Phytochemical and Biological Investigation of *Commelina diffusa*

A project submitted

by

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Dedication

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

Certification statement

This is to certify that, this project titled ‘Phytochemical and Biological Investigation of '*Commelina diffusa*’ submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Ridwan Islam, Senior Lecturer, Department of Pharmacy, BRAC University and that appropriate credit is given where I have used the language, ideas or writings of another.

Signed,

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Countersigned by the Supervisor

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Acknowledgement

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

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

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Rubina Ahmed Mou

Abstract

Present study was performed to find out important phytochemical constituents, and establish the scientific basis of the use of the leaves of *Commelina diffusa* as therapeutic agent in traditional medicine in Bangladesh. The leaves of the plant were extracted by using methanol and the crude methanolic extract was subjected to phytochemical screening and *in vitro* biological investigation. The phytochemical screening tests confirmed the presence of phenols, sterols, and glycosides, which made the plant a promising source of new natural drugs. The antimicrobial activity testing, done by agar diffusion method, revealed good activity against different bacteria and fungi, with the maximum potential against *Staphylococcus aureus.* The study also confirmed mild total phenolic content and DPPH free radical scavenging activity that in turn confirmed mild antioxidant potential of the plant under experiment, and moderate cytotoxicity in brine shrimp lethality bioassay. Nevertheless, further investigation is needed to develop new drugs, especially anti-microbial agents, to treat a wide variety of diseases.

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Abbreviations

DPPH - 1, 1-diphenyl-2-picrylhydrazyl

ASA - Ascorbic Acid

DMSO - Dimethylsulphoxide

GAE - Gallic Acid Extract

VS - Vincristine Sulphate

TPC - Total Phenol Content

WHO - World Health Organization

ME - Methanolic extract

Chapter One: Introduction

1.1 Rationale and objective of the present study

Medicinal plants are different types of plants used in the study or practice of the medicinal and therapeutic use of plants and medicinal activities. In drug development and synthesis, medicinal plantcan be used because it contains a high resource of ingredients. These medicinal plants also have a strong role in human values development all over the world (Mantry *et al*., 2014).

About half million therapeutic plants are available all around the world and their medicinal activities have not investigated yet, so these plants have a splendid future and they could be significant key for present and future treatment (Hassan, 2012).In many developing countries, a large number of people depend on traditional consultants and their methods of using medicinal plants in order to encounter health care needs (S. Hosseinzadeh *et al*., 2015).

Conventional medicinal drug refers to fitness practices, procedures, awareness and beliefs incorporating plant, animal and mineral based medications, spiritual treatments, implemented singularly or in mixture to treat, diagnose and save ailments or preserve well-being (Hussain and Malik, 2013).The therapeutic properties of the medicinal plants are partially assigned to essential oils which have been presented to have biological properties such as antiviral, antibacterial, insecticidal and antioxidant properties (Keawsaardand Kongtaweelert, 2012). The worldwide interest for restorative plants is communicated from four identifiable sources: (i) pharmaceutical productions, (ii) traditional healthcare systems (iii) individual traditional health specialists and (iv) women in family home care (Lambert *et al*., 1997).

The plants were the fundamental source of medications for the world's population. Today, 75% of the total population, the poor 3/4ths, still depends on those plants and other tools of conventional drug. Plants have given a source of motivation to novel medication combinations, as plant derived medication have made substantial commitments to human wellbeing and prosperity (Haque, 2015). The quantity of higher plant species (angiosperms and gymnosperms) on this planet is assessed at 250,000 (Ayensu, 1978), with a lower level at 215,000 (Cronquist, 1981; Cronquist, 1988) and an upper level as high as 500,000 (Tippo, 1977; Schultes, 1972). Of these, exclusive around

6% have been partitioned for biologic movement, and a detailed 15%have been assessed phytochemically (Verpoorte, 2000).

Bangladesh, having an expansive assortment of plant kingdom gave the old culture to the act of herbal medications. The high price of imported customary medications as well as unavailability to western medicinal services office, suggest that conventional method of social insurance is the main type of human services that is reasonable and accessible to our rural people. Then again, though when western wellbeing offices are accessible, customary medication is seen as a productive and a worthy framework from a social point of view.

Bangladesh contains about 5000 plant species due to its fruitful soils, climate and seasonal variety (Kadir 1990; Yusuf *et al*., 1994). About 500 of these species are appealed to have medicinal properties (Ghani 2004; Hossain 2005). About 85% of the population lives in rural areas (Halim *et al*., 2007) and almost 80% are reliant on medicinal plants for primary healthcare (Hossain 2005). It was stated that many people regularly use such treatment because herbal medication is sometimes considered comparatively low-cost. However, its popularity stems from the effectiveness of the treatment in most cases (Mukul *et al*., 2007) and comparatively safety, with few or no side effects (Mukul *et al*., 2007). In addition, herbal medications, because of their dispersed nature, are mostly easily and rapidly available (Elliot and Brimacombe 1986).

The utilization of restorative plants in primary health frameworks is imperative, particularly in remote provincial groups and inadequately available ranges. The arrangement of Unani and Ayurvedic prescription was additionally brought under the National Drug Policy of Bangladesh in 1982 to guarantee accessibility, business assembling and promoting of value Unani and Ayurvedic Medicine and Drugs (Ahsan *et al*., 1997).

1.2 The Plant Family: Commelinaceae

Commelinaceae is a group of blooming plants. Sometimes the group is stated to as the dayflower family or spiderwort family. It is one of five families and by a long shot the biggest of these with around 731 known species in 41 genera (Christenhusz and Byng, 2016).Renowned genera include *Commelina* (dayflowers) and *Tradescantia* (spiderworts). The family is different in both the Old World tropics and the New World

tropics, sometimes some genera display in the both place (Faden, 1983). The variation in morphology, predominantly which of the flower and inflorescence, is assumed to be especially high among the angiosperms (Evans *et al*., 2000).

Herbs are yearly or perennial, infrequently woody at base. Stems are with noticeable nodes and internodes. Leaves alternate, distichous or, then again spirally organized, sessile or petiolate; leaf sheath noticeable, open or closed; leaf sharp edge basic or the whole. Flowers are bisexual, actinomorphic orzygomorhpic, trimerous in nature. There are 6 tepals, in 2 whorls of 3, external tepals for the most part green and uniform while the internal tepals are normally free, equivalent or unequal, white, pink, blue or purple. The androceum normally comprises six particular stamens distributed in two whorls, all functional or some of them are missing or characterized by staminodes. The gynoecium consists of a single basic pistil of three carpals and a predominant ovary containing three or once in a while by abortion just two locales, each having one couple of axile ovules. The fruit is in loculicidal or indehiscent capsule shape *(*Faden, 1998).

1.3 The plant genus: *Commelina*

Commelina is a genus of around 170 species usually called dayflowers because of the short existences of their flowers. They are more rarely recognized as widow's tears. *Commelina* is the largest genus of Commelinaceae family (Faden, 2006). They are dispersed over the tropical and sub-tropical areas with core centers in Africa and Asia. Members of this genus concerned in consideration of both cytologists and cytotaxonomists for varieties in chromosome number and morphology (Jones and Jopling 1972).

A substantial number of types of this genus have been researched cytologically from various parts of the world (Bhattacharya 1975, Jones and Jopling 1972, Jones *et al*., 1975, Lewis 1964, Morton 1967, Patwary *et al*., 1985, Rao *et al*., 1968). In India, the cytology of 19 species out of a total of 23 species has been observed (Rao and Kammathy, 1970). In Bangladesh, nine species are known which are similarly common in India (Khan and Alam, 1976).

1.4 Some reported species

- *Commelina benghalensis* L.
- *Commelina virginica* L.
- *Commelina communis*

- *Commelina cyanea* .
- *Commelina diffusa* R.Br
- *Commelina kotschyi* Hassk.
- *Commelina erecta* L.
- *Commelina lukei* Faden.
- *Commelina mascarenica* C.B.Clarke
- *Commelina sphaerorrhizoma* Faden& Layton
- *Commelina tuberosa* L.

1.5 Description of *Commelina diffusa*

Commelina diffusa, occasionally recognized as the climbing dayflower or spreading dayflower, is a pantropical herbaceous plant in the dayflower family. It has been familiarized with the southeastern United States where it is most common in wet soils. There are two familiar varieties, one being the sort and the other being C. diffusa var. gigas, which is local to Asia and has been acquainted with Florida. It blossoms from spring to fall and is most basic in disturbed environments, wet places and forests. In China the plant is utilized therapeutically as a febrifuge and a diuretic. A blue color is additionally removed from the blossom for paints. In the Hawaiian Islands, it is known as "honohono grass", despite the fact that it is actually not a grass."Honohono" introduces to the exchanging structure of the leaves.

Fig1.1 A whole plant

Fig 1.2 leaves with stems

Fig 1.3Flower with seeds

1.5.1Taxonomic hierarchy

Domain: Eukaryota

Kingdom: Plantae

Phylum: Spermatophyta

Subphylum: Angiospermae

Class: Monocotyledonae

Order: Commelinales

Family: Commelinaceae

Genus: *Commelina*

Species: *Commelina diffusa*

Scientific name: *Commelina diffusa* Burm. f.

Local name:

- **Bangladesh:** manaina
- **Cuba:** canutillo
- **Indonesia:** brangbangan
- **Japan:** Shimatsuyukusa
- **Philippines:** alibangon
- **Thailand:** phak-prap

1.5.2 General Botanical Data

Commelina diffusa is a yearly, or all the more prostrate to ascending, greatly branched herb which can grow 1 meter or more high. It is collected from the remote area for nearby use as food, drug and region of dyestuff.

Table 1.1: Description of different plant parts of *Commelina diffusa*

Part of plant	Description
Roots	White stringy roots, fasciculate, also present at the nodes in contact with the soil.
Stem	Thick, cylindrical and glabrous
Leaves	Simple alternate leaves, ovate to lanceolate, bright green, glabrous, 2 to 5cm long and 0.5 to 2cm wide.
Fruit	Capsule three-celled. Long from 4 to 5mm. Lower one indehiscent with 1 seed; dorsal one dehiscent with 2 seeds.
Seeds	Each capsule has 5 seeds, reticulate-ribbed, elongated and brownish, the length is from 2 to 3mm.

1.6 Chemistry of *Commelina diffusa*

1.6.1 Chemical constituents

- anthocyanins
- dammaranetriterpene
- stigmasterol,
- n-octacosanol,
- n-triacontanol,
- ß-sitosterol
- alkanols,
- n-dotriacontanol,
- sterols,
- campesterol (Ghani, 2003)

1.7 Uses of *Commelina diffusa*

1.7.1 Edibility

- The small flowers can be used as salad green.
- The stems are cooked and eaten as green vegetables in India.
- The leaves of the plants are used as fresh salads.

1.7.2 Folkloric

- The juice of the plant is used in the treatment of Mumps.
- The fresh juice is used for the treatment of poisonous snake bites. This drug may be used with antidote preparation and applied on the bite.
- It is used in the treatment of frequent urination, diarrhea and vomiting, laryngopharyngitis, tonsillitis and colds.
- It can be used on external wound bleeding.
- The people of Africa and Asia use this plant to treat high blood pressure and renal diseases.
- In Nigeria, it is used for relieving constipation. Leaf-infusion used as eyewash. Eye lotion is used for eye complaints.
- In China, decoction of whole plants is used for lowering the body temperature and detoxification, for leucorrhea and health protection.
- In Mexico, the plant is used for the treatment of pink eye, dysmenorrhea and dermatitis.
- In Paraguay, the whole plant is used for gonorrhea and infertility treatments.
- In Hawaii, the plant is used as blood purifier and wound healing.
- In Ecuador and Peru, the decoction is used as a tea to treat headaches.
- In the Guianas, the juice from the whole plant is used in a decoction against lumps. The infusion of the leaves is used to treat hair loss and fever.
- In Santa Lucia, the suppository of stem treated with castor oil is used in moving infant's bowels. The tea of the plant is used to treat bladder infection and hypertension.

1.7.2 Commercial Uses

- Petal juice is used as a dye painting.
- In Africa, India and Asia, the plant is used as food for low income people.
- Sticky plant juice is used as glue.

Chapter Two: Methodology

2.1 Chemical investigation of the experimental plants

Commelina diffusa belongs to the family of Commelinaceae and it was tested for its chemical constituents.

2.1.1 Collection and preparation of the plant material

The leaves of *Commelina diffusa* were collected from Dhaka in April, 2016. After proper washing, the leaves were sun dried for several days. The dried leaves of the plant were blended to a rough powder by using grinding machine. The powder was then stored in an air tight container and kept in dry and cool place.

2.1.2 Extraction of the plant material

300gm of the coarse powder material was taken in a fresh, round bottomed flask (5 liters) and soaked in 2.5 liter of methanol. Then the container was sealed by foil and kept for a period of 15 days accompanying random stirring and shaking. Then the mixture was filtered by a fresh cotton plug and finally with a Whatman No.1 filters paper. The total volume of the filtrate mixture was then reduced using a Buchii Rotavapour at low temperature and pressure. The weight of the crude extract was 25 gm.

2.2 Phytochemical Identification

Phytochemical identification was completed on the methanolic extract of *Commelina diffusa* in order to identify its chemical compositions like alkaloids, glycosides, phenols, flavonoids, sterols etc.

2.2.1 Preparation of Extracts

2-3gms of powder was taken into a 100 ml conical flasks and 50 ml of solvent (Methanol) was added. The flask was labeled and allowed to stand for 1-2hrs, filtered using Watmann No.1 filter paper. The filtrate obtained was used for the screening of secondary metabolites following standard procedures (Kokate *et al*., 2009; Evans and Trease 2002; Khandelwal, 1995; De *et al*., 2010). The following qualitative tests were performed:

I. **Tests for Alkaloids:**
   A. **Mayer's test:** 1ml of extract was mixed with a few drops of Mayer's reagent (Potassium Mercuric Iodide Solution). Formation of cream color precipitate indicates the presence of alkaloids.

B. **Wagner's test:** To 1ml of each extract was mixed with equal volumes of Wagner's reagent (Iodine in potassium iodide). Formation of reddish brown precipitate indicates the presence of alkaloids.

C. **Dragendorff's reagent test:** To 1ml of each extract, 2 ml of Dragendorff's reagent was added and mixed. To this 2 ml of dilute HCl was added. Formation of an orange colored precipitate indicates the presence of alkaloids.

D. **Hager's test:** To 2ml of each extract, a few drops of Hager's (Saturated picric acid solution) reagent were added. Formation of a bright yellow colored precipitate indicates the presence of alkaloids.

II. Tests for glycosides:

A. **Keller Killiani test:** 1ml of the extract was dissolved in 1ml of glacial acetic acid and cooled, after cooling, 2-3 drops of ferric chloride was added. To this solution 2ml of conc. H_2SO_4 was added carefully along the walls of the test tube. Appearance of reddish brown colored ring at the junction of two layers indicates the presence of glycosides.

B. **Conc. H_2SO_4 test:** To 1ml of the extract, 1ml of conc. H_2SO_4 was added and allowed to stand for 2 min. a reddish color precipitate indicates the presence of glycosides.

C. **Molisch's test:** 2-3 drops of Molisch's reagent was added to the extract and mixed well. Then a few drops of conc. $H_2SO_4$was added carefully. Formation of reddish-purple colored ring at the junction of two layers indicates the presence of glycosides.

III. Test for anthraquinones: 1-2ml of the extract was mixed with equal volumes of benzene and then about 1ml of 10% ammonia solution was added. Formation of red color on addition of ammonia indicates the presence of anthraquinones.

IV. Tests for Phenols:

A. Ellagic acid test: The extract was treated with few drops of 5% (w/v) glacial acetic acid followed by 5% (w/v) $NaNO_2$ solution. Formation of muddy brown color indicates the presence of phenols.

B. Phenol test: 2ml of the extract was separately treated with 1ml of $FeCl_3$ solution. Development of an intense color confirmed the presence of phenols.

V. Tests for Flavonoids:

A. **Zinc-HCl reduction test:** To the extract add a pinch of Zinc dust and a few drops of Conc. HCl. Formation of deep red color indicate the presence of Flavonoids.

B. **Lead-acetate test:** To 1-2 ml of the extract, add few drops basic lead acetate solution. Formation of reddish brown precipitate indicates the presence of flavonoids.

C. **$FeCl_3$ test:** To 1-2 ml of the extract, add few drops of neutral ferric chloride solution. Deposition of blackish red precipitate indicates the presence of flavonoids.

D. **Alkaline Reagent test:** To 1-2 ml of the extract, a solution of sodium hydroxide is added. Appearance of yellow to red color indicates the presence of flavonoids.

VI. Test for Sterols:

A. **Liebermann-Burchard test:** To 1-2 ml of the extract, a few drops of acetic anhydride solution were added. To this mixture, a few drops of Conc. H_2SO_4 were added carefully along the walls of the test tube. Formation of reddish brown ring at the junction of two layers indicates the presence of steroids.

B. **Salkowski test:** to 1-2 ml of the extract, 5ml of chloroform was added. To the above mixture, 1ml of conc. H_2SO_4 was added carefully along the walls of the tube and mixed. The formation of reddish color in the lower layer indicates the presence of steroids.

2.3 Evaluation of Antioxidant Activity

The current development in the information of free radicals and reactive oxygen species (ROS) in science is delivering a therapeutic revolution that guarantees another period of health and disease management. The vast majority of the possibly harmful impacts of oxygen are because of the arrangement and movement of a number of chemical compounds, known as ROS, which have a tendency to provide oxygen to different substances. Free radicals and antioxidants have turned out to be commonly used terms in modern discussions of disease mechanisms (Lobo *et al.*, 2010).

The purpose of this study was to evaluate methanolic extract of *Commelina diffusa* as new potential sources of natural antioxidants and phenolic compounds. The therapeutic properties of plants have been researched in the recent scientific developments in

throughout the whole world, because of their intense antioxidant activity exercises, no side effects and economic feasibility.

The antioxidant activity analyzed in terms of

- Determination of total phenolic content.
- Determination of antioxidant properties: DPPH assay

2.3.1 Determination of total phenolic content

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

The phenolic compounds employ their antioxidant properties by redox properties, which enable them to act as reducing agents, hydrogen donators and singlet oxygen quenchers (Proestos *et al*., 2017).

Antioxidant compounds can be found in various foods and medicinal plants which play an important part in the prevention and treatment of chronic diseases which are caused by oxidative pressure. They frequently have strong antioxidant and free radical scavenging abilities and anti-inflammatory action too, which are similarly the basis of other bioactivities and health benefits, such as anti-aging, anticancer, and defensive activity for cardiovascular diseases, diabetes mellitus, obesity and neurodegenerative diseases (An-Na Li *et al*., 2014).

2.3.2 Principle

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

Phenols + Na_2CO_3 complete ionization → Ionized phenols

Ionized reagents + phenols + Folin-Ciocalteu Oxidation → Folin-Ciocalteu reagent complex

(Yellow) **(Blue)**

2.3.3 Materials and Methods

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

2.3.4 Materials

- Folin-Ciocalteu reagent (10 fold diluted)
- Na_2CO_3 solution (7.5 %)
- Distilled water
- Pet ether
- Methanol
- Chloroform
- Ethyl acetate
- UV-spectrophotometer
- Vial
- Beaker (100 and 200 ml)
- Ascorbic acid
- Pipette (1ml and 5 ml)
- Micropipette (50-200 µl)

Table 2.1 Composition of Folin-Ciocalteu reagent

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

2.3.5 Standard curve preparation

Here, Gallic acid was used as standard. Gallic acid solutions were prepared which have different concentrations ranging from 100 µg / ml to 0 µg / ml. 2.5 ml of Folin-Ciocalteu reagent which was diluted 10 times with distilled water and 2.0 ml of Na_2CO_3 (7.5 % w/v) solution was added to 0.5 ml of gallic acid solution. The mixture was incubated for

20 minutes at room temperature. The absorbance was measured at 760 nm, after 20 minutes.

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

2.3.6 Sample preparation

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

2.3.7 Total phenolic compound analysis

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

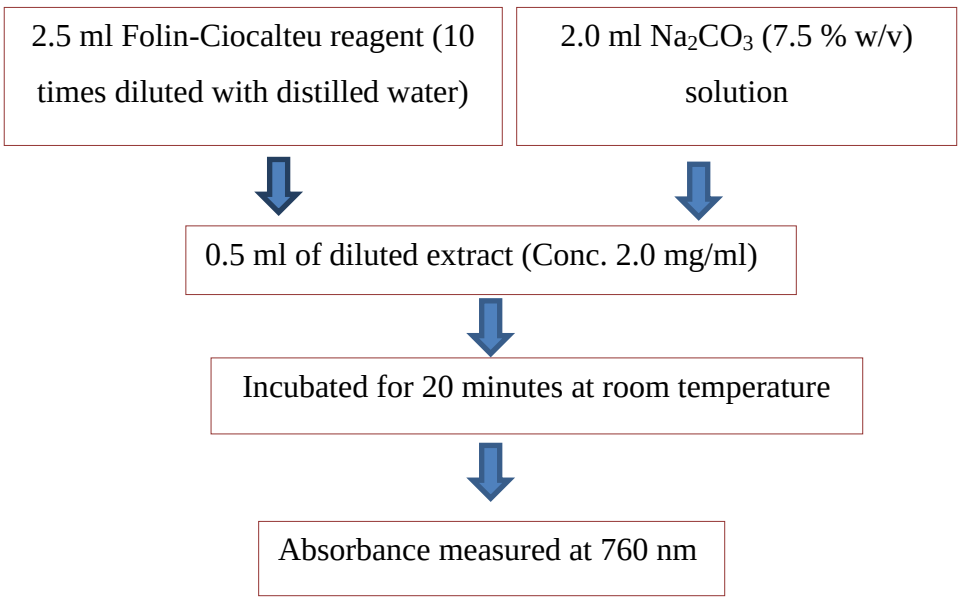


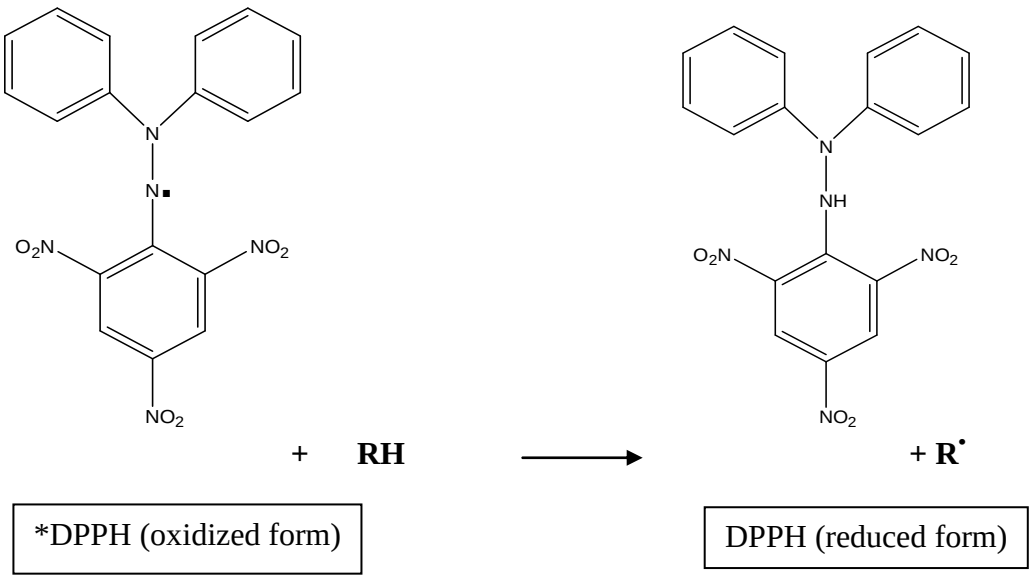
Figure 2.1: Schematic representation of the total phenolic content determination

2.3.8Antioxidant activity: DPPH assay

2.3.9 Principle

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

Then 2.0 ml of a methanol solution of the sample (extract/ control) at different concentration (500µg/ml to 0.977µg/ml) were mixed with 3.0 ml of a DPPH methanol solution (20µg/ml). The bleaching of purple colored DPPH methanol solution by the plant extract is compared with a standard ascorbic acid.



* DPPH = 1, 1-diphenyl-2-picrylhydrazyl

2.3.10 Materials and Methods

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

Table 2.2: Reagents and apparatus used in DPPH assay

1,1-diphenyl-2-picrylhydrazyl	UV-spectrophotometer
Micropipette (50-200 µl)	Beaker (100 and 200ml)
Ascorbic acid	Amber reagent bottle

Distilled water	Test tube
Methanol	Light-proof box
Chloroform	Pipette (5ml)
Ethyl acetate	

2.3.11 Control preparation for antioxidant activity measurement

Here, Ascorbic acid (ASA) and BHT (*tert*-butyl-1-hydroxytoluene) were used as positive control. Calculated amount of ASA and BHT were dissolved in methanol to get a mother solution having a concentration 1000 µg/ml. Serial dilution were made using the mother solution to get different concentration ranging from 500.0 to 0.977 µg /ml.

2.3.12 Test sample preparation

Calculated amount of methanolic extract was measured and dissolved in methanol to get the mother solution (Conc. 1000 µg/ml). Serial dilution of the mother solution gave different concentration ranging from 500.0 to 0.977 µg /ml which were kept in the marked flasks.

2.3.13 DPPH solution preparation

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

2.3.14 Assay of free radical scavenging activity

2.0 ml of a methanol solution of the sample (extract/ control) at different concentration (500 µg/ml to 0.977 µg/ml) were mixed with 3.0 ml of a DPPH methanol solution (20 µg/ml). After 30 min reaction period at room temperature in dark place the absorbance was measured at 517 nm against methanol as blank by UV spetrophotometer.

Inhibition of free radical DPPH in percent (I%) was calculated as follows:

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

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

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

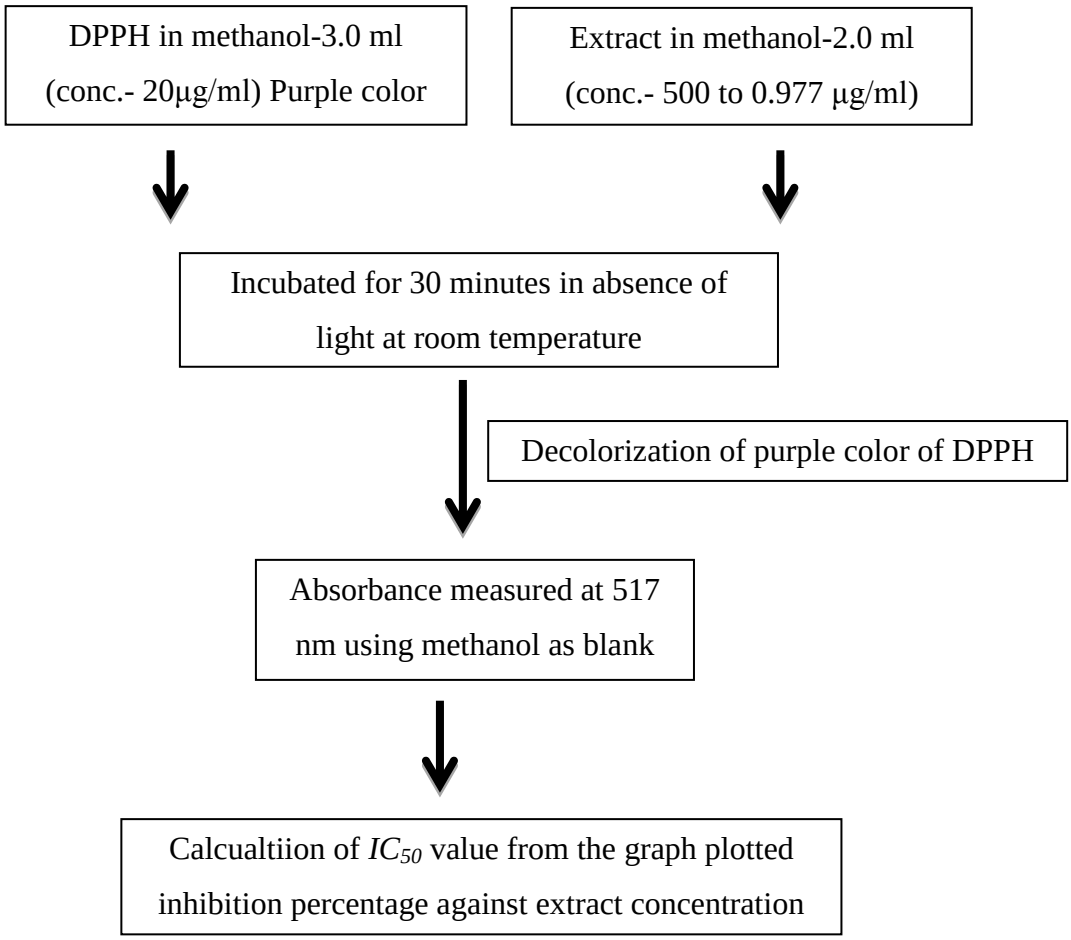


Figure 2.2: Schematic representation of the method of assaying free radical scavenging activity

2.3. Brine Shrimp Lethality Bioassay

2.3.1 Introduction

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

such as antiviral, pesticidal, antimicrobial and anti-tumor etc. of the plant extract (Meyer *et al*., 1982; McLughilin *et al*., 1998).

Brine shrimp lethality bioassay system stands better than other cytotoxicity testing methodology since it is quick in process, cheap and requires no exceptional equipment or aseptic procedure. It uses a huge number of organisms are used for statistical validation and small amount of sample. It doesn't require animal serum just like other methods.

2.3.2 Principle

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

2.3.3 Materials

- Sea salt (NaCl)
- *Artemia salina* leach (brine shrimp egg)
- Small tank with perforated dividing dam to hatch the shrimp
- Lamp to attract shrimps
- Pipettes
- Micropipette
- Glass vials
- Magnifying glass
- Test tubes
- Test samples of experimental plants

2.3.4 Experimental Procedure

2.3.4.1 Preparation of seawater

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

2.3.4.2 Hatching of brine shrimps

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

2.3.5 Preparation of test samples of the experimental plant

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

Table 2.3: Test samples with concentration values after serial dilution

Test Tube No.	Concentration (µg/ml)
1	400.0
2	200 .0
3	100 .0
4	50 .0
5	25 .0
6	12.5
7	6.25
8	3.125
9	1.5625
10	0.78125

2.3.6 Preparation of control group

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

i) Positive control
ii) Negative control

2.3.7 Preparation of the positive control group

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

2.3.8 Preparation of the negative control group

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

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

2.3.9 Counting of nauplii

After 24 hours, the vials were inspected using a magnifying glass and the number of survivors were counted. The percent (%) mortality was calculated for each dilution. The concentration-mortality data were analyzed statistically by using linear regression using

a simple IBM-PC program. The effectiveness or the concentration-mortality relationship of plant product is usually expressed as a median lethal concentration (LC_{50}) value. This represents the concentration of the chemical that produces death in half of the test subjects after a certain exposure period.

2.4 Antimicrobial Screening

2.4.1 Introduction

Microscopic organisms and parasites are responsible for several infectious diseases. The expanding clinical implications of drug resistant parasite and bacterial pathogens have allowed further implication to antimicrobial drug studies. Around the world, infectious disease is one of fundamental reasons of death which representing approximately 50% of all deaths. Maybe it is not shocking to see these statistics in developing countries, but the surprising is that mortality rates due to infectious disease are so high in developing countries like United States. This is shocking specified that it was once thought that we would reduce infectious disease by the end of the era. The increases are referred to increases in HIV/AIDS and respiratory tract infections. Other contributing aspects are a proliferation of antibiotic resistance in nosocomial and community acquired infections. Moreover, the most intensive increases are taking place in between 25–44 year old age people ((Pinner *et al*., 1996).

Antimicrobial screening is the major phase of antimicrobial drug research which is implemented to determine the susceptibility of many fungi and bacteria to any agent. This experiment measures the capability of each test sample to inhibit the *in vitro* bacterial and fungal growth. This ability may be estimated by any of the following three methods (Ayafor, 1972).

- ✓ Bio autographic method
- ✓ Disc diffusion method
- ✓ Serial dilution method

Among the above stated methods the disc diffusion (Bayer *et al*., 1966) is a most popular in vitro study for antimicrobial activity. It is basically a quantitative or qualitative test representing the sensitivity or resistance of the microorganisms to the test materials.

However, no dissimilarity between bacteriostatic and bactericidal activity can be completed by this method (Roland R, 1982).

2.4.2 Principle of disc diffusion method

In this usual method, antibiotics diffuse from a restrained source through the nutrient agar gel and produce a concentration gradient. A fixed amount of dried and sterilized filter paper discs having the test samples are placed on nutrient agar medium homogeneously seeded with the testing microorganisms. Here, the standard antibiotic (kanamycin) discs and the blank discs are using as positive and negative control. These discs are reserved at 4°C for 24 hours to permit the maximum diffusion of the test materials to the encompassing media (Barry, 1976). The discs are then reversed and incubated at 37°C for 24 hours for the development of the organisms. The test samples having antimicrobial property constrain microbial growth in the media adjacent to the discs and thus produce a clear, separate area which is defined as zone of inhibition. The antimicrobial activity is done by determining the diameter of zone of inhibition which is expressed in millimeter (Barry, 1976; Bayer *et al*., 1966).

In this study by disc diffusion method, the crude extract was tested for antimicrobial activity. This experiment is done more than one time and the mean of the readings is calculated (Bayer *et al*., 1966).

2.4.3 Experimental

2.4.3.1 Apparatus and reagents

Filter paper discs	Petri dishes
Sterile cotton.	Autoclave
Inoculating loop	Nutrient Agar Medium
Laminar air flow hood	Micropipette
Spirit burner	Refrigerator
Chloroform	Ethanol
Screw cap test tubes	Incubator

Sterile forceps	Nose mask and Hand gloves

2.4.3.2 Test organisms

The bacterial and fungal strains which were used for the experiment (both the gram positive and the gram-negative organisms) were taken for the test and they are listed in the Table 2.4.

Table 2.4 Different strains used in antimicrobial screening

Gram positive Bacteria	**Gram negative Bacteria**	**Fungi**
Bacillus subtilis	*Escherichia coli*	*Aspergillusniger*
Bacillus megaterium	*Shigella boydii*	*Sacharomyces cerevacae*
Bacillus cereus	*Pseudomonas aeruginosa*	
Staphylococcus aureus	*Vibrio parahemolyticus*	
Sarcina lutea	*Vibrio mimicus*	

Table 2.5: List of Test materials

Plant part	**Sample code**	**Test Sample**
Leaves of *Commelina diffusa*	ME	Methanolic extract of *Commelina diffusa*

2.4.4 Composition of culture medium

The subsequent media was used generally to determine the antimicrobial activity and to make subculture of the test organisms.

a) Nutrient agar medium

Ingredients	**Amount**
Bacto peptone	0.5 gm
Sodium chloride	0.5 gm
Bacto agar	2.0 gm
Bacto yeast extract	1.0 gm
Distilled water q.s.	100 ml
pH	7.2 + 0.1 at 25^0C

b) Nutrient broth medium

Ingredients	**Amount**
Bacto peptone	0.5gm
Bacto beef extract	0.3gm
Distilled water q.s.	100 ml
pH	7.2 + 0.1 at 25^0C

c) Muller – Hunton medium

Ingredients	**Amount**
Starch	0.15 gm
Bacto agar	1.70gm
Beef infusion	30 gm
Casamino acid	1.75gm
Distilled water q.s.	100 ml
pH	7.3 + 0.2 at 25^0C

d) Tryptic soya broth medium (TSB)

Ingredients	**Amount**
Bacto tryptone	1.70 gm
Bacto dextrose	0.25gm
Bactosoytone	0.30gm
Di potassium hydrogen Phosphate	0.25gm
Sodium chloride	0.50gm
Distilled water q.s	100 ml
pH	7.3 + 0.2 at 25^0C

Nutrient agar medium is commonly used and it is used in the present review for determining the sensitivity of the organisms to the test materials and to make fresh cultures.

2.4.5 Preparation of the medium

The calculated amount of each of the ingredients was taken in a conical flask and distilled water was added to it to make the required volume of the medium. A clear solution was made by heating the contents in a water bath. The pH (at 25^0C) was maintained at 7.2-7.6 by using NaOH or HCl. In screw cap test tubes, 10 ml and 5 ml of the medium was added to prepare plates and slants respectively. The test tubes were then covered and sterilized by autoclaving at 15-lbs. at 121^0Cpressure for 20 minutes. The slants were actually used for preparing fresh culture of bacteria and fungi which were used for sensitivity study.

2.4.6 Sterilization procedure

The antimicrobial activity was done in Laminar Hood to avoid any type of cross contamination and precautions were strictly maintained. UV light was switched on one hour before working in the Laminar Hood. Petridishes and other glassware were sterilized by autoclaving at 121^0C and a pressure of 15-lbs/sq. inch was maintained for 20 minutes. Micropipette tips, forceps, cotton, blank discs were also sterilized by UV light.

2.4.7 Preparation of subculture

In a sterilized condition under laminar air flow, the test organisms were shifted from the pure cultures to the agar slants through a transfer loop to prepare fresh pure cultures. The inoculated strains were incubated at 37^0C for 24 hours for their optimal development. These cultures were used for the sensitivity test.

2.4.8 Preparation of the test plate

The test organisms were moved from the subculture to the test tubes that were containing about 10 ml of dissolved and sterilized agar medium through a sterilized transfer loop in a sterilized zone. The test tubes were shaken by spinning to get a constant suspension of the organisms. The bacterial and fungal suspension was instantly shifted to the sterilized petridishes. The petridishes were revolved many times clockwise and anticlockwise to reassure consistent distribution of the test organisms in the media.

2.4.9 Preparation of discs

The calculated amount of test sample was dissolved in Chloroform or methanol to get the desired concentrations in a sterilized condition. Filter paper discs were reserved in a

blank petridish under the laminar hood. Then the discs were soaked with solutions of test samples and dried.

Table 2.6: Preparation of sample Discs

Plant part	Test Sample	Dose (μg/disc)
Leaf of *Commelina diffusa*	Methanolic extract of Bark	400

Standard Kanamycin (30 μg /disc) plates were used as positive control to confirm the activity of standard antibiotic against the test organisms as well as for comparison of the reaction delivered by the identified antimicrobial agent with that of made by the test sample. Blank discs were used as negative controls to confirm that the filter paper and the residual solvents were not active themselves.

2.4.10 Diffusion and Incubation

All the discs were placed gently on the previously distinguished zones in the agar plates that were pre-inoculated with test bacteria and fungi. The plates were reserved in a refrigerator at low temperature (4^0C) for 24 hour to allow adequate diffusion of the samples to the surrounding medium. And finally they were kept in the incubator for 24 hours at 37^0C.

2.4.11 Determination of the zone of inhibition

The antimicrobial strength of the test samples were measured by their activity to inhibit the growth of the microorganisms which gave a clear zone of inhibition. After incubation, the antimicrobial screening of the test materials were determined by calculating the diameter of the zones of inhibition in millimeter by a transparent scale.

Figure 2.3: Clear zone of inhibition

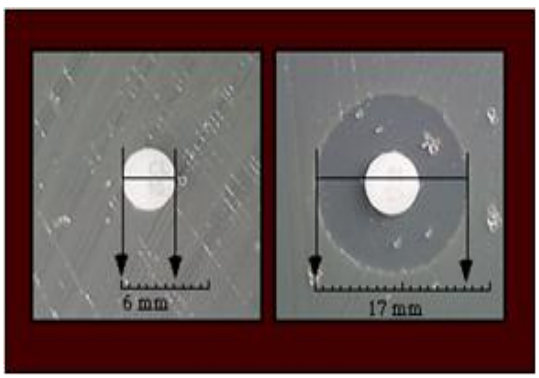


Figure 2.4: Determination of clear zone

Chapter Three: Result and Discussion

3.1 Phytochemical Identification

Phytochemical screening of the plant material established the identification of therapeutically important compounds like glycosides, phenol, sterols which were found but alkaloids, anthraquinones and flavonoids were absent in the methanolic extract of *Commelina diffusa*. The following table will give an idea about the phytochemical compounds present in the plant.

Table 3.1: Phytochemical identification of *Commelina diffusa*

Serial No.	Test	Reagent involved	*Commelina diffusa*
1.	**Alkaloids**		
	Mayer's test	HgI and KI	-
	Wagner's test	I_2 and KI	-
	Dragendorff's reagent test	Bismuth Nitrate, Tartaric Acid and Potassium Iodide	-
	Hager's test	Picric Acid	-
2.	**Glycosides**		
	Keller Killiani test	Glacial acetic acid, $FeCl_3$ and conc. H_2SO_4	+
	Conc. H2SO4 test	Conc. H_2SO_4	+
	Molish's test	α-naphthol, ethanol and conc. H_2SO_4	+
3.	**Anthraquinones**	benzene and 10% NH_3solution	-
4.	**Phenols**		
	Ellagic acid test	5% glacial acetic acid and 5% (w/v) $NaNO_2$ solution	++
	Phenol test	$FeCl_3$ solution	++
5.	**Flavonoids**		
	Zinc-HCl reduction test	Zinc dust and Conc. HCl.	-
	Lead-acetate test	lead acetate solution	-

	FeCl3 test	$FeCl_3$ solution	-
	Alkaline Reagent test	NaOH solution	-
6.	**Sterols:**		
	Liebermann-Burchard test	Acetic anhydride and Conc. H_2SO_4	++
	Salkowski test	chloroform and conc. H_2SO_4	++
'++' = Present in moderate amount, '+' = Present in Trace Amount, '-'=Absent			

3.2 Determination of Total Phenolic Content

The methanolic extract (ME) of the leaves of *Commelina diffusa* was subjected to total phenolic content determination. Folin-Ciocalteu reagent was used for this test. Depending on the absorbance value of the extract solutions, the total phenolic content of the extract was determined and compared with the standard solutions of gallic acid (table 3.1). The total phenolic content of the sample was expressed as mg of GAE (gallic acid equivalent)/ gm of extract and are given in table 3.2. The total phenolic content found in ME was 21.815mg of GAE / gm of extract.

Table 3.2: Standard curve prepration by using gallic acid

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

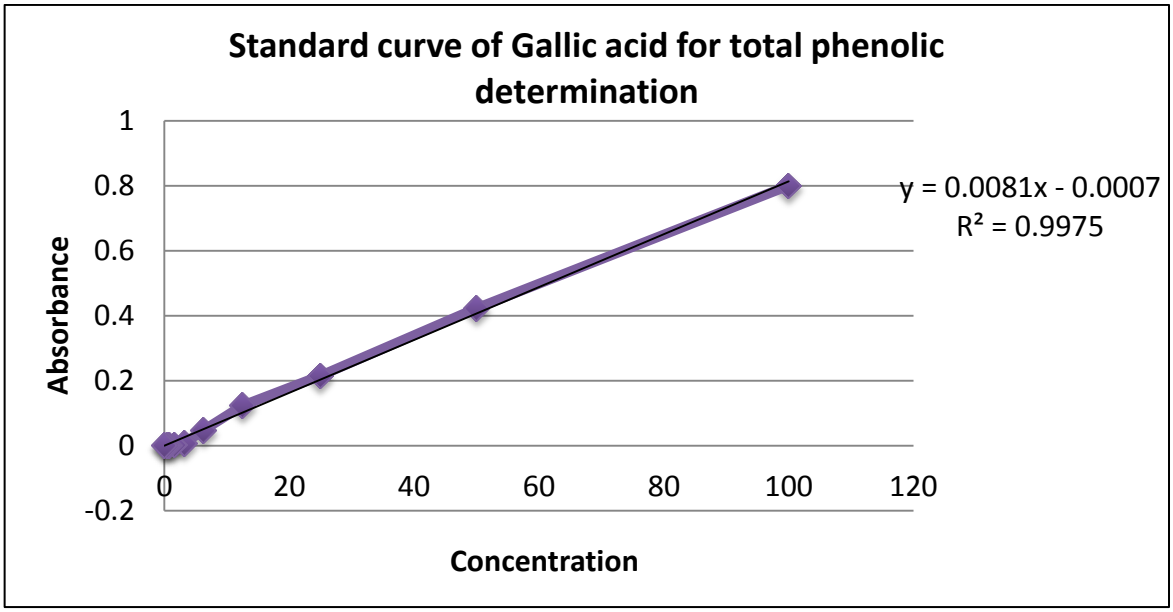


Figure 3.1: Standard curve of Gallic acid for total phenolic determination

Table 3.3: Test samples for total phenolic content determination

Plant part	Sample code	Test Sample	Total phenolic content (mg of GAE / gm of extract
Leaves of *Commelina diffusa*	ME	Methanolic extract	21.815

3.3 DPPH Assay

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

In this research, Methanolic extract solution (ME)presented a notable free radical scavenging activity with IC_{50} value of 401.57µg/ml which is very high than standards. So we can sum up that the methanolic extract of leaves of *Commelina diffusa* has mild free radical scavenging activity.

Table 3.4: IC_{50} values of the standards and methanolic extract of leaves of *Commelina diffusa*

Plant part	**Sample code**	**Test Sample**	**IC_{50} (µg/ml)**
Leaves of *Commelina diffusa*	ME	Methanolic extract	401.57
ASA (Ascorbic acid) (standard)			3.01
BHT (*tert*-butyl-1-hydroxytoluene) (standard)			21.17

Table 3.5: IC_{50} value of Ascorbic acid (ASA)

Absorbance of the blank	**Conc. (µg/ml)**	**Absorbance of the extract**	**% inhibition**	**IC_{50} (µg/ml)**
0.325	500	0.005	98.46	3.01
	250	0.006	98.15	
	125	0.015	95.38	
	62.5	0.024	92.61	
	31.25	0.068	79.07	
	15.625	0.098	69.84	
	7.813	0.139	57.23	
	3.906	0.186	42.76	
	1.953	0.175	46.15	
	0.977	0.193	40.61	

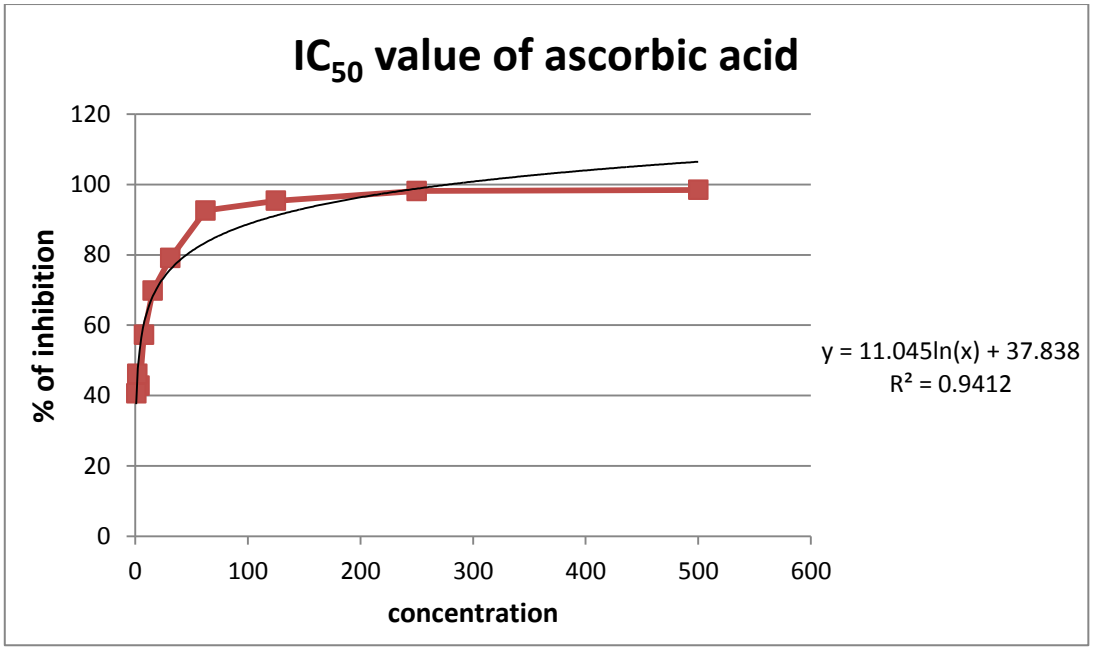


Figure 3.2: IC_{50} value of ascorbic acid

Table 3.6: IC_{50} value of *tert*-butyl-1-hydroxytoluene (BHT)

Absorbance of the blank	Conc. (µg/ml)	Absorbance of the extract	% inhibition	IC_{50} (µg/ml)
0.325	500	0.018	94.46	21.17
	250	0.068	79.07	
	125	0.097	70.15	
	62.5	0.135	58.46	
	31.25	0.159	51.07	
	15.625	0.175	46.15	
	7.813	0.206	36.61	
	3.906	0.225	30.76	
	1.953	0.238	26.76	
	0.977	0.287	11.69	

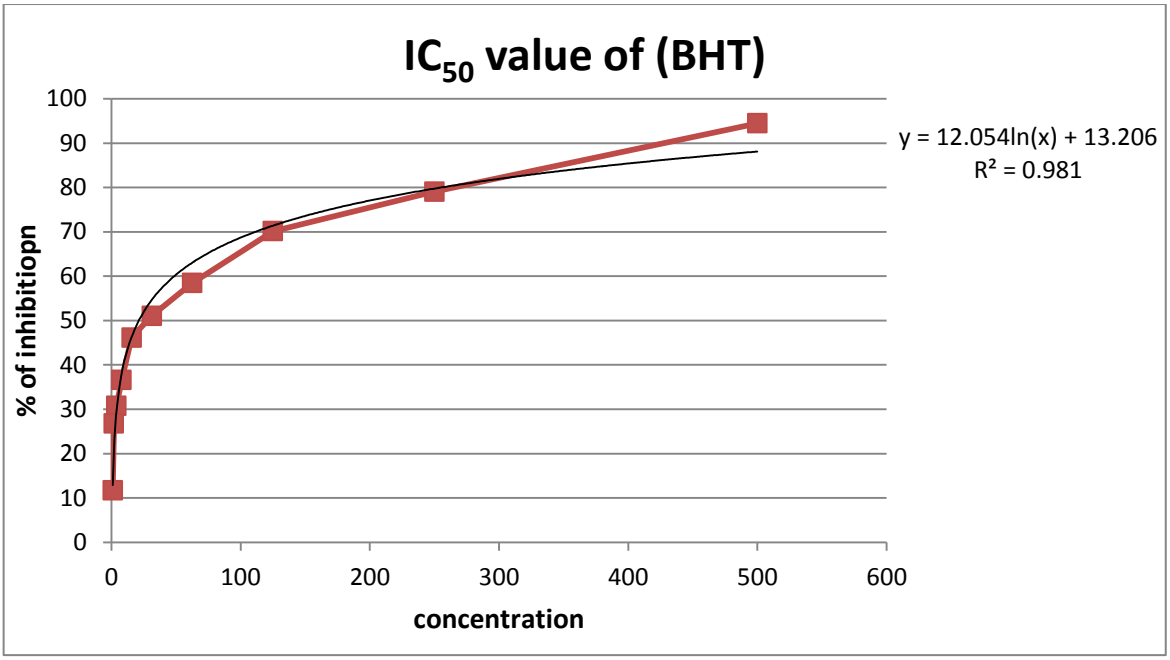


Figure 3.3: IC_{50} value of tert-butyl-1-hydroxytoluene (BHT)

Table 3.7: IC_{50}value of Methanolic extract (ME)

Absorbance of the blank	Conc. (µg/ml)	Absorbance of the extract	% Inhibition	IC_{50}
0.325	500	0.152	53.23	401.57
	250	0.182	44	
	125	0.199	38.78	
	62.5	0.214	34.15	
	31.25	0.226	30.46	
	15.625	0.229	29.54	
	7.813	0.23	29.23	
	3.906	0.232	28.62	
	1.953	0.233	28.31	
	0.977	0.235	27.69	

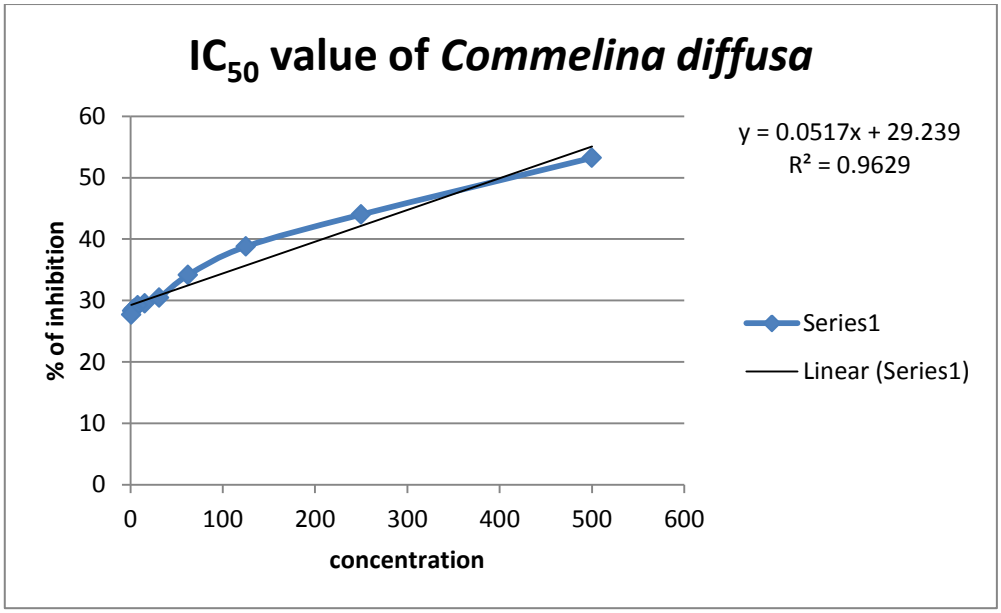


Figure 3.4: IC_{50} value of ME of leaves of *Commelina diffusa*

3.4 Brine Shrimp Lethality Bioassay

The methanolic extract (ME) of leaves of *Commelina diffusa* was examined for brine shrimp lethality bioassay. The cytotoxicity of the extract to brine shrimp was observed and the results are given in Table 3.10.

The lethal concentration (LC_{50}) of the test sample was determined by plotting the percentage of mortality rate of shrimps against the logarithm of concentration. The curve of regression analysis helps in gaining the best-fit line. Vincristine sulfate (VS) was used as positive control and the LC_{50} was found to be 0.45 µg/ml. The LC_{50} of the methanolic extract of leaves of *Commelina diffusa* was 1.43µg/ml. So we can say that the methanolic extract of *Commelina diffusa* has high cytotoxic activity.

Table 3.8: LC_{50} values of the test samples of leaves of *Commelina diffusa*

Test samples	Regression line	R^2	LC_{50} (µg/ml)
VS	y = 30.799x + 60.653	0.973	0.45
ME	y = 21.261x + 19.604	0.8885	1.43

Table 3.9: Effect of Vincristine sulfate (positive control) on shrimp nauplii

Conc.(µg/mL)	Log_{10}Conc.	%Mortality	LC_{50}(µg/mL)
0	-	0	0.35
0.039	-1.4089	20	
0.078125	-1.1072	30	
0.15625	-0.8061	30	
0.3125	-0.5051	40	
0.625	-0.2014	50	
1.25	0.09691	70	
2.5	0.39794	80	
5	0.6989	80	
10	1.00	90	
20	1.30102	100	

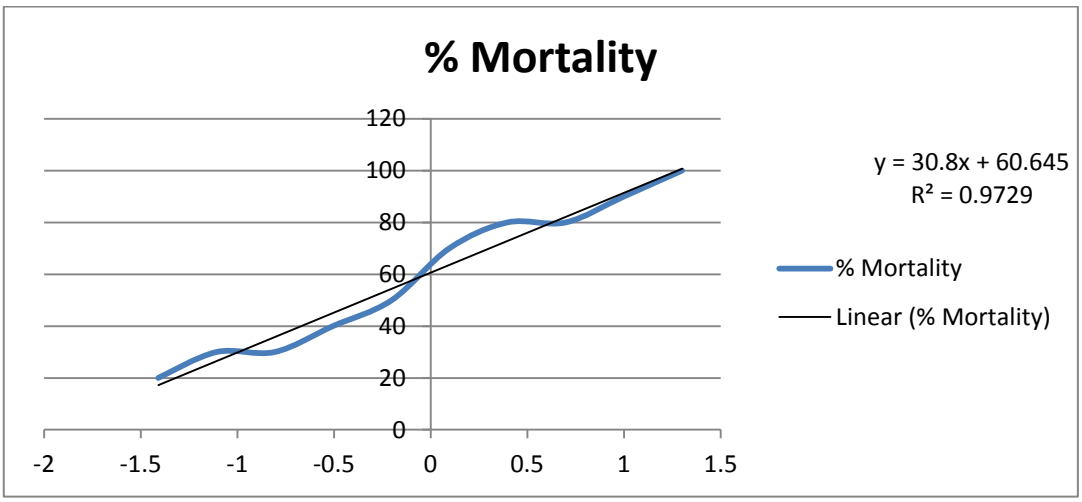


Figure 3.5: Plot of % mortality and predicted regression line of VS

Table 3.10: Effect of the methanolic extract (ME) of leaves of *Commelina diffusa* shrimp nauplii

Conc. (µg/mL)	$Log_{10\ conc.}$	% of mortality	LC_{50}
0	-	0	1.43
0.78125	-1.1072	10	
1.5625	0.19382	20	
3.125	0.49485	20	
6.25	0.79588	30	
12.5	1.09691	40	
25	1.39794	50	
50	1.69897	50	
100	2	60	
200	2.30103	70	
400	2.60206	90	

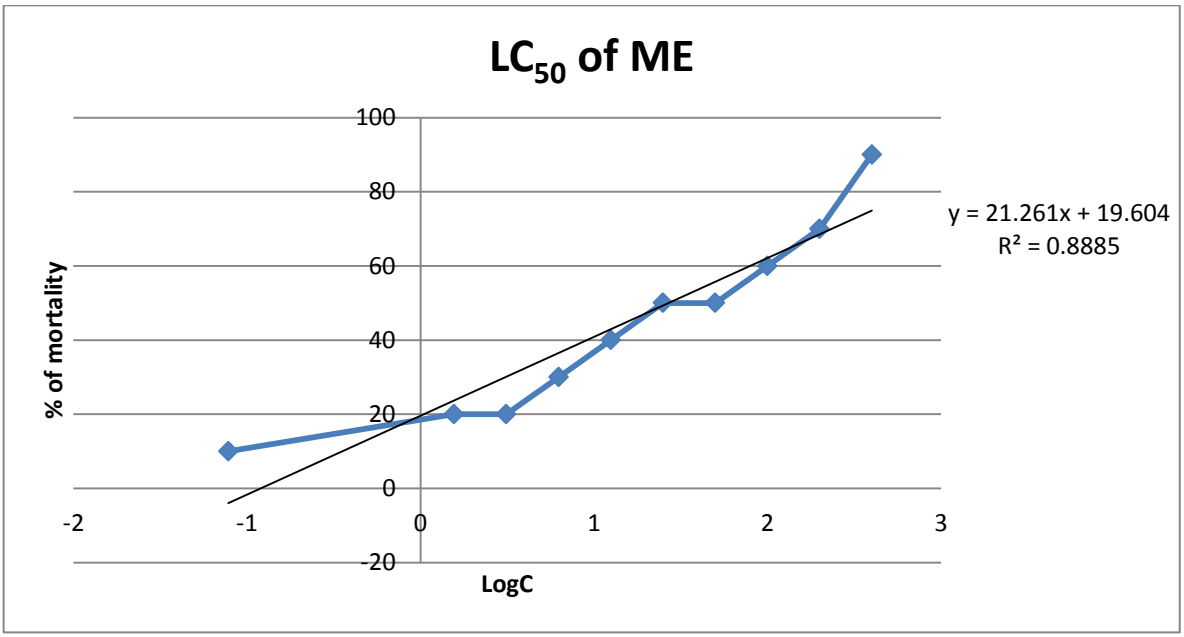


Figure 3.6: Plot of % mortality and predicted regression line of ME

3.5 Antimicrobial Screening

The methanolic extract (ME) of leaves of *Commelina diffusa* was investigated to antimicrobial activity with a concentration of 400 μg/disc in every case. The methanolic extract of the leaves of *Commelina diffusa* showed good antimicrobial activity against different microorganisms.

The maximum zone of inhibition exhibited by ME was found to be 25mm against *Staphylococcus aureus.* Moderate to high antimicrobial activities were against *Escherichia coli, Sacharomyces cerevacae* and *Shigella boydii* (having zone of inhibition of 24, 22, and 21 mm respectively). Some mild antimicrobial activity was found against *Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Sarcinalutea, Pseudomonas aeruginosa, Vibrio mimicus, and Aspergillus niger* (having zone of inhibition of 19, 18, 19, 17, 14, and 13 mm respectively). Among all of the test microorganisms, the lowest activity was exhibited against *Vibrio parahemolyticus* (having zone of inhibition of 12 mm).

Table 3.11: Antimicrobial activity of test samples of bark of *Commelina diffusa*

Test microorganisms	Diameter of zone of inhibition (mm)	
	Extract	**Kanamycin**
Gram Positive Bacteria		
Bacillus cereus	19	37.6
Bacillus megaterium	18	38.3
Bacillus subtilis	19	35.0
Staphylococcus aureus	25	35.0
Sarcina lutea	17	37.3
Gram negative bacteria		
Escherichia coli	24	37.0
Pseudomonas aeruginosa	14	35.6
Shigella boydii	21	35.1
Vibrio mimicus	13	37.3
Vibrio parahemolyticus	12	38.0
Fungi		
Aspergillus niger	20	37.0
Sacharomyces cerevacae	22	38.6

Chapter Four: Conclusion

Chapter Four: Conclusion

The crude methanolic extract of the leaves of *Commelina diffusa* can be a source of herbal medicine to efficiently treat various human diseases. The phytochemical screening revealed the presence of important phytochemical constituents like phenols, glycosides, and sterols. The biological investigation of *Commelina diffusa* showed that it has mild antioxidant properties that can treat oxidative stress. A high cytotoxicity of the methanolic extract of leaves of *Commelina diffusa* was also confirmed as compared with the standards. On the other hand, the leaf extract showed moderate to high antimicrobial activity against gram-positive and gram negative bacteria and fungi.

Therefore, the study established that the extract of the leaves of *Commelina diffusa* might be a significant contributor to drug development with potential antimicrobial activity. However, chemical investigations and pharmacological studies in animal models should be carried out to further establish the biological effects.

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