PHYTOCHEMICAL AND PHARMACOLOGICAL SCREENING OF LEAF OF Dillenia indica Linn. (Chalta).

(Family-Dilleniaceae)

A Thesis By

Md. Azizur Rahman

Student I.D. M.S-070703

Session: 2006-2007.

Submitted To

Biotechnology and Genetic Engineering Discipline, Khulna University, in partial fulfillment of the requirements for the degree of Masters of Science in Biotechnology and Genetic Engineering.

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DECLARATION

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 The findings of the research has not been/will not be published anywhere without the concurrence of the concerned supervisor/co-supervisor.

.....

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Examination Roll No.: MS 070703
March, 2010.

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ABSTRACT

Dillenia indica a tall tree (15-20m), belonging to the family Dilleniaceae was investigated for its phytochemical & biological activities. For these purpose the concentrated methanolic extract of leaves was used. Reducing sugar, alkaloids, gums, steroids and tannins were identified when crude extract was subjected to screening for different chemical groups. Antibacterial activity was assayed against a number of selective bacteria. *Bacillus cereus, Bacillus subtilis, Shigella dysenterriae* type-1, *Shigella boydii, Vibrio cholera, Salmonella typhi, Salmonella typhi*-13690 and *Salmonella paratyphi* A-141203 were found to be inhibited by methanolic extract. Minimum Inhibition Concentration (MIC) value was found to be 200µg/ml for each of *Shigella boydii* and *Bacillus subtilis*, while 300µg/ml and 450µg/ml for *Shigella dysenterriae* type-1 and *Vibrio cholerae* respectively. Minimum Bactericidal Concentration (MBC) value was found to be 500µg/ml for each of *Shigella boydii, Vibrio cholerae* and *Shigella dysenterriae* type-1 and 300µg/ml for *Bacillus subtilis*. This extract did not show any inhibitory effect against *Psedomonas* and *Streptococcus agalactiae*. The brine shrimp lethality assay is considered a useful tool for preliminary assessment of toxicity. The Methanolic extract of leaves of *Dillenia indica* (Linn) showed the moderate toxicity (LC_{50}=3µg/ml and LC_{90}=32 µg/ml) in the brine shrimp lethality assay.

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Nothing should be performed alone successfully. Contribution of other person must present behind every successful work as boost. So this successful research work is also not exceptional to this natural law.

First I would like to praise to the Almighty God, by the kind of whom I am alive & have got chance to study in this subject & have become enable to complete this research work & finally write up the outcome of the research work drive towards the fulfillment of the degree of Masters of Science in Biotechnology and Genetic Engineering.

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The Author

Table of Contents

List of Tables

List of Figures

Figure no. **Page no.**

List of Figures

Figure no. **Page no.**

DEDICATION

To my Beloved
Parents

List of Abbreviations

DMSO = Dimethylsulfoxide

g. = Gram

gr. = Group

h. = Hour

LAF = Laminar Air Flow

MIC = Minimum Inhibitory Concentration

MBC = Minimum Bactericidal Concentration

min. = Minute

ml. = Milliliter

mm. = Millimeter

SEM = Standard Error of Mean

UV = Ultra-violet

WHO = World Health Organization

wt. = Weight

admin. = Administration

Cont. = Continue

Fig. = Figure

D.indica = *Dillenia indica*

CHAPTER ONE

INTRODUCTION

".....for every disease that afflicts mankind there is a treatment or a core occurring naturally on this Earth."

----------Norman R.Farnsworth,

University of Illinois.

CHAPTER ONE

INTRODUCTION

The plants that possess therapeutic properties or exert beneficial pharmacological effects on the animal body are generally designated as "Medicinal plants" (Ghani, 2003). 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).

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.

Since the dawn of civilization, human being used plants for remedies of their ailments. These plants were sometimes toxic and sometimes medicines. They chose medicines by trial & error basis. By this way, medicinal plants come forward.

Most of the people of our country have no or little access to allopathic medication due to their low income in respect to the high cost of allopathic medicine. They can hardly afford to spend much money for the prevention and cure of their diseases. As a result, about 70-80% of the

population of our country still has to depend on the indigenous systems for the maintenance of their health. The survey indicates an extensive use of medicinal plants, most of which are consumed in a crude and substandard form by people. The use of such crude and substandard herbal drugs is dangerous and threatens public health. Thus the analysis of plants for exploring the boundary of chemical entities and their biological screening is the current need for standardization of herbal medication.

1.1 Medicinal plants : Basis of modern medicine

Throughout the world, plants have been in continuous use in one way or the other for the treatment of various ailments. In India, the sacred Vedas, which date back between 3500 B.C. and 800 B.C., give many references of medicinal plants. One of the remotest works in traditional herbal medicine is "Virikshayurveda," compiled even before the beginning of Christian era and formed the basis of medicinal studies in ancient India. The Rig Veda, dating between 3500 B.C. to 1800 B.C., seems to be the earliest record available on medicinal plants. Herbs seem to be very important component of medicine in other cultures too; Greek, African and Chinese medicines., etc. Nearly 80% of the world population depends upon traditional system of health care. Allopathic drugs have brought a revolution throughout the world but the plant base medicines have its own unique status.(Behera,K.K,*et al.*, 2006) Surveys had revealed that 50% of the top prescription drugs in the USA are based on natural products and the raw materials are locked up in the tropical world –interiors of Africa, Asia and Latin America. The local uses of plants as a cure are common particularly in those areas, which have little or no access to modern health services, such as the innumerable tribal villages and hamlets in Bangladesh

(Behera,K.K,2003). The indigenous traditional knowledge of medicinal plants of various ethnic communities, where it has been transmitted orally for centuries is fast disappearing from the face of the earth due to the advent of modern technology and transformation of traditional culture. The collection of information about natural flora, classification, management and use of plants by the people holds importance among the ethnobotanists. The earliest mentioned of the medicinal use of plants is found in the Rig Veda (4500-1600 BC), which reported that the Indo-Aryans used the Soma plant as medicinal agent.

The famous Papyrus Ebers recorded the use of many plants as far that as in 1500 BC. Ayurvedic system of medicine described the use of 127 plants as curative agents as early as in 1200 BC. The first Chinese Pharmacopoeia containing a list of 135 different medicinal plants with their uses and method of preparation appeared around 1122 BC. More than 400 recipes of plant medicines used in Greek system of medicine were described by Hippocrates around 400 BC. These are the starting materials from which modern medicine gradually evolved to reach the present state of its development [Ghani, 2003].

1.2 Medicinal Plants : Exploration of medicinal properties of plants

The exploration of medicinal properties of plants throughout the ages was accomplished principally through careful observation, trial and error and accidental discovery. Observation of animals' instinctive discrimination between toxic and palatable plants might also have helped primitive man in choosing those plants which are beneficial from nutritive and medicinal standpoints. And in this process, the human race, over the countries, has created a vast heritage

of knowledge and experience on medicinal plants in different cultures and civilizations. Most of such indigenous knowledge was handed down, through the ages, by at first orally and later in written form as papyri, baked clay tablets, parchments, manuscripts, herbal and finally printed herbals, pharmacopoeias and other works.

1.3 Medicinal Plants : Indirect contribution to modern synthetic drugs

Plants have contributed and are still contributing to the development of modern synthetic drugs and medicine in a number of ways such as (1)novel structures of biologically active chemical compounds, isolated from plant sources, often prompt the chemist to synthesis similar or better semi-synthetic compounds,(2)Synthetic drugs with similar or more potent therapeutic activity are often prepared by structural modification of the plant-derived compounds with known biological activity and (3)Various analogues and derivatives of plant constituents with similar or better pharmacological actions and therapeutic properties are often prepared by chemists for use as potent drugs. Homatropine (a synthetic tropane alkaloid similar to atropine), syrosingopine (a synthetic derivative of reserpine) chloroquine (a synthetic derivative of quinine), dihydromorphinone, methyldihydromorphinone, oxymorphine, ethyl morphine and N-allylnormorphine (synthetic derivatives of morphine) are some of the example of such synthetic drugs which plants have contributed indirectly. Even in this age of synthetic drugs, there are some naturally occurring drugs, such as the Digitalis glycosides used in cardiac complications and the Catharanthus alkaloids used in cancers, which have no synthetic alternatives. In such cases, plants continue to remain as their principal and only sources [Ghani, 2003].

1.4 Herbal drug research : Bioactivity guided approach

Plants, the molecular architect still offers a great potentiality for drug search. Plants contain compounds having interesting skeletons. Bioactivity guided phytochemical investigation of medicinal plants may yield newer chemical constituents of remarkable therapeutic interest.

Occasionally, native lore provides clue to plants with pharmacologic activity. Digitalis, opiates and the cinchona alkaloids (quinine and quinidine) came into modern medicine by this route. Curare was obtained from a South American plant long used by natives to prepare arrow poison. *Dillenia indica* is used for a variety of illnesses; only in recent years were its tranquilizing properties recognized in Western medicine and its active principle, reserpine, isolated.

Books on herbal medicinal practice report numerous medicinal plants, which are still not investigated. These plants can be subjected to pharmacologic screening as per their medicinal use to evaluate their utility. In case of significant result, chromatographic and spectroscopic methods can be applied to isolate the responsible agent.

Bioactivity guided approach has three characteristic phases of investigation.

First: Biological activity is detected in crude material, and a bioassay system is set up to permit the identification of active fractions and discarding the inactive ones.

Second: The crude material is fractionated by the most appropriate chemical procedures, all fractions are tested, active fractions are further fractionated, and so on, until pure compounds are obtained.

Third: The chemical structures of pure compounds are determined.

1.5 Procedure: Drug development from plant source

Disease is as old as life itself, and man has always been in search of agents to cure diseases. Medicinal plants and herbs have been in use for eradication of diseases and human suffering since antiquity. The exploitation of the medicinal properties of plants throughout the ages was accomplished principally through careful observation, trial and error, and accidental discovery. In this process, the human race, over the centuries, has created a vast heritage of knowledge and experience on medicinal plants in different cultures and civilizations. The major portion of the present day knowledge of the medicinal properties of plants is the sum total of some observations and experiences. Some of the wonder drugs of modern medicine have their roots in their early knowledge of medicinal plants. According to some generous estimates, almost 80% of the present day medicines are directly or indirectly obtained from plants [Ghani, 2003].

Modern drug development from plant source is carried out according to a systemic investigation as ascribed [Ghani, 2003].

1. Selection and correct identification of the proper medicinal plant.
2. Extraction with suitable solvent(s).
3. Detection of biological activity of crude extract and establishment of a bioassay system to permit the identification of the active fractions and rejection of the inactive ones.
4. Fractionation of crude extract using the most appropriate chromatographic procedures, biological evaluation of all fractions and separation of the active fractions.
5. Repeated fractionation of active fractions to isolate pure compound(s).
6. Elucidation of chemical structure of pure compound(s) using spectroscopic methods.
7. Evaluation of biological activity of pure compound(s).
8. Investigation of other pharmacological properties of the active agent(s).
9. Toxicological tests with pure compound(s).
10. Production of drug in appropriate dosage forms.

1.6 Research in medicinal plants

Medicinal plants constitute the major constituents of indigenous medicines. Thus the quality and effectiveness of medicinal preparations depend solely in case of indigenous medicines, on the genuineness and quality of the medicinal plants or products that are used in their preparation. So it is very important to ensure that these medicinal plants or their products really posses the clamed properties and exert the desired therapeutic effects. But there is hardly any medicinal

plant, which is used for a single therapeutic purpose. Again all medicinal plants do not really posses the claimed therapeutic virtues and medicinal properties, because most of these claims are based on old literature, folk saying, occasional experiences and traditional uses, but not on any significant clinical or pharmacological studies and statistical data. Thus the claims of medicinal properties may not be true or scientifically valid on case of all the so-called medicinal plants.

In order to substances the above assertion about their validity, these medicinal plants must be subjected to extensive study. Allempls must be made to separate the real medicinal plants from the useless ones, and for this extensive phytochemical and pharmacological investigation must be carried out on these plants. This will help in making the indigenous systems more scientific and more reliable. At the same time much of the superstition, wrong impressions and over estimations associated with the medicinal plants will be removed.

1.7 Plant Preview

Taxon: **Dillenia indica Linn.**

Genus: *Dillenia*

Family: Dilleniaceae.

Nomen number: 14122

Place of publication: Sp. pl. 1:535. 1753

Typification: View record from Linnaean Plant Name Typification Project of the Natural History Museum of London.

Name verified on: 12-May-1995 by ARS Systematic Botanists. **Last updated:** 12-May-1995

Species priority site is: Natl. Germplasm Repository - Miami (MIA),

Accessions: 1 in National Plant Germplasm System.

Habit: tree

Description: "Tree is 15-20m tall or more with dense, rounded, spreading crown and study trunk 2-4 dm thick; leaves obovate, 1-3 dm long, serrate, a lateral vein running to each tooth; buds 12-16 cm tick, three-fourths as long, sepals tightly imbricated, rounded, thick at base; petals broadly obovate, about twice as long as sepals, broad-clawed; carpels usually 20" (Wiggins & Porter, 1971).

Distributional range:

Native:

Indian Subcontinent:Bangladesh, India; Sri Lanka

Indo-China: Thailand; Vietnam

Malesia: Indonesia; Malaysia.

Native range: East Indies and Malaysia (Wiggins & Porter, 1971).

Propagation: Seed

Economic importance:

- **Food additives:** flavoring
- **Environmental:** ornamental

Dillenia indica Linn. (Family- Dilleniaceae) is a medicinal plant which posses important active principals that have special medicinal value and various parts of plants are traditionally used against a variety of diseases by the rural people [Ghani, 2003]. The plants of Dilleniaceae family grow well in hilly areas of Bangladesh such as Chittagong, Modhupur, Mymensingh, Tangail, Chittagong, Rangpur, Sylhet and Dhaka Districts. A wide range of pharmacological and biological activities was exhibited by the secondary metabolites isolated from plants belonging to Dilleniaceae. Among them terpenoids and flavonoids were the most well known, though a large amount of alkaloid was also searched. Betulinic acid was isolated from *Dillenia indica & D. return*, which exhibited high antitumor activity (Enamul Haque *et.al.,)*. Plant sample was collected from Shachebunia, Khulna, Bangladesh.

Classification:

Dillenia indica L.

Kingdom: Plantae – Plants

Subkingdom: Tracheobionta – Vascular plants

Superdivision: Spermatophyta – Seed plants

Division: Magnoliophyta – Flowering plants

Class: Magnoliopsida – Dicotyledons

Subclass: Dilleniidae

Order: Dilleniales

Family: Dilleniaceae – Dillenia family

Genus: Dillenia L. – Dillenia

Species: *Dillenia indica* L. – chulta

Synonnyms;

Dillenia, Elephant-apple, Chalta, Karambel,Fruta-estrela, Arvore-da-pataca ,Arvore-do-dinheiro Dilênia, Flor-de-abril.

Dillenia indica (Dilleniaceae) is a tall tree reaching approximately 15 meters in height. Its local name is Chalta (Bangali). It has alternate glossy shiny green leaves and fragrant white flowers; however the flowers have a yellow throat instead of red. The fruits are green when young and turn bright red at maturity. The dried fruits (drupes) are 5-10 cm in length. When they fall from the branches, the papery, outer layer falls off exposing a thick fibrous husk.This fibrous layer fruits are very tight and heavy weight. The fruits separate into severals parts.

Dillenia indica is native to muddy river deltas of Southeast Asia and tropical Pacific Islands as far east as French Polynesia, also distributed widely throughout Bangladesh, India, Malaysia, China, Australia and Philippine etc. The tree is a hydrohalophyte and occurs in wet areas. It grows along the sandy coasts, riverbanks and near mangrove swamps. It tolerates full-strength sea water.

Fig 1: Leaf of *Dillenia indica.*

1.8 Traditional Uses

The bark and leaves of the plant are traditionally used as emetic and cathartic; kernels are used as emetic; fruit is used as a cure for hydrophobia (Kirtikar and Basu, 1987). Its bark and fruits are purgative and used for the treatment of rheumatism (Rollet, 1981).

Medicinal plants constitute a precious natural wealth of a country and contribute a great deal to its health care programs. They play a significant role in providing primary health care services to rural people. They serve as important therapeutic agents as well as important raw materials for the manufacture of traditional and modern medicines. Being naturally gifted by a suitable tropical climate and fertile soil, Bangladesh possesses a rich flora of tropical plants. Bangladesh imports a large quantity of pharmaceutical raw materials including medicinal plants and semi processed plant products to produce drugs and medicines. This huge foreign exchange can be saved if the indigenous medicinal plants or their semi-processed products are utilized by the manufacturers to satisfy their needs.

Bioactivity guided phytochemical investigation of medicinal plants may yield newer chemical constituents of remarkable therapeutic interest. The presence of diverse bioactive compounds like steroids, terpenoids, flavonoids, alkaloids, glycosides, etc. in plants has formed the therapeutic basis of herbal medication. Occasionally, native folk lore provides clue to plants with pharmacologic activity. Books on herbal medicinal practice report numerous medicinal plants, which are still not investigated. These plants can be subjected to pharmacologic screening as per their medicinal use to evaluate their utility. In case of significant result, chromatographic and spectroscopic methods can be applied to isolate the responsible agent. The responsible agent or active component would be tested for their selective efficacy and toxicity. In the present study the leaf of *D. indica* were selected for phytochemical and pharmacological screening as a part of bioactivity guided separation.

1.9 Objectives

The present study was conducted to carry out the following objectives-

1. To find out the phytochemical groups of the plant *Dillenia indica.*
2. To find out the antibacterial activity of the plant *D.indica.*
3. To confirm the uses of the plant as traditional medicine.

CHAPTER TWO

REVIEW OF LITERATURE

CHAPTER TWO

LITERATURE REVIEW

2.1: Previous research findings with *Dillenia indica*

Leaf contains tannic acid, resins, waxs, coloring materials, alkaloid substance, saponin-like glycoside, gum, glucose and minerals **(Barbosa-Filho *et al.*, in 2000).**

Biologically active compounds from the leaf of *Dillenia indica* have had a dramatic impact in medicine including: quinine for the treatment of malaria; reserpine for controlling hypertension; cocaine as a muscle relaxant; and vincristine for treating children with leukemia. Tropical plants have an enormous but as yet untapped potential to yeild novel drugs and medecine (Deans & Svoboda, 1990).**Udom Kokpol *et al.*,** (1992) reported that the rice growth inhibitors and fish poison from leaf of *Dillenia indica.*

Gaysorn Veerachato *et al.*, (1992) studied the chemical constituents from the leaf of *Dillenia indica* namely dichamantin, pinocembrin, polycarpol, benzylbenzoate, turpentine and stimasterol/sitosterol.**Udom Kokpol *et al.*,** (1993) reported that the chemical constituients namely 5, 6, 7-trimethoxyflavone and 5, 6, 7, 8-tetramethoxyflavone and biological activities of *Dillenia indica.*New flavonoids glycosides, naringenin7-galactosyl,diterpine, dipoloic acid and dihedral quetcetin 5- galactoside were isolated from the leaf of *D. indica* have been found to exhibit cytotoxic and lympholytic activity **(Rosangkima *et al.*, in 2004).**

New chemopreventive rotenoids, 6 aalpha, 12 aalpha-12 a-hyddroxyelliptone (3), together with five other known rotenoids were identified from the leaves of *Dillenia indica* plant reported in 1992 (**Ito C *et al.,*** 1992).Among seventy-two extracts (methanol) obtained from the leaves, barks, and roots have been screened for antibacterial and antifungal activities. *Dillenia indica* displayed the broadest spectrum of activity (C.Wiart ***et al.,*** in 2004).**Bernadete P. da Silva, *et.el.,*** reported two flavonol diglycosides, tamarixetin 3-*O*-neohesperidoside, kaempferide 3-*O*-neohesperidoside and the known quercetin 3-*O*-neohesperidoside, together with six other known flavonoids were isolated from the leaves of *Dillenia indica.*

Two acylated flavonol glycosides 3-*O*-(4″-*O*-acetyl)-α- -rhamnopyranoside of mearnsetin (myricetin 4′-methyl ether) and myricetin 3-*O*-(4″-*O*-acetyl-2″-*O*-galloyl)-α- -rhamnopyranoside and 15 known polyphenols have been isolated and identified from the leaves of *Dillenia indica.* The complete structure of all isolated metabolites were elucidated based on chemical and spectroscopic methods of analysis (**Ibrahim I. Mahmoud,*et el.,*** 2001).

Four new flavonol glycosides namely kaempferide 3-*O*-β-xylosyl (1⇒2)-β-glucoside, kaempferol 3-*O*-α-rhamnoside-7,4′-di-*O*-β-galactoside, kaempferol 3,7,4′-tri-*O*-β-glucoside and quercetin 3-*O*-[α-rhamnosyl (1→6)] [β-glucosyl (1→2)]-β-glucoside-7-*O*-α-rhamnoside, were characterized from a methanolic leaf extract of *Dillenia indica.* Structures were established by spectroscopic and chemical methods and by comparison with authentic samples (**Lawrence O. Arot Manguro,*et el.,*** 2003).

Mizuo Mizuno,*et el.,*(1992) reported two known glycosides, ikarisoside F and epimedin A, two new glycosides of a flavonol with a γ,γ-dimethylallyl group were isolated from the underground and the aerial parts of *Dillenia indica.* The structures were determined to be des-*O*-methylanhydroicaritin 3,7-diglucoside and anhydroicaritin 3-glucosyl (1 → 3)rhamnoside-7-glucoside by means of spectral analysis.

CHAPTER THREE

MATERIALS AND METHODS

CHAPTER THREE

MATERIALS AND METHODS

The plant selected for present work was *Dillenia indica*.The experiment was conducted at Microbiology laboratory of Biotechnology and genetic Engineering discipline, Khulna University, Khulna.

3.1 : Collection and Source

For the present investigation, the plant *Dillenia indica* (Family- Dilleniaceae) was collected from Khulna District, Bangladesh in December 2007.The plants leaves were washed with water to remove muds and other unwanted material prepared for crude extraction.

3.2: Preparation of crude extract

3.2.1: Drying and grinding

The collected plant parts (leaves) were separated from undesirable materials or plants or plant parts. They were shed-dried for four week after cutting into small pieces. The plant parts were ground into a coarse powder with the help of a suitable grinder. The powder was stored in an airtight container and kept in a cool, dark and dry place until analysis commenced.

3.2.2: Hot extraction (Methanol extraction)

The powdered material was successively extracted using methanol (80-90%) for 72 hours in soxhlet apparatus. The extract was evaporated under reduced pressure to obtain a solid mass. The phyto constituents in the extracts were identified by treating the extracts with various chemical reagents.

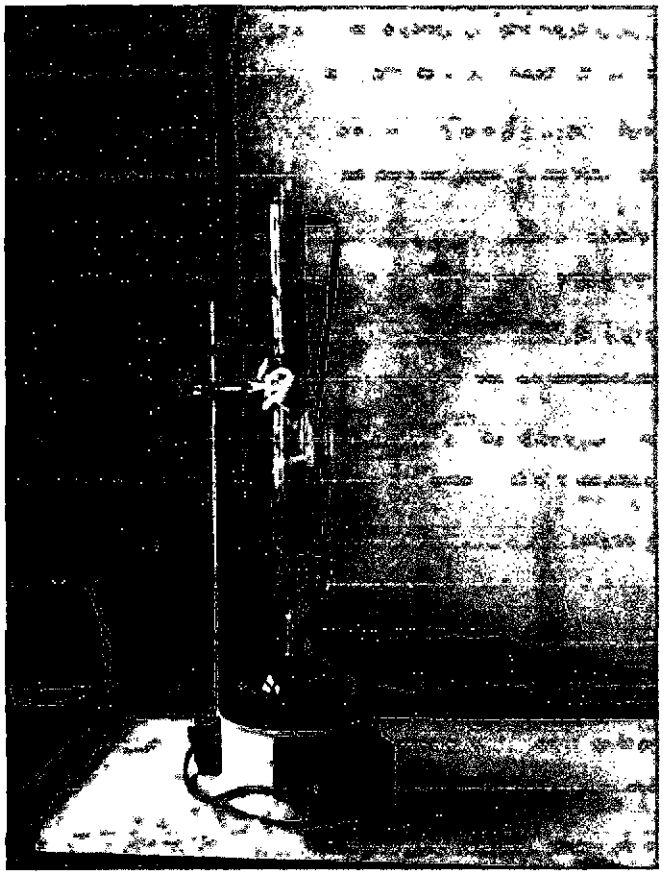

Fig-1: Soxhlet apparatus

3.2.3: Evaporation of the solvent

The filtrate (Methanol extract) obtained was evaporated under ceiling fan and oven until dried. It rendered a concentrate (12.63gm) of greenish black color. The concentrate was designated as crude extract of methanol.

Therefore, yield value (%) = 12.63/200 ×100 % = 6.315%.

3.3: Phytochemical Screening

Phytochemistry or plant chemistry has developed in recent years as a distinct discipline. It is related to 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.

To find out these different constituents present in plants methods are needed for separation, purification and identification. As a result of modern extraction isolation techniques and pharmacological testing procedures, new plant drugs usually find their way into medicine as purified substances rather than in the form of galenical preparations.

The precise mode of extraction naturally depends on the texture and water content of the plant material being extracted and on the type of substance that is being isolated. Generally, two type of procedure are used for obtaining organic constituents-

1. Cold extraction and

2. Hot extraction

The extract obtained is then carried out in a rotary evaporator which will concentrate bulky solution down to small volumes. In most cases, mixtures of substances will be present in the extract and these are subjected to chromatographic fraction (J.B Harbone, 1984). If a single substance is present, the crystals can be purified by recrystalization. As standard precaution against loss of material, concentrated extracts should be stored in the refrigerator and a trace of toluene added to prevent fungal growth.

The separation and purification of plant constituents is mainly carried out using Thin Layer Chromatography (TLC) and column chromatography. The choice of techniques depends largely on the solubility properties and volatilities of the compounds to be separated.

3.3.1: Chemical Group Tests

Testing of different chemical groups present in extract represent the preliminary phytochemical studies. The chemical group test, which were performed are follows (Trease *et al.,* 1983; Walls, 1985; Plummer, 1985). In each test 10% (w/v) solution of extract in methanol was taken unless otherwise mentioned in individual test.

3.3.2: Reagents used for the different chemical group test

The following reagents were used for the different chemical group test (Trease *et al., 1983;* Ali, 1998; Dev, 2002).

i) Mayer's reagent

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

ii) Dragendroff's Reagent

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

iii) 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.

iv) 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.

v) 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.

vi) Molish Reagent:

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

vii) Wagner's Reagent:

1 m of iodine solution.

viii) Hanger's Reagent: 1 ml of picric acid solution

3.3.3: Tests procedure for identifying different chemical groups

Alkaloid tests were performed by Dragendroff's reagent, Wagner's reagent,Hanger's reagent and Mayer's reagent (Mayer,1982). Sulfuric acid test was done to identify steroids. Gum test was performed by using Molish reagent and sulfuric acid. Hydrochloric acid was used to identify flavonoids. Benedict's solution and Fehling's A and B solution were used to detect reducing sugar. Tanins were identified by using lead acetate solution. Distilled water was used for identifying saponins (Table-1). The following tests were performed for identifying different chemical groups (Trease*et al.*, 1983; Ghani, 1998; Dev, 2002).

Table-1: Different chemical group tests performed and the results are mentioned

Sample	Test solution	Observation	Inference
Tests for Alkaloids:			
# 2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid	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.	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 iodine solution (Wagner's reagent).	Reddish brown Precipitate was observed.	Presence of alkaloid.
# 2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid.	1 ml of picric acid solution (Hager's reagent)	Yellowish precipitate was observed.	Presence of alkaloid.

Test for Steroids:

# 1 ml solution of chloroform extract was taken.	1 ml sulfuric acid.	Chloroform layer. Acquired reddish brown color and acid layer showed green fluorescence.	Presence of steroid.

Test for Gums :

# 5 ml solution of extract.	1ml Molish reagent and 1ml sulfuric acid.	Red-violet ring produced at the junction of two liquids.	Presence of gums.

Test for Flavonoids: # 1 ml solution of ethanolic extract.	Few drops of conc. HCl was added to the extract	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 15 minutes.	One centimeter layer of foam present.	Presence of Saponins.

Tests for Reducing sugars: # 5 ml solution of extract.	5 ml Fehling's A and B solution boiled for 5 minutes on a boiling water bath.	Brick red colored precipitate was obtained	Presence of reducing sugars
# 5 ml solution of extract.	5 ml Benedict's reagent and boiled for 5 minutes on a boiling water bath	Brick red color was precipitate was found.	Presence of reducing sugars

Test for Tannins:

# 5 ml solution of extract.	1 ml of 10% Lead acetate solution.	Yellow precipitate was obtained	Presence of tannins.

3.4: Antimicrobial Activity

Because of available antimicrobials failure to treat infectious diseases, many researchers have focused on the investigation of natural products as source of new bioactive molecules. A variety of methods are found for this purpose and since not all of them are based on same principles, results obtained will also be profoundly influenced not only by the method selected, but also by the microorganisms used to carry out the test, and by the degree of solubility of each test-compound. The test systems should ideally be simple, rapid, reproducible, and inexpensive and maximize high sample throughput in order to cope with a varied number of extracts and fractions. The complexity of the bioassay must be defined by laboratory facilities and quality available personnel. The currently available screening methods for the detection of antimicrobial activity of natural products fall into three groups, including bioautographic, diffusion, and dilution methods. The bioautographic and diffusion methods are known as qualitative techniques since these methods will only give an idea of the presence or absence of substances with antimicrobial activity. On the other hand, dilution methods are considered quantitative assays once they determine the minimal inhibitory concentration. Antimicrobial activities reported in the literature have been evaluated with diverse sets of methodologies, degrees of sensitivity, amount of test compounds and microbial strains, often difficult to compare. For this reason, our purpose is to suggest some recommendations and establish criteria for the use of bioautographic and diffusion methods. In this study, screening of leaf extracts of *Dillenia indica* for their antibacterial activity were carried out by disc diffusion method (Bauer *et.al.*, 1966) and then Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) was determined.

3.4.1: Apparatus

1. Filter paper discs (Matricel BBL, Cockville, USA)
2. Petri dish

3. Inoculating loop
4. Sterile cloth
5. Sterile forceps
6. Sterile scissor
7. Tissue paper
8. Micropipette
9. Test tubes
10. Beaker
11. Nose masks and hand glove
12. Laminar air flow (LAF) (ESCO, EQU 03 EHTL, ESCO micro Pte Ltd, Singapore)
13. Incubator (WTB binder, Titling, Germany)
14. Autoclave
15. Water bath (Thermostatic water bath, Shanghae, China)
16. Digital slide callipers (SM 208-10, UK)
17. Lamp
18. 10 ml volumetric flask
19. Hot air oven (WTB binder, Titling, Germany)
20. Marker (Read leaf, Japan)

3.4.2: Microorganisms used for the activity test

In this screening test for antibacterial activity eleven pathogenic bacteria which cause common enteric diseases in our country. Both gram-positive and gram-negative bacterial strains were taken for the test. The bacterial strains used for the investigation are listed below. The bacterial strains were collected from the microbiology laboratory, Biotechnology and Genetic Engineering Discipline, Khulna University, Bangladesh.

Table-2: Organisms used in study

Name of organisms
1.*Bacillus cereus*
2.*Bacillus subtilis*
3.*Psedomonas*
4.*Shigella dysenterriae* type-1
5. *Shigella boydii*
6.*Vibrio cholerae*
7.*Streptococcus agalactiae*
8.*Salmonella typhi*
9. *Salmonella typhi*-13690
10. *Salmonella paratyphi* A-141203

3.4.3: Sterilization of different equipments and media

- Media, Petri dishes, blank discs and other glass wares were sterilized by autoclaving at a temperature of 121^0 C and a pressure of 15 lbs/sq inch. for 20 minutes.
- Blank discs kept in a covered Petri dish, loop and forceps were subjected to dry heat before use it.
- Laminar hood was started 15-20 minutes before start our work to avoid accidental contamination.

3.4.4: Culture media

The following media were used normally to demonstrate the antibacterial activity and to make subculture of the test organisms.

A) Nutrient agar media

B) Nutrient broth media

C) Mullar-Hinton agar media

D) Tryptic soy broth (TSB)

Among these, nutrient agar is most frequently used which was also used for the present antibacterial screening.

3.4.5: Preparation of culture media

For preparation of nutrient agar slant, required amounts of nutrient broth were taken in a conical flask. Distilled water (volume was less than the required final volume) was added and the contents were heated in a water bath to make a clear solution. The pH was adjusted at 7.2 ± 0.2 using NaOH or HCl.The required amount of agar was added to the solution and the distilled water was added to make the final volume. Again the total volume was heated in a water bath to obtain a clear solution. Two ml of the prepared media was transferred to each of the required number of one drum vial. The vials were autoclaved at 15-Ibs.pressure/sq. inch for 15 minutes at 121 ^{0} C.

Dehydrated Tryptone–soy agar media was used for routinely culture of the bacteria under test. The calculated amount of medium was weighed. It was then suspended in the required volume of distilled water in a conical flask with required amount of agar (1.5%) and mixed thoroughly. The conical flask was plugged with cotton and then autoclaved at 15 Ibs.pressure for 15 minutes at 121 ^{0} C.Twenty five ml of the sterilized media was poured to each of the required number of petridishes,which were previously washed and sterilized. After solidified, the petridishes were stored at 4 ^{0} C by covering aluminum foil.

For the preparation of Mullar-Hinton broth, calculated amount of dehydrated media was suspended in distilled water and three ml aliquots of suspension were taken into required number of screw-cap test tube prior to autoclaving.

Mac-conkey agar media was prepared in the same way as Tryptone-soy agar using dehydrated media.

3.4.6: Inoculation

The test organism from the pure culture (preserved at -5 ^{0} C and 4 ^{0} C) were streaked on agar plates with the help of an inoculating loop. For the growth of the test organism the inoculated plates were incubated at 37 ^{0} C overnight (18 to 22hrs).

3.4.7: Preparation of the discs

Sterilized discs were placed in a sterilized blank petri dish. The discs were impregnated separately with the solution of test material so that each disc contain 200 µg and left for a period of time in an aseptic condition for the complete evaporation of the solvents. Control discs were also prepared in order to determine whether remaining solvent (if any) in disc shows any antimicrobial activity. For the preparation of control disc equal volume of solvent without test material was used to soak the paper disc. The standard antibiotic discs were obtained from market. Kanamycin was used as standard antibiotic discs.

3.4.8: Procedure of discs diffusion method for screening antibacterial activity

From overnight culture plate small portion of a fresh colony was transferred to test tube containing Muller-Hinton broth and incubated at 37 ^{0} C until the growth reached the log phase (~5×10^6 cfu/ml).After optimum growth plates were seeded properly inoculated by pouring the culture broth with a Pasteur pipette to make a bacterial lawn. The excess culture broth was discard from the plates with dropper and allowed to dry for 5 minutes. Disc impregnated with different solvent extracts concentration was placed at a proportionate distance from each other using a sterile needle. The plates were incubated invertedly over night at 37 ^{0}C and checked for the inhibition zone. A transparent scale was used to measure the zones (in mm).

3.4.9: Test for *In vitro* determination of Minimal inhibitory concentration (MIC) of the extracts of the leaf of *Dillenia indica.*

The minimum inhibitory concentration (MIC) of methanol extract was determined by the broth dilution test tube method (Blair *et.al.*1997).In this method an increasing concentration of solvent extract added to different test tube.

Thirteen sterilized screw cap tubes each containing 3ml of nutrient broth were taken. Then a calculated amount of methanol extract was added from the stock solution (1mg/ml) to the test tube to obtain different concentrations (100, 150, 200, 250, 300, 400,450, 500, 600, 700, 800,

900, 1000 and 1100 µg/ml respectively) of the extract. Then 10 µl of inoculum (from log phase of growth ~5×10^6 cfu/ml) added. Now all the tubes were incubated at 37 ^{0} C overnight. After incubation, tubes having lowest concentrations of extract showing no turbidity considered MIC of the respective solvent extract.

3.4.10: *In vitro* determination of Minimum Bactericidal Concentration (MBC) of Methanol extract.

The minimum bactericidal concentration (MBC) of methanol extract was determined by measuring the viability loss of bacterial culture with increasing concentration of extract. Test tubes containing higher concentrations of leaf extracts than MIC were plated out on nutrient agar plate to check the viability. Suspension from the tube containing lowest concentration extracts producing no colonies after overnight incubation indicated MBC.

3.5: Bioassay for Cytotoxic effect by brine shrimp lethality test

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, pesticide 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 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. (Lee *et.al.* 1988)

3.5.1: Materials

1. *Artemia salina* Leach (brine shrimp eggs)
2. Pipettes (5 ml & 1 ml)
3. Sea salt (mixture of pure & crude Sodium Chloride)
4. Micro-pipette (10-200 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. Magnifying glass
   Volumetric flask. (10 ml)
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

3.5.2: Preparation of sea water (brine)

38g Sea salt (mixture of pure NaCl 20 gm & crude NaCl 18 gm) was weighed accurately, dissolved in distilled water to make one liter and then filtered off to get a clear solution.

3.5.3: 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 eggs 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.

3.5.4: Preparation of the sample solution

10 mg of crude methanol extract of *Dillenica indica* was weighed accurately in a vial and dissolved in 10 ml of dimethyl sulfoxide. The concentration of this solution was 1 mg/ml.

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

Twenty four clean test tubes were taken. Twelve of these were for the samples in six concentrations (two test tubes for each concentration) and twelve for control test. Then a calculated amount of methanol extract was added from the stock solution (1mg/ml) to the test tube to obtain different concentration of the sample in the vials were 2, 4, 8, 16, 32, 64 µg/ml respectively.

For the control, same volumes of DMSO (as in the sample test tubes) were taken in the rest twelve test tubes (two 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, 1982).

CHAPTER FOUR

RESULTS AND DISCUSSION

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1: Results of phytochemical Screening

Methanolic extract of leaf of *Dillenia indica* Linn. was subjected to screening for various phytochemical groups.

Table -4: Results of different group tests are given bellow

Extract	Alkaloid	Steroid	Gums	Reducing sugars	Tannins	Flavonoids	Saponins
Methanolic	+	+	+	+	+	-	+

+ = Presence **- = Absence**

Phytochemical study showed that alkaloids, Steroid, Gums, reducing sugars, tannins, and Saponins are present in the methanolic extract.

4.2: Results of Antimicrobial Activity

All the gram-positive strains showed sensitivity to methanolic extract, but promising activity found against *Bacillus subtilis, Bacillus megaterium* and *Staphylococcus β-haemolyticus* (Table 5). *Dillenia indica* showed satisfactory activity against organisms tested.

4.2.1: Results of disc diffusion test

In this preliminary experiment methanol extracts showed positive result. Disc of methanol extract with the concentration of 200µg/disc produced zones of 8 mm for each of *Bacillus subtilis*, *Shigella boydii*, *Vibrio cholerae* and *Salmonella typh*-13690, 6 mm for each of *Bacillus cereus* and *Salmonella typhi*, 10 mm for each of *Shigella dysenterriae* type-1 and *Streptococcus agalactiae* and 0 mm for each of *Pseudomona*and *Salmonella paratyphi* A-141203.

Effect of methanolic extract was found significant against *Shigella dysenterriae* type-1 and *Streptococcus agalactiac* (zone of 10 mm diameter for each). Antibacterial activity of methanolic extract against *Bacillus subtilis*, *Shigella boydii*, *Vibrio cholera*, and *Salmonella paratyphi* A-141203 were also noteable (8 mm zone) (Table -5).*Pseudomonas* and *Salmonella paratyphi* A-141203 are fully resistant to methanol extract (Table-5)

Though the extract are crude but the zones of inhibition were very clear which indicate the presence of active compound against bacteria.The methanolic extract of the plant demonstrated strong antibacterial activity against *Shigella dysenterriae* type-1, *Streptococcus agalactiac* with zone of 10 mm for each. *Bacillus subtilis*, *Shigella boydii*, *Vibrio cholerae* and *Salmonella paratyphi* A-141203 with zone of 8 mm for each. This was comparable to the valuves of ampciline 34, 35, 44, 39, 35, 34mm respectively.The extract was proved to be resistant against .*Psedomonas* and *Salmonella paratyphi* A-141203.

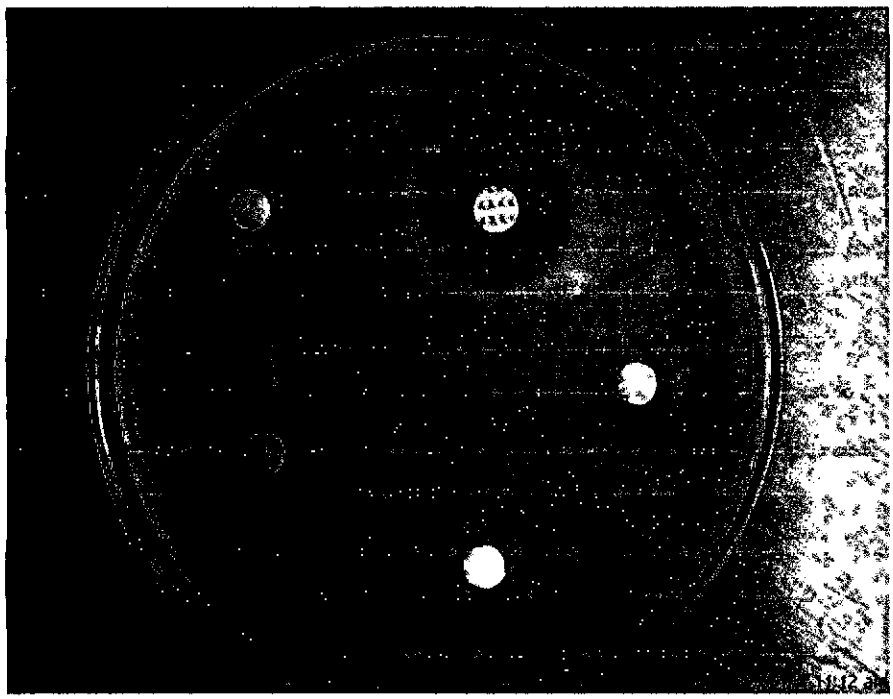

Fig-6: Plates shows zones of inhibition of organism by extract of *Dillenia indica.*

Table-5: Sensitivity of enteric pathogen against methanolic extract of *Dillenia indica.*

Bacterial species	Ampiciline (30 µg/disc)	Diameter of Zone of inhibition (mm)
1.*Bacillus cereus*	44	6
2.*Bacillus subtilis*	43	8
3.*Psedomonas*	33	-
4.*Shigella dysenterriae* type-1	34	10
5. *Shigella boydii*	39	8
6.*Vibrio cholerae*	35	8
7.*Streptococcus agalactiae*	35	10
8.*Salmonella typhi*	32	6
9. *Salmonella typhi*-13690	36	8
10. *Salmonella paratyphi* A-141203	34	-

4.2.2: Results of MIC test

Some selected bacteria from those showed sensitive against methanolic extract in preliminary screening were included for MIC test.The MIC value was found 200µg/ml for each of *Bacillus subtilis* and *Shigella boydii*,, 300µg/ml for *Shigella dysenterriae* type-1 and 450µg/ml for *Vibrio cholerae* respectively.In the MIC test a gradual decrease of bacterial growth were observed from the initial concentration.

4.2.3: Results of MBC

By measuring the viability loss of inoculums from the tubes above MIC value of the extract, the minimum bactericidal concentration (MBC) tests were carried out against *Shigella boydii*, *Bacillus subtilis*, *Vibrio cholerae* and *Shigella dysenterriae* type-1.Gradual loss of viability of bacteria was observed with increasing the concentration of extract and from a certain concentration no viable bacteria was found. In case of methanol extract MBC values were found to be 300µg/ml for *Bacillus subtilis,* Similarly, MBC value for each of *Shigella dysenterriae* type-1, *Shigella boydii, Vibrio cholerae* is 500 µg/ml.

Table-6: MIC value of *Dillenia indica* leaf extract against *Bacillus subtilis, Shigella dysenterriae* type-1, *Shigella boydii* and *Vibrio cholerae* with increasing concentrations.

Concentration of Me/Cr µg/ml	*Bacillus subtilis*	*Shigella dysenterriae* type-1	*Shigella boydii*	*Vibrio cholerae*
100	G	G	G	G
150	G	G	G	G
200	NG	G	NG	G
250	NG	G	NG	G
300	NG	NG	NG	G
400	NG	NG	NG	G
500	NG	NG	NG	NG
600	NG	NG	NG	NG
700	NG	NG	NG	NG
800	NG	NG	NG	NG
900	NG	NG	NG	NG
1000	NG	NG	NG	NG
1100	NG	NG	NG	NG

Me/Cr=Methanol Extract of leaf of Dillenia indica,G=Growth,NG= No Growth

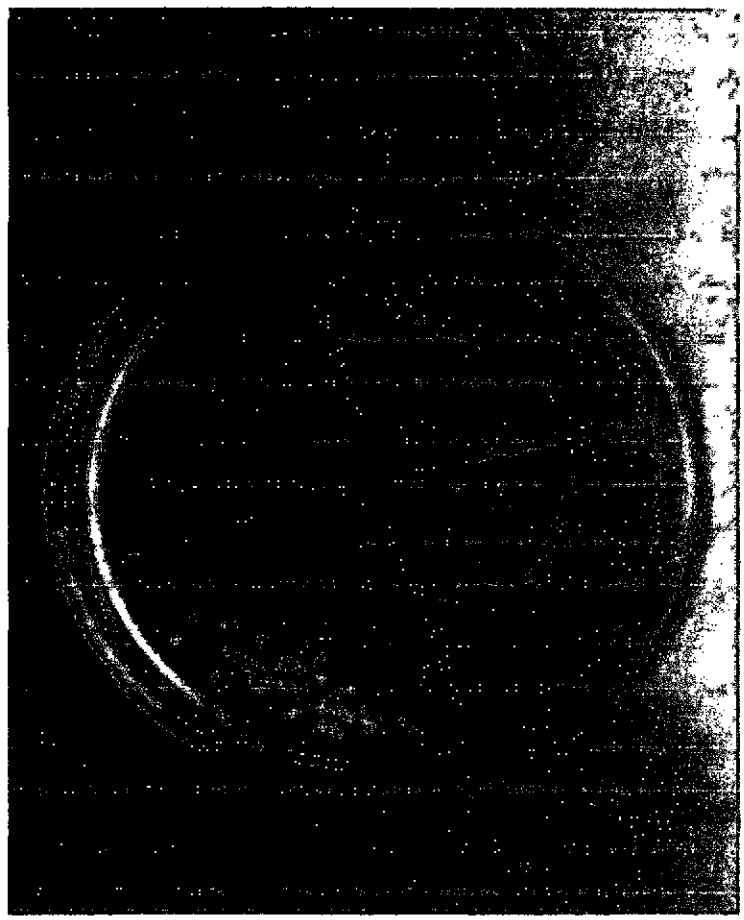

Fig-7: Increasing Concentration which destroy the bacteria as a result no colony form on Nutrient Agar plate.

In table 7 shows loss of viability in MBC assay. Loopful suspension from tubes with 200 µg/ml and above concentration (300 µg/ml, 400 µg/ml, 500 µg/ml, 600 µg/ml, 700 µg/ml, 800 µg/ml, 900 µg/ml, 1000 µg/ml) were streaked on to Nutrient Agar plate and incubated overnight at 37^0C and after incubation. Presence of Bacterial colony was observed. Table-7 shows that the above concentration from MIC tubes produced no colony in Nutrient Agar plate. Hence viability of *Bacillus subtilis* ceased in 300µg/ml i.e., no colony was produced hence the MBC value of *Bacillus subtilis* is 300µg/ml. Similarly, MBC value for each of *Shigella dysenterriae* type-1, *Shigella boydii, Vibrio cholerae* is 500 µg/ml.

Table-7: MBC value of *D. indica* leaf extract against *Bacillus subtilis, Shigella dysenterriae* type-1, *Shigella boydii* and *Vibrio cholerae.*

MIC tubes with different concentration	***Bacillus subtilis***	***Shigella dysenterriae*** type-1,	***Shigella boydii***	***Vibrio cholerae***
200	+	+	+	+
300	-	+	+	+
400	-	+	+	+
500	-	-	-	-
600	-	-	-	-
700	-	-	-	-
800	-	-	-	-
900	-	-	-	-
1000	-	-	-	-

+ = Growth of bacteria on nutrient agar plate, - = No growth of bacteria on nutrient agar plate

Viability of *Bacillus subtilis* ceased in 300µg/ml i.e., no colony was produced hence the MBC value of *Bacillus subtilis* is 300µg/ml. Similarly, MBC value for each of *Shigella dysenterriae* type-1, *Shigella boydii, Vibrio cholerae* is 500 µg/ml.

by plotting the percentage of lethality of brine shrimp nauplii which will kill, or inactivate 50% of the test animal. IC_{50} is inversely proportional to the toxicity of a compound, i.e. lower is the IC_{50}, higher is the activity.

Test sample showed different mortality rate 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 log concentration on the graph paper produced an approximate linear correlation between them. From the graph (figure- 9 & 10) the concentrations at which 50% (LC_{50}) at 95% confidence intervals and 90% (LC_{90}) mortality of brine shrimp nauplii occurred were obtained by extrapolation (3 and 32 µg/ml respectively). It was found from the result of the brine shrimp lethality test (Table 13) that the crude methanolic extract exhibited toxicity towards brine shrimp. Test samples showed different mortality rate at different concentrations. The mortality rate of brine shrimp was found to be increased with the increase of the concentration for each sample. The percent mortality of the brine shrimp nauplii was calculated for every concentration for each sample.

Conclusion

The methanol extract of leaf of *D.indica* possess moderate cytotoxic activity that indicates the extract may have anticancer, antiviral activities. And it was preliminary investigation, so further studies is required to find out active principle responsible for these activities.

Table-13: Results of Brine Shrimp lethality bioassay of methanol extract of *D.indica*

Conc. (µg/ml)	Log conc	Avg. no of alive shrimp (sample)	Avg. no of alive shrimp (control)	mean± SEM	% mortaliy	LC_{50} (µg/ml)	LC_{90} (µg/ml)
2	0.30	7		6.67±0.332	30%		
4	0.60	4		3.93±0.332	60%		
8	0.90	3		3±0.577	70%		
			10			3	32
16	1.20	2		2.03±0.332	80%		
32	1.50	1		1.3±0.332	90%		
64	1.80	0		0.33±0.332	100%		

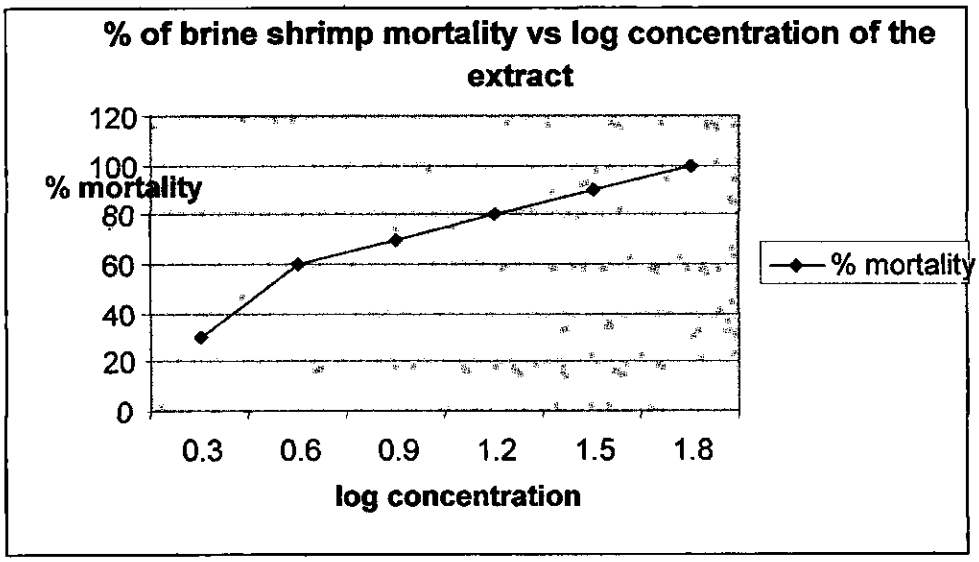

Fig-8: % mortality vs log concentration

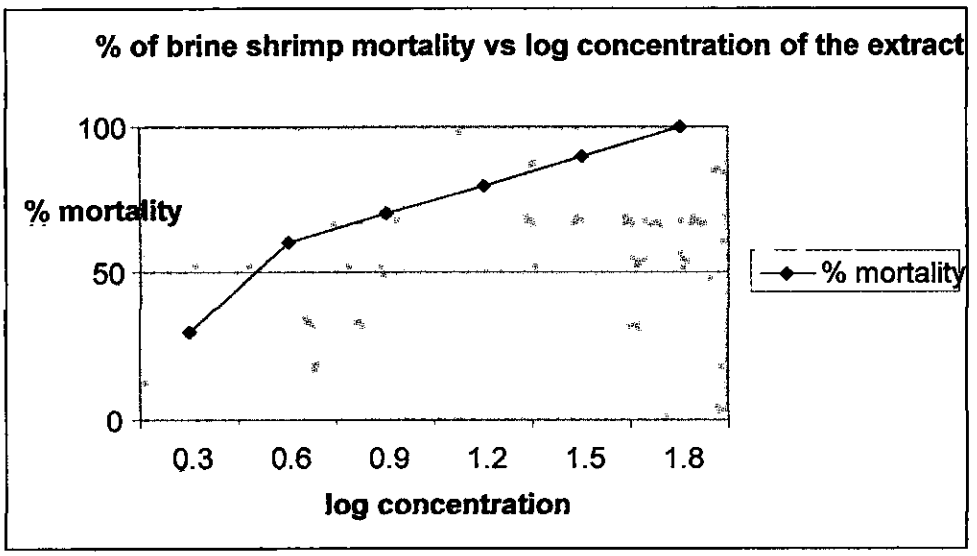

Fig-9: LC_{50} of leaf extract of *D.indica*

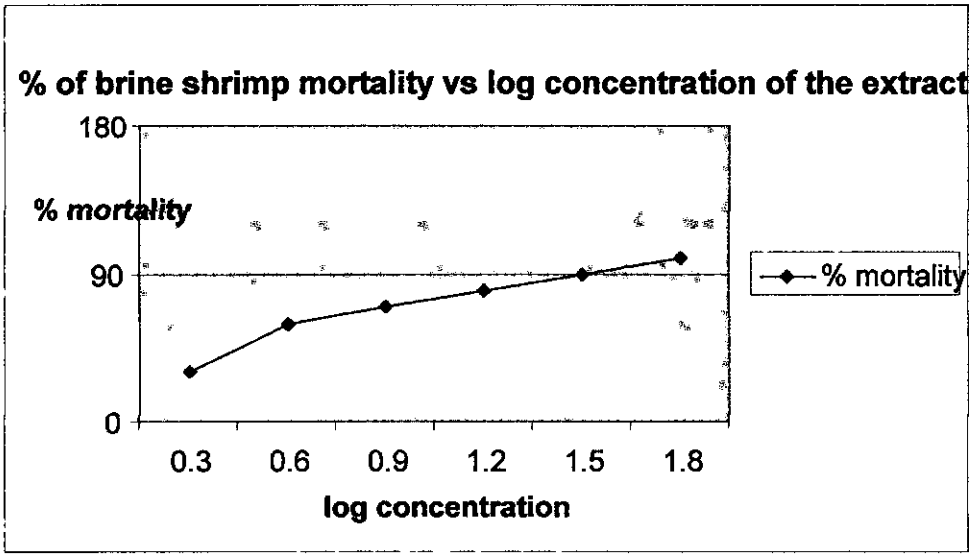

Fig-10:LC_{90} of leaf extract of *D.indica*

CHAPTER FIVE

SUMMARY AND CONCLUSION

CHAPTER FIVE

SUMMARY AND CONCLUSION

Antibacterial activity of D.indica was tested by using the agar diffusion method. Agar diffusion method is widely acceptable for the preliminary screening of antimicrobial activity.It is essentially a qualitative or semi-qualitative test indicating the sensitiviy or resistance of microorganisms to the test materials (Pelczar *et al.,* 1993).The extract showed strong antibacterial activity aganist all the test microorganisms with exception of *Pseudomonas* and *Salmonella paratyphi* A-141203. As the extract showed antibacterial activity, this plant extract may be used in remedy of different microbial diseases such as diarrhea, dysentry and skin diseases. The antibacterial activity of the plant may be attributable to the presence of the phytochemical constituents observed. The presence of tannins, saponins, flavonoids and reducing sugars has eariler been associated with antibacterial activity (Hostettman and Nakanishi, 1979; Sodipo *et al.,* 1991; Okwute and Mann, 1999).On the basis of this result it can be concluded that the extract of *D. indica* possesses antibacterial and cytotoxic activity and this may be due to the phytochemical constituents.

The probable antibacterial activity of *D.indica* leaf extracts was tested by the disc diffusion method measuring the inhibitory zone produced. In this method paper disc containing plant material were placed on Nutrient agar plate freshly sedded for making bacterial lown. Plant materials having antibacterial activity produced a clear zone around it, which are measured by a transparent scale.

In the screening test for probable antibacterial activity of plant extracts 10 entero pathogenic bacteria were used (Table-1). Among them *Bacillus cereus, Bacillus subtilis, Shigella*

dysenterriae type-1, *Shigella boydii, Vibrio cholerae, Salmonella typhi, Salmonella typhi*-13690, *Salmonella paratyphi* A-141203 were found to be sensitive to methanol extract and *Psedomonas* and *Streptococcus agalactiac* were found fully resistant to methanol extract.

So the MIC of the extract was determined against selective bacteria. This test was accomplished by broth dilution method. In the MIC test a gradual decrease of bacterial growth were observed upto the concentration just below MIC value. The lowest concentration of extract from the tube showing no visible growth was considered MIC.

The MIC values were found 200µg/ml for each of *Shigella boydii* and *Bacillus subtilis*, 450 µg/ml for *Vibrio cholerae* and 300µg/ml for *Shigella dysenterriae* type-1.

The minimum bactericidal concentration (MBC) test was conducted by the viability loss of inoculam. Counts were made from the tube showing MIC value and following tubes (having concentration higher than MIC) for determination of MBC.The MBC test were done against *Shigella boydii, Bacillus subtilis, Vibrio cholera* and *Shigella dysenterriae* type-1.

The MBC values of methanol extract were found to be 500µg/ml for each of *Shigella boydii, Vibrio cholera and Shigella dysenterriae* type-1 and 300µg/ml for *Bacillus subtilis.*

The MIC and MBC result argued that antibacterial compound is very much accumulated in the methanol extract. It is anticipated that isolation of active ingredients from methanol extract will clarify the assumption.

Last of all, in brine shrimp lethality of the extract showed moderate toxicity and it was LC_{50} for 3 µg/ml and LC_{90} for 32 µg/ml. Toxicity tests are essential for selective activity investigation (e.g. antibacterial or antifungal tests; avoid falsepositives), to detect possible safety of medicinal

plants and for further development of active extracts or isolated compounds. Since this experimental plant product has it cytotoxicity so can be suggested that methanolic extract of leaf of *D. Indica* possess moderate toxicity on brine shrimp lethality test which support in favour of many pharmacological effects e.g., antinociceptive, antidiarrhoreal, antimalarial, antirheumatic etc.Therefore further research is essential to find out the active principles responsible for these activites.

CHAPTER SIX

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ANNEXURES

APPENDIX

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

Media Composition

a. Nutrient agar media

Ingredients	Amounts (Gram/Liter)
Peptone A	6.0
Yeast Extract	2.0
Beef Extract	1.0
Sodium Chloride	5.0
Agar A	14.0
pH	7.3 (approximately)

b. Nutrient agar media

Ingredients	Amounts (Gram/Liter)
Bacto Beef Extract	0.3
Bacto peptone	0.5
Distilled Water q.s.	100ml
pH	7±0.1 at 25°C