Evaluation of antibacterial, antioxidant and toxicological activities of crude extracts of *Calotropis gigantean, Mimosa pudica, Achyranthes aspera* and *Hibiscus rosa-siensis* variety: *Kalyani* from Faridpur, Bangladesh

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APPROVAL CERTIFICATE

This thesis entitled **"Evaluation of antibacterial, antioxidant and toxicological activities of crude extracts of *Calotropis gigantean, Mimosa pudica, Achyranthes aspera* and *Hibiscus rosa-siensis* variety: *Kalyani* from Faridpur, Bangladesh"** is submitted by Nayeema Nushrat in partial fulfillment of the requirement for the degree of Master of Science in Biotechnology, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. This work was performed at Laboratories of the MNS department, BRAC University, and department of Microbiology, University of Dhaka and the department of pharmacy, University of Asia Pacific, Dhaka, Bangladesh.

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DEDICATION

I dedicate this dissertation to my dear parents, for encouraging my curiosity and teaching me that the key to success is perseverance and hard work.

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ABBREVIATIONS

A_{ctr}	absorbance of control
A_{sample}	absorbance of sample
A_{std}	absorbance of standard
A_{test}	absorbance of test sample
°C	degree Celsius
DPPH	1,1-diphenyl-2-picrylhydrazyl
DMSO	Di-methyl-sulphoxide
l	litre
mg	milligram
ml	milliliter
mM	milimolar
MHA	Muller Hinton Agar
MHB	Muller Hinton Broth
M.W.	molecular weight
UV	ultraviolet
MeOH	methanol
µg	microgram
µl	microliter
λ	wavelength

ABSTRACT

Recent years have witnessed a revival of the drug discovery of drugs from medicinal plants for the maintenance of health in all parts of the world. The aim of this work was to investigate 4 plants belonging to 4 families (*Calotropis gigantean, Mimosa pudica, Achyranthes aspera* and *Hibiscus rosa-siensis* variety:*Kalyani,* which were collected from different places in Bangladesh (Faridpur) for their *in vitro* antibacterial, antioxidant and toxicological evaluation.

The4 plants (leaves) were extracted with methanol and chloroform to yield 8 extracts by Soxhlet extraction. Antimicrobial activity was tested against 3 Gram-positive clinical isolates namely, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus* and 3 Gram-negative clinical isolates namely, *Escherichia coli, Vibrio cholerae, Salmonella typhi* using agar diffusion method. Determination of MIC was done against *Escherichia coli 0157:H7* and clinical isolate of *Staphylococcus aureus* by using broth micro-dilution assay. Antioxidant activity was investigated by measuring the scavenging activity of the DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical. Evaluation for in vitro toxicological screening was done against MDCK (Madin-Darby Kanine Kidney) cells by using an established microtiter plate assay based on cellular staining with trypan blue.

None of the crude extracts showed growth inhibition against the test microorganisms. In addition, the methanol extracts of *Calotropis gigantean, Mimosa pudica, Hibiscus rosa-siensis* (Variety: *Kalyani*) *and Achyranthes aspera* exhibited a great antioxidant effect at 500 µg/ml compared with the effect of three standards of Ascorbic acid, BHT (Butylated hydroxyl toluene) and Propyl Gallate at this concentration. Notable cell death was observed for methanol extract from *Calotropis gigantean* and *Mimosa pudica* while the methanol extracts of *Hibiscus rosa-siensis (Variety: Kalyani)* and *Achyranthes aspera* provided viable condition for the growth of MDCK cell as it demonstrates similar appearances to that of untreated MDCK control.

The results will guide the selection of some plant species for further pharmacological and phytochemical investigations.

CHAPTER 1

INTRODUCTION and LITERATURE REVIEW

1.1 Plants: A therapeutic goldmine

The study of medicinal plants has attracted many researchers, owing to the useful applications of plants for the treatment of various diseases in humans and animals. To date, medicinal plants have been used in all cultures as a source of medicine for the treatment of various diseases including stomach complaints, malaria, depression, cancer and AIDS (Hoareau, 1999). Natural products and related drugs are used to treat 87% of all categorized human diseases including bacterial infection, cancer and immunological disorders (D. C. Newman, GM, 2007). About 25% of prescribed drugs in the world originate from plants (Rates, 2001) and over 3000 species of plants have been reported to have anticancer properties (Graham, 2000). In developing countries and particularly in Bangladesh about 80% of the population relies on traditional plant based medicines (folk medicine) to treat serious diseases including infections, cancers and different types of inflammation (FAO, 2004). Bangladesh has a rich heritage of herbal medicines among the South Asian countries. More than 500 species of medicinal plants are estimated to be growing in Bangladesh and about 250 species of them are used for the preparation of traditional medicines. However, the majority of these plants have not yet undergone chemical, pharmacological and toxicological studies to investigate their bioactive compound(s) (Ghani, 2003). Traditional records and ecological diversity indicate that Bangladeshi plants represent an exciting resource for possible lead structures in drug design.

In this study 4 plants belonging to 4 families (*Calotropis gigantean, Mimosa pudica, Achyranthes aspera* and *Hibiscus rosa-siensis variety: Kalyani*) were collected from different locations of Faridpur district of Bangladesh to screen them for possible antioxidant, antimicrobial and cytotoxic activity. All of these plants have been used in traditional medicine of Bangladesh for the treatment of various diseases such as cancer, inflammation or infectious diseases (Table 1.5). Only limited research has been performed on these plants to evaluate their antioxidant, antimicrobial and cytotoxic potential.

In previous studies using extracts from *Hygrophila auriculata, Bruguiera gymnorrhiza, Clerodendron inerme, Blumea lacera, Hibiscus tiliaceous* and *Argemone Mexicana* NFk-B inhibition, cytotoxic or cytoprotective activities have been observed (Chen *et al.*, 2006; Chiang 2004; Kim *et al.*, 2008; Lambertini *et al.*, 2004; Lampronti *et al.*, 2005; Lampronti *et al.*, 2008; Manoharan *et al.*, 2008; Owen *et al.*, 2007; Roome *et al.*, 2008; Rosa *et al.*, 2007).

For other plants anti-oxidant activity (i.e. *Clerodendron inerme, M. pentaphylla* and *Aegiceras corniculatum*) anti-inflammatory activity (Kim, et al., 2008; Roome, et al., 2008; Somasundaram & Sadique, 1986), (*Hygrophila auriculata, Bruguiera gymnorrhiza, X. moluccensis and Hibiscus tiliaceous*) (Banerjee *et al.*, 2008; Rosa *et al.*, 2006; Uddin *et al.*, 2005; Vijayakumar *et al.*, 2006) and antibacterial activity (*F. religiosa, M. pentaphylla, Argemone mexicana* and *Adiantum caudatum)* have been reported (Bhattacharjee *et al.*, 2006; Kim, et al., 2008; Singh *et al.*, 2008).

The majority of plant-based natural products are phenolic compounds (Kirakosyan, 2006). Anticancer activity has been shown to be associated with a variety of classes, such as polyphenols, flavonoids and catechins (Park *et al.*, 2004). A number of flavonoids and polyphenols have previously been isolated from different parts of *Hygrophila auriculata, L. indica, Bruguiera gymnorrhiza, Clerodendron inerme, X. moluccensis* and *Blumea lacera* (Bheemasankara Rao *et al.*, 1977; Ghani, 2003; Uddin, et al., 2005), which may be involved in their reported cytotoxic activity.

Since a literature search indicated limited information regarding biological and phytochemical investigations of selected plants, this study was carried out as a part of our continued exploration of Bangladeshi medicinal plants for interesting biological activities.

1.2 Medicinal plants and their applications to healthcare

Plants have been used for medicinal purposes for thousands of years. Ancient Egyptian, Chinese, and Indian documents show that medicine in these societies included numerous plant-based remedies and preventives. As a matter of fact, the Chinese traditional medicine (TCM) system and the traditional Indian system of medicines (Ayurveda) are considered to have developed systematic compilation of herbal ingredients used for health benefits, as early as 5000 BC (Khan & Balick, 2001; Yang *et al.*, 2003). The Greeks and Arab civilizations also have contributed substantially to the assimilation and development of plant-based medicines. As early as 2600 BC, the first record of use of plants for therapeutic purposes was

found in Mesopotamia. It is reported that the people in Mesopotamia used oils from cedar, cypress, licorice, and myrrh, to help cure infections. Apart from these, records of traditional systems of medicine can be found have originated in various ancient cultures across the world. Unani systems of medicine, African traditional system of medicine, as well as shamanism can be stated as few examples (Cragg *et al.*, 1997).

The knowledge base for the traditional systems of medicine was mostly empirical and the medicinal properties of plants were explored by "trial and error" basis. An individual practitioner of traditional system of medicine would practice based on such empirical knowledge and experience. Although there were only a few, who documented their studies, this accumulated knowledge about medicinal properties of plants was then passed down to next generations. This is one of the reasons why the traditional system of medicine could not be recognized by the western world as systematic knowledge based science. In the western world, documentation of the folk knowledge about herbal medicines began around 15th century. One of the well-known examples is The Herbal, General Histrory of Plants, written by John Gerard in 1597. In this compilation, he documented the folklore use of herbs, foxglove (*Digitalis purpurea*) being one of them. It was reported by Gerard that a decoction of foxglove in hot water can be consumed to obtain beneficial effects for dropsy (congestive heart failure). This report later helped William Withering, a British physician to explore the therapeutic value of the extracts of *Digitalis purpurea*. Cardiac glycosides isolated from the digitalis plant later in late 19century are still used as one of the most widely used treatments for heart conditions (Mahon & Forster, 1998). Thus the traditional systems of medicine provided a foundation for the development of modern systems of medicine. With the expanse in knowledge of organic chemistry, isolation of the active principles from the plants such as strychnine, morphine, and colchicines was carried out in the early 1800s (Fallarino, 1994; D. J. Newman *et al.*, 2000).

During early 20th century, most of the drug discovery efforts were focused on extraction of the traditionally important medicinal plants and isolation of bioactive constituents from these extracts via *in vivo* studies on animals. As described before, the psychoactive plant constituents such as morphine, codeine and papaverine were isolated from the popular hallucinogenic plant *Papaversomni ferum*. These compounds were later developed as drug candidates and are still clinically used. Discovery of anti-malarial compounds, quinine from the bark of the Peruvian tree of Cinchona and artemisin in from the traditionally used Chinese

herb *Artemisia annua* L. are other examples of success of pharmacognostic evaluation of medicinal plants.

During the first half of 20th century, the central idea behind new drug discovery from medicinal plants was to explore the ethnobotanical evidence about the medicinal properties of a plant and to purify the pharmacologically significant constituents. Although the paucity of advanced bioassay methods was evident, the drug discovery efforts were upholded by the basic organic chemistry and chromatographic techniques (Balunas & Kinghorn, 2005).

With the advent of modern science, availability of advanced in vitro screening methods and sophisticated separation techniques were helpful in speeding up the drug discovery process from medicinal plants. This led to development of new chemical entities (NCEs) from the medicinal plants. With the adaptability of *in vitro* bioassays to screen hundreds of samples in a single run, it became possible for the scientists to pursue a high volume of leads from ethnobotanical literature for screening against a particular disease target. After the preliminary screening, positive lead extracts would be subjected to chromatographic separations, followed by secondary screening procedures to obtain the pharmacologically relevant molecule(s) as the drug candidate. Moreover, with modern synthetic methods it also became possible for the researchers to synthetically mimic the chemical constituents obtained from natural sources. This has enabled large scale production of such NCEs and in turn helped the diminishing natural resources.

In last 50 years, there are many successful stories, in which, medicinal plants, especially those with ethnobotanical evidence of use for medicinal purposes, have become the sources of new drug candidates.

Thus, it can be reasonably argued that medicinal plants have contributed a lot to the modern drug discovery. Several reviews recognize the role of traditionally used plants for development of clinically used drugs (Chin *et al.*, 2006). Out of a total of 520 new drugs approved for commercial use between 1983 and 1994, 30 were new natural products and 127 were chemically modified natural products. Furthermore, it was estimated by Newman et al. in 2003 that about 25% of the single drug molecules that are currently under clinical use are obtained from natural sources, i.e. from medicinally important plants, directly or is synthetic derivatives of the natural product (D. J. Newman *et al.*, 2003).Despite the great successes already achieved in natural products chemistry and drug development, the potential of molecular diversity has not been fully tapped. Only an estimated 15% of the 250,000 species

of higher terrestrial plants in existence have been chemically and pharmacologically investigated in systematic fashion. On the other hand, in past two decades, the tools of combinatorial chemistry and in silico molecular modeling techniques have helped in design of new drugs based on the natural ligands of known drug targets. For example, structure of albuterol is based on the hormone eadrenaline and binds to the same receptor. This has led to a shift in the drug discovery paradigm less towards the use of ethnobotanical leads for new drug discovery. In many cases, preliminary screening of a traditionally used medicinal plant or extract showed good activity. However, no NCE could be developed out of such extract due to the loss of activity upon dereplication of the extract. At many instances, the activity of an extract could be attributed to more than one chemical constituent. Consequently, upon chromatographic separations the activity was lost (Williamson, 2001). Further the cost of drug discovery has put restrictions on the use of ethnic evidences or reports of traditional use of a plant species, as a pursuable lead for the development of NCEs. As a result, most of the pharmaceutical companies have been compelled to reduce their research spending on the drug development from medicinal plants (Bajorath, 2002).

At the current juncture, it can be postulated that the potential of medicinal plants used in the traditional systems of medicines, or those having ethnobotanical use for attaining beneficial effects on human health cannot be fully explored by the pharmaceutical drug development industry.

1.3 Hypothesis

The working hypothesis was that the five medicinal plants for this study (*Calotropis gigantean, Mimosa pudica, Achyranthes aspera* and *Hibiscus rosa-siensis*, variety: Kalyani) have some potential antioxidant, antimicrobial and toxicological activity and depending on the activities, possibly lead to the development of alternate new therapeutic modalities.

1.4 Justification of the study

The results of this study will contribute towards the growing database of knowledge on herbal medicines and help to advocate the safe and effective use of traditional herbal remedies. Ineffective remedies will be identified and excluded as treatment modalities in future. The

use of plants for treatment of various diseases in developed countries, although rooted in the traditional method of treatment, is also largely due to the inability to afford consultation or the use of registered pharmaceutical products. It is hoped that the approval of their use will allow doctors to use the best crude drugs suited to their needs. It is believed that the screening of these drugs will also add to the ever increasing scientific database of medicinal plants, not only in Bangladesh but also globally. Any plant showing substantial activity will be studied further including the isolation and elucidation of the active component(s).

1.5 Aims and objectives of study

The aims and objectives of this research were:

(1) To evaluate naturally-occurring substances for antibacterial activity using a microtiter plate assay against *Staphylococcus aureus, Escherichia coli* and using a Agar Disk diffusion assay and Agar Well diffusion assay against 3 Gram-positive clinical isolates namely, *Staphylococcus aureus, Bacillus subtilis, Bacillus cereus;* 3 Gram-negative clinical isolates namely, *Escherichia coli, Vibrio cholerae, Salmonella typhi.*

(2) To assess the antioxidant capacity of methanol extracts of the collected plants,

(3) To evaluate the *in vitro* toxicological effect of methanol extracts of the collected plants on MDCK (Madin-Darby Kanine Kidney) cells.

Thus, the main aim of the present study was to carry out a phytochemical and biological investigation on selected plants especially on those that are endemic and those that find use in the traditional medicine.

1.6Plants Selected in the study

In this study 4 plants belonging to 4 families were collected for evaluation of their cytotoxic, antimicrobial and antioxidant activities and a list of plants screened in the study has been given in Table 1.5.

1.6.1 Taxonomy of the four plants

Table 1.1 Systematic classification (taxonomy) of Akonda

Kingdom	Plantae – Plants
Subkingdom	Tracheobionta – Vascular plants
Superdivision	Spermatophyta – Seed plants
Division	Magnoliophyta – Flowering plants
Class	Magnoliopsida – Dicotyledons
Subclass	Asteridae
Order	Gentianales
Family	Asclepiadaceae – Milkweed family
Genus	Calotropis R. Br. – calotropis
Species	*Calotropis gigantea* (L.) W.T. Aiton – giant milkweed

Table 1.2 Systematic classification (taxonomy) of LajjabotiLata

Kingdom	Plantae – Plants
Subkingdom	Tracheobionta – Vascular plants
Superdivision	Spermatophyta – Seed plants
Division	Magnoliophyta – Flowering plants
Class	Magnoliopsida – Dicotyledons
Subclass	Rosidae
Order	Fabales
Family	Fabaceae – Pea family
Genus	Mimosa L. – sensitive plant
Species	*Mimosa pudica* L. – shameplant

Table 1.3 Systematic classification (taxonomy) of Bilaiakra

Kingdom	Plantae – Plants
Subkingdom	Tracheobionta – Vascular plants
Superdivision	Spermatophyta – Seed plants
Division	Magnoliophyta – Flowering plants
Class	Magnoliopsida – Dicotyledons
Subclass	Caryophyllidae
Order	Caryophyllales
Family	Amaranthaceae – Amaranth family
Genus	Achyranthes L. – chaff flower P
Species	*Achyranthes aspera* L.

Table 1.4 Systematic classification (taxonomy) of Holudzoba

Kingdom	Plantae – Plants
Subkingdom	Tracheobionta – Vascular plants
Superdivision	Spermatophyta – Seed plants
Division	Magnoliophyta – Flowering plants
Class	Magnoliopsida – Dicotyledons
Subclass	Dilleniidae
Order	Malvales
Family	Malvaceae – Mallow family
Genus	Hibiscus L. – rosemallow
Species	*Hibiscus rosa-sinensis* L. – shoeblack plant

1.6.2 Habit and distributionof the plants

1.6.2.1 Habit and distribution of akonda

A moderate to large sized perennial shrub abounding in milky latex, with opposite- decussate oblong-articulate thick leaves, inodorous purplish white flowers and oblong follicles; grows commonly in the waste lands in all areas of the country (Ghani, 2003) .

1.6.2.2Habit and distribution of lajjabotilata

Lajjabati Lata is a stout strangling prostrate shrubby plant with compound leaves sensitive to touch, spinous stipules and globose pinkish flower heads, grows as a weed in almost all parts of the country (Ghani, 2003).

1.6.2.3Habit and distribution of bilaiakra/apang

A common herbaceous spreading weeds with woody base, ovate, short-petiole and wavy-margined leaves, small reddish-green flowers in long spinous spikes, grow widely in all districts.

1.6.2.4 Habit and distribution of holudzoba

An evergreen, much-branched, 5-8 feet high woody shrub with short-petioled, ovate-lanceolate serrate leaves and hanging showy yellow flowers, commonly planted as ornamental flower-plant in gardens all over the country.

1.6.3 Ethnomedical uses of the screened plants

The Ethnomedical uses of the plants are given in the Table 1.5:

Table 1.5: List of plants screened in the study.

Plant species	Family	Local name	Traditional uses
Calotropis gigantea	Asclepiadaceae	Akonda	Rb: used as purgative, diaphoretic and emetic; F: stomachic, useful in treatment of cold, cough, asthma and loss of appetite; L: used against the skin diseases, insect bites, piles, boils, cancers, abdominal tumors, wounds and tuberculous leprosy.
Mimosa pudica	Mimosaceae	Lajjaboti	WP: Regarded as astringent, diuretic, blood purifier antispasmodic, and in the treatment of convulsions, sore gums; L: used in the piles and fistula; R: used in the piles and fistula.
Achyranthes aspera	Amaranthaceae	Bilaiakra	WP: Regarded as anti-fungal; anti-bacterial, embolic, purgative, astringent and diuretic
Hibiscus rosa-siensis variety: Kalyani	Malvaceae	Holudzoba	R:demulcent & used in cough, L: used as emollient, anodyne and aperiant; AP: extract of aerial parts is spasmolytic, analgesic and hypotensive.

AP: Aerial parts; B: Bark; L: Leaves; F: Flowers; R: Roots; S: Seed; WP: Whole Plant.

1.7 Antioxidants

Antioxidants (oxidation inhibitors) represent a class of substances that vary widely in chemical structure that reduce oxidative damage. They can be defined as any substance that when present at low concentrations compared to those of an oxidizable substrate significantly delay or prevent the oxidation of substrate (Gutteridge & Quinlan, 1993; Vaya *et al.*, 2001). Antioxidants have been found to act as defensive and protective agents against oxidative species in the human body, food and plants, inhibiting the decomposition of oxidation products which result in decreased nutritional value and sensory quality (Pokorny *et al.*, 2001). Even though, oxygen is an essential element for life, it can create damaging by-products during normal cellular metabolism, (Gerschman *et al.*, 1954) proposed that the damaging effects of O_2 could be attributed to the formation of oxygen radicals. This hypothesis was popularized and converted into the "superoxide theory of O_2 toxicity" after the discovery of a class of enzymes, superoxide dismutases (SODs), which appear specific for catalytic removal of superoxide free radical, $O_2^{\cdot}$. In its simplest form, the superoxide theory states that O_2 toxicity is due to excess formation of $O_2^{\cdot}$ and that the SOD enzymes are major antioxidant defenses (Halliwell, 1996).Table 1.6 presents a list of disorders and diseases associated with free-radical pathology.

Table 1.6 Examples of some disorders and diseases associated with free-radical pathology

Cancers	CCl4 and other halogeno alkanes	Thrombosis
Coronary-heart disease/atherosclerosis	Redox cycling mechanisms	Organ storage
Diabetes	Quinones	Transplantation
Cataract	Nitroimidazoles	Neurological degeneration
Adverse drug reactions	Arthritis	Aging
Toxic liver injuries	Immune hypersensitivity	Traumatic inflammation

1.7.1 Categories of antioxidants and their mechanisms of action

There are two basic types of antioxidants, primary and secondary. Primary antioxidants intercept and stabilize free radicals by donating active hydrogen atoms. Phenols represent the two main types of primary antioxidants. Secondary antioxidants prevent formation of

additional free radicals by decomposing the unstable hydroperoxides into a stable product. Antioxidants have diverse mechanisms of action (Table1.7).

The most important mechanism is their reaction with lipid free radicals, forming inactive products (Pokorny, et al., 2001). In other words, antioxidants can be classified into two groups according to their solubility; hydrophilic antioxidants (water-soluble), such as the majority of phenolic compounds and ascorbic acid, and lipophilic antioxidants (fat-soluble) such as carotenoids and vitamin E.

Table 1.7 Mechanisms of antioxidant activity (Pokorny, et al., 2001)

Antioxidant class	Mechanism of antioxidant activity	Examples of antioxidants
Proper antioxidants	Inactivating lipid free radicals	Phenolic compounds
Hydroperoxide Stabilisers	Preventing decomposition of hydroperoxides into free radical	Phenolic compounds
Synergists	Promoting activity of proper antioxidants	Citric acid Ascorbic acid
Metal chelators	Binding heavy metals into inactive compounds	Phosphoric acid Maillard compounds
Singlet oxygen quenchers	Transforming singlet oxygen into triplet oxygen	Carotenes
Substances reducing hydroperoxides	Reducing hydroperoxides in a nonradical way	Proteins, amino acids

1.7.2 Synthetic Antioxidants

Most of the antioxidants occurring naturally in food stuff exhibit comparatively weak antioxigynic properties. Consequently a number of substances possessing marked antioxigenic properties have been developed and put in the market for use in food. Different antioxidants vary in their effectiveness to stabilize fats or fats products are used which are discussed as under.

1.7.2.1 Butylated Hydroxytoluene (BHT)

Butylated hydroxyl toluene, commonly known as BHT is 2,6-di-tert-butyl-4-methyl phenol or2,6-di-tert-butyl-p-cresol. It is also synthetic antioxidant, originally developed for use in petroleum products and rubber, which has been adopted for use in food products. Like BHA, BHT belongs to group of compounds called "hindered phenols" [Butylated hydroxyl toluene (BHT)".

1.7.2.2 Propyl Gallate

Propyl gallate is the one of the most widely used antioxidant at present and is a component of many commercial antioxidant preparations.

1.7.3Potential sources of natural antioxidants

In recent years, numerous report have been published on the identification of novel, naturally occurring antioxidants from plants, animals, microbial sources, and processed food products. Table 1.8 presents a list of plant antioxidant sources. Recent reports in this area discuss young green barley leaves (Osaw, 1992); leaves of *Polygonum hydropiper*, a medicinal herb (Haraguchi, 1992) and pea bean (Tsuda, 1994). In addition to identification of the sources, numerous reports have appeared on further identification and isolation of the active compounds from various sources. Most natural antioxidants are phenolic compounds that, with the exception of the tocopherol, contain ortho-substituted active group, whereas the synthetic antioxidants, with the exception of the gallates, are para-substituted.

Table 1.8 Some Potential Sources of Natural Antioxidants from Plants.

Apple cuticle	Oats
Birch bark	Olives
Carob pod	Oregano
Chia seed	Peanut seed coat
Cloves	Pepper
Cocoa shells	Red onion skin
Garlic	Rice hull
Korum rind	Rosemary
Leaf lipids	Sesame seed oil
Licorice	*Silybiumm arianum* seed oil
Mustard leaf seed	Tea
Myristica fragrans	Wheat gliadin

1.7.4 Superiority of natural antioxidants over synthetic

The oils with higher content of unsaturated fatty acids, especially polyunsaturated FA, are most susceptible to oxidation. In order to overcome the stability problems of oils and fats synthetic antioxidants, such as butylated hydroxyl anisole (BHT), butylated hydroxy toluene (BHT), tertiary butyl hydroquinone (TBHQ) have been used as food additives. But recent reports reveal that these compounds may be implicated by health risks, including cancer and carcinogenesis. Therefore the most powerful synthetic antioxidant (TBHQ) is not allowed for food application in Japan, Canada and Europe. Similarly, BHA has also been removed from the generally recognized as safe (GRAS) list of compounds (Farag, 1989). Due to these safety concerns, there is an increasing trend among food scientists to replace these synthetic antioxidants with natural ones, which in general are supposed to be safer.

1.7.5 Plants with Antioxidant Activity

Many plants are studied about their antioxidant activities such as *Allium satiam, Centella asiatica, Azadirachta indica A. Juss*,var, *siamensis* Valeton, *Ginkgo bilogba Linn. Curcuma longa* Linn.(Scartezzni, 2000). It has been reported that natural antioxidants such as α - tocopherol (vitamin E), ascorbic acid (vitamin C), β - carotenids, flavonoids and polyphenolic compounds present in variety of vegetables and fruits.

1. 8Microbial resistance

1.8.1Microbial cell targets

Many antimicrobial agents are able to control harmful microorganisms. However, infectious diseases, such as those caused by *Staphylococcus aureus,* are becoming difficult to treat (Mazel & Davies, 1999) as a result of microbial genetic flexibility and the misuse of antimicrobial products. Identifying interactions between antimicrobial agents and microorganisms is an essential step in microbial resistance research (Cos *et al.*, 2006). The cell wall, cell membrane, genetic material, protein synthesis, and metabolic pathways are the most useful targets in a viable microbial cell. Antimicrobial agents act on these targets and their respective actions such as cell wall synthesis, DNA and RNA function and/or protein synthesis, to ultimately eliminate the infectious organisms. For example, ciprofloxacin, a drug effective against bacteria, targets topo isomerase enzymes preventing the replication of DNA.

1.8.2 Chromosomal mutations and acquisition of new genes

Resistance is said to occur following a mutation in a critical chromosomal gene, or following the acquisition of a new gene via horizontal gene transfer from another species. Mutations are caused by a number of factors including damage to the genome by environmental factors, point-mutations, base-pairing errors, and frame shift mutations caused by the insertion or deletion of genetic material by transposons (Kenward *et al.*, 1978). In some cases, plasmids called resistance (R) factors transfer resistance from one microbe to another by conjugation, transformation or less commonly transduction. For example, the RP1 plasmid originating in *Pseudomonas* species encodes resistance to carbenicillin, ampicillin, cephaloridine, kanamycin, and tetracycline (Kenward, et al., 1978). In a study conducted by Alexander and Jollick (1977), this plasmid was found to confer resistance in *Caulobacter vibrioides.* The bacterium acquired three antibiotic resistance markers located in RP1. Nonetheless, microorganisms have the genetic flexibility to develop resistance to any antimicrobial agent (Mazel & Davies, 1999).

1.8.3 Microbial mechanisms of resistance

When a target is no longer affected by an antimicrobial agent, following a mutation or acquisition of a new gene, some of the possible mechanisms of resistance include enzymatic transformation, modification of the target, sequestration of the antimicrobial agents, efflux from the cell interior, and prevention of compound entry into the cell (Wright & Sutherland, 2007). To control antimicrobial resistance, it is imperative to carry out routine and effective monitoring of infectious microorganisms, such as *Staphylococcus aureus, Escherichia coli, Candida albicans, and Pseudomonas aeruginosa,* to track the worldwide movement of resistance genes (Mazel & Davies, 1999).

1.9 Naturally-occurring antimicrobial substances

Plants as alternative sources of antimicrobial substances represent a starting point for drug discovery (Cseke *et al.*, 2006) due to their disease preventive properties. Although some plants remain to be the source of many powerful antimicrobial products for the global population, many species remain unexplored (Mohana *et al.*, 2009). More awareness is being drawn towards the discovery, promotion and use of naturally-occurring substances as antimicrobial agents because in most cases they continue to be the only form of medicine available in less developed countries. They are also quickly becoming a popular form of

medicine in developed countries due to their active chemicals referred to as secondary metabolites, or phytochemicals.

1.10Plant secondary metabolites

Phytochemicals are the organic substances that are generated and accumulated by plants (Harborne, 1973). Over time, plant metabolism has evolved and the enzymes catalyzing metabolic reactions have been refined to yield specific products. For instance, primary metabolic pathways, also known as target-oriented pathways, yield small single products with no side products. Conversely, secondary metabolic pathways, also known as diversity-oriented pathways, typically synthesize a variety of products in response to cues (Fischbach & Clardy, 2007). Secondary metabolites, in contrast to primary metabolites, are not directly involved in the growth and/or reproduction of the plant (Harborne, 1973). They are nonnutritive plant chemicals that are synthesized to protect the plant from adverse circumstances, such as a microbial or parasitic attack, or harsh weather conditions. A single plant may produce an arsenal of protective chemicals in response to their lack of motility as well as their stresses and interactions with the environment (Fernie *et al.*, 2007).

The major groups of antimicrobial secondary metabolites include alkaloids, flavonoids (flavones and flavonols), quinones, essential oils, lectins, phenolics, polyphenols, tannins and terpenoids (Samy *et al.*, 2008). Advances in plant genomics research continues to unfold a better understanding of the genetic and biochemical mechanisms involved in the synthesis of plant secondary metabolites. The identification and isolation of genes involved in the different metabolic pathways are also examined (Sumner *et al.*, 2007). According to some authors, at least 200,000 secondary metabolites are known within the plant kingdom (Sumner, et al., 2007) and roughly 4,000 new secondary metabolites are being discovered every year from different plant species.

Recent studies continue to describe some of the secondary metabolic pathways leading to active antimicrobial phytochemicals. The most significant pathways are the shikimic acid pathway, phenyl propanoid pathway, mevalonic pathway and DXP pathway.

1.11 Significance of Toxicological Assay

Commercially utilizable compounds are being produced using modern biotechnology for use as food additives, chemotherapeutic agents, and pesticides. Traditionally, animal testing has always played an important role in the safety evaluation of such agents. However, financial and ethical considerations, together with an increased awareness of the limitations of animal models in relation to human metabolism, now warrant the development of alternative testing methods. Therefore, it is fitting that the potential of biotechnology should provide mammalian cell systems for in vitro testing. The ultimate aim of *in vitro* toxicity testing is the replacement of animals in testing protocols, but in the short term, procedures are refined to reduce the numbers of animals required. This "three Rs" philosophy of reduction, refinement, and replacement was first proposed by Russell and Burch as early as 1959, and is now recognized in the UK Animals in Scientific Procedures Act, 1986 and EC Directive 86/609/ECC.

In this study, a toxicological screening assay was done to evaluate the effect of selected plants on the epithelial cells of Madin- Darby canine kidney (MDCK cells). By this assay it is possible to confirm whether the imminent therapeutic agents produced from the plants are toxic on normal host cells or not. From the upcoming results we can also assure the safety of the imminent therapeutic agents on the kidney since, MDCK cells are both normal and mammalian kidney cells.

CHAPTER 2

MATERIALS and METHODS

2.1 Plant materials

The four medicinal plants for the current study are *Calotropis gigantean, Mimosa pudica, Achyranthes aspera and Hibiscus rosa –siensis variety: Kalyani.*

2.1.1 Collection of plant materialsand identification

Fresh leaves of *Calotropis gigantean, Mimosa pudica, Achyranthes aspera and Hibiscus rosa -siensis variety: Kalyani were* collected from different locations of Faridpur in January, 2010 in air tight polyethylene bags. The plants were identified by Bushra Khan and Ahsan Habib (Scientific Officers) from Bangladesh National Herbarium, Dhaka.

2.1.2 Plant storage

Collected fresh plant materials were examined and old, insect and fungus infected leaves were removed and to ensure effective drying the collected leaves were kept in open mesh bag and kept apart from individual plants.

2.1.3 Preparation of plant materials

Plant leaves were placed in diffused sunlight for 60 days until they were totally dried. The dried plant materials were ground to fine powder using laboratory grinder and kept in air tight polyethylene bags in the dark until extraction.

2.2 Extraction of plant materials through Soxhlet extraction method

2.2.1Chemicals

Methanol and Chloroform were obtained from Merck, Germany.

2.2.2 Equipments

Soxhlet apparatus (Glasscolabs, UK), Filter paper (Whatman, England) and Hot plate were obtained from Daihan, Labtech.

2.2.3 Extraction of plant materials with chloroform

Air-dried and powdered plant materials were extracted with chloroform (CH_3Cl,) using Soxhlet extractor (Glasscolabs, UK). The Soxhlet extraction procedure is a semicontinuous process, which has been found to yield an optimal extraction of similar products (Schmitt, 2006). The protocol followed was the standard method of extraction published by Current Protocols (Shahidi, 2001). 30 g of each of powdered plant materials were weighed into extraction chamber. Plant material was extracted with 400 ml chloroform at the boiling point (61^0 C) using a heating mantle. The condensation rate for the chloroform was set at about 100-120 drops per minute and the extraction was continued for 6 hours. After the extraction the extract was allowed to cool and filtered with Whatman grade 1: 11 µm cellulose filter paper. Filtered extract was concentrated by using a hot plate at low temperature (40-50^0 C). Dried extract was weighed and expressed in percentage of original sample. All extracts were stored at 4^0 C until used.

2.2.4 Extraction of plant materials with methanol

The plant material residue after chloroform extraction was dried overnight and then extracted with methanol (CH_3OH) using Soxhlet extractor. Dried plant material residues were weighed into extraction chamber. Plant material was extracted with 400 ml methanol at the boiling point (65^0 C) using a heating mantle. The condensation rate for the chloroform was set at about 60-80 drops per minute and the extraction was continued for 8 hours. After the extraction the extract was allowed to cool and filtered with Whatman grade 1: 11 µm cellulose filter paper. Filtered extract was concentrated by using a hot plate at low temperature (40-50^0 C). Dried extract was weighed and expressed in percentage of original sample. All extracts were stored at 4^0 C until used.

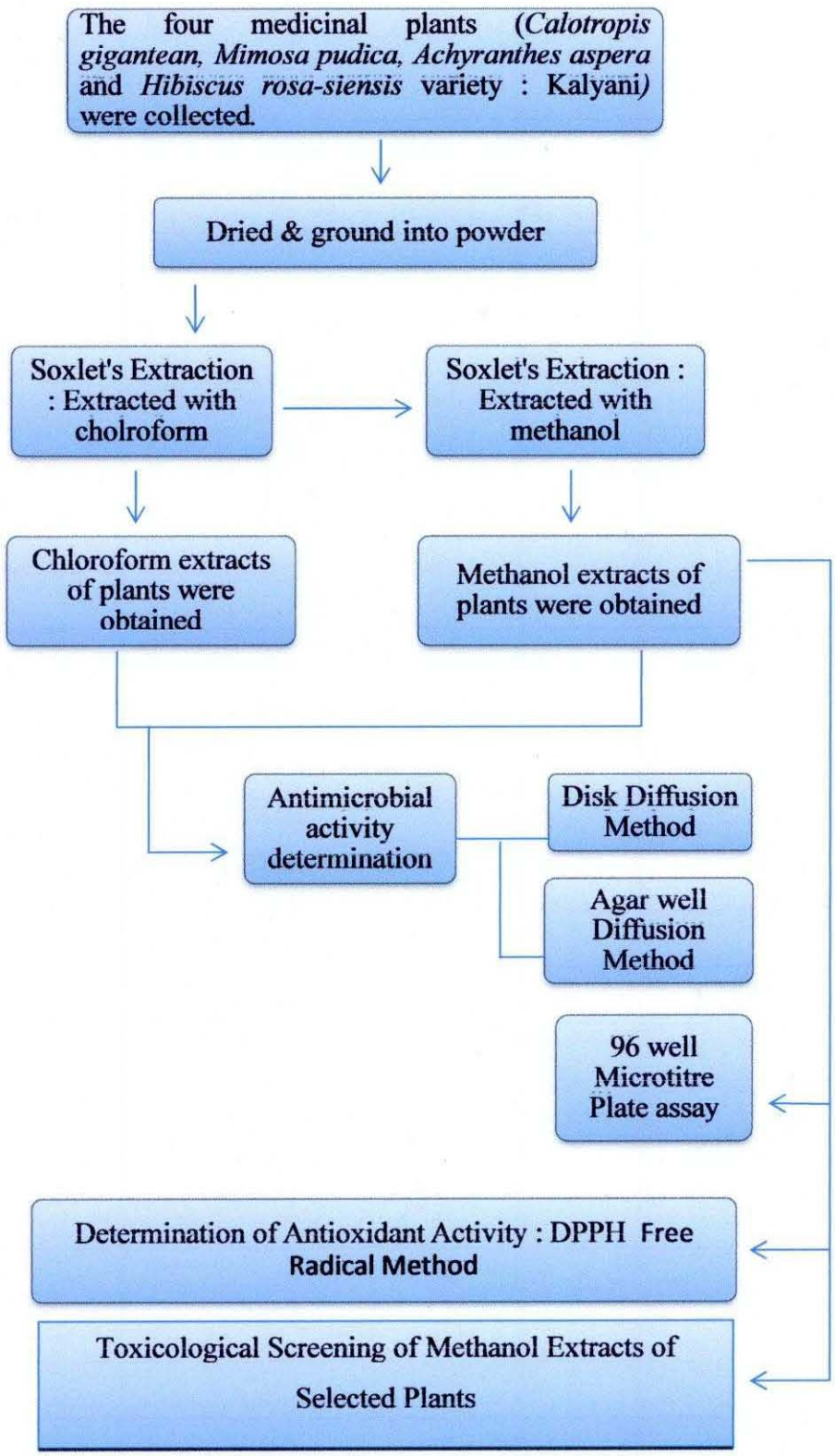


Figure 2.1 Schematic Presentation of Process involved in Study

2.3 Assessment of antimicrobial activity of selected plants by disk diffusion method

The antimicrobial activity test of each extract was carried out by the Kirby-Bauer disc diffusion method (Bauer *et al.*, 1966). For the antimicrobial assessment, conventional methods of testing antibiotic abilities are usually applied. There are two basic techniques used for the assessment of antibacterial activity -

1. The agar diffusion method (paper disc or well),
2. The dilution method (agar or liquid broth).

The agar diffusion method is the most widespread technique of antimicrobial activity assessment. The method is recognized as precise and reliable, even though it produces semi-quantitative results, and according to some authors, only qualitative (Janssen *et al.*, 1987)and not always repeatable (Kalodera *et al.*, 1997). Nonetheless, it makes it possible to estimate the degree of microorganism's growth inhibition and their morphological changes in a simple way.

2.3.1Microorganisms and media preparation

Three Gram-positive clinical isolates — *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus* and three Gram-negative clinical isolates — *Escherichia coli, Vibrio cholerae, Salmonella typhi* were obtained for agar disk diffusion assay from the Microbiology Department of Dhaka University. Media were prepared aseptically following the instructions indicated on the container, the required amount were measured and then media were autoclaved, left to cool down and poured into petri dishes for further use.

2.3.2 Inoculum preparation

Stock cultures of bacterial isolates were streaked onto nutrient agar plates and incubated at 37^0C overnight. From overnight subcultures of each isolates three to five morphologically similar colonies were transferred to 5 ml of normal saline, mixed using a vortex mixer. The suspension's turbidity was adjusted at 0.08-0.13 measured by a spectrophotometer (Shimadzu) at OD 625 nm to obtain a bacterial cell concentration of 1×10^8cfu/ml. Turbidity was adjusted by adding sterile normal saline, if the turbidity is too high, or by adding more bacterial material if is too low. The inoculum suspension was used within 15 min of preparation.

2.3.3 Agar disk diffusion

Methanol and chloroform extracts were redissolved into the respective solvents and mixed using a vortex mixer. The plant extract solutions were filtered using a 0.22μm Millex Millipore filters (Carrigtwohill, Ireland). Sterile filter paper disks of 6 mm diameter were impregnated with 25 μl, 50 μl and 75 μl of extract solution to produce three disks for each plant extracts equivalent to 0.5, 1 and 1.5 mg of the dried extract respectively. Disks were dried for 6 hours to allow complete evaporation of solvent.

Suspension of each bacterial isolate was inoculated onto the Muller-Hinton agar plate by dipping a cotton swab into the suspension and streaking over the surface of the plates so as to create a confluent lawn of bacterial growth. The inoculated plate is allowed to dry with the lid left ajar for no more than 15 min. Using sterile forceps the discs were placed onto the agar surface gently pressed down to ensure contact. Plates were kept for 2 hours in refrigerator to enable prediffusion of the extracts into the agar. Then the plates were incubated overnight at 37°C. Kanamycin (30 μg) was used as positive control. Negative controls were performed with paper discs loaded with organic solvents (chloroform and methanol) and dried. At the end of the incubation period the antimicrobial activity was evaluated by measuring the inhibition zones.

2.4Antimicrobial activity of selected plants by agar well diffusion method

In the burn arena, prior to the advent of topical antibacterial agents, the overall mortality rate in a typical burn population would be reported a 38% to 45%. However after the use of topical antimicrobial therapy the overall mortality was reduced to 14% to 24% (Mason *et al.*, 1986).This enhanced survival was probably due to a susceptibility assay developed at the Cincinnati Shriners Burns Hospital in 1978 by Nathan and his colleagues called Nathan's Agar Well Diffusion Assay (Nathan, 1978). This assay has become the "gold standard" among many burn centers throughout the world.

2.4.1 Microorganisms

Three Gram-positive clinical isolates — *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus* and three Gram-negative clinical isolates — *Escherichia coli*, *Vibrio cholerae*, *Salmonella typhi* were obtained for agar well diffusion assay from the Microbiology Department of Dhaka University.

2.4.2 Inoculum preparation

Stock cultures of bacterial isolates were streaked onto nutrient agar plates and incubated at 37^0C overnight. From overnight subcultures of each isolates three to five morphologically similar colonies were transferred to 5 ml of normal saline, mixed using a vortex mixer. The suspension's turbidity was adjusted at 0.08-0.13 measured by a spectrophotometer (Shimadzu) at OD 625 nm to obtain a bacterial cell concentration of 1×10^8cfu/ml. Turbidity was adjusted by adding sterile normal saline, if the turbidity is too high, or by adding more bacterial material if is too low. The inoculum suspension was used within 15 min of preparation.

2.4.3 Agar well Diffusion

Methanol and chloroform extracts were redissolved into the respective solvents and mixed using a vortex mixer. The plant extract solutions were filtered using a 0.22μm Millex Millipore filters (Carrigtwohill, Ireland).

By sterile technique, 6 mm wells were made on Muller-Hinton agar plates. Suspension of each bacterial isolate was inoculated onto the Muller-Hinton agar plate by dipping a cotton swab into the suspension and streaking over the surface of the plates so as to create a confluent lawn of bacterial growth. The inoculated plate is allowed to dry with the lid left ajar for no more than 15 min. Using sterile micropipette wells were loaded with 25 μl, 50 μl and75 μl of extract solution to produce three well for each plant extracts equivalent to 0.5 mg, 1 mg and 1.5 mg of the dried extract respectively. Plates were kept for 2 hours in refrigerator to enable prediffusion of the extracts into the agar. Then the plates were incubated overnight at 37°C. Kanamycin was used as positive control. Negative controls were performed with wells loaded with organic solvents (chloroform and methanol). At the end of the incubation period the antimicrobial activity was evaluated by measuring the inhibition zones.

2.5Minimum inhibitory concentration determination by broth microdilution

2.5.1 Microorganisms

The following microorganisms were used in this study: *Escherichia coli 0157:H7* and clinical isolate of *Staphylococcus aureus* were obtained from Microbiology Department of The University of Dhaka.

2.5.2 Inoculum preparation

Stock cultures of bacterial isolates were streaked onto nutrient agar plates and incubated at 37^0C overnight. From overnight subcultures of each isolates three to five morphologically similar colonies were transferred to 5 ml of Muller-Hinton broth, mixed using a vortex mixer. The suspension's turbidity was adjusted at 0.08-0.13 measured by a spectrophotometer (Shimadzu) at OD 625 nm to obtain a bacterial cell concentration of 1×10^8cfu/ml. Turbidity was adjusted by adding sterile Muller-Hinton broth, if the turbidity is too high, or by adding more bacterial material if is too low. The turbidity adjusted solution was diluted by a factor of 1:100 by adding 500 µl bacterial suspension to 49.5 ml sterile Muller-Hinton broth to achieve a concentration of 1×10^6cfu/ml.

2.5.3 Microtiter plate assay

Methanol extract of the plants were dissolved in Muller-Hinton broth containing 5% DMSO to obtain a concentration of 2mg/ml and sterilized using a 0.22µm Millex Millipore filters (Carrigtwohill, Ireland). Sterilized methanol extract solutions were stored at -20^0 C until used. Ampicillin solution of 2mg/ml was prepared in the similar manner.

Row A, B, C and D of a sterile 96-well microtiter plate was used to determine minimum inhibitory concentration of *Calotropis gigantean, Mimosa pudica, Achyranthes aspera and Hibiscus rosa -siensis variety: Kalyani* respectively against one bacterial isolates. Row E is used for the positive control Ampicillin. Column 11 & 12 were used as growth control well & sterility control well respectively.

200 µl of Muller-Hinton broth containing 5% DMSO was pipetted into sterility control wells (column 12) and 100 µl into growth control wells. 100 µl of 5% DMSO in MHB was added to each well of 1 to 10 columns. 100 µl sterilized methanol extract solutions were pipetted into wells of column 1 for respective plants. After proper mixing 100 µl of the mixture was

transferred from wells of column 1 to wells of column 2. The process was repeated with a multichannel pipette until column 10 was reached from where 100 µl of the mixture was discarded to achieve a final volume of 100 µl in each wells of column 1 to 10 of row A to D. Wells of column 1 to 10 of row E was similarly filled with ampicillin solution. 100 µl of 1×10^6cfu/ml bacterial suspension was pipetted into each well of column 1 to 11 of row A to E, making the final volume of each well 200 µl. The final concentration of bacteria in each well was 5×10^5cfu/ml and the concentration of extracts ranged between 1000 to 1.953 µg/ml. Plates were then incubated at 37°C for overnight. One plate (sterile round-bottom 96-well plates) was used per bacterial isolates. This was to prevent cross contamination during the initial stage of culture inoculation and the incubation period. After incubation, the MIC of each extract was determined as the lowest concentration at which no growth was observed.

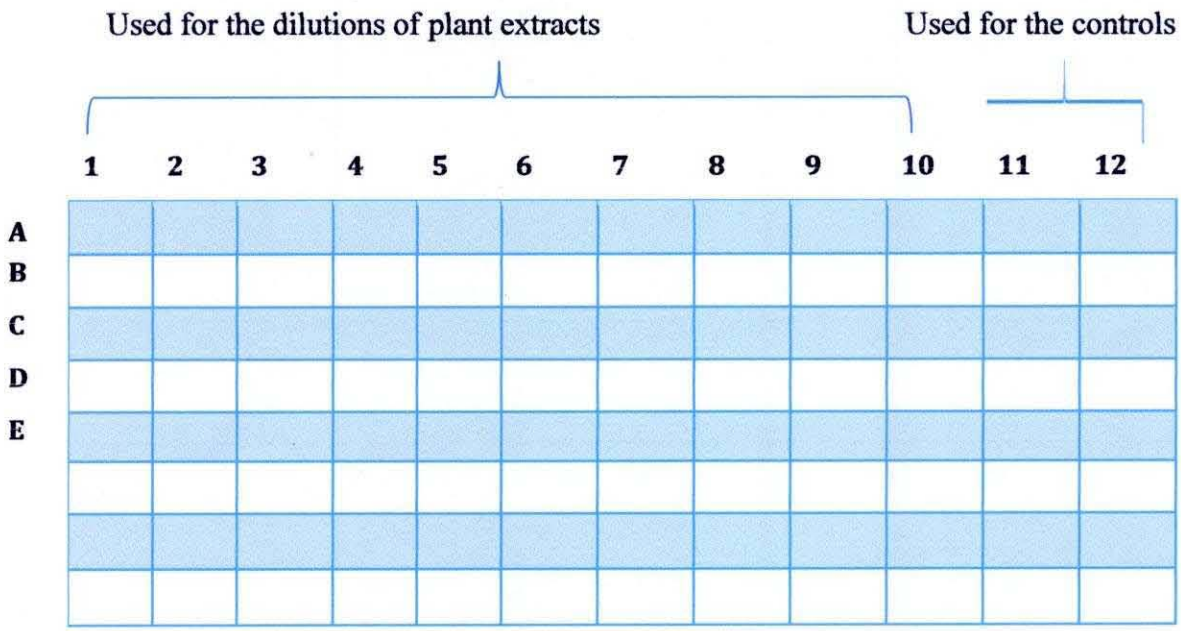


Illustration of the microtitre plate, made up of 12 columns (1-12) and 8 rows.

Row A-D represents four different plant extract

Row E represents Ampicillin (positive control)

Column 11 = Growth Control

Column 12 = Sterility Control

Figure 2.2: Illustration of the microtitre plate

2.6 Antioxidant activity of methanol extracts of selected plants

2.6.1 Chemicals

All chemicals and reagents (analytical) used were of high grade purity. Methanol, Ascorbic acid, Propyl galate and BHT (Butylated Hydroxy toluene) were obtained from Fisher. DPPH (1, 1-diphenyl-2-picrylhydrazyl) was obtained from Sigma.

2.6.2 Determination of antioxidant activity of methanol extracts of selected plants with the DPPH free radical method

The DPPH free radical scavenging assay was carried out for the evaluation of the antioxidant activity. This assay measures the free radical scavenging capacity of the investigated extracts. DPPH is a molecule containing a stable free radical. In the presence of an antioxidant, which can donate an electron to DPPH, the purple colour typical for free DPPH radical decays, and the absorbance change at λ = 517 nm is measured. This test provides information on the ability of a compound to donate a hydrogen atom, on the number of electrons a given molecule can donate, and on the mechanism of antioxidant action. The method was carried out as previously described by(Brand, 1995). The methanol extracts of four plants (*Calotropis gigantean, Mimosa pudica, Achyranthes aspera,* and *Hibiscus rosa-siensis variety: Kalyani)* were redissolved in methanol and various concentrations (10, 50, 100, 500 and 1000 µg/ml) of each extract were used. Similar concentrations of ascorbic acid, propyl galate and BHT were used as positive control. The assay mixture contained in a total volume of 1 ml, 500 µl of the extract, 125 µl prepared DPPH (1 mM in methanol) and 375 µl solvent (methanol). After 30 min incubation at 25°C, the decrease in absorbance was measured at λ = 517 nm.

The radical scavenging activity was calculated from the equation:

% of radical scavenging activity = $Abs_{control}$ - $Abs_{sample}/Abs_{control} \times 100$

2.7 Toxicological Evaluation of Four plants

Application of *in vitro* techniques to the assessment of cytotoxic potential in drug development continues to be a rapidly growing area. The use of continuous cell lines is an important component in this discovery process and may provide fundamental information about *in vivo* xenobiotic toxicity (Bacon *et al.*, 1990).

2.7.1 Cell Culture

The toxicity of methanol plant extracts was examined on distal kidney (MDCK) cell line. MDCK cell line, provided by the Microbiology Laboratory of University of Dhaka, was cultured in DMEM (Sigma Aldrich) supplemented with 200 mM of glutamine, 10% of fetal bovine serum (SIGMA), 3.7g/L NaHCO3 (MERCK), 20mM HEPES, 50 µg/ml Penicillin, 50 µg/ml Streptomycin and 50 µg/ml Gentamycin. The cultures were maintained in 37°C in 5% of humidity CO_2 atmosphere. After 60% - 80% confluence, the cells were trypsinized with 0.25% Trypsin, counted and passaged.

2.7.2 Morphological Study

Methanol extracts were dissolved in 1% DMSO and sterilized using 0.22µm Millex Millipore filter. Sterilized methanol extract solutions were stored at -20^0 C until used. MDCK cells suspended in DMEM (SIGMA) containing 10% heat-inactivated fetal calf serum were cultured in 96-Well flat bottom Microtiter plate (IWAKI, SCITECH) at a density of 10^5 cell/well in the presence of 5% CO_2 at 37°C.

The cells are then treated with 37.5, 75, 150 µg/ml of methanol extracts dissolved in DMSO and incubated for 24 h. Untreated cells receiving only media were used as control. After the treatment period, morphological observations were made with an inverted phase contrast microscope using standard criteria for non-specific cytopathological effects (i.e. membrane bleb formation, cell detachment, cellular crenation and cell lysis). Viable MDCK cells retained normal shape, size and attachment as a complete monolayer toxic effect of methanol extract and was characterized by cell detachment with the appearance of floating, rounded cells and debris (Halliday, 2009).

CHAPTER 3

RESULTS

In the course of screening for antimicrobial, antioxidant and toxicological activities, a number of plants from different locations of the Faridpur used in Bangladeshi traditional medicine were evaluated. Extracts representing 4 plant species belonging to 4 families were subjected to biological screening. The botanical names, plant part used and the traditional uses of the plants in the collected areas are presented in Table1.5.

3.1 Results

3.1.1 Collection and extraction of plant material

Records of the collected plants are adapted in table 3.1 presenting the habit and habitat of the plants. Plant materials extracted with Soxhlet extractor are presented in table 3.2 showing % yield of the different plant extract.

3.1.2 Antimicrobial activity

Antimicrobial activity of the plant extracts was screened using the Kirby-Bauer disk diffusion method and Nathan's agar well diffusion assay. In addition to that, MIC of plant methanol extracts was determined by broth microdilution assay using 96-well microtiter plate. The results of disk diffusion assay, agar well diffusion assay, and broth microdilution assay are recorded in table 3.3, 3.4 and 3.5 respectively.

No inhibition zone was observed for any extracts in disk diffusion assay using different concentrations of disks against either Gram-positive or Gram-negative isolates. No inhibition was also observed for disks loaded with the solvent (negative control). However, kanamycin disks of 30 μg (positive control) showed strong inhibition against every Gram-positive or Gram-negative isolates.

No zone of inhibition was observed for any extracts in agar well diffusion assay using different concentrations of extract loaded in each well against either Gram-positive or Gram-negative isolates. No inhibition was also observed for wells loaded with the solvent (negative control). However, wells laden with kanamycin solution (30 μg/well) (positive control) exhibited strong inhibition against every Gram-positive or Gram-negative isolates.

No inhibition was seen for any methanol extracts of different concentrations used against *S. aureus* isolate and *E. coli 0157:H7* strain for the determination of MIC. However, no growth was noted in the wells loaded with Ampicillin (positive control). No growth was observed for the wells of column 12 (sterility control) whereas distinct growth was observed in the wells of column 11 (growth control).

3.1.3 Radical scavenging activity

The methanol extracts of *Adiantum capillus-veneris*, *Blumea lacera,* and *Cassia alata* showed a high effective free radical scavenging activity in the DPPH assay. These extracts exhibited a noticeable antioxidant effect at low concentrations compared with the standards (Table 3.6). The methanolic extracts of *Cissus quadrangularis* demonstrated moderated free radical scavenging activity with diminutive antioxidant activity at lower concentration and high antioxidant activity at increasing concentration.

3.1.4 Cytotoxic activity

Toxicological evolution of methanolic extracts was performed by *in vitro* cytotoxicity assay against MDCK cell line. Morphological study of methanolic extract treated MDCK cell under inverted phase contrast microscope revealed the viability. MDCK cultures treated with different concentrations of *Calotropis gigantean* and *Mimosa pudica* methanolic extracts demonstrated the characteristics of dead cells with disappearance of monolayer and normal shape and size, cell detachment, presence of floating, rounded cells and debris. This indicates the toxic effect of *Calotropis gigantean* and *Mimosa pudica* on MDCK cells at the investigated concentration. The methanolic extracts of *Achyranthes aspera* and *Hibiscus rosa-sinensis* (Kalyani variety) on the other hand, provided viable condition for the growth of MDCK cell as it demonstrates similar appearances to that of untreated MDCK control.

Table 3.1 Selected plant varieties and their collection record

Plant No.	Plant's Local Name	Scientific Name	Location of Collection	Tropology	Abundance	Plant Type	Leaf type	Date of Collection	Time Collection
1.	Akanda	*Calotropis gigantea*	Faridpur, Bangladesh	Plains	Few	perennial shrub	opposite-decussate oblong-articulate thick	21/1/2010	Afternoon
2.	Lozzaboti	*Mimosa pudica*	Faridpur, Bangladesh	Plains	Abundant	strangling prostrate shrub	sensitive to touch	23/1/2010	Morning
3.	Holudzoba	*Hibiscus rosa-siensis variety: Kalyani*	Faridpur, Bangladesh	Plains	Very few	woody shrub	short-petioled, ovate-lanceolate serrate leaves	22/1/2010	Morning
4.	Bilaiakra	*Achyranthes aspera*	Faridpur, Bangladesh	Plains	Occasional	herbaceous spreading weed with woody base	ovate, short-petiole and wavy-margined leaves	21/1/2010	Noon

Table 3.2 Percent yield of extracts of plant materials in methanol and chloroform

Plant	Family	Parts used	Extract	% yield
Calotropis gigantea	Asclepiadaceae	Whole plant	Chloroform	4.03
			Methanol	21.2
Mimosa pudica	Mimosaceae	Leaves	Chloroform	2.93
			Methanol	20.73
Hibiscus rosa –siensis variety: Kalyani	Malvaceae	Leaves	Chloroform	2.2
			Methanol	13.20
Achyranthes aspera	Amaranthaceae	Whole plant	Chloroform	1.44
			Methanol	9.4

Table 3.3 Antimicrobial activity of plant extracts observed in disk diffusion assay expressed in terms of zone of inhibition (mm)

Evaluated Item	Extract	Concentration per disk	Zone of Inhibition Against Different Microorganisms in mm					
			SA	BS	BC	EC	VC	ST
Calotropis gigantea	Chloroform	0.5 mg	-	-	-	-	-	-
		1.0 mg	-	-	-	-	-	-
		1.5 mg	-	-	-	-	-	-
	Methanol	0.5 mg	-	-	-	-	-	-
		1.0 mg	-	-	-	-	-	-
		1.5 mg	-	-	-	-	-	-
Mimosa pudica	Chloroform	0.5 mg	-	-	-	-	-	-
		1.0 mg	-	-	-	-	-	-
		1.5 mg	-	-	-	-	-	-
	Methanol	0.5 mg	-	-	-	-	-	-
		1.0 mg	-	-	-	-	-	-
		1.5 mg	-	-	-	-	-	-
Hibiscus rosa-siensis variety: Kalyani	Chloroform	0.5 mg	-	-	-	-	-	-
		1.0 mg	-	-	-	-	-	-
		1.5 mg	-	-	-	-	-	-
	Methanol	0.5 mg	-	-	-	-	-	-
		1.0 mg	-	-	-	-	-	-
		1.5 mg	-	-	-	-	-	-
Achyranthes aspera	Chloroform	0.5 mg	-	-	-	-	-	-
		1.0 mg	-	-	-	-	-	-
		1.5 mg	-	-	-	-	-	-
	Methanol	0.5 mg	-	-	-	-	-	-
		1.0 mg	-	-	-	-	-	-
		1.5 mg	-	-	-	-	-	-
Kanamycin (+ Control)	n/a	30 µg	-	-	-	-	-	-
Chloroform (- Control)	n/a	5 ml	-	-	-	-	-	-
Methanol (- Control)	n/a	5 ml	-	-	-	-	-	-

SA= *Staphylococcus aureus*, BC= *Bacillus cereus*, BS= *Bacillus subtilis*, EC= *Escherichia coli*, VC = *Vibrio cholerae*, ST= *Salmonella typhi*

Table 3.4 Antimicrobial activity of plant extracts observed in agar well diffusion assay expressed in terms of zone of inhibition (mm)

Evaluated Item	Extract	Concentration per disk	Zone of Inhibition Against Different Microorganisms in mm					
			SA	BS	BC	EC	VC	ST
Calotropis gigantea	Chloroform	0.5 mg	-	-	-	-	-	-
		1.0 mg	-	-	-	-	-	-
		1.5 mg	-	-	-	-	-	-
	Methanol	0.5 mg	-	-	-	-	-	-
		1.0 mg	-	-	-	-	-	-
		1.5 mg	-	-	-	-	-	-
Mimosa pudica	Chloroform	0.5 mg	-	-	-	-	-	-
		1.0 mg	-	-	-	-	-	-
		1.5 mg	-	-	-	-	-	-
	Methanol	0.5 mg	-	-	-	-	-	-
		1.0 mg	-	-	-	-	-	-
		1.5 mg	-	-	-	-	-	-
Hibiscus rosa-siensis variety: Kalyani	Chloroform	0.5 mg	-	-	-	-	-	-
		1.0 mg	-	-	-	-	-	-
		1.5 mg	-	-	-	-	-	-
	Methanol	0.5 mg	-	-	-	-	-	-
		1.0 mg	-	-	-	-	-	-
		1.5 mg	-	-	-	-	-	-
Achyranthes aspera	Chloroform	0.5 mg	-	-	-	-	-	-
		1.0 mg	-	-	-	-	-	-
		1.5 mg	-	-	-	-	-	-
	Methanol	0.5 mg	-	-	-	-	-	-
		1.0 mg	-	-	-	-	-	-
		1.5 mg	-	-	-	-	-	-
Kanamycin (+ Control)	n/a	30 µg	-	-	-	-	-	-
Chloroform (- Control)	n/a	5 ml	-	-	-	-	-	-
Methanol (- Control)	n/a	5 ml	-	-	-	-	-	-

SA= *Staphylococcus aureus*, BC= *Bacillus cereus*, BS= *Bacillus subtilis*, EC= *Escherichia coli*, VC = *Vibrio cholerae*, ST= *Salmonella typh*

Table 3.5 MIC of methanol extracts of investigated plants

Investigated Element	**MIC against different microorganisms (µg/ml)**	
	Escherichia coli 0157:H7	*Staphylococcus aureus*
Calotropis gigantea	-	-
Mimosa pudica	-	-
Hibiscus rosa –siensis variety: Kalyani	-	-
Achyranthes aspera	-	-
Ampicillin	1.953 µg/ml	1.953 µg/ml

Table 3.6 Free radical scavenging activity of methanol extracts of investigated plants

Investigated Element	% Radical scavenging activity at different concentrations				
	10 µg/ml	50 µg/ml	100 µg/ml	500 µg/ml	1000 µg/ml
Calotropis gigantea	71.68	74.19	85.66	93.19	99.64
Mimosa pudica	21.51	77.78	82.08	85.66	88.17
Hibiscus rosa-siensis variety: Kalyani	25.09	36.56	54.48	83.51	83.51
Achyranthes aspera	28.32	41.58	49.82	92.11	93.87
Ascorbic Acid (Standard)	95.34	97.13	97.49	98.57	99.28
BHT (Standard)	83.87	94.98	97.13	98.92	99.28
Propyl Gallate (Standard)	96.77	98.92	99.28	99.64	100.00

Figure 3.1 Free radical scavenging activity of the investigated plants.

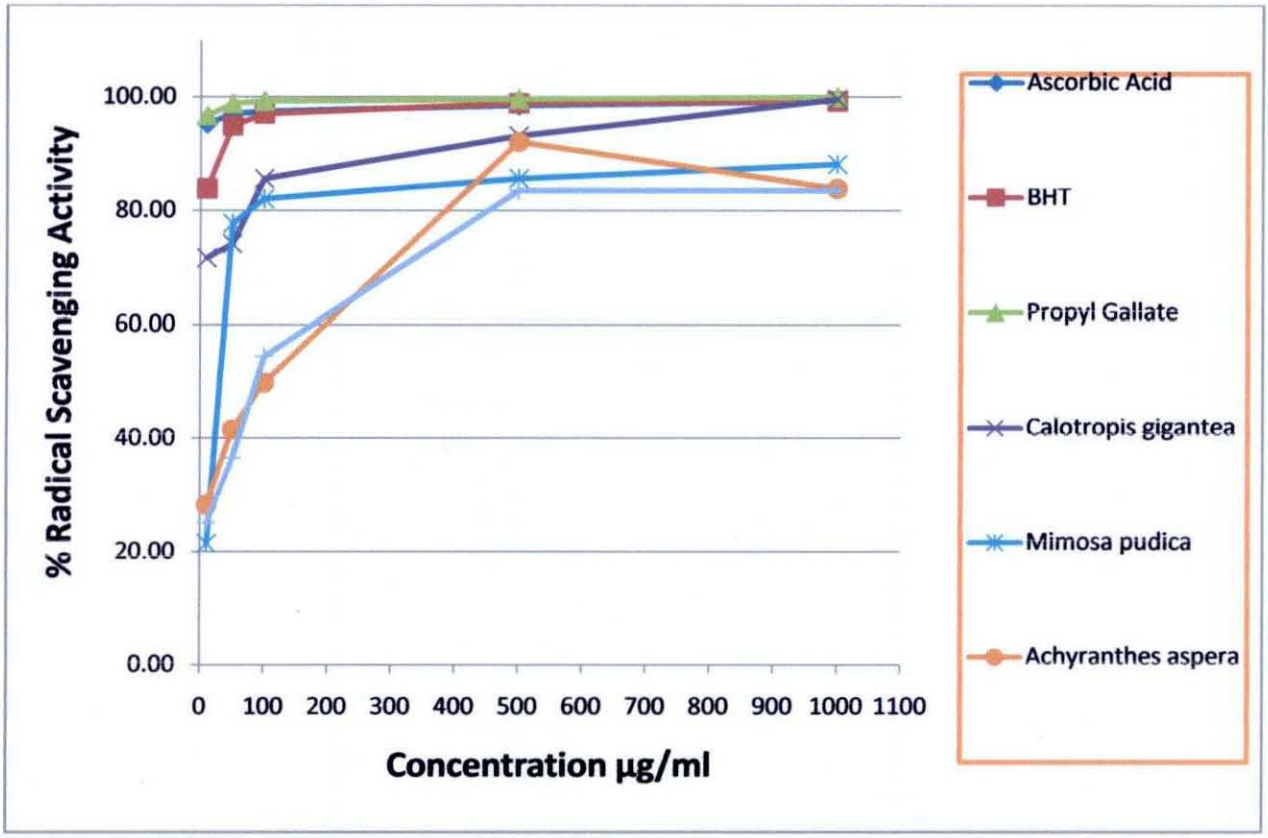


Figure 3.2 Pictures of collected plants

Plate 1: A view of Lozzaboti (*Mimosa pudica*) found in the collection area

Plate 2: A view of Akanda (*Calotropis gigantea*) found in the collection area

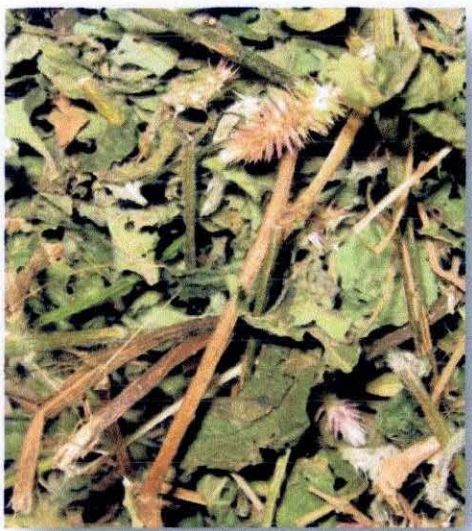
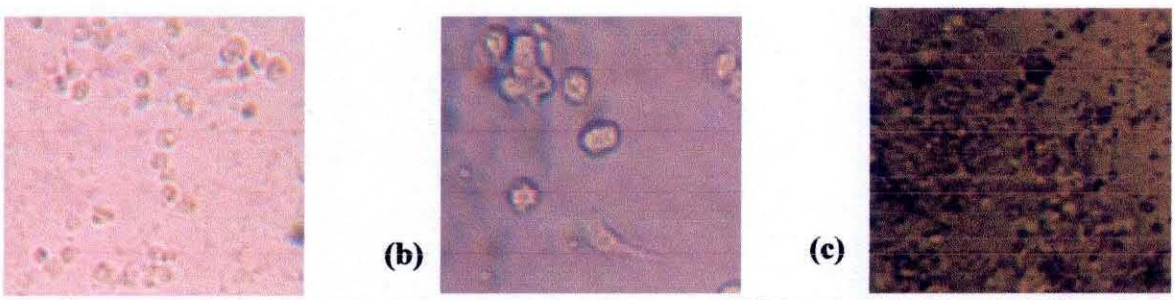


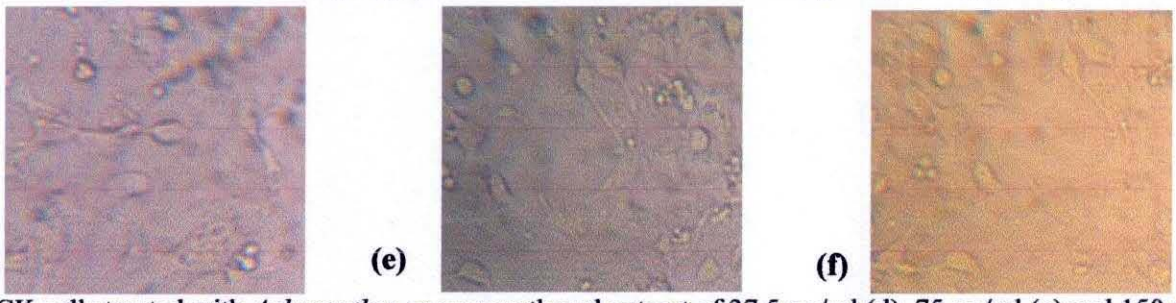
Plate 3 : A view of Bilaiakra (*Achyranthes aspera*) found in the collection area

Plate 4: A view of Holudzoba (*Hibiscus rosa-siensis variety: Kalyani*) found in the collection area

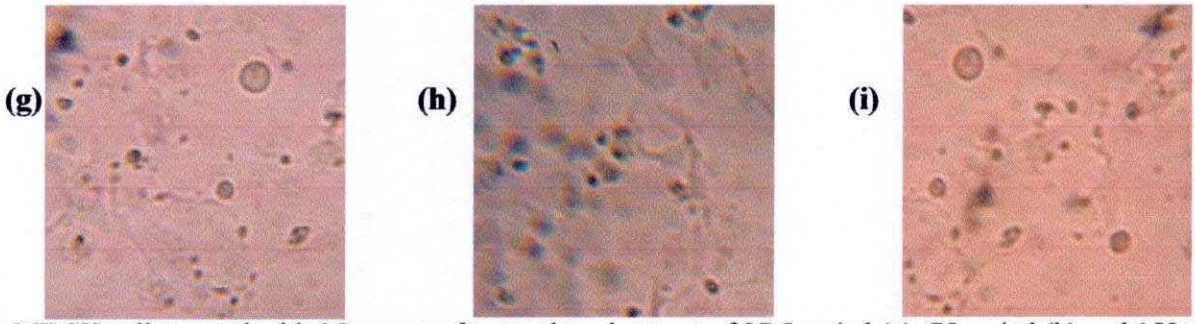
Figure 3.3 Micrographs of MDCK cultures treated with methanol extracts of different concentrations



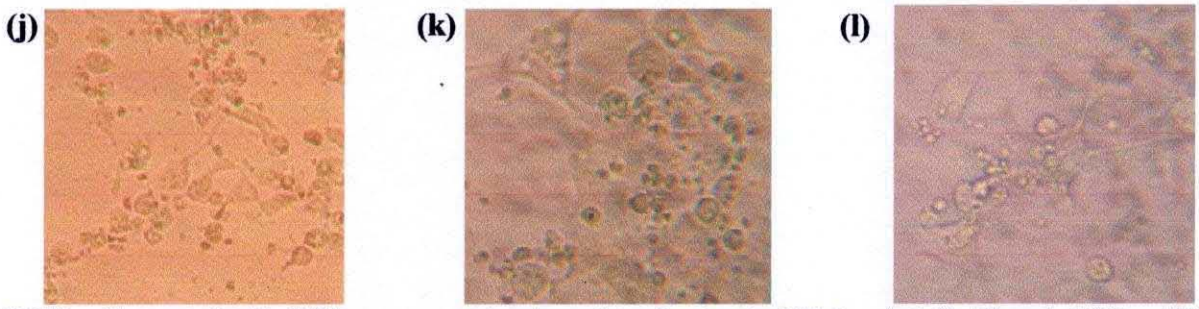
MDCK cells treated with *Calotropis gigantean* methanol extract of 37.5 µg/ml (a), 75 µg/ml (b) and 150 µg/ml (c)



MDCK cells treated with *Achyranthes aspera* methanol extract of 37.5 µg/ml (d), 75 µg/ml (e) and 150 µg/ml (f)



MDCK cells treated with *Mimosa pudica* methanol extract of 37.5 µg/ml (g), 75 µg/ml (h) and 150 µg/ml (i)



MDCK cells treated with *Hibiscus rosa –siensis* methanol extract of 37.5 µg/ml (j), 75 µg/ml (k) and150 µg/ml (l)

Untreated MDCK control (m)

CHAPTER 4

Discussion

Plants have been the traditional sources of raw materials for medicine. A rich legacy of knowledge to preventive and curative medicines was available in ancient scholastic works included in the Atharvaveda, Charaka, Sushruta, etc. An estimate suggests that about 13,000 plant species worldwide are known to be used as drugs. The trend of using natural products has increased and the active plant extracts are frequently used for new drug discoveries and for the presence of antimicrobials (Das *et al.*, 1999). In recent years one of the areas which attracted a great deal of attention is the possible therapeutic potential of antioxidants in controlling degenerative diseases associated with marked oxidative damage. Several plant extracts and different classes of phytochemicals have been found to have quite prominent antioxidant activity (Larson, 1988; Sreejayan & Rao, 1997; Tripathi, 1996;Vani, 1997).

The present study included screening of 4 plants collected from Faridpur, Bangladesh for their toxicological evaluation, antimicrobial and antioxidant activities. Identification of the investigated plants was done by Bushra Khan and Ahsan Habib (Scientific Officers) of Bangladesh National Herbarium, Dhaka, Bangladesh. The existing knowledge about the selected plants is very limited.

Methanol and ethanol extraction yield of each of investigated plants presented in Table 3.2 shows that the yield of methanol extract of *Calotropis gigantean* was the highest (21.2%) while chloroform extract of *Achyranthes aspera* yielded the lowest (1.44 %).

It was demonstrated previously that methanolic fraction of *Mimosa pudica* leaves have antibacterial activity against *Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia, Pseudomonas aeuroginosa and Salmonella typhi* and the phytochemical screening of the extracts revealed presence of phytoconstitutents namely alkaloids, carbohydrates, steroids, flavonoids and glycosides which possibly might have contributed to the anti-bacterial activity (Rekha, 2009), whereas, the investigated *Mimosa pudica* showed no growth inhibitory effect on the tested microorganisms. Moreover, few chemical and biological studies have been carried out on this plant (Balakrishnan, 2006; Dinda, 2006; Genest, 2008;Yuan *et al.*, 2007). Leaves and stems have been reported to contain an alkaloid, mimosine; leaves also contain an adrenaline-like substance and seeds contain mucilage and roots contain tannins. The plant also contains turgorins (Schaller & Schildknecht, 1992).

Free radicals are involved in many disorders like neurodegenerative diseases, cancer and AIDS. Antioxidants through their scavenging power are useful for the management of those diseases. DPPH stable free radical method is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extracts (Koleva *et al.*, 2002). Hence, the search for additional free radical scavengers or antioxidants, especially from plant sources, is of prime importance.

Prior work indicated that methanol extract of the aerial part of *Mimosa pudica* showed moderate antioxidant activity (IC_{50} 296.92 µg/ml) against DPPH free radical (Chowdhury *et al.*, 2008)and this finding may be correlated with the presence of flavonoids and terpenoids. The antioxidant effects of methanol extract of the leaves of *Mimosa pudica* observed in the present study are in harmony with these data. Moreover, none of the methanol (MeOH) extracts of *Mimosa pudica* and *Mimosa rubicaulis* exhibited any significant toxicity towards brine shrimps (Genest, 2008), however the investigated plants caused minute cell death on the tested MDCK cells.

Previous work indicated that a leaf extract from *A. aspera* collected in different areas of the United Arab Emirates was tested and showed inhibition against *Staphylococcus aureus, Bacillus subtilis, E. coli and Aspergillus terreus*, respectively (Bashir *et al.*, 1992), while, the investigated *A. aspera* showed no growth inhibitory effect on the tested microorganisms. It was also reported that the plant contains an alkaloid achyranthine, large amount of potash, hormones, ecdysterone and inokosterone, sterols, saponins and amino acids (Ghani, 2003). Leaves, stems and roots contain ecdysterone, alkaloid, sterols and saponins and roots contain hentriacontane, saponins and oleanolic acids (Ghani, 2003).

In an *in vitro* assay the methanolic extract of *A. aspera* leaves (100 µg) revealed significant inhibitory effects on the Epstein-Barr virus early antigen induced by the tumour promoter 12-O-tetradecanoylphorbol-13-acetate in Raji cells. In the *in vivo* two stage mouse skin carcinogenesis test, the total methanolic extract possessed a pronounced anticarcinogenic effect and the total extract and the fraction are believed to be valuable antitumour promoters in carcinogenesis, (Chakraborty *et al.*, 2002)while the investigated plants caused microscopic cell death on the tested MDCK cells.

Flowers of *Hibiscus rosa-siensis* have been reported to contain cyaniding diglucoside, flavonoids and vitamins – thiamine, riboflavin, niacin and ascorbic acid. Leaves and stems of this plant have been found to contain beta sitosterol, sigma sterol, taraxerol acetate and three

cyclopropane compounds and their derivatives (Ghani, 2003). The antioxidant effects we have found with methanol extract of the leaves of *Hibiscus rosa-siensis* are in harmony with these data because these phytoconstitutents may be liable for the antioxidant potential of the investigated Kalyani variety of *Hibiscus rosa-siensis*. The investigated *Hibiscus rosa-siensis* showed no growth inhibitory effect on the tested microorganisms but it caused minute cell death on the tested MDCK cells.

It has been stated that the active principle of the leaves and stems of *Calotropis gigantea* is milky latex, which contains protease enzymes, calotropain FI, FII (Abraham & Joshi, 1979) and calotropain DI, DII (Pal & Sinha, 1980). Leaves also contain glycolipids, phospholipids, waxes and fatty acids (Rudravarapu *et al.*, 1993). Earlier work indicated that both methanol extract of *Calotropis gigantean* and its chloroform fraction showed activity against *Sarcinalutea, Bacillus megaterium* and *Pseudomonas aeruginosa* which were determined by serial dilution technique (Alam *et al.*, 2008), however the investigated *Calotropis gigantean* showed no growth inhibitory effect on the tested microorganisms.

In addition, a new pregnanone, named calotropone was isolated from the EtOH extract of the roots of *Calotropis gigantean* L. together with a known cardiac glycoside and the structures were elucidated by a study of their physical and spectral data. These compounds displayed inhibitory effects towards chronic myelogenous leukemia K562 and human gastric cancer SGC-7901 cell lines (Wang *et al.*, 2008), whereas the investigated methanol extract of *Calotropis gigantea* in our screens showed a remarkable antioxidant effect and an extraordinary cytotoxic effect on MDCK cells.

The results in the present study are in agreement with the traditional uses of the plants studied. The results further support the idea that medicinal plants can be promising sources of potential antioxidants and the toxicological screening can be used to evaluate the safety of potential anticancer agents on host cells.

The present results will form the foundation for selection of plant species for further investigation in the potential innovation of new natural bioactive compounds.

APPENDIX

DPPH FREE RADICAL SCAVENGING ASSAY

DPPH Solution

1,1-diphenyl-2-picrylhydrazyl	**1 g**
Methanol to	**100 ml**

1 g of DPPH (1,1-diphenyl-2-picrylhydrazyl) was transferred 100 ml volumetric flask and methanol was added up to 100 ml . The solution was then solicited for 2 min and kept in the refrigerator and protected from light.

TOXICOLOGICAL SCREENING

Composition of media for the MDCK cells

For 500 ml media:
50 ml of 10 % FCS
5 ml of 100x Antibiotic mixture
20 ml of 200x mM Glutamine
1.85 gof $NaHCO_3$
20mM of HEPES

Composition of Antibiotic mixture

Antibiotic mixture Penicillin, Streptomycin and Gentamycin prepared by in such manner that each ml contains 50 µg of each antibiotic.

Composition of Nutrient Agar :

Ingredients	Grams / Litre
Peptic digest of animal tissue	5.000
Sodium chloride	5.000
Beef extract	1.500
Yeast extract	1.500
Agar	15.000

Composition of Mueller Hinton Agar

Ingredients	Grams/Litre
Beef infusion solids	4.0
Starch	1.5
Casein hydrolysate	17.5
Agar	15.0

Composition of Mueller Hinton Broth

Ingredients	Grams/Litre
Beef infusion solids	4.0
Starch	1.5
Casein hydrolysate	17.5

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