and antibacterial activity of *Lindenbergia indica* Vatke: A common wall flora against some human pathogens in Doon Valley, Uttarakhand:

This study was performed to evaluate phytochemical as well as antibacterial property of *Lindenbergia indica* Vatke. The phytochemical analysis of this dried plant extract revealed the existence of proteins, alkaloids, carbohydrates, cardiac glycosides, triterpenoids, saponins, phenols and tannins. Agar disc diffusion assay was performed in order to evaluate antimicrobial properties of various concentrations of crude extracts against twelve bacterial strains pathogenic to humans. Among the seven extracts of this plant, those were assayed in this study, only two of them- ethanol and acetone extracts were found to be the most active, since they demonstrated antibacterial activity against the majority of the bacterial strains which were studied. Moreover, the result of this study revealed that *Lindenbergia indica* Vatke had a huge potential in the treatment of bacterial infection, which had probably justified the traditional use of this plant in curing infections like sore throats, chronic bronchitis, eruptions in skin as well as toothache (Singh *et al.*, 2013).

iii) Two New Steryl Glycosides from *Lindenbergia indica*:

This study showed the presence of two new saponin compounds namely: Alpha-L-rhamnopyranosyl (1→4) beta-D-glucopyranosyl (1→3) sitosterol and Alpha-L-rhamnopyranosyl (1→5) alpha-L-arabinofuranosyl (1→3) sitosterol in this plant (Tiwari & Choudhary, 1979).

Chapter 2

Materials and Methods

2.1 Investigation of Chemical Compounds of *Lindenbergia indica* by Phytochemical Screening

2.1.1 Introduction:

Historically it has been evident from different observations that before the beginning of synthetic era of medicines, humans were entirely dependent on medicinal plants for the avoidance, alleviation and treatment of variety of diseases. Since the ancient time medicinal plants and herbs have been used to cure diseases in India, which are locally termed as "Ayurveda". But these days, the worth of medicinal plants and herbs as herbal treatment is gradually decreasing because of the lack of awareness, combinatorial chemistry, genomics and also because of the deforestation. Consequently, a lot of precious medicinal herbs and their species are becoming rare day by day. It is estimated that about 500000 plants are present in our earth and amongst them only around 10000 plants are commonly consumed for medicinal use worldwide (Srujana *et al.*, 2012)

In the current dissertation, the plant *Lindenbergia indica* under the family Scrophulariaceae has been investigated.

Table 2.1: Name and family of the plant:

Name of the Plant	**Family**	**Plant Part**
Lindenbergia indica	Scrophulariaceae	Leaf and Branch

2.1.2 General Approaches to Drug Discovery from Natural Sources:

Basically, there are the following three approaches for discovering new medicines (Rahman, 2012):

a) Traditional Approach: The traditional approach of discovering medicine has been used from many years in various cultures as well as systems of medicine. In this approach,

drugs are discovered by means of trial and error basis. For example, drugs like ephedrine, morphine which are being used for a many years were discovered by this approach (Rahman, 2012).

b) Empirical Approach: This approach is based on the physiological procedure which creates therapeutic agents from naturally derived lead compound. Examples of some drugs which are obtained from empirical approach include Propranolol, Tubocurarine, Cimetidine etc.

c) Molecular Approach: This approach of discovering medicine is founded on the accessibility or perceptive of a target molecule for the medicinal agent. The mainstream of drug discovery is founded on the molecular pattern which is in accordance with the progress of molecular biological method and the progress in genomics.

Following is a figure (Figure 2.1) showing the steps of searching process for lead compounds (Howlader *et al.,* 2012)**:**

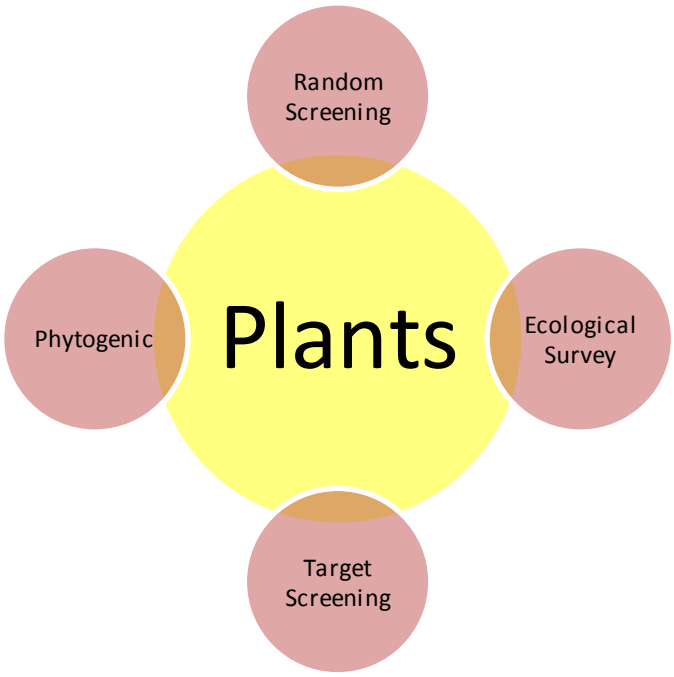

Figure 2.1: Lead compound search

2.1.3 Design of Biological Investigation of Plant Extracts:

The term "Herbal Medicine" or "Phytomedicine" refers to the use of leaves, fruits, roots, barks or flowers of plants or herbs for the purpose of medicinal use. At present, the real origin of a lot of vital pharmaceutical drugs and ingredients which are presently being used by patients throughout the world, are basically the plants that have been used by the native people. Globally, an incomparable expansion has been flourished in the formulations obtained from plant source, medicines and health-care goods. For this reason, the market coverage of products derived from plant origin is globally more than 60%. The prospective of medicinal plants and herbs can be evaluated by discovering various new chemical constituents of broad structural variety. Additionally, these newly invented chemical entities can be provided as an outline for generating more efficient drugs by synthetic or semi-synthetic method (Howlader *et al.,* 2012).

The study of naturally obtained plants is advantageous compared to that of the synthetically obtained drugs because it results into compounds possessing new structural attributes with new biological activities and exhibit minimal resistance as well as negligible side effects. Moreover, almost every pharmacological group of drugs comprises a natural product model. The determination of structure and screening of biological activity of natural compounds having a medicinal use has turned into a significant function in medicinal chemistry (Srujana *et al.*, 2012).

2.1.4 Collection and Preparation of the Plant Material:

At first, the whole plant sample *Lindenbergia indica* was collected from Savar area, Bangladesh. Then the sample plant was identified by an expert taxonomist (Accession Number of the plant is 42233). After that the leaves of the sample were cut into tiny pieces in order to allow a better drying under direct sunlight. The plant sample was then sun dried for 8 days, after which, the dried leaves were smashed into coarse powder (Figure 2.2) with the help of a high capacity grinding machine that was washed as well as cleaned previously to prevent any kind of cross contamination.

Figure 2.2: Coarse powder of the plant *Lindenbergia indica*

2.1.5 Extraction of the Plant Material:

Initially, two amber containers each of 2.5 liters were used to soak 500gm of powdered plant leaves in 2.2 liters of Methanol. After that these two amber containers with their contents were sealed by cotton plugs and aluminum foil. Then these amber containers were reserved in a clean and dry place for 14 days with gentle shaking and stirring occasionally. After 14days, the whole mixture was filtered three times. At first the mixture was filtered using thin fabric (Figure 2.3A) which consumed 1hour and 30 minutes and then was filtered through cotton (Figure 2.3B) that took 10 minutes. Finally the mixture was filtered through filter paper (Figure 2.3C) that consumed approximately 2 hours. The filtrate was then evaporated with the help of a "Rotary Evaporator" (Figure 2.4A) at 40°C with 100 rpm for 126 minutes. After performing the evaporation, the final volume of the mixture was 100ml. After that the filtrate was taken in a beaker and was dried using a hair drier (Figure 2.4B) for about 16 hours. The crude extract (Figure 2.4C) finally obtained weighed approximately 62gm.

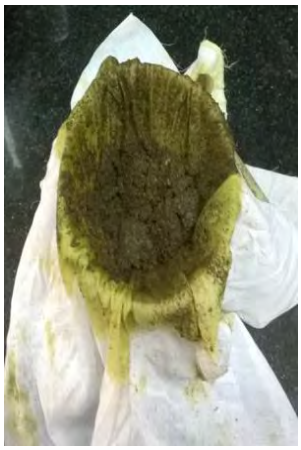

Figure 2.3A: Filtration through thin fabric.

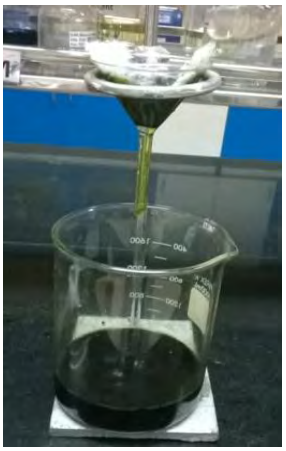

Figure 2.3B: Filtration through cotton.

Figure 2.3C: Filtration through filter paper.

Figure 2.3: Three times filtration of the plant extract

Figure 2.4A: Evaporation of the plant extract

Figure 2.4B: Drying of the plant extract

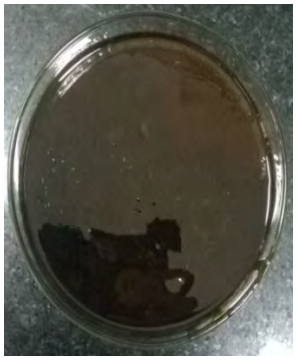

Figure 2.4C: Crude plant extract

Figure 2.4: Evaporation, drying and the crude plant extract

2.1.6 Methods for Phytochemical Screening:

2.1.6.1 Introduction:

Pharmacological properties of medicinal plants as well as different products which are of plant origin basically depend on their chemical components. As a result, the main goal of phytochemical investigation of plants as well as natural substances is to separate, characterize as well as to detect their chemical constituents. But this process is a lengthy as well as arduous. Since the chemical constituents that are present in plants and natural compounds are very diverse, those range from simple hydrocarbons to complex steroids, so no consistent technique of analysis can be commonly used to separate and detect them. Additionally, the chemical components of plants include compounds such as carbohydrate, tannins, proteins, steroids, terpenes, glycosides, flavonoids, carotinoids, amines, resins, volatile oils and numerous other substances with various types of chemical composition and characteristics. As a consequence, a variety of different phytochemical investigation as well as physico-chemical methods are applied to investigate them (Ghani, 2003).

2.1.6.2 Phytochemical Screening of the Plant *Lindenbergia indica*:

There is a diversity of qualitative tests for performing the phytochemical screening of plants. Following are some of the most commonly used tests that are easy, quick and available, for which they have been performed for the phytochemical screening of the extract of the plant *Lindenbergia indica* (Ghani, 2003).

2.1.6.2.1 Test for Carbohydrates:

- **Molisch's Test:**

In order to perform the Molisch's test for the identification of carbohydrates, initially, 2mL of the aqueous extract of the plant *Lindenbergia indica* was taken in a test tube and then 2 drops of 10% (1gm alpha-naphthol in 10mL Ethanol) freshly prepared ethanolic solution of alpha-naphthol was mixed thoroughly with it. Consequently, under a fume hood 2mL of concentrated sulphuric acid was added to the mixture and permitted to pour down the surface of the test tube in order to allow the acid to create a layer under the aqueous solution.

Then, a red circle was created between the junctions of two layers which indicated the presence of carbohydrates (Figure 2.5A). In addition, on standing or shaking, a dark purple solution was seen (Figure 2.5B). After that, the mixture was shaken and permitted to stand for 2 minutes. Then it was diluted with 5mL of water which instantly resulted in a dull violet precipitate, which was indicative of the presence of carbohydrate in the plant extract.

Figure 2.5A: Formation of red circle between the junctions of two layers.

Figure 2.5B: On standing or shaking a dark purple solution was formed.

Figure 2.5:Molisch'sTest for the identification of carbohydrates

2.1.6.2.2 Test for Proteins:

- **Biuret's Test:**

In order to perform the Biuret's test for identifying proteins in the plant extract, initially, 7 drops of 10% sodium hydroxide solution and 3% of copper sulphate solution was added to 1mL of the hot aqueous extract of the plant *Lindenbergia indica*. If protein was present in the test sample, then a red or violet color would be produced but in the current test sample, no color change observed (Figure 2.6) which indicated the absence of protein in the plant extract.

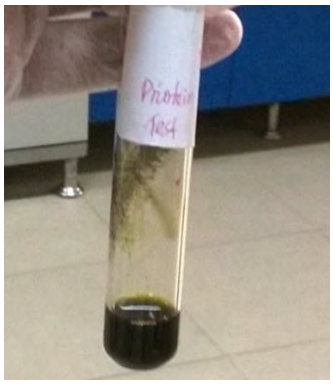

Figure 2.6: Biuret's test for protein (no color change was found)

2.1.6.2.3 Test for Glycosides:

- **General Test for Glycosides:**

At first, a very small amount (0.5gm) of the methanolic extract of the dried plant *Lindenbergia indica* was dissolved in 1mL of distilled water and additionally a few drops of 5% previously prepared sodium hydroxide solution was added to the mixture. A yellow color developed which was the indicative of the presence of glycosides in the plant extract.

- **Test for Glucosides:**

Initially, a small amount of the methanolic extract of the plant *Lindenbergia indica* was dissolved in a mixture of 5mL methanol with 5mL of distilled water and then this solution was divided into two equal parts and kept into two different test tubes which were treated in the following ways:

i) One of the test tubes were boiled with a mixture of equal volumes of "Fehling's Solution A and B" and any brick red precipitate was noted.

ii) After that, in the other test tube with the solution few drops of dilute sulphuric acid was added and the mixture was carefully boiled for about 5 minutes with the help of a "Bunsen Burner". The mixture was then neutralized with sodium hydroxide solution and using a litmus paper the neutralization of the mixture was confirmed. Finally, an equal volume of a mixture of "Fehling's Solution A and B" was added to the mixture and boiled.

Now, formation of a brick-red precipitate in the (ii) experiment, which was carried out with the hydrolysed extract and no production of such a precipitate in the (i) experiment would be an indication of the presence of glucosides and in this current study a brick-red precipitate was produced (Figure 2.7B) in (ii) experiment, but in the (i) experiment, no such precipitate was formed (Figure 2.7A) which confirmed the presence of glucosides in the sample extract.

Figure 2.7A: No production of break-red color

Figure 2.7B: Production of brick-red color

Figure 2.7: Test for glycosides

2.1.6.2.4 Test for Saponins:

- **Frothing Test:**

At first, 0.1gm of the powdered plant material was boiled with 10mL of distilled water for about 5 minutes and was filtered. After that, the filtrate was cooled and diluted with 5mL of distilled water with vigorous shaking. Finally, formation of a constant frothing (Figure 2.8), which remained unchanged on heating pointed to the presence of saponin in the plant extract.

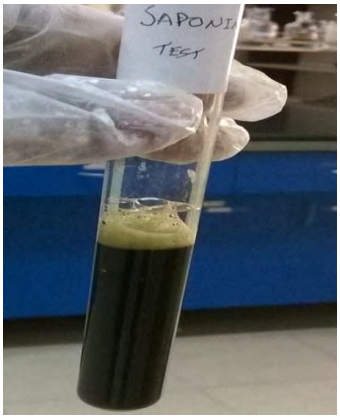

Figure 2.8: Frothing Test for saponins (formation of constant frothing)

- **Alternative Test for Saponins:**

Initially, 0.5 gm of the methanolic extract of the plant was dissolved in 10mL of distilled water and the above mentioned similar result was obtained which indicated the presence of saponins in the plant extract.

2.1.6.2.5 Test for Flavonoids:

- At first, a few drops of concentrated hydrochloric acid were added to the methanolic extract of the plant. If flavonoids were present an immediate development of red color would be formed but in this current experiment no such red color was formed rather a blackish green color was obtained which indicated the absence of flavonoid in the test sample.
- In order to perform this test, 0.5mL of the methanolic extract of the plant *Lindenbergia indica* was taken in a test tube and with it, a tiny amount of zinc and 8 drops of concentrated hydrochloric acid were added. Then the solution was boiled using a steam bath for about 5 minutes. In this experiment, formation of orange or red color points to the presence of flavones, red to crimson color is indicative of the presence of flavanols, crimson to magenta color specifies the presence of flavones and a crimson to green or blue color is indicative of the presence of flavonoids. But in this current experiment, no such previously mentioned color was formed; rather an ash color on the upper portion of the mixture and beneath it a brown color was obtained which pointed to the absence of flavonoids in the plant extract.

2.1.6.2.6 Test for Steroids:

- **Liebermann Burchard's Test:**

A small amount of methanolic extract (instead of petroleum ether extract, methanolic extract was used) of the plant *Lindenbergia indica* was dissolved in 1mL of chloroform in a test tube. Additionally, 2mL of acetic anhydride as well as 1mL of concentrated sulphuric acid was added to the mixture. Immediately, a greenish color (Figure 2.9) was formed which turned into blue on standing indicating the presence of steroid in the test sample.

Figure 2.9: Liebermann Burchard's Test for steroids

2.1.6.2.7 Test for Tannins:

- **Ferric Chloride Test:**

Initially, 0.5 gm of methanolic extract was dissolved in 10 mL of distilled water and the solution was filtered using a filter paper. After that, a few drops of 5% ferric chloride solution was added to the filtrate. If a blue, blue-black, blue-green or green color or a precipitation would produce, it would be an indicative of tannins; and in the current experiment a blue-green color was obtained which pointed to the presence of tannins in the plant extract. Furthermore, on addition of a few milliliter of dilute sulphuric acid the color vanished; subsequently a yellowish brown precipitate was formed.

- **Lead Acetate Test:**

At first, 5mL of an aqueous extract of the plant was taken in a test tube and then a few drops of 1% solution of lead acetate was added .The production of a yellow or red precipitate is an indicative of the presence of tannin in the test sample; and in this present experiment a yellow color was obtained which pointed to the presence of tannin in the test sample.

2.1.6.2.8 Test for Resins:

In order to perform this test, a small amount of methanolic extract was dissolved in 5mL of acetic anhydride using a gentle heat. The solution was then cooled and 0.05mL of sulphuric acid was added to it. If resins were present, a bright purplish red color would produce which would rapidly change into violet color. But in the current study, no change of color was obtained which indicated the absence of resins in the plant extract.

2.1.6.2.9 Test for Alkaloids:

- **General Laboratory Test for Alkaloid:**

Approximately 0.5gm of the extract was stirred with 5mL of 1% hydrochloric acid on a steam bath and then this mixture was filtered using a filter paper. After that the filtrate was treated with a few drops of each of the following reagents separately. Turbidity or the production of respective colors indicated presence of alkaloids in the plant extract:

- **Mayer's Reagent:**

The filtrate was treated with few drops of Mayer's Reagent (potassio-mercuric iodide solution). If a white or creamy white precipitate was produced it was an indicative to the presence of alkaloids in the test sample, but in this present experiment no change of color in the solution was found which pointed to the absence of alkaloids in the plant extract.

- **Hager's Reagent:**

The filtrate was treated with few drops of 1% solution of picric acid (Hager's Reagent). If a yellow crystalline precipitate was produced it would pointed to the presence of alkaloids in the test sample, but in this current study no change of color in the solution was found which pointed to the absence of alkaloids in the extract material.

- **Dragendroff's Reagent:**

The filtrate was treated with few drops of bismuth potassium iodide solution (Dragendroff's solution). If an orange or orange-red precipitate was created it would pointed to the presence of alkaloids in the plant extract, but in this current study no change of color in the solution was found which indicated to the absence of alkaloids in the sample extract.

2.2 Evaluation of Antioxidant Activity

2.2.1 Introduction:

Antioxidants are the substances which have the ability to prevent oxidative damage occurring due to the effect of free radicals. Antioxidants function by interfering with the oxidation process which is achieved by their reaction with free radicals, chelating metals and working as scavengers as well. Some recent scientific studies have revealed that antioxidants have a great potential for the treatment of various diseases such as diabetes mellitus, atherosclerosis, cancer, aging process, neurodegenerative diseases etc. There are some commercially available synthetic antioxidants such as BHA (Butyl Hydroxy Anisole), BHT (Butyl Hydroxy Toluene) etc. but are they are detrimental to human health due to their toxicity. Antioxidants which are derived from plant source for example vitamin C, vitamin E, phenolic acids, carotenes etc. have the potential to decrease the risk of diseases. Moreover, most of the compounds having antioxidant property in diet are obtained from plant source which are the member of different classes of substances having a wide range of physical as well as chemical properties (Shekhar & Anju, 2014). So, plants have an immense potential for being used as a source of naturally found antioxidants which can be utilized in the treatment of a variety of chronic diseases as well as for the treatment of cancer, which has turned into an enormous concern worldwide (Shekhar & Anju, 2014).

The purpose of the current study was to evaluate the methanolic extract of the leaf of the plant *Lindenbergia indica* in order to identify the antioxidant content in it and to establish this plant as a new potential source of naturally found antioxidant.

2.2.2 Principle:

Evaluation of antioxidant activity by DPPH method by Brand-Williams *et al.*, (1995) is basically based on the estimation of the free radical scavenging capacity of the plant extracts on the free radical 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH).

According to DPPH method of evaluation of antioxidant activity, 1mL of methanolic solution of the plant extract at various concentrations is mixed with 3mL of methanolic solution of DPPH. Ascorbic Acid or Butyl-1-Hydroxy Toluene (BHT) in a concentration

within 1-100 µg/mL is used as standard and blank sample is also prepared. Then after keeping the standard and the sample in dark place for 30 minutes, the antioxidant activity of the plant extract is evaluated from the lightening of the purple color of the methanolic solution of DPPH free radical by the extract of the plant, compared to that of Ascorbic Acid or Butyl-1-Hydroxy Toluene (BHT) with the help of analyzing the absorbance by UV-Spectrophotometer at the wavelength of 517nm.

After that, the inhibition % is calculated by means of the following formula:

Inhibition % = [(Absorbance$_{Control}$ - Absorbance$_{Sample}$) / Absorbance$_{Control}$] × 100.

Basically, the DPPH assay is founded on the reduction of the free radical DPPH. This free radical DPPH, having an odd electron shows a highest absorption at 517nm.After reacting with the stable free radical DPPH, antioxidants get paired off in the participation of a hydrogen donor and consequently, get reduced to DPPHH, which results in the decrease of the absorbance from that of DPP-H. The radical that is in the DPPH-H form shows yellow color as a consequence of decolorization, with regard to the total number of electrons caught. Likewise, an increase in decolorization will indicate the more reducing capacity. Evaluation of antioxidant activity by DPPH assay has been the most accepted as well as used method for assessing the free radical scavenging action of any potential and new drug (Brand-Williams *et al*., 1995).

2.2.3 Materials:

Table 2.2: List of materials:

2,2-Diphenyl-1-Picrylhydrazyl (DPPH)	Pipette (1mL and 5mL)
Ascorbic Acid	Test tubes
Methanolic extract of the plant *Lindenbergia indica*	Light proof box
Methanol	UV-spectrophotometer
Distilled water	Volumetric flask (25mL)

2.2.4 Preparation of Control for the Evaluation of Antioxidant Activity:

In order to perform this method, ascorbic acid was used as positive control. About 0.005g of Ascorbic Acid was dissolved in 1mL of methanol to have the desired concentration of 5000 µg/mL. Then the mother solution was serially diluted to obtain various concentrations ranging from 1250 µg/mL to 78.125 µg/mL.

2.2.5 Preparation of Test Sample:

For the preparation of test sample, 0.005gm of methanolic extract of the plant was weighed. Then this calculated amount of plant extract was dissolved in 1 mL of methanol in order to obtain the desired concentration of 5000 µg/mL. After that, this mother solution was serially diluted to obtain various concentrations ranging from 1250 µg/mL to 78.125 µg/mL.

2.2.6 Preparation of DPPH Solution:

In order to prepare the DPPH solution, 2 mg of DPPH was weighed accurately and then dissolved in 50mL of methanol to obtain a concentration of 40µg/mL. This solution was then covered with aluminum foil and kept in dark place.

2.2.7 Assay for Free Radical Scavenging Activity:

Now, 2.0mL were taken from each of the methanolic solution of the sample (control) having various concentrations, ranging from 1250 µg/mL to 78.125 µg/mL and each of these was mixed with 3.0mL of the DPPH methanol solution (40 µg/mL). After that, all the samples were kept in the dark place for a reaction period of about 30 minutes at room temperature. Then, using the UV-spectrophotometer, the absorbance of the sample was measured at 517 nm against methanol which was used as blank.

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

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

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

2.3 Brine Shrimp Lethality Bioassay

2.3.1 Introduction:

Brine Shrimp Lethality Test is carried out in order to assess the cytotoxicity level of plants. This test is a very helpful instrument for the preliminary evaluation of toxicity of medicinal plants. This bioassay can detect a broad spectrum of bioactivity, which is present in crude plant extracts. The purpose of this scheme is to render a front-line screening method which can act as a support for more precise as well more costly bioassays. Brine Shrimp Lethality Test has various advantages for example it is cheap, easy to perform and requires small amount of test material (Meyer *et al*., 1982).

In this existing investigation, Brine Shrimp Lethality Test has been performed in order to evaluate the level of cytotoxicity of the plant *Lindenbergia indica*. This bioassay will operate as a basis to quantify the level of toxicity, safety as well as to evaluate the potential of the plant *Lindenbergia indica* to be used as a new drug.

2.3.2 Principle:

In order to perform the brine shrimp bioassay method, brine shrimp eggs (*Artemia salina* leach) are hatched in simulated seawater to obtain nauplii. After that, a calculated amount of DMSO (Dimethyl Sulphoxide) is added in order to prepare preferred concentration of the sample under test (Meyer *et al*., 1982).

The tank is kept for about 48 hours with a constant supply of oxygen and light. When the hatching is achieved, nauplii were counted by means of visual inspection using magnifying glass and are then taken in test tubes containing 5mL of simulated seawater. Consequently, samples of various concentrations are added to previously marked test tubes using micropipette. After that, these test tubes are left for 24 hours and then total number of survived nauplii was counted after 24 hours (Meyer *et al*., 1982).

2.3.3 Materials:

Table 2.3: Test materials of experimental plant:

4.0 mg of test sample of the experimental plant *Lindenbergia indica*	Test tubes
Brine Shrimp Eggs (*Artemia salina* leach)	Micropipette (0.1 mL) and Pipette (5mL)
Sodium Chloride (NaCl) salt	Magnifying glass
Small tank for the hatching of eggs	Lamp to attract brine shrimps
Distilled water	Beaker (10mL)

2.3.4 Preparation of Seawater:

At first, 76.0 gm of pure Sodium Chloride (NaCl) was weighed using balance machine and then mixed with 2.0 liter of distilled water. Then the mixture was stirred vigorously to dissolve the salt into the distilled water. After that this solution was filtered using cotton in order to acquire a clear solution. Then this seawater was poured into the small tank.

2.3.5 Hatching of Brine Shrimp:

Initially, brine shrimp eggs (*Artemia salina* leach) were collected from pet shops. After that, a very small amount (about 4-5 mg) of these brine shrimp eggs was added to the seawater containing tank. Subsequently, constant oxygen supply was generated to the tank containing brine shrimp eggs using an oxygen pump. A lamp was used to generate the light of 25W was used to initiate hatching (Figure 2.10). Then the tank was kept for 48 hours with a constant supply of oxygen as well as light. After 48 hours eggs were hatched and naupliis were visible in the tank. After that, 10 living nauplii were added to all of the 10 test tubes having 5mL of seawater.

Figure 2.10: Hatching of brine shrimp

2.3.6 Preparation of Test Samples of the Experimental Plant:

In order to prepare the stock solution, 4.0 mg of the methanolic extract of the plant *Lindenbergia indica* was dissolved in 200µL of pure DMSO (Dimethyl Sulfoxide).At first,10 test tubes were filled with 5mL of simulated seawater, containing 10 brine shrimp nauplii. Then, in order to make a concentration of 400 µg/mL, in the first test tube 100µL of the stock solution was taken which possessed previously contained 5mL of simulated sea water with 10 nauplii. Subsequently, a series of solutions of different concentrations ranging from 200µg/mL to 0.781µg/mL were prepared by means of serial dilution method from the stock solution. In addition, in every case, 100µL of sample solution was added to each of the test tube and fresh DMSO was added to the beaker containing the solution of extract and DMSO. As a consequence, various concentration ranging from 400 µg/mL to 0.781 µg/mL were obtained in various test tubes (Table 2.4).

Table 2.4: Test samples with different concentration values after serial dilution:

No. of Test Tube	Concentration (µg/mL)
1	400
2	200
3	100
4	50

5	25
6	12.5
7	6.25
8	3.125
9	1.563
10	0.781

2.3.7 Preparation of Control Group:

In order to validate the test method, control groups are applied in cytotoxicity study. Therefore, control groups ensure that the results which have been obtained only due to the action of test sample as the effect of other factors are abolished. Normally, following two types of control groups are used in cytotoxicity studies:

- Positive control
- Negative control

2.3.8 Preparation of the Positive Control Group:

The result obtained from the test sample is compared against the result which has been acquired by assessing the positive control. In this study, vincristine sulphate has been used as a positive control. An accurately measured amount of vincristine sulphate was dissolved in DMSO in order to obtain an initial concentration of 20 µg/mL and from this, serial dilution was carried out using DMSO in order to obtain various concentrations ranging from 10 µg/mL, 5 µg/mL, 2.5 µg/mL, 1.25 µg/mL, 0.625 µg/mL, 0.3125 µg/mL, 0.156 µg/mL, 0.078 µg/mL and 0.0390 µg/mL. After that, in order to have the positive control group, the positive control solutions were poured into the previously marked test tubes that contained 5mL of simulated seawater with 10 living nauplii.

2.3.9 Preparation of the Negative Control Group:

In order to prepare the negative control group, 100 µL of DMSO was added to each of the three test tubes containing 5mL of simulated seawater with 10 living nauplii in each. This

negative control group is used to indicate that if the nauplii in these test tubes demonstrate a swift mortality rate, then this assay will be assumed as invalid since the nauplii died due to the reason except for the cytotoxicity of the sample under test.

2.3.10 Counting of the Nauplii:

After 48 hours all the samples were examined and the total number of survived nauplii was counted using a magnifying glass. Then the % (percent) mortality for each dilution was calculated. The LC_{50} value was also determined which represent the concentration of the chemical which causes death in half of the subjects under test after passing a certain exposure period.

2.4 Evaluation of Antimicrobial Activity

2.4.1 Introduction:

Before the preamble of antimicrobial agents and other new drugs, natural compounds have been consumed as traditional medicine for thousands of years throughout the world. Almost 50% of modern drugs are derived either from natural sources and these naturally obtained drugs play a very significant role in the development of drugs in the sector of pharmaceutical industry (Thenmozhi *et al.*, 2013).

The antimicrobial screening test evaluates the capability of every test sample to restrain the *in vitro* bacterial as well as fungal growth. This evaluation can be performed by any one of the following three methods (Rahman, 2012):

i) Serial dilution method
ii) Disc diffusion method
iii) Bioautographic method

In addition, amongst all of the above mentioned three methods, disc diffusion is extensively used and one of the most accepted methods (Bayer *et al.*, 1966) since it is both qualitative as well as quantitative test that indicates the resistance and sensitivity of the microorganisms to the test sample.

The purpose of this investigation is to evaluate the antimicrobial activity of the methanolic extract of the plant *Lindenbergia indica* by Disc Diffusion Method in order to identify the antimicrobial activity of it.

2.4.2 Principle of Disc Diffusion Method:

This method involves the diffusion of antibiotics from a controlled source through the nutrient agar gel and then the formation of a concentrated gradient. In order to perform this method, a filter paper disc of 6mm diameter containing the test sample of known amounts is at first dried. After that, it was sterilized and finally placed on nutrient agar medium, which was homogeneously seeded with the test microorganisms. Standard antibiotic discs of Ciprofloxacin and blank discs are used a positive control and negative control respectively.

Then these plates are held at a low temperature of 4° C for a time period of 24 hours in order to permit the utmost diffusion of the test samples to the surrounding media (Barry, 1976). Consequently, the plates carrying the media are inverted and then incubated at 37°C for a time period of 24 hours in order to allow the optimum growth of the microorganisms. After that, it is observed that the test samples which have the antimicrobial property hinder microbial growth surrounding the discs in the media and thus results in a clear and separate area which is termed as zone of inhibition. Accordingly, the antimicrobial action of the test material is evaluated by calculating the diameter of zone of inhibition which is termed in millimeter (mm).

2.4.3 Apparatus and Reagents:

Table 2.5: Apparatus and reagents:

Petri dish	Test tube	Filter paper disc
Laminar air flow hood	Micropipette	Sterile cotton
Nutrient agar media	Distilled water	Sterile forceps
Refrigerator	Spirit burner	Screw cap test tubes
Methanol	Autoclave	Conical flask
Nose mask and hand gloves	Incubator	Inoculating loop

2.4.4 Test Organisms:

The test organisms, i.e. bacterial and fungal strains that were used for this experiment were collected as the form of pure cultures from the Institute of Nutrition and Food Science, University of Dhaka. In order to perform this test, both gram positive as well as gram negative microorganisms were collected, which are listed below (Table 2.6):

Table 2.6: List of bacteria and fungi:

Gram Positive Bacteria	Gram Negative Bacteria	Fungi
Bacillus cereus	*Escherichia coli*	*Candida albicans*
Bacillus megaterium	*Pseudomonas aeruginosa*	*Saccharomyces cerevisiae*
Bacillus subtilis	*Salmonella typhi*	*Aspergillus niger*
Staphylococcus aureus	*Shigella boydii*	
Sarcina lutea	*Shigella dysenteriae*	
	Vibrio mimicus	
	Vibrio parahaemolyticus	
	Salmonella paratyphi	

2.4.5 Culture Medium and Its Composition:

In this study, in order to prepare the fresh culture, nutrient agar medium was used which is the most commonly used culture medium. Composition of the nutrient agar medium is given in the following Table 2.7:

Table 2.7: Composition of nutrient agar medium:

Ingredients	Amount
Sodium chloride	0.5 gm
Bacto peptone	0.5 gm
Bacto yeast extract	1.0 gm

Distilled water	100 mL
Bacto agar	2.0 gm
pH	7.2+ 0.1, at 25°

2.4.6 Preparation of the Test Medium:

In order to prepare the required volume of the medium, calculated amount of the methanolic extract of the plant *Lindenbergia indica* was taken into a conical flask and then distilled water was added to it to prepare the desired volume. After that, the contents were heated in a water bath in order to obtain a clear solution. Accordingly, at 25°C, the pH was adjusted within 7.2-7.6 using Sodium Hydroxide (NaOH) or Hydrochloric Acid (HCl). Then, in order to prepare plates and slants, 10mL and 5mL of the medium were transferred respectively into screw cap test tubes. Consequently, the test tubes were capped and then sterilized by means of autoclaving at 15-lbs pressure for 20 minutes at 121° C temperature. In order to prepare fresh culture of bacteria and fungi, the slants were used.

2.4.7 Sterilization Procedure:

Laminar Hood and all other types of safety measures were maintained to evade any contamination as well as cross contamination by the test microorganisms. Ultra-Violet (UV) light was switched on before using the Laminar Hood. Moreover, petri dishes as well as other glassware were sterilized by autoclave at 121°C for 20 minutes at a pressure of 15-lbs./square inch. Additionally, micropipette tips, blank discs, forceps and cotton were sterilized as well.

2.4.8 Preparation of Bacterial Subculture:

To prepare the bacterial subculture, 2.4gm of agar media was dissolved in 100 mL of distilled water. Consequently 5mL of the solution was taken into a screw cap test tube and was solidified at normal temperature. Then after solidification, bacteria were added to the media. After that, inoculated strains were incubated for a day (24 hours) at 37°C for the

optimum growth of microorganisms. Then the fresh cultures were used to perform the sensitivity test.

2.4.9 Preparation of the Test Plate:

The test microorganisms were shifted from the subculture to the test tubes which contained 10mL of melted as well as sterilized agar medium by means of a sterilized transfer loop and this process was done in an aseptic area. In order to obtain a uniform suspension of the microorganisms, the test tubes were shaken. Consequently, the suspension of the bacteria was shifted to the sterilized Petri dishes. After that, the Petri dishes were rotated clockwise as well as anticlockwise for a number of times in order to confirm homogenous dispersion of the test microorganisms.

Figure 2.11A: Freshly prepared culture medium

Figure 2.11B: Pouring of culture medium to the Petri dishes

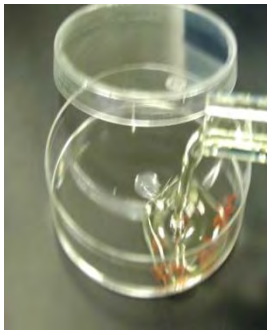

Figure 2.11C: Pouring of culture medium to the petri dishes

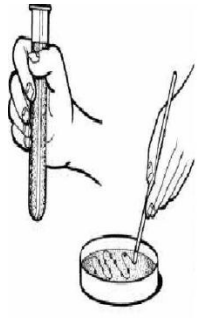

Figure 2.11D: Transfer of bacterial and fungal suspension to petri dishes

Figure 2.11: Preparation of the test plates

2.4.10 Standard Discs:

With a view to assure the activity of standard antibiotic against the test microorganisms and to compare the response which was generated by the known antimicrobial agent with the antimicrobial activity produced by that of the test sample, standard disks were used as a

means of positive control groups. In order to perform this study, Ciprofloxacin standard disc in an amount of 5µg/disc was used as the reference antimicrobial agent.

2.4.11 Blank Discs:

Here, blank discs were used as negative controls which assured that the residual solvents were found to be left over the discs although air-drying was achieved and also the filter papers were not activated.

2.4.12 Preparation of Sample Disc with Test Sample:

To prepare the sample disc with test sample, 8.0 mg of the sample was taken and then dissolved in 200 µL of methanol. After that 10.0 µl of this was taken in per filter paper disc whose concentration was 400 µg.

Table 2.8: Preparation of sample disc with test sample:

Plant Part	Test Sample	Dose µg/disc	Required Amount for Each Disc (mg)
Leaves and Branches of the plant *Lindenbergia indica*	Methanolic extract of the leaves and branches of *Lindenbergia indica*	400	8.00

2.4.13 Diffusion and Incubation:

At first, 15mL of prepared agar media was taken in each test tube and bacteria were then transferred to it, after which they were allowed for inoculation. After that, the media containing bacteria was transferred to the Petri dish and was solidified at room temperature for half an hour. After solidification, the sample disc was transferred to the Petri dish at

definitely marked point. Consequently, the petri dishes were collected from the refrigerator and were kept at 37°C for 24 hours.

2.4.14 Determination of Antimicrobial Activity by Measuring the Zone of Inhibition:

The antimicrobial effectiveness of the test sample was measured by their action in order to avoid the growth of the microorganisms which surrounded the discs and gave distinct zone of inhibition. Then, when incubation was completed the antimicrobial actions of the test samples were evaluated by measuring the width of the zones of inhibition in millimeter (mm).

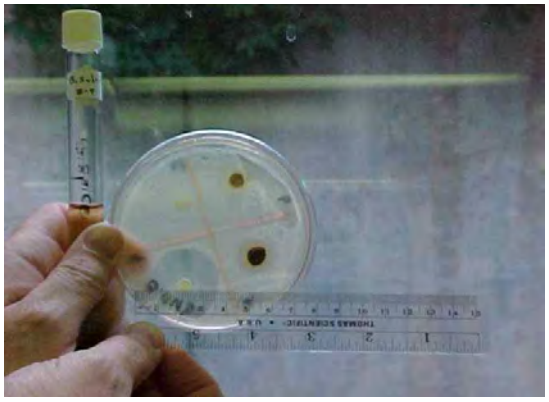

Figure 2.12A: Determination of clear zone of inhibition

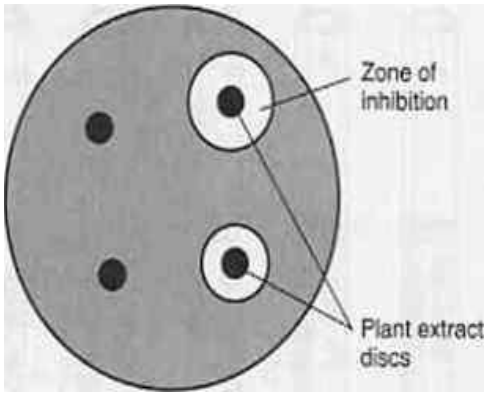

Figure 2.12B: Clear zone of inhibition

Figure 2.12: Determination of zone of inhibition.

Chapter 3

Results

3.1 Phytochemical Screening of *Lindenbergia indica*

Following is a table (Table 3.1), showing the result of phytochemical screening of the methanolic extract of the leaves and branches of the plant *Lindenbergia indica*:

Table 3.1: Result of phytochemical screening of the plant *Lindenbergia indica*:

Tests	**Methanolic Extract of the Leaf and Branch**
Carbohydrate	+
Proteins	-
Glycosides	+
Glucosides	+
Saponins	+
Flavonoids	-
Steroids	+
Tannins	+
Resins	-
Alkaloids	-

(+) Signifies presence of the compound

(-) Signifies the absence of the compound

3.2 Evaluation of Antioxidant Activity

The result of evaluation of antioxidant activity of the methanolic extract of the leaves and branches of the plant *Lindenbergia indica* is given below:

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

Absorbance of the Blank	**Concentration of the Standard (µg/mL)**	**Absorbance of the Standard (nm)**	**% Inhibition**	**IC_{50} (µg/mL)**
0.855	78.125	0.032	96.26	3.40
	156.25	0.027	96.84	
	312.5	0.025	97.08	
	625	0.022	97.43	
	1250	0.020	97.66	

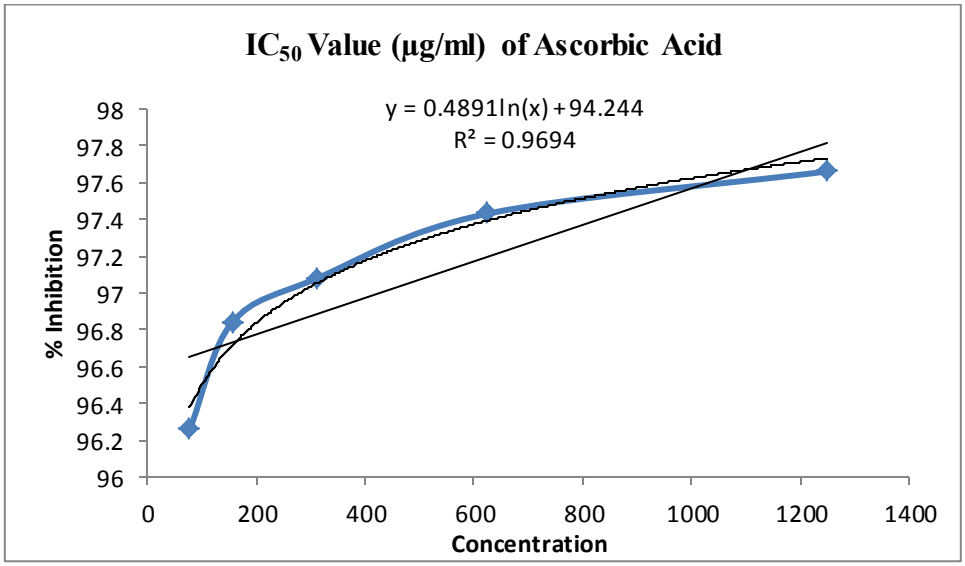

Figure 3.1: IC_{50} value (µg/mL) of ascorbic acid

Table 3.3: DPPH free radical scavenging activity of crude methanolic extract of the plant *Lindenbergia indica* :

Absorbance of the Blank	**Concentration of the Extract (µg/mL)**	**Absorbance of the Extract (nm)**	**% Inhibition**	**IC_{50} (µg/mL)**
	78.125	0.842	89.82	
	156.25	0.546	83.63	
0.855	312.5	0.178	79.18	243.484
	625	0.140	36.14	
	1250	0.087	1.52	

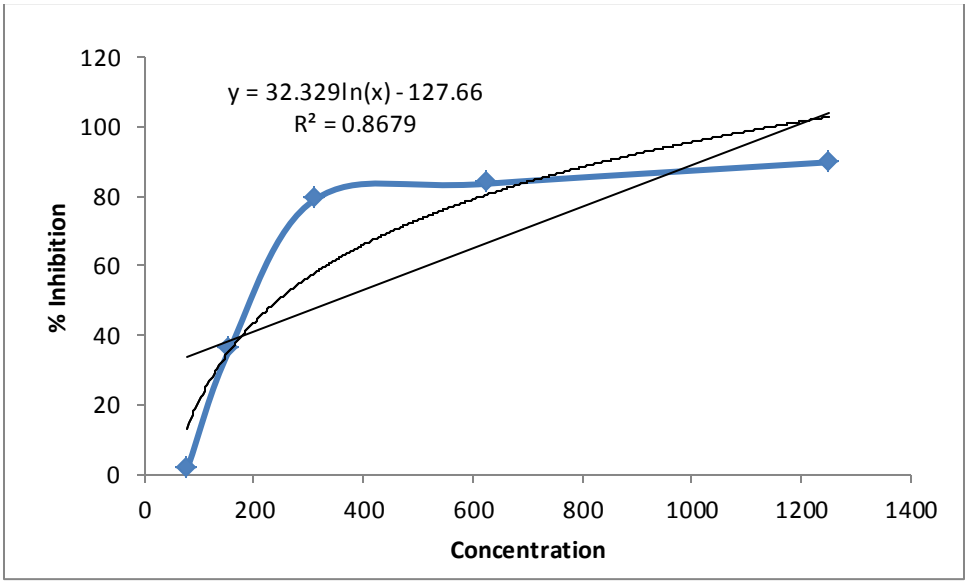

Figure 3.2: IC_{50} value of methanolic extract of the plant *Lindenbergia indica*

3.3 Brine Shrimp Lethality Bioassay

The result of brine shrimp lethality bioassay of methanolic extract of the leaves and branches of the plant *Lindenbergia indica* is given below:

Table 3.4: Effect of vincristine sulphate on brine shrimp nauplii:

Concentration (µg/mL)	**Log_{10} Concentration**	**% Mortality**	**Regression Equation**	**R^2 Value**	**LC_{50} Value (µg/mL)**
0.0390	-1.4089	20	Y=30.80X + 60.65	0.973	0.451
0.07816	-1.1072	30			
0.15625	-0.8061	30			
0.3125	-0.5051	40			
0.625	-0.2041	50			
1.25	0.0969	70			
2.5	0.3979	80			
5	0.6980	80			
10	1	90			
20	1.3010	100			

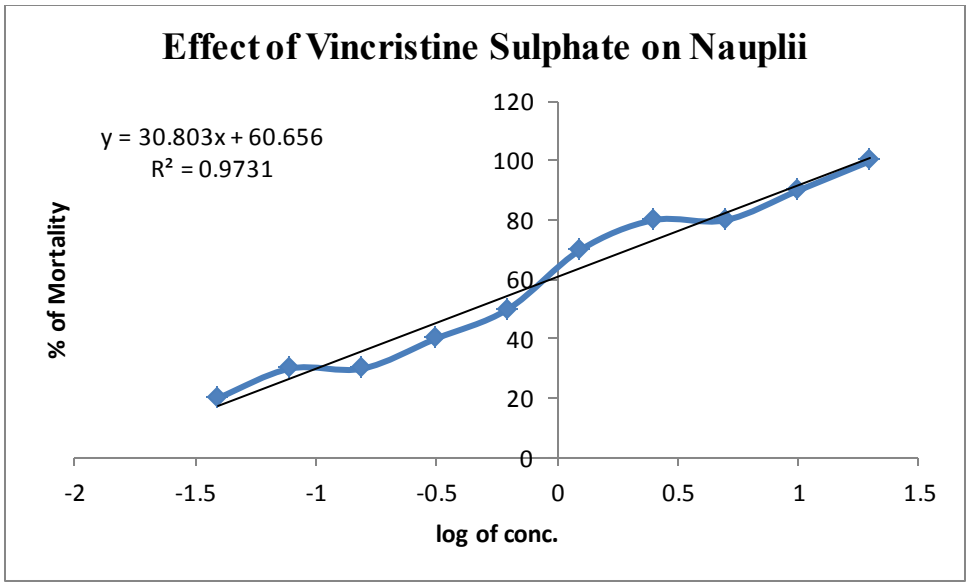

Figure 3.3: Effect of vincristine sulphate on brine shrimp nauplii.

Table 3.5: Effect of methanolic extract of the plant *Lindenbergia indica* on brine shrimp nauplii:

Concentration (µg/mL)	**Log_{10} concentration**	**Total no. of nauplii taken**	**Total No. of dead nauplii**	**% of Mortality**	**Regression Equation**	**R^2 Value**	**LC_{50} Value (mg/mL)**
0.7813	-0.10721	10	1	10	Y= 6.039X + 14.46	0.757	767.503
1.5625	0.19382	10	2	20			
3.125	0.49485	10	2	20			
6.25	0.79588	10	2	20			
12.50	1.09691	10	2	20			
25	1.39794	10	2	20			
50	1.69897	10	2	20			

100	2.0	10	3	30			
200	2.30103	10	3	30			
400	2.60202	10	3	30			

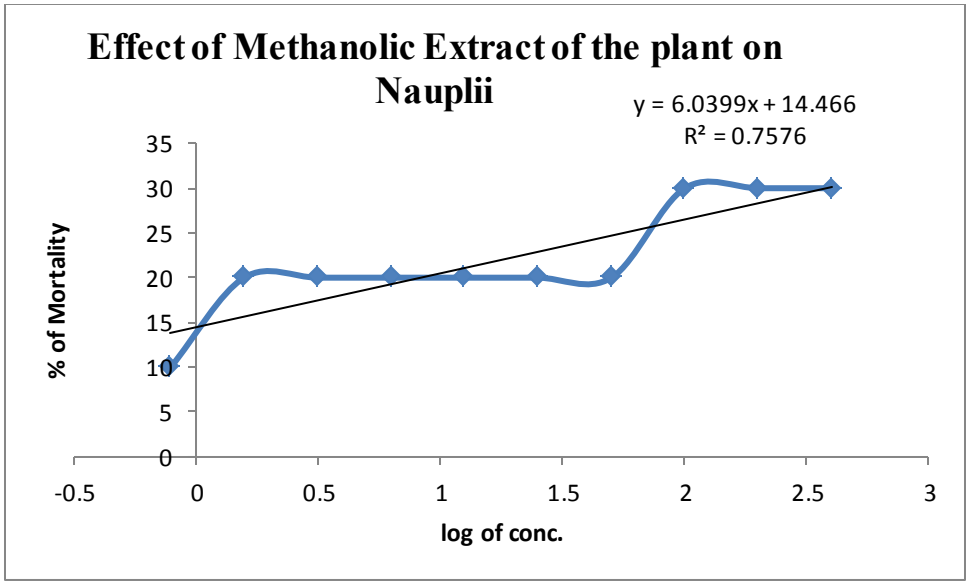

Figure 3.4: Effect of methanolic extract of the plant *Lindenbergia indica* on brine shrimp nauplii.

3.4 Antimicrobial Activity Test

The result of antimicrobial activity test of methanolic extract of the leaves and branches of the plant *Lindenbergia indica* is given below:

Table 3.6: Antimicrobial Activity of Test Sample of *Lindenbergia indica*:

Gram Positive Bacteria	**Diameter of Zone of Inhibition for the Methanolic Extract (mm)**	**Diameter of Zone of Inhibition for Ciprofloxacin (mm)**
Bacillus cereus	8	40
Bacillus megaterium	8	40
Bacillus subtilis	8	40
Staphylococcus aureus	8	40
Sarcina lutea	8	40
Gram Negative Bacteria		
Escherichia coli	9	41
Pseudomonas aeruginosa	9	41
Salmonella paratyphi	9	41
Salmonella typhi	9	42
Shigella boydii	9	42
Shigella dysenteriae	9	42
Vibrio mimicus	8.5	42
Vibrio parahaemolyticus	8.5	42
Fungi		

Candida albicans	8	41
Saccharomyces cerevisiae	7.5	41
Aspergillus niger	7.5	41

Chapter 4

Discussion

4.1 Phytochemical Screening of *Lindenbergia indica*

After performing the phytochemical screening tests of the methanolic extract of the leaves and branches of plant *Lindenbergia indica*, presence of some bioactive compounds such as carbohydrates, glycosides, glucosides, saponins, steroids and tannins have been identified from the extract of leaves and branches of this plant. But after performing the relevant tests absence of proteins, flavonoids, resins and alkaloids have been identified. Since the presence of some significant chemical compounds like carbohydrates, glycosides, glucosides, saponins, steroids and tannins have been identified from this plant extract, so further investigation on this medicinal plant *Lindenbergia indica* may be valuable to identify the presence of some other new important chemical constituents in it which may help to establish this plant as a new medicine.

4.2 Evaluation of Antioxidant Activity

In this experiment, ascorbic acid was used as a reference standard. After performing the experiment of evaluation of antioxidant activity of methanolic extract of the leaves and branches of the plant *Lindenbergia indica*, IC_{50} value of the plant extract was compared to that of the IC_{50} value of ascorboic acid (positive control) in order to evaluate the free radical scavenging activity of the test sample. In this investigation, it has been obtained that the methanolic extract of the plant *Lindenbergia indica* gave an IC_{50} value of 243.484µg/mL while the positive standard ascorbic acid showed an IC_{50} value of 3.40µg/ml. So, from the comparison of the IC_{50} value of the plant extract with Ascorbic Acid, it can be suggested that the methanolic extract of the leaves and branches of the plant *Lindenbergia indica* has a moderate antioxidant activity and further research on this plant may work as a basis to introduce the medicinal plant *Lindenbergia indica* as a new source of naturally occurred antioxidant.

4.3 Brine Shrimp Lethality Bioassay

After performing the study of Brine Shrimp Lethality Bioassay of the methanolic extract of the plant *Lindenbergia indica*, the LC_{50} (lethal concentration) value of the investigated sample after passing 24 hours was determined by means of creating a plot of percentage of the died (% of Mortality) brine shrimp (nauplii) against the logarithm of the concentration of the test sample. After that, with the help of "Regression Analysis", the best fitted line has been found from the data which were given in the curve. In this investigation, an anticancer drug vincristine sulphate has been used as a positive control that showed the LC_{50} value of 0.451μg/mL. On the other hand, methanolic extract of the leaves and branches of the plant *Lindenbergia indica* gave the LC_{50} value of 767.503 mg/mL. Finally, from the comparison of the LC_{50} value of the methanolic extract of the plant *Lindenbergia indica* with the LC_{50} value of the reference standard vincristine sulphate, it can be proposed that this test plant *Lindenbergia indica* has no considerable cytotoxicity. Since this medicinal plant has no significant cytotoxicity, more investigation on this plant may be effective to establish it as a new medicine.

4.4 Evaluation of Antimicrobial Activity

After performing the microbiological investigation of the methanolic extract of the plant *Lindenbergia indica* it has been obtained that this extract showed moderate activity (zone of inhibition within 8mm) against Gram Positive bacteria i.e. *Bacillus cereus* , *Bacillus megaterium*, *Bacillus subtilis*, *Staphylococcus aureus* and *Sarcina lutea*. In addition, this methanolic extract was moderately active (zone of inhibition within 8.5- 9mm) against Gram Negative bacteria i.e. *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella paratyphi*, *Salmonella typhi*, *Shigella boydii*, *Shigella dysenteriae*, *Vibrio mimicus* and *Vibrio parahaemolyticus.* The methanolic extract of the plant *Lindenbergia indica* also showed a moderate activity (zone of inhibition between 7.5mm-8mm) against some fungi i.e. *Candida albicans*, *Saccharomyces cerevisiae* and *Aspergillus niger*. Thus, further research on the plant *Lindenbergia indica* may function as a basis to introduce this medicinal plant as a new antimicrobial agent.

Conclusion

The methanolic extract of the leaf and branch of the plant *Lindenbergia indica* (Scrophulariaceae family) has been studied for the evaluation of phytochemical as well as its biological profiles.

After performing the phytochemical investigation of the plant *Lindenbergia indica* it has been found that this plant contained some of the significant bioactive substances such as carbohydrates, tannins, saponins, glycosides and glucosides. From the biological investigation, it has been found that this plant extract showed a negative response in the brine shrimp lethality bioassay having LC_{50} value of 767.503 mg/ml. On the other hand, this plant extract demonstrated a moderate antioxidant activity with an IC_{50} value of 243.484μg/ml and in antimicrobial activity test, this extract showed a moderate level of antimicrobial activity with a zone of inhibition within 7.5mm -9mm.

Accordingly, the current study has demonstrated a potential bioactivity of the plant *Lindenbergia indica* offering further detailed investigations which will assist to invent the unfamiliar effectiveness of this plant, as well as to introduce it as a potential new medicine.

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