Investigation of *in-vitro* antioxidant and cytotoxic potential of methanol extract of *Begonia roxburghii* leaves

A project submitted

by

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This work is dedicated to my parents and siblings for their love and constant support…

Certification statement

This is to certify that this project titled "Investigation of *in-vitro* antioxidant and cytotoxic potential of methanol extract of *Begonia roxburghii* leaves" submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (Hons.) from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Dr. Raushanara Akter, Assistant Professor, Department of Pharmacy, BRAC University and that appropriate credit is given where I have used the language, ideas or writings of another.

Signed,

_____

Countersigned by the supervisor,

_____

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Abstract

Begonia roxburghii (B. roxburghii) is a medicinal plant that belongs to Begoniaceae family which is commonly known as East Himalayan Begonia. Literature review revealed that, the leaves of this plant have been widely used as drug or component of drug to cure various gastrointestinal disorders like diarrhea, bile dysentery, stomach ache and skin diseases. Literature data also revealed that, the leaf extract possess therapeutic effects such as antimicrobial activity in order to prevent the growth of harmful microorganisms as well as antioxidant and cytotoxic potential in order to suppress the growth of cancer cells in human body. The current study was aimed to investigate the *in-vitro* antioxidant and cytotoxic potential of methanol extract of *B. roxburghii* by standard methods that had not been previously conducted. In this study, the methanolic extract of the leaf of *B. roxburghii* was screened for the identification of certain phytocompunds named as tannins, alkaloids, flavonoids, glycosides, resins, steroids, carbohydrates and phenols which provide definite pharmacological actions on human body. In this study, four methods were used for instance, DPPH free radical scavenging assay, total phenolic content, total flavonoid content and total antioxidant capacity for the determination of *in-vitro* antioxidant potential. With the increase in concentration, the % of inhibition of DPPH, total phenolic content, total flavonoid content and total antioxidant capacity also increased. In case of DPPH free radical scavenging assay, the highest % of inhibition, 89.92 % was obtained at the highest concentration of 1200 µg/mL. At the highest concentration of 1200 µg/mL the leaf extract showed remarkable value of total phenolic content and antioxidant capacity which was expressed as 100.58 mg of gallic acid per gram of dried extract and 98.58 mg of ascorbic acid per gram of dried extract respectively. Along with that, *in-vitro* cytotoxic potential of the leaf extract was also assessed by MTT assay at different concentrations of (25 mg/mL, 2.5 mg/mL, 0.25 mg/mL, 0.025 mg/mL) of *B. roxburghii* leaf extract against HeLa cell line (Cervical cancer cell line). The cytotoxicity test revealed that, at the highest concentration of 25 mg/mL, methanolic extract of *B. roxburghii* leaves showed 80 % of the cell death whereas HeLa cell survival was only 20 % at that concentration. Therefore, antioxidant potential was significant in three methods whereas cytotoxic potential was not that much significant. However, further investigation can also assist to invent unfamiliar properties and unexplored pharmacological activities of this plant as well as to familiarize the plant as a new source of medicine.

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CHAPTER: 1

INTRODUCTION

Chapter 1: Introduction

From the very beginning of their existence, human beings are relying upon natural resources in order to make their life comfortable. Human beings are subjected to the nature's elements for their endurance and in addition for the treatment of different diseases. From the ancient time, an enormous number of different diseases were generally treated with agents that are collected from various parts of plants and trees and these agents are known as "Herbal medicine'' which is considered to be the most effective source of medicine. However, the success of herbal medicine in the treatment of various ailments is affluent as well as very much effective.

The utilization of herbal medicines is essential nowadays as it involves the use of the advance technology, clinical researches and analytical tools alongside with quality control assurance (Altschuler et al., 2007). Many people feel secure in utilizing herbal medicines since as it has negligible or minor side effects (Hassan, 2012).

World Health Organization (WHO) defines medicinal plant as "A medicinal plant is a plant where one or more of its parts that contain active ingredients are utilized for various therapeutic purposes or which acts as predecessors in order to synthesize the useful drugs" (Sofowora, 1982). Medicinal plants comprise an abundant source of constituents which are utilized for the improvement as well as to synthesize new drugs. The World Health Organization (WHO), assessed that approximately three fourth of the population of the world finds easier in utilizing herbal medicines as they are more consistent as well as less expensive.

According to the World Health Organization (WHO), approximately 75 to 95 % of the population of the world predominantly relies upon conventional medicines and includes the utilization of the plant extracts or their active ingredients (Moly, 2011). The petition for an ever increasing number of medications from plant sources are consistently expanding, which consequently requires the screening of therapeutic plants with expected biological activity (Sumathi & Parvathi, 2010).

1.1. A brief history of medicinal plant

In the initial 3000 BC ancient Chinese and Egyptian papyrus used medicinal plants for the treatment of various diseases. Medicinal plants are utilized for the beneficial of health in many countries of the world (Birks et al., 2007).

Despite the fact that the overview of the previous study on herbal medicines is new however, the utilization of herbal medicines is a gift provided to the humanity for its minimal side effects.

When all the advanced technologies were definitely not presented, people used to live so closed to nature that they use the plants for therapeutic purposes. From the prehistoric times, people did not have proper information about the main cause of their ailments. Moreover, they did not have proper knowledge to choose the right plant that could be used to cure their diseases.

It is expected that in order to cure various ailments, medicinal plants were most likely utilized. However, no one precisely knows the exact period of time and place where medicinal plants were utilized for the first time. It is assumed that, our ancestor started the use of herbal medicine at first. On a Sumerian clay slab at Nagpur, nearly 5000 years old written confirmation for utilization of medicinal plants has been found. Approximately it contains 12 formularies that use more than 250 medicinal plants for the preparation of medication.

The Egyptians used to utilize the principle of herbal medicine in a precised and more organized way. The most intriguing printed record is the Papyrus of Ebers from 1800 A.C. With known plants, there were more than 700 recipes. The Chinese book "Pen T' Sao," that mostly depends on roots and grasses is a decent instance of medicinal plants. The book incorporates the investigation of more than 300 plants (Botanical-online, 1999-2016).

The ancient history shows us that the famous book “De Materia Medica” was written by “the father of pharmacognosy”, Discorides. Approximately 944 drugs described on that book among which 657 are of plant origin. Along with the illustration of appearance, mode of collection and preparation as well as their therapeutic effects (Petrovska, 2012). From the ancient times, Indian ayurvedic such as Vagabhatta, Sushruta, Charaka plants were used to treat wound healing.

In present days there are national and international pharmacopeias that incorporates the medications and other preparations, illustration, analytical composition, formulation, dosage, standards of purity, various chemical properties for identification and

investigation, and so on. Various countries for instance, the United Kingdom, Russia, Germany they have their distinct pharmacopeias. Unauthorized medications got ousted from the field of medicine due to the use of conventional and popular medicines.

1.2. Significance of medicinal plants as traditional medicine

Natural medications are broadly utilized for its safety, efficacy, therapeutic effect and accessibility. On the other hand, these drugs are observed to be more pecuniary and are less costly than the other allopathic medications.

From the ancient times, medicinal plants are being utilized to cure various ailments consequently it will play a major role in establishing the history of drug discovery.

Medicinal preparations are prepared from various parts of the plants and trees such as leaves, stems barks, roots, fruits and flowers which comprises various chemical constituents as well as the desired constituent. These chemical constituent will provide certain biological action to the human body and give therapeutic effect as well as it will expand the value of the particular plant as medicinal plant. Some bioactive constituents of medicinal plants are glycosides, phenols, saponins, flavonoid, and alkaloids which have definite pharmacological action on human body.

On the other hand, by the isolation of the desired component as well as by addition of the bulk, preparation of the dosage form leads to drug discovery. Despite that, lots of modern technologies are being utilized for instance, computer based molecular modeling design and combinatorial chemistry to produce synthetic drug molecule progressively but the medications that are derived from the plant are highly acceptable by the patients. Besides, Medicinal plants are being used for various clinical purposes which make these more dependable (Veresham, 2012).

According to a report that is published by The World Health organization (WHO), approximately 85 % of the population of the world uses natural medications for their prime health care. In addition, by analyzing the history of the former 20 years of United States showed that, the uses of natural medications have been expanded considerably as the patients have less tolerance to the synthetic drug and the high cost of the drug (Derwich et al., 2009).

1.3. Medicinal plants as alternative of synthetic drugs

Plants are the largest source of biochemical and pharmaceuticals in our universe. These living stores can create interminable biochemical mixes. In their living, human what's more, creatures are utilizing just a little portion (1 to 10 %) of plants accessible on Earth (250,000 to 500,000 species) (Borris, 1996).

The collected confirmation got from hundreds of years of use in conventional prescription or from information of how types of plants have advanced and adjusted to their condition can significantly lessen the opportunity to identify advancement hopefuls. Now a days, a significant number of plant- derived products are suggested for patients by the specialists (Gwynn & Hylands, 2000).

Table 1.1: Some of the medicinal plants used for treating common diseases

Scientific Name (Family)	**Part used**	**Traditional ailment**	**References**
Aloe vera L.	Leaf gel	Skin diseases including eczema, irritation, wounds, abrasions, psoriasis, cold sores, sun burns, microbial skin diseases, cosmetic application and hair loss	Zari, S. T., & Zari, T.A. (2015).
Curcuma longa L.	Rhizome paste, powder	Skin diseases like eczema, wounds, irritation, inflammation, erythema, burns, itching, sunburn	Zari, S. T., & Zari, T.A. (2015).
Acanthus ilicifolius L.	Root	Leucorrhoea	Hossan et al., (2010).
Holarrhena antidysenterica Wall.	Bark	Used in the treatment of dysentery, dropsy, fever, diarrhea and intestinal worm infections	Chopra, R.N., Nayer, & Chopra, I.C. (1992).
Plumbago zeylanica L.	Root	Used in paralysis, secondary syphilis, leprosy and ophthalmics	Chopra, R.N., Nayer, & Chopra, I.C. (1992).

Table 1.2: Some drugs previously isolated from natural sources

Drug	Chemical class	Source	Medical use	Mechanism of action
Penicillin	Penicillin	Fungus, *Penicillium niger*	Antibiotic	Inhibition of Peptidoglycan synthesis
Paclitaxel (Taxol TM)	Terpenoid	Pacific yew tree, *Taxus brevifolia*	Tubulin polymerization stabilizer, Sneader (2005).	Tubulin polymerization stabilizer
Morphine	Alkaloid	Dried latex of Opium poppy, *Papaver somniferum* (1804)	Potent Painkiller/ Analgesic, Sertürner (1805).	Opioid agonist by binding to opiate receptors (mu, delta, and kappa)
Quinine	Alkaloid	Cinchona bark, *Cinchona officinalis* (1820)	Anti-malarial agent (Meshnick & Dobson, 2001, p. 396).	Protein synthesis inhibitor
Salicylic acid	Beta-hydroxy acid	Barks of willow tree	Aspirin is used as an analgesic, anti-inflammatory and antipyretic (NSAID)	Inhibition of COX enzymes in the COX pathway
Digoxin	Cardiac glycoside	*Digitalis purpurea*	Atrial fibrillation and congestive heart failure	Inhibition of the Na + /K + - ATPase membrane pump

(Edeoga, Okwu, & Mbaebie, 2005)

1.4. Antioxidants

Antioxidants are the compounds that guard the cells from the harmful effects of reactive oxygen species, for instance, singlet oxygen, superoxide, peroxyl radicals, peroxy nitrite that causes oxidative stress which leads to the damage of the cell (Hamid, 2010).

These agents occur naturally in plants. They are found in various dietary supplements such as vitamins, minerals etc. Vegetables, fruits, nuts and grains are rich sources of antioxidants. Antioxidant impedes the oxidation of other molecules in the body. They shield the body from destructive molecules that are known as free radicals.

Oxidation is a one type of biochemical reaction which occurs in the body in order to produce free radicals which in turn lead to the initiation of extemporaneous chain reaction that causes damage to the cells of human body. Antioxidants halt the chain reaction by eliminating the free radicals as well as oxidation reaction by oxidizing themselves. There are extensive varieties of antioxidants that are derived from natural and as well as synthetic origin cause has been suggested for the treatment of different ailments.

1.4.1. Mechanism of three main types of antioxidants

(1) Primary antioxidants

Natural antioxidants are known as primary antioxidants or chain breaking antioxidants. Various minerals such as manganese, zinc, copper, selenium, iron are the natural antioxidants. Vitamins for instance, vitamin B, vitamin C and vitamin E are also natural antioxidants. Phytocompounds such as flavonoids also belong to natural antioxidants.

(2) Secondary antioxidants

Synthetic antioxidants are known as secondary antioxidants due to their capacity to catch the free radicals which is responsible for demolishing the chain reaction. Examples include ascorbic acid, Beta carotene, albumin, bilirubin, butylated hydroxyl toluene, butylated hydroxyl anisole, propyl gallate, metal chelating agent, nordihydroguaretic acid (Perez, 2013)

(3) Tertiary antioxidants

Tertiary antioxidant causes damage to the biomolecules that are repaired by free radicals. For instance, DNA repairs enzymes and methionine sulfoxide reductase (Hamid, 2010).

Moreover, polyphenolic compounds and flavonoids are strong antioxidants that have potential and effective consequences for human wellbeing and combat against various ailments. The antioxidant potential of flavonoid relies upon the chemical structure. The

position of hydroxyl groups and other features in the chemical structure of flavonoids plays a vital role for their antioxidant and free radical scavenging activities.

1.5. Free radicals

Free radicals are the molecules that contain unpaired electron in the outer shell ls which are capable to occur freely (Sarma, 2010). Although the free electron free radicals are very much unstable, they are highly reactive at the same time that means they can react with other molecules that are closer to them. This incorporates DNA, carbohydrates, protein, lipid. There are stable radicals, persistent radicals and di-radicals. As free radicals cause damage to our human body, it is necessary to counter them. Synthesized antioxidants have minor effects on countering free radicals (Halliwell & Gutteridge, 1986). Use of various drugs, toxins, dietary factors is also responsible for the free radical generation. Three steps are involved for the generation of free radical radicals which involves initiation reaction, propagation reaction and termination reaction. The main focus of free radical is DNA, lipids and proteins. They are responsible to cause diseases such as Alzheimer's disease, kidney disease, cancer cardiac reperfusion abnormalities, fibrosis (Sarma, 2010).

1.5.1. Types of free radicals as a result of metabolic reaction

Free radicals are classified on the basis of their type. For instance, singlet oxygen ($^1O_2$), triplet oxygen, hydroxyl radical (OH•), superoxide anion radical ($O_2^{-\ -}$), hydrogen peroxide, lipid peroxyl free radical, nitric oxide radical (NO•), alkoxyl radical, peroxynitrite radicals.

1.5.2. Common diseases caused by free radicals

Free radicals are accountable for various diseases like rheumatoid arthritis, type II diabetes mellitus, adult respiratory disease syndrome, atherosclerosis, myocardial infarction, vascular diseases, chronic inflammatory diseases, hypercholesterolemia, Alzheimer disease and Parkinson disease.

1.6. Significance of antioxidant potential of medicinal plants

Oxidative stress is termed as a condition which occurs due to the imbalance between oxidants and antioxidants which actually leads to the damage to the cell. It has been reported that oxidative stress is the leading cause of ageing (Hyun et al., 2006) and other diseases such as cancer (Kinnula & Crapo, 2004). Oxidative stress is accountable for the development of a many ailments. Plants have a natural capacity to biosynthesize an extensive variety of non-enzymatic cancer prevention agents fit for lessening ROS-prompted harm that is caused by oxidative stress. There are several *in-vitro* methods that can be used for the screening of the plants for the prevention of cancer (Kasote et al., 2015).

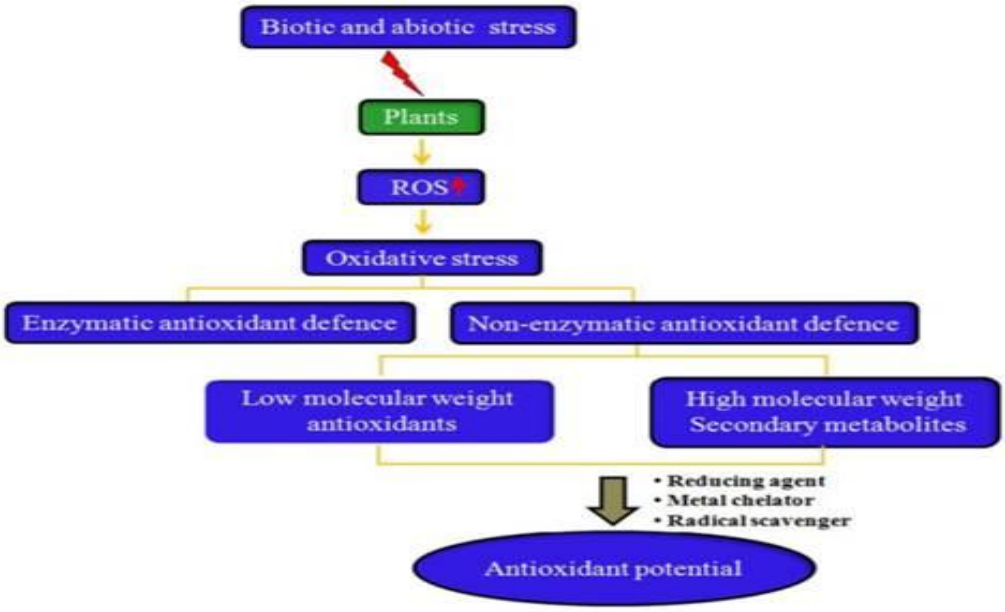


Figure 1.1: Antioxidant potential of plants

1.7. Prevalence of cancer

Cancer is considered as the life-threatening diseases in humans. In the present time, a significant number of anticancer agents are obtained from various natural sources. According to the World Health Organization (WHO), cancer is considered to be the foremost causes of death throughout the world, that accounted for 7.5 million deaths (approximately 12 %) of the population of the world in 2007.

1.8. Overview of cervical cancer

Cervical cancer is a cancer which progresses in the cervix of woman (the entrance to the womb from the vagina). This type of cancer usually develops when there is an uncontrolled growth of the abnormal cells on the cervix. Cells that lines the cervical surface goes through sequential changes. In exceptional cases, the precancerous cells may be transformed into cancerous cells. It has been reported that, cervical cancer accounted for 503000 newly diagnosed cases, 1.5 million cases that are prevalent and 273000 deaths throughout the world in 2003 (Steenbergen et al., 2014).

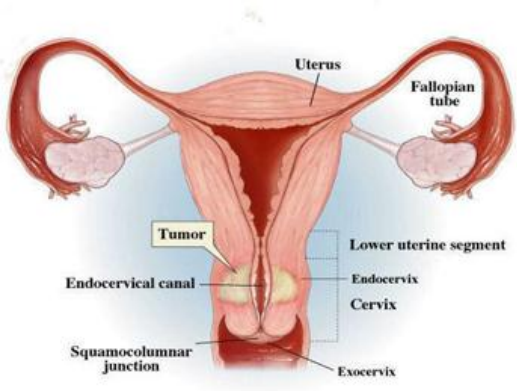


Figure 1.2: Cervical cancer

1.9. Causes of cervical cancer

Human papillomavirus (HPV) is responsible for causing the cervical cancer. It has been reported that, two strains of the HPV virus namely HPV 16 and HPV 18 are accountable for 80 % cases of cervical cancer. HPV infection doesn't show symptoms (Steenbergen et al., 2014). However, treatment option can minimize the potential risk of the development of cervical cancer.

1.10. Types of cervical cancer

There are two main types of cervical cancer.

Squamous cell cervical cancer

Squamous cells are accounted for approximately 80 % of cervical cancer. The cancer cells comprises of the flat cells which lines the exterior of the cervix that frequently initiates at the place in which the external surface links with the cervical canal.

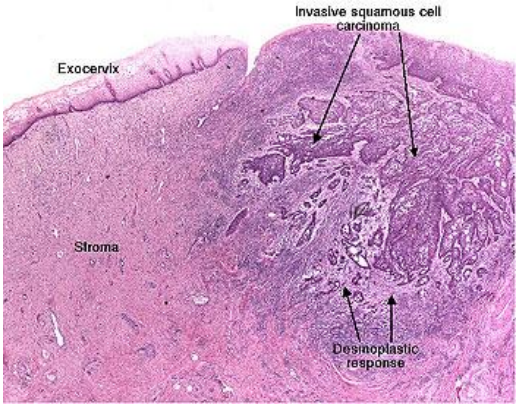


Figure 1.3: Squamous cell cervical cancer

Adenocarcinoma

Adenocarcinoma accounted for approximately 15–20 % of cervical cancer. This disease progresses within the glandular cells thatis lined by the cervical canal. It is very challenging to identify the cancer cells by cervical screening tests as they mature in the cervical canal.

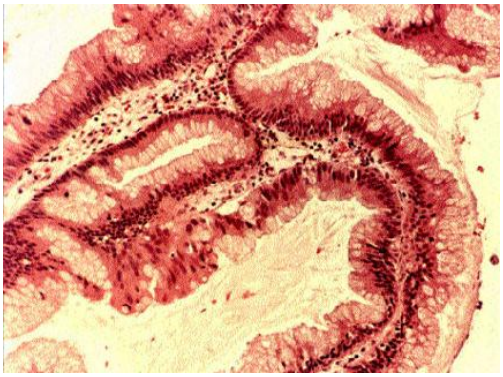


Figure 1.4: Adenocarcinoma

1.11. Stages of cervical cancer

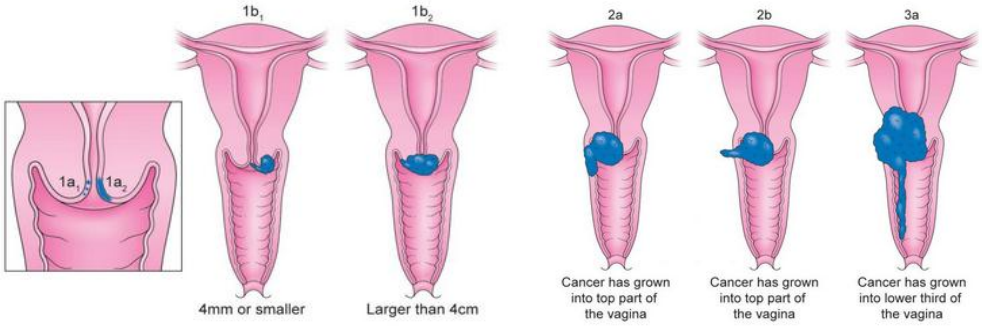


Figure 1.5: Stages of cervical cancer

The following stages are involved in the progression of cervical cancer:

In stage I A cervical cancer, a minor amount of cancer cell is observed under a microscope in the cervical tissues but the cancer does not spread to the adjacent tissues whereas, in stage I B, a significant number of cancer cell is observed in cervical tissues.

In stage II A, cancer usually spread away from the cervix to the upper two third part of the vagina whereas, in stage II B, cancer spreads throughout the cervical tissues.

In stage III, cancer spreads all over the pelvic area as well as to the lower two third part of the vagina in order to block the tubes which connect the kidneys to the bladder (the ureters).

In stage IV A, cancer spreads to other body parts, for instance the bladder or rectum whereas, stage IV B cancer spreads to distant organs, for example the lungs (Steenbergen et al., 2014).

1.12. Diagnosis of cervical cancer

Cervical cancer can be treated by cryosurgery or laser surgery. Other options include radiation therapy, chemotherapy, surgery or a combination of these methods. Besides, several tests are performed for the diagnosis of cervical cancer for instance, Pap test, pelvic examination, HPV typing. If abnormal results are shown by the Pap test as well as a positive result is shown by the HPV test, several diagnostic tests are performed for instance, colposcopy, biopsy, computed tomography (CT or CAT) scan, x-ray, cystoscopy, positron emission tomography (PET) or PET-CT scan, proctoscopy (sigmoidoscopy), laparoscopy.

1.13. Treatment of cervical cancer

A cone biopsy with pelvic lymph nodes dissection is usually performed for treating stage I A cervical cancer. Stage I B, II A, II B, III, IVA cervical cancer can be treated with chemo radiation. Cisplatin or cisplatin alongside with fluorouracil can be used. Stage IV B cervical cancer is treated by radiation therapy or chemotherapy in order to slow the progression of cancer and to relief symptoms. The chemo can be cisplatin or carboplatin alongside with other drug. For instance, topotecan, paclitaxel (Taxol), gemcitabine and bevacizumab (Avastin).

1.14. Medicinal plant as anticancer agent

Medicinal plants are considered to possess anticancer activities thereby they are being used for health benefits from the ancient time The use of medicinal plants is believed to contain a wide spectrum of polyphenolics, alkaloids, flavonoids, terpenoids that have therapeutic effect and that might hinder the formation of cancer (Dwidevedi et al.,2011). The antioxidant property of a plant is very useful to prevent and cure cancer and other diseases by protecting the cells which is mainly triggered by free radicals. It has been assessed that, 80-85% of worldwide population depend solely on plant extracts or their active principles (Siegel et al., 2015). More than 150000 plant species has been examined and a huge number of them contain useful substances (Loc & kiet, 2011). There are a few known metabolites that possess anticancer properties such as alkaloid, flavonoids, terpenoids, phenylpropanoids that has been separated from characteristic sources (Kumar, George & Suresh, 2012). Cytotoxic phytochemicals such as vinca alkaloids, vinblastine (VLB) and vincristine (VCR) isolated from the *Catharanthus roseus* (Apocynaceae) are frequently utilized as a part of oncology with strong medications and filled in as model for engineered mixes (Park et al., 2008).

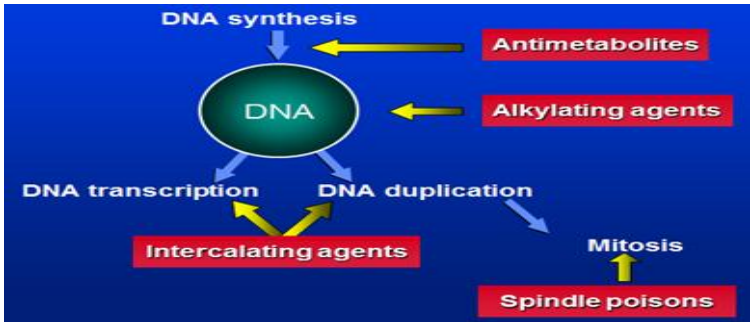


Figure 1.6: Mechanism of action of anticancer activity

1.15. Phytochemical evaluation of crude extract of medicinal plants

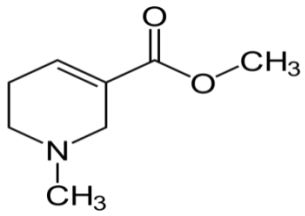
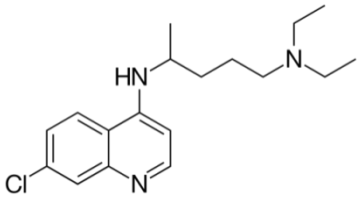
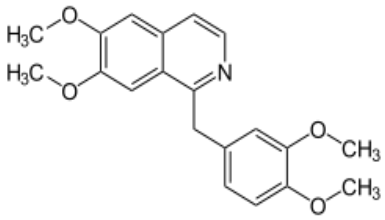
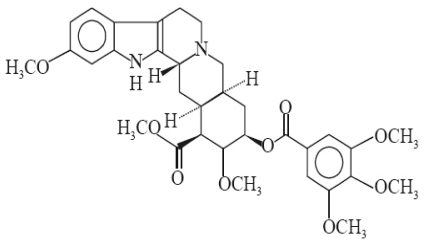
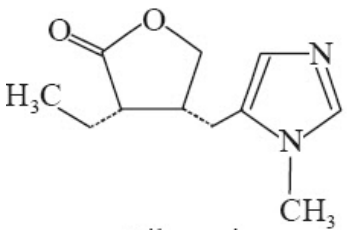
Medicinal plants possess significant bioactive compounds that have certain pharmacological actions in human body. These compounds incorporate substances like flavonoids, alkaloids, saponins, glycosides, tannins and phenolic compounds. Polyphenolic compounds are also known as "polyhydroxy phenols are a structural class of compounds that can be natural, synthetic or semi-synthetic. They comprise of different class of compounds namely glycosides, tannins, flavonoids, phenolic compounds/ phenols. These types of chemical substances retain or hold medicinal values that can be used for phytochemical investigation and pharmacology. The isolation of these chemical substances from the medicinal plants exerts various pharmacological actions to the human body such as antimicrobial activity, antioxidant activity, analgesic activity, cytotoxic activity and so on. Various studies shows that the compounds that are isolated from the natural sources are superior to those of the synthetic analogues due to their environmental safety and biodegradability.

1.15.1. Alkaloids

Alkaloids belong to the largest class of nitrogenous organic compounds found in the plant origin which has definite pharmacological actions on human body (Ghani, 2003). They incorporate numerous drugs (morphine, quinine) as well as poisons (atropine, strychnine).

Table 1.3: Classification of alkaloids along with examples

Alkaloids are classified on the basis of biosynthetic precursors from which they are obtained.

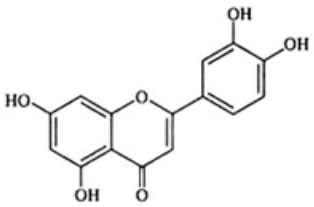
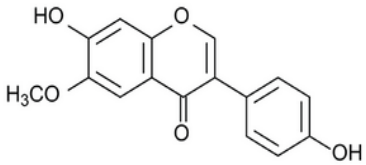
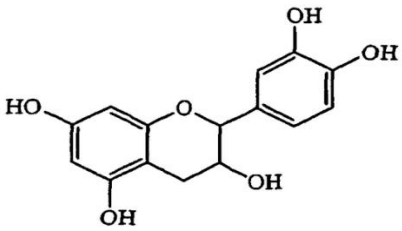
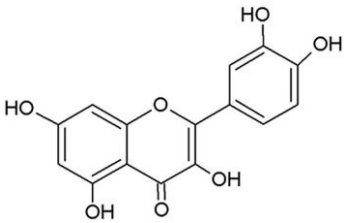
Class of alkaloids	Schematic Structure	Example
Pyridine group		Arecoline , Melicopicine
Quinoline group		Quinine, Quinidine
Isoquinoline group		Papaverine, Morphine
Indole group		Reserpine, Catarantine, Tabersonine,
Imidazole group		Pilocarpine

1.15.2. Flavonoids

Flavonoids belong to a large family of polyphenolic plant compounds. Chemically, flavonoids are 15-carbon containing compounds that involve two phenyl rings (A and B) and a heterocyclic ring (C). The B-ring structure of flavonoid possesses a hydroxyl group that is essential in scavenging ROS at the time of "oxidative stress" (Rice-Evans, 2001). Flavonoid possesses anti-oxidant, anti-inflammatory and anti-cancer potential. One of the most abundant dietary flavonoid is quercetin. The favorable structural feature of quercetin provides it free radical scavenging activity (Sannigrahi et al., 2009).

Flavonoids are classified into flavones, flavanones, isoflavones, catechins, anthocyanidins flavonols and chalcones. The positioning of the hydroxyl group in their structure, determines the nature of the pharmacological activity.

Table 1.4: Classification of flavonoids

Class of flavonoids	Schematic structure	Examples
Flavones		Luteoline, Apigenine
Isoflavones		Glycitein, Genistein
Anthocyanins		Catechin, Epicatechin (EC), Epicatechin gallate
Flavonol		Quercetin, Myricetin

(Barrington, 2011)

1.15.2.1. Flavonoid as anticancer agent

Flavonoid functions as an anticancer agent. Flavonoids act on both the single stage as well as the entire process of cancer development.by several mechanisms such as modulation of mitogenic signaling as well as apoptotic signaling, regulation of cell cycle, angiogenesis, and metastatic impacts in the cell. Thereby, the mechanism of action of DNA topoisomerase I/II, arrival of cytochrome C from mitochondria as well as subsequent triggering of caspases-3, 8 and 9 are impeded by flavonoids (Yadegarynia, 2012).

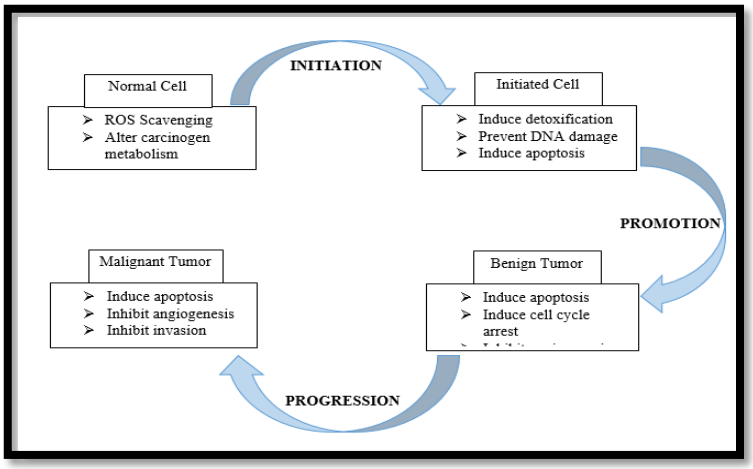


Figure 1.7: Model of carcinogenesis and potential consequences of flavonoids on cancer progression

1.15.3. Phenols or phenolic compounds

Phenols belong to a class of organic compounds that contain a hydroxyl group (-OH) that is directly attached to an aromatic hydrocarbon group. Phenols are present in various dietary supplements and in plants that are used in traditional medicines. They play a vital role in human health and disease which is an important subject of research. Thymol is one type of phenol that devoid of antimicrobial as well as anti-inflammatory properties.

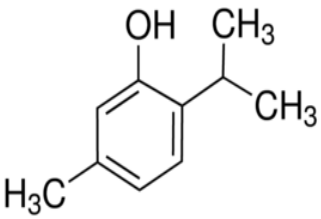


Figure 1.8: Structure of thymol

1.15.3.1. Antioxidant potential of phenolic compounds

Phenolic compounds possess antioxidant potential that includes scavenging of free radicals, reduction of hydro peroxide formation, inhibition of lipid oxidation,

1.15.3.2. Anticancer activity of phenolic compounds

Polyphenols acts as anti-cancer agents. Numerous polyphenols for instance, flavonoids, tannins, gallic acid, resveratrol, proanthocyanidins, anthocyanin and epigallocatechin-3-gallate. All of them are effective for the treatment of cancer.

1.15.3.3. Anti-microbial, anti-fungal and anti-viral activity of phenolic compounds

Phenol possesses effective anti-microbial, anti-fungal and anti-viral activity.

1.15.3.4. Cardio protective activity of phenolic compounds

Phenolic compound possess cardio protection activity. Postprandial hyperlipidemia and oxidative stress can be minimized by using poly phenols.

1.16. Tannins

Chemically, tannins are polyhydroxy phenolic substances. Tannin compounds are widely distributed in various plant species. Tannins demonstrate anti-carcinogenic, antiseptic and antibiotic activity. They inhibit lipid peroxidation. Tannins are also directed for numerous gastrointestinal disorders. For instance, ulcer, bile dysentery, diarrhea (Ghani, 2003, p. 20). Plants that are devoid of tannin involve *Eucalyptus* species (Eucalyptus), *Cinnamomum cassia* (Cassia bark).

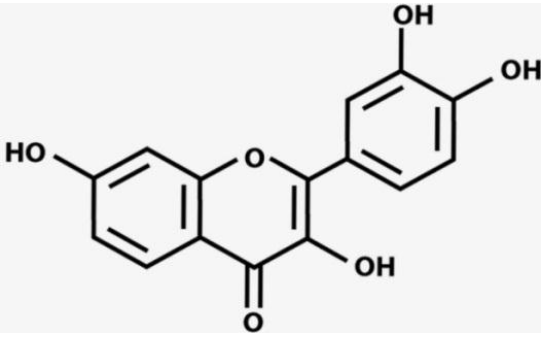


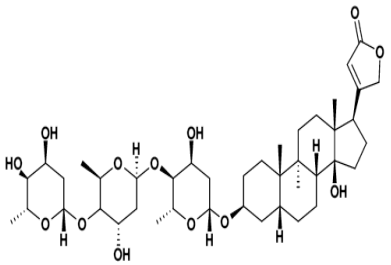
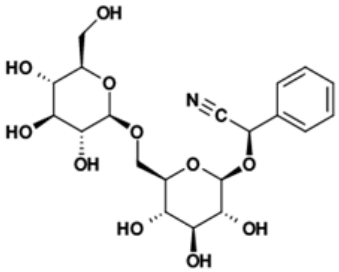
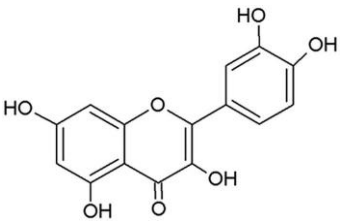
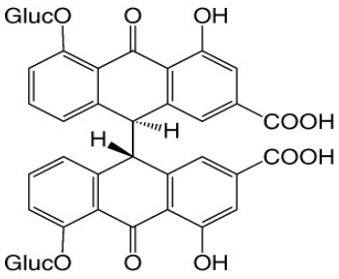
Figure 1.9: Structure of tannin

1.17. Glycosides

Glycosides are usually termed as compounds that involve a carbohydrate molecule (sugar) which is mainly contained in various parts of the plants. This carbohydrate molecule is transformed to a sugar molecule by means of hydrolytic cleavage as well as a non-sugar element (aglycone). They are termed particularly for the sugar that is contained in them for instance, glucoside or glucose, fructoside or fructose (Ghani, 2012). Glycosides are the very important class of drugs for the modern medicine. They are the class of compounds that are most abundant than the alkaloids.

Glycosides can be classified into major classes' namely cardiac glycosides, cyanophore glycosides, flavonoid glycosides, anthraquinonone glycosides, thiocyanate glycosides.

Table 1.5: Classification of glycosides

Glycoside Class	Schematic structure	Examples
Cardiac Glycosides		Digitalis, Digitoxin, Digoxin
Cyanophore glycosides		Amygdalin
Flavonoid glycosides		Quercetin
Anthraquinone glycosides		Sennoside A Sennoside B

1.18. Introduction to the Genera of Begoniacea family

There are two genera of Begoniaceae family. One of them is *Hillebrandia* and it has just a single species. They are indigenous to the Hawaiian Islands. Another genera is known as *Begonia* which comprises of around 1400 species all over the world (Wendy et al., 2003).

1.19. Classification of various plants of Begoniaceae family

Plants of Begoniaceae family are classified on the basis of their difference in the structure of leaves, roots etc.

Fibrous Begoniaceae

Figure 1.10: Fibrous Begoniaceae

Fibrous Begoniaceae are so named as their root has structural resemblance to the fiber (Jackson & Perkins, 2014).

Tuberous Begoniaceae

Figure 1.11: Tuberous Begoniaceae

The name Tuberous Begoniaceae was given because the structure of the root resembles tuber. In addition, this tuberous Begoniaceae can be additionally characterized depending upon whether they are single or double flowered, as far as their smell and so forth (Jackson & Perkins, 2014).

Rhizomatous Begoniaceae

Figure 1.12: Rhizomatous *B. acetosa*

Rhizomatous Begoniaceae are distinguished from other varietiesof Begonias as they arise from the stems (rhizomes) that develop alongside the surface of the soil (Jackson & Perkins, 2014).

Thick-stem Begoniaceae

Figure 1.13: Thick-stem Begoniaceae

Thick-stem Begoniacea comprises leaves that surround the ascending axis of the plant (Jackson, 2014).

1.20. Most common species of Begoniaceae family

Begonia malabarica

B. malabarica is a herb that contains stem. They are usually 2 ft. in length that are repetitively dividing, alongside with numerous leaves. Leaves are 3-5 inches, heart-shaped that are usually uneven at the base.

Medicinal uses

B. malabarica is an essential medicinal plant and its fundamental secondary metabolites are quercetin, luteolin, and β-sitosterol. The leaves are very effective to cure diarrhea, blood cancer as well as respiratory infections,

Figure 1.14: *B. malabarica*

Begonia sparreana

Leaves of *B. sparreana* are 2-4 inches long. This plant contains a rhizome that is essential for the multiplication or reproduction of this plant.

Medicinal uses

Leaves are effective to cure gastrointestinal disorders like diarrhea, jaundice.

Figure 1.15: *B. sparreana*

Begonia peltafolia

Figure 1.16: *B. peltafolia*

B. peltafolia comprises of juicy stem which are freely branched and the ascending axis of the plant is twisted with white colored substance. Leaves are usually 2-5 inches long (Otto & Dietrich, 1841).

Medicinal uses

Leaves are used in the treatment of stomach ulcer, stomachache, bile dysentery, diarrhea and respiratory infections, blood cancer and skin diseases.

1.21. Introduction to *B. roxburghii*

1.21.1. Plant description

B. roxburghii is a kind of herbal medicinal plant that belongs to *Begoniaceae* family. It is also known as East Himalayan Begonia as they are highly available in the Himalayan region of India, Nepal and Indo-china. In Bangladesh, its common name is Khatredoi and this name is derived from Chakma language. *B. roxburghii* grows in the shady moist places of Chittagong hilly tracts region (Mark Hughes, 1829).

1.21.2. Botanical description

B. roxburghii is a herb that has succulent stems. They are 35-80 cm in length. Leaves are usually cordate, uneven and sharp at the base. The main characteristics that differentiate this plant from other species of Begoniaceae family are the red pigmented dots over the ascending axis of the plant. The dimensions of the leaves are about 15-22.8 cm. Cymes are short, dichotomous, axillary, few-flowered, sepals and petals are white in color. Flowers are white in color and have a yellow bunch at the center. Flowers have four petals which show up with constrained space at the end. Fruits are usually sub pyramidal,

4-celled and very succulent and divided into four roundish projection and those are encompassed by red dots. The juicy stem of Begonia *i*s about 10 cm long (Mark Hughes, 1829)**.**

Figure 1.17: Leaves of *B. roxburghii*

Figure 1.18: Flowers and stems of *B. roxburghii*

Botanical name: *Begonia roxburghii*

Common Name: East Himalayan Begonia

Synonyms: *Heterophragma roxburghii*

1.21.3. Geographical distribution

B. roxburghii is commonly native to the Indian subcontinent. They are highly available in Himalayan region of India. In Bangladesh *B. roxburghii* grows in the shady moist places of Chittagong hilly tracts and Sylhet region in shady moist places (Mark Hughes, 1829).

1.21.4. Taxonomical classification of *B. roxburghii*

Kingdom : Plantae

Division : Magnoliophyta

Class : Magnoliopsida

Order : Cucurbitales

Family : Begoniaceae

Genus : Begonia

Species : *Begonia roxburghii*

(Agardh, 1824)

1.22. Traditional use of *B. roxburghii*

- Rootstock is given in fecal discharge in bile dysentery.
- Leaves are also effective against throat, mouth and various skin diseases.
- Root and leaf is used in the treatment of gastrointestinal disorders like diarrhea, bile dysentery, stomach ulcer and stomach ache.
- Leave has antimicrobial activity that prevents the growth of harmful microorganisms in the body.
- The plant is also effective against respiratory tract and HIV infections.
- Leaves also possess antioxidant and cytotoxic activity to suppress the growth of cancer cells and helps in the treatment of cancer (Joshi, Shukla & Nailwal, 2014).

1.23. Rationale of the study

Various biological activities of medicinal plants have been extensively studied over the past decades. Some medicinal plants have been reported to exhibit antioxidant potential that are utilized to prevent and cure complex ailments namely atherosclerosis, stroke, Alzheimer's disease, and cancer. They usually neutralize the free radicals in human body thereby they perform their action (Joshi & Nailwal, 2014).

It is also reported other medicinal plant possess cytotoxic potential that are used to cure cancer. In addition to that, some medicinal plants devoid of anti-microbial activity which is responsible for the destruction and suppression of growth of harmful microorganism in human body. Widespread phytochemical analysis and in addition, the segregation of active ingredients from the plant plays a vital role to evade the adverse effects which in turn will ensure the safe and effective utilization of herbal medicines.

The main focus of the present study was to identify and illustrate the chemical constituents by means of phytochemical screening as well as to analyze the potential bioactivities of methanolic extract of *B. roxburghii* (*Begoniaceae*) leaves. From the previous studies on various species of these plants showed that, the plant has strong antimicrobial, antifungal, antidiabetic, antitumor, antioxidant and cytotoxic potential. Along with that, the leaves of this plant are also effective to cure complex ailments including various gastrointestinal disorders such as bile dysentery, diarrhea and jaundice. Since, no significant amount of work has been performed on the particular plant so there might be some unexplored biological activity of this plant. Therefore, the current study will function as a premise to identify some unknown properties as well as to describe the unexplored biological activities of the *B. roxburghii* leaf extract. The study will focus on the phytochemical screening and investigation of *in-vitro* antioxidant and cytotoxic potential of methanolic extract of *B. roxburghii* leaves.

1.24. Aim of the study

The aim of this study was to investigate *in-vitro* antioxidant and cytotoxic potential of methanolic extract of *B. roxburghii* leaves.

1.25. Objective of the study

The literature review was studied that comprises of essential findings of *B. roxburghii*. Thereafter, the objectives of the project were established by using methanolic extract of *B. roxburghii* leaves:

- To carry out phytochemical screening with the purpose of qualitative determination of the existence of phytocompounds in the selected medicinal plant extract.
- To investigate antioxidant as well as cytotoxic potential of the selected medicinal plant extracts using various *in-vitro* methods.
- To evaluate free radical scavenging assays comparable to that of standard ascorbic acid using DPPH free radical scavenging method.

CHAPTER: 2

LITERATURE REVIEW

Chapter 2: Literature review

2.1. Previously studied pharmacological properties of *Begonia species*

Begonia (Family: Begoniaceae) is a large genus of about 1,400 species widely distributed in tropical and subtropical areas, especially in Central and South America .The leaves of *Begonia* species are used for the treatment of respiratory tract infections, diarrhea, blood cancer, and skin diseases and anti HIV activity. The juice of *B. tricocarpa* is used to treat opacity of eyes.

2.1.1. Antimicrobial activity

Previous studies showed that *B. radicans* possess antimicrobial activity. Greek word anti means against, microbes means little and bios means life combined to form the word antimicrobial. These are the compounds which are responsible for the destruction and suppression of growth of harmful microorganisms in human body. Some essential volatile oils of these species were assumed to possess antimicrobial activity (Agardh, 1824).

It has been reported that, *B. erythrophylla*, *B. heracleifolia*, *B. samperflorens* and *B. fuchsioides* devoid of antimicrobial activity (Urban & Frisby). In addition, the antibacterial activity of the leaf extract of *B. albo-coccinia, B. cordifolia, B. dipetala, B. fallax* and *B. floccifera* were determined by disk- diffusion method against *S. aureus, B. subtilis, E. coli* and *P. aeruginosa* (Agardh, 1824). Previous study showed that, *B. fallax* and *B. floccifera* is highly effective against *S. aureus* and *P. aeruginosa.* All the species of Begonia possess strong antibacterial activity due to the presence of active constituents namely alkaloids and anthraquinones. Previous studies also showed that, *B. tricocarpa* possess a significant antibacterial activity against the bacteria *P. aeruginosa.*

2.1.2. Antifungal activity

B. malabarica is devoid of antifungal activity against the tested fungi (Catalano et al., 1998). Previous study showed that, *B. tricocarpa* also possess significant antifungal activity against *Aspergillus nigar* and *Candida albicans.*

For determining the antifungal activity of *B. malabarica*, agar-well diffusion method was followed (Perez et al., 1990).

2.1.3. Antitumor activity

From the previous studies, it has been reported that *B. tuberhybrida*, *B. heracleifolia*, *B. plebeja* devoid of antitumor activity (Doskotch & Fuller, 1995).

2.1.4. Antioxidant activity

From the previous study, it was showed that *B. peltata* and *B. conchifolia* possess antioxidant property. Nutrients like vitamins and minerals including some enzymes which protect human body from harmful effect of damage are known as antioxidants. These antioxidants chemical compounds are highly effective in the prevention of stroke, cancer, heart disease, Alzheimer's disease, Rheumatoid arthritis. They actually act by neutralizing the free radical in the body. Vitamins, minerals, selenium and flavonoid are type of compounds which are responsible for showing antioxidant property (Agardh, 1824).

2.1.5. Anti-diabetic activity

Previous studies showed that, the stem of *B. malabarica* was used traditionally by the Malasar tribe to treat diabetes. Therefore, *B. malabarica is considered* as a hypoglycemic and anti-hyperglycemic agent. The methanol extract of *B. malabarica* had shown a significant hypoglycemic effect.

2.2. Phytocompounds previously isolated from *Begonia species*

From the previous studies, it was found that *B. floccifera* contains saponin. Saponin is a polycyclic aglycone attached to one or more sugar side chain. Saponin is a naturally occurring chemical substance in plants they have highly foam forming properties in a homogenously dispersed system. More 100 families of plants have been found with saponin. Saponin possesses anti-cancer activity and they act through anti-tumor pathway. Saponin causes the down regulation of cancer cells by inducing apoptosis and by modulating ERK- independent NF-kB signaling pathway (Solomon Jeeva, 2012). It was reported that, *B. malabarica* possess stigma sterol, luteolin, friedelin, glucopyranoside, β-sitosterol, quercetin, epi-friedelinol, and β -sitosterol–3-β-D- (Desaiet et al., 1975).

In addition, the phytochemical evaluation of leaf extracts of *B. tricocarpa* revealed the presence of flavonoids .Besides, phytocompounds like lipids and steroids and significant amount of carbohydrates, oxalic acid, amino acids, coumarin were also identified from the previous studies. Besides, the previous study also showed that *B. albococcinia, B. cordifolia, B. dipetala, B. floccifera, B. fallax* also possess phytocompounds like alkaloids, anthraquinones, flavonoids, glycosides, phenolic compounds and steroids. Saponins were absent in these *Begonia* species.

CHAPTER: 3

METHODOLOGY

Chapter 3: Methodology

3.1. Collection and authentication of plant material

In order to investigate *in-vitro* antioxidant and cytotoxic potential, the medicinal plant *B. roxburghii* was chosen. As no significant amount of work had been conducted on this plant, thereby the plant was decided to be chosen for investigating the antioxidant and cytotoxic potential by the assistance of comprehensive literature and also its availability.

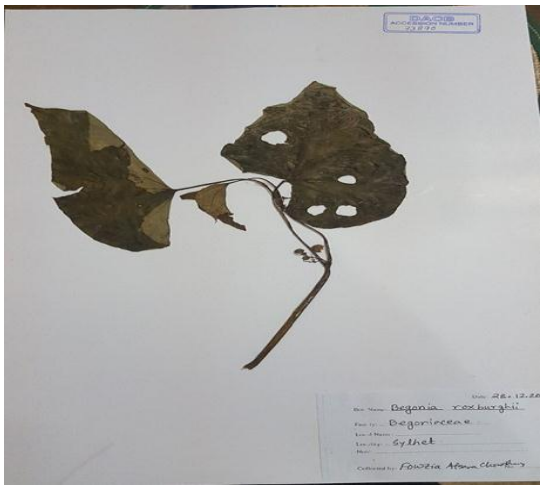


Figure 1.19: *B. roxburghii* leaves obtained from the National Herbarium of Bangladesh, Mirpur, Dhaka.

3.2. Chemical investigation of *B. roxburghii*

Name of the plant: *B. roxburghii*

Family: Begoniaceae

Plant part: Leaves

At first, the whole plant parts of *B. roxburghii* was collected from Sylhet, Bangladesh in December, 2016. For the authentication of the plant, the plant was given to the National Herbarium of Bangladesh (NHB), Mirpur, Dhaka. After one week, the leaf was taxonomically identified as (ACCESSION NO: DACB-73890). After that, the plant was authenticated by the taxonomist of Bangladesh National Herbarium, Mirpur, Dhaka.

3.3. Extraction procedure

The following processes are involved in the extraction procedure.

A. Plant material preparation and drying (2-steps)

B. Extraction process (5-steps)

3.4. Preparation of the plant material

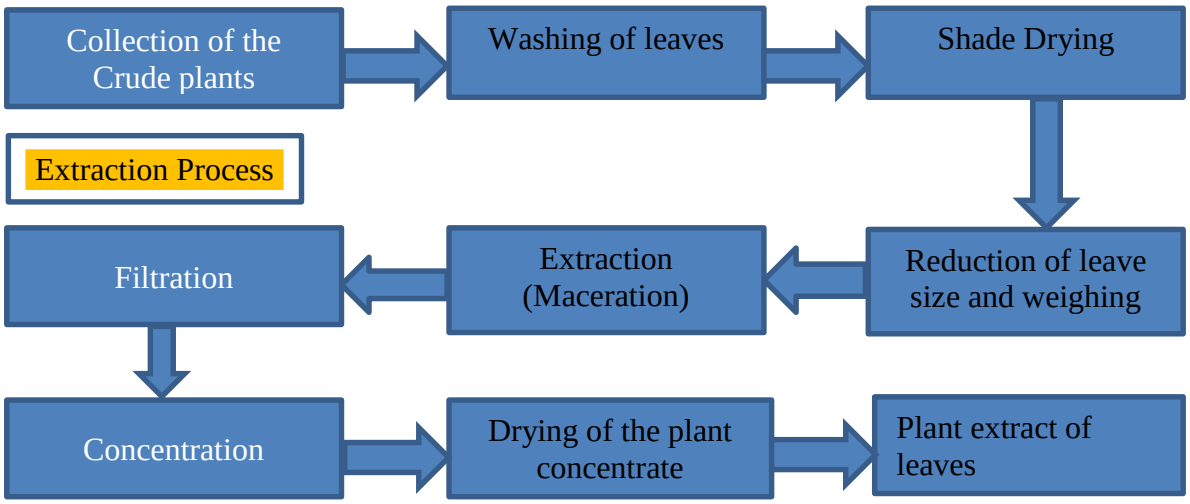


Figure 1.20: Flow chart of the extraction procedure of the crude medicinal plant

3.5. Plant material preparation and drying

This process occurs in two steps.

a. Washing of leaves

b. Shade drying

At first, the leaves were separated from the stem of the plant. After that, it was washed carefully with clean water in order to remove any dirt. After that, the plant was shade-dried for several days until it is completely dried. Then, the dried leaves were used in the extraction process.

3.6. Extraction process

It occurs in five steps.

- Size reduction and weighing
- Extraction
- Filtration
- Concentration
- Drying of the plant concentrate

3.6.1. Size reduction and weighing

The clean dried leaves were grounded by using a high capacity grinding machine in order to make a coarse powder. After that, the coarse powder poured in an air-tight plastic container in order to avoid the risk of cross-contamination. Thereafter, the plastic container was properly labeled with all the necessary information and then it was kept in dried, cooled and dark place until it is needed for further investigation.

Then, the powder of the leaves was poured into a beaker and the amount was determined by weighing. Finally, the amount was recorded.

3.6.2. Extraction

Extraction of plant materials are performed by maceration process in which methanol was used as the organic solvent. The powdered leaves of *B. roxburghii* were soaked in 900 mL of methanol in two separate beakers for five days at room temperature (22-25°C) and agitation was performed occassionally.

Table 1.6: The weight of powdered plant material and the volume of methanol used for the maceration process

	Beaker (1)	**Beaker (2)**
Weight of plant powder/g	72	65
Volume of methanol/mL	900	900

Two layer phases was observed after the maceration process. The upper-most layer contains the methanol solution of the extract that is separated by filtration whereas in the lower-most layer sediment was observed.

3.6.3. Filtration

At first, the contents of the beaker were decanted by using a Whatman filter paper. It was then filtered after three days of maceration process.

Figure 2.1: Filtration of the methanolic extract of *B. roxburghii* leaves

3.6.4. Concentration

After filtration, the filtrate was collected and then concentrated with the help of a rotary evaporator (Heidolph) at 100 rpm at 30°C, until concentrated methanol extract was produced. After that, in order to dry under LAF (Laminar Air Flow), the mixture was transferred onto the petri-dishes.

Figure 2.2: Concentrating the filtrate of *B. roxburghii* leaves using a rotary evaporator

3.6.5. Drying

In order to evaporate the solvent from the extract as well as to avoid the growth of microorganisms at the time of drying the extract, the petri dishes were kept under Laminar Air Flow (LAF).When the extract was successfully dried, the petri-dishes were safely covered with aluminum foil and it was kept in the refrigerator for further investigation .

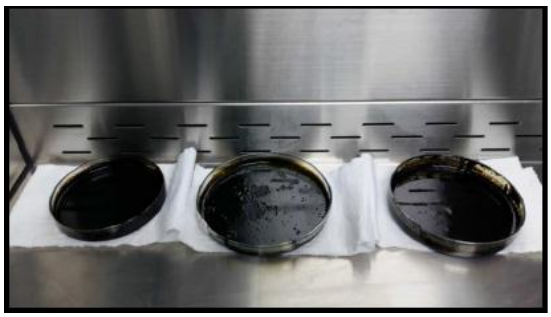


Figure 2.3: The petri-dishes that contains the methanolic leaf extract was placed under LAF for drying

3.7. Phytochemical screening of leaf extract of *B. roxburghii*

In order to investigate the phytochemical compositions, for instance, tannins, alkaloids, flavonoids, phenols, glycosides, resins, steroids, carbohydrates and phenols. Phytochemical screening was performed on the leaf extracts of *B. roxburghii* by various test methods.

3.7.1. Detection of alkaloids

Three tests were performed for the qualitative determination of alkaloids. At first, 0.5 gm of methanolic extract of *B. roxburghii* was dissolved in 5 mL of 1 % Hydrochloric acid. Subsequently, they were boiled in a water bath and filtration was performed. The filtrate was used in order to perform the following tests.

Hager's test

At first, a few drops of Hager's reagent (1 % picric acid solution) were added to 2 mL of the filtrate. After that, the presence of alkaloids was confirmed by the formation of yellow precipitate (Waldi, 1965).

Mayer's test

According to Evans (1997), Mayer's Reagent (10mL) was prepared by dissolving 0.135 gm of Mercuric (II) Chloride and 0.5 gm of Potassium Iodide in 10 mL distilled water. After that, a few drops of Mayer's reagent were added to a 2 mL of the filtrate along the sides of the test tube. The presence of alkaloids was detected by the formation of white or creamy precipitate.

Wagner's test

Wagner's Reagent (10 mL) was prepared by dissolving 0.2 gm of Iodine crystals and 0.6 gm of Potassium Iodide in 10 mL distilled water. The test was performed by adding a few drops of Wagner's reagent to a 2 mL of the filtrate. The presence of alkaloids was confirmed by the formation of brownish-black precipitate in the sample (Wagner, 1993).

3.7.2. Detection of flavonoids

Lead acetate test

At first, a few drops of lead acetate solution were added to the methanolic leaf extract. The presence of flavonoids was confirmed by the formation of yellow colored precipitate.

Zinc ribbon test

The presence of flavonoids can be confirmed by another method. At first, a test tube was taken that contains 0.5 mL of alcoholic extract. After that, 5-10 drops of concentrated Hydrochloric acid and a small piece of Zinc was added to the test tube. Followed by that, the solution was boiled for a few minutes and then left to stand. The formation of a red to crimson color solution signified the presence of flavonoids (Sindhu et al., 2013).

3.7.3. Detection of carbohydrates

At first, 0.5 gm of methanol extract of *B. roxburghii* was dissolved in 5 mL of distilled water and the mixture was filtered. In this way, carbohydrate was qualitatively detected. After that, the following two tests were performed in the filtrate (Ramkrishnan et al., 1994).

Molisch's test

At first, to a 2 mL filtrate, two drops of Molisch's Reagent (alcoholic solution of α-napthol) was added. After that, 2 mL of concentrated sulfuric acid was pipetted along the sides of the test tube and was allowed to stand for a while. The presence of carbohydrates was indicated by the formation of a violet ring.

Fehling's test

At first, 1 mL of each of the Fehling's solution A and B were added in a 1:1 ratio to 2 mL of the filtrate. After that, it was boiled for a few minutes. The presence of reducing sugar was indicated by the formation of a brick-red precipitate.

3.7.4. Detection of tannins

Lead acetate test

At first, a few drops of 1 % Lead acetate solution were added to 1 mL of the leaf extract. The presence of tannins was confirmed by the formation of a yellow-colored precipitate (Tiwari & Bimlesh, 2011).

Potassium dichromate test

First of all, 1 gm of Potassium dichromate was dissolved in 10 mL distilled water in order to form 10 % Potassium Dichromate solution. After that, the crude extract of leaf was converted into 5 mL aqueous solution and it was dissolved in 1 mL of 5 % ferric chloride solution. The presence of tannins was confirmed by the formation of yellow precipitation indicates (Ghani, 2003).

Ferric chloride test

At first, 0.5 gm of ferric chloride was dissolved in 10 mL distilled water and then 5 % Ferric chloride solution was prepared. In order to perform the test, 5 mL aqueous solution of crude extract was dissolved in 1 mL of 5 % ferric chloride solution. The presence of tannins was indicated by the formation of greenish black precipitation (Ghani, 2003).

3.7.5. Detection of phenols/phenolic compounds

Ferric chloride test

At first, 2 mL of extract was measured in a test tube and 3-4 drops of 15 % (w/v) Ferric chloride solution was added in it. The formation of a bluish-black precipitate signified the presence of phenols (Soni & Sosa, 2013).

3.7.6. Detection of glycosides

At first, before subjecting to Borntrager's Test, the methanolic extract of *B. roxburghii* was hydrolyzed with dilute Hydrochloric acid (Mariappansenthilkumar, 2013).

Borntrager's test

At first, 5 mL of 5 % Ferric (III) chloride and 5 mL of dilute Hydrochloric acid were added to the 5 mL of filtrate. The mixture was then heated in a boiling water bath for 5 mins and it was cooled down. After that, 5 mL of benzene was added to the mixture and it was shaken thoroughly. Thereafter, using a separating funnel the organic layer was separated by and an equivalent volume of dilute ammonia solution was added. The presence of glycosides was confirmed by the formation of a pinkish-red color in the ammonical layer (Kamalakar et al., 2014).

3.7.7. Detection of phytosterols

Libermann Burchard's test

At first, 1 mL of chloroform was added to a small amount of leaf extract and it was then filtered. After that, 2 mL of acetic anhydride was added to the filtrate and it was then boiled and cooled. Finally, the solution was treated with 1 mL of concentrated sulfuric acid. The presence of phytosterols was indicated by the formation of a brown ring at the junction (Soni & Sosa, 2013).

3.7.8. Detection of resins

The presence of resins can be identified by the addition of 5-10 drops of acetic anhydride to 2 mL of the extract followed by gently heating the solution. After that, 0.5 mL of sulfuric acid was added to the solution. The presence of resin was identified by the formation of a bright purple color (Soni & Sosa, 2013).

3.7.9. Detection of saponins

Froth test

At first, the extract was diluted with distilled water and 20 mL of the volume was made. After that, the contents of the cylinder were shaken for about 15 mins in a graduated cylinder. 2 cm in height foam layer was observed which indicated the presence of saponins (Kokate, 1999).

3.7.10. Detection of steroids

Salkowski test

At first, 2 mL of chloroform, 1 mL of sulfuric acid were gradually added to a 1 mL of leaf extract. The presence of steroids was confirmed by the formation of red color (Ghani, 2003).

3.8. *In-vitro* antioxidant potential

In order to determine *in-vitro* antioxidant activities of leaf extracts, several methods are used. Among them, four methods were chosen in order to determine the *in-vitro* antioxidant activity in the leaf extract of *B. roxburghii* which are as follows:

- DPPH (1, 1-diphenyl-2-picryl hydrazyl) free radical scavenging assay
- Total phenolic content (TPC)
- Total flavonoid content (TFC)
- Total antioxidant capacity (TAC)

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

The DPPH free radical scavenging assay of leaf extract *B. roxburghii* was determined by using the method (Braca et al., 2001).

Reagents and chemicals

Reagents and chemicals required for determining DPPH scavenging activity

Name of reagent

1. DPPH **(**1, 1-diphenyl-2-picryl hydrazyl)
2. Methanol
3. L-ascorbic acid

Reagent preparation

At first, 2 mg of DPPH was dissolved in 50 mL of distilled water in order to prepare 0.004 % (w/v) DPPH solution. After that, the solution was stored in the refrigerator at -4°C till before use.

Sample and standard preparation

At first, the sample stock solution was made by dissolving 120 mg of extract in 10 mL of methanol in order to produce 12 mg/mL concentration. By serial dilution of the sample stock solution, six serially diluted concentrations of the sample were prepared as 1200, 800, 400, 200, 100, 50 µg/mL.

By using L-ascorbic acid, the standard was prepared in the same manner. After that, the extract was made by six serially diluted concentrations which range from 1200 to 50 µg/mL.

Preparation of blank solution

3mL methanol was used in order to prepare the blank solution.

Experimental procedure

a) At first, in clean test tubes, 1 mL of each of the fractions of sample and standard (L-ascorbic acid) were taken.

b) After that, 2 mL of 0.004 % (w/v) DPPH solution was added to each of the test tube.

c) Then, at room temperature for 30 minutes, the test tubes were incubated. Thereafter, the absorbance of the resulting solutions and control (DPPH and methanol) was measured at 517 nm against blank (Methanol) using a spectrophotometer (U-2910 UV-Vis Spectrophotometer).

d) Finally, the percentage of free radical scavenging activity (% FRS) was then calculated from the equation as follows:

% Inhibition of free radical scavengers = $(A_0 – A_1) / A_0$ x 100

Whereas, $\mathbf{A_0}$ = The absorbance of the control

$\mathbf{A_1}$ = The absorbance of the sample/standard

3.8.2. Determination of total phenolic content (TPC)

According to Wolfe, Wu & Liu (2003), the TPC of the plant extract of *B. roxburghii* was determined by using Folin- Ciocalteu method.

Reagents and chemicals

Reagents and chemicals required for determining total phenolic content

Name of reagent

1. Folin-Ciocalteu Reagent (FCR)
2. Gallic acid monohydrate (Standard)
3. Sodium carbonate
4. Methanol

Reagent preparation

At first, 25 mL of FCR was taken in a 250 mL volumetric flask and then it was diluted with distilled water up to 250 mL mark. In this way, 250 mL of 10 % FCR solution was prepared. Followed by this, 100 mL of 7.5 % (w/v) Sodium carbonate was prepared by measuring out 7.5 gm of Sodium carbonate in a 100 mL volumetric flask and then diluting it with distilled water up to 100 mL mark.

Sample and standard preparation

At first, the sample stock solution was prepared by dissolving 120 mg of leaf extract of *B. roxburghii* in 10 mL of methanol in order to produce 12 mg/mL concentration. After that, by serial dilution of the sample stock solution, four serially diluted concentrations of the sample were prepared as 1200, 800, 400, 200 µg/mL. By using Gallic acid, the standard was prepared in the same manner. After that, the extract was made by four serially diluted concentrations which ranges from 1200 to 200 µg/mL.

Preparation of the blank

The blank solution was prepared by 5 ml of FCR solution and 4 ml Sodium carbonate. After that, 1 mL of methanol was used in order to make the volume up to 10 mL.

Experimental procedure

a) At first, in clean test tubes, 1 mL of each of the fraction of sample and standard (Gallic acid) concentrations were taken.

b) After that, 5 mL of FCR solution was added in each test tube.

c) Followed by this, 4 mL of Sodium Carbonate solution was added.

d) Then, each of the mixture was vortexed in a vortex machine for about 15 s and followed by stand for 30 mins at 40°C in a water bath.

e) Finally, the absorbance of the standard and sample solutions was measured against blank at 765 nm by using spectrophotometer (U-2910 UV-Vis Spectrophotometer).

f) The total phenolic content, C, for each of the fractions was expressed as gallic acid Equivalents using the following equation:

$C = (c \times V)/m$

Where,

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

c = Concentration of Gallic acid obtained from calibration curve (µg/mL)

V = Volume of sample solution (mL)

m = weight of the sample (g)

3.8.3. Determination of total flavonoid content (TFC)

TFC of the extracts of *B. roxburghii* was determined by using this method (Kumaran & Karunakaran, 2007).

Reagents and chemicals required

Reagents and chemicals required for determining total flavonoid content

Name of reagent

1. Quercetin (Standard)
2. Methanol
3. Potassium Acetate
4. Aluminium Chloride

Reagent preparation

At first, by taking 10 gm of Aluminium chloride in a 100 mL volumetric flask and diluting it with distilled water up to 100 mL mark, 100 mL of 10 % Aluminium chloride solution was prepared. After that, by taking 9.815 gm of Potassium acetate in a 100 mL volumetric flask and diluting it with distilled water up to 100 mL mark, 100 mL of 1M Potassium acetate solution was prepared.

Sample and standard preparation

At first, the sample stock solution was made by dissolving 120 mg of leaf extract of *B. roxburghii* in 10 mL of methanol in order to produce 12 mg/mL concentration.

After that, by serial dilution of the sample stock solution, four serially diluted concentrations of the sample were prepared as 1200, 800, 400, 200 µg/mL.

By using Quercetin, the standard was prepared in the same manner. After that, the extract was made by four serially diluted concentrations which ranges from 1200 to 200 µg/mL.

Quercetin was used as the standard and the stock solution was prepared in the same manner as the extract resulting in four serially diluted concentrations, ranging from 1200, 800, 400, 200 µg/mL.

Preparation of the blank

The blank solution was prepared by 200 µL of 10 % Aluminium chloride solution, 200 µL of 1M Potassium acetate solution, 5.6 mL of distilled water and 4 mL of methanol in order to make the final volume of the solution upto 10 mL.

Experimental procedure

a) At first, in clean test tubes, 1 mL of each of the fraction of sample and standard (Quercetin) concentrations were taken.

b) 3mL of methanol was added in each test tube.

c) After that, by using 1000 µL micropipette, 200 µL of 10 % Aluminium chloride solution and 200 µL of 1M Potassium acetate solution were added to each of the test tubes.

d) Followed by this, in order to make the final volume of the solution up to 10 mL, 5.6 mL of distilled water was added to the test tube.

e) The test tubes were then incubated at room temperature for about 30 mins.

f) Afterwards, the sample and standard absorbance was measured against blank at 415 nm by using spectrophotometer (U-2910 UV-Vis Spectrophotometer).

g) Finally, the total flavonoid content C, for each of the fractions were expressed as Quercetin Equivalents using the following equation:

$C = (c \times V)/m$

Where,

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

c = concentration of quercetin obtained from calibration curve (µg/mL)

V = Volume of sample solution (mL)

m = weight of the sample (g)

3.8.4. Determination of total antioxidant capacity (TAC)

The TAC of the leaf extract of *B. roxburghii* was determined by using this method (Prieto et al., 1999).

Reagents and chemicals required

Reagents and chemicals required for determining total antioxidant capacity

Name of reagent

1. Ammonium Molybdate
2. L-Ascorbic Acid (Standard)
3. Sodium Triphosphate ($Na_3PO_4.12H_2O$)
4. Concentrated sulfuric acid (98%)
5. Methanol

Reagent preparation

At first, by taking 3.28 mL of 98 % concentrated sulfuric acid in a 100 mL volumetric flask and diluting it with distilled water up to 100 mL mark, 100 mL of 0.6 M Sulfuric acid was prepared.

After that, by taking 4.494 gm of Potassium acetate in a 100 mL volumetric flask and diluting it with distilled water up to 100 mL mark, 100 mL of 0.004 M Ammonium Molybdate solution was prepared.

Consequently, by measuring 1.0645 gm of Aluminium chloride in a 100 mL volumetric flask and diluting it with distilled water up to100 mL mark, 100 mL of 0.028 M Sodium Phosphate solution was prepared.

Sample and standard preparation

At first, the sample stock solution was made by dissolving 120 mg of leaf extract of *B. roxburghii* in 10 mL of methanol in order to produce 12 mg/mL concentration.

After that, by serial dilution of the sample stock solution, four serially diluted concentrations of the sample were prepared as 1200, 800, 400, 200 µg/mL.

By using Ascorbic acid, the standard was prepared in the same manner. Thereafter, the extract was made by four serially diluted concentrations which range from 1200-200 µg/mL.

Preparation of the blank

For preparing the blank solution, 3 mL of reagent solution and 300 µL of methanol were used.

Experimental procedure

a) At first, in clean in test tubes, 300 µL of each of the fraction of sample and standard (L-ascorbic acid) concentrations were taken.

b) After that, 3 mL of Reagent solution (0.6 M sulfuric acid, 0.028 M Sodium phosphate and 0.004 M Ammonium molybdate) was added into the test tubes.

c) The test tubes (sample, standard and blank) were then incubated at 95°C in a water bath for about 90 mins.

d) Finally, the standard and sample absorbance was measured against blank at 695 nm using spectrophotometer (U-2910 UV-Vis Spectrophotometer).

e) The total antioxidant capacity, A, for each of the fractions was expressed as Ascorbic Acid Equivalents (AAE) using the equation as:

$A = (c \times V)/m$

Where,

A = Total antioxidant capacity, milligram 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)

3.8. Cytotoxic activity study by MTT assay

3.8.1. Principle of *in-vitro* cytotoxic potential

In-vitro cytotoxicity incorporates a cell arrangement which is performed in a specific medium alongside nutrients and antibiotics. These allow the cells to remain sound and sterilized and in addition anchor of the surface. Cytotoxicity examines vary depending on the type of analysis. Cell is cultured in a particular no per well or microtiter plate and concentrates are added further to decide the feasibility of cells under a magnifying lens. Apoptosis will be caused at the cultured cells after the addition of concentrates although it can cause secondary necrosis. Slowly the ingestion systems of cells stay down which drives the cells to lose their membrane integrity. Lastly, the cells discharge the cytoplasmic substance into the medium. The quantity of suitable cells in the medium decides the effect of the concentrate on the cell line. To be more particular, it demonstrates whether the concentrates had any cytotoxic movement or not (Nema & Khare, 2012).

HeLa cell (Cervical cancer cell)

Description: HeLa cell- line

Tissue Species: Human

Cell Lines: HeLa

Storage instruction: HeLa cell slide can be stored for up to 4 months at 4°C under desiccate conditions.

3.8.2. Methodology

Solutions preparation

1% Penicillin-streptomycin solution

In MTT assay, penicillin -streptomycin solution, also known as pen-strep is used in order to control the bacterial contamination as well as to thoroughly maintain a sterile condition throughout the process. Penicillin-Streptomycin solution contains a mixture of 10000 unit of penicillin per mL and 10 mg of streptomycin per mL.

10 % Fetal bovine serum (FBS)

In MTT assay, 10 % fetal bovine serum (FBS) was prepared by the addition of 50 mL of fetal bovine serum to the 500 mL of DMEM (Dulbecco's Modified Eagle's medium).

Trypsin:

0.25 % trypsin was added in the medium.

2 % DMSO (Dimethyl sulfoxide) solution

In MTT assay, 2 % DMSO solution was prepared by the addition of 60 µL of DMSO in 2940 mL of distilled water for control.

Celltiter 96 assay kit

Celltiter 96 assay kit is an accumulation of qualified reagents that give a fast furthermore advantageous system with determination of the amount of proliferation and cytotoxicity (Ifere et al., 2010). It is a view of change of a cell division with tetrazolium salt under a formazan product that is undoubtedly distinguished utilizing a 96-well plate. A mixer of dye solution was added in 96 well plates to culture cells to perform this assay.

3.8.3. Instruments required analyzing the cytotoxic potential of the plant extract

- Biological Bio Safety Cabinet (Model : NU-400E, Nuaire, USA)
- CO_2 Incubator (Nuaire, USA)
- Trinocular microscope with camera (Optika, Italy)
- Hemocytometer

3.8.4. Consumables to analyze the cytotoxic potential of the plant extract

- 96-well plate
- 15-mL tubes
- Culture flask
- Tips
- Gloves
- Cell culture media
- Antibiotics(1% penicillin-streptomycin)solution
- Gentamycin (0.2%)
- Trypsin
- Serological pipette

3.8.5. HeLa cell line

At first, the HeLa cell line was collected from the Centre for Advance Research and Science (CARS) in the University of Dhaka. After that, by using a cell culture flask, this cell line was cultured and maintained in DMEM (Dulbecco's Modified Eagle's medium). Then, the cell was stored in liquid nitrogen.

3.8.6. Preparation of the different concentrations of the plant extract

The assay was performed by using four concentrations that are respectively 25 mg/mL, 2.5 mg/mL, 0.25 mg/mL and 0.025 mg/mL of the *B. roxburghii* leaf extract. A concentration of 25 mg/mL was made by dissolving 25 mg of leaves extract in 1mL DMSO. This was the stock solution. After that, 2.5 mg/mL concentration was made by diluting 25 mg/mL solution for 10 times by DMSO. In order to make 2.5 mg/mL concentration, 10 µL of sample 1 was added to the 90 µL of DMSO. In the same manner, 0.25 mg/mL and 0.025 mg/mL concentrations were made by serial dilution. At last, the samples were filtered through 0.45 µm syringe filter prior to examination.

3.8.8. Cell culture

Preparation of assay plates

HeLa cell was maintained in DMEM (Dulbecco's Modified Eagle's medium) along with the addition of 1 % penicillin-streptomycin, 0.2 % gentamycin, 10 % fetal bovine serum (FBS).

Procedure

Cytotoxic potential was performed in the Centre for Advanced Research in Sciences (CARS) by using their commercial services. The MTT colorimetric assay was performed by using celltiter 96 non-radioactive cell proliferation assay kit (Promega, USA). In brief, HeLa, a human cervical carcinoma cell line was maintained in DMEM (Dulbecco's Modified Eagle's medium) containing 1 % Penicillin-Streptomycin (1:1), 0.2 % gentamycin and 10 % fetal bovine serum (FBS). After that, cells (2.0×10^4/100 µL) were seeded onto 96 well plates and incubated at 37^0C and 5 % of CO_2 atmosphere. On the next day, 25 µL of sample (filtered) was added into each well.

After 48 hours of incubation, cytotoxicity was examined by using celltiter 96 non-radioactive cell proliferation assay kit (Promega, USA).Then the absorbance was measured at 570 nm using a 96-well plate reader. Same procedure had been followed for all the concentrations, both negative control and positive control. Negative control was contained medium with 2 % DMSO solution and blank was contained only the medium. Duplicate cells were used for each sample.

CHAPTER: 4

RESULT AND CALCULATION

Chapter 4: Result and calculation

4.1. Phytochemical screening of methanolic extract of *B. roxburghii* leaves

In order to investigate the phytochemical compositions, for instance, tannins, alkaloids, flavonoids, phenols, glycosides, resins, steroids, carbohydrates and phenols. Phytochemical screening was performed on the leaf extracts of *B. roxburghii* by various test methods. The obtained results are given in the following table (1.7).

Table 1.7: Phytochemical screening of methanolic extract of *B. roxburghii* leaves

Class of compound	Result
Tannins	+++
Alkaloids	+++
Flavonoids	++
Glycosides	+
Resins	+
Steroids	+
Carbohydrates	+
Phenols	+
Phytosterols	-
Saponins	-

(+) indicates the existence of phytocompounds in a single method, (++) indicates the existence of phytocompounds in two methods, (+++) indicates the existence of phytocompounds in three methods and (-) means the absence of phytochemicals respectively.

Interpretation

The phytochemical screening of *B. roxburghii* showed the presence of alkaloids, flavonoids, tannins, phenolic compounds, glycosides, resins, carbohydrates, and steroids. On the other hand, it showed the absence of phytosterols and saponins (Table 1.7).

4.2. *In-vitro* antioxidant potential

For the investigation of *in-vitro* antioxidant potential of leaf extracts, several methods are used. Among them, four methods were selected in order to investigate *in-vitro* antioxidant potential of methanolic extract of *B. roxburghii* leaves which are as follows:

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

In-vitro antioxidant potential was performed by DPPH free radical scavenging assay (Braca et al., 2001) and the result obtained was recorded in the following table (1.8).

Table 1.8: DPPH free radical scavenging assay by standard L-ascorbic acid

Standard (L-ascorbic acid) concentration (µg/mL)	**Absorbance at 517 nm**	**% of inhibition of DPPH by L-ascorbic acid**
50	0.006	94.95
100	0.005	95.79
200	0.004	96.64
400	0.003	97.48
800	0.002	98.31
1200	0.001	99.16

Graphical presentation of % of inhibition of DPPH by L-ascorbic acid

The percentage of inhibitions that was found from the above table (1.8) was plotted in a graph shown below. The graph was drawn against the concentrations that were used in the above table (1.8).

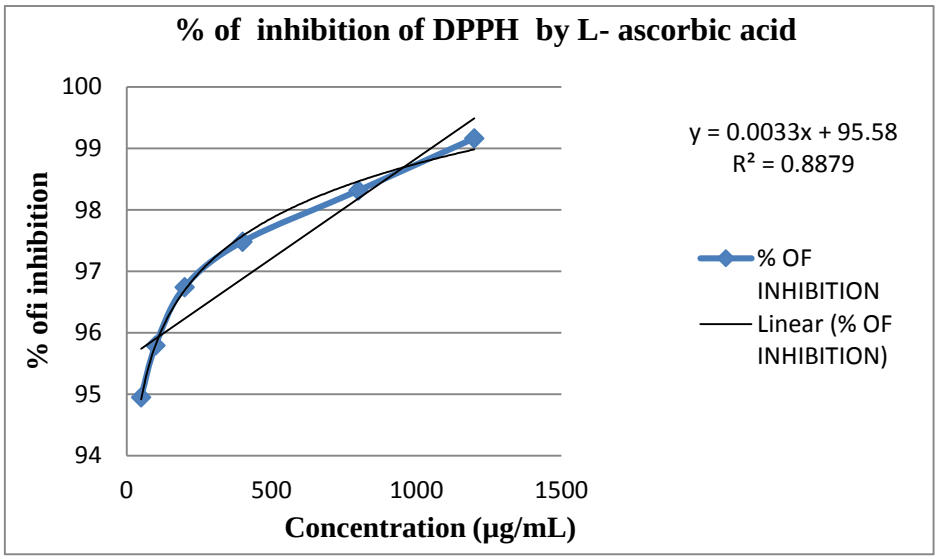


Figure 2.4: Graphical presentation of % of inhibition of DPPH by L-ascorbic acid

Table 1.9: DPPH free radical scavenging assay by *B. roxburghii*

Extract concentration (µg/mL)	**Absorbance at 517 nm**	**% of inhibition of DPPH by *B. roxburghii* (µg/mL)**
50	0.017	85.71
100	0.016	86.55
200	0.015	87.39
400	0.014	88.24
800	0.013	89.08
1200	0.012	89.92

Graphical presentation of % of inhibition of DPPH by *B. roxburghii*

The percentage of inhibitions that was found from the above table (1.9) was plotted in a graph shown below. The graph was drawn against the concentrations that were used in the above table (1.9).

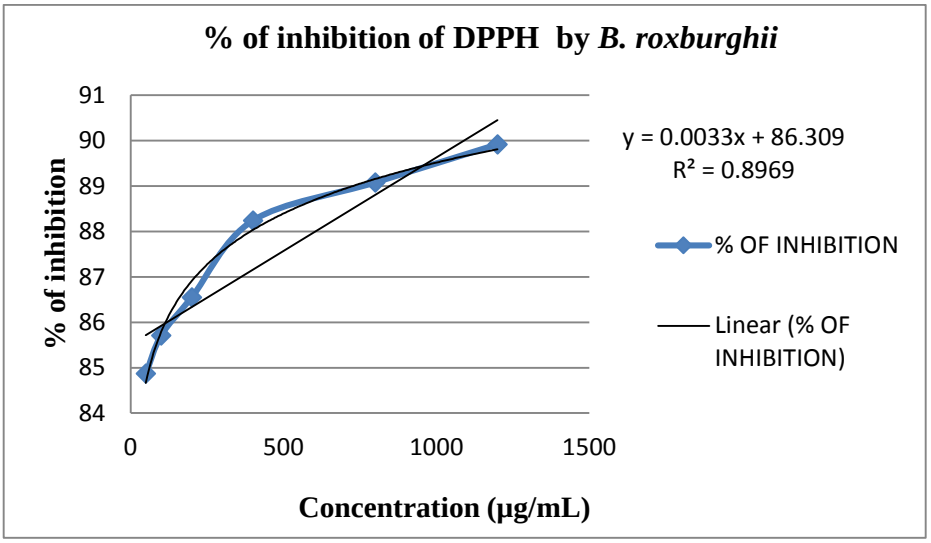


Figure 2.5: Graphical presentation of % of inhibition of DPPH by *B. roxburghii*

Interpretation

In order to investigate *in-vitro* antioxidant potential of methanolic extract of *B. roxburghii* leaves, DPPH free radical scavenging assay was performed by using L-ascorbic acid as the standard. From the table 1.9 and figure 2.5, the obtained result exhibited that, with the increase in concentration of methanolic extract of *B. roxburghii* leaves from 50 to 1200 µg/mL, the % of inhibition was also increased from 85.71 to 89.92 µg/mL. The highest % of inhibition, 89.92 µg/mL was exhibited at the highest concentration which was expressed as 1200 µg/mL. At the lowest concentration of 50 µg/mL, the methanolic extract of *B. roxburghii* leaves exhibited notable % of inhibition which was 85.71 %. Thus, it can be inferred that, DPPH free radicals are scavenged by the antioxidants present in the sample.

4.2.2. Determination of total phenolic content (TPC)

The total phenolic content of methanolic extract of *B. roxburghii* leaves was investigated by the method described by Folin-ciocalteu (Wolfe, Wu & Liu, 2003). The obtained result was recorded in the following table (1.10).

Table 1.10: Total phenolic content (TPC) in *B. roxburghii*

Concentration of *B. roxburghii* (µg/mL)	**TPC (GAE) mg of gallic acid per gram of dried extract**
200	40.17
400	64.25
800	90.92
1200	100.58

Calibration curve of standard gallic acid

The absorbance that was found from the above table (1.10) was plotted in a graph shown below. The graph was drawn against the concentrations that were used in the above table (1.10).

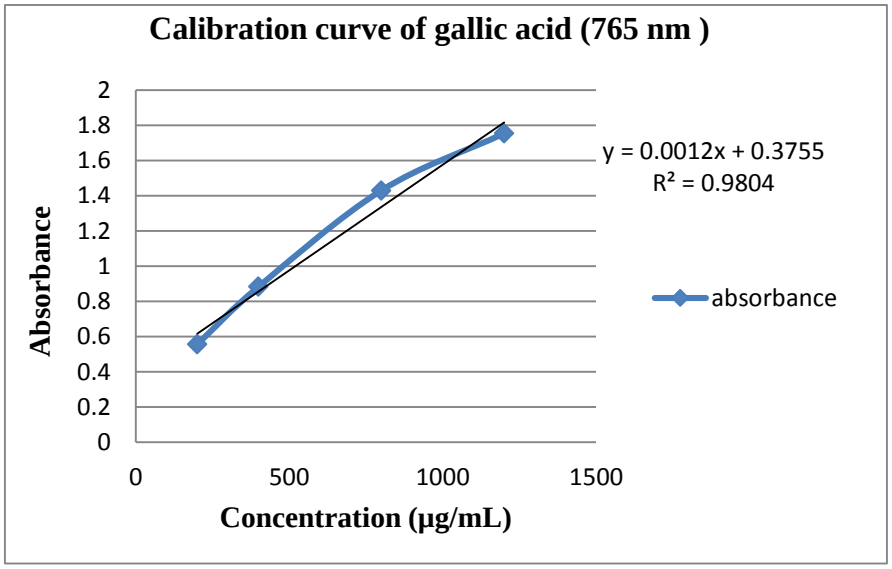


Figure 2.6: Calibration curve of gallic acid (GA) at 765 nm for determining (TPC) in *B. roxburghii*

Interpretation

In order to analyze *in-vitro* antioxidant potential of methanolic extract of *B.* roxburghii leaves, the total phenolic content was determined by the method described by Folin-Ciocalteu by using gallic acid as the standard. From table 1.10 & figure 2.6, the obtained result exhibited that, with the increase in concentration of methanolic extract of *B. roxburghii* leaves from 200 to 1200 µg/mL, the total phenolic content also increased from 40.17 to 100.58 mg of gallic acid per gram of dried extract. At the highest concentration of 1200 µg/mL, the leaf extract of *B. roxburghii* exhibited the highest value of total phenolic content which was expressed as 100.58 mg of gallic acid per gram of dried extract. It can be said that, with the increase in total phenolic content, its antioxidant potential also increases. Thus, it can be inferred that, the leaf extract of *B. roxburghii* comprises of adequate amount of gallic acid to exhibit strong antioxidant potential.

4.2.3. Determination of total flavonoid content (TFC)

In order to investigate *in-vitro* antioxidant potential of methanolic extract of *B. roxburghii* leaves, the total flavonoid content was determined (Kumaran & Karunakaran, 2007). The obtained result was recorded in the following table (2.1).

Table 2.1: Total flavonoid content (TFC) in *B. roxburghii*

Concentration of *B. roxburghii* (µg/mL)	**TFC (QE) mg of quercetin per gram of dried extract**
200	24.67
400	27.41
800	35.58
1200	41.42

Calibration curve of quercetin

The absorbance that was found from the above table (2.1) was plotted in a graph shown below. The graph was drawn against the concentrations that were used in the above table (2.1).

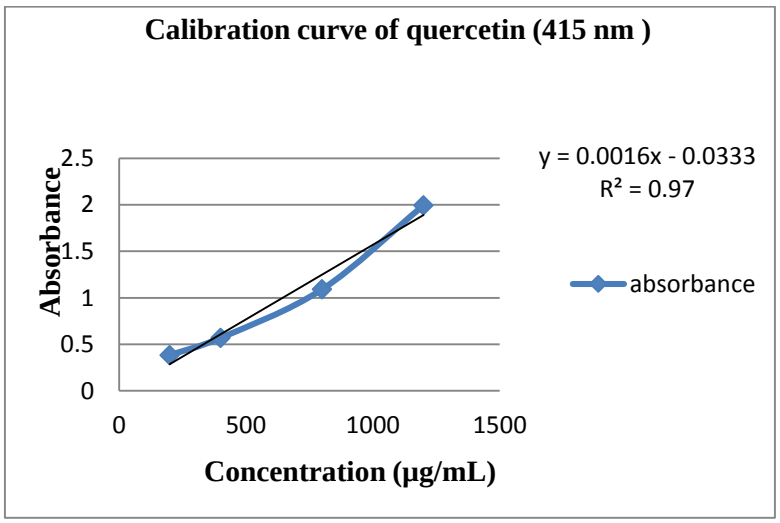


Figure 2.7: Calibration curve of quercetin at 415 nm for determining (TFC) in *B. roxburghii*

Interpretation

In order to investigate *in-vitro* antioxidant potential of methanolic extract of *B. roxburghii* leaves, total flavonoid content was determined by using quercetin as the standard. From the table 2.1 and figure 2.7, the obtained result exhibited that, with the increase in concentration of methanolic extract of *B. roxburghii* leaves from 200 to 1200 µg/mL, the total flavonoid content also increased from 24.67 to 41.42 mg of quercetin per gram of dried extract. At the highest concentration of 1200 µg/mL, the leaf extract exhibited only 41.42 mg of total flavonoid content per gram of dried extract. Thereby, it can be predicted that, a positive correlation exists between total flavonoid content of *B. roxburghii* and its antioxidant potential.

4.2.4. Determination of total antioxidant capacity (TAC)

In order to investigate *in-vitro* antioxidant potential of methanolic extract of *B. roxburghii* leaves, total antioxidant capacity was determined (Prieto et al., 1999). The obtained result was recorded in the following table (2.2).

Table 2.2: Total antioxidant capacity (TAC) in *B. roxburghii*

Concentration of *B. roxburghii* (µg/mL)	**TAC (AAE) mg of L-ascorbic acid per gram of dried extract**
200	54.59
400	67.25
800	84.16
1200	98.58

Calibration curve of L-ascorbic acid

The absorbance that was found from the above table (2.2) was plotted in a graph shown below. The graph was drawn against the concentrations that were used in the above table (2.2).

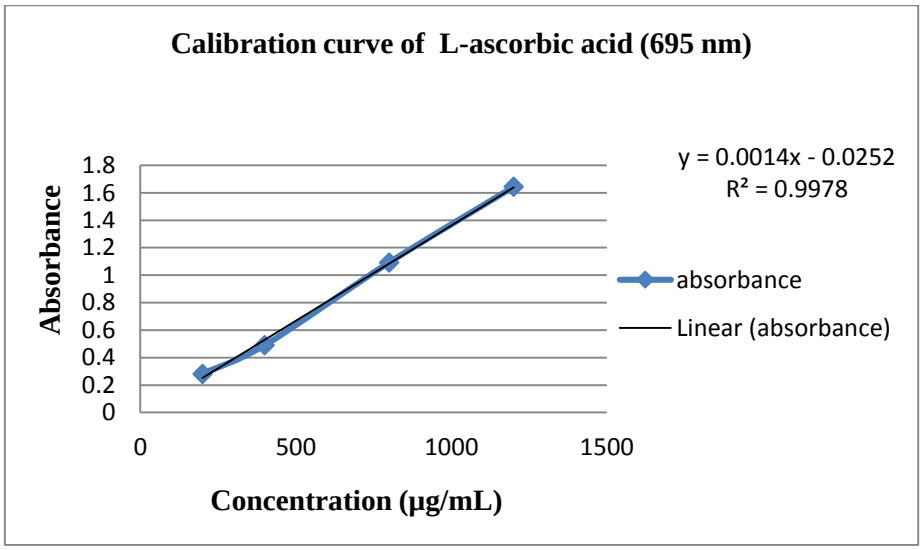


Figure 2.8: Calibration curve of L-ascorbic acid (AA) at 695 nm for determining (TAC) in *B. roxburghii*

Interpretation

In order to analyze *in-vitro* antioxidant potential of methanolic extract of *B. roxburghii* leaves, total antioxidant capacity was determined by using L-ascorbic acid as the standard. From the table 2.2 and figure 2.8, the obtained result exhibited that, with the increase in concentration of methanolic extract of *B. roxburghii* leaves from 200 to 1200 µg/mL, the total antioxidant capacity also increased from 54.59 to 98.58 mg of L-ascorbic acid per gram of dried extract. At the highest concentration of 1200 µg/mL, the leaf extract exhibited the highest value of total antioxidant capacity which was expressed as 98.58 mg of L-ascorbic acid per gram of dried extract. Thus it can be inferred that, that the leaf extract of *B. roxburghii* comprises of adequate amount of ascorbic acid to exhibit strong antioxidant potential.

4.3. Results of cytotoxic potential shown by methanolic extract of *B. roxburghii* leaves

In-vitro cytotoxicity potential was performed against HeLa cell (Cervical cancer cell line) by using MTT assay at various concentrations of (0.025 mg/mL, 0.25 mg/mL, 2.5 mg/mL, 25 mg/mL) of methanolic extract of *B. roxburghii* leaves. As a positive control, 2 % DMSO was used. For each concentration, absorbance was observed.

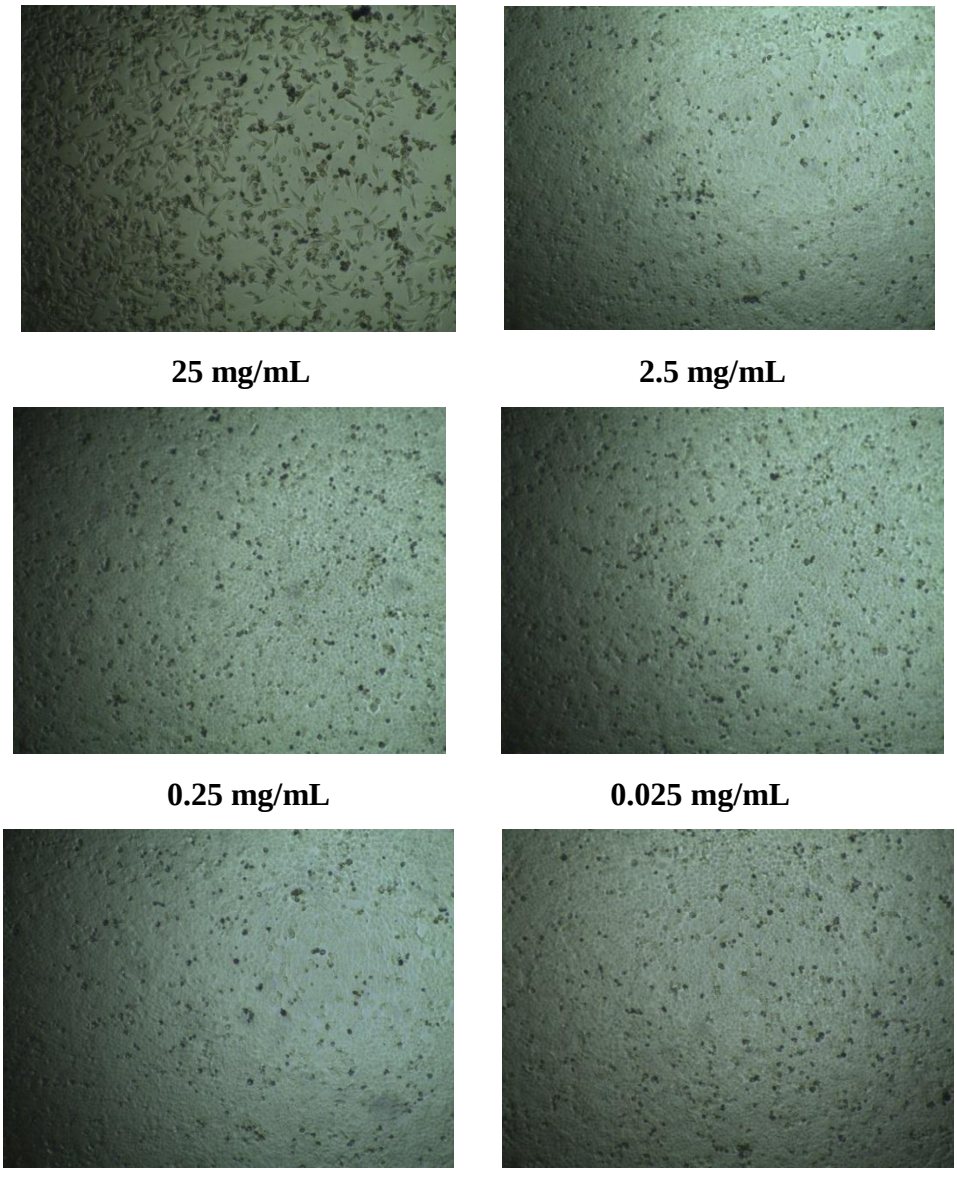


Figure 2.9: Viability of cells of methanolic extract of *B. roxburghii* leaves at various concentrations after incubation for at least 48 hours, as a positive control 2% DMSO was used.

Table 2.3: Cytotoxic analysis result

Sample ID	Absorbance at 570 nm		Remarks
Solvent (-)	2.24	2.13	At 25 mg/mL concentration : Though nearly 80 % cell death was observed in microscopic observation, it was not found in MTT. Background at 25 and 2.5 mg/mL is high suggesting that the sample color has interfered in MTT result.
Solvent (+)	2.09	1.33	
Background	0.169	0.10	
25	2.591	1.93	
Background	0.764	0.56	
2.5	2.536	2.00	
Background	0.242	0.23	
0.25	2.232	1.81	
Background	0.125	0.12	
0.025	1.822	2.36	
Background	0.104	0.11	

Background: only sample

Solvent (-): only cell

Solvent (+): solvent (DMSO)

25 mg/mL: sample + cell

Table 2.4: % of cell survival and % of inhibition of cells by methanolic extracts of *B. roxburghii* leaves

Sample Conc. (mg/mL)	% of survival of cells	% of inhibition of cells
Control 2 % DMSO	≥ 85	15
0.025	≥85	15
0.25	≥ 85	15
2.5	≥ 85	15
25	≥20	80

Interpretation

In-*vitro* cytotoxicity potential was performed against HeLa cell (Cervical cancer cell line) by using MTT assay at various concentrations In the medium, HeLa cell was subjected to 0.025 mg/mL, 0.25 mg/mL, 2.5 mg/mL, and 25 mg/mL concentrations of methanolic extract of *B. roxburghii* leaves. HeLa cell was incubated for at least 48 hours. After examining the cells under a microscope, it was exhibited that, at the highest concentration of 25 mg/mL, methanolic extract of *B. roxburghii* leaves showed 80 % of the cell death whereas HeLa cell survival was only 20 %. In contrast, at the lowest concentrations of 0.025 mg/mL and 0.25 mg/mL, 2.5 mg/mL, the survival of HeLa cell was 85 % which indicated that, only 15 % of cell death was occurred at that concentration. From the above discussion, it can be suggested that, methanolic extract of *B. roxburghii* leaves devoid of weak cytotoxic potential. Therefore, the findings of the current study exactly matched with the previous studies. Therefore, this study exhibited that, there was no cell death at lower concentrations whereas at the highest concentration of 25 mg/mL, significant number of cell death occured which is 80 %. Therefore, it indicates that *B. roxburghii* leaf extract exhibits cytotoxic potential at the highest concentration.

CHAPTER: 5

DISCUSSION

Chapter 5: Discussion

B. roxburghii is a medicinal plant of Begoniaceae family. This plant is indigenous to Indian subcontinent there by it is also known as East Himalayan Begonia. The leaves of this plant are effective to cure various gastrointestinal disorders like diarrhea, dysentery as well as for the treatment of various skin diseases. Literature review showed that, leaf extract of *B. roxburghii* devoid of antimicrobial activity that prevents the growth of harmful microorganisms in human body as well as antioxidant and cytotoxic potential in order to suppress the growth of cancer cells in our body.

The current study was aimed in order to investigate *in-vitro* antioxidant and cytotoxic potential of methanolic extract of *B. roxburghii* leaves that had not been previously conducted. In this study, the antioxidant potential was performed by using four different test methods on methanolic extract of *B. roxburghii* leaves which was DPPH free radical scavenging assay, total phenolic content, total flavonoid content and total antioxidant capacity. Along with that, *in-vitro* cytotoxic potential of methanolic extract of *B. roxburghii* leaves was also performed by using MTT assay on HeLa cell lines (Cervical cancer cell).

Literature review revealed that, phytochemical screening of the leaf extracts of *B. roxburghii* exhibited that, the leaf extract comprises of tannins, alkaloids, flavonoids, glycosides, resins, steroids, carbohydrates and phenols that have definite pharmacological actions on human body. In the current study, phytochemical screening of methanolic extracts of *B. roxburghii* leaves exhibited that, the leaf extract devoid of tannins, alkaloids, flavonoids, glycosides, resins, steroids, carbohydrates and phenols that have definite pharmacological actions on human body. In contrast, it was also revealed that, methanolic extract of *B. roxburghii* leaves does not comprise of phytosterols and saponins. Therefore, the current study findings exactly matched with the literature review.

Literature data also exhibited that, leaf extracts of *B. roxburghii* devoid of antioxidant potential. Therefore, methanolic extract of the leaves of *B. roxburghii* was introduced to various *in-vitro* antioxidant assays. In order to investigate *in-vitro* antioxidant potential of methanolic extract of *B. roxburghii* leaves, DPPH free radical scavenging assay was performed by using L-ascorbic acid as the standard (Braca et al., 2001). From table 1.9 and figure 2.5, the obtained result exhibited that, with the increase in concentration of methanolic extract of *B. roxburghii* leaves from 50 to 1200 µg/mL, the % of inhibition also increased from 85.71 to 89.92 µg/mL. The highest % of inhibition of 89.92 µg/mL

was obtained at the highest concentration which was expressed as 1200 µg/mL. At the lowest concentration of 50 µg/mL, methanolic extract of *B. roxburghii* leaves exhibited notable % of inhibition which was 85.71 %. A notable % of inhibition was also exhibited by other concentrations. The obtained result was then compared with the standard, which exhibited that, a notable % of inhibition was showed by the methanolic extract of *B. roxburghii* leaves that was close to the standard.

Thus, it can be inferred that, DPPH free radicals are scavenged by the antioxidants present in the extract. In other words, it can be said that, methanolic extract of *B. roxburghii* leaves devoid of strong antioxidant potential.

Thereafter, in order to analyze *in-vitro* antioxidant potential of methanolic extract of *B. roxburghii* leaves, the total phenolic content was also determined by the method described by Folin-Ciocalteu by using gallic acid as the standard (Wolfe, Wu & Liu, 2003). From table 1.10 and figure 2.6, the obtained result exhibited that, with the increase in concentration of methanolic extract of *B. roxburghii* leaves from 200 to 1200 µg/mL, the total phenolic content or in other words, the antioxidant potential also increased from 40.17 to 100.58 mg of gallic acid per gram of dried extract. At the highest concentration of 1200 µg/mL, the leaf extract of *B. roxburghii* showed the highest value of total phenolic content which was expressed as 100.58 mg of gallic acid per gram of dried extract. It can be said that, with the increase in total phenolic content, its antioxidant potential also increases. By analyzing the result, it can be inferred that, leaf extract of *B. roxburghii* comprises of adequate amount of gallic acid to exhibit strong antioxidant potential.

Also, the total flavonoid content was determined in order to investigate *in-vitro* antioxidant potential of methanolic extract of *B. roxburghii* leaves, by using quercetin as the standard (Kumaran & Karunakaran, 2007). From table 2.1 and figure 2.7, the obtained result exhibited that, with the increase in concentration of methanolic extract of *B. roxburghii* leaves from 200 to 1200 µg/mL, the total flavonoid content also increased from 24.67 to 41.42 mg of quercetin per gram of dried extract. At the highest concentration of 1200 µg/mL, the leaf extract exhibited only 41.42 mg of total flavonoid content per gram of dried extract. Thereby, it can be predicted that a positive correlation exists between total flavonoid content of *B. roxburghii* and its antioxidant potential.

Afterwards, in order to analyze *in-vitro* antioxidant potential of methanolic extract of *B. roxburghii* leaves, total antioxidant capacity was also determined, by using L-ascorbic acid as the standard (Prieto et al., 1999). From table 2.2 and figure 2.8, the obtained

result exhibited that, with the increase in concentration of methanolic extract of *B. roxburghii* leaves from 200 to 1200 µg/mL, the total antioxidant capacity also increased from 54.59 to 98.58 mg of L-ascorbic acid per gram of dried extract. At the highest concentration of 1200 µg/mL, the leaf extract exhibited the highest value of total antioxidant capacity which was expressed as 98.58 mg of L-ascorbic acid per gram of dried extract. Thus, it can be inferred that, leaf extract of *B. roxburghii* comprises of adequate amount of ascorbic acid to exhibit strong antioxidant potential.

Therefore, the higher DPPH scavenging, TPC and TAC of leaf extract of *B. roxburghii* can be attributed to the fact that the leaf extract possess strong antioxidant potential (Keen et al., 2005). Therefore, the findings of the current study exactly matched with the literature review.

Moreover, from the previous study it was also found that, the leaf extract of *B. roxburghii* devoid of cytotoxic potential. As no cytotoxic study of leaf extract of *B. roxburghii* was conducted previously, in the current study, *in-vitro* cytotoxicity potential was performed against HeLa cell (Cervical cancer cell line) by using MTT assay at various concentrations of methanolic extract of *B. roxburghii* leaves in order to analyze the viability of cells. Afterwards, the medium was incubated for at least 48 hours by utilizing micro plate reader and the viable cells were determined. As a positive control, 2 % DMSO was used.

Afterwards, in the medium, HeLa cell was subjected to 0.025 mg/mL, 0.25 mg/mL, 2.5 mg/mL, and 25 mg/mL concentrations of methanolic extract of *B. roxburghii* leaves. HeLa cell was incubated for at least 48 hours. By analyzing the result, it was exhibited that, no cell death occurred at lower concentrations whereas at the highest concentration, significant number of cell death occurred.

After examining the cells under a microscope, it was exhibited that, at the highest concentration of 25 mg/mL, methanolic extract of *B. roxburghii* leaves showed 80 % of the cell death whereas the cell survival was only 20 % at that concentration. In contrast, at the lowest concentrations of 0.025 mg/mL and 0.25 mg/mL, 2.5 mg/mL, survival of HeLa cell was 85 % which indicated that, only 15 % of cell death was occurred at that concentration. From the above discussion, it can be suggested that, methanolic extract of *B. roxburghii* leaves devoid of weak cytotoxic potential. Therefore, the findings of the current study exactly matched with the previous studies.

CHAPTER: 6

CONCLUSION AND FUTURE DIRECTION

Chapter 6: Conclusion & future direction

Conclusion

B. roxburghii has been traditionally utilized to cure various gastrointestinal disorders including bile dysentery, diarrhea and jaundice. The plant is also effective against throat, mouth and various skin diseases, respiratory tract and HIV infections and blood cancer. Leaves of *B. roxburghii* has been reported to possess different therapeutic application such as, antimicrobial activity that prevents the growth of harmful microorganism in human body as well as antioxidant and cytotoxic potential in order to suppress the growth of cancer cells in our body. In this study, four methods were used for instance, DPPH free radical scavenging assay, total phenolic content, total flavonoid content, total antioxidant capacity in order to investigate *in-vitro* antioxidant potential. From DPPH free radical scavenging assay, the obtained result showed that, the highest % of inhibition 89.92 % was found at the highest concentration of 1200 µg/mL which signified that methanolic extract of *B. roxburghii* leaves possess notable antioxidant potential. Also, the leaf extract exhibited remarkable value of total phenolic content which was expressed as 100.58 mg of gallic acid per gram of dried extract at the highest concentration of 1200 µg/mL. Antioxidant capacity also exhibited remarkable result which was expressed as 98.58 mg of ascorbic acid per gram of dried extract at the highest concentration of 1200 µg/mL. Along with that, *in-vitro* cytotoxic potential of the methanolic extract of *B. roxburghii* leaves was also assessed by MTT assay at various concentrations on HeLa cell line (Cervical cancer cell). From the cytotoxicity test, it was revealed that, the leaf extract exhibited 80 % of the cell death at the highest concentration which was expressed as 25 mg/mL. In contrast, at that concentration, the survival of the cell was only 20 % which was not that much significant to utilize the leaf extract of *B. roxburghii* as an anticancer agent. Finally, it was established that, *in- vitro* antioxidant potential exhibited significant results in three methods namely DPPH free radical scavenging assay, TPC, TAC whereas cytotoxic potential was not that much significant.

Future directions

Pharmacological investigation of *in-vivo* antioxidant potential would be performed on *B. roxburghii* as it has significant antioxidant potential. However, further study can be conducted on this plant for inventing unfamiliar properties and unexplored pharmacological activities and to familiarize the plant as a new source of medicine.

REFERENCES

References

Alam, M. N., Bristi, N. J., & Rafiquzzaman, M. (2013). Review on *in-vivo* and *in-vitro* methods evaluation of antioxidant activity. *Saudi Pharmaceutical Journal*, *21*(2), 143–152.

Ali, N.A., Julich, W.D., Kusnick, C., & Lindequist, U. (2001). Screening of Yemini medicinal plants for antibacterial and cytotoxic activities. *Journal of Ethnopharmacology*, *74*(2), 173-179.

Alam, M. N., Bristi, N. J., & Rafiquzzaman, M. (2013). Review on *in-vivo* and *in-vitro* methods evaluation of antioxidant activity. *Saudi Pharmaceutical Journal*, *21*(2), 143–152.

Amutha, S., & Sreedevikumari ,T. (2016). Evaluation of antibacterial activity of different solvent extracts of *Begonia cordifolia*. *International journal of Zoology and Applied Biosciences, 1 (*3), 144-147.

Bolton, J.L., Trush, M.A., Penning, T.M., Dryhurst, G., & Monks, T.J. (2000). Role of quinones in Toxicology. *Chemical Research in Toxicology, 13*(3), 135–160.

Burton, G.W., & Ingold, K.U. (1984). Beta-carotene: An unusual type of lipid antioxidant. *Journal of Medicinal Plant Studies, 22*(6), 569–573.

Ramesh, N., Viswanathan, M., Saraswathy, A., Balakrishna, K., Brindha, P., & Lakshmanaperumalsamy, P. (2002). Phytochemical and antimicrobial studies of Begonia malabarica. *Journal of Ethnopharmacology*, *79*(1), 129-132.

Fuller, R.W., Cardellina, J.H., Cragg, G.M., Boyd, M.R., (1994). Cucurbitacins differential cytotoxicity, dereplication and first isolation from *Begonia*. *4* (7), 59-78.

Jose, S., & Kumar, T. S. (2016). Preliminary Studies on Phytochemical Properties and Antimicrobial Activity of *Begonia trichocarpa*. *International Journal of Research in Ayurveda & Pharmacy, 7*(5), 91-96.

Kalpanadevi, V., & Mohan, V. R. (2012). In vitro antioxidant studies of *Begonia malabarica* Lam. and *Begonia floccifera*. *Asian Pacific Journal of Tropical Biomedicine, 2*(3), 142-156

Punithavathi, M., Aranganathan, J., & Janani, M. (2014). Antibacterial Activity and Phytochemical Screening of Begonia roxburhii Linn. (Leaves). *LS: International Journal of Life Sciences, 3*(1), 28.

Rajani, M. K. (2013). DPPH Free Radical Scavenging Activity. *Journal of medicinal plant Studies*, *7*(6), 60-88.

Sumathi, P., & Parvathy, A. (2010). Antimicrobial activity of some traditional medicinal plants. *International journal of Zoology and Applied Biosciences, 4(*4), 316-32.

Thatoi, H., & Dutta, S. (2008). Antimicrobial activity and ethano medicinal uses of some medicinal plants from Similipal Biosphere Reserve. *Asian Medical Journal Plant Science, 3* (7), 260-267.

Vadlapudi, P., & Chandrasekar, N. (2010). *In-vitro* bioactivity of Indian medicinal plant *Begonia roxburghii Journal of Global Pharmaceutical Technology, 2*(2), 43-45.