Phytochemical and Biological Evaluation of *Begonia roxburghii*

A project submitted

by

Ahsania Akter

ID: 12146011

Session: Spring 2012

to

The Department of Pharmacy

in partial fulfillment of the requirements for the degree of

Bachelor of Pharmacy (Hons)

Dhaka, Bangladesh

September, 2016.

CERTIFICATION STATEMENT

This is to certify that this project titled “Phytochemical and Biological investigation of *Begonia roxburghii”* submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy, BRAC University constitutes my own work under the supervision of Mir IshrunaMuniyat, Lecturer, Department of Pharmacy, BRAC University and checked by Dr. Sharmind Neelotpol, Assistant Professor, Department of Pharmacy, BRAC University. The credit has been given where I have used the language, ideas or writings of another.

Signed by,

_____

Counter signed by,

_____

ACKNOWLEDGEMENT

I would like to thank almighty Allah for the strength, wisdom and courage to pursue such a huge endeavor. I know that this accomplishment was achieved because of the prayers of myself and others, along with Allah's grace mercy on my life. I want to express my gratitude to those people with due respect who helped me in every possible way to complete this project. I have truly drawn upon my experiences and knowledge as a student of Pharmacy to complete this project. I have also received help from number of persons to complete this project and I would like to thank them all to finish this huge task within its deadline. I would like to express my gratitude to the honorable chairperson Dr. Eva Rahman Kabir, Department of Pharmacy, BRAC University. Dr. SharmindNeelotpol, Assistant Professor, Department of Pharmacy, BRAC University and my respected Course instructor Mir IshrunaMuniyat, Lecturer, Department of Pharmacy, and BRAC University who has supported and gave me the chance to prepare the project on this topic.

ABSTRACT

The aim of this study was to investigate the Phytochemical and Biological properties of *Begonia roxburghii* from *Begoniaceae* family. To meet the goal of this study several chemical tests was performed such as test for tannin, resin, saponin, glycoside and alkaloids. By observing the results from required chemical tests it has been found that this medicinal plant contains some essential phytochemicals such as tannin, saponin, resin, glycoside and alkaloids. To investigate the biological property such as antioxidant activity the DPPH assay was performed. To investigate the cytotoxicity of the plant extract the Brine shrimp lethality bioassay was performed. At the end, moderate antioxidant property has been observed and slightly higher cytotoxic property has been found in this evaluation.

Table of contents

Chapter: 1 - Introduction

Chapter: 2 - Methodology

List of table

List of figure

List of Abbreviations:

- mg = Milligram
- gm = Gram
- mm = Millimeter
- ml = Milliliter
- µg = Microgram
- BHA = Butyl Hydroxy Anisole
- BHT = Butyl Hydroxy Toluene
- IC_{50} = Median Inhibition Concentration
- LC_{50} = Lethal Concentration
- DPPH = 2,2-Diphenyl-1-Picrylhydrazyl
- UV = Ultraviolet
- DMSO = Dimethyl Sulfoxide
- GI = Gastrointestine
- HCl = Hydrochlorc Acid
- FRS= Free Radical Scavengers/Scavenging
- R^2= Regression Coefficient
- *A.scaphula= Anisopterascaphula*
- WHO= World Health Organization
- m= Meter
- cm= Centimeter

Dedicated to my parents

1. INTRODUCTION

The name ‘phytochemical’ is derived from Greek culture where the Greek word “Phyton” means plant. Phytochemicals are cellular active compounds found in plants that appear naturally and provide biological functions in human and other living beings. These chemical compounds shield plants from danger and injury and furnish the plant with unique color, aroma and flavor. Practically, these cellular active chemical components that shield the plant cells from climatic trouble like the introduction of harmful products, exertion, scarcity and microbial attack, are known as phytochemicals. Day by day the use of chemical components which are obtained from plant for medicinal purposes has been considerably increased. (Ellof, 1998; saxena et al, 2013)

Plants with medicinal activities have been the origin of classical medicine amongst the country field inhabitant across the globe. The Sumerian and the Akkad Ian civilizations are assumed to be few of the first civilizations to use medicinal plants with their biological aspects. Around 400 herbal plants were placed in a ranking by an ancient researcher who elaborated naturally occurring products of plants with their biological application (Doughari et al, .2009). In Chinese, Egyptian and Ayurvedic products formed by nature have been fundamental part of the old cultural medicine system (Sarkar and Nahar, 2007). It is assumed that approximately 3.4 billion people in the world are highly depended on medicines produced by plants. This dimension corresponds to the 88 percent of the dwellers in earth who depend on ancient traditional medicines for their basic relevant biological problems. (Doughari et al, 2009).

According to the World Health Organization (WHO), a medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for pharmaceutical semi synthesis. Biologically active chemical compounds can often be found in various parts of these medicinal plants including its leaves, roots, rhizomes, stems, barks, flowers, fruits, grains or seeds and they are being used in the manipulation or analysis of disorders of human body.

Variation among plant species can be observed all over the world. The allowance of degree of tendency to study the species abundance describes the progressive development of the branch of knowledge dealing with past events of diversity among and within plant and animal species in the environment. Furthermore, these information from many investigations are ranked in the

background of history of geology (Mittelbach et al., 2007). The study of plants in the particular geographical distribution dealing with the organisms mark with relative magnitude of time. In addition, this development point out that the augmentation of this degree of tendency is both ancient ("museum" model) and recent ("cradle" model) within groups (Birmingham& Dick, 2001; McKenna & Farrell, 2006). A further step of dilemma that makes it difficult to put in order the progressive development of study that motivates the equatorial biological evolution are cellular intercommunication like herbal clashes and organism intercommunication (Berenbaum and Zangerl, 2006). However , the study of progressive development of the science of living materials come up with some basic questions like what are the aspects that motivating the biological evolution can be known by elaborated studies of branch of biology.

Phytochemical technique is a technique which deals with the quality control of medicinal plants such as saponins, alkaloids, flavonoids, volatile, oils and anthraquinones etc. In case of herbal medicines, most of the time biologically active compounds are not detected properly and therefore their action cannot be predicted accurately. To investigate the phytochemical compounds of medicinal plants properly it is essential to have adequate knowledge regarding phytochemicals. (Houghton Mifflin, 2002)

In 1690, a French botanist named Charles Plumier represented *Begonias* for the first time and the name *Begonias* were given after Michel Begon, one of the fellow French botanists. Many other names had been used before for *Begonias* but the name was finally established when Linnaeus used this name in his species Plantarum in 1753. Furthermore, in 1777 the first living *Begonia* arrived in Europe and by 1847 almost 70 to 80 species were cultivated. (Charles Valin, 2014)

1.1 Genera of *Begoniacea* family

There are two genera of *Begoniaceae*family. One of them is *Hillebrandia* and it has only one species. This species of *Hillebrandia*is indigenous plant to the Hawaiian Islands and this is the only member of the *Begoniaceae* family which can survive in those islands. Another one is the genera named *Begonia* which consists of around 1400 species with worldwide distribution. The relationships between *Begoniaceae* and the *Cucurbitales* were suggested by following datas from "The study of *Begoniaceae*". Accordingly, the approximate age of both *Begonia* and the *Begoniaceae*were described. *Hillebrandia*as are one of the familiar group to the *Begoniaceae*and their ranking were sustained by much analysis. Investigation indicates that *Hillebrandia* lineage is approximately 51-65 million years old. In addition, the *Hillebrandiasandwicensis* had survived on the Hawaiian island from the very ancient period. (Wendy et al; 2003)

1.2 Various plants of *Begoniaceae* family

The *Begonia* genera of *Begoniaceae* family contains approximately 1400 species and this *Begoniaceae* family can be classified on the basis of various factors (Wendy et al; 2003). Classification on the basis of their difference in the structure of root, leaves, etc. are discussed below:

1.2.1 Tuberous:

The name tuberous was given due to their tuber like structure of root. It can be assumed that tuberous *Begoniaceae*are most famous all over the world. Moreover, this tuberous *Begoniaceae*can be further classified based on whether they are single or double flowered, in terms of smell etc. (Jackson and Perkins, 2014).

Figure 1.1: Tuberous *Begoniaceae* (collected from American Begonia Society 2016)

1.2.2 Fibrous:

Fibrous *Begoniaceae*are next in fame followed by the tuberous *Begoniaceae* especially in America. Fibrous *Begoniaceae*are used to decorate house in America. The root of this *Begoniaceae* resembles the structure of fiber (Jackson and Perkins, 2014).

Figure1.2: Fibrous *Begoniacea*(collected from American Begonia Society 2016)

1.2.3 Rhizomatous:

Rhizomatous *Begoniaceae*is indigenous to Asia and America. The modern strain of Rex *Begoniaceae*are descented from this rhizomatous *Begoniaceae*. The name rhizome is apparently due to their property of rhizome which is attached to soil like reptiles. (Jackson and Perkins, 2014)

Figure1.3: Rhizomatous *Begoniaceae*(*collected* from American Begonia Society 2016)

1.2.4 Bulb:

Bulb *Begoniaceae* belongs to small classes of *Begoniaceae* and the root structure is likely the structure of bulb. This *Begoniaceae*was derived from arid island in the Indian Ocean. Bulb *Begoniacea* were first discovered in 1880 (Jackson and Perkins, 2014).

Figure1.4: Bulb *Begoniaceae*(collected from American Begonia Society 2016)

1.2.5 Cane-like:

Cane- like *Begoniaceae* is very close to the structure of bamboo and contain a long internodes. These plants are very popular as they are easy to grow and enhances the natural beauty with their various colorful heavily flowers. Bright red, orange, pink, white and all shades in between these colors are common of cane-like *Begoniaceae.* (Jackson and Perkins, 2014)

Figure1.5: Cane-like *Begoniacea* (collected from American Begonia Society 2016)

1.2.6 Thick-stem:

The length of thick-stem *Begoniacea*is five or six feet. These plants grow upward with tall appearance. Sometimes they are branched and sometimes non-branched. Thick-stem *Begoniacea*contains leaves surrounding the ascending axis of the plant (Jackson and Perkins, 2014).

Figure1.6: Thick-stem *Begoniaceae*(collected from American Begonia Society 2016)

Begoniaceae are usually plants which contain stem with abundance of juice and grow in wet and suitable for warm and gloomy climate and are exceptionally adapted to an arid environment. There are apparently wide variations among species of *Begoniaceae* family and most of them are greenish in color, some are red in color, some are combination of both and some appears with so many additional colors. Besides, there are other relevant factors which differ from each other. Their structure of root, shape of leaves, individual smell, length of ascending axis of the plant, nature of the male and female flower etc. In spite of having these variations *Begoniaceae*are

classified from different point of view. There are some most common species of *Begoniaceae* family that occurs in nature. They are –

1.3.1 *Begonia conchifolia*:

Begonia conchifolia contains a rhizome which is supposed to help in the multiplication or reproduction of the plant. The lustrous surface of shield shaped leaves is considered to protect the leave. This species is indigenous to rainy forest region of Costa Rica and Panama. (Dietrich, 1851)

Figure1.7: *Begonia conchifolia*(collected from The Begonia page, Botanical glossary)

1.3.2*Begonia peltata:*

Begonia peltata appears with juicy stem which are freely branched and the ascending axis of the plant is matted with white colored substance. The combination of leaves of this plant surrounding this white colored substance which are green in color and round in shape appears as silver color. (Otto and Dietrich, 1841).

Figure1.8: *Begonia peltata (*collected from The Begonia page, Botanical glossary)

1.3.3 *Begonia radicans*:

Begonia radicans contain sub-shrub which is filled with juicy substances. The leaves of this plant are dark green colored with a bunch of light pink colored flowers. They are familiar to grow in the coastal region of Eastern Brazil. (Vellozo, 1831)

Figure1.9: *Begonia radicans*(collected from The Begonia page, Botanical glossary)

1.3.4 *Begonia fuschioide:*

*Begonia fuschioide*contains small leaves highly lustrous green leaves. The leaves of this plant are pendulous in shape. This plant blooms flowers which are pink, red or combination of both in color with attractive smell. Their growing nature is spreading. (Vellozo, 1831)

Figure1.10: *Begonia fuschioide*(collected from The Begonia page, Botanical glossary)

1.4 *Begonia roxburghii:*

Begonia roxburghii is a kind of herbal medicinal plant from *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. In Bangladesh *Begonia roxburghii* grows in the shady moist places of Chittagong hilly tracts region. (Mark Hughes, 1829)

Figure1.11: *Begonia roxburghii (*collected from East Himalayan Begonia)

The feature which differentiates this plant from other species of *Begoniaceae* family is the red pigmented dots over the ascending axis of the plant. Leaves are round and sharp at the end. Flowers are white in color with a yellow bunch at the center. Flowers have four petals which appear with limited space at the end. Its fruits are square in shape and divided into four roundish projection and those are enveloped by red dots. The length of the juicy stem of *Begonia*

roxburghii is approximately 30-90 cm long and the dimension of leaves are 15-22.8 cm. (Mark Hughes, 1829)

1.4.1 Taxonomical hierarchy –

Kingdom – plantae

Division – Magnoliophyta

Class – Magnoliopsida

Order – Cucurbitales

Family – Begoniaceae

Genus – Begonia

Species – Roxburghii

(Agardh, 1824)

1.4.2 Other species with phytochemical properties and descriptions:

Previous phytochemical studies on *Begoniaceae*family investigated that few number of species of *Begoniaceae* family contain phytochemicals which have biological activity. Such as-

It has been found that *Begonia floccifera* contains saponin. It is a polycyclic aglycon 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)

Tannins are plant polyphenols which is produced by nature. It is composed of very different form of oligomers and polymers. Chemical formula of tannin is $C_{76}H_{52}O_{46}$. One of the most important characteristic of tannin is that they attach with protein and causes the precipitation of protein.

Tannin solution is acidic in nature and contains astringent property. Tannin is also known as tannic acid. It is responsible for astringency. Tannins are found in the region of root, bud, leaf and stem of plant tissue. (Solomon jeeva, 2012)

Coumarin is a compound of heterocyclic organic group. Chemical formula of coumarin is $C_6H_9O_2$. Orthohydroxylation of cinnamic acid produces coumarin. Coumarin stimulates macrophages and is used in High Protein Edema disorder. In addition, coumarin also causes the activation of other immune system. Hence, used in the chronic inflammation. The main property of coumarin is it is an anticoagulant. It is responsible for the block of multiple steps in coagulation cascade. Coumarin is inhibitors of vitamin k which motivate the conversion of prothrombin to thrombin (Goodman and Gilman, 2006). Thus, it is used worldwide in the blood coagulation disorder.

Begonia peltata and *Begonia conchifolia* gives 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 serious chronic diseases such as cancer, heart disease, stroke, Alzheimer's disease, Rheumatoid arthritis and cataracts. 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)

In addition, *Begonia radicans* give antimicrobial activity. Greek word anti means against, micros 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 microorganism in human body. Some essential volatile oils are assumed to possess antimicrobial activity. (Agardh, 1824)

The variable color of leaves and flowers among species of *Begoniaceae*family occur because of the presence of different chemicals in the individual plants. (Blair and Kilsyth, 1824)

White Begonia means there is generally no color is present. Accordingly there is no light to be scattered which will be seen. Thus it appears as white color in nature. Moreover, in case of blue Begonia the fabrics of flower pigmentation are because of the presence of substance with chemical nature. Researchers have showed that genes in the chromosome influence the quantity

and properties of these chemical substances. The attractive colors of *Begoniaceae*which grab the attention is due to the presence of soluble glycosides. The flower cell of *Begoniaceae* contains glycoside which is homogenously dispersed in vacuoles and there are protoplasm which surrounds those vacuoles. There are presence of some plastids which cannot be combined with glycosides and produce orange or yellow color in white *Begoniaceae*. (Blair and Kilsyth 1824)

Glycosides are divided into Anthoxanthins and Anthocyanins according to the color they reflect in a particular plant.

Colors from pale ivory to deep yellow occurs due to the presence of Anthoxanthins and the crimson, scarlet and red to all shades of yellow colors occur due to the presence of Anthocyanins.

So, *Begonia roxburghii* being in the same family it can be assumed that these properties are also present in this plant. As the aim of this study is to evaluate the phytochemical properties of *Begonia roxburghii*some particular properties are selected to conduct the elucidation. Those are –

- Tannin content
- Saponin content
- Glycoside content
- Resin content
- Cytotoxicity
- Antioxidant activity.

1.4.3 Significance of solvents:

It is necessary to convert the blended powder into solution to perform all the tests required for the elucidation of those selected properties. Hence, solvents are needed for making a solution. Some specific solvents are being used to perform the relevant tests like – methanol, petroleum ether, chloroform and carbon tetra chloride.

Methanol is an organic solvent and it is also termed as methyl alcohol. Chemical formula of methanol is CH_3OH. It is one of the simplest alcohols. Methanol is produced by some bacteria during their anaerobic metabolism. Methanol appears with polar liquid property at room temperature. It is polar and volatile in nature. Chemical formula of methanol is CH_3OH. Methanol is reactive with air and by reacting with air it gives carbon di-oxide and water. Because of the strength of OH group methanol is completely miscible and highly dissolved in water. It is widely used as a solvent because of its high solubility.

Petroleum ether is an organic solvent. It is highly volatile and flammable in nature. It is also known as benzene. Chemical formula of petroleum ether is C_7H_7BrMg. From the pentane family it is a light hydrocarbon. In the nature it is produced by refining process of gasoline. The main application for using petroleum ether in laboratory is that fats and lipids are highly suspended in this solvent without hampering their basic chemical properties. Thus, it is used in plant extraction.

Chloroform is an organic solvent and it is nonflammable. Chemical formula of chloroform is $CHCl_3$. It shows reaction with strong oxidants. It is unable to be dissolved in water. Chloroform is highly dissolved in alcohol, ether, acetone, gasoline and other organic solvents. It is an important solvent for organic compound as well as gums, fats, resins, sulfur and iodine etc.

2. Methodology

2.1 Preparation of methanolic extract:

Collected leaves of *Begonia roxburghii*was dried by washing properly. These dried leaves were blended. After blending 500 gm of powder was obtained. This dry powder powder was soaked with 2.5 liter of methanol for seven days in two tightly sealed amber bottles. After observing for seven days the mixture was filtered. Filtration process was done in two steps. At first it was filtered using cotton and secondly it was filtered using the filter paper. Total one thousand (1000) ml of the sample was obtained. Then this sample was subjected to rotary evaporator for the evacuation. Using this process used methanol was separated from the plant extract. After the separation of methanol the amount of remaining plant extract was 25 ml which was dried by keeping in the fume hood. Then drying for five days the methanolic extract was prepared. Weight of the methanolic extract was 12.5 gm.

Figure2.1: Filtration of the methanolic extract

Figure2.2: Evaporation in the rotary evaporator

Figure 2.3: After evaporation in the rotary evaporator plant extract placed in petri dish

2.2 Partitioning of methanolic extract using different solvent

Partition of crude extract from plant leaves was performed by Kupchan and Tsou (1973) method and their modified version Wagenen et al. (1993). Total 5 gm of the crude extract was dissolved with 10% aqueous methanol. Then it was extracted with petroleum ether, chloroform and ethyl acetate sequentially. All the fractions obtained by partitioning using different solvents were dried and was used for performing several test required in the study.

2.2.1 Partitioning using petroleum ether

A separating funnel was taken and the mother solution was placed in the separating funnel. About 100ml of petroleum ether was added to the mother solution in a separating funnel. It was shaken vigorously and was kept for a while. The upper portion of the separating funnel is an organic portion which is soluble in petroleum ether. This upper portion was collected. The process was repeated thrice. At the end all the fractions obtained by petroleum ether partition was dried.

2.2.2 Partitioning using chloroform

About 16ml of distilled water was added to the mother solution that was left after partitioning with petroleum ether and was mixed properly. Then 100 ml of chloroform was added to the mother solution in the separating funnel and was shaken. It was kept for separation. The bottom layer was the expected portion which was collected and the process was repeated for thrice. All the fractions obtained by chloroform partition were dried.

2.2.3 Partitioning using ethyl acetate

About 20ml of distilled water was added to the remaining mother solution after partitioning with petroleum ether and chloroform. Then 100 ml of ethyl acetate was added to the mother solution in the separating funnel and was shaken. It was kept for separation. The bottom layer was the expected portion which was collected and the process was repeated for thrice. The fractions obtained by ethyl acetate were dried.

Figure 2.4: Partitioning in separating funnel

2.3 Phytochemical screening:

2.3.1 Test for glycoside:

2.3.1.1 Materials and reagents:

a) Test tube

b) Pipette

c) Sodium hydroxide

d) Distilled water

e) Methanolic extract

2.3.1.2 Procedure:

In a test tube, 0.001 gm of methanolic extract was taken. Then 1 ml of water and few drops of Sodium hydroxide were added to the test tube. Yellow colored precipitation was observed.

2.3.2 Test for Saponin:

2.3.2.1 Materials and reagents:

a) Beaker

b) Pipette

c) Distilled water

d) Electric Heater

e) Methanolic extract

2.3.2.2 Procedure:

In a small beaker, 0.001 gm of methanolic extract was taken. Then 1 ml of distilled water was added and this mixture was heated for a while. Persistent froth was observed.

2.3.3 Test for Tannin:

2.3.3.1 Materials and reagents:

a) Beaker

b) Test tube

c) Electric heater

d) Filter paper

e) Distilled water

f) Ferric Chloride

g) Methanolic extract

2.3.3.2 Procedure:

About 0.5 gm of methanolic extract was taken in a beaker and 10 ml of distilled water was added to the beaker. Then it was subjected to heat. After heating it was kept for 3 minutes. This heated extract was filtered and 1 ml of filtrate was placed in a test tube. Then 4 ml of distilled water and few drops of 5% Ferric chloride were added to the test tube. Green colored precipitation was observed.

2.3.4 Test for Resin:

2.3.4.1 Materials and reagents:

a) Beaker

b) Pipette

c) Acetic anhydride

d) Sulphuric acid

e) Methanolic extract

2.3.4.2 Procedure:

Total 0.001 gm of Methanolic extract was taken in a beaker and 5 ml of acetic anhydride was added to the beaker. Then it was heated and was kept for cooling of the extract. About 0.05 ml of sulphuric acid was added to the extract. The extract change purplish red color to violet color.

2.3.5 Test for Alkaloids:

2.3.5.1 Materials and reagents:

a) beaker

b) Test tube

c) Mayer's reagent

d) Hager's reagent

e) Methanolic extract

f) Hydrochloric acid

2.3.5.2 Procedure:

In a beaker 5 ml of diluted hydrochloric acid was added to 50 mg of methanolic extract to prepare the stock solution. Mayer's reagent was added to the stock solution. White colored precipitation was observed.

2.4 Evaluation of antioxidant activity

2.4.1 Antioxidant activity

Oxidation process has been estimated as the main reason of several serious diseases in human body. Free radicals are mostly related with this oxidation process to cause cellular damage. Free radicals are the atom or group of atoms with odd number electron which can be formed when oxygen interacts with other molecule. Free radicals are highly reactive and appear with significant chain reactions which result in damage of cellular components like DNA and other cell membrane. Human beings thirst for knowledge invented that medicinal plants contain the components which can alter chain reaction of free radical and prevent the cellular damage. This free radical scavenging activity is known as antioxidant activity.

2.4.2 DPPH (Di phenyl pecrylhydragiene) assay

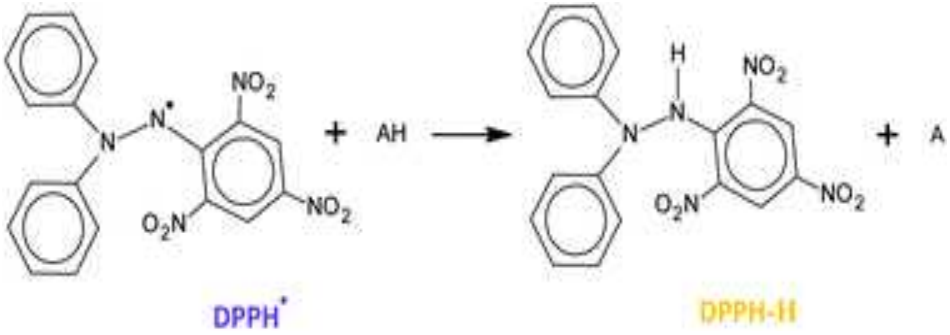

Figure 2.4.1: Free radical scavenging activity (collected from analytical methods issue 17, 2013)

The method of Brand-Williams *et al.*,1995 was estimated to investigate the free radical scavenging activity of the plant *extracts* on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) which is a stable free radical.

DPPH assay is an accepted method worldwide in which DPPH act as a free radical scavenged hydrogen donor. DPPH assay is based on the reduction of DPPH. When antioxidants react with DPPH it forms pair with the hydrogen and produces reduced DPPHH which altered free radical reaction potential. Formation of DPPHH results in decolorization or the appearance of yellow color based on the electron capture capacity.

The decolorization of the sample is based on the degree of reduction of the free radical.

2.4.3 Principle

According to the method of DPPH assay (Brand-William et al, 1995), 3.0 ml of methanolic solution of DPPH was mixed with 2.0 ml methanolic solution of plant extract. Ascorbic Acid or Butyl-1-Hydroxy Toluene (BHT) in a concentration within 1- 100 µg/ml was used as standard and blank sample was also prepared which was methanol. After that keeping the standard and the sample in dark place at room temperature for 30 minutes, the antioxidant activity of the plant extract was investigated from the decolorization of the DPPH solution by the plant extract which was compared with the standard like ascorbic acid or Butyl-1-Hydroxy Toluene (BHT) by using UV- Spectrophotometer to measure the absorbance at the wavelength of 517nm. (Brand-William 1995)

The formula for calculating the inhibition%:

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

2.4.4 Materials and Reagents

a) Pipette

b) Volumetric flask

c) Test tubes

d) Light proof box

e) Ascorbic Acid

f) Methanol

g) Distilled water

h) Methanolic extract of the plant

i) Fraction of petroleum ether partioning

j) Fraction of chloroform partitioning

k) Fraction of ethyl acetate partitioning

l) UV- Spectrophotometer

m) 2, 2-diphenyl-1-picrylhydrazyl (DPPH)

2.4.5 Preparation of control for antioxidant activity investigation

In this study, ascorbic acid was used as the positive control to perform the DPPH assay. Total 0.005 gm of ascorbic acid was dissolved in 1 ml of methanol to have the desired concentration of 5000 µg/ml. This solution was diluted sequentially to obtain various concentrations ranging from 1250 µg/ml to 78.125 µg/ml.

2.4.6 Preparation of Test Sample

0.005gm of methanolic extract, fraction of petroleum ether partitioning, fraction of chloroform partitioning and the fraction of ethyl acetate partitioning of the plant was dissolved in 1 ml of methanol in separated test tube for each solution individually for the desired concentration of 5000 µg/ml. Then these solutions were sequentially diluted to obtain several concentrations ranging from 1250 µg/ml to 78.125 µg/ml.

2.4.7 Preparation of DPPH solution

To prepare the DPPH solution 2 mg of DPPH was dissolved in 50 ml methanol to get the solution having concentration of 40 µg/ml. Then this solution was kept in dark place.

2.4.8 Assay for free radical scavenging activity

To assay the free radical scavenging activity 2.0 ml of methanolic solution from each of the sample with several concentrations ranging from 1250 µg/ml to 19.50 µg/ml. Each of these taken samples was mixed with 3.0 ml of DPPH solution with the concentration of 40 µg/ml. Then, all of the samples were kept in dark place at room temperature for 30 minutes. This causes the reduction reaction. Using the UV- spectrophotometer absorbance of each of the sample were measured at 517 nm against methanol used as blank.

The formula for calculating the inhibition of free radical of DPPH is:

$$\textbf{Inhibition \% (I \%)} = [(\text{Absorbance}_{\text{blank}} - \text{Absorbance}_{\text{sample}}) / \text{Absorbance}_{\text{blank}}] \times 100$$

The (IC_{50}) value for each of the sample was determined from the graph by plotting inhibition percentage (I %) against the concentration of extract (µg/mL).

2.5 Evaluation of cytotoxicity by brine shrimp lethality bioassay

2.5.1 Cytotoxicity

Cytotoxic refers to a substance or process which results in cell damage or cell death. The prefix "cyto" refers to cell and "toxic" to poison (Lynne Eldridge, 2016). To establish a drug from medicinal plants with chemical constituents which are pharmacologically active it is necessary to estimate its cytotoxicity. The chemical components which possess the pharmacological activity often cause serious side effects and are known as toxic to cell. To use a drug with proper precaution in order to prevent its toxicity to cell evaluation of cytotoxicity is necessary.

2.5.2 Brine shrimp lethality bioassay

The brine shrimp lethality bioassay technique is very easy to perform and this technique is not that much expensive. In addition, this method does not require highly aseptic environment and with very small amount of materials it can be performed. It appears that brine shrimp lethality bioassay is predictive of cytotoxicity and pesticidal activity (Ghisalberti, 1993). This in vivo lethality test has been effectively used for bioassay-guide fractionation of active cytotoxic and antitumor agents since its introduction in 1982 (Meyer et al., 1982),

2.5.3 Principle

According to brine shrimp lethality bioassay (Meyer et al., 1982), in simulated sea water Brine shrimp eggs were hatched to get nauplii. To obtain the desired concentration of the test sample required amount of dimethyl sulphoxide (DMSO) was calculated and was added. Then nauplii were counted by visual inspection. These nauplii were taken in vials containing 5 ml of simulated sea water. By using micropipette samples of different concentrations were added to labeled vials. After that, observing for 24 hours survived nauplii were counted.

2.5.4 Materials and reagents

a) *Artemiasalina leach* (brine shrimp egg)

b) Sea salt (NaCl)

c) Small tank with perforated dividing dam to hatch the shrimp

d) Lamp to attract the shrimps

e) Pipettes

f) Micropipette

g) Glass vials

h) Magnifying glass

i) Test tubes

j) Test samples of experimental plants

Table 2.5.1: Test samples of experimental plants

Sample code	Test sample	Calculated amount (mg)
ME	Methanolic Extract	4.0
PESF	Petroleum Ether Soluble fraction	4.0
CSF	Chloroform soluble fraction	4.0
EASF	Ethyl Acetate soluble fraction	4.0

2.5.5 Preparation of seawater

One liter of distilled water was taken in a beaker and 38 gm of sea salt (pure Nacl) was weighed and dissolved in it. To get a clear solution it was filtered.

2.5.6 Hatching of brine shrimps

In the small tank sea water was placed. Shrimp eggs were added to the sea water in the tank. Oxygen supply was provided during the hatching time. After one day shrimp eggs were matured as nauplii. Then these matured shrimps were taken from the tank with the help of Pasteur pipette. 10 living shrimps were added to each of the test tube containing 5 ml of seawater.

2.5.7 Test sample preparation

The calculated amount of all the test sample were taken in vials and were dissolved in 100 µl of pure dimethyl sulphoxide (DMSO). Then 50 µl of solution was taken in the first test tube containing 5 ml of simulated sea water and then 10 shrimp nauplii were added to the test tube. So, the final concentration of the solution in the first test tube was 400 µg/ml. Thus a series of serial dilution were performed to prepare solutions of varying concentration. About 50 µl samples were added to test tube and 50 µl DMSO was added to vial in each stage of dilution.

2.5.8 Preparation of positive control group

In this study to perform the brine shrimp lethality bioassay vincristine sulphate was used as positive control. Required amount of vincristine sulphate was dissolved in DMSO to get an initial concentration of 20 µg/ml. Then serial dilution was performed to get concentrations of 10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1.25 µg/ml, 0.625 µg/ml, 0.3125 µg/ml, 0.15625 µg/ml, 0.078125 µg/ml, 0.0390 µg/ml sequentially. Then positive control solutions were added to the labeled vials containing 10 brine shrimp and 5 ml simulated sea water.

Table 2.5.2: After serial dilution concentration in the Test sample

Test Tube no.	Concentration (µg/ml)
1	400.0
2	200.0
3	100.0
4	50.0
5	25.0
6	12.5
7	6.25
8	3.125
9	1.5625
10	0.78125

3. Result and Discussion

The chemical investigation of Begonia roxburghii shows that there is presence of tannin, saponin, resin, glycoside and alkaloid.

3.1 Result of DPPH assay

Table 3.1: (IC_{50}) value of ascorbic acid (standard)

To use ascorbic acid as standard for the DPPH assay its absorbance was measured with different concentration by performing serial dilution. The absorbance, % of inhibition and IC_{50} µg/ml values are given in the following table:

Absorbance of the blank	Concentration of the extract µg/mL	Absorbance of the extract	% of inhibition	IC_{50} µg/ml
	78.125	0.789	7.3	
	156.25	0.601	29.46	
0.852	312.5	0.370	56.5	378.08
	625	0.020	97.7	
	1250	0.019	97.8	

By plotting the values of concentration to the X axis and the % of inhibition values to the Y axis, an equation has been found from which the IC_{50} value is obtained for Ascorbic acid. The graph is given below:

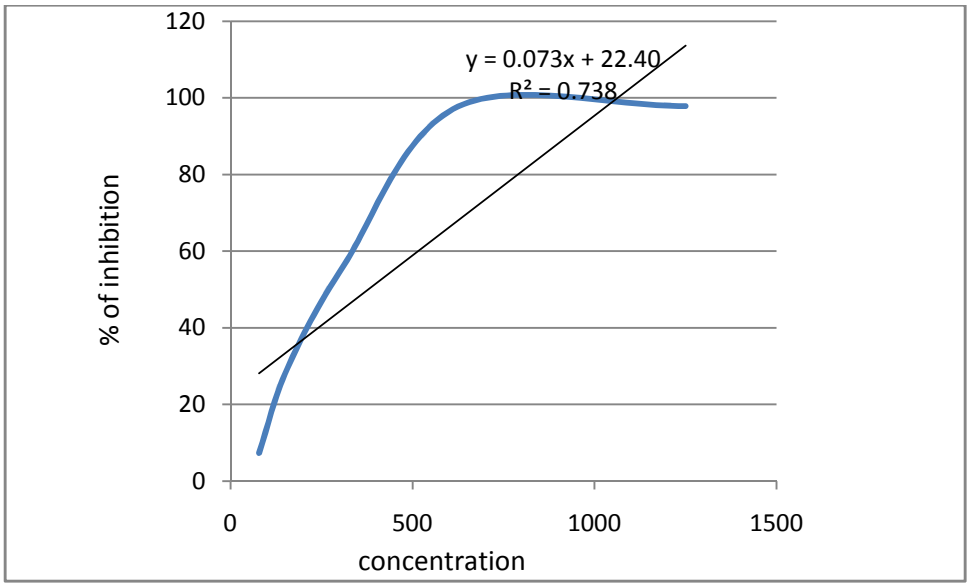

Figure 3.1: (IC_{50}) value of ascorbic Acid

Table 3.2: (IC_{50}) value of methanolic extract of the plant

Absorbance of the methanolic extract of the subjected plant was measured with its different concentration by performing serial dilution. The absorbance, % of inhibition and IC_{50} µg/ml values are given in the following table:

Absorbance of the blank	Concentration of the extract µg/mL	Absorbance of the extract	% of inhibition	IC_{50} µg/ml
	78.125	0.840	1.5	
	156.25	0.456	46.5	
0.852	312.5	0.258	70	362.94
	625	0.153	82.1	
	1250	0.147	82.8	

By plotting the values of concentration and the values of % of inhibition an equation has been found from which the IC_{50} value of methanolic extract is obtained. The graph is given below:

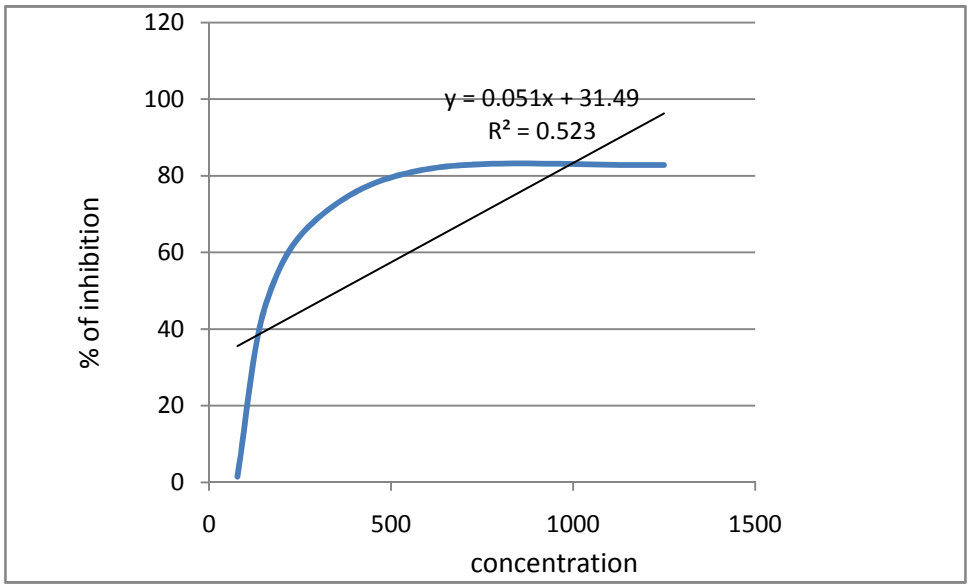

Figure 3.2: (IC_{50}) value of methanolic extract of the plant

Table 3.3: (IC_{50}) value of fraction with petroleum ether partitioning

Absorbance of fraction with petroleum ether partitioning was measured by performing serial dilution. The absorbance, % of inhibition and the IC_{50} µg/ml is given below:

Absorbance of the blank	Concentration of the extract µg/mL	Absorbance of the extract	% of inhibition	IC_{50} µg/ml
	78.125	0.745	12.5	
	156.25	0.398	53.2	
0.852	312.5	0.286	66.4	285.43
	625	0.173	79.6	
	1250	0.132	84.5	

By plotting the values of concentration to the X axis and the value of % of inhibition to the Y axis an equation has been found from which the IC_{50} value of fraction with petroleum ether partitioning is obtained. The graph is given below:

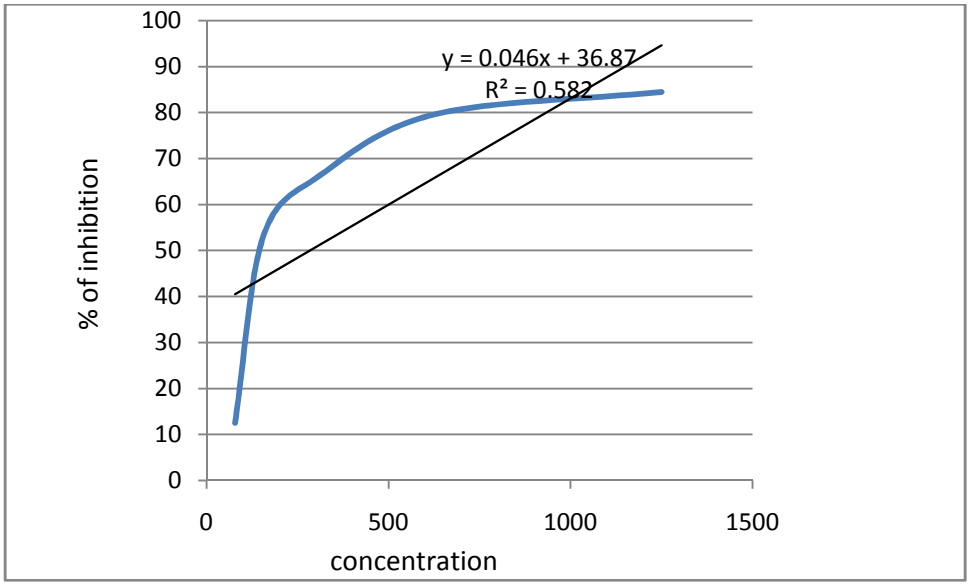

Figure 3.3: (IC_{50}) value of fraction with petroleum ether partitioning

Table 3.4: (IC_{50})value of fraction with chloroform partitioning

Absorbance of fraction with chloroform partitioning was measured by performing serial dilution. The absorbance, % of inhibition and the IC_{50} value are given in the following table:

Absorbance of the blank	Concentration of the extract µg/mL	Absorbance of the extract	% of inhibition	IC_{50} µg/mL
	78.125	0.621	27.1	
	156.25	0.310	63.6	13.68
0.852	312.5	0.202	76.2	
	625	0.129	84.8	
	1250	0.096	88.7	

By plotting the values of concentration to the X axis and the value of % of inhibition to the Y axis an equation has been found from which the IC_{50} value of fraction with chloroform partitioning is obtained. The graph is given below:

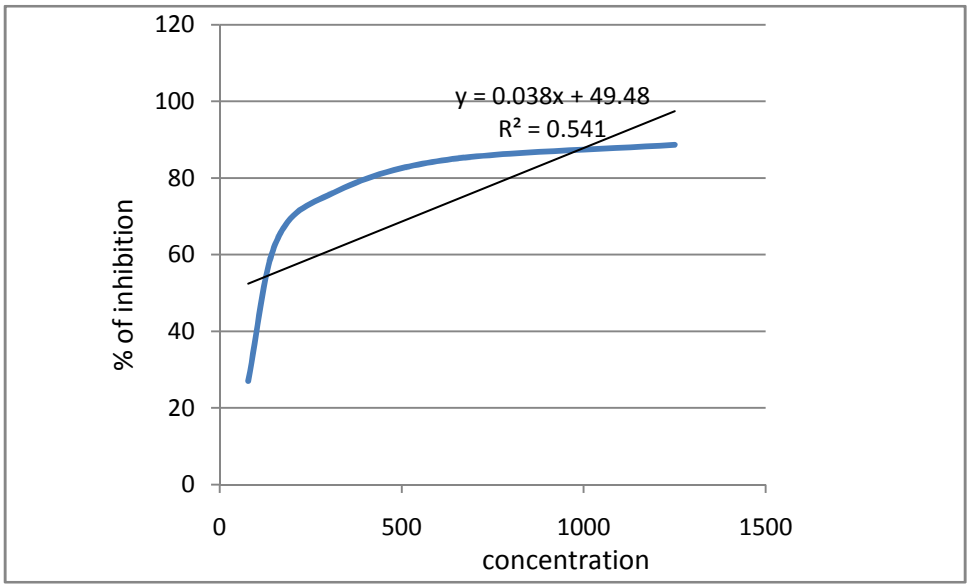

Figure 3.4: (IC_{50}) value of fraction with chloroform partitioning

Table 3.5: (IC_{50}) value of fraction with ethyl acetate partitioning

Absorbance of fraction with ethyl acetate partitioning was measured by performing serial dilution. The absorbance, % of inhibition and the IC_{50} value are given in the following table:

Absorbance of the blank	Concentration of the extract µg/mL	Absorbance of the extract	% of inhibition	IC_{50} µg/mL
	78.125	0.551	35.3	
	156.25	0.410	51.8	
0.852	312.5	0.267	68.6	88.68
	625	0.139	83.6	
	1250	0.117	86.2	

By plotting the values of concentration to the X axis and the value of % of inhibition to the Y axis an equation has been found from which the IC_{50} value of fraction with Ethyl acetate partitioning is obtained. The graph is given below:

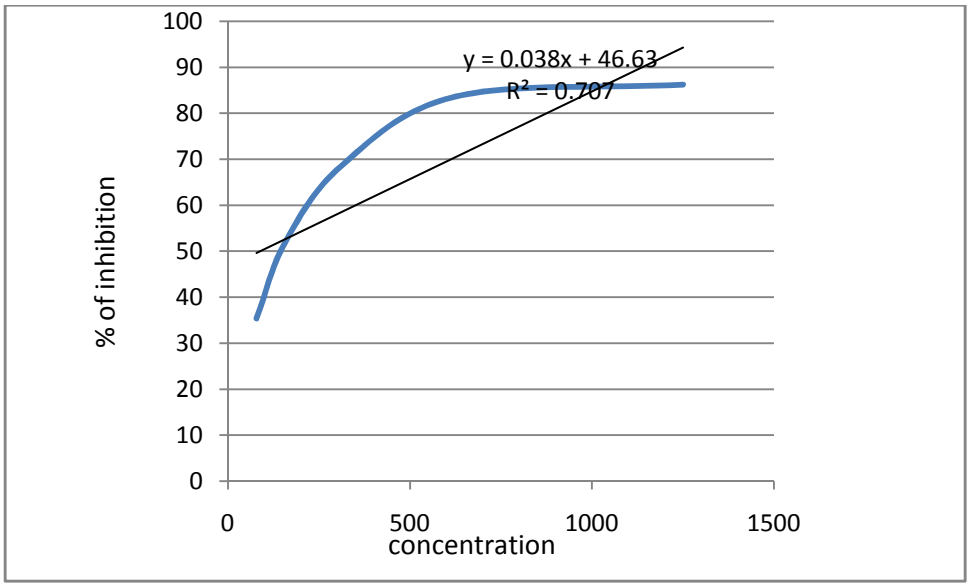

Figure 3.5: (IC_{50}) value of fraction with ethyl acetate partitioning

Table 3.6: IC_{50} values of Test samples

The IC_{50} µg/ml values for ascorbic acid used as standard, methanolic extract and different fractions obtained from partitioning are given below:

Sample code	Test sample	IC_{50} µg/ml
ASA	Ascorbic acid	378.08
ME	Methanolic extract	362.94
PESF	Petroleum ether soluble fraction	285.43
CSF	Chloroform soluble fraction	13.68
EASF	Ethyl acetate soluble fraction	88.68

By plotting the standard and different fractions to the X axis and the obtained IC_{50} values for each fraction and standard to the Y axis the difference of values from the standard is observed. The graph is given below:

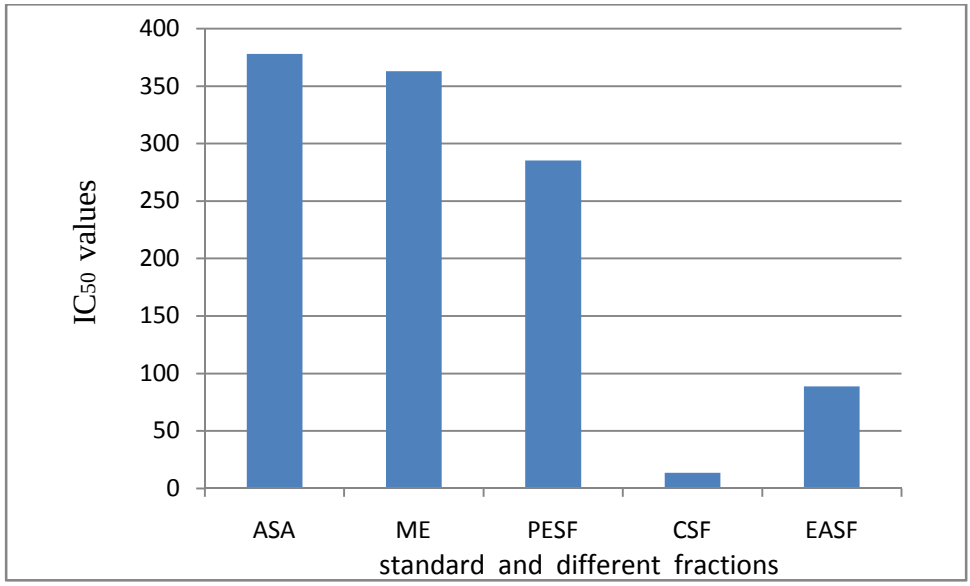

Figure 3.6: IC_{50} values of test samples

In the antioxidant activity investigation of *Begonia roxburghii*, the methanolic plant extract and its different fraction soluble in petroleum ether, chloroform and ethyl acetate was subjected for DPPH assay. Ascorbic acid was used as standard. EASF showed the highest free radical scavenging activity with IC_{50} value of 88.68µg/ml.TheIC_{50} value of other fractions such as ME is 362.94 µg/ml, PESF is 285.43 µg/ml and CSF showed the IC50 value of13.68 µg/ml respectively.

3.2 Result of brine shrimp lethality bioassay

Table 3.2.1: Effect of vincristine sulphate on shrimp nauplii

Addition of 10 shrimp nauplii to 10 test tube individually containing different concentration of vincristine sulphate causes death of shrimp naupli. Consequently, % of mortality and LC_{50} value is given below:

Concentration (µg/ml)	Log_{10} concentration	% mortality	LC_{50} (µg/ml)
0		0	
0.039	-1.4089	10	
0.078125	-1.1072	20	
0.15625	-0.8061	30	
0.3125	-0.5051	40	5.53
0.625	-0.2014	40	
1.25	0.09691	50	
2.5	0.39794	60	
5	0.6989	70	
10	1.00	70	
20	1.30102	90	

By plotting the value of $\log_{10}$ concentrations to the X axis and the % of mortality to the Y axis an equation and R^2value has been found from which the LC_{50} value of vincristine sulphate is obtained. The graph is given below:

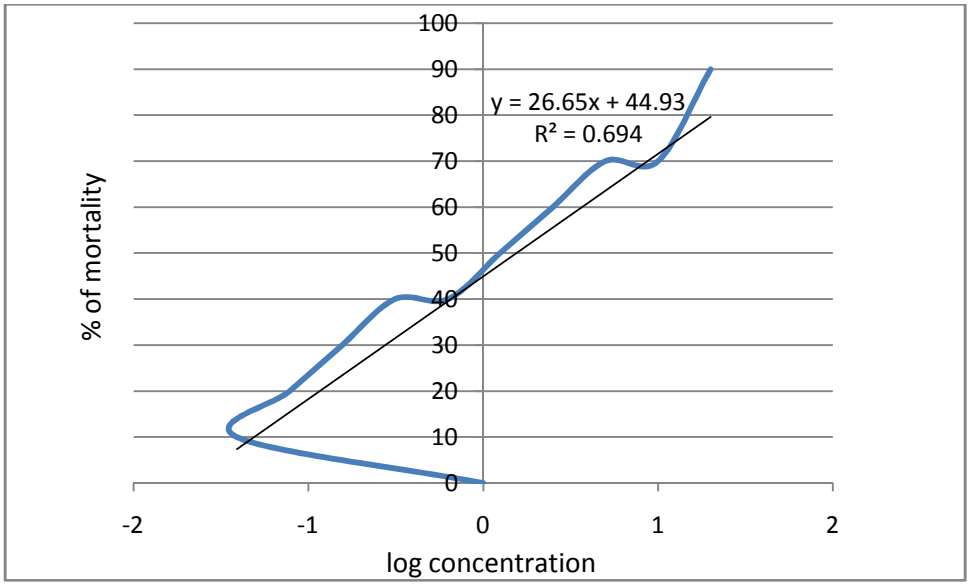

Figure 3.2.1: LC_{50} of vincristine sulphate on shrimp nauplii

Table 3.2.2: Effect of methanolic extract on shrimp nauplii

Addition of 10 shrimp nauplii to 10 test tube individually containing different concentration of methanolic extract and DMSO causes death of shrimp nauplii. Consequently, % of mortality and LC_{50} value is given below:

Concentration (µg/ml)	Log_{10} concentration	% mortality	LC_{50} (µg/ml)
0		0	
0.78125	-0.10720997	50	
1.5625	0.193820026	40	
3.125	0.494850022	50	
6.25	0.795880017	70	0.622
12.5	1.096910013	60	
25	1.397940009	80	
50	1.698970004	80	
100	2	70	
200	2.301029996	90	
400	2.602059991	100	

By plotting the value of $\log_{10}$ concentrations to the X axis and the % of mortality to the Y axis an equation and R^2value has been found from which the LC_{50} value of methanolic extract is obtained. The graph is given below:

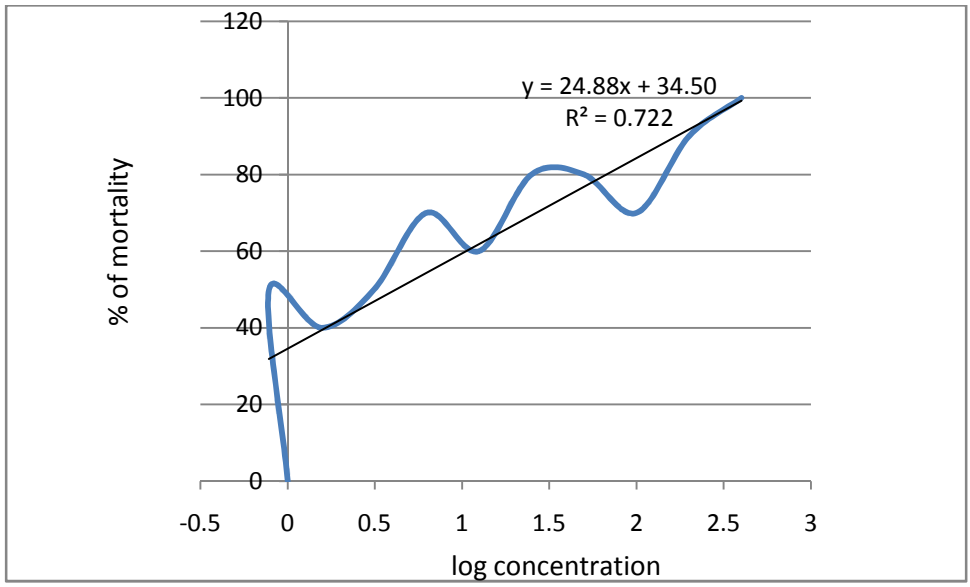

Figure 3.2.2: LC_{50} of methanolic extract on shrimp nauplii

Table 3.2.3: Effect of petroleum ether soluble fraction on shrimp nauplii

Addition of 10 shrimp nauplii to 10 test tube individually containing different concentration of fraction with petroleum ether partitioning and DMSO causes death of shrimp naupli. Consequently, % of mortality and LC_{50} value is given below:

Concentration (µg/ml)	Log_{10} concentration	% of mortality	LC_{50} (µg/ml)
0		0	0.66
0.78125	-0.10720997	40	
1.5625	0.193820026	50	
3.125	0.494850022	60	
6.25	0.795880017	60	
12.5	1.096910013	50	
25	1.397940009	70	
50	1.698970004	80	
100	2	80	
200	2.301029996	100	
400	2.602059991	100	

By plotting the value of $\log_{10}$ concentrations to the X axis and the % of mortality to the Y axis an equation and R^2value has been found from which the LC_{50} value of fraction with petroleum ether partitioning is obtained. The graph is given below:

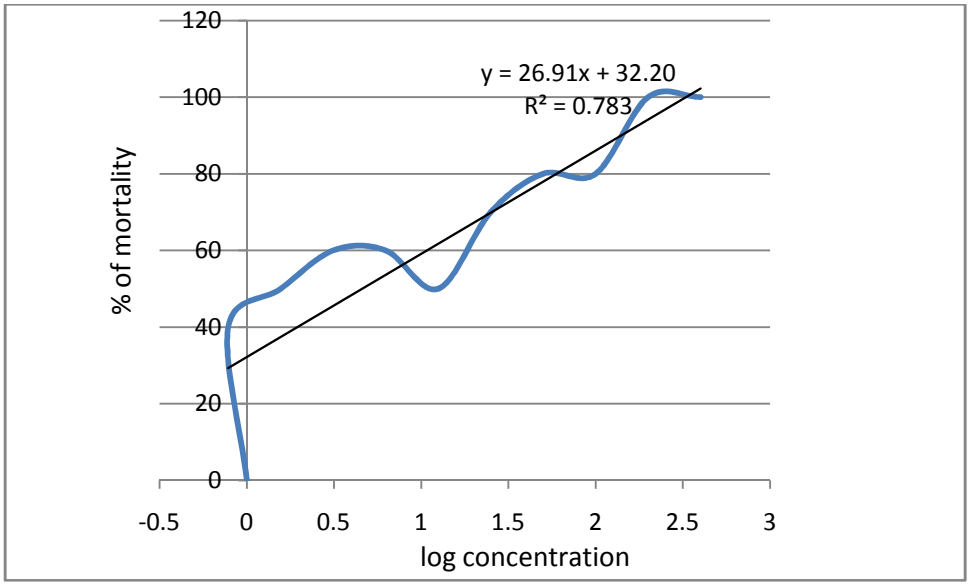

Figure 3.2.3: LC_{50} of petroleum ether soluble fraction on shrimp nauplii

Table 3.2.4: Effect of chloroform soluble fraction on shrimp nauplii

Addition of 10 shrimp nauplii to 10 test tube individually containing different concentration of fraction with chloroform partitioning and DMSO causes death of shrimp nauplii. Consequently, % of mortality and LC_{50} value is given below:

Concentration (µg/ml)	Log_{10} concentration	% of mortality	LC_{50} (µg/ml)
0		0	0.46
0.78125	-0.10720997	50	
1.5625	0.193820026	50	
3.125	0.494850022	60	
6.25	0.795880017	70	
12.5	1.096910013	70	
25	1.397940009	80	
50	1.698970004	70	
100	2	90	
200	2.301029996	80	
400	2.602059991	100	

By plotting the value of log_{10} concentrations to the X axis and the % of mortality to the Y axis an equation and R^2value has been found from which the LC_{50} value of fraction with chloroform partitioning is obtained. The graph is given below:

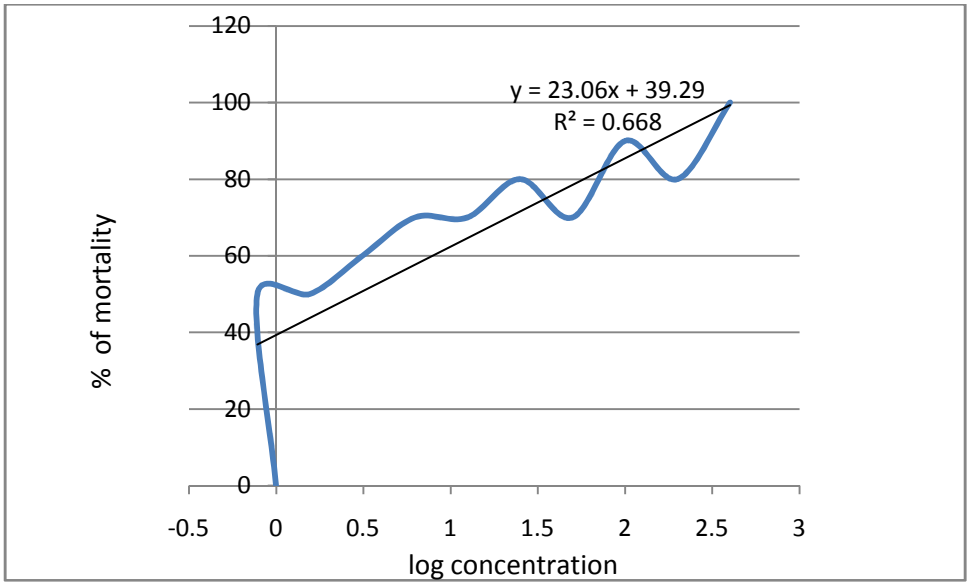

Figure 3.2.4: LC_{50} of chloroform soluble fraction on shrimp nauplii

Table 3.2.5: Effect of ethyl acetate soluble fraction on shrimp nauplii

Addition of 10 shrimp nauplii to 10 test tube individually containing different concentration of fraction with ethyl acetate partitioning and DMSO causes death of shrimp nauplii. Consequently, % of mortality and LC_{50} value is given below:

Concentration (µg/ml)	Log_{10} concentration	% of mortality	LC_{50} (µg/ml)
0		0	0.75
0.78125	-0.10720997	50	
1.5625	0.193820026	40	
3.125	0.494850022	50	
6.25	0.795880017	60	
12.5	1.096910013	50	
25	1.397940009	60	
50	1.698970004	80	
100	2	80	
200	2.301029996	90	
400	2.602059991	90	

By plotting the value of log_{10} concentrations to the X axis and the % of mortality to the Y axis an equation and R^2 value has been found from which the LC_{50} value of fraction with ethyl acetate partitioning is obtained. The graph is given below:

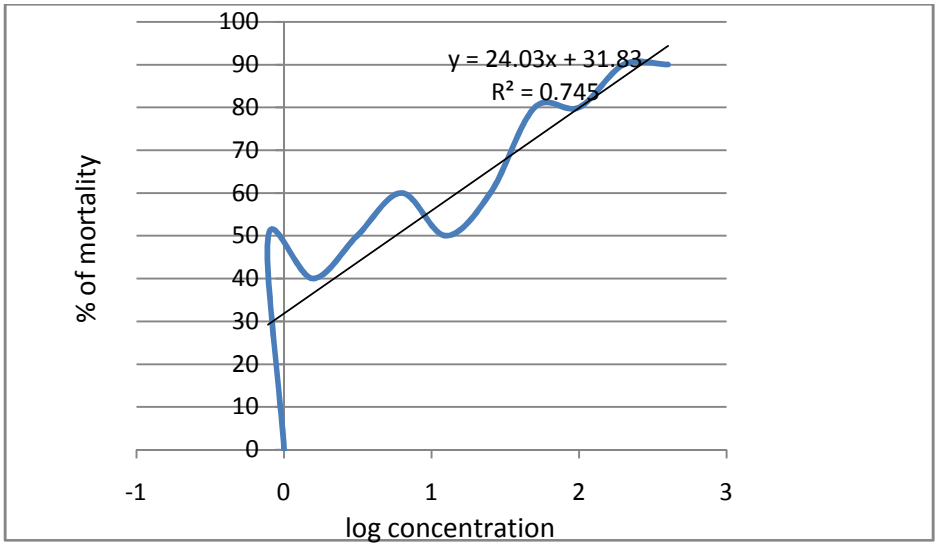

Figure 3.2.5: LC_{50} of ethyl acetate soluble fraction on shrimp nauplii

Table 3.2.6: LC_{50} values of the test samples

Test sample	Regression line	R^2	LC_{50}(µg/ml)
VS	Y= 26.65X + 44.93	0.694	0.190
ME	Y= 24.88x + 34.51	0.722	0.622
PESF	Y = 26.91x+ 32.20	0.783	0.66
CSF	Y = 23.06x + 39.29	0.668	0.46
EASF	Y = 24.03x + 31.83	0.745	0.75

By plotting the positive control vincristine sulphate and different fractions to the X axis and the values of LC_{50} to the Y axis shows the variation of values with the positive control. The graph is given below:

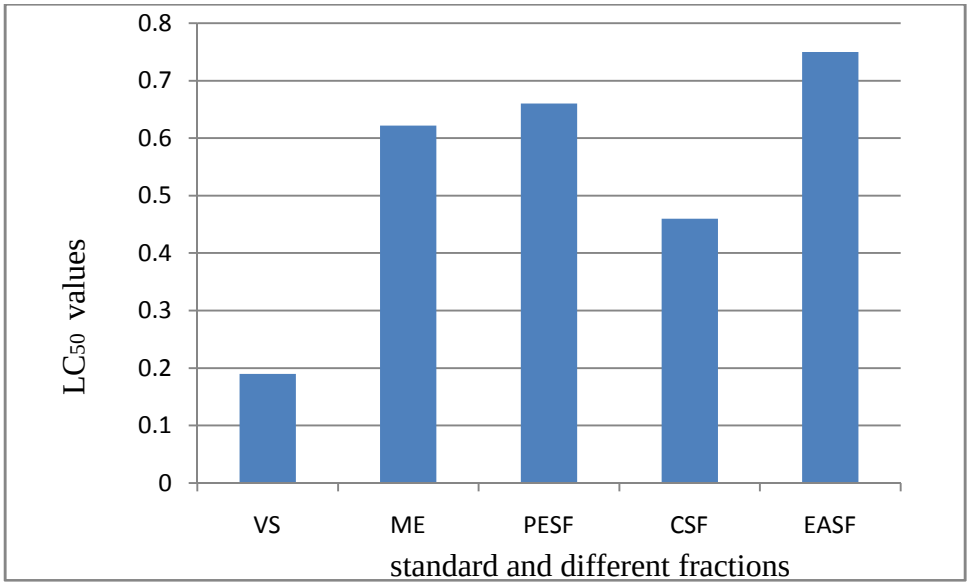

Figure 3.2.6: LC_{50} values of different fraction and standard

To investigate the cytotoxicity of *Begonia roxburghii*, the methanolic extract of the plant and its other partitions soluble in petroleum ether, chloroform and ethyl acetate were subjected for brine shrimp lethality bioassay. Vincristine sulphate was used as positive control in this study. The LC_{50} values of vincristine sulphate is 0.190 µg/ml, methanolic extract is 0.622µg/ml, petroleum ether soluble fraction is 0.66µg/ml, chloroform soluble fraction is 0.46µg/ml, and ethyl acetate soluble fraction 0.75 µg/ml respectively.

4. Conclusion:

After performing the above chemical tests with the plant leaf of *Begonia roxburghii*it is found that this plant contains some essential chemicals like tannin, resin, saponin, glycoside and alkaloids. There is presence of moderate antioxidant property like *Begonia conchifolia* and *Begonia fluccifoid.* In addition, the cytotoxic property of various fraction of this plant is higher than the positive control Vincristine sulphate. At the end, it can be expected that the results obtained from the phytochemical investigation of this plant can be helpful in further developed medicinal study of *Begonia roxbnurghii.*

Reference:

Agardh, (1824). The Begonia page. Family of succulent plants. Antioxidants and free radicals.(n.d.). Retrieved from medical tents: http://www.rice.edu/~jenky/sports/antiox.html

Bauer, M. (1932). American Begonia Society.

Brown, D. (2015). Plants Poisonous to Livestock and other Animals.

Clement, W. L. (2004). Phylogenetic position and biogeography of Hillebrandiasandwicensis(Begoniaceae): A rare Hawaiian relict1.

Dewitte, A. (n.d.). The Origin of Diversity in, 27.

Doughari, J. H. (n.d.). Phytochemicals: Extraction Methods, 33.

DR. A.C. BLAIR, K. (n.d.). The story of Begonia, 14.

Ghani, A. Medicinal plants of Bangladesh.

Joshi, P. K. (2012). Coumarin: Chemical and Pharmacological Profile, 5.

MamtaSaxena, J. S. (2013). Phytochemistry of medicinal plants. Journal of Pharmacognosy and Phytochemistry, 15.

MD, L. E. (2016). Cytotoxic Chemotherapy and Other Cytotoxic Agents.

Medical discoveries. (n.d.). Retrieved from chloroform:

http://www.discoveriesinmedicine.com/Bar-Cod/Chloroform.html

Methanol institute. (n.d.). Retrieved from Properties of methanol:

http://www.methanol.org/technical-information/properties-of-methanol.aspx

Pharmacology. (n.d.).Retrieved from Antimicrobial resistance learning site:

http://amrls.cvm.msu.edu/pharmacology/antimicrobials/antimicrobials-an-introduction

Rajani, M. K. (2013). DPPH Free Radical Scavenging Activity of. Journal of medicinal plant studies, 7.

Shuli man, W. G. (2010). Chemical study and medical application of saponins as anti-cancer agents.

Sirintorn, P. P. (2004). Brine shrimp lethality activity of Thai medicinal plants, 6.valin, C. (n.d.). The breeding history of begonias