# Phytochemical and Pharmacological Evaluation of *Crassocephalum crepidioides*

By

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Bachelor of Pharmacy (Hons.)

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## Declaration

It is hereby declared that

1. The thesis submitted is my/our own original work while completing degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help

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# Approval

"Phytochemical and Pharmacological Evaluation of *Crassocephalum crepidioides"* submitted by Mayesha Samiha of Spring, 2015 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy (Hons.) on August, 2019.

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## Ethics Statement

This study does not involve any human trial.

## Abstract

In the development of modern medicine, the natural sources have been introduced because of their bioactive compounds and pharmacological effects. The herbs and plants are producing secondary metabolites from which semi synthesized or synthesized drugs are manufactured. Hence, the phytochemical screening of the plant *Crassocephalum crepidioides* was carried out to evaluate the antioxidant property in which DPPH assay and total phenolic content were measured. Besides, thrombolytic activity was conducted and to determine the toxicity of the plant brine shrimp lethality test was done. The study revealed, the moderate antioxidant effect in which the value was 637.22 in total phenolic content and in DPPH assay the half maximal inhibitory value is 136.016. At the same time, the toxicity of the plant was also moderate. Brine shrimp lethality test was performed to find out the toxicity level. However, the plant confirmed a satisfactory outcome of thrombolytic activity in which clopidogrel bisulphate was used as reference. It can be concluded that drugs can be prepared by the phytochemical screening of the methanolic extract of the plant Crassocephalum crepidioides.



**Keywords:** Phytochemical screening, Antioxidant properties, Toxicity, Thrombolytic activity.

## Dedication

*Dedicated to my parents*

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## List of Acronyms:

| | |
|---|---|
| ROS | Reactive Oxygen Species |
| DPPH | 1,1 diphenyl-2-picrylhydrazyl |
| DMSO | Di-methyl sulfoxide |
| TPC | Total phenolic content |
| GAE | Gallic acid equivalent |
| ASA | Ascorbic acid |
| ME | Methanolic extract |
| WHO | World health Organisation |
| $LC_{50}$ | Lethal concentration |
| IC50 | Half maximal inhibitory concentration |

# Chapter One

## Introduction:

In our environment, plants are an integral part and from these plants, human health is benefited. Previously, people have been traditionally using collected compounds of medicinal plants to treat various types of diseases. Many of the ancient scholars used to believe that heath related issues and diseases could be treated only by the herbal medicines produced from plants. For this in mind, many of the researches were conducted by the ancient researchers, the attempts were made to assess the efficacy of different medicinal herbs on the basis of scientific findings that delivers therapeutic cure of several diseases.

For the procurement process in the treatment of diseases, medicinal plants are used which supplies different kinds of therapeutic agents. As these therapeutic agents are extracted from medicinal herbs and plants, these are used directly to produce semi synthesized or synthesized drug. Earlier, the herbalists used to know about the correct remedies and treatment of diseases and they produced medicines by concoctions, preparations and extracts.

However, the ancient people or herbalists had given importance on wound healing, as a result whole two chapters are allocated on wound healing in the book named “Sushruta Samhita” and where it is mentioned that to revive from wound, hundreds of plants have been used which possesses effective medicinal properties, as well as these medicines are required to achieve a wound which is neat and clean (Baghde, 2017).

Here, these traditional medicinal plants are termed as “Herbology” which defines different herbs, medicinal plants are used for medicinal purposes to treat diseases. The foundation of drug therapy was developed from the concept of using traditional herbal medicine to treat and cure diseases. Therefore, from early to modern era, it is regarded as the most advantageous

sources of medicine. So, for the advancement of human race, it is fundamental to recognize the potential pharmacological and therapeutic effect of medicinal plants from natural sources.

The earth is brimming with different kinds of plant species of total 374,000 among which the amount of vascular plants are approximately 308,312 and the number of flowering plants are 295,383 (Marteen, Christenhugz & W. Byng, 2016)(Pimm & Joppa, 2015). The medicines obtained from natural sources and their derivatives have been clinically used and their percentage is about 60%.

In this modern world, the popularity of naturally derived products is increasing and around 80% of the people are dependent on them due to their effectiveness (Musa, Adekomi, Tijani, &Muhammed, 2011). Till now, for therapeutic purposes around 35 000 to 70,000 plants were examined to derive compounds from them. According to WHO (World Health Organization), 11% of drugs are regarded as fundamental drugs among 252 drugs (Veeresham, 2014). Additionally, 2100 types plants are there which are potent and useful in the treatment of diseases.

The natural plants remained a fundamental asset of new compound which are therapeutically active and from these plants commercially efficacious drugs are obtained directly or indirectly. The active ingredients of about 121 plants are used recently and the drugs derived from the herbal plants are appreciated around the world is approximately 25%.

Several drugs are produced from medicinal drugs, for example, Digoxin from *Digitalis* species, vincristine and vinblastine gotten from the *Catharanthus roseus*, *Atropa belladonna* provide atropine along with morphine and also codeine from *Papaver somniferum* (Yue-Zhong Shu, 1998). In a recent analysis, the use of medicine from natural sources are enhancing rapidly (Goldfrank et al., 1982; Mentz and Schenkel, 1989). It was reported Liu et al. (2000) by that naturally derived medicines are 50% of which are marketed.

Likewise, to produce the drugs from medicinal herbs, phytochemical screening is important to detect the properties as the plants produce secondary metabolites. Such as, alkaloids, tannins, terpenoids, flavonoids, phenols, amines, lectins, steroids and saponins etc. (Guerriero et al., 2018). These phytochemicals or molecules helps to protect against the microorganisms and herbivores.

Recently, the antibiotic resistance is a global threat for the people around the world. Hence, the requirement to produce new antimicrobial drugs leads to phytochemical screening of medicinal herbs in search of essential and potent agents which acts against these microbes. Besides, in our body, the free radicals are formed to assist in different function and the growth of cells.

Excessive production of these derivatives of free radicals triggers various diseases including inflammation, cancer, damage to the lung. To inhibit the oxidation process, the significance of antioxidants is noteworthy. Usually, plants are the great sources which contains antioxidants. Using the antioxidant property of the plants, various diseases can be cured which occurs due to the oxidation process.

## 1.1 Selection of the plant *Crassocephalum crepidioides* for this project:

*Crassocephalum crepidioides* was selected for this project due to the availability of the plant in Bangladesh and this plant exhibits some medicinal properties which are useful and productive for therapeutic purposes. In addition, the bioactive compounds present in this plant are alkaloids, anthracenes, quinones, saponins, terpenoids etc. which are potent medicinal substances. Recently crude drugs are popular around the world and many pharmaceutical industries are producing medicines from herbs. As a result, this plant can be used for manufacturing of medicines. The extract of this plant was used for determining the properties, such as, antibacterial property, antioxidant cytotoxicity and thrombolytic activity.

## 1.2 Introduction to the selected plant: *Crassocephalum crepidioides*

Crassocephalum crepidioides is also known as Eyukula, ebolo, thickhead or fireweed plant. It belongs to the family Asteraceae (Fauziana & Susandarini, 2019).This plant is called as "Buror chul" by the local people of Bangladesh. It is mainly an indigenous plant of tropical Africa, South Africa, Senegal East, Ethiopia, Fiji, Australia, subtropical Asia, Madagascar, Mauritius but also abundant in our country, specifically at hill tracts.

## 1.3 Description of the morphology of the plant: *Crassocephalum crepidioides*

Plant is widespread in tropical or subtropical areas but it is mostly available in tropical Africa. Leaves are slightly succulent and oval, spiral or elliptical in shape he leaves are having an uneven edge and these mucilaginous leaves and stems are taken as vegetables, Thickhead or fireweed is a fleshy and erect annual plant which can grow up to 180 cm in height and sometimes it can be branched (Adjatin, Dansi, Yêvidé, …, & 2013, 2013).

The parts of the plants are also used for treatment purposes. Flowers are the most appealing part of this plant. They are covered with green bracts and having red or maroon at the top. They are bisexual, equally distributed, having tubular corolla and around 9 to 11 mm in length. The color of the flowers varies from orange to yellow and at the top the color remains as brown reddish. After blooming properly, the flowers become white in color and puffy in nature.

Fruits have hair, dark purple in color and 9 to 12 mm in length and they considered as ribbed achene, linear or cylindrical in shape and minutely toothed.(Backer & Merr, n.d.)However, the seeds make the plant more beautifying part of the plant. They come into sight after 9 to 10 days of sowing and their very fast-growing seeds and 2 cm in length. It looks like snow white ball when it is matured. The feather of the seeds is assumed as the hair of an old man. Thus, it

helps to float on the air and disperses the seeds. The germination of the *Crassocephalum crepidioides* at a temperature ranges from 10 to 30 or 10 to 40°C and the pH level lies within 4 to 10.

## 1.4 Taxonomy of the Plant:

The plant *Crassocephalum crepidioides* belongs to the kingdom of Plantae. The order and family of this plant are respectively Asterales and Asteraceae. Hence, it is a part of Senecioneae tribe. The genus is regarded as *Crassocephalum* and species of the plant is *crepidioides* (Pineland & Preserve, 2015).

**Classification:**

**Kingdom:** Plantae

**Order:** Asterales

**Family:** Asteraceae

**Genus:** *Crassocephalum*

**Species:** *crepidioides*

## 1.5 The family of the plant: Asteraceae

Asteraceae is a significant family which has higher economical value as it supplies herbal teas, replacement of coffee ingredients, sweetening agents, essential oils, sunflower seeds etc. This family is considered as the most diverse and one of the largest which possesses mostly flowering plants having around 1620 genera and 23,000 species (Barreda et al., 2015).

In general, the plants in this family are annual and perennial herbs. Besides there are shrubs, trees and climbers which are present in an appreciable number. The members of this family

have great importance for their use in garden ornamentals. Some of the plants are marigolds, asters, cosmos, ageratums and dahlias, zinnias, ragweed, sunflower, safflower, burdock etc.

The flowering plants of this family contain many small flowers, seems like clustered together and known as florets which are encircled with bracts. These bracts act as a shield to the flowers. The florets of the flower can be disk or ray in some of the plant. In flowering plants, the petals are outermost part whereas the corolla are elongated and the calyx are different from other plants and called as pappus.

The fruits of Asteraceae family are familiar as achene that are single seeded. In fact, these achenes carry the seeds until maturation and dispersed. The pollination system in these plants are usually secondary and the stamen of the flowers contain the pollen grains inwardly. If cypsela is present, then fruit is absent in this category of plant.

Asteraceae family generates secondary metabolites such as, alkaloids, terpenoids, tannins, acetylenes, alcohols, methylated flavanols, inulin fructans( Lajter, 2015).Moreover, in this largest family , the genera and species are widely distributed and about 950 genera including 20000 species belong to this vast family (Rahman et al., 2008). Among the plants, *Dahlia pinnata, Eclipta prostrata, Blumera lacera, Tagetes erecta, Calendula officinalis, Gaillardia pulchella, Callistephus chinensis, Eclipta alba* are common and easily available plants.

## 1.6 Ethnopharmacological use of the plant:

Folk medicines are popular around the world. The use of *Crassocephalum crepidioides* as traditional medicine have been noticed in several cases. For instance, the juice of the leaves is consumed for the treatment of indigestion and stomachache. Also, application of leaf paste on

the injured area serves as healing agent and protects from microbes as well (Adjatin et al., 2012).

Moreover, in the treatment of epilepsy, sickness in sleep and in case of swollen lips these herbs are used. People have been using different parts of the plant as herbal medicine in the treatment of fever, hepatitis and other inflammations. During the pregnancy of women it is taken so that it helps to move the fetus (Malan & Neuba, 2011).

## 1.7 Therapeutic uses of the plant:

This plant attributes several pharmacological effects. Among these effects, the noteworthy effects are antioxidant and hepatoprotective effects along with antihyperlipidemic activity (Aniya et al., 2005). Other uses of this plant are beta cell protection and antidiabetic activity (Bahar et al., 2017). Hence, toxicity is another property of this this plant (Adjatin, Dansi, Badoussi, et al., 2013).Among the other species of *Crassocephalum crepidioides*, approximately 24 numbers of plants which possesses medicinal properties (Adjatin, Dansi, Yêvidé, et al., 2013).

**Antioxidant property:**

Reactive oxygen species and free radicals are harmful for our body. When excessive amount of these free radicals is produced by our body, causes different inflammation, Parkinson's diseases, diabetes, atherosclerosis as well as many other diseases. Antioxidants helps to prevent the oxidation process which produces free radicals as by products (Mahdi-Pour, Jothy, Latha, Chen, & Sasidharan, 2012).

In addition, the liver is an essential part of our body which works in the metabolism, secretion and detoxification of endogenous and exogenous substances. Because of the free radicals and oxidative dress, the liver starts to damage and the function of the liver

deteriorates which can be prevented by the antioxidants (Sobeh et al., 2019). The plant *Crassocephalum crepidioides* has antioxidant property which can be used to produce drugs to fight against these diseases.

**Antidiabetic activity:**

Diabetes is regarded as chronic disease which leads to hyperglycemia due to insufficient production of insulin. There are two types of diabetes, hence diabetes mellitus is the most prominent and causes severe difficulties to the patient.

To control the diabetes, antidiabetic drugs are produced which targets to metabolite the glucose in our bloodstream (Sagbo, Van De Venter, Koekemoer, & Bradley, 2018). The antidiabetic activity is present in the plant *Crassocephalum crepidioides* which helps to reduce the glucose level in our blood, also helps in metabolism (Bahar et al., 2017).

**Cytotoxicity:**

Cytotoxic drugs work by destroying the cancer cells or inhibiting the growth and cell division. Because of the abnormal cell division and mutation of genes, cancer spreads in our body which can be prevented using cytotoxic drugs. In plants and herbs, the cytotoxic properties are prevalent. Through phytochemical screening, cytotoxic drugs can be formulated from plants and herbs.

**Other uses:**

The leaves and the roots of the plant are edible and used as vegetable in some parts of Africa because of its sharp taste. Not only Africa, in Nigeria, Japan, Vietnam and Tanzania the leaves are popular food items which is cooked in a delicious way. Hence, the nutritional value of the leaves contains protein 3.1 gm, carbohydrates 14.1 gm, water 79.8 gm, fat 0.7 gm, fiber 1.8 and calcium 260 mg (Arawande, Komolafe, & Imokhuede, 2013).

# Chapter Two

# Methodology:

## 2.1 Collection and Identification of the plant:

The plant *Crassocephalum crepidioides* was collected from Moulvibazar at Sylhet region in Bangladesh. Afterwards, the plant was identified and verified by the National Herbarium Bangladesh. The identification number of the plant is 48251.

## 2.2 The Procedure of Extracting Phytochemicals from the Plant:

The leaves were separated from the plant and dried for a week. After that, the dried leaves were grinded to get fine particles of plant leaves. Around 199.5 gm of powdered leaves were soaked in solvent such as, methanol for 3 to 4 days and the methanol extract of powdered plant was filtered using filtration paper.

Then, rotary evaporator named Heidolph was used to dry the methanol extract to get the desired plant extract. The total weight of plant obtained from this procedure is 3.469. The plant extract is ready for experimental use.

## 2.3 Evaluation of Antioxidant properties:

Antioxidants are important for our health and also plays a vital role in preventing diseases. In general, antioxidants are inhibitory substances that are used to prevent oxidation which produces free radicals. These free radicals cause harm to the cells of our body. Oxidative stress is produced due to the formation of reactive oxygen species (ROS) which is also responsible for cell damaging.

To assay antioxidant activity, two experimental methods were followed. They are:

1.Determination of total phenolic content

2.Analysis of antioxidant property using DPPH assay.

## 2.3.1Determination of Total phenolic content:

**Principle:**

Folin-Ciocalteu reagent is used in the determination of total phenolic compound which is based on oxidation reduction reaction process (Vardin et al., 2005). Phenols in presence of alkaline solution reacts with Folin-Ciocalteu reagent which is completely ionized to produce blue color under standard conditions. The change of color at this concentration is noted down and the absorbance is measured at 760 nm which will provide the total phenolic content of the plant extract (Blainski, Lopes, & De Mello, 2013).

## 2.3.2 Materials and reagents:

In this experiment, Folin-Ciocalteu reagent (LOBA Chemicals Pvt. Ltd, India), sodium carbonate (Merck Specialties Pvt. Ltd, Mumbai), gallic acid monohydrate (Sigma Aldrich, USA), methanol (Active Fine Chemicals Ltd, Bangladesh), pipette, distilled water, UV spectrophotometer, beaker, volumetric flask and glass rod are used.

## 2.3.3 Reagent preparation:

In a volumetric flask, 2 mL of Folin-Ciocalteu reagent is taken and which was diluted with 20 mL of distilled water so that the percentage of the reagent becomes 10% in 20 mL of distilled water.

Taking 7.5gm of sodium carbonate in a100mL volumetric flask, distilled water was poured down in it and filled up to the mark of the flask and the percentage was 7.5% in 100mL of distilled water.

### 2.3.4 Standard preparation:

In this experiment, gallic acid is considered as standard. The stock solution was prepared by measuring 0.12gm of gallic acid which was dissolved in 10 mL of methanol in a volumetric flask and the concentration of the solution was 12mg/mL. To dilute the preparation, serial dilution of the solution was made in the respective concentration 100 µg/mL, 50 µg/mL, 25 µg/mL 12.5 µg/mL and 6.25 µg/mL using solvent.

### 2.3.4 Preparation of the sample:

The plant extract was measured on a balance about 0.12gm and it was taken in a volumetric flask in which 10 mL of solvent was added to dilute the sample properly. The initial concentration of the sample was 12mg/mL. Then, the sample solution was serially diluted using the concentration 1200 µg/mL, 800 µg/mL, 400 µg/mL and 200 µg/mL respectively.

### 2.3.5 Blank preparation:

To prepare the blank solution, 5mL of Folin-Ciocalteu reagent was taken and 4mL of sodium carbonate was also added to it. To make the volume up to 10mL, methanol was added which was 1mL.

### 2.3.6 Procedure of the experiment:

1mL of sample and 1mL of standard solution in different concentration was taken in different test tubes. Then, in each test tube, 2.5mL of Folin-Ciocalteu reagent was poured and same amount of sodium carbonate was added to each test tubes.

Each test tubes were shaken and mixed properly and kept in water bath for half an hour at 40°C.Lastly, the reading of absorbance was taken at 765nm using UV-spectrophotometer against blank for each sample and standard solution.

Total phenolic content was determined using the standard curve of gallic acid prepared from the reading taken during the experiment. It was expressed as gallic acid equivalent per gm plant extract.

## 2.3.7 Analysis of antioxidant property using DPPH assay:

## 2.3.8 Principle:

According to Blois, DPPH (1,1diphenyl-2-picrylhydrazyl) is a strong free radical, the antioxidant property of the plant extract is determined based on this free radical. 1 mL of the plant extract was mixed with 2 mL of DPPH solution due to which hydrogen atom is donated and nitrogen atom of DPPH is reduced. As a result, discoloration of violet color DPPH solution is compared with the ascorbic acid to analyze the antioxidant activity (Kedare & Singh, 2011).

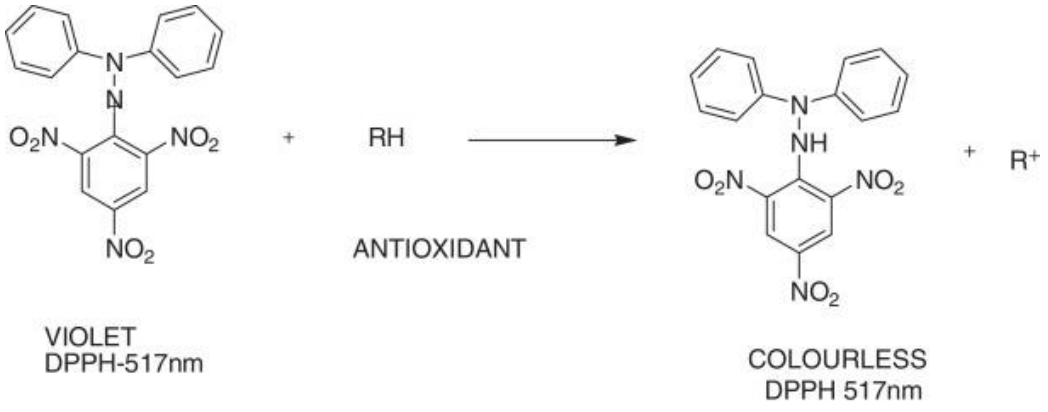


**Figure 1:** Reduction reaction of DPPH (Kedare & Singh, 2011).

## 2.3.9 Reagents and materials:

In this study, DPPH (1,1diphenyl-2-picrylhydrazyl) (Sigma Aldrich, USA), methanol, L-ascorbic acid (Merck, Germany), balance (Model: XY6002G), beaker, UV-spectrophotometer (U-2910), volumetric flask is required.

### 2.3.10 Preparation of reagent:

In 50 mL of distilled water 0.002gm of DPPH was added. After dissolving the solution was kept in the refrigerator at -4°C so that it could be used during the experiment.

### 2.3.11 Standard solution preparation:

To prepare the stock solution of standard, 0.12gm of L-ascorbic acid was taken and dissolved in 10mL of distilled water in which the concentration is 12mg/mL. For dilution the solution was serially diluted based on the following concentration 500 µg/mL, 250 µg/mL, 125 µg/mL and 62.5 µg/mL respectively.

### 2.3.12 Preparation of sample solution:

0.12gm of plant extract was measured and taken in a 10mL volumetric flask and 10mL of distilled water was added up to the mark. After dissolving the solution was serially diluted using the concentration 500 µg/mL, 250 µg/mL, 125 µg/mL and 62.5 µg/mL respectively. The sample solution was ready for use.

### 2.3.13 Procedure of the experiment:

1 mL of each concentration of sample and standard solution was taken in different test tubes. Then,0.004% of DPPH solution that is 2 mL in amount was added to each of the solution. After that, the solution was kept in incubator about half an hour at room temperature.

Finally, the absorbance of sample and standard were taken at 517nm using UV-spectrophotometer against blank. To find out the antioxidant property, % of inhibition of

DPPH was calculated. The following formula was used to calculate the percentage of inhibition:

% of inhibition=[( Control absorbance – Sample absorbance) / Control absorbance)] x 100(Aniya et al., 2005).

## 2.4 Evaluation of in vitro thrombolytic activity:

Thrombolytic agents are those helps to lysis the blood clot by converting the plasminogen into plasmin which breaks down the fibrinogen and dissolves the blood clot (Hossain & Mahmood, 2015). To analyze this activity in the plant sample the experiment is carried out.

### 2.4.1 Reagents and apparatus required:

In the experiment, blood sample from donor, distilled water, standard: Pidogrel (generic name: Clopidogrel bisulphate), micropipette, Eppendorf tube (1.5mL) are required to carry out the process.

### 2.4.2 Preparation of sample solution:

0.4gm of plant extract was mixed in 10 mL of distilled water and the suspended solution was kept for overnight. The next day, using cotton plug filtration was done to get the sample solution.

### 2.4.2 Preparation of standard solution:

In this experiment, pidogrel was regarded as standard which was taken 75mg of it and dissolved in 10 mL of distilled water in a beaker. Then, it was shaken and mixed properly and made standard stock solution.

### 2.4.3 Preparation of blood sample:

In aseptic condition, 3 mL of blood was taken from a volunteer who had never gone through anticoagulant therapy or taken oral contraceptive. After that, the blood was transferred into micro centrifuge tubes and each tube contains 1 mL of blood. Then, the blood sample was incubated at 37 °C for 45 minutes.

### 2.4.4 Procedure for thrombolytic activity test:

The weight of the microcentrifuge tubes was measured separately and the blood drawn from the volunteer was taken in these tubes, each tube contains 1 mL of venous blood. Then, the microcentrifuge tubes were placed into an incubator maintain the temperature at 37 °C and kept for 45 minutes.

After the formation of clot, the weight of the tubes was measured containing the clot and serum and then the serum was completely withdrawn from the tubes. Again, the weight of the clot was noted down.

Then, 100 µL of distilled water which was regarded as blank, 100 µL standard that is, the pidogrel solution and 100 µL of sample preparation was added to individual tubes. Again, the tubes were taken for incubation for 90 minutes at the same temperature at 37°C.

Finally, after the incubation was over, disruption of clot containing blank, standard and sample the weight of the microcentrifuge tubes was measured. Moreover, after removing the released clot the final weight is taken. The percentage of clot lysis was measured using the following formula:

**% of clot lysis**= (weight of released clot / clot weight) ) × 100 (Ramjan, Hossain, Runa, Md, & Mahmodul, 2014).

### 2.5.1 In vitro cytotoxicity assay:

### Principle:

For the test of cytotoxicity, Brine shrimp lethality bioassay is an effective method through which bioactive substance of plant sources or natural origin can be analyzed (Sarah, Anny, & Misbahuddin, 2017). Bioactive compounds are the secondary metabolites of plant having useful effects on human health which contains many phytochemicals within it.

This procedure permits in finding the bioactive substance of natural sources as well as pure compounds. Using brine nauplii entity in this brine shrimp lethality test provides advantageous method for screening and fractionation in the revelation of advanced bioactive compounds (Pimentel Montanher, Pizzolatti, & Costa Brighente, 2002).

For obtaining the brine nauplii, they are hatched in recreated seawater. After calculating the required amount of di-methyl sulfoxide (DMSO), the plant extract sample is prepared using different concentration by diluting it. Then, withdrawing the nauplii from the tank, using Pasteur pipette in a vial containing 5mL of seawater and different concentrations of plant extract are included in it. Afterwards, they are kept for 24 hrs. under observation and the next day the nauplii are counted and calculate the result for this test.

### 2.5.2 Brine shrimp lethality test procedure:

### 2.5.3 Required materials:

In this study, Brine shrimp egg (Artemia salina), hatching tank, lamp for attracting the nauplii, DMSO (Di- methyl sulfoxide), unrefined salt, Pasteur pipette, micropipette, test tubes are needed.

### 2.5.4 Preparation of seawater:

In 1L distilled water, 38gm of salt (unrefined NaCl) was added, dissolved properly to make the seawater and the solution was filtered to obtain clear solution.

### 2.5.5 For test procedure, hatching of brine shrimp:

From the laboratory, brine shrimp eggs were collected and the prepared seawater was poured down in a small hatching tank. Then, the collected eggs were added to the tank containing seawater. In this condition, the tank was left for 2 days providing constant oxygen supply to grow them. Additionally, a lamp was introduced so that the nauplii get enticed to free them from the shells and they were collected from the tank using pipette.

### 2.5.6 Preparation of the sample solution for the test:

0.12gm of plant extract was measured. Placing the sample in test tubes, 200 µL of DMSO was added to dissolve it and made up to 20mL using seawater. The sample solution was diluted serially in the following concentration 1200 µg/mL, 800µg/mL, 400 µg/mL and 200 µg/mL respectively.

| **Concentration (µg/mL)** | **Sample solution mL** | **20nauplii containing seawater** | **Total volume** |
|---|---|---|---|
| 1200 | 2.5 | 2.5 | 5mL |
| 800 | 2.5 | 2.5 | 5mL |
| 400 | 2.5 | 2.5 | 5mL |
| 200 | 2.5 | 2.5 | 5mL |

**Table 1:** 2.5mL seawater containing 20 nauplii and 2.5 mL of sample solution (Asaduzzaman, Sohel Rana, Raqibul Hasan, Monir Hossain, & Das, 2015)

### 2.5.7 Positive control preparation:

Positive control is required in this method to measure the result of test sample comparing with the standard. Here, vincristine sulfate is considered as positive control and very cytotoxic in nature. By dissolving in DMSO, the initial concentration of vincristine sulfate was 20µg/mL Later, the solution was diluted at the following concentration that is 10 µg/mL, 5 µg/mL, 2.5 µg/mL and 1.5 µg/mL.

### 2.5.8 Preparation of negative control:

Negative control is necessary to determine that if the shrimps indicates the fast death rate, then the test procedure is not supportable. They considered to be died with some other possible reason. To prepare the negative control, 100 µg/mL of DMSO was poured into three test tubes holding 5 mL of seawater which were marked previously.

### 2.5.9 Counting of nauplii:

By means of a magnifying glass, after the observation of 24 hours, the nauplii was counted placing the test tubes against a black background.

The percentage of death rate was calculated from the following equation:

% of death = [ number of dead / (number of dead + number of alive)] × 100 (Sathasivam & Lakshmi, 2017).

### 2.6.1 Analyzing antimicrobial activity on *Crassocephalum crepidioides:*

Microorganisms are the causative agents of various diseases around the world. To treat these communicable diseases, antimicrobial agents are required to fight against them. The death rate due to infection caused by bacteria has been reduced because of better sanitation, use of

antibiotics and vaccines. On the other hand, the bacterial strains developing resistance which enables the regeneration of some diseases (Kowti et al., 2010).

According to a statistic carried out by EARS-net (European Antimicrobial Resistance Surveillance Network) in December 31- January 1, 2015, after analyzing it was found that among 671689 infections having antibacterial resistance against bacteria, around 63.5% was related to health issues (Cassini et al., 2019). To overcome this problem, replacement of new antibiotic agents would be benefitted.

Plant produces secondary metabolites which contains chemically active compounds having therapeutic effects. These medicinal properties of plants are safe and efficacious for human health. In recent years, based on these properties of plant, the usage of drugs by the people from natural sources have increased.

As a result, development of antimicrobial agent from natural plant sources is effective solution. In search of new antimicrobial agents, screening is an important aspect and with the aid of it, the vulnerability of many bacteria against these plant extract could be confirmed. There are three methods to determine the antibacterial effect of any agent. Such as,

1.Serial dilution method.

2. Disc diffusion method.

3. Bio autographic method.

In this experiment, disc diffusion method was followed to determine the antimicrobial activity.

### 2.6.2 Disk diffusion method:

### Principle:

In this analysis, antimicrobial agent infused in disc and placed on the agar containing the test sample is used as a basis. The antibiotic spreads through the agar plates in which creates zone of inhibition to exhibit the effectiveness of the antibiotics(Shryock et al., 2008).

### 2.6.3 Materials and reagents used in the test:

In this procedure, autoclave, incubator (LT-36/37), petri dish, forceps (sterile), micropipette, microorganisms, filter paper disc, laminar air flow hood are needed.

### 2.6.4 List of microbes used in this test:

1. *Shigella dysenteriae* (gram negative bacteria)

2. *Bacillus subtilis* (gram positive bacteria)

3. *Pseudomonas aeruginosa* (gram negative bacteria)

4. *Streptococcus pyogenes* (gram positive bacteria)

5. *Bacillus cereus* (gram positive bacteria)

6. *Escherichia coli* (gram negative bacteria)

### 2.6.5 Preparation of nutrient broth:

0.13gm of nutrient broth powder was added to 10 mL of distilled water. For 6 different bacteria, 6 conical flasks were taken and the desired amount of broth was prepared. For dilution purposes, 1.3gm of nutrient broth powder was added to 100 mL of distilled water.

### 2.6.6 Preparation of agar medium:

To prepare the media, 1.4gm of nutrient agar powder was added in 50 mL of distilled water. Then, the media was heated to dissolve it properly and sterilized using autoclave about 15 to 20 minutes

### 2.6.7 Preparation of discs using test sample:

1gm of the plant extract was dissolved in 10 mL of methanol and the filter paper discs were soaked in the plant extract under laminar flow and then after drying the discs were prepared for use.

### 2.6.8 Sterilization procedure:

All the apparatus used in this method such as, the petri dish, conical flask, cotton plug, micropipette, forceps were sterilized by autoclave maintaining the pressure around 15-lbs/sq. and temperature at 121 degree Celsius. To maintain aseptic condition, all the works were operated within the laminar air flow hood.

### 2.6.9 Dilution of bacteria:

To dilute the bacterial concentration, Mc- Farland standard was followed and the absorbance was measured using UV spectrophotometer.

**Table 2:** Dilution of bacteria using Mc-Farland standard

| **Serial number of test tubes** | **Nutrient broth containing bacteria µg/mL** | **Nutrient broth mL** | **Absorbance** |
|---|---|---|---|
| 1 | 350 | 5.0 | 0.012 |
| 2 | 240 | 4.9 | 0.109 |
| 3 | 400 | 4.0 | 0.075 |
| 4 | 370 | 5.0 | 0.057 |
| 5 | 300 | 3.86 | 0.096 |
| 6 | 320 | 4 | 0.031 |

### 2.6.10 Preparation of inoculum:

From a pure culture of bacteria, using a loop, bacteria were taken into the broth.Then, the broth containing bacteria was incubated at 30°C.

Afterwards, by the application of Mc-Farland standard the turbidity of bacteria was adjusted adding nutrient broth to it.

### 2.6.11 Procedure of antimicrobial test:

### Inoculating the plates:

With the help of a cotton swab, bacteria were collected from the suspension of bacteria in this procedure. The agar plates prepared earlier was inoculated by the swab having the bacteria. After streaking the plates were allowed for drying about 3 to 5 minutes.

After finishing inoculation of plates, the antimicrobial discs prepared from the plant extract were placed into the plates along with the control group. Here, streptomycin and amoxicillin were used to compare the result.

Finally, the plates were kept at the incubator maintaining the optimum temperature at 37 °C for 24 hours

### 2.6.12 Determination the zone of inhibition:

The zone of inhibition is determined by the effect of antimicrobial agent in which there is no growth of bacteria and this zone is termed as the zone of inhibition. Using a scale, the diameter of the zone of inhibition is measured and find out the result.

# Chapter Three

## Result and Discussion:

## 3. Result and observation:

## 3.1 Analysis of antioxidant property:

## 3.2 Evaluation of total phenolic content of *Crassocephalum crepidioides:*

**Table 3:** Absorbance of gallic acid

| **Concentration (µg/mL)** | **Absorbance** | **Regression line** | **R2** |
|---|---|---|---|
| 100 | 0.795 | | |
| 50 | 0.411 | | |
| 25 | 0.216 | Y=0.0078x+0.0198 | 0.9994 |
| 12.5 | 0.126 | | |
| 6.25 | 0.057 | | |

From the above table, by preparing the standard curve using the values of concentration and absorbance, the regression value was obtained 0.9994.

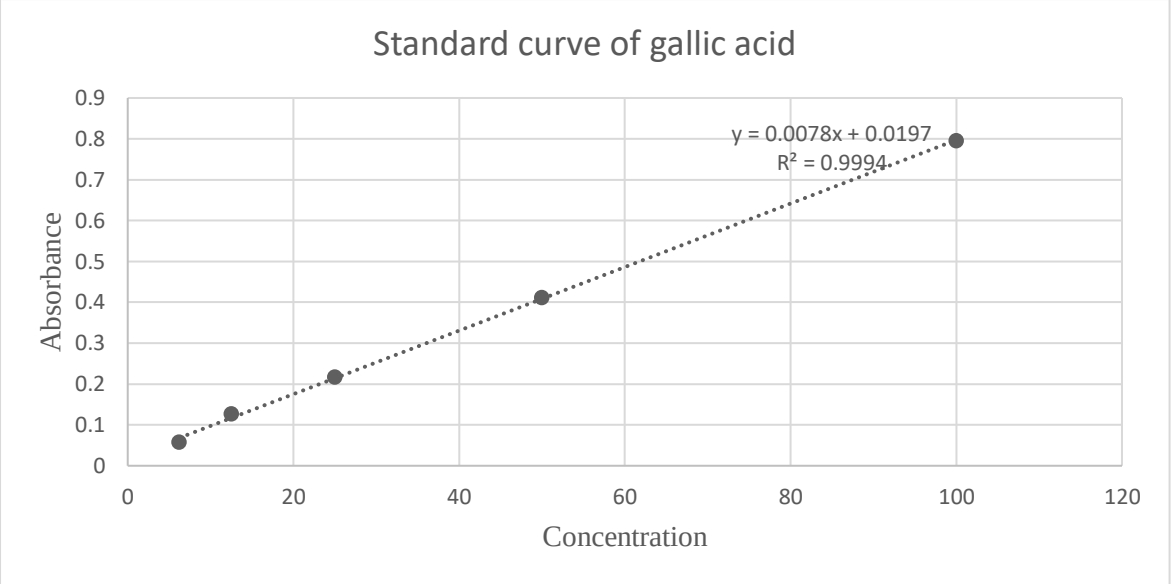


**Figure 2:** Standard curve of gallic acid

**Table 4**: Total phenolic content determination

| **Extract name** | **Sample code** | **Part of plant** | **Total phenolic content (mg of GAE/gm of extract** |
|---|---|---|---|
| Methanol extract | ME | Leaves of the plant (*Crassocephalum crepidioides)* | 637.22 |

According to the chart, the total phenolic content of the methanolic extract of the plant *Crassocephalum crepidioides* is 637.22 which is an acceptable result and it indicates the presence of antioxidant property of the plant. As a result, the methanolic extract of the plant exhibits antioxidant property.

## 3.4 DPPH assay:

**Table 5:** $IC_{50}$ value of methanol plant extract and standard

| Sample code | Extract name | Parts of plant | IC50 value |
|---|---|---|---|
| ME | Methanol | Leaves of the plant (*Crassocephalum crepidioides)* | 136.016 |
| Ascorbic acid | | | 94.12 |

**Table 6**: $IC_{50}$ value of Ascorbic acid.

From the table, it is evident that the $IC_{50}$ value of methanol extract of the plant and ascorbic acid are 136.016 and 94.12 respectively. So, it depicts that the half maximal inhibitory concentration of the plant extract is higher than the ascorbic acid which shows the presence of antioxidant property of the plant.

| **Concentration (µg/mL)** | **Absorbance of standard** | **% of inhibition** | **$IC_{50}$ µg/mL** |
|---|---|---|---|
| 500 | 0.034 | 97.82 | |
| 250 | 0.041 | 97.37 | 94.12 |
| 125 | 0.060 | 96.16 | |
| 62.5 | 0.171 | 89.05 | |

The chart gives us information about the $IC_{50}$ value of ascorbic acid which is obtained by placing the reading of concentration and calculating the percentage of inhibition and after preparing the standard curve, the desired value was obtained which is 94.12

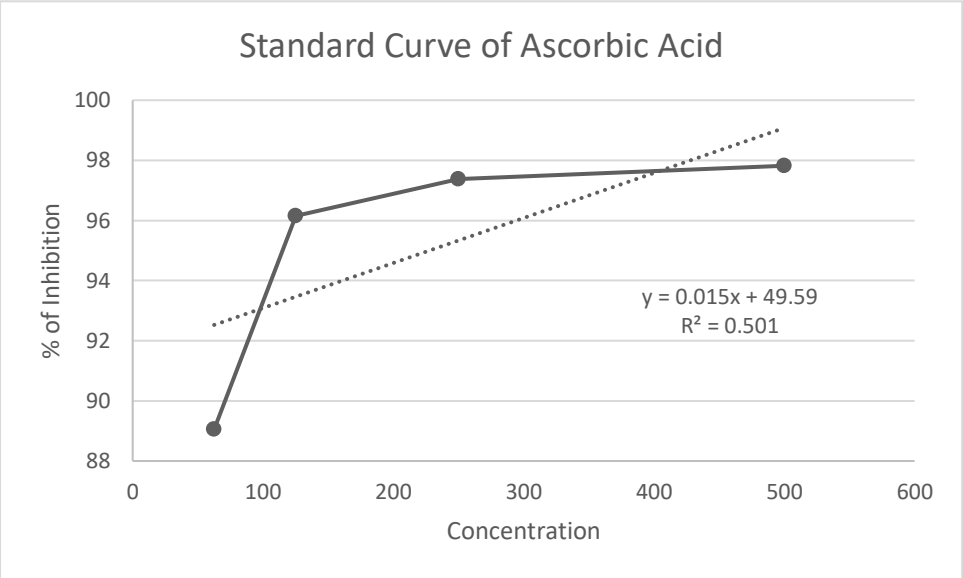


**Figure 3:** Standard curve of ascorbic acid

**Table 7**: $IC_{50}$ value of methanol plant extract:

| **Concentration (µg/mL)** | **Absorbance of methanol extract** | **% of inhibition** | **$IC_{50}$** |
|---|---|---|---|
| 500 | 0.105 | 98.78 | |
| 250 | 0.076 | 97.44 | 136.016 |
| 125 | 0.040 | 95.13 | |
| 62.5 | 0.019 | 93.28 | |

In this chart, the $IC_{50}$ value of the plant extract is 136.016 which is obtained by calculating the percentage of inhibition and later, the standard curve was prepared to get the result.

## 3.7 Analysis of thrombolytic activity:

**Table 8:** Result of thrombolytic activity

| **Name of the sample** | **$W_1$ (weight of the micro tube** | **$W_2$ (weight of the micro tube containing clot** | **$W_3$ (weight of clot)** | **$W_4$ (weight of the tube with clot after lysis** | **$W_5$ (weight of lysis)** | **% of clot lysis** |
|---|---|---|---|---|---|---|
| Blank | 0.837 | 1.800 | 0.963 | 1.718 | 0.082 | 8.52% |
| Standard | 0.837 | 1.811 | 0.974 | 1.750 | 0.061 | 6.26% |
| Methanol extract | 0.837 | 1.742 | 0.905 | 1.662 | 0.08 | 8.81% |

In this experiment, for comparative study, thrombolytic activity of the blank, standard and plant extract was found and recorded. Then, calculating the weight taken during the experiment, the result of the evaluation was obtained. Here, $W_1$ is the weight of the microtube without any substance, $W_2$ is the weight of the microtube containing the clot. To obtain the weight of the clot, $W_1$ is subtracted from $W_2$. Next, to get the weight of lysis, weight of the tube containing clot $W_2$ is subtracted from $W_4$. Using the equation, percentage of clot lysis was found. From the experiment, the effect of the plant extract showed satisfactory result on clot lysis compared to standard.

## 3.8 Evaluation of brine shrimp lethality test:

**Table 9**: $LC_{50}$ value of methanol plant extract

| **Concentration (µg/mL)** | **Log concentration** | **Nauplii taken** | **Nauplii dead** | **Nauplii Alive** | **% of mortality** | **$LC_{50}$** |
|---|---|---|---|---|---|---|
| 1200 | 3.079 | 20 | 20 | 0 | 100 | |
| 800 | 2.903 | 20 | 4 | 16 | 20 | 69.245 |
| 400 | 2.602 | 20 | 8 | 12 | 40 | |
| 200 | 2.301 | 20 | 6 | 14 | 30 | |

**Table 10:** $LC_{50}$ value of vincristine sulfate.

| **Concentration (µg/mL)** | **Log concentration** | **Nauplii taken** | **Nauplii dead** | **Nauplii alive** | **% of mortality** | **LC50** |
|---|---|---|---|---|---|---|
| 10 | 1 | 10 | 8 | 2 | 80 | |
| 5 | 0.698 | 10 | 6 | 4 | 60 | 3.064 |
| 2.5 | 0.397 | 10 | 5 | 5 | 50 | |
| 1.5 | 0.176 | 10 | 4 | 6 | 40 | |

From this evaluation, it can be revealed that the lethal concentration of vincristine sulfate is 3.064 and the plant extract has the value 69.245 respectively. Here, vincristine sulfate acts as standard and the toxicity effect of the vincristine sulfate on nauplii was detected through the experiment as well as the effect of the plant extract was also recorded. Later, calculating the

percentage of mortality rate, the lethal concentration was found and compared with the standard for obtaining the result.

## 3.9 Antimicrobial effect on *Crassocephalum crepidioides*:

Antimicrobial agents are used for the purpose of destroying or inhibiting the microorganisms. From this study, the plant extract did not exhibit any effect on microbes. However, the environment may have effect on the microbes as well as the genetical stress could be another reason for which the microbes did not show any effect in the experiment.

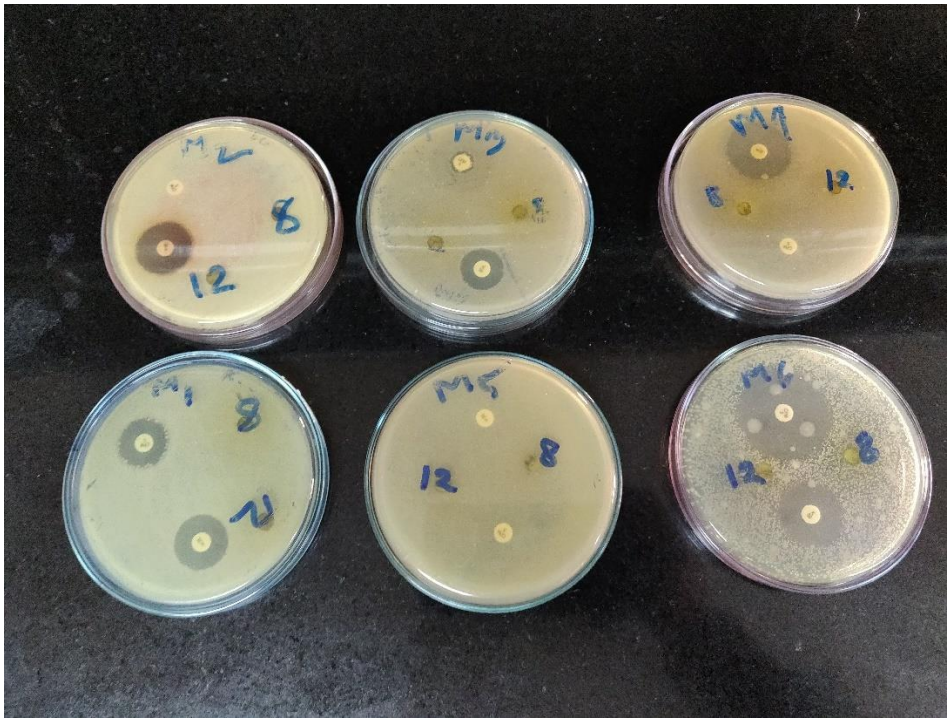

**Figure 4:** Antimicrobial test

## 3.10 Discussion:

*Crassocephalum crepidioides* is one of the traditional medicinal plant which exhibits several pharmacological effects such as antitumor, hepatotoxic effect, antibacterial, antioxidant anthelmintic, local anesthetic properties etc. These medicinal properties can be used in the development of phytomedicine as they have the potential sources of bioactive compounds.

The notable phytochemicals produced by this plant are tannins, coumarins, steroids and anthracene derivatives.

The phytochemical screening of this plant revealed the outcome after analyzing the antioxidant property, thrombolytic activity, the brine shrimp lethality test and antimicrobial test. At first, the antioxidant activity was evaluated. In this analysis, two methods were operated to determine the activity (DPPH assay and total phenolic content determination).

The total phenolic content of the methanolic extract of *Crassocephalum crepidioides* was 637.22 mg/gm which is a satisfactory value and gallic acid was standard in this procedure. The $IC_{50}$value of the plant extract of DPPH assay was 136.016 using ascorbic acid as reference standard, the obtained value from this study which is a moderate value if compared with Awang-kanak, abubaka rand Mohamed (Awang-kanak, Fadzelly, & Bakar, 2019). The concentration of the extract ranged from 1 to 100 µg/mL in case of total phenolic content and 1 to 500 µg/mL in DPPH assay.

Next, the thrombolytic activity of the plant extract was evaluated against blank that is distilled water and as a standard clopidogrel bisulphate was used. The percentage of clot lysis of distilled water 8.52%, whereas the standard provided 6.26% and the plant extract had a value of 8.81%. After analyzing the values, the plant extract showed an effective and satisfactory result of thrombolytic activity.

Then again, the evaluation of brine shrimp lethality test displayed the $LC_{50}$value of plant extract as 69.245 which was moderately toxic and the $LC_{50}$value of vincristine sulfate was 3.064 which was very toxic according to Meyer index followed by the article (Hamidi, Jovanova, & Panovska, 2014).

Moreover, the antimicrobial test was also performed but due to some reason the result was not found. In the procedure, both gram- positive and gram-negative bacteria are used. At first,

the experiment was carried out using low concentration but did not get the result. Then, increasing the concentration of the plant extract, 1gm is used to evaluate the test. Even though the outcome is disappointing. The possible reasons might be the microbes might have gone through Horizontal Gene Transfer during some generation or the other, thus acquiring genes that make them resistant against the antimicrobial agent.

Another reason could be the plant might have been influenced by the environment. Thus, in a foreign environment, it might have produced antimicrobial agents to combat microbes that affect it. Those microbes might have been absent in this environment. Thus, the plant might not have produced the antimicrobial agents.

The phytochemical evaluation of methanolic extract of the plant *Crassocephalum crepidioides* provides antioxidant effect, thrombolytic activity as well as cytotoxicity which are important information for the isolation of active compounds and production of the drugs from the medicinal plants. In this study, leaves of the plant were used to determine the properties. Hence, other parts of the plant such as, roots, stems, flower and fruits can be used for the evaluation purpose. Therefore, this study aims to provide active constituents from natural sources to produce upgraded medicines to fight against diseases.

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