Investigation of Cytotoxic and Antibacterial Activity of Methanol Extract of *Callicarpa macrophylla* Leaves.

A project submitted
by
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This work is dedicated to my parents, my younger brother and my husband for their love and constant support…

Certification Statement

This is to certify that this project titled "Investigation of Cytotoxic and Antibacterial Activity of Methanol Extract of *Callicarpa macrophylla* Leaves" submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (Hons.) from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Dr. Raushanara Akter, Assistant Professor, Department of Pharmacy, BRAC University and the contents of this dissertation, in full or in parts, have not been submitted to any other Institute or University for the award of any Degree or Diploma of Fellowship.

Signed,

Countersigned by the Supervisor,

Acknowledgement

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Abstract

Callicarpa macrophylla (family: Verbenaceae) has been commonly used in traditional medicine for a wide range of ailments related to the circulatory, digestive, endocrine, respiratory and skeletal systems as well as to infectious diseases. The purpose of the study was to evaluate the cytotoxic and antibacterial effect of *Callicarpa macrophylla (C. macrophylla)* leaf extract. The cytotoxic potential of the extract was examined on HeLa (a human cervical carcinoma cell line) cells using MTT assay and the antibacterial activity was assessed by disc diffusion method. The highest cytotoxic potential (92% cell growth inhibition) was evident at the concentration of 2.5 mg/mL and the lowest cytotoxic potential (7% cell growth inhibition) was found at the concentration of 0.25 mg/mL. The extract exhibited the cytotoxic activity in a concentration dependent manner. This extract showed potent cytotoxic activity with an IC_{50} value of 1.38 mg/mL. The antibacterial activity of methanol extract of the plant was investigated and low to moderate antibacterial activity against the tested bacterial strains was showed by the extract compared to the kanamycin that was used as a positive control. The highest antibacterial activity (zone of inhibition 22 mm) was found for *Bacillus cereus* at the concentration of 350 µg/disc. The results of study clearly indicated the presence of cytotoxic and antibacterial properties of the methanol extract. Phytochemical screening of this plant extract showed the presence of flavonoids, tannins, steroids and glycosides. The potent cytotoxic effect may be due to the presence of flavonoids, tannins, and glycosides and the potent antibacterial effect may be due to the presence of tannins and steroids. Thus, this study finding provide a scientific basis for the use of this plant as traditional medicine in the treatment of cancer and infectious disease and its further investigation and commercial exploitations of the plant. Future work will be focused on isolation of compounds which are responsible for cytotoxic and antibacterial activity and on new bioactivity investigation of the plant extract of *C. macrophyllay*.

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List of Abbreviations

B.C.: Before Christ

NCE: New Chemical Entity

COX: Cyclooxygenase

MTT: 3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyltetrazolium Bromide

COPD: Chronic Obstructive Pulmonary Disease

AChE: Acetyl Cholinesterase

nAChR: Nicotinic acetylcholine receptor

HPPD: 4-hydroxyphenylpyruvate dehydrogenase

NSAID: Non-Steroidal Anti-inflammatory Drug

CAM: Complementary and Alternative Medicine

IC_{50}: Half maximum inhibitory concentration

LAF: Laminar Air Flow

DMSO: Dimethyl Sulfoxide

CARS: Centre for Advanced Research in Sciences

FBS: Fetal Bovine Serum

DMEM: Dulbecco's Modified Eagles' Medium

NHB: National Herbarium of Bangladesh

AIDS: Acquired Immune Deficiency Syndrome

NICRH: National Institute of Cancer Research and Hospital

CHAPTER ONE

INTRODUCTION

Chapter 1: Introduction

1.1 Natural Products

Population is increasing, so millions of people are suffering from various types of diseases worldwide and demand of medicine is also rising. To treat different diseases there are various formulations are available for producing medicine commercially but most of the time they show toxic effects or side effects in human body, highly expensive and sometimes less effective. Therefore, it is necessary to identify alternative and naturally available herbal medicinal product with less toxic effect and economically helpful for all the people.

At 2600 B.C. from Mesopotamia the earliest records of natural products were depicted on clay tablets in cuneiform. Oils from *Cupressus sempervirens* (cypress) and *Commiphora* species (myrrh) which are still used today to treat coughs, colds and inflammation were documented here. Records of the uses of natural products are documented in The Chinese Materia Medica at nearly 1100 B.C. (Wu Shi Er Bing Fang, contains 52 prescriptions), Shennong Herbal (~100 B.C., 365 drugs) and the Tang Herbal (659 A.D., 850 drugs). It was the Arabs who were the first to privately own pharmacies (8th century) with Ibn Sina (known as Avicenna as well), a Persian pharmacist, physician, philosopher and poet, contributing much to the sciences of pharmacy and medicine through works such as the ―The Canon of Medicinae" (Dias, Urban, & Roessner, 2012).

Around 7.5 lakhs species of plants on earth has been identified by human, of which 5 lakhs are classified as ―higher plants" and 2.5 lakhs as ―lower plants" (Devi, 2015). Plant kingdoms are used from the ancient time for removing different type of disease by human being. Before discovering modern drugs human depends on leaves, seeds, bark, fruits and other parts of the plant. Use of medicinal plant to treat disease is very effective and traditional process. About 60% of all drugs clinically used worldwide are produced from natural products and their derivatives and from natural products of medicinal plants 25% of total drugs are produced. About 87% of all categorized drugs including antibiotic, anticancer, anticoagulant, antiparasitic and immunosuppressant agents are derived from natural products and their derivatives. New chemical entities introduced into the market are more the 28% comes from natural products (Akter, 2013).

1.2 New Chemical Entities from Natural Products

As a new chemical entity (NCEs) natural products have played a significant role. Between 1981 and 2002 around 28% of NCEs were natural products or natural product-derived (Newman et al., 2000). In this time period another 20% of NCEs were considered natural product mimics that mean the synthetic compound was derived from the study of natural products (Newman et al., 2003). After Combination of these categories, research on natural products accounts for approximately 48% of the NCEs reported from 1981–2002. For a new synthetic compounds, with diverse structures and often with multiple stereo centers was provided by natural products that can be challenging synthetically (Nicolaou & Snyder, 2004; Peterson & Overman, 2004). Many structural features common to natural products (e.g., chiral centers, aromatic rings, complex ring systems, degree of molecule saturation, and number and ratio of heteroatoms) have been shown to be highly relevant to drug discovery efforts (Balunas & Kinghorn, 2005; Feher & Schmidt, 2003; Lee & Schneider, 2001).

Preparing extracts from the plant materials is done by phytochemists (natural product chemists), these extracts are subjected to biological screening in pharmacologically relevant assays, and through bioassay-guided fractionation the process of isolation and characterization of the active compound (s) are started. For discovering medicinal plant drug molecular biology has become necessary and through the determination and implementation of appropriate screening assays directed towards physiologically relevant molecular targets (Balunas & Kinghorn, 2005).

In 1805, morphine was isolated in pure form *Papaver somniferum* L. plant became the first pharmacologically active compound, although its structure was not elucidated until 1923 (Sneader, 2005). The 19th century marked the isolation of numerous alkaloids from plants (species in parentheses) used as drugs, namely, atropine (*Atropa belladonna*), caffeine (*Coffeaarabica*), cocaine (*Erythroxylum coca*), ephedrine (*Ephedra* species), morphine and codeine (*Papaver somniferum*), pilocarpine (*Pilocarpus jaborandi* Holmes), physostigmine (*Physostigma venenosum*), quinine (*Cinchona cordifolia* Mutis ex Humb.), salicin (*Salix* species), theobromine (*Theobroma cacao*), theophylline (*Camellia sinensis*), and (+)-tubocurarine (*Chondodendron tomentosum* Ruiz & Pav.). After these discoveries, bioactive

secondary metabolites from plants both in their original and modified forms were later utilized more widely as medicines (Salim, Chin, & Kinghorn, 2008).

In pharmacological, physiological and biochemical studies Muscarine, physostigmine, cannabinoids, yohimbine, forskolin, colchicines and phorbol esters, all compounds obtained from plants, are plays as an important tools (Sneader, 1996; Williamson, Okpako, & Evans, 1996).

Pervilleine A (10), along with eight other tropane alkaloids, was isolated from the roots of *Erythroxylum pervillei Baill.* (Erythroxylaceae) (Silva et al., 2001). Betulinic acid was isolated from *Ziziphus mauritiana Lam.* (Rhamnaceae) (Pisha, G.A., & M.C., 1996). The ethyl acetate-soluble extract displayed selective cytotoxicity against human melanoma cells (MEL-2). Between 1940 and 2002, from all available anticancer drugs 40% were natural products or natural product-derived and another 8% considered natural product mimics (Newman et al., 2003). Some drugs isolated previously from natural sources are given in Table 1.1.

Table 1.1: Some drugs isolated previously from natural sources (Meshnick & Dobson, 2001; Serturner, 1805; Hartunf, 1954; Seader, 2005).

Drug	**Chemical class**	**Source**	**Medical use**	**Mechanism of action**
Quinine	Alkaloid	Cinchona bark, *Cinchona officinalis*	Anti-malarial agent	Protein synthesis inhibitor
Artemisinin	Sesquiterpene lactone with an endoperoxide group	Chinese medicinal herb, *Artemisia annua* (Sweet worm wood)	Anti-malarial drug	Heme-mediated decomposition of the endoperoxide bridge to produce Carbon-centered free fadicals
Penicillin	Penicillin	Fungus, *Penicillium niger*	Antibiotic	Inhibition of Peptidoglycan synthesis

Table 1.1: Some drugs isolated previously from natural sources (continued).

Drug	**Chemical class**	**Source**	**Medical use**	**Mechanism of action**
Morphine	Alkaloid	Dried latex of Opium poppy, *Papaver somniferum*	Potent Painkiller/Analgesic	Opioid agonist by binding to opiate receptors (delta, and kappa)
Digoxin	Cardiac glycoside	*Digitalis purpurea*	Atrial fibrillation and Congestive heart failure	Inhibition of the Na^+/K^+-ATPase membrane pump
Colchicine	Alkaloid	Autumn crocus flowering plant, *Colchicum autumnale*	Anti-inflammatory agent in acute gout flares and as an alternative to NSAID	Tubulin polymerization inhibitor,
Paclitaxel (Taxol^{TM})	Terpenoid	Pacific yew tree, *Taxus brevifolia*	Tubulin polymerization stabilizer,	Tubulin polymerization stabilizer
Salicylic acid (Later chemically modified to Aspirin)	Beta-hydroxy acid	Barks of willow tree	Aspirin is used as an analgesic, anti-inflammatory and antipyretic (NSAID)	Inhibition of COX (Cyclooxygenase) enzymes in the COX pathway.

Recently in U.S. market four new medicinal plant derived drugs have been introduced. They are-

1. Arteether (trade name Artemotil)- it is a potent anti-malarial drug and is derived from artemisinin, a sesquiterpenelactone isolated from a plant used in traditional Chinese medicine (TCM) *Artemisia annua* L. (Graul, 2001; van Agtmael, 1999).

2. Galantamine (also known as galanthamine, trade name Reminyl) - it is a natural product discovered through anethno botanical lead and first isolated from *Galanthus woronowii* Losinsk. (Amaryllidaceae) (Heinrich & Teoh, 2004; Pirttila, 2004). Galantamine is approved for the treatment of Alzheimer's disease, by inhibiting acetyl cholinesterase (AChE) slowing the process of neurological degeneration as well as binding to and modulating the nicotinic acetylcholine receptor (nAChR) (Heinrich & Teoh, 2004; Pirttila, 2004).

3. Nitisinone (trade name Orfadin)- it is a newly released medicinal plant derived drug that works on the rare inherited disease, tyrosinaemia, demonstrating the usefulness of natural products as lead structures (Frantz & Smith, 2003). All three of these triketones inhibit the same enzyme, 4-hydroxyphenylpyruvate dehydrogenase (HPPD), in both humans and maize (Balunas & Kinghorn, 2005; Mitchell, 2001).

4. Tiotropium (trade name Spiriva)- recently has been released to the United States market for treatment of chronic obstructive pulmonary disease (COPD) (Balunas & Kinghorn, 2005; Mundy & Kirkpatrick, 2004)

1.3 Traditional Medicines

Within local area plant based medical systems was introduced primarily based on different parts of the plant and created the traditional medicinal treatment system, the Ayurvedic and Unani of the Indian subcontinent, the Chinese and Tibetan of other parts of Asia, the Native American of North America, the Amazonian of South America and several local systems within Africa. In C. 3000 B.C. Shen Nung's ―Pen T'Sao" or ―Shennong Ben Cao Jing" is known as the oldest list of medicinal herbs (Petrovska, 2012).

Bangladesh has remarkable enormous variety of flora including medicinal plant because of its location and suitable climate. In the country more than 5700 angiosperms are present and more than 500 are believed to have medicinal properties. Traditional health care system mainly based on these plants. Pastoral and ethnic people those live in remote areas are strictly like to maintain and follow the traditional health care system to cure any disease (Sayed et al., 2009).

1.4 Medicinal Plants

Because of adverse drug reaction of synthetic products day by day herbal products are gaining good reputation in the world market. –Medicinal plants mean those plants that are usually used to treat and prevent different types of disease and that may play a significant and beneficial role in health care" (Chandra, 2013).

According to the latest report from the World Health Organization up to 80% of the population in Africa uses traditional medicine, which includes herbs, for primary health care. In China, traditional herbal preparations account for 30-50% of total medicinal consumption. The use of herbs and herbal products is rapidly spreading in industrialized countries as well (WHO, 1992) For instance, 90% of the German population has used a natural remedy at some point in their life. The global market for herbal medicines currently stands at over US $60 billion annually and is growing steadily (Soni, Dixit, Irchhaiya, & Alok, 2014).

Medicinal plants should have some properties to use these for therapeutic treatment such as:

Synergic medicine: Use of these plants can harmonize or damage others and can neutralize their possible adverse effect because all the component of this plant interacts concurrently.

Support of official medicine: The ingredients of the plant give a very successful effect to treat any cancer like complex cases.

Preventive medicine: Depending on the ability to prevent the manifestation of some diseases characteristics of the component of the plants can also be confirmed. When the disease is already present these will help to decrease the use of the chemical remedies which will be used i.e., reduce the synthetic treatment side effect (Hassan, 2012).

1.5 Significance of Medicinal Plants in Drug Discovery

Plants as a source of therapeutic agents serve different significant purpose as follows:

a) Isolating bioactive compounds for using directly as drugs, e.g., digoxin, digoxin, morphine, reserpine,taxol, vinblastine, vincristine.

b) To produce bioactive compounds of novel or known structures as lead compounds for semi synthesis to produce patentable entities of higher activity and/or lower toxicity, e.g.,

metformin, nabilone, oxycodon (and other narcotic analgesics), taxotere, teniposide, verapamil, and amiodarone, which are based respectively on galegine, 9-tetrahydrocannabinol, morphine, taxol, podophyllotoxin, and khellin.

c) It is important as a pharmacologic tools to use the agents, e.g., lysergic acid diethylamide, mescaline, yohimbine.

d) As a herbal remedy using the whole plant or parts of it, e.g., cranberry, Echinacea (Fabricant & Farnsworth, 2001).

It is estimated that 60% of anti-tumor and anti-infectious drugs already on the market or under clinical trial are of natural origin (Fabricant & Farnsworth, 2001). The vast majority of these are still obtaining from wild or cultivated plants and lot of them yet not synthesize economically (Rates, 2001). Following Table 1.2 shows some list of drug produced from plant extract–

Table 1.2: Example of some medicinal plants used for treating common ailments (Bobbarala, Katikala, Naidu, & Penumajji, 2009).

Scientific Name (Family)	**Part used**	**Traditional Ailment**
Acacia farnesiana (L.) Willd	Bark, roots	Astringent, demulcent, poultice, stomachic.
Acalypha indica Linn.	Aerial parts	Skin diseases, ulcers, bronchitis, head ache, snake bite.
Cleome viscosa Linn.	Leaves and seeds	Anthelmintic, carminative, diaphoretic and rubefacient.
Plumbago zeylanica L.	Root	Used in paralysis, secondary syphilis, leprosy and ophthalmics.
Coriandrum sativum Linn.	Fruits	Colic, laxative, blood purifier, indigestion, sore throat.

Table 1.2: Example of some medicinal plants used for treating common ailments (continued).

Scientific Name (Family)	**Part used**	**Traditional Ailment**
Hildegardia populifolia (Roxb.)	Stem bark	Dog bite, Malaria.
Melia azedarach L.	Leaf, seed oil, flower	Vermifuge, insecticide, astringent, antiseptic, antidiabitic, antiviral, antibiotic
Tephrosia tinctoria Pers.	Root	Antisyphilitic.
Tinospora cordifolia (Willd.)	Stem	Analgesic and anti-inflammatory.
Curcuma longa L	Rhizome paste, powder	Skin diseases like eczema, wounds, irritation, inflammation, erythema, burns, itching, sunburn.

1.6 Scientific Basis of Herbal Drug

As a non-scientific, inactive and erroneous medicine herbal drug was often classified. Herbal drug has some medicinal value and therapeutic utility which was proved by phytochemical and biological investigation of herbal drug. Tannin is chemical having antiseptic and astringent property which was traditionally used topically to treat skin disease. It produces a thin but strong barrier on infected area with react the proteins when used topically. This layer helps to protect the infected area from micro-organism. As well as tannin has antibiotic property. So it is said that between herbal drug and allopathic medicine there is no basic difference (Devi, 2015).

1.7 Rationale of Herbal Drug Research: Special Reference to Bangladesh

We know Bangladesh is a developing country and most of the people in our country life in under poverty line. As allopathic medicine is costly so it is very difficult for them to buy allopathic medicine to treat their disease due to their uncompromisable low income. In 1990

a survey was conducted in different villages of Bangladesh shows that if people suffering any disease only 14% can contact qualified allopathic doctor, 29% contact unqualified village doctors, 10% contact mollahs, 29% contact quack and 19% contact homeopaths. An extensive use of medicinal plants has been indicated by the survey and people take most of which are served in a crude and substandard form. Such crude and substandard herbal drug is dangerous and use of such drug might be threatening public health. To explore the chemical entities and their biological screening by analyzing the plants is the current need for standardization of herbal medication (Devi, 2015).

A proper health care system can be established in Bangladesh by supplying low cost medicines to its population as it is a country of low economic growth. By utilizing our natural resources of medicinal plants and their constituents properly this might be only possible. So, it is an urgent need for revolutionizing our drug sector to do scientific exploration and standardization of these potential crude drugs (Devi, 2015).

A large quantity of pharmaceutical raw materials including medicinal plants and semi-processed plant products to produce drugs and medicines is imported by Bangladesh. During the last five years more than 1500 core Taka has been spent by Bangladesh for importing chemicals, raw materials and semi-processed drugs of plant origin from neighboring and other countries and day by day this is increasing. If the indigenous medicinal plants or its semi processed products are utilized by the manufacturer properly this huge foreign exchange can be saved (Devi, 2015; Ghani, 1998).

1.8 Cancer and Role of Plant Extract to Treat the Cancer Disease

Cancer is a leading cause of death worldwide. Cancer is one of the most severe health problems in both developing and developed countries. It is a general term applied to malignant diseases characterized by rapid and uncontrolled proliferation of abnormal cells which may mass together to form a solid tumor or proliferate throughout the body, and when progress it causes death. Cancer cells are able to grow, invade neighboring tissues and may also affect other organs through the lymphatic system or bloodstream. Two factors primarily cause cancer disease they are external factor and internal factor. According to WHO, 2010 statistics evident that around 12 million peoples in each year are diagnosed with cancer (Akter, 2013).

Chemotherapy and radiotherapy are known as conventional cancer treatments have shown some effectiveness for reducing or eradicating cancers. However, different types of unpleasant side effects can be produced by these treatments, e.g. nausea, vomiting, changes in bowel habits, fatigue and hair loss. Moreover, for reducing or managing side effects of conventional cancer treatments complementary and alternative medicine (CAM), herbals and multivitamin supplements, or herbal medicine is increasingly used as an adjunctive treatment for cancer patients (Azarifar, Mortazavi, Farhadian, Parvari, & Roushnadeh, 2015).

1.8.1 Overview on Cancer Disease and Anticancer Drug Mechanism in Cancer

About 8.2 million people annually died by the cancer disease throughout the world. More than 2-3% of the annual deaths recorded globally accounted. In the human body all the cells have the same DNA, which contain 23 pairs of chromosomes. Cell division is the process by which the chromosomes are copied, but sometimes mutations occur in the DNA. Apoptosis or cell death is the common for most of these mutation results. If the mutant cell survives, during cell division process it will pass its mutated DNA to the next generation. These mutant DNA cells accumulations affect the cell's behavior. As a result a series of molecular changes occur which alter the normal properties of a cell and cancer cell is produced. It is difficult to control the cell division of cancer cell whereas Normal cell division is precisely controlled and cancer cell crowd out other normal cells and function abnormally. Abnormal growing of cancer cell can change the normal cell characteristics. For example, they can changes in normal cell structure, immortality and decreased cell adhesion, production of new enzymes, ability to divide infinitely, and random migration. Cancer cells can divide and grow faster and spread because of this cellular changes as a result it damage the correct function of major organs. These abnormally growing lots of cells are called tumors (Pathiranage, 2016).

Tumors can be malignant (cancerous) or benign (non- cancerous). Benign tumors are one type of tumors where they do not spread but they grow. On the other hand malignant tumors by metastasis process can spread and invade other tissues (WHO, 2016).

According to the tissue from which they arise cancers can be classified. Such as-

Carcinomas are cancers that occur in epithelial tissues, including breast, lung and skin. Sarcomas are cancers that begin in the connective tissues such as bone, cartilage, fat, and muscle. Leukemia are cancers of the bone marrow and blood cells (Pathiranage, 2016).

Based on mechanism and chemical structure chemotherapy drugs can be divided into several groups. Drugs that affect DNA, mitosis inhibitors and the newer synthetic kinase inhibitors are known as major types of chemotherapy drugs. Below Figure 1.1 shows that the drugs that affects DNA including anti metabolites, topoisomerase inhibitors and alkylating agent and affects mitosis include taxanesand vinca alkaloids (Pathiranage, 2016).

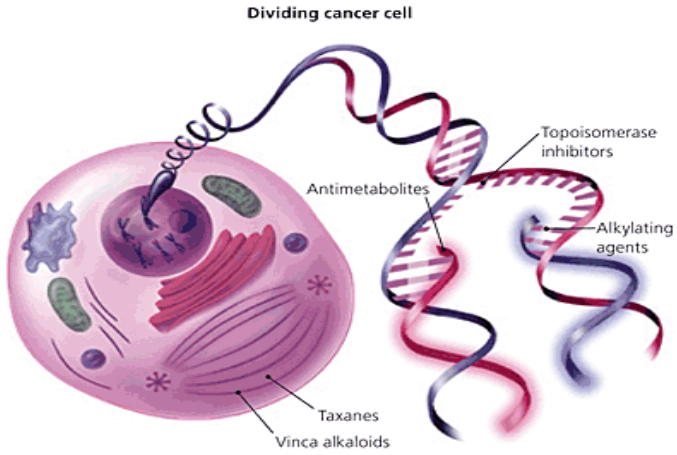


Figure 1.1: Mechanism of traditional chemotherapy.

These drugs helps to stop or slow the growth of cancer cells but these drugs also stop or slow the growth of healthy cells that grow and divide rapidly, such as cells that line intestines and hair follicles. As a result some common side effects occur such as bone marrow suppression, nausea, hair loss etc. (Gerber, 2008; Pathiranage, 2016).

1.8.2 Anticancer Drugs Isolated from Plants

Now a day, much attention has been received by herbal medicines as substitute anticancer drugs. Plant kingdom which synthesizes a variety of structurally diverse bioactive compounds is a potential source of chemical constituents with antitumor and cytotoxic activities. Recently, for pharmaceutical application and the interest in plants as a source of potential therapeutic agents, particularly as anticancer agents there has been a great deal of attention and discoveries in exploiting plant kingdom. For understanding of carcinogenesis

and identification of compounds with specific antitumor activities, investigation of the cellular growth control mechanisms is very important (Raina et al., 2014).

Therefore, to select plant extracts with potential antitumor properties cytotoxicity screening models provide important preliminary data for future studies. Following folklore claims of their efficacy many modern drugs with plant origin have been discovered by combination with the extensive ethnobotanical knowledge of local peoples. Table 1.3 shows some anticancer drug developed from plant extract. The selection of plants species based on two main strategies in anticancer drug discovery:

1) First approach is random screening and ethnomedical knowledge and

2) Second approach includes plants used in organize traditional medical systems like herbalism and folklore (Raina et al., 2014).

Above 60% of currently used anti-cancer agents are derived from natural source including plants, marine organisms and micro-organisms (Gordaliza, 2007). Due to potential benefits of cancer treatment in plant based drugs, globally their use is increasing from 10% to 40% and on the Asian continent, it has reached 50% (Cassileth & Deng, 2004; Raina, Soni, Jauhari, Sharma, & Bharadvaja, 2014). Following table shows some cytotoxic drug obtained from medicinal plant sources (Akter, 2013).

Table 1.3: List of some anticancer drug obtained from plant extract.

Drug	**Mechanism of action**	**Plant source**
Vinblastine, vincristine	Inhibition of tubuline polymerization	*Catharanthus roseus* (Apocynaceae)
Etoposide, teniposide	Inhibition of topoisomerase	*Podophyllum eltatum, p. emodi* (Berberidaceae)
Paclitaxel, docetaxel	Promotion of tubulin stabilization	*Taxus species*
Irinotecan, topotecan, 9-minocampothecin,	Inhibition of topoisomerase	*Taxus brevifolia* (Taxaceae)
9-nitrocamptothecin		*Camptotheca acuminate.*
Homoharringtonine	Inhibition of protine synthesis	*Cephalotaxus harringtonia*

Table 1.3: List of some anticancer drug obtained from plant extract (continued).

Drug	Mechanism of action	Plant source
Flavopiridol	Inhibition of cell cycle progression at G1 or G2 phase	*Dysoxylum binectariferum*
4-Ipomeanol	Cytochrome p-450-mediated conversion into DNA binding metabolites.	*Ipomoea batatas*

1.9 Method of Cytotoxic Study

Different type of experiment are used to determine the cytotoxic effect of plant extract but the basic principle is to compare the rate of proliferation of cancer cell line in the presence or absence of the test substance after a certain period of time. Usually several cancer cell lines and healthy cell lines are used to measure the selectivity between the healthy and cancer cell lines. Cytotoxic test are based on cell viability and cell survival. MTT assay is one of the most common and useful methods to test the cytotoxic effect on the other hand apoptosis or necrosis and cell analysis can be used to identify the mechanism of the cytotoxicity observed (Akter, 2013).

1.9.1 Principle of MTT Assay Method

MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyltetrazolium Bromide) assay method is a standard calorimetric laboratory test used to measure the color change and it is used for the analysis of cell proliferation. In case of cell cytotoxicity of cell against several potential toxic and medical agents MTT assay is popular to use. MTT is a yellow color solution. By the –Succinate Tetrazolium Reductase" MTT turns in to insoluble formazan MTT which fall in to the enzymes of respiratory chain in mitochondria to reduce metabolically active cells to purple formazan (Figure 1.2).

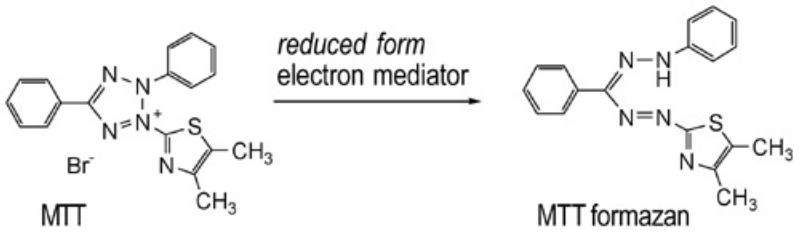


Figure 1.2: Principle of MTT assay.

By using DMSO the purple formazan is dissolved to form purple colored solution and reading is taken at 570 nm and data is analyzed by the graph obtained between cell number and absorbance. The IC_{50} (half maximum inhibitory concentration) can understand from the curve. Here the rate of tetrazolium reduction proportional to the rate of cell proliferation.

It has lots of advantages such as no radioactivity, use of adherent cell lines and high throughout analysis using scanning multi wall spectrophotometer can possible by MTT assay method. On the other hand some disadvantages like difficulty in analysis of heterozenous population and few non-proliferating cells can metabolize MTT and give the results is also present. Sometimes by this method the false results can produce when the cell lines are infected or contaminated with mycoplasma (Talupula, 2011; Tim, 1983).

1.10 Antibacterial Effect of Plant

Medical microbiology is the study of science which is related to the prevention and management of disease caused by micro-organism. Its sub disciplines are virology (study of viruses), bacteriology (study of bacteria), mycology (study of fungi), phycology (study of algae) and protozoology (study of protozoa. Antimicrobial agents are inhibitory chemical which are used to treat disease caused by micro-organism employed to kill micro-organism or prevent their growth. There are three types' antibacterial agents:

1. Antibiotics and chemically synthesized chemotherapeutic agents.
2. Non-antibiotic chemotherapeutic agents (Disinfectants, antiseptics and preservatives)
3. Immunological products.

1) Antibiotics: Micro-organisms are used to produce this or they might be fully or partly prepared by chemical synthesis. In minimal concentrations they inhibit the growth of micro-organisms. Antibiotics may be of microbial origin or purely synthetic or semi synthetic. Synthetic antimicrobial agents include sulfonamides, diamino pyrimidin derivatives

antitubercular compounds, nitrofuran compounds, 4-quinoline antibacterials, imidazole derivatives, flucytosine etc.

2) Non-antibiotics: Non-antibiotic chemotherapeutic agents such as acids and their derivatives, alcohols and related compounds etc are included in the second category of antibacterial agents.

3) Immunological products: Certain immunological products such as vaccines and monoclonal antibodies are used to control the diseases as a prophylactic measure.

Antimicrobial drugs may either kill microorganisms outright or simply prevent their growth (Cruickshank, 1962).

These agents could exhibit their antimicrobial activity in various ways. They may inhibit-

(1) Cell-wall synthesis
(2) Protein synthesis
(3) Nucleic acid synthesis
(4) Enzymatic activity
(5) Folate metabolism or
(6) Damage cytoplasmic membrane (L. D. Gebbharadt; J. G. Bachtold, 1955).

Following table 1.4 shows some antimicrobial compounds obtained from different plants (Nasir, Fatima, Ahmad, & Ihsan-ul-Haq, 2015)–

Table 1.4: Some antimicrobial bioactive compounds obtained from medicinal plants.

Compound	**Species**	**Antimicrobial activity**
Aloe emodin Chrysophanol Aloin	*Aloe ferox*	Antibacterial
Anolignan B	*Terminalia sericea*	Antibacterial
3,5-bis-O-caffeoyl quinic acid 4,5-bis-O-caffeoyl quinic acid	*Lonicera japonica Thunb*	Antibacterial
Catechol 2-hydroxybenzyl alcohol	*Salix capensis*	Antibacterial

Table 1.4: Some antimicrobial bioactive compounds obtained from medicinal plants (continued).

Compound	Species	Antimicrobial activity
Carnosol	*Salvia chamelaeagnea*	Antibacterial
Ceanothic acid Ceanothetric acid	*Caenothus americanus*	Antibacterial
Diospyrin Isodiospyrin Mamegakinone 7-Methyljuglone Neodiospyrin Shinanolene	*Euclea natalensis*	Antimycobacterial
Ent-beyer-15-en-19-ol 4-Epimer *ent*beyer-15-en-18-ol 15b,16b-Epoxide-*ent*-beyeran-19-ol Nidoanomalin	*Helichrysum tenax var tenax*	Antibacterial and antifungal (yeasts)
Epicatechin Catechin	*Schotia latifolia*	Antibacterial
d-Friedoolean-14-en-oic acid (3-acetyl aleuritolic acid)	*Spirostachys africana*	Diarrhoeal pathogens
Genkwanin 5-Hydroxy-7,4-dimethoxyflavone Rhamnocitrin Rhamnocitrin	*Combretum erythrophyllum*	Antibacterial and antifungal (moulds)
Helihumulone	*Helichrysum cymosum*	Antibacterial and antifungal (yeasts)
Lanosol ethyl ether	*Osmundaria serrata*	Antibacterial and Antifungal
Linoleic acid	*Helichrysum pedunculatum*	Antibacterial
2-Methyl-4-[2,4,6-trihydroxy-3-(2-methylpropanoyl) phenyl]but-2-enyl acetate	*Helichrysum caespititium*	Antibacterial and antifungal (moulds)
2-Methyl-6-(-3-methyl-2-butenyl)benzo-1,4-quinone Benzopyran,6-hydroxy-8-methyl-2,2-dimethyl-2Hbenzopyran	*Gunnera perpensa*	Antibacterial and antifungal (yeasts)

Table 1.4: Some antimicrobial bioactive compounds obtained from medicinal plants (continued).

Compound	Species	Antimicrobial activity
Muzigadial	*Warburgia salutaris*	Antibacterial
Prenyl-butryl phloroglucinol Kaurenoic acid	*Helichrysum kraussii*	Antibacterial
2,4,6-Trihydroxychalcone (pinocembrin chalcone) 5,7-Dihydroxyflavanone (pinocembrin)	*Helichrysum trilineatum*	Antibacterial
3,5,7-Trihydroxyflavone (galangin)	*Helichrysum aureonitens*	Antibacterial and antifungal (moulds)
Vernolide Vernodalol	*Veronia amygdalina*	Antibacterial and antifungal (moulds)

Approximately among one half of all deaths in tropical countries infectious disease are one of main causes of death worldwide. The increases are credited to increases in respiratory tract infections and HIV/AIDS. This test helps to measures the ability of each test sample to inhibit the in vitro fungal and bacterial growth. The following three methods are available to estimate the test result-

- Disc diffusion method
- Serial dilution method
- Bio autographic method (Devi, 2015).

1.11 Principle of Disc Diffusion Method

As a simple and well-standardized method the disk diffusion method is well known and susceptible. Therefore, in our study, disc diffusion method was used to screen the antibacterial activity. On the surface of a large agar plate bacterial inoculums are applied. Antibiotic discs and disc of test materials are placed on the inoculated agar surface. To determine the results plates are incubated for 16–24 hr at 35°C. If the test material having antimicrobial property it will inhibit the microbial growth in the media surrounding the discs

and a clear area will be found which is known as zone of inhibition. Then the zones of microbial growth inhibition are measured from the nearest millimeter around each of the antibiotic discs. The susceptibility of the isolate and to the diffusion rate of the drug through the agar medium is related with the diameter of the zone of inhibit (Barry, 1976; Devi, 2015).

1.12 The Plant *Callicarpa macrophylla*

The plant which was studied belongs to the family Verbenaceae. The Verbenaceae family is commonly known as the verbena family or vervain family. It is mainly tropical flowering plants. It contains trees, shrubs, and herbs distinguished for heads, spikes, or clusters of small flowers and most of them have an aromatic smell (Stevens, 2012). There are about 75 genera and 3000 species worldwide, mostly in tropical and subtropical regions, with only a few in the temperate regions. After narrow down the Verbenaceae family now a day it includes some 35 genera and 1,200 species (Heywood, Brummitt, Culham, & Seberg, 2007).

1.12.1 Taxonomic Hierarchy of *Callicarpa macrophylla* (Soni et al., 2014)

Kingdom: Plantae

Unranked : Angiosperms

Unranked : Eudicots

Unranked : Asterids

Order: Lamiales

Family: Verbenaceae

Genus: Callicarpa

Species: *Callicarpa macrophylla*

1.12.2 Description of the Plant

Callicarpa macrophylla is a large shrub. Leaves are 12.5-23 cm. long and ovate or ovate-lanceolate, acuminate, white-tomentose beneath (Figure 1.3). Flowers are small and rose-colored, corowded in axillary globose, peduncled cymes, 2.5-7.5 cm across. Drupes white (Ghani, 2003). *C. macrophylla* is an accepted name and it was first collected by A.F. Judd in

1928 with specimen BISH 71847 and deposited in Royal Botanical Garden, Kew. The synonyms of these plants are *Callicarpa dunniana* and *Callicarpa incana Roxb*. *C. macrophylla* is a small tree 3-5 mm high. Stem and branches densely covered with a greyish tomentum of stellate hairs (Soni et al., 2014).

Figure 1.3: Plant leaves of *C. macrophylla.*

1.12.3 Medicinal Uses

Wood paste is used to treat mouth and tongue sores. Leaves are smoked to relieve headache. Seed paste is used in stomatitis and leprosy. The leaves are warmed and applied to rheumatic joints (Ghani, 2003). The tincture and decoction is also used in the treatment of diarrhea and dysentery. The root of this plant is useful in relieving rashes on the tongue if chewed. A poultice of root is curative in fever and its juice is thought to ease indigestion. The aromatic oil of the root is used in the treatment of stomach disorders. The inner bark of the plant is used to apply on cuts and wounds. The fruit berry is edible and consumed when ripened fully (Ghani, 2003). Traditionally *C. macrophylla* has been used to treat tumor (Soni et al., 2014).

1.12.4 Chemical Constituents

Aerial parts contain 2-tetracyclic diterpenoids, calliterpenone and its mono-OAc. Leaves contain sitosterol, calliterpenone and its mono-OAc, luteolin, apigenin and its 7-glucuronides; ursolic acid, its 2-OH derivetives, crategolic acid. Seeds contain calliterpenone and its acetate (Asolkar et al*.,* 1992).

1.12.5 Common Names of the Plant

C. macrophylla is commonly known as –*Velvety Beauty Berry*". It is known by various vernacular names in different geographical regions (Soni et al., 2014). Bengali or Vernacular Name of this plantisMathara, Barmala, Dhalahuja, Aplotan, Fulujha, Jugga Harina and the tribal Name is Hozagach (Chakma) (Ghani, 2003).

1.12.6 Distribution/Habitat of the Plant

In Bangladesh, *C. macrophylla* is found in forests of Sylhet, Sal forests and Chittagong Hills Tracts (Ghani, 2003). *C. macrophylla* is globally distributed across India, China, Bhutan, Myanmar, South East Asia, and Nepal. In India it is distributed in Jammu & Kashmir, Himachal Pradesh, Uttar Pradesh, Bihar, Sikkim, West Bengal, Arunachal Pradesh, Assam, Meghalaya, Nagaland, Manipur, Mizoram, Tripura, and Andhra Pradesh, up to an altitude of 1800 meters (Rajesh, 2014).

1.13 Rationale of the Study

To minimize the side effects and to make sure the safe as well as effective use of herbal medicines extensive bioactivity screening of plants is necessary. The rational of this study was phytochemical screening and to assess the potential biological activity of the medicinal plant *C. macrophylla* (Verbenaceae family). Day by day cancer situation in Bangladesh is getting extremely alarming. There are 1300 to 1500 thousand cancer patients in Bangladesh has been diagnosed. In Bangladeshi cervical cancer is the second leading cause of deaths of women. According to the National Institute of Cancer Research and Hospital (NICRH) around thirteen thousand women die every year in Cervical Cancer in Bangladesh. A few research works so far have been done on this plant, so there was a scope to evaluate this plant for other bioactivities which have not been examined scientifically. Globally HeLa cells are used in scientific researches like cancer, AIDS, gene mapping and countless other scientific study. Literature review on the phytochemical screening of this plant showed that *C. macrophylla* contains Flavonoids, Glycosides, Tannins and Steroids which are responsible to provide cytotoxic and antibacterial activity. *C. macrophylla* has been traditionally used in the treatment of dysentery, to relieve headache, used in stomatitis and leprosy and applied to rheumatic joints (Ghani, 2003). This present study may work as a

basis to identify some unfamiliar properties and medicinal uses of this plant as there was no previous research found on cytotoxic and antimicrobial effect of this plant against *Bacillus cereus, Streptococcus pneumonia and Shigella dysenteriae*. So, this study is focused on the determination of cytotoxic and antibacterial properties of *C. macrophylla* methanol leaf extract of leave of this plant.

1.14 Aim of the Project

The aim of the study is the investigation of cytotoxic and antibacterial activity of methanol extract of *C. macrophylla* Leaves.

1.15 Objectives of the Project

After reviewing the literature pertaining to the previous findings of *C. macrophylla,* the objectives of the project was made as follows using methanol leaf extract of *C. macrophylla:*

a) Carrying out phytochemical screening in order to qualitatively determine the presence of chemical constituents.
b) Determination of its cytotoxic potential using MTT assay method.
c) Investigation of antibacterial effect by disc diffusion method.

CHAPTER TWO

LITERATURE REVIEW

Chapter 2: Literature Review

2.1 Pharmacological Activities Previously Studied

2.1.1 Antibacterial Activity

By using agar disc diffusion assay techniques *ex vivo* antibacterial activity studies on ethanolic and aqueous stem back extracts of *C. macrophylla* was done against some gram positive and gram negative strains. Results showed SEE can inhibit moderately growth of against all the bacterial strains, but except *Salmonella typhimurium* SAE was exceptionally inactive against all strains. Because of the phytoconstituents in SAE might be responsible for the inhibition of *S. typhimurium* growth (Soni et al., 2014).

2.1.2 Antidiabetic Activity

The antidiabetic activity of flower extract of this plant was performed in dexamethacin induced diabetic rats. By using dexamethasone at a dose of 5 mg/kg, rats were treated and it was continued 10 days for developing insulin resistance. The blood glucose level, body weight and lipid profile was estimated. The blood glucose level decreases gradually in the animals treated with *C. macrophylla* flower extract (100 mg/kg b.w. and 200 mg/kg b.w., orally) and Glibenclamide (1 mg/kg) was used to compare the antidiabetic effect. Concluded with the results that *C. macrophylla* shows antidiabetic activity in dexamethasone model (Soni et al., 2014).

2.1.3 Analgesic and Antipyretic Activity

Respectively by using Tail Immersion Model and Brewer's Yeast Induced Pyrexia Model analgesic as well as anti-pyretic effect was evaluated by aqueous and ethanolic extracts of *C. macrophylla* leaves. After comparing with the standard drugs result showed that aqueous extract of leaves induced better analgesia and have anti-pyretic potential than ethanolic extract. In case of infection induced fever combination of analgesia as well as anti-pyretic effect will ascertain its significant role**.** Tail immersion test in albino rats, respectively was done by aqueous & ethanolic extracts of roots at two concentrations of 200 & 400 mg/kg to evaluate its analgesic potentials. Ethanolic extract have less analgesic activity than the aqueous extract of roots (Soni et al., 2014).

2.1.4 Antifungal Activity

The antifungal activity of ethanolic and aqueous extracts of the stems of *C. macrophylla* Vahl were investigated by Agar disc diffusion method. It was performed against seven fungal strains. In the treatment of fungal infection the overall results provide hopeful baseline information for the potential use of the crude antifungal extracts from *C. macrophylla* (Soni et al., 2014).

2.1.5 Anti-inflammatory Activity

Anti-inflammatory activity of aqueous and ethanolic extracts of leaves of *C. macrophylla* wase valuated using Carrageenan paw edema method where diclofenac sodium was considered as standard drug. Results showed that ethanolic extract of *C. macrophylla* leaves have better anti-inflammatory profile than the aqueous extract and can be the choice to be used as anti-inflammatory drug and ethanolic root extract have superior anti-inflammatory spectrum than aqueous one. Results are highly promising and establish that roots of *C. macrophylla* have anti-inflammatory potential, comparable to that of standards (Soni et al., 2014).

2.1.6 Anti-arthritic Activity

By protein denaturation model and human red blood cell membrane stabilization model *in-vitro* anti-arthritic activity of ethanolic extract of *C. macrophylla* flower were evaluated. As a standard drug diclofenac sodium was used. Results showed that compared to standard drug used as diclofenac sodium the ethanolic extract of *C. macrophylla* at different concentrations possessed significant anti-arthritic activity. So, it indicates that ethanolic extract of *C. macrophylla* flower have anti-arthritic activity (Soni et al., 2014).

2.2 Previously Isolated Photochemical Compounds of *C. macrophylla*

C. macrophylla leaves contains *α*-amyrenol, α-amyrin, ursolic acid, 2*α*, 3*α*, 19*α*-trihydroxy -12-dien-28- ursolic acid, betulinic acid, *β*-sitosterol, daucosterol, flavanoids such as luteolin, apigenin, luteolin-7-O-glucuronide, apigenin-7-O-glucuronide, *β*-sitosterol-*β*-*D*-glucoside, 2*α*-hydroxyursolic acid, crategolic acid, docosanoic acid, tricosanoic acid, tetracosanoic acid, ethyl tricosanoate, 3,7,3′-trimethoxy-4′, 5-dihydroxyflavone, The bark contain

betulinic acid. The roots and aerial part contains essential oil, (diterpene) calliterpenone, calliterpenone monoacetate & Seeds contains calliterpenone, calliterpenone-17-acetate, oleanolic acid. Several compounds were isolated from diterpenoid- 16α, 17-Isopropylideno-3-oxo-phyllocladane, 3β, 16α, 17-trihydroxyPhyllocladane, 16, 17-dihydroxy kauranoids, Terpenoids, fatty acids & other constituents (Soni et al., 2014). Some compounds are isolated previously from this plant listed in the Table 2.1.

Table 2.1: Some compounds isolated previously from *C. macrophylla* (Soni et al., 2014).

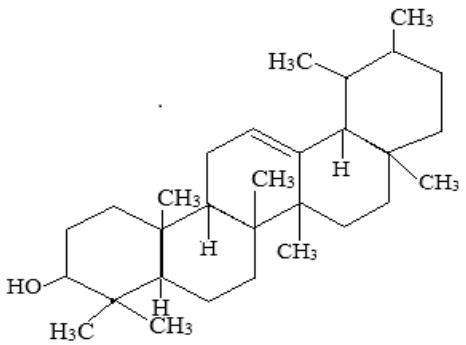
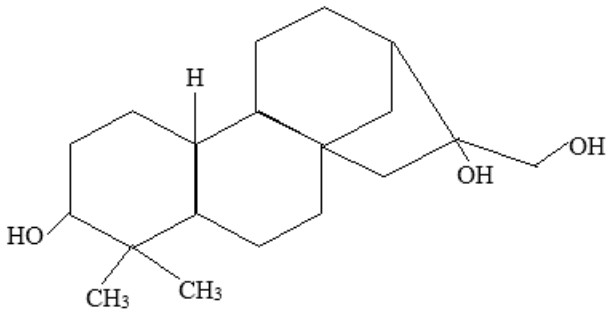
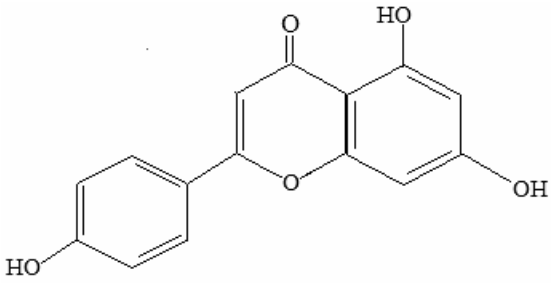
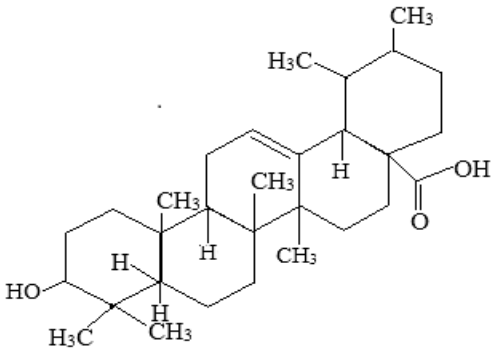
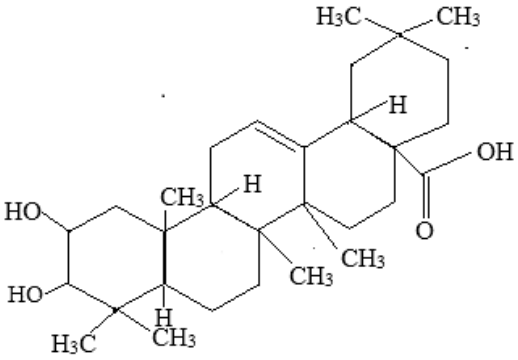
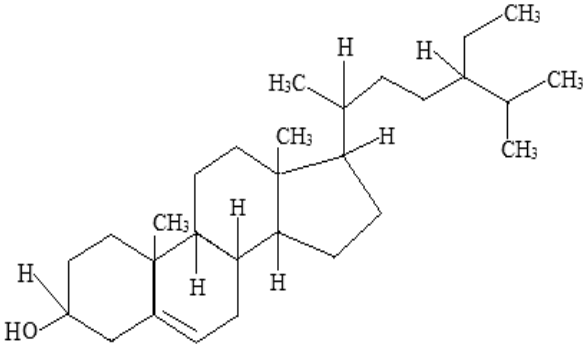
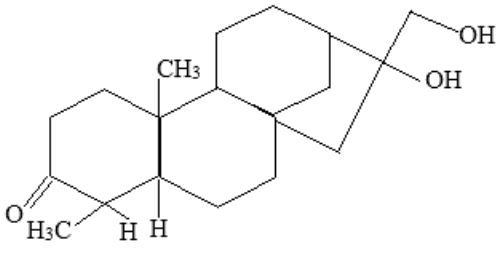
Compounds	**Isolated from part**	**Structures**
α-amyrenol	Leaves	
3β, 16α, 17-trihydroxy Phyllocladane	Leaves	
Apigenin	Leaves	

Table 2.1: Some compounds isolated previously from *C. macrophylla* (continued).

Compounds	Isolated from part	Structures
Ursolic acid	Leaves	
Crategolic acid	Leaves	
Daucosterol	Leaves	
Calliterpenone	Seeds & Aerial	

CHAPTER THREE

METHODOLOGY

Chapter 3: Methodology

3.1 Collection and Authentication of Plant Material

Callicarpa macrophylla was as the plant for this study since no previous investigation was found on its cytotoxic bioactivity and antibacterial study. After reviewing the literature about this plant and comparing its availability it was decided to be chosen *C. macrophylla* plant leaves which are of Verbenaceae family for our investigation. In February 2016, the whole plant was collected from Sylhet, Bangladesh and it was submitted to the National Herbarium of Bangladesh, Mirpur and Dhaka for its authentication. Ten days later its voucher specimen (ACCESSION NO.: DACB-43486) was collected and the plant was identified and confirmed by the taxonomist of National Herbarium of Bangladesh, Mirpur, Dhaka.

3.2 Extraction Procedure

There were few steps involved in the extraction process of this plant. The whole extraction process can be divided into two parts:

a) Plant material preparation and drying
b) Extraction process (chloroform and methanol extract)

The overall procedure can be show in a flowchart Figure 3.1 given below:

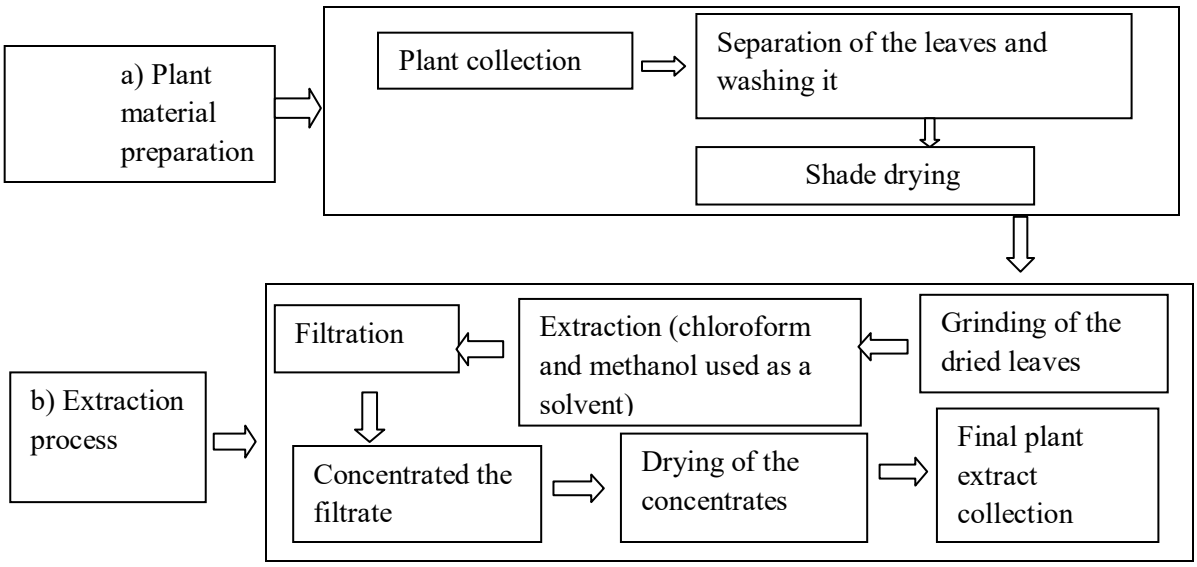


Figure 3.1: The overall extraction procedure.

3.2.1 Plant Material Preparation and Drying

After collecting the plant and authenticated by National Herbarium of Bangladesh (NHB) the leaves were plucked off from the plant stem and washed thoroughly with clean water for removing plant wreckage and other dust particles. Then this clean wet leaves were shade dried for several days. Science sunlight can damage the chemical compounds containing the leaves which are heat and light sensitive. The dried leaves were ready to go for the next step.

3.2.2 Extraction Process

3.2.2.1 Size Reduction

After drying the leaves properly, the crispy fresh leaves were grounded to make powder by using a high capacity grinding machine. The weight of the powdered plant leaves were taken and kept record properly. Air tight plastic container was used to package the powder of leaves which was properly labeled with necessary information. Then the container was kept in a cool, dry and dark place for future investigation. To avoid cross-contamination and to remove undesirable things during grinding and packaging all the materials were properly cleaned and necessary measures were taken carefully.

3.2.2.2 Extraction

To perform of this study, total powdered plant leaves were taken in equal amount in two beakers and as a solvent methanol and chloroform were used. Each beaker contained 122 gm of leaves powder. In one beaker 122 gm powdered plant material of *C. macrophylla* was soaked in 1 litter of methanol and in another beaker 122 gm powdered plant material of this plant was soaked in 1 litter of chloroform. At normal room temperature (22-25°C) both beakers were kept for 3 days in dark place with occasional agitation.

3.2.2.3 Filtration

By using Whatman filter (pore size: 110 mm), after three days of maceration, methanol and chloroform extract were filtrated (Figure 3.2). Before filtration the content of the beaker were decanted.

Figure 3.2: Filtration of the plant extract.

3.2.2.4 Concentration

Rotary evaporator (Heidolph) was used to concentrate the collected filtrate at 100 rpm at 37°C and the process was continuing until the concentrated mathanolic and chloroform extract were produced (Figure 3.3). After that the concentrated extract were transferred on separate Petri-dishes for drying under Laminar Air Flow (LAF).

Figure 3.3: Concentrating the filtrate of *C. macrophylla* plant using rotary evaporator.

3.2.2.5 Drying

At the last stage, for evaporating the solvent from the extract the Petri-dishes were placed under LAF until the dry or semi-solid extract was found. After drying of the extract,

aluminum foil was used to cover the Petri-dishes properly and refrigerated for further use with proper labeling.

3.3 Phytochemical screening

Phytochemical screening was performed on the crude extracts of *C. macrophylla* in order to access its qualitative chemical compositions namely, alkaloids, carbohydrates, tannin, flavonoids, steroids, triterpenes, glycoside etc (Figure 3.4).

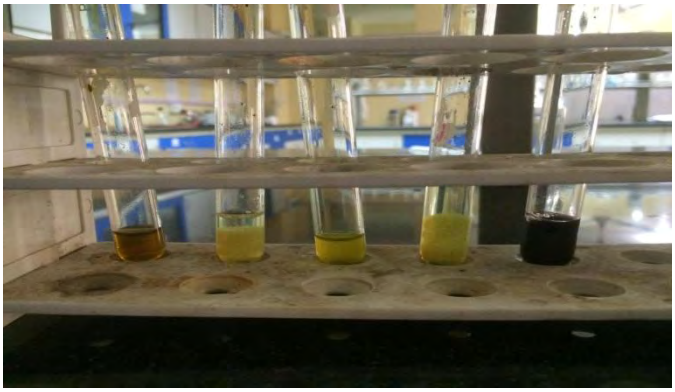


Figure 3.4: Various phytochemical tests performed on the methanolic extract of *C. macrophylla.*

The following qualitative tests were performed:

3.3.1 Detection of Alkaloid

For the qualitative determination of alkaloids, three tests were performed. 0.5 gm of methanolic extract of *C. macrophylla* was dissolved in 5 mL of 1% Hydrochloric acid, boiled in a water bath followed by filtration. Using the filtrate obtained the following tests were performed:

3.3.1.1 Hager's Test

To 2 mL of the filtrate, a few drops of Hager's reagent (1% picric acid solution) were added and the presence of alkaloids was confirmed by the formation of yellow precipitate (Waldi, 1965).

3.3.1.2 Mayer's Test

10 mL Mayer's Reagent is prepared by dissolving 0.1358 gm of Mercuric (II) Chloride and 0.5 gm of Potassium Iodide in 10 mL distilled water.

Then, to a 2 mL of the filtrate, a few drops of Mayer's reagent were added along the sides of the test tube. The formation of a white or creamy precipitate indicates the presence of alkaloids (Evan, 1997).

3.3.1.3 Wagner's Test

10 mL Wagner's Reagent is prepared by dissolving 0.2 gm of Iodine crystals and 0.6 gm of Potassium Iodide in 10 mL distilled water.

To a 2 mL of the filtrate, a few drops of Wagner's reagent were added. Formation of a brownish-black precipitate confirms the presence of alkaloids in the sample (Wagner, 1993).

3.3.2 Detection of Flavonoids

3.3.2.1 Lead Acetate Test

The methanolic extract was treated with a few drops of lead acetate solution and the formation of yellow colored precipitate signifies the presence of flavonoids.

3.3.2.2 Zinc Ribbon Test

The presence of flavonoids can be confirmed by another method. To a test tube containing 0.5 mL of alcoholic extract, 5-10 drops of concentrated Hydrochloric acid and a small piece of Zinc was added. The solution was then boiled for a few minutes and then left to stand. The formation of a red to crimson color solution indicates the presence of flavonoids (Sindhu, Uma and Manorama, 2013).

3.3.3 Detection of Saponins

3.3.3.1 Forthing Test

To run this test, a very first small amount (0.1 gm) of powdered plant material was heated to boil for 55 minutes with 10 mL of distilled water. Then the solution was cooled and filtered.

The filtration was diluted with 5 mL of distilled water with vigorous shaking and was left to stand for 10 minutes. A dense persistent forth will indicate the presence of saponins.

3.3.4 Detection of Phytosterols

Libermann Burchard's Test

To a small amount of extract, 1 mL of chloroform was added and filtered. The filtrate was then treated with a 2 mL of acetic anhydride, boiled and cooled. Finally, 1 mL of concentrated sulfuric acid was added to the solution. Formation of a brown ring at the junction indicates the presence of phytosterols (Soni & Sosa, 2013).

3.3.5 Detection of Steroids

Salkowski Test

To 1 mL of extract, 2 mL of chloroform, 1 mL of sulfuric acid were added. The appearance of red color indicates the presence of steroids (Ghani, 2003).

3.3.6 Detection of Tannins

3.3.6.1 Lead acetate test

To 1 mL of the extract, a few drops of 1% Lead acetate solution were added and the formation of a yellow-colored precipitate indicates the presence of tannins (Tiwari and Bimlesh, 2011).

3.3.6.2 Ferric Chloride Test

A 5% Ferric chloride solution is prepared by dissolving 0.5 gm of ferric chloride in 10 mL distilled water. 5 mL aqueous solution of crude extract was dissolved in 1 mL of 5% ferric chloride solution and the formation of greenish black precipitation indicates the presence of tannin (Ghani, 2003).

3.3.7 Detection of Glycosides

General test for Glycosides

At first 1 ml distilled water was taken in a test tube, a very small amount (0.5 gm) of methanolic extract was added to the test tube and dissolved. After that previously prepared

5% sodium hydroxide solution was added to the test tube solution. Glycoside compound will show yellow coloration.

3.3.8 Detection of Phenols/Phenolic compounds

Ferric Chloride Test

This test is performed by measuring 2 mL of extract in a test tube followed by adding 3-4 drops of 15% (w/v) Ferric chloride solution. The formation of a bluish-black precipitate signifies the presence of phenols (Soni & Sosa, 2013).

3.4 *In-vitro* Cytotoxic Activity

There are various *in-vitro* methods are available to determine the cytotoxic effect of leaves extract. In our study cytotoxic effect of *C. macrophylla* methanolic extract was used and evaluated on HeLa cell line at different concentrations of the plant extracts using MTT assay and cell viability was quantified.

3.4.1 Preparation of Sample from Plant Extracts

For screening the cytotoxic activity of this plant extract methanolic extract was used at four different concentrations (2.5, 0.25, 0.025 and 0.0025 mg/mL). Two percent (2%) DMSO was used as a solvent. 2.5 mg/mL concentrated sample was prepared by adding 25 mg leaves extract in 1 mL 2% DMSO solvent as 1st concentration which was the stock solution. Second concentration was made by adding 10 µL from stock solution and 90 µL 2% DMSO solutions, 3rd concentration was made by adding 10 µL from 2nd concentration and 90 µL 2% DMSO. Finally the 4th concentration was prepared by mixing 10 µL from 3rd concentration solution and 90 µL 2% DMSO.

3.4.2 Instruments Used in This Study

Biological Bio Safety Cabinet (Model: NU-400E, Nuaire, USA), CO_2 Incubator (Nuaire, USA), Trinocular microscope with camera (Olympus, Japan), Hemocytometer.

3.4.3 Materials Used

96-well plates, 15 mL tubes, Tips, Gloves, Culture flask, Cell culture media, Antibiotics, Gentamycin, Serological pipette, Trypsin etc.

3.4.4 Procedure

Cytotoxic activity was examined in Centre for Advanced Research in Sciences (CARS) using their commercial services. In brief, HeLa, a human cervical carcinoma cell line was maintained in DMEM (Dulbecco's Modified Eagles' medium) containing 1% penicillin-streptomycin (1:1) and 0.2% gentamycin and 10% fetal bovine Serum (FBS). Cells ($1x10^4$/90 µl) were seeded into 96-well plates and incubated at 37°C+5% CO_2. After 24 hr, 10 µL of sample of given concentrations was added into each well. Cytotoxicity was examined after 48 hr of incubation using Cell Titer 96 Non-Radioactive Cell Proliferation Assay kit (Promega, USA). Samples were filtered through 0.45 µm syringe filter prior to examination. Duplicate wells were used for each sample. Two percent (2%) DMSO was used as a negative control. The cytotoxic activity was calculated with the measured absorbance values using the following equation:

% of cytotoxic activity =100-(Absorbance of test sample/Absorbance of negative control) *100

3.5 Antibacterial Activity by Disc Diffusion Method

3.5.1 Apparatus & Reagents

- Filter paper discs
- Screw cap test tubes
- Petri dishes
- Nose mask and Hand gloves
- Inoculating loop
- Laminar air flow
- Sterile cotton
- Autoclave
- Sterile forceps
- Incubator
- Spirit burner
- Ethanol
- Micropipette
- Nutrient Agar Medium

3.5.2 Test Sample of *C. macrophylla*

Methanol extract of *C. macrophylla* leaves were taken as test sample because from the literature reviewed we found methanol extract showed better results.

3.5.3 Test Organisms

For this experiment pure bacterial strain cultures (Table 3.1) was used and those were collected from BRAC University Microbiology laboratory.

Table 3.1: List of micro-organisms used in this study.

Type of Bacteria	**Bacterial Strain**
Gram positive	*Streptococcus pneumonia*
	Bacillus cereus
Gram negative	*Shigella dysenteriae*

3.5.4 Procedure

3.5.4.1 Preparation of the Medium

4.25 gm nutrient agar medium was taken in a bottle with a cap and dissolved in 100 mL distilled water for preparing required volume of medium. For sterilization the contents were autoclaved for 40 to 45 minutes.

3.5.4.2 Sterilization Procedure

The antibacterial screening was done in laminar air flow hood and all types of precautions were highly maintained for avoiding any type of contamination and cross contamination by the test organisms. At 121°C temperature Petri dishes and other glassware were sterilized by autoclaving for 40 minutes. Micropipette tips, cotton, forceps, blank discs etc. were also sterilized in the same way.

3.5.4.3 Preparation of the Test Plate

Three petri dishes were used where each of the Petri dishes was containing about 10 mL of melted and sterilized agar medium. By using vortex machine, 3 separate bacterial suspensions were mixed with normal saline and bacterial suspensions were taken by loop. Then sterilized cotton bud was taken and dipped into the bacterial suspension. With the help of this cotton bud 3 separate bacterial sample were applied to 3 Petri dishes. At the same concentration duplet test were done by the same procedure.

3.5.4.4 Preparation of Discs

Three types of discs were used for 3 separate antibacterial screening.

Standard Discs: In this investigation, kanamycin disc was used as the reference. Kanamycin was used as a positive control to ensure the activity of standard antibiotic against test organism. These were also used to compare the effect produced by known antibacterial agent and that of test sample.

Blank Discs: Methanol was used as negative controls. These were used for ensuring that the residual solvent (left over the discs even after air-drying) and the filter paper were not active themselves.

Sample Discs: These discs were soaked with solutions of test samples of known concentration, dried and used to determine the antibacterial activity of the samples.

3.5.4.5 Preparation of Test Sample

Different concentrations of the test sample solution (350 µg/disc, 250 µg/disc and 150 µg/disc) were prepared for each 3 antibacterial screening. Sterilized metrical filter paper dishes were taken in a blank Petridis under the laminar hood. Then discs were soaked with solutions of test samples and dried.

3.5.4.6 Application of Test Samples

Test sample were placed in each bacterial suspension containing Petri dishes. One kanamycin containing disc, one disc was contained methanol solvent as a blank disc and another one contained test sample (Figure 3.4).

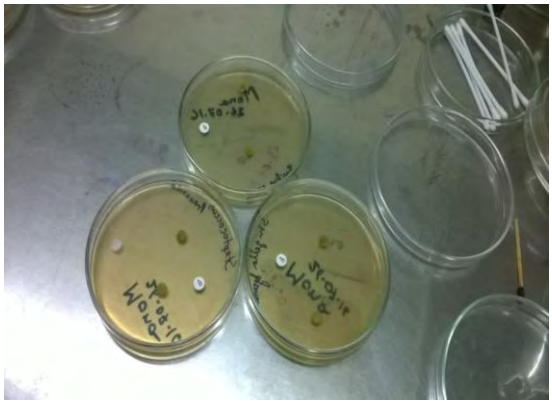


Figure 3.4: Petridis containing sterile agar media and test sample discs.

3.5.4.7 Diffusion & Incubation

The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test bacteria. The plates were then kept in a refrigerator for about 5 hr upside down to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at 37°C for 24 hr.

3.5.4.8 Determination of Antibacterial Activity by Measuring the Zone of Inhibition

The antibacterial potency of the test agents are measured by their activity to prevent the growth of the bacterial strains surrounding the discs which gives clear zone of inhibition (Fugure 3.5). After incubation, the antimicrobial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale.

Figure 3.5: Determination of clear zone of inhibition.

CHAPTER FOUR

RESULT AND DISCUSSION

Chapter 4: Results and discussion

4.1 Result of Phytochemical Screening of *C. macrophylla*

The phytochemical screening was performed using different test like Hager's test, Mayer's test, Wagner's test, Lead acetate test, Zinc ribbon test, Salkowski test , Ferric Chloride test etc. and the results are summarized in the Table 4.1.

Table 4.1: Phytochemical screening of *C.macrophylla.*

Class of compound	Result
Alkaloids	---
Flavonoids	++
Phenols/Phenolic compounds	-
Glycosides	+
Tannins	++
Phytosterol	+
Resins	+
Steroids	+
Saponin	+
(+) means presence in a single method test, (++) means presence experimented in two methods, (+++) means presence experimented in three methods, (-) means absence experimented in a single method, and (---) means absence experimented in three methods.	

Interpretation: The phytochemical screening of *C. macrophylla* showed the presence of flavonoids, glycosides, tannins, phytosterol, resins, saponin and steroids whilst showing the absence of alkaloids and phenolic compounds (Table 4.1).

4.1.2 Discussion of Phytochemical Screening of *C. macrophylla*

Previous evaluation of the phytochemical constituents present in *C. macrophylla* reported that the methanol extract of this plant did not possess alkaloids and phenolic compounds but

this plant posses glycoside, tannins, resins, flavonoids, saponins (Gupta, 2013). In our study after phytochemical screening we found the presence of same classes of compounds that were previously investigated. glycosides, tannins have the ability to inhibit abnormal growth of cancer cell and flavonoids, steroids helps to inhibit bacterial infection (Soni et al., 2014).

Qualitative determination evident the presence of flavonoids and polyphenolic compounds of dried extract of *C. macrophylla*, the extract was then subjected to various *in-vitro* antioxidant assays.

4.2 Result of Cytotoxic Activity Test

To evaluate the cytotoxic effect of methanolic extract of *C. macrophylla* a MTT assay with HeLacell line was performed at different concentrations of 0.0025 mg/mL, 0.025 mg/mL, 0.25 mg/mL and 2.5 mg/mL. IC_{50} (50% growth inhibition) value was determined which was 1.38 mg/mL. With the increase of concentration from 0.25 mg/mL to 2.5 mg/mL of leave extract, % of cell growth inhibition of HeLa cells increased from 7% to 92% (Figure 4.2 and Table 4.1). Thus the cytotoxic effect was concentration dependent.However, at 0.0025 mg/mL and 0.025 mg/mL concentration no cell growth inhibitions were found. As a negative control 2% DMSO was used and 100% cell survival result was found that means 2% DMSO have no cytotoxic effect. The IC_{50} (50% cell growth inhibition) for HeLa cell line was found 1.38 mg/mL for methanolic leave extract of *C. macrophylla.*

4.2.1 Percentage of Survival Cells and the Inhibition of Cell growth

Table 4.2 summarized the cytotoxic activity result of methanolic extract of *C. macrophylla* leaves-

Table 4.2: Cytotoxic activity (% of cell growth inhibition) of *C. macrophylla* methanol leaf extract.

Sample concentration (mg/mL)	**Absorbance at 570 nm**	**%Survival of cells HeLa cells**	**% of HeLa cell inhibition**	**Standard deviation (±)**	**IC_{50} (mg/mL)**
0.0025				0	1.38
0.025				0	
0.25	2.56	93%	7	0.15	
2.5	0.226	8%	92	0.53	
Negative Control (2% DMSO)	2.74	100	0	0	

*IC_{50}= The half maximal inhibitory concentration

4.2.2 Graphical Representation of Cytotoxic Activity.

Figure 4.1 was found after plotting the concentration of sample against % of cells growth inhibition. Concentration was plotted in X axis and percentages of cells inhibition was plotted in Y axis.

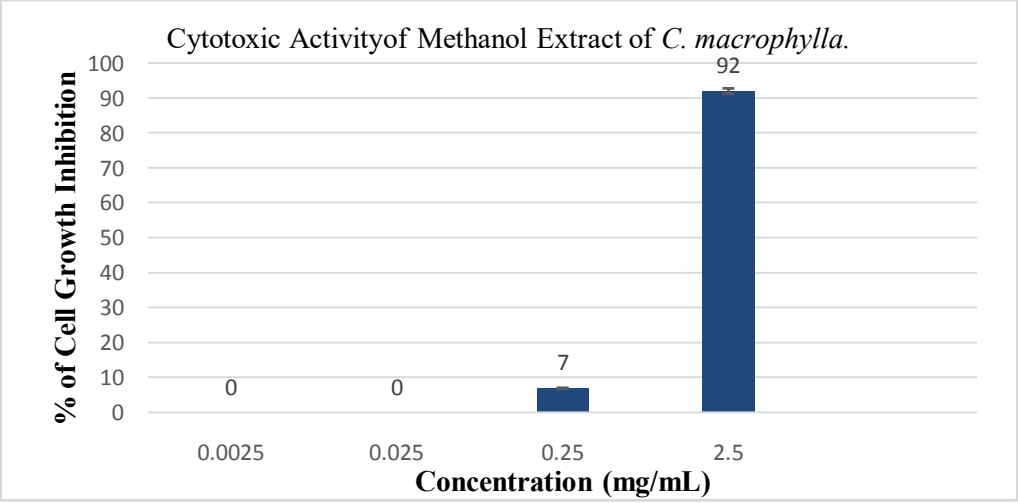


Figure 4.1: Cytotoxic activity of *C. macrophylla* leave extracts.

4.2.3 Discussion

Traditionally used many medicinal plants have anticancer property. Plant has some chemicals of different classes such as flavonoids, tannins, glycosides which have the ability to inhibit abnormal growth of cancer cells (Soni et al., 2014). The mode of action of the drug is unknown but proper utilizing the knowledge of medicinal plant and investigation of new compounds can give good anticancer drug from natural source of plants. Methanol extract of *C. macrophylla* showed higher cytotoxic effect at the higher concentration thoughat lower concentration showed no cytotoxic effect.

Previous study on the cytotoxic activity of another species named *C. nudiflora* was performed using chronic myelogenous leukaemia K562 cell line. The study was conducted with ethanol extract of *C. nudiflora* leaves using MTT assay and 50% - 70% ethanol extract of *C. nudiflora* leaves showed moderate cytotoxic activity (IC_{50} value was 36.0 µg/mL) (Ma Y et al., 2014). In our study, at the lower concentrations of 0.0025 mg/mL and 0.025 mg/mL concentration 100% cell was survived whereas with the increasing of concentration % of survival cells were decreased. At the concentration of 0.25 mg/mL 93% cells were survived and at 2.5 mg/mL only 8% cells were survived that means 92% cell growth was inhibited at the concentration of 2.5 mg/mL with the methanol extract. The cytotoxic effect of the methanolic leave extracts of *C. macrophylla* shows in Figure 4.2. IC_{50} value 36.0 µg/mL was found for another species named *C. nudiflora* ethanol extract and in our study IC_{50} value is 1.38 mg/mL for methanolic extract of *C. macrophylla*.

The highest cell growth was found at 2.5 mg/mL concentration and the IC_{50} value was 1.38 mg/mL. This potent (92% cell growth inhibition) cytotoxic activity establishes the scientific basis for the use of cancer treatment. Furthermore, phytochemical screening of this extract revealed the presence of flavonoids, tannins, glycosides and steroids. Previous study showed that anticancer activity of plant extract is attributed to different phytochemicals such as flavonoids and glycosides (Soni et al., 2014). So, this potent cytotoxic activity of *C. macrophylla* may be due to possessing of these compound classes.

Figure 4.2 shows the cytotoxic effect of the methanolic leave extracts of *C. macrophylla*.

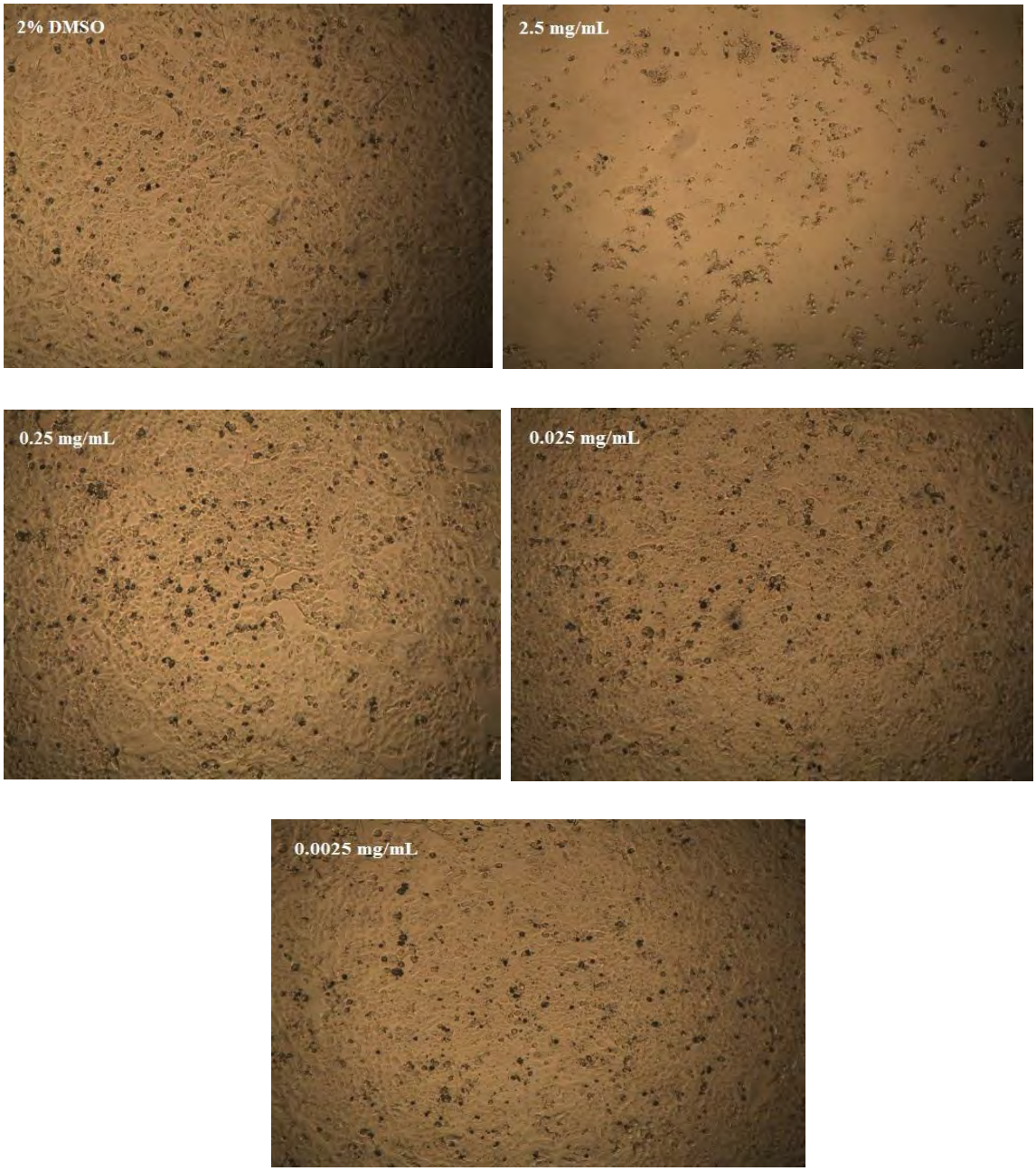


Figure 4.2: Percentage of cell growth inhibition by methanolic extract of *C. macrophylla* at different concentrations after 48 hr incubation.

4.3 Results of Antibacterial Activity Test

The antibacterial activity of methanol extract of *C. macrophylla* leaves were examined in this study against Gram positive bacteria *Bacillus cereus* and *Streptococcus pneumonia* and Gram negative bacteria *Shigella dysenteriae.* Zone of inhibition was measured after the

methanol extract was subjected to the selected bacterial strains kanamycin was used as a standard reference drug.

4.3.1 Zone of Inhibition of Methanol Extract of *C. macrophylla*

Antibacterial activity of methanolic extract of *C. macrophylla* leaves is shown in the Table 4.3.

Table 4.3: Percentages of zone of inhibition by methanol extract of *C. macrophylla* leaves.

Bacteria		**Zone of inhibition(mm)**			
		Concentrations (µg/disc)	Standard sample	Standard deviation (±)	Methanol extract
Gram positive bacteria	*Bacillus cereus*	350 µg/disc	31 mm	1	22 mm
		250 µg/disc	31 mm	1.258306	14 mm
		150 µg/disc	31 mm	0.763763	6 mm
	Streptococcus pneumonia	350 µg/disc	20 mm	No significant result found	
		250 µg/disc	20 mm		
		150 µg/disc	20 mm		
Gram negative bacteria	*Shigella dysenteriae*	350 µg/disc	21 mm	No significant result found	
		250 µg/disc	21 mm		
		150 µg/disc	21 mm		

Figure 4.3 shows antibacterial activity against *Streptococcus pneumonia, Shigella dysenteriae and Bacillus cereus* and Figure 4.4 shows antibacterial activity against *Bacillus cereus* at three different concentrations of 350 µg/disc, 250 µg/disc and 150 µg/disc.

Figure 4.3: Antibacterial activity against *Streptococcus pneumonia*, *Shigella dysenteriae and Bacillus cereus*.

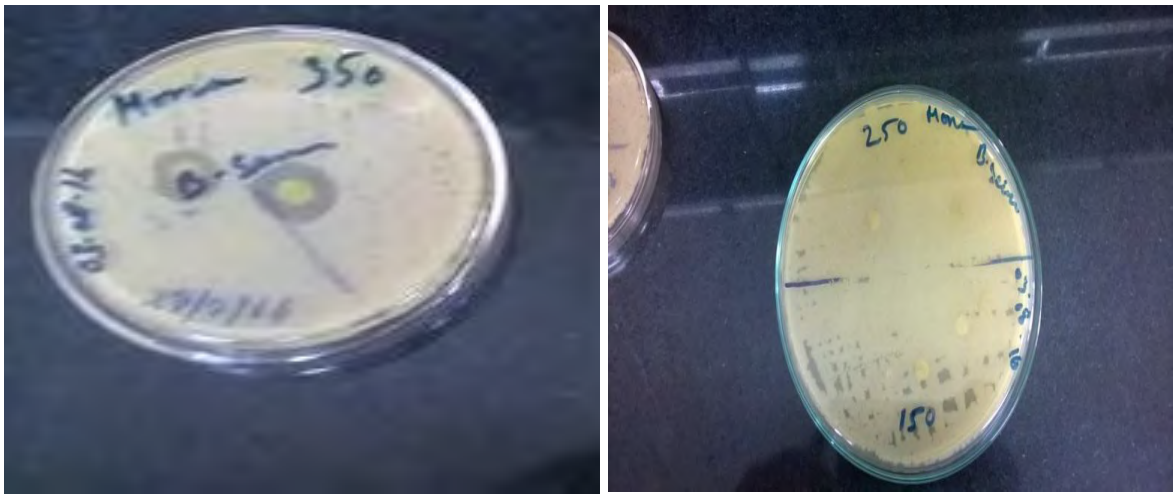


Figure 4.4: Antibacterial activity against *Bacillus cereus* at three different concentrations of 350 µg/disc, 250 µg/disc and 150 µg/disc.

4.3.2 Graphical Representation of Antibacterial Activity Test

Figure 4.5 was found after plotting the data of antibacterial activity against *Bacillus cereus.* The concentration of sample was plotted against zone of inhibition. Concentration was plotted at X axis and zone of inhibition was plotted at Y axis.

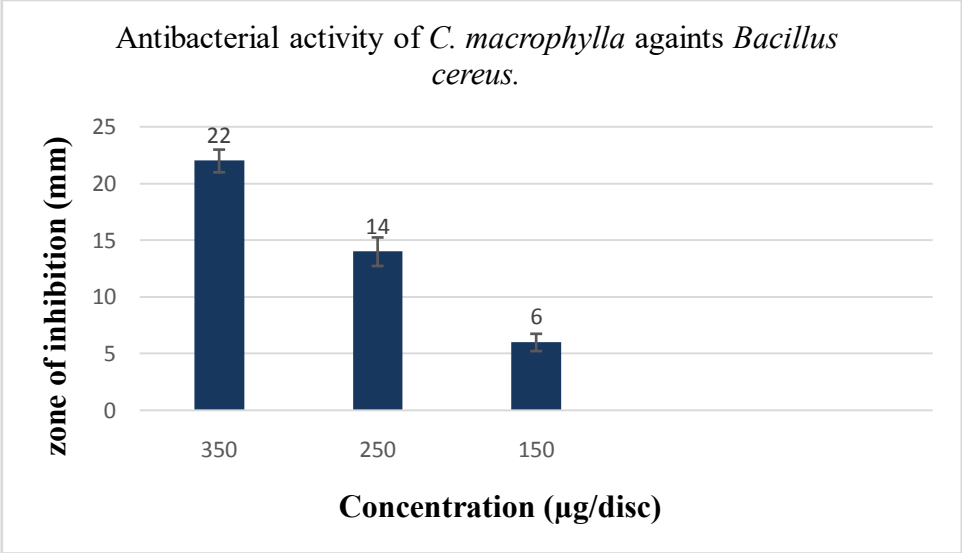


Figure 4.5: Graphical representation of antimicrobial test against *Bacillus cereus.*

4.3.3 Discussion

The result of the antibacterial screening of methanol extract of *C. macrophylla* against 3 different bacterial strains is summarized in the Table 4.3. Finding showed that in case of *Bacillus cereus* methanol extract of *C. macrophylla* exhibited moderate to low antibacterial activity when compared to kanamycin (a standard antibacterial drug) at different concentrations. At the concentration of 350 µg/disc showed higher antibacterial activity (zone of inhibition 22 mm) comparing to 250 µg/disc (14 mm) and 150 µg/disc (6 mm). Though zone of inhibition of any concentration of plant extracts were not equal to the zone of inhibition of kanamycin (zone of inhibition 31 mm) against *Bacillus cereus* but was comparable to the standard. So, this result inferred that the extract has activity against *Bacillus cereus* bacteria in particular. On the other hand, no significant zone of inhibition was found against *Streptococcus pneumonia* and *Shigella dysenteriae* bacterial strain at the tested concentrations of methanol extract of plant leaves. So, *C. macrophylla* did not show any antibacterial activity against *Streptococcus pneumonia* and *Shigella dysenteria.* Among

all the bacterial strains, against *Bacillus cereus* methanol extracts of *C. macrophylla* showed the highest antibacterial activity comparable to the standard drug kanamycin at 350 µg/disc concentration. The antibacterial activity of this plant is concentration dependent; with the increase of concentration it showed increased antibacterial effect.

From the previous time, as antibacterial drug plant extract of *C. macrophylla* was used. From the literature review revealed that aqueous extract of this plant had antibacterial activity against *S. typhimurium* and zone of inhibition was found 17 mm at 200 µg/disc concentration (Soni et al., 2014). In this study concentration dependent antibacterial activity was found against *Bacillus cereus* and 22 mm zone of inhibition was found at 350 µg/disc. Plant extract containing flavonoids and steroids show antibacterial activity (Soni et al., 2014). In phytochemical screening of *C. macrophylla* reviewed the presence of those compound classes in our study. Therefore, these classes of compounds may be responsible for the exhibited antibacterial activity of *C. macrophylla* against *B. cereus*. Thus, the leaves extract of *C. macrophylla* shows antibacterial study to specific microorganism.

CHAPTER FIVE

CONCLUSION

Chapter 5: Conclusion

Over use of any medicine is dangerous for life. Proper use of medicine is safe for life. Studying of medicinal plant and their chemical constituents helps to isolate important chemical compound for human and animal by which less toxic and more effective drug can be produced. Researcher's explore building block of plants which helps to identify compounds belongs to different classes that can be used as life saving drugs. So, plant chemical compound plays an important role to new drug or new molecule investigation. The present study was focused on the evaluation of cytotoxic and antibacterial effect of methanol extract of *C. macrophylla* leaves since these activities were not studied before. Moreover this plant has been used to treat cancer. Cytotoxic and antibacterial activity test evident that with the highest concentration this extract showed the highest cytotoxic and antibacterial activity. The highest (92%) cells growth inhibition occurred at the 2.5 mg/mL concentration and maximum zone of inhibition (22 mm) was found against *Bacillus cereus* bacterial strain at the highest concentration of 350 µg/disc. To conclude, this present study finding demonstrate that the methanolic extract of *C. macrophylla* leaves have potent cytotoxic but moderate antibacterial activity.

Traditionally various parts of *C. macrophylla* has been used as a medicine to treat various types of disease such as cancer, polydipsia, diarrhea, diabetes, dysentery, fever, as blood purifier, anti-pyretic, analgesic, anti-ulcer, gastric stimulants etc. Previous scientific research on this plant has shown the presences of various important classes of chemical compounds are present in different parts of the plant. Over the years, the plant has been used traditionally without any documented serious side effects. As the extract showed potent cytotoxic and moderate antibacterial activity, this extract has proved it scientific basis of being used as anticancer and anti-infective agent traditionally through our study finding. This cytotoxic and antibacterial potential of the extract could be related to its phytochemical constituents since the phytochemical screening of *C. macrophylla* showed the presence of flavonoids, glycosides, tannins, resins, saponin and steroids. So, further investigations are warranted to isolate and identify the active compounds present in the plant extract and their efficacy need to be tested. It will help in the development of novel and safe drugs for the treatment of various diseases like cancer and infection.

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