PHYTOCHEMICAL AND PHARMACOLOGICAL SCREENING OF (LEAVES, ROOTS, AND BARK) *Clerodendrum viscosum*

MD. SAMIM HOSSAIN.

**BIOTECHNOLOGY AND GENETIC ENGINEERING DISCIPLINE,
LIFE SCIENCE SCHOOL, KHULNA UNIVERSITY,
KHULNA-9208, BANGLADESH.**

NOVEMBER, 2008

PHYTOCHEMICAL AND PHARMACOLOGICAL SCREENING OF (LEAVES, ROOTS, AND BARK) *Clerodendrum viscosum*

A Thesis Submitted

By

Md. Samim Hossain.
Examination Roll No.MS 070733
Session: 2006-2007

Submitted to

Biotechnology and Genetic Engineering Discipline, Khulna University, in partial fulfillment of requirements for the degree of Bachelor of Science (M.Sc.) in Biotechnology and Genetic Engineering.

Biotechnology and Genetic Engineering Discipline,
Life Science School, Khulna University,
Khulna-9208, Bangladesh
November, 2008.

PHYTOCHEMICAL PHARMACOLOGICAL SCREENG OF *(LEAVES, ROOTS, AND BARK) Clerodendrum viscosum*

DECLARATION

This thesis is the original research work of the author, except as otherwise stated. The material presented has not been submittrd either in whole or in part for a degree for this or any other university. The findings of the research has not been /will not be published anywhere without the concurrence of the supervisor or co-supervisor.

MD. Samim Hossion
..... 5-04-11

Author

MD. Samim Hossain

I.D. No. MS-070733

April, 2011.

Acknowledgements

It's my pleasure to express the greatest and deepest gratitude to the supreme of the universe, the almighty Allah, to whom all praises go for enabling me to complete my research work.

The present work was carried out in the Biotechnology and Genetic Engineering Discipline, Khulna University. Therefore, I am grateful to the authority for providing the facilities.

I would like to express my indebtedness to my respected supervisor, Mahabub-e-Sobhani, Lecturer, Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna for his continuous guidance, keen interest, strong inspiration, time to time help and constructive criticism to carry on the research work.

I express my best possible gratefulness, sincere appreciation and regards to his sincere and experienced Co-supervisor, Md. Saifuzzaman Lecturer, Pharmacy Discipline, Khulna University, Khulna.

I express my deepest gratitude to honorable teacher, Professor Md. Raihan Ali, Head, Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna, Bangladesh, for his inspiration, affectionate suggestion and guidance to perform this research work.

I am grateful to all of the teachers of Biotechnology and Genetic Engineering Discipline for their assistance and encouragement to perform the research work.

My cordial thanks and gratitude to Mr. Rabiul Islam, Lab, Technician and Mr. Moniruzzaman, Lab Assistant, Pharmacy Discipline, Khulna University for their kind and friendly cooperation.

I would like to Thanks my seniors Reza vi, Razu vi, Jumu apu, Milon vi, Boloram da, and Kaberi didi, senior class met for their co-operation in various areas of my work.

I want to acknowledge my friend, Ajoy, Sobuj, Sonia, Rasel, for their pleasant company during the research work and preparation of this thesis.

My special heartful thanks extend to all my classmates and friends for their friendly cooperation and occasional help.

With the heartiest feelings, I remember my parents, whose blessings accelerated me at every steps of life.

Finally, I would like to express my gratitude to my parents and brothers for their moral support, inspiration and encouragement throughout the work.

The Author

PHYTOCHEMICAL AND PHARMACOLOGICAL SCREENING OF (LEAVES, BARK, AND ROOT) *Clerodendrum viscosum*

A Thesis Submitted

By

Examination Roll No. M.S 070733

ABSTRACT

This study was designed to investigate phytochemical nature (group determination of plant constituent) and selected pharmacological activities (cytotoxicity, anti-oxidant and antibacterial) of the ethanol extract of leaves, bark and root of *Clerodendrum viscosum.* Phytochemical study of the extract of *Clerodendrum viscosum* leaves indicated the presence of reducing sugar, tannin, alkaloid, flavanoid, carbohydrate, saponin, and steroid types of compounds. Photochemical study of the extract of bark indicated alkaloid, saponin, reducing sugar, carbohydrate, and gum types of compounds. Root indicates alkaloid, saponin, reducing sugar, carbohydrate, types of compounds. In the free radical scavenging activity study (DPPH assay) the extract (leaves, roots, and bark) showed mild antioxidant activity at the concentration of 100 μg/ml. A general cytotoxicity of (leaves, roots, and bark) the extracts was assessed by a simple and low cost assay using brine shrimp lethality as an indicator of cytotoxicity. The extract of leaves of *Clerodendrum viscosum* showed moderate level of general toxicity in the brine shrimp lethality assay (LC_{80} = 40μg/ml for leaves, and LC_{90} = 40μg/ml root and bark). The extract showed significant antibacterial activity against both a number of gram positive and gram negative bacteria such as *Proteus spp, Bacillus cerius, Staphylococcus agalactiae, Shigella dysenteriae and Salmonella paratyphi-A1.* The obtained results provide a support for the uses of this traditional medicine.

ABBREVIATIONS USED IN THE TEXT

et al.,	*et alia* =and other people
etc.	*et cetra*= and the others
gm	Gram(s)
gm/l	Gram(s) per liter
i.e.	*id est* = that is
mg	Milligram(s)
mg/l	Milligram(s) per liter
ml	Milliliter(s)
L	Liter
mm	Millimeter(s)
µL	Micro liter
µg	Micro gram
Viz.	Videlicet = namely
Conc	Concentration (s)
DPPH	1,1-diphenyl-2picrylhydrazyl
DMSO	Dimethyl sulfoxide
ED	Effective Dose
LC	Lethal Concentration
IC	Inhibition Concentration

List of the Figure

List of the Table

List of the Graph

Chapter One

1. Introduction

Bangladesh is a country of plant diversity. Medicine is the wonder of the world and blessings for mankind. Most of the plants possess different kinds of secondary metabolites which are used as drugs, medicine or therapeutic agents for the treatment of different kinds of diseases. So Medicinal plants hold an importance in the economy of any country as well as can brings a tremendous change in medicine sector. The World Health Organisation (WHO) estimated that 80% of the population of developing countries relies on traditional medicines, mostly plant drugs, for their primary health care needs. Also, modern pharmacopoeia still contains at least 25% drugs derived from plants and many others which are synthetic analogues built on prototype compounds isolated from plants. Demand for medicinal plant is increasing in both developing and developed countries due to growing recognition of natural products, being non-narcotic, having no side-effects, easily available at affordable prices and sometime the only source of health care available to the poor. The history of drug from natural sources is very significant and well known. Illness, physical discomforts, injuries, wounds and fear of death had forced prehistoric man to use any natural substance that he could lay his hands on.

According to WHO consultative group on medicinal plants, "A medicinal plant is any plant which, in one or more of its organs contains substances that can be used for therapeutic purposes or which, is a precursor for synthesis of useful drugs." Medicinal plants may be defined as a group of plants that possess some special properties or virtues that qualify them as article of drugs and therapeutic agent and are used for medicinal purposes (Ghani, 2003).

Plant kingdom, the storehouse of thousands of unexplored compounds, possesses a great potentiality for drug search even in the day of synthetic chemistry. About 90% of medicinal plant used by the industries is collected from the wild. While

over 800 species are used in production by industry, less than 20 species of plants are under commercial cultivation. Over 70% of the plant collections involve destructive harvesting because of the use of parts like roots, bark, wood, stem and the whole plant in case of herbs .This possess a definite threat to the genetic stocks and to the diversity of medicinal plants if biodiversity is not sustainable used.

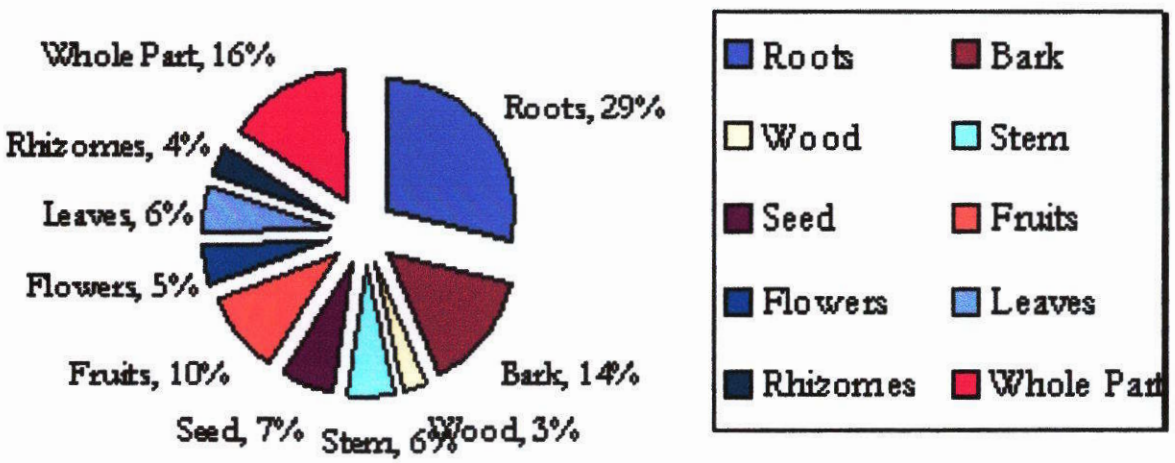


Figure 1.1: Break up of medicinal plant by their parts utilized (Source: Report of the Task Force on Conservation & Sustainable use of Medicinal Plants, Govt. of India).

As part of our continuing efforts to study the chemical and pharmacological aspects of the medicinal plants of Bangladesh, *Clerodendrum Viscosum* was investigated. Successive extraction of dried powdered Leaves root and Bark of *Clerodendrum Viscosum* and the effects of all extracts were investigated for chemical group test, cytotoxic effect, and antibacterial activity.

1.1 Plant Preview

The species is indigenous throughout the tropics and subtropics. It is not known if the species originated in the Africa, America and tropical Asia. This plant that gives us white flower and red fruits. Due to its many traditional uses, *C. Viscosum* has doubtless been spread by humans

C. Viscosum is an evergreen, sprawling tree that typically grows to 3-7 ft in height. It is not indigenous to many parts of the tropics and has been introduced to new regions by people. Once established, the tree often persists and spreads, especially in moist gullies, streambeds, and other wet areas. The tree can grow in a wide range of soils, including in hospitable brackish swamps, and limestone. It stands up well, side of the road. High winds *C. Viscosum* can cause fall over. This *C. Viscosum* does not give us any wood. Total plant part that is used as firewood, and medicine.

Clerodendrum is a genus of about 400 species of flowering plants in the family *Verbenaceae (Lamiaceae).* The genus is native to tropical and warm temperate regions of the world, with most of the species occurring in tropical Africa and southern Asia, but with a few in the tropical Americas and northern Australasia, and a few extending north into the temperate zone in eastern Asia. Common names include glorybower, bag flower and bleeding-heart.

They are shrubs, lianas, and small trees, growing to 1-12 m tall, with opposite or whorled leaves.

Clerodendrum species are used as food plants by the larvae of some Lepidoptera species including *Endoclita malabaricus* and *Endoclita sericeus*

1.2 Botanical Features

Common Name / Local Name:	Vat tree (Bangladesh).
Scientific Name:	*Clerodendrum Viscosum*
Synonyms:	glorybower, bagflower and bleeding-heart
Family:	Verbenaceae.
Used:	Medicinal Plant
Country of Origin:	The genus is native to tropical and warm temperate regions of the world, with most of the species occurring in tropical Africa and southern Asia, but with a few in the tropical Americas and northern Australasia, and a few extending north into the temperate zone in eastern Asia
Habitat:	common on beaches and mangrove communities

1.3 Plant Morphology

- Flowers

The flowers are typical of the *Clerodendrum* genus, showy, fragile, and short-lived, falling the same day that they open Individual flowers white in color, with the corolla consisting of five radiating, obovate.White petals are (2–3 cm) [1.6–2.4 in] long. It contents black anthers. In color flowering and fruiting may occur at May to June of the year. The time of flowering when plant is 120 days and fruiting is typically when plant is 130 days.

Fig 1.2: Flower of *C. Viscosum*

- Leaves

The leaves are simple, heart shaped, rather large 20–28 cm long, 14–20 cm width. When discolor plant, grayish green on the upper surface, in some Pacific islands there is a variant with bronze-green shoots and new leaves.

- Fruits

The fruits are a light brown, ovoid, dry capsule, about 1 cm round.

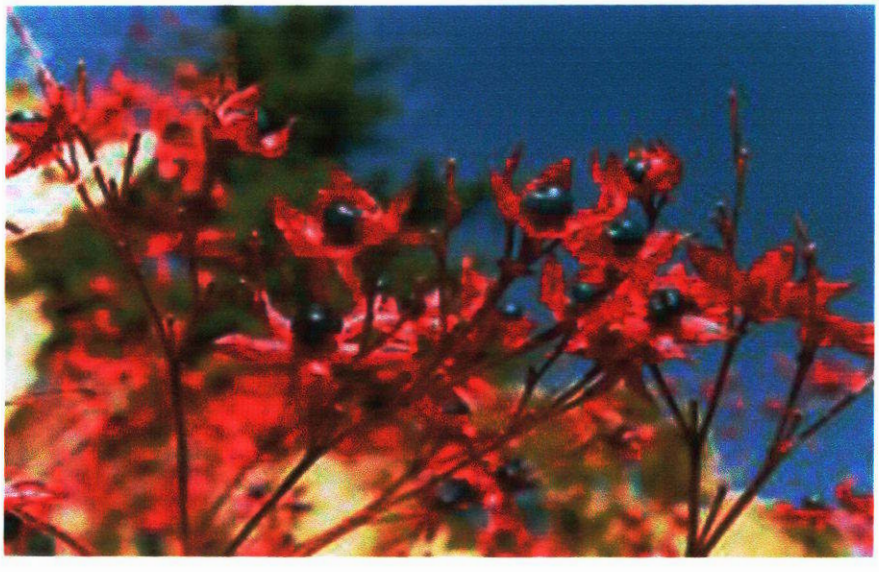


Fig 1.3: Fruit of *C. Viscosum*

- Bark

The bark is gray to light brown, smooth to slightly longitudinally fissure with horizontal cracks.

- Rooting habit

The plant has a highly spreading, near-surface, natural root system, often comprised of only main roots.

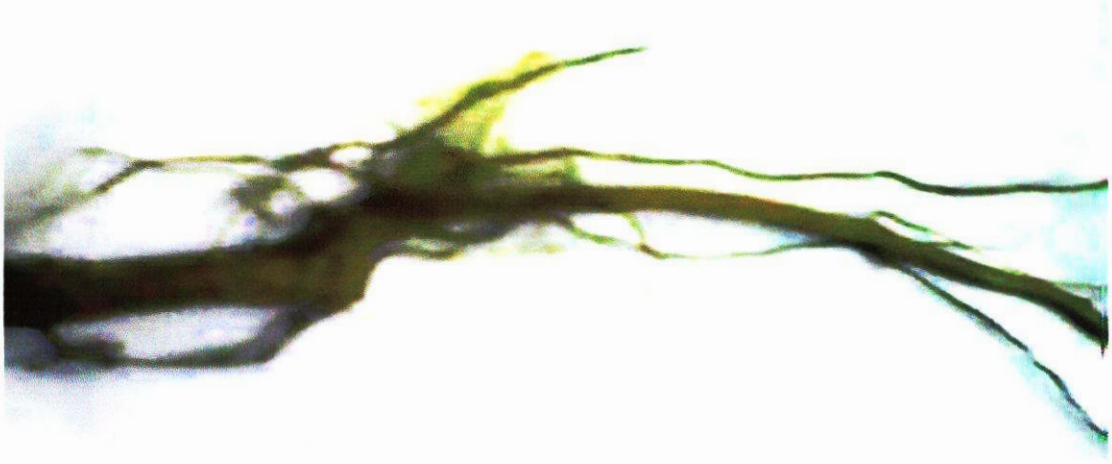


Figure 1.4: Root section of *C. viscosum*

1.4. Traditional medicinal Uses

The plant is used in traditional medicine by the people and possesses various interesting pharmacological activities.

Leaves are used to cool fevers, soothe coughs and remove phlegm (Malaysia, Indonesia); the leaf buds were chewed/swallowed for dry throat. The young leaves are edible and various parts of the plant have historically been used for medicinal purposes. Fresh bark and leaves soaked in water is used for worm action. Total parts of *Clerodendrum viscosum* works for gastric ulcer, stomach pain, disease. So that total part of this plant is beneficial for human being.

1.5. Aim and of Objectives the study

Plant secondary metabolites have been used for mankind as remedies since the beginning of civilization. Now a day, they still play an important role in health care for about 80% of the world's population. Diverse bioactive metabolites like steroids, flavonoids, alkaloids, glycosides, etc. in plants have formed the therapeutic basis of herbal medication. Thus emphasis is given on the biological screening of medicinal plants for further exploration of their active constituents.

The aim of the present study was to investigate the scientific basis of the traditional uses of the plant *C viscosum*. The traditional use of this plant leads to the investigation of the following pharmacological action.

1. Chemical Group Test
2. Cytotoxicity Screening
3. Antimicrobial Screening
4. In-vitro Antioxidant activity.

Chapter Two

Literature review

2. Biological Investigation.

Labo reported (2006) that a detailed pharmacognostical study of the root and leaf of the crude drug *Clerodendrum viscosum* Vent. (Verbenaceae). It is an important plant in the Indian system for medicine. The root and leaf samples were studied using light and confocal microscopy, WHO recommended-physicochemical determinations, and authentic phytochemical procedures. The physicochemical, morphological, and histological parameters presented in this paper may be proposed as parameters to establish the authenticity of *C. viscosum* root and leaf, and may possibly help to differentiate the drug from its other species.

Bird (1961) studied that the capacity of certain plant proteins closely simulate the action of various human blood group specific agglutinins is well known. Almost all strong blood-group-specific plant agglutinins have been obtained from the genus Leguminosae. Examination of other non-leguminous plants has now revealed specific agglutinins in the fruit pulp of *Clerodendrum viscosum.*

Sajem and Gosai (2006) reported that *Clerodendrum viscosum* leaves are taken raw or are mixed with vegetable for curing diabetes, high blood pressure and asthma (82%). Medicinal plants used by Jaintia tribes of the North Cachar Hills district of Assam, northeast India.

Hossan, et *al.* (2006) reported the Chakmas form the largest ethnic minority group inhabiting the Chittagong Hill Tracts forest region of Bangladesh. They have their own traditional medicinal practitioners and have a long tradition of using plants to cure diseases. Gastrointestinal disorders like diarrhea and dysentery are prevalent amongst the Chakmas. They undertook an ethno botanical survey of the Chakma tribe to identify plants used for treatment of gastrointestinal disorders. The objective of this study was to create a database of medicinal plants, which could be further analyzed for identification of novel compounds. This in turn, can lead

newer formulations for cure of endemic diseases like diarrhea and dysentery, which affects a large number of people, particularly in the under-developed countries. A total of 17 plant species were identified as to their being used by the of them *Clerodendrum viscosum* works for gastric ulcer, stomach pain, disease. This plant parts used, mode of preparation of medicine and dosages will be presented.

Rahman *et al.* (2006) reported that the crude ethanolic extract of the leaves of *Clerodendrum viscosum* (Family: Verbenaceae) was evaluated for its antioxidant and analgesic activities to investigate the scientific basis of the traditional uses. The antioxidant property of the extract was assessed by 1, 1-diphenyl -2- picryl hydrazyl (DPPH) free radical scavenging assay. The extract showed prominent antioxidant activity which was comparable to standard drug ascorbic acid. The extract produced significant ($P<0.001$) writhing inhibition in acetic acid induced writhing in mice at the dose of 125 mg, 250 mg and 500 mg/kg body weight respectively, which were comparable to the standard drug diclofenac sodium. The results tend to suggest that the crude leaves extract at the above doses have antioxidant and analgesic activities and indicate that it might possess biologically active constituents having free radical scavenging and analgesic activities respectably.

Das, A.K. and Roy, B.A. (1981) reported that Anti-inflammatory, antinociceptive, and neuropharmacological activities of *Clerodendron viscosum.*

Snigdhi Ror and zashim Md. (2006) reported that disease symptoms sores, diabetes to be treated by *Clerodendron viscosum.*

Richad lobo and ISR Punitha, (2006) reported that *Clerodendron viscosum* has antisnake venome activity. A plant traditional used in India for the treatment of a snake bite was evaluated by in vitro and in vivo methods. While *in vitro* studies were perform using human blood. *In vivo* studies were carried out using mice administered three different doses of the extract 5 minutes before the

to newer formulations for cure of endemic diseases like diarrhea and dysentery, which affects a large number of people, particularly in the under-developed countries. A total of 17 plant species were identified as to their being used by the Chakmas most of them *Clerodendrum viscosum* works for gastric ulcer, stomach pain, disease. This plant parts used, mode of preparation of medicine and dosages will be presented.

Rahman *et al.* (2006) reported that the crude ethanolic extract of the leaves of *Clerodendrum viscosum* (Family: Verbenaceae) was evaluated for its antioxidant and analgesic activities to investigate the scientific basis of the traditional uses. The antioxidant property of the extract was assessed by 1, 1-diphenyl -2- picryl hydrazyl (DPPH) free radical scavenging assay. The extract showed prominent antioxidant activity which was comparable to standard drug ascorbic acid. The extract produced significant ($P<0.001$) writhing inhibition in acetic acid induced writhing in mice at the dose of 125 mg, 250 mg and 500 mg/kg body weight respectively, which were comparable to the standard drug diclofenac sodium. The results tend to suggest that the crude leaves extract at the above doses have antioxidant and analgesic activities and indicate that it might possess biologically active constituents having free radical scavenging and analgesic activities respectably.

Das, A.K. and Roy, B.A. (1981) reported that Anti-inflammatory, antinociceptive, and neuropharmacological activities of *Clerodendron viscosum.*

Snigdhi Ror and zashim Md. (2006) reported that disease symptoms sores, diabetes to be treated by *Clerodendron viscosum.*

Richad lobo and ISR Punitha, (2006) reported that *Clerodendron viscosum* has antisnake venome activity. A plant traditional used in India for the treatment of a snake bite was evaluated by in vitro and in vivo methods. While *in vitro* studies were perform using human blood. *In vivo* studies were carried out using mice administered three different doses of the extract 5 minutes before the

administration of *Naja naja* snake venom. The results of the in vitro studies showed that the extract probably interacts. With but dose not stabilize membrane protein. In the *in vivo* studys the extract showed significance antisnake venome activity.

Chapter Three

Phytochemical Screening

3. Introduction

The subject of phytochemistry or plant chemistry has developed in recent years as a distinct discipline, somewhere in between natural product organic chemistry and plant biochemistry and is closely related to both. It is concerned with the enormous variety of organic substances that are elaborated and accumulated by plant and deals with the chemical structures of these substances, their biosynthesis, turnover and metabolism, their natural distribution and their biological function. In all these operations, methods are needed for separation, purification and identification of many different constituents present in plants.

3.1. Collection and Identification:

The plant *Clerodendrum Viscosum* was collected from different suitable place of (Ambaria, sengram, khoksha kushtia) Bangladesh during the month of 2nd April 2008. During collection any type of adulteration was strictly prohibited. The plant sample was identified by the experts at Bangladesh (Accession No. 32898) National Herbarium, Mirpur, Dhaka and also by the authorities of Botanical Garden, Mirpur, Dhaka and a voucher specimen was deposited in the National Herbarium Centre, Mirpur, Dhaka.

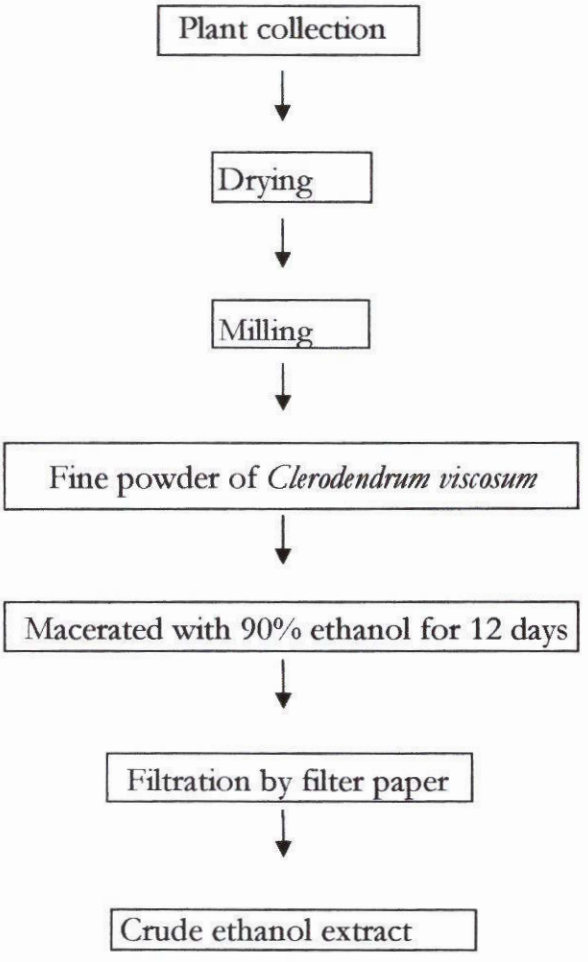
3.2. Drying and grinding:

The collected plant parts (Roots, leaves, and bark) were washed with water, separated from undesirable materials or plants or plant parts. They were sun-dried for fifteen days after cutting into small pieces. The plant parts were ground into a fine powder with the help of a suitable grinder (Capacitor start motor, Wuhu motor factory, China). The powder was stored in an airtight container and kept in a cool, dark and dry place until analysis commenced.

3.3. Extraction:

The powdered materials (400gm of leaves, 200gm of bark and 100gm of roots) was taken in three clean, flat bottomed glass containers (4 liters) and soaked in 1650ml, 1200ml and 500ml of 90% of ethanol respectively. The containers with its contents was sealed and kept for a period of 12 days accompanying occasional shaking and stirring. The whole mixture then underwent a coarse filtration by a piece of clean, white cotton material. Then it was filtered through Whatman filter paper. The extract was concentrated by evaporation process.

3.4. Extraction at a glance



3.5. Evaporation of the solvent:

The filtrate (ethanol extract) obtained was evaporated by three ceiling fans. The leave extract rendered a dark green color and the bark extract turned into brown

color and root extract white green color. The concentrated was designated as crude extract or ethanol extract. The yield of the leaves extract was 6 gm and bark extract was 3 gm and roots extract was 1gm.

3.6. Chemical Groups Tests:

Testing of different chemical groups in extract represent the preliminary studies. In each test 10% (w/v) solution of extract in Ethanol was taken unless otherwise mentioned in individual test. The chemical group tests, which were performed and the reagents needed in these tests are as follows (Evans *et al.*, 1989).

3.7. Reagents:

The following reagents were used for the different chemical group tests.

- Mayer's reagent

1.36 gm mercuric iodide in 60 ml of water was mixed with a solution contains 5 gm of potassium iodide in 20 ml of water.

- Dragendroff's Reagent

1.7 gm basic bismuth nitrate and 20 gm tartaric acid ware dissolved in 80 ml water. This solution was mixed with a solution contains 16 gm potassium iodide and 40 ml water.

- Fehling's solution A

34.64 gm copper sulphate was dissolved in a mixture of 0.50 ml of sulfuric acid and sufficient water to produce 500 ml.

- Fehling's solution B

176 gm of sodium potassium tartarate and 77 gm of sodium hydroxide were dissolved in sufficient water to produce 500 ml. Equal volume of above solution were mixed at the time of use.

➢ Benedicts Reagent

1.73 gm cupric sulphate, 1.73 gm sodium citrate and 10 gm anhydrous sodium carbonate were dissolved in water and the volume was made up to 100 ml with water.

➢ Molish Reagent

2.5 gm of pure α-naphthol was dissolved in 25 ml of ethanol.

➢ Libermann-Burchard Reagent

5 ml acetic anhydride was carefully mixed under cooling with 5ml concentrated sulfuric acid. This mixture was added cautiously to 50 ml absolute ethanol with cooling

3.8. Tests performed for identifying different chemical groups

The following tests were performed for identifying different chemical groups (Trease and Evans, 1983; Ghani, 1998;)

3.8.1. Tests for reducing sugar:

There are three tests have been carried out for Reducing Sugar. They are given below.

➢ Benedict's Test

0.5 ml of aqueous extract of the plant material is taken in a test tube. 5ml of benedict's solution is added to the test tube, boiled for 5 minutes and allowed to cool spontaneously. A red color precipitate of cuprous oxide is formed in the presence of a reducing sugar.

➢ Fehling's Test (Standard Test)

2ml of an aqueous extract of the plant material is added to1ml of a mixture of equal volumes of Fehling's solutions A and B. Boiled for few minutes. A red or brick red color precipitate is formed in the presence of a reducing sugar.

➢ Combined Reducing Sugar test

1ml of an aqueous extract of plant materials is boiled with 2ml of dilute hydrochloric acid for 5 minutes. Cooled and neutralized the mixture with sodium hydroxide solution and perform the Fehling's test as described above. A brick-red precipitate is formed in the presence of a reducing sugar due to the release of reducing sugars on hydrolysis.

3.8.2. Tests for Tannins:

➢ Ferric Chloride Test

5 ml solution of the extract is taken in a test tube. Then 1 ml of 5%Ferric chloride solution is added. Greenish black precipitate is formed in the presence of tannins.

➢ Potassium dichromate test

5 ml solution of the extract is taken in a test tube. Then 1 ml of 10% Potassium dichromate solution is added. A yellow precipitate is formed in the presence of tannins.

➢ Lead Acetate Test

1 ml. of 10% Lead acetate solution is added to 5 ml of extract solutions. A yellow precipitate is formed. It indicates the presence of tannins.

3.8.3. Test for Flavonoids:

A few drops of concentrated hydrochloric acid is added to a small amount of an alcoholic extract of the plant material. An Immediate red color is developed which indicates the presence of Flavonoids.

3.8.4. Test for Saponins:

1 ml solution of the extract is diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 minutes. One centimeter layer of foam is formed in the presence of saponins.

3.8.5. Test for Gums:

5 ml solution of the extract is taken and then molish reagent and sulphuric acid are added. Red violet ring is produced at the junction of two liquids in the presence of gums and carbohydrate.

3.8.6. Test for Steroids:

- Libermann-Burchard test

1ml solution of chloroform extract is taken and then 2 ml Libermann-Burchard reagent is added. Reddish purple color is produced which indicates the presence of steroid.

- Sulphuric acid test

1ml solution of chloroform extract is taken and then 1ml Sulphuric acid is added. Red color is produced in the presence of steroid.

3.8.7. Test for Alkaloids:

- Mayer's test

2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid is taken in a test tube. Then 1 ml of Mayer's reagent is added. Yellow color precipitate is formed in the presence of alkaloids.

➢ Dragendroff's test

2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid are taken in a test tube. Then 1 ml of Dragendroff's reagent is added. Orange brown precipitate is formed in the presence of alkaloids.

➢ Wagner's test

2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid are taken in a test tube. Then 1 ml of iodine solution (Wagner's reagent) is added. Reddish brown precipitate is formed in the presence of alkaloids.

➢ Hager's test

2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid are taken in a test tube. Then 1 ml of picric acid solution (Hager's reagent) is added. Yellowish precipitate is formed in the presence of alkaloids.

Table1. Different chemical group tests of leaves extract performed and the results are mentioned.

Sample	Test solution	Observation	Inference
Test for Alkaloids			
2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid.	0.1 ml of Mayer's reagent.	Yellowish buff colored precipitate was obtained.	Presence of alkaloid.
2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid.	0.1 ml of Dragendroff's reagent.	Orange brown precipitate was observed.	Presence of alkaloid.
2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid	1 ml of Wagner's reagent.	Reddish brown precipitate was not observed.	Presence of alkaloid.

Tests for Flavonoids			
1 ml solution of ethanolic extract.	Few drops of conc. HCl	Immediate red color was formed.	Presence of Flavonoids.
Tests for Saponins			
1 ml solution of the extract was diluted with distilled water to 20 ml.	Shaken in a graduated cylinder for 20 min.	One centimeter layer of foam was formed.	Presence Saponins
Tests for Reducing sugars			
2 ml solution of aqueous extract.	1 ml equal volume of Fehling's A and B solution. Boiled for 5 minutes on a boiling water bath.	Brick red colored was precipitate	Presence reducing sugars.
0.5 ml solution of extract.	5 ml Benedict's reagent and boiled for 5 minutes on a boiling water bath.	Brick red colored was precipitate.	Presence of carbohydrates.
1 ml solution of aqueous extract.	Boiled with 2 ml of dil. HCl acid for 5 min. after cooling neutralized with NaOH solution and performed Fehling's test as described above.	Brick red precipitate was formed.	Presence of combined reducing sugars.
Tests for Tannins			
5 ml solution of extract.	1 ml of 5% Ferric chloride solution.	Greenish black precipitate was formed.	Presence of tannins.

5 ml solution of extract.	1 ml of 10% potassium dichromate solution.	Yellow precipitate was obtained.	Presence of tannins.
5 ml solution of extract	1 ml of 10% Lead acetate	Yellow precipitate was obtained.	Presence of tannins.
Tests for Gums			
5 ml solution of extract.	Molish reagent and sulfuric acid were added.	Red –violet ring was not produced at the junction of two liquids.	Absence of gums
Test for Steroids			
1 ml solution of chloroform extract.	2 ml Libermann – Burchard reagent was added.	Chloroform layer not acquire reddish purple colour.	Absence of steroids
1 ml solution of chloroform extract	1 ml Sulphuric acid was added.	Red color was not produced.	Absence of steroid.

Table2. Different chemical group tests of bark extract performed and the results are mentioned.

Sample	Test solution	Observation	Inference
Test for Alkaloids			
2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid.	0.1 ml of Mayer's reagent.	Yellowish buff colored precipitate was obtained.	Presence of alkaloid.
2 ml solution of the extract and 0.2 ml of	0.1 ml of Dragendroff's	Orange brown precipitate was	Presence of alkaloid.

dilute hydrochloric acid.	reagent.	observed.	
2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid	1 ml of Wagner's reagent.	Reddish brown precipitate was observed.	Presence of alkaloid.
Tests for Flavonoids			
1 ml solution of ethanolic extract.	Few drops of conc. HCl	Immediate red color was not formed.	Absence of Flavonoids.
Tests for Saponins			
1 ml solution of the extract was diluted with distilled water to 20 ml.	Shaken in a graduated cylinder for 20 min.	One centimeter layer of foam was formed.	Presence of saponins.
Tests for Reducing sugars			
2 ml solution of aqueous extract.	1 ml equal volume of Fehling's A and B solution. Boiled for 5 minutes on a boiling water bath.	Brick red colored was precipitate	Presence of reducing sugars.
0.5 ml solution of extract.	5 ml Benedict's reagent and boiled for 5 minutes on a boiling water bath.	Brick red colored was precipitate.	Presence of carbohydrates.
1 ml solution of aqueous extract.	Boiled with 2 ml of dil. HCl acid for 5 min. after cooling neutralized with NaOH solution and performed Fehling's test as described above.	Brick red precipitate was formed.	Presence of combined reducing sugars.

Tests for Tannins			
5 ml solution of extract.	1 ml of 5% Ferric chloride solution.	Greenish black precipitate was not formed.	Absence of tannins.
5 ml solution of extract.	1 ml of 10% potassium dichromate solution.	Yellow precipitate was not obtained.	Absence of tannins.
5 ml solution of extract	1 ml of 10% Lead acetate	Yellow precipitate was not obtained.	Absence of tannins.
Tests for Gums			
5 ml solution of extract.	Molish reagent and sulfuric acid were added.	Red –violet ring was produced at the junction of two liquids.	Presence of gums
Test for Steroids			
1 ml solution of chloroform extract.	2 ml Libermann –Burchard reagent was added.	Chloroform layer not acquire reddish purple colour.	Absence of steroid.
1 ml solution of chloroform extract	1 ml Sulphuric acid was added.	Red color was not produced.	Absence of steroid.

Table3. Different chemical group tests of roots extract performed and the results are mentioned.

Sample	Test solution	Observation	Inference
Test for Alkaloids			
2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid.	0.1 ml of Mayer's reagent.	Yellowish buff colored precipitate was obtained.	Presence of alkaloid.
2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid.	0.1 ml of Dragendroff's reagent.	Orange brown precipitate was observed.	Presence of alkaloid.
2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid	1 ml of Wagner's reagent.	Reddish brown precipitate was observed.	Presence of alkaloid.
Tests for Flavonoids			
1 ml solution of ethanolic extract.	Few drops of conc. HCl	Immediate red color was not formed.	Absence of Flavonoids.
Tests for Saponins			
1 ml solution of the extract was diluted with distilled water to 20 ml.	Shaken in a graduated cylinder for 20 min.	One centimeter layer of foam was formed.	Presence of saponins.

Tests for Reducing sugars			
2 ml solution of aqueous extract.	1 ml equal volume of Fehling's A and B solution. Boiled for 5 minutes on a boiling water bath.	Brick red colored was precipitate	Presence of reducing sugars.
0.5 ml solution of extract.	5 ml Benedict's reagent and boiled for 5 minutes on a boiling water bath.	Brick red colored was precipitate.	Presence of carbohydrates.
1 ml solution of aqueous extract.	Boiled with 2 ml of dil. HCl acid for 5 min. after cooling neutralized with NaOH solution and performed Fehling's test as described above.	Brick red precipitate was formed.	Presence of combined reducing sugars.
Tests for Tannins			
5 ml solution of extract.	1 ml of 5% Ferric chloride solution.	Greenish black precipitate was not formed.	Absence of tannins.
5 ml solution of extract.	1 ml of 10% potassium dichromate solution.	Yellow precipitate was not obtained.	Absence of tannins.
5 ml solution of extract	1 ml of 10% Lead acetate	Yellow precipitate was not obtained.	Absence of tannins.

Tests for Gums			
5 ml solution of extract.	Molish reagent and sulfuric acid were added.	Red –violet ring was not produced at the junction of two liquids.	Absence of gums
Test for Steroids			
1 ml solution of chloroform extract.	2 ml Libermann –Burchard reagent was added.	Chloroform layer not acquire reddish purple colour.	Absence of steroid.
1 ml solution of chloroform extract	1 ml Sulphuric acid was added.	Red color was not produced.	Absence of steroid.

Table 4. Summary of Chemical group test results.

Plant extract	*Alkaloids*	*Reducing sugars*	*Tannins*	*Gums*	*Flavonoids*	*Saponins*	*Steroids*	*Carbohydrate*
Ethanol extract of leaves	+	+	+	−	+	+	−	+
Ethanol extract of bark	+	+	−	+	−	+	−	+
Ethanol extract of root	+	+	−	−	−	+	−	+

+ =Presence; − =Absence.

3.9. Results

The ethanolic extract of *C viscosum* was subjected for chemical group test. Alkaloid, Flavanoids, Saponins, Reducing sugar, Carbohydrates, Tennins found in the leaves extract. Ethanolic extract of bark extract showed the presence of alkaloids, reducing sugar and Saponins Carbohydrates, Gum. In root extract Alkaloid, Saponins, Reducing sugar, Carbohydrates, found. The experimental findings form the study showed that the ethanolic extract has organic compounds which can show extensively pharmacological activity.

Chapter Four

Screening For In-*vitro* Antioxidant Activity

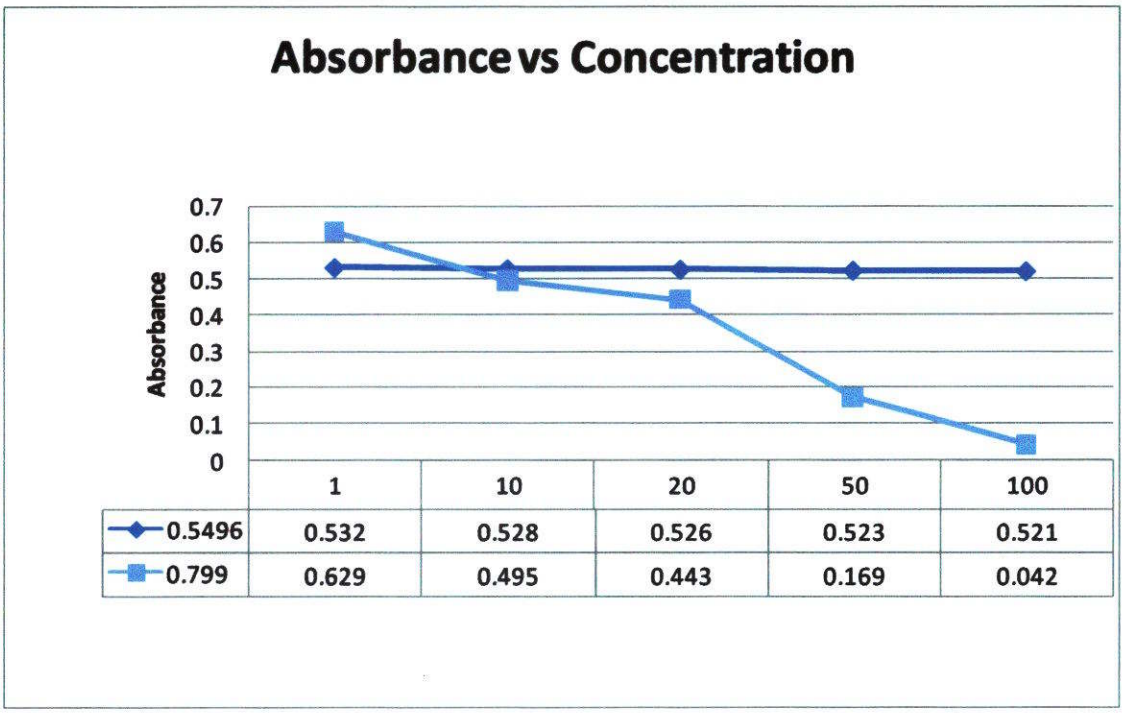
4. Introduction

The DPPH antioxidant assay is based on the ability of 1, 1-diphenyl-2picrylhydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron which is responsible for the absorbance at 517 nm, and also for visible purple yellow color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized which can be quantitatively measured from the change in the absorbance.

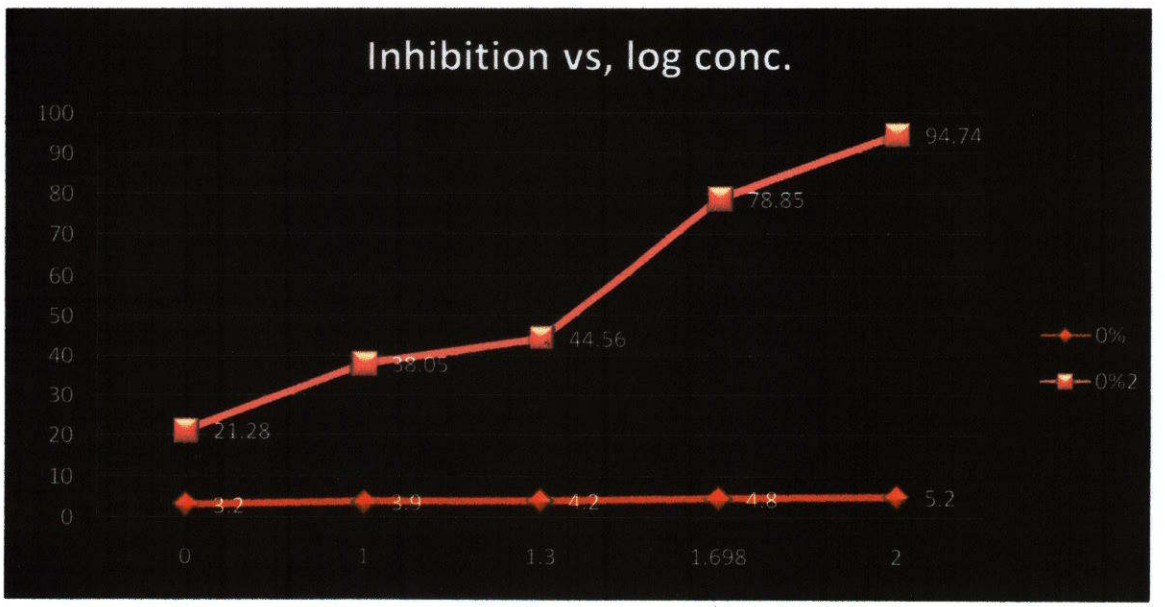
The plant extract exhibited positive results for flavonoid, reducing sugar, tannin, gum and saponin. It has previously been outlined in a number of scientific publications that plants contain polar groups especially phenolic groups of compounds such as tannins and flavonoids can act as primary antioxidants or free radicals terminator (Miliauskas *et al.*, 2004). Probable cause of absence of antioxidant activity in the ethanol extract is that the phenolic group of compounds had evaporated during evaporation of ethanol.

Table 5. DPPH Scavenging Assay of *C. viscosum leaves*

Conc.of extract (µg / ml)	Absorbance 1	Absorbance 2	Absorbance 3	Average $\overline{X}$	$\overline{X} \pm SD$	$\overline{X} \pm SE$	% inhibition	log Conc.
Blank	0.556	0.5498	0.5497	0.5496	0.549 ± 0.0036	0.549±0.00057	0%	0
1	0.531	0.532	0.533	0.532	0.532 ± 0.001	0.532 ± 0.00208	3.2	0
10	0.529	0.527	0.528	0.528	0.528±0.001	0.528±0.000578	3.9	1
20	0.525	0.526	0.527	0.526	0.526±0.001	0.526±0.000578	4.2	1.3
50	0.522	0.523	0.524	0.523	0.523± 0.001	0.523 ± 0.000578	4.8	1.698
100	0.520	0.521	0.523	0.521	0.521±0.00152	0.521±0.000883	5.20	2



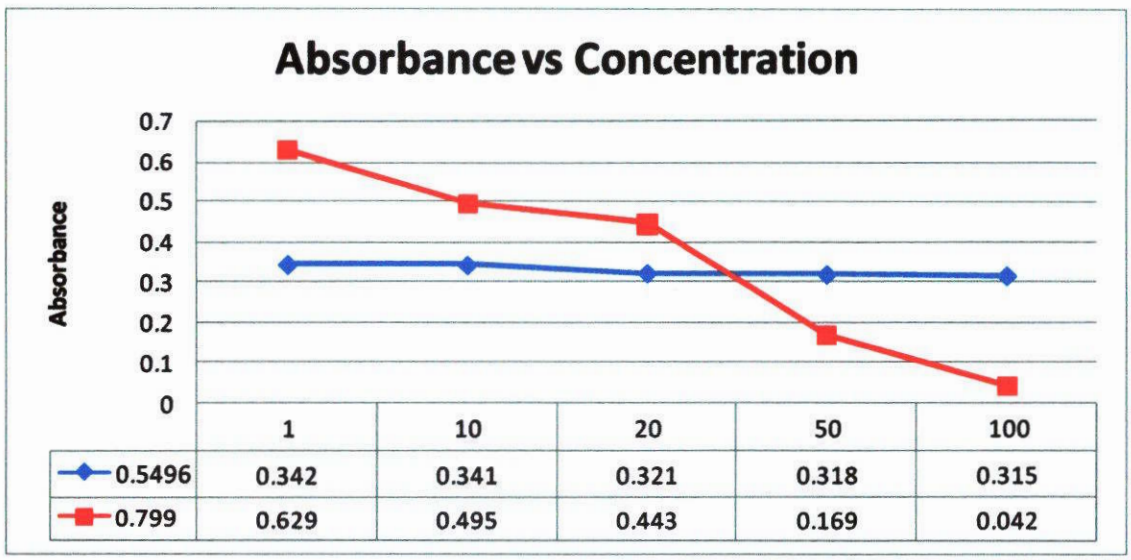
Graph1. DPPH Scavenging Assay *C. viscosum* leaves (Absorbance vs Conc of leaves and ascorbic acid.)



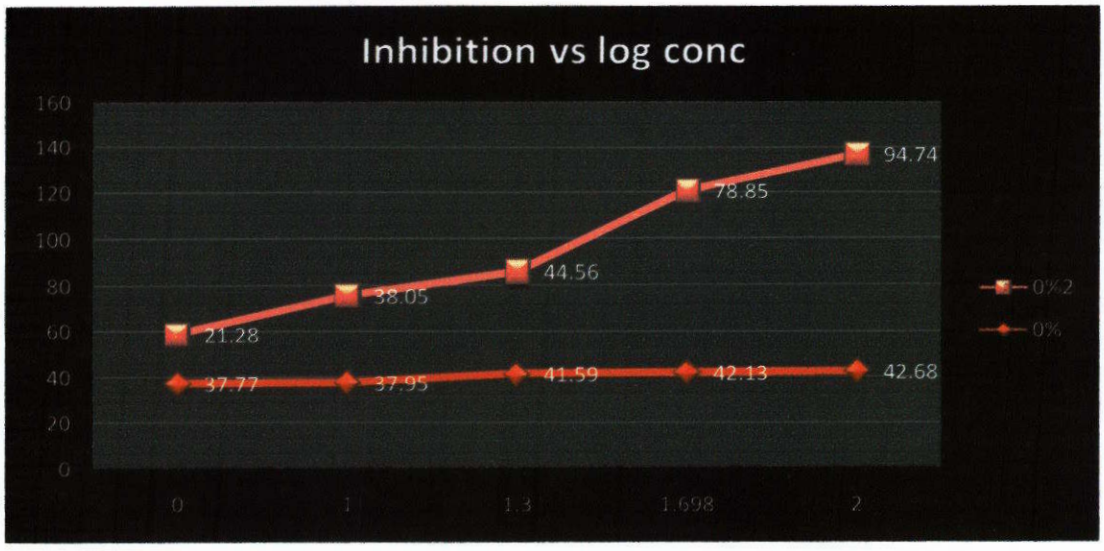
Graph 2. DPPH Scavenging Assay of *C. viscosum* leaves (% inhibition vs. log Conc.)

Table 6. DPPH Scavenging Assay of *C. viscosum* roots.

Conc.of extract (µg / ml)	Absorbance 1	Absorbance 2	Absorbance 3	Average $\overline{X}$	$\overline{X} \pm SD$	$\overline{X} \pm SE$	% inhibition	log Conc.
Blank	0.556	0.5498	0.5497	0.5496	0.549 ± 0.0036	0.549±0.00208	0%	0
1	0.342	0.343	0.341	0.342	0.342± 0.001	0.342± 0.000578	37.77	0
10	0.340	0.342	0.341	0.341	0.341±0.001	0.341±0.000578	37.95	1
20	0.320	0.321	0.323	0.321	0.321±0.0015	0.321±0.000578	41.59	1.3
50	0.318	0.319	0.317	0.318	0.318± 0.001	0.318 ± 0.000578	42.13	1.698
100	0.315	0.316	0.314	0.315	0.315±0.001	0.315±0.000578	42.68	2



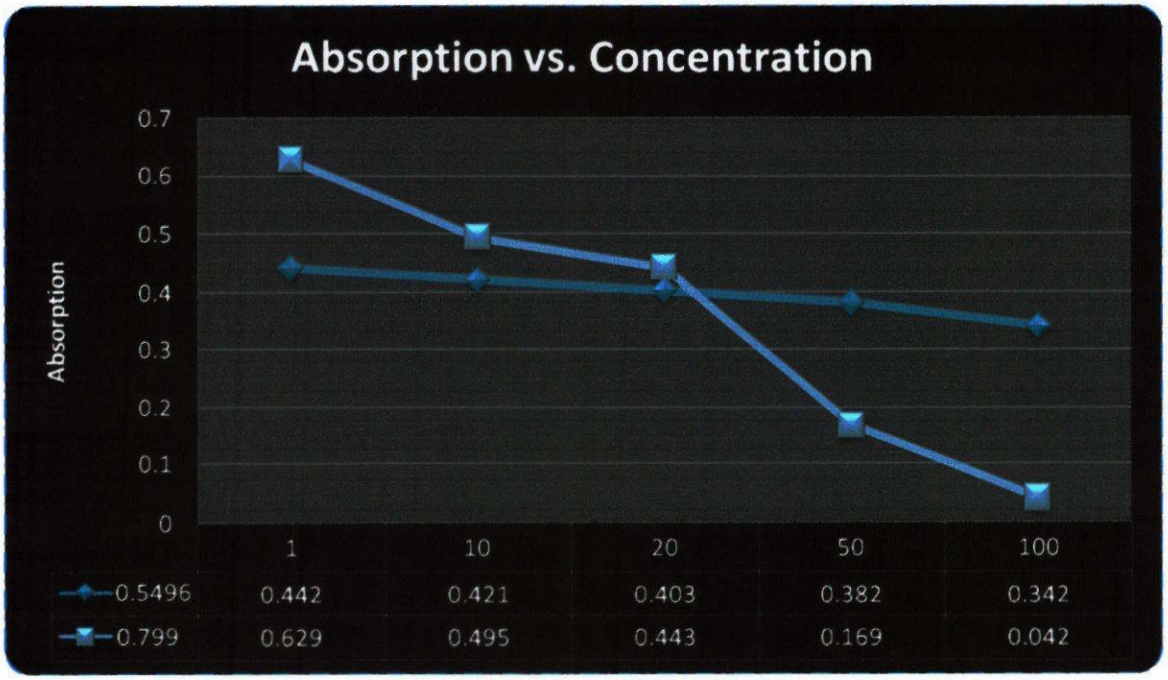
Graph 3. DPPH Scavenging Assay of *C. viscosum* root (Absorbance vs Conc.)



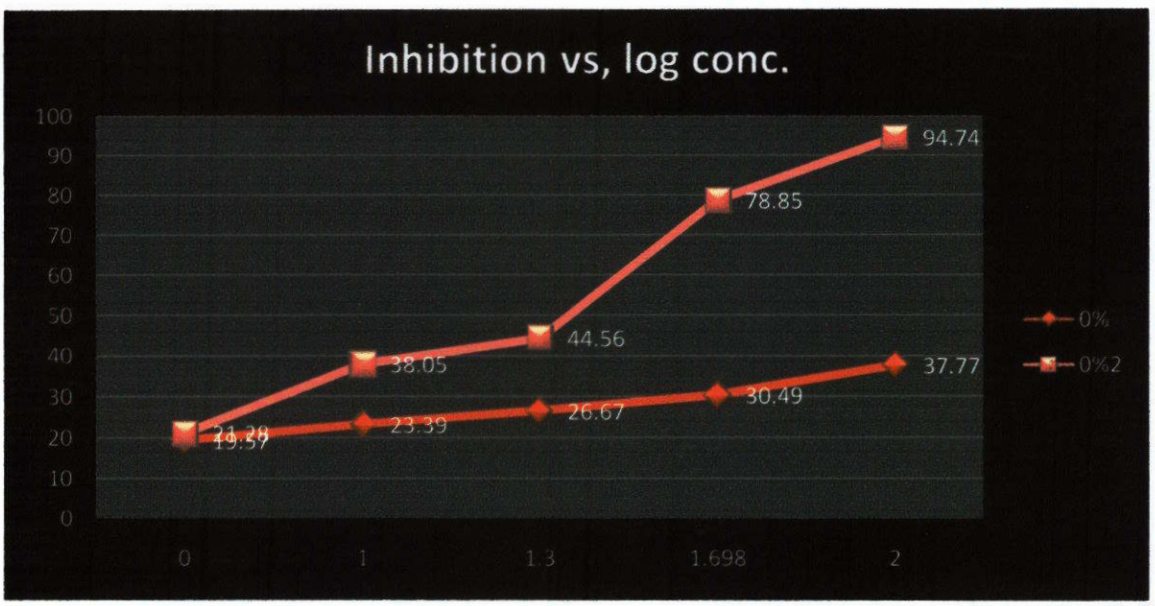
Graph 4. DPPH Scavenging Assay of *C. viscosum root* (% inhibition vs. log Conc.)

Table 7. DPPH Scavenging Assay of *C. viscosum Bark*

Conc. of extract (µg / ml)	Absorbance 1	Absorbance 2	Absorbance 3	Average $\overline{X}$	$\overline{X} \pm SD$	$\overline{X} \pm SE$	% inhibition	log Conc.
Blank	0.556	0.5498	0.5497	0.5496	0.549 ± 0.0036	0.549±0.002086	0%	0
1	0.441	0.442	0.443	0.442	0.442± 0.001	0.442± 0.000578	19.57	0
10	0.421	0.422	0.420	0.421	0.421±0.001	0.421±0.000578	23.39	1
20	0.404	0.402	0.403	0.403	0.403±0.001	0.403±0.000578	26.67	1.3
50	0.382	0.383	0.381	0.382	0.382± 0.001	0.382± 0.000578	30.49	1.698
100	0.343	0.342	0.341	0.342	0.342±0.001	0.342s±0.000578	37.77	2



Graph 5. DPPH Scavenging Assay of *C. viscosum* barks (Absorbance vs. Conc.)



Graph 6. DPPH Scavenging Assay of *C. viscosum bark* (% inhibition vs. log Conc.)

Table8. DPPH Scavenging Assay of Ascorbic acid.

Conc.of ascorbic acid(µg / ml)	Absorbance 1	Absorbance 2	Absorbance 3	Average $\overline{X}$	$\overline{X} \pm SD$	$\overline{X} \pm SE$	% inhibition	log Conc.
Blank	0.80	0.799	0.798	0.799	0.799±0.001	0.799±0.0005	0%	0
1	0.632	0.629	0.626	0.629	0.629±0.003	0.629 ± 0.0017	21.28	0
10	0.488	0.495	0.502	0.495	0.495±0.007	0.495 ± 0.0040	38.05	1
20	0.441	0.443	0.445	0.443	0.443±0.002	0.443 ± 0.0011	44.56	1.3
50	0.165	0.169	0.173	0.169	0.169±0.004	0.169 ± 0.0023	78.85	1.698
100	0.047	0.042	0.173	0.042	0.042±0.074	0.042±0.0429	94.74	2

4.1. Result

In the quantitative assay, the extracts of *C. viscosum* (leaves, roots, Bulk) displayed mild free radical scavenging activity in the DPPH assay and which is not significantly comparable to that of ascorbic acid, a well-known standard antioxidant.

Generally plant parts which have antireumatic activity (as *C.viscosum* is traditionally used) also show antioxidant activity. But the ethanol extract shows mild antioxidant activity.

Chapter Five

Bioassay for Cytotoxic Effect by Brine Shrimp Lethality Test

5. Introduction:

In this bioassay, the crude extract of leaves roots, bark showed lethality indicating the biological activity of the compound present in C. *viscosum* extract. Completely Randomized Design was followed during this bioassay (Kothari CR. 1978).

Brine shrimp lethality bioassay is a recent development in the assay procedure for the bioactive compounds and natural product extracts, which indicates cytotoxicity as well as a wide range of pharmacological activities e.g. anticancer, antiviral, pesticidal etc. Bioactive compounds are almost always toxic in high doses. Pharmacology is simply toxicology at a lower dose or toxicology is simply pharmacology at higher dose. Thus, in vivo lethality in a simple zoological organism (brine shrimp nauplii) can be used as a convenient monitor for the screening and fractionation in the discovery of new bioactive natural products. Natural product extracts, fractions or pure compounds can be tested for their bioactivity by this method. This bioassay is indicative of cytotoxicity and a wide range of pharmacological activities of natural products.

5.1. Materials:

1. Artemia salina Leach (brine shrimp eggs)
2. Pipettes (5 m1 & 1 ml)
3. Sea salt (mixture of pure & crude Sodium Chloride)
4. Micro-pipette (5-200 µl adjustable)
5. Test tube (15 ml)
6. Small tank for perforated dividing dam to grow shrimp
7. Cover and lamp to attract shrimp
8. Volumetric flask. (10ml)

9. DMSO (Dimethyl sulfoxide)
10. Spoon
11. Electric water blower to produce current
12. Electric bulb to produce heat
13. Stand to hold the bulb
14. Petri dish
15. Test tube stand
16. Beaker (1 liter)

5.2. Methods:

5.2.1. Preparation of sea water (brine):

Sea salt (mixture of pure NaCl 20 gm & crude NaCl 18 gm) was weighed accurately, dissolved in distilled water to make 1 litre and then filtered off to get a clear solution.

5.2.2. Hatching of brine shrimp eggs:

Sea water was taken in a small tank and shrimp eggs were added to one side of the divided tank and this side was covered. The shrimps were allowed for two days to hatch and mature as nauplii (larvae). The hatched shrimps were attracted to the lamp on the other side of the divided tank through the perforations in the dam. These nauplii were taken for bioassay.

5.3.3. Preparation of the sample solution:

500 mg of crude ethanol extract of leaf and bark was weighed accurately in a vial and dissolved in 6 ml of dimethyl sulfoxide and 4 ml of distilled water. The concentration of this solution was 50 mg/ml.

5.3.4. Application of test solution and brine shrimp nauplii to the test tubes:

18 clean test tubes were taken. Nine of these were for the samples in three concentrations (three test tubes for each concentration) and nine for control

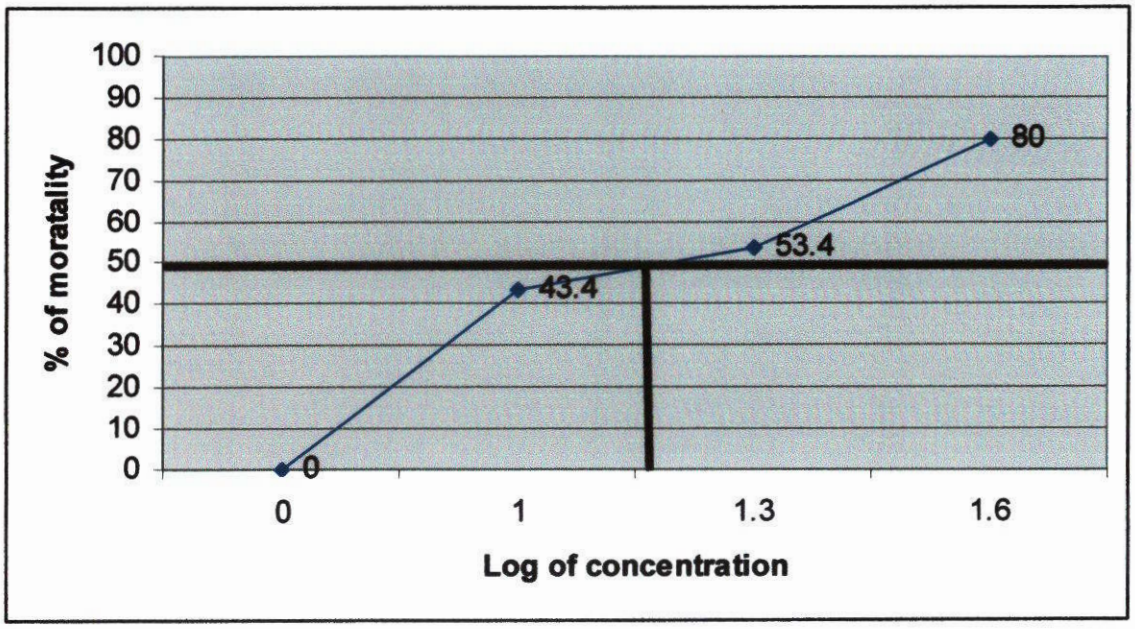
test.10, 20, 40µl of the test solution were taken in test tubes and 10ml of the sea water was added to each test tube containing 10 brine shrimp nauplii. For the control, same volumes of 6 ml DMSO (as in the sample test tubes) and 4 ml distilled water mixture were taken in the rest fifteen test tubes (three test tubes for each concentration). Finally with the help of a Pasteur pipette 10 living shrimps were kept to each of the test tube (Meyer *et al.*, 1982).

5.3.5. Counting of nauplii:

After 24 hrs the test tubes were observed and the number of survived nauplii in each test tube was counted and the results were noted. From this, the percentage of lethality of brine shrimp nauplii was calculated at each concentration for each sample.

Table 9. Result of Brine Shrimp lethality bioassay of ethanolic extract of C. *viscosum* leaves

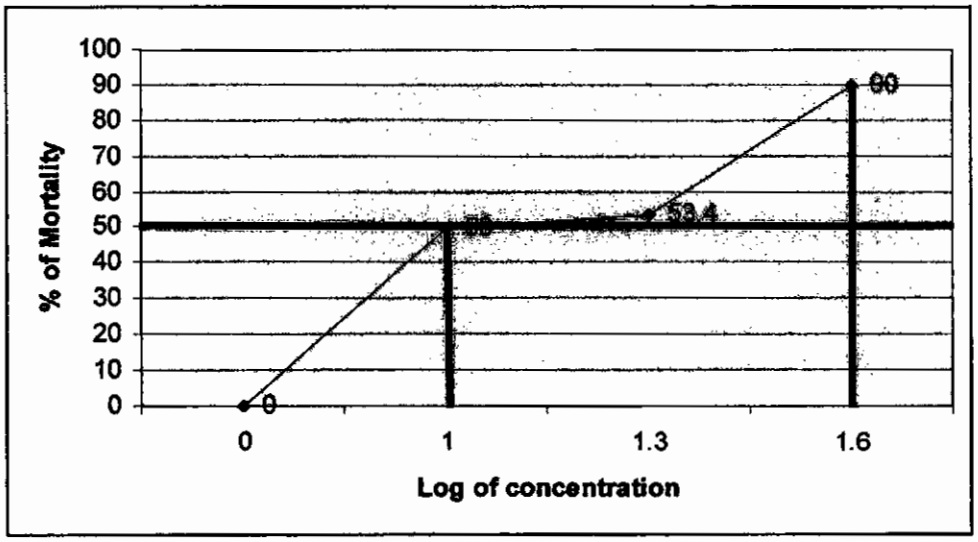
Conc. (µg/ml)	Log (Concentration)	T-1	T-2	T-3	Avg. no of alive shrimp (in sample)	Avg. no of alive shrimp (in control)	$\bar{X}\pm SD$	$\bar{X}\pm SE$	Percentage of mortality
10	1	4	8	5	5.66	10.00	5.66 ± 2.08	5.66 ± 1.203	43.4
20	1.30	4	6	4	4.66		4.66 ± 1.155	4.66 ± 0.667	53.4
40	1.60	0	4	2	2.00		2.00 ± 2	2.00 ± 1.156	80



Graph 7. Graphical representation of Lethality (LC_{50}) test of leaves extract of *C. viscosum.*

Table 10. Result of Brine Shrimp lethality bioassay of ethanolic extract of C. *viscosum* bark

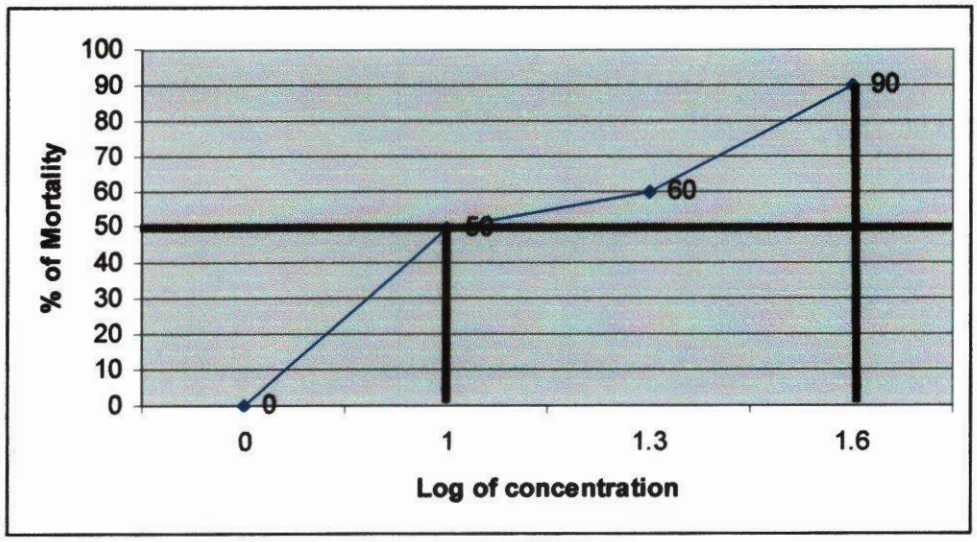
Conc. (µg/ml)	Log (Conc.)	T-1	T-2	T-3	Avg. no of alive shrimp (in sample)	Avg. no of alive shrimp (in control)	$\bar{X} \pm SD$	$\bar{X} \pm SE$	Percentage of mortality
10	1	5	6	4	5	10.00	5± 1	5± 0.578	50
20	1.30	4	6	4	4.66		4.66 ± 1.15	4.66 ± 0.667	53.4
40	1.60	0	1	2	1.00		1.00 ± 1	1.00 ± 0.578	90



Graph 8. Graphical representation of Lethality (LC_{50}) and (LC_{90}) test of bark extract of *C. viscosum.*

Table 11. Result of Brine Shrimp lethality bioassay of ethanolic extract of *C. viscosum* root

Conc. (µg/ml)	Log (Conc.)	T-1	T-2	T-3	Avg. no of alive shrimp (in sample)	Avg. no of alive shrimp (in control)	$\bar{X}\pm SD$	$\bar{X}\pm SE$	Percentage of mortality
10	1	6	4	5	5.00	10.00	5.00 ± 1	5.00 ± 0.578	50
20	1.30	4	5	3	4.00		4.00 ± 1	4.00 ± 0.578	60
40	1.60	0	1	2	1.00		1.00 ± 1	1.00 ± 0.578	90



Graph 9. Graphical representation of lethality (LC_{50}) and (LC_{90}) test of root extract of *C. viscosum.*

5.4. Result

In this bioassay, the crude extract of *C viscosum* leaves root and bark showed lethality indicating the biological activity of the compound present in the extract. Completely randomized design was followed during this bioassay sample showed various mortality rates at different concentrations. The mortality rate of brine shrimp was found to be increased with the increase in concentration of the sample and plot of percent mortality versus concentration of extract that are used, on the graph paper produced an approximate linear correlation between them. The final values were found to be 40 µg/ml for the crude extract of leaves (80 % mortality), 40 µg/ml of crude extract of bark (90% of mortality) and 40 µg/ml crude extract of roots concentration 90 % mortality of brine shrimp was found. In the table result of Brine Shrimp lethality bioassay of ethanol extract of *C viscosum* leaves roots, and bark is given.

Chapter Six

Screening for In-Vitro Antibacterial activity

6. Introduction

In vitro antibacterial activity of plants can be detected by observing the growth response of various microorganisms to those plant extracts which are placed in contact with them. This can be done in three ways (diffusion, dilution, and bioautographic methods) and a great number of factors viz, the extraction method, inocula volume, culture medium composition, pH, and incubation temperature can influence the results.

Agar well diffusion technique is widely acceptable for the preliminary screening of Antibacterial activity. It is essentially a qualitative or semi-quantitative test indicating the sensitivity or resistance of microorganisms to the test materials. However, no distinction between bacteriostatic and bacteriocidal activity can be demonstrated by this method. The sensitivity of Ethanol extract of *Clerodendrum viscosum vent* against a number of pathogenic microorganisms was investigated in vitro by agar well diffusion method.

6.1. Apparatus and reagent

1. Nutrient agar medium
2. Petridishes
3. Inoculating loop
4. Sterile cloth
5. Sterile forceps
6. Sterile scissor
7. Tissue paper
8. Micropipette
9. Pure ethanol

10. 80% ethanol
11.Test tubes
12. Beaker
13. Nose masks and hand gloves
14. Standard antibiotic discs (kanamycin)
15. Laminar air flow
16. Incubator
17. Autoclave
18. Water bath
19. Digital slide calipers
20. Lamp
21.10 ml volumetric flask
22.Hot air oven
23.Marker
24.Cork borer
25. Agar powder

6.2. Procedure

6.2.1. Preparation of media

Media are prepared by compounding the required individual ingredients or more conveniently, by adding water to a dehydrated product which contains all the ingredients. Practically all media are available commercially in powdered form.

The following steps were followed in the preparation of bacteriological nutrient agar media

- Definite amounts of nutrient agar (14 gm) were accurately weighed.
- It was taken in volumetric flask (500 ml) containing distilled water (half of the required volume).

- A clear medium was obtained by thorough dissolving agar over a water bath with occasional shaking.
- Then the final volume was adjusted.
- The medium was then transferred in 16 ml and *5* ml volume respectively, to prepare plates and slants, in a number of required test tubes.
- The test tubes were then plugged with cotton and sterilized in an autoclave at a temperature of 121°c and pressure of 15 lbs/sq inch for 20 minutes.

6.2.2. Sterilization of different equipments and media

- Media, petridishes, cork borer and other glasswares were sterilized by autoclaving at a temperature of 121°c and a pressure of 15 lbs/sq inch for 20 minutes.
- UV light was switched on before one hour working in a laminar hood to avoid accidental contamination.

6.2.3. Preparation of sub-culture:

- The autoclaved medium was transferred in 5 ml volume to prepare slants in a number of required test tubes.
- These slants were used for fresh culture of microorganisms, which in tern would be used for sensitivity tests.
- With the help of an inoculating loop, the test organisms from the pure cultures were transferred to the agar slants in an aseptic condition using laminar air hood.
- The inoculated slants were then incubated at 37°c for 24 hours to assure the growth of test organisms. This culture was used within two days.

6.2.4. Preparation of the seeded test plates

- 16 ml autoclaved medium was poured to each test tube under laminar air hood.

- Each of the test organisms were transferred from the subculture to the test tube containing 16 ml autoclaved media with the help of the sterilized inoculating loop at *45°c* in an aseptic area.
- The test tubes were shaken by rotation to get a uniform suspension of organisms. The bacterial suspensions were immediately transferred to the sterile petridishes aseptically.
- The petridishes were rotated several times, first clockwise and then anticlockwise, to assure homogeneous distribution of the test organisms.
- The medium was poured into petridishes in such a way as to give a uniform layer of depth of approximately 4 mm. after the medium became cooled to room temperature, it was stored in a refrigerator (4°c).

6.2.5. Preparation of test sample:

The crude extract was dissolved in methanol to get the concentration of 60 mg ml.

6.2.6. Preparation of standard:

Pure kanamycin was dissolved in water to get a concentration of 1 µg/ml and amount of 30 µl was used in each hole.

6.2.7. Preparation of blank:

30 µl of ethanol was used as blank in each case.

6.2.8. Application of test samples:

The seeded test plates were bored by a sterilized cork borer in an aseptic condition the test samples (10 µl), blank and standard samples were applied into the hole.

The test plates were kept sometimes to allow the diffusion of the materials into the agar and then transferred in a refrigerator and kept there for 60 minute for complete diffusion of the sample.

6.2.9. Incubation of the test plates:

The test plates then incubated in a suitable incubator at 37° C for 24 hours.

6.2.10. Measurement of zone of inhibitor:

The diameter of zone of inhibition observed around the hole was measured with help of a slide calipers.

Table 12. *In vitro* anti-bacterial activity of Ethanol extract (leaves, Bark, Root) of *c.viscosum*

Bacterial sample	Extract root	Extract leaf	Extract bark	Kanamycin
S-1	–	2	–	20
S-2	–	–	–	18
S-4	6	5	–	20
S-5	–	–	12	25
S-7	–	–	–	18
S-15	–	6	7	20
S-16	–	–	4	24
S-17	–	–	–	16
S-18	6	–	–	18

1. S-1 *Bacillus cerius*
2. S-2 *Bacillus subtilis*
3. S-4 *Proteus*
4. S-5 *Shigella dysentriae*
5. S-7 *Shigella boydiis*
6. S-15- *Staphylococcus agalactiaes-*
7. *S-17 Shigella typhi-136402*
8. S-18 *Shigella ParatyphiA-141203*

6.3. Result

The result of the antibacterial activity measured in term of diameter of zone of inhibition in mm. The crude ethanol extract of the leaf Roots, Bark of *c.viscosum* was tested for antibacterial activity against a number of bacteria. Standard antibiotic Kanamycin was used for comparison purpose. The extract showed some extent of antibacterial activity. The ethanol extract showed strong antibacterial activity against *Bacillus cerius, Shigella dysenteries, Staphylococcus agalactiae, Proteus, and Salmonella Paratyphi-A* Plant extract producing more than 12 mm zone of inhibition against bacteria has strong antibacterial activity which is significantly comparable to a standard antibiotic. (Pelczar *et al.*, 1993). As the extract showed some extent of antibacterial activity, this plant extract may be used in remedy for different microbial diseases such as diarrhoea and dysentery. *Shigella dysenteriae* is a dysentery causing pathogen for human. And the test sample showed significant zone of inhibition against this bacteria. It is a preliminary investigation. Further study should be done for the fulfillment of this test.

Chapter Seven

7. Conclusion

The project work presented here deals with different studies on *C. viscosum*. The investigations were carried out on phytochemistry and pharmacology.

The objective has been to explore and evaluate the Chemical Group Test, Cytotoxicity Screening, Antimicrobial Screening, In-vitro Antioxidant activity. To provide a suitable lead, which may be utilized in future to pursue a new line of investigation.

The ethanolic extract was tested for the presence of different chemical groups and the following groups were identified- Alkaloids, Tannins, Reducing sugars, gum, steroid in bark, root and in leaves extract. The final values were found to be 40μg/ml for the crude extract of leaves (80 % mortality), 40 μg/ml of crude extract bark (90%of mortality) and 40 μg/ml crude extract of roots concentration 90 % mortality of brine shrimp was found.

In the quantitative assay, the extracts of *C. viscosum* (leaves, roots, Bulk) displayed mild free radical scavenging activity in the DPPH assay and which is not significantly comparable to that of ascorbic acid, a well-known standard antioxidant.

The ethanol extract of leaves, root and bark were tested for their activity against a number of both gram positive and gram negative bacteria. This antibacterial activity was compared to that of a standard antibiotic kanamicin. The ethanolic extract of leaves root and bark of *C viscosum* were showed high activity against supplied test bacterial strains.

The leaves and bark of *C viscosum* were taken for preliminary investigation. As all the experiments were conducted with some limitations, further pharmacological and toxicological study is required to establish the therapeutic uses of the plant.

Reference

Abou-Issa, H. M (1995). Relative efficacy of glucarate on the initiation and promotion phase of rate mammary carcinogenesis. *Anticancer Res.* May-Jun: **15(3):**805-10.

Abou-Issa,H.M (1988). Putative metabolites derived from dietary combinations of calcium glucarate and N-(4-hydroxyphenyl) retinamide act synergistically to inhibit the induction of rat mammary tumors by 7, 12-dimethylbenz(a) anthracene. *Proc Natl Acad Sci,* USA. Jun: 85(12):4181-4.

Ahmed, F., Rahman, K.M and Rahman, M.M. (2003). Antibacterial Activity of *Vitis trifolia* Linn. *Khulna University Studies* 5(1):11-13.

Ali, M. (1998). *A Text Book of Pharmacognosy,* Second edition, CBS Publishers and Distributor, India.

Ames, B.N., Shigenaga, M.K, Hagen, T.M. (1993). Oxidants, antioxidants, and the degenerative diseases of aging. *Proc Natl Acad Sci.* 90:7915-7922.

Ali, S., Singh, P., Thomson, R.H. (1980). Naturally occurring quinones. Part 28. Sesquiterpenoid quinones and related compounds from *Hibiscus tiliaceus. Journal of the Chemical Society,* Perkin Transactions 1: *Organic and Bio-Organic Chemistry.* (1972-1999) (1): 257-9.

Bauer, A.W, Kirby, W.M.M, Sherris, J.C.and Turck, M. (1996). Antibiotic susceptibility testing by a standardized single disc method. *Am.J.Clin. Pathol.* 45: 493-496.

Blot, W.J., Li, J.Y. and Taylor, P.R. (1993). Nutrition intervention trials in Linxian, China: supplementation with specific vitamin/mineral combinations, cancer incidence, and disease-specific mortality in the general population. *J Natl Cancer Inst.* 85:1483-91.

Bird, G. W. G. (1961). Hæmagglutinins from *Clerodendrum viscosum. Nature* 191, 292; DOI:10.1038/191292a0

Chawla, H. S. (2004).*Introduction to Plant Biotechnology.* Second Edition. Oxford and IBH Publishing Co. Pvt. Ltd. India. pp. 67

Chopra, R.N., Chopra, I.C., Hunda, K.I. and Kapur, L.D. (1982). *Chopra's Indigenous Drugs of India.* Academic publishers. New Delhi. India. pp. 1-15.

Claus, E. P. and Tyler, V. E. (1993). *Pharmacognosy.* 5th edition, Lea & Febiger Publisher. Philadelphia, pp. 1-3.

Cordell, G.A. (2000) Biodiversity and drug discovery-a symbiotic relationship. *Phytochemistry.* 55: 463-480.

Chao, S.C., Ku, Y.C., Hang, L.Y, Lin, N.T., Lin,S.T. (1962). Mixed cooking of Taiwan hardwoods for pulp and paper making. Taiwansheng Linye Shiyanso Hezuo Baogao Zhongguo Nongcun Fuxing Lianho Weiyuanhui Hezuo. (3): 11 pp

Das, A. Kand Roy, B.A (1981) *Allelopathic potential of Clerodendrum viscosum . in relation to* Flora; morphologie, geobotanik, oekophysiologie. Flora Morphol Geobot Oekophysiol 1982. v. 172 (1) pp421-427

Elevitch and Thomson, (2006). Biodiversity and drug discovery-a symbiotic relationship. *Phytochemistry.* 55: 463-480.

Evans, W.C. (1989).Trease and Evan's *Text book of Pharmacognosy.* University Press, Cambridge.13th ed. pp. 546.

Ghani, A. (1998). *Medicinal Plants of Bangladesh,* the Asiatic Society of Bangladesh, pp. 1-7, 215.

Harborne, J .B. (1984). Phytochemical methods (*A guide to modern techniques to plant analysis*). 3rd ed. Chapman and Hall, London.

Heerdt, A.S. (1995). Calcium Glucarate as a chemo-preventive agent in breast cancer. Isr J Med Sci; 31(2-3):101-5.

Mahato, R.B. and Chaudhary, R.P. (2005). Ethnomedicinal Study And Antibacterial Activities of Selected Plants Of Palpa District, Nepal. Scientific World, Vol. 3, No. 3 pp: 26

Hess, S.C. (1995). Antibacterial activity and phytochemical analysis of Vochysia divergens (Vochysiaceae). J. Ethnopharmacol. 47:97-100.

Jaroszewski ,J.W, Stroem-Hansen, T., Hansen, S.H, Thastrup, O., Kofod, H.. (1992.) On the botanical distribution of chiral forms of gossypol. Planta Medica. 58(5): 454-8.

Keng, H. (1990). *The Concise Flora of Singapore (Gymnosperms & Dicotyledons).* ELBL Publishers. First edition; pp 148 - 149.

Kothari, C.R. (2005). *Research Methodology.* Second Edition.New Age International Publishers. pp 41.

Lobo, Richard; Kumar, C. Dinesh; Punitha, I.S.R.; Rajendran, K.; Shirwaikar, Arun; Shirwaikar, Annie (2006). Pharmacognostical Evaluation of *Clerodendrum viscosum.* Natural Product Sciences. 12(2); pp: 79-84.

Melecchi, M.I.S., Peres, V.F, Dariva C., Zini, C.A., Abad, F.C., Martinez, M.M, Caramao, E.B. (2006). Optimization of the sonication extraction method of *Hibiscus tiliaceus* L. flowers. *Ultrasonics Sonochemistry*, 13(3): 242-250.

Myers (1982). Phytochemical methods (*a guide to modern techniques to plant analysis*). Third edition.Champan and Hall: pp.335-337.

Masuda,T., Yamashita, D., Takeda ,Y., Yonemori, S. (2005). Screening for tyrosinase inhibitors among extracts of seashore plants and identification of

potent inhibitors from *Garcinia subelliptica. Bioscience, Biotechnology, and Biochemistry*, 69(1): 197-201.

Miliauskas (2004). Nutrition intervention trials & supplementation with specific vitamin/mineral combinations, cancer incidence, and disease-specific mortality in the general population.

Richad lobo and Dinesh Kumer, (2006) Pharmacognostical evalution of *Clerodendron viscosum.*Natural product science12 (2):79-84.

Richad lobo and ISR Punitha, (2006), Deparment of pharmaceuties, Manipul college of pharmaceutical sciences, Minipal-576104, India.

Rahman, Shafiur; Sarder, Mokaddez; Shilpi, Jamil Ahmad; Hasan, Choudhury Mahmud (2006) investigated the Antioxidant and analgesic activity of *Clerodendrum visconsum* leaf.

Navarro, V., Villarreal, M.L, Rojas, G. and Lozoya, X. (1996), Antimicrobial evaluation of some plants used in Mexican traditional medicine for the treatment of infectious diseases. *J. Ethnopharmacol.* 53:143-147.

Nair, A.G.R, Subramanian, S.S, Swamy, M.N. (1961). Glycosides from the flowers of *Hibiscus tiliaceus. Journal of Scientific and Industrial Research*, Section B: *Physical Sciences.* 20B: 553-4.

Oda, K .and Nakasone, H. (1976). Distribution of calcium oxalate crystals in the stems of some species grown in Okinawa. Mokuzai Gakkaishi. **22(12):** 703-6.

Paquin, D., Ogoshi, R., Campbell, S., Li, Q.X. (2002). Bench-scale phytoremediation of polycyclic aromatic hydrocarbon-contaminated marine sediment with tropical plants. *International Journal of Phytoremediation,* 4(4): 297-313.

Prasad, T.K (1981) *Physiological studies in the regenerating root cuttings of Clerodendrum viscosum* Current science, Curr Sci Oct 20, 1981. v. 50 (20) pp, 510-530

Popp, M., Larher, F., Weigel, P. (1984). Chemical composition of Australian mangroves. III. Free amino acids, total methylated onium compounds and total nitrogen. *Zeitschrift fuer Pflanzenphysiologie,* 114(1): 15-25.

Planning Commission, Government of India. (2000). Report of the Task Force on Conservation & Sustainable use of Medicinal Plants. Published on internet .

Pelczar, M.J., Chan, J.R., ECS and Krieg NR. (1993). *Microbiology.* Fifth edition. Tata McGraw-Hill Publishing Company Ltd. New Delhi. Pp.103-105. 510-539.

Report of the Task Force on Conservation & Sustainable use of Medicinal Plants, Govt. of India

Sadhu (2003). Antibacterial activity and phytochemical analysis of Vochysia divergens (Vochysiaceae). J. Ethnopharmacol.

Sajem and Gosai (2006), *Journal of Ethnobiology and Ethnomedicine* 2006 **2**:33 doi:10.1186/1746-4269-2-33

Subramanian, S .and Nair, A.G.R. (1973). Chemical constituents of the fruits of *Hibiscus tiliaceus. Current Science.* 42(21): 770-1.

Trease, G.E. and Evans, W.C. (1983). *Pharmacognosy.* 12th edition. ELBS Publishers. India.pp.377.

Tripathi, K.D. (2001). *Essentials of Medical Pharmacology.* 3rd ed. Jaypee Brothers Medical Publishers Pvt. Ltd. India.pp-432.

Wallis TE. (1985). *Text Book of Pharmacognosy,* 5th Edition. CBS Publishers. New Delhi. India.

APPENDICES

Appendix I

Statistical Equations:

1. Arithmetic Mean $(\overline{X}) = \frac{\Sigma X}{n}$

Where, ΣX = Summation of Observed Value

n = No. of Observation

2. Standard Deviation (SD) = $\sqrt{\frac{\Sigma(X-\overline{X})^2}{n}}$

Where, X = individual Value

$\overline{X}$ = Mean Value

n = No. of Observation

3. Standard Error (SE) = $\frac{SD}{\sqrt{(n-1)}}$

Where, SD = Std. Deviation

n = No. of observation

4. Student's t-Test

$$t = \frac{M_1 - M_2}{\sqrt{(SE_1)^2 + (SE_2)^2}}$$

Where, M_1 = Mean Value of Group- 1 (Control)

M_2 = Mean Value of Group- 2 (Test)

SE_1 = Std. Error of Group- 1

SE_2 = Std. Error of Group- 2