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Screening of Selected Medicinal Plants of Bangladesh for Biological

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SCREENING OF SELECTED MEDICINAL PLANTS OF BANGLADESH FOR BIOLOGICAL

A thesis submitted for the degree of
Master of Philosophy
in the Department of Botany
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By

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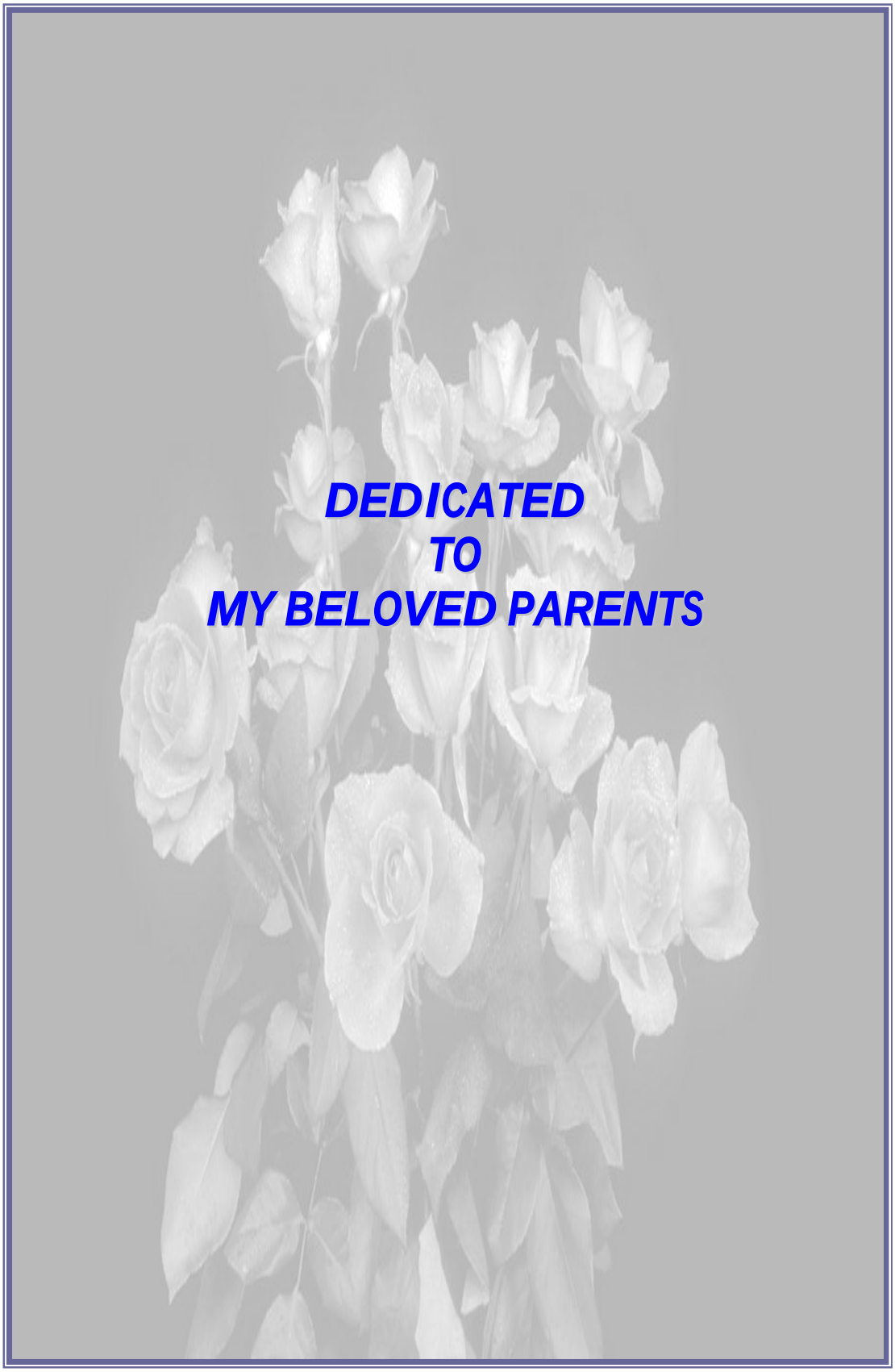
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DECLARATION

I do hereby declare that the MPhil thesis entitled "**SCREENING OF SELECTED MEDICINAL PLANTS OF BANGLADESH FOR BIOLOGICAL ACTIVITY**" has been carried out by me in the Plant Biotechnology and Microbiology Laboratory under the guidance and supervision of Dr. M. Firoz Alam, Professor, Department of Botany, University of Rajshahi is now submitted as a thesis towards the fulfillment for the degree of **Master of Philosophy** in the Department of Botany, University of Rajshahi, Rajshahi-6205, Bangladesh. I also declare that, I have never submitted the thesis or any part of this thesis for any degree or diploma elsewhere. This is the original work of mine.

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CERTIFICATE

It is my pleasure to certify that the research work (Thesis) presented in this dissertation entitled "**SCREENING OF SELECTED MEDICINAL PLANTS OF BANGLADESH FOR BIOLOGICAL ACTIVITY**" is submitted by Md. Mostafizur Rahman in fulfillment for the requirements of the degree of **Master of Philosophy** in Botany in the field of Biotechnology and Microbiology, to the Department of Botany, University of Rajshahi, Rajshahi-6205, Bangladesh. The work or part of it has not been submitted before as candidature for any other degree.

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ABSTRACT

In the current study three plants namely *Cassia sophera* (L.) Roxb, *Cassia fistula* (L.) and *Cassia tora (*L.) Roxb. were evaluated for their bioactivities. For these antibacterial, antitumor, phytotoxic and phytochemical properties of leaves extracts of plants were evaluated. In case of antibacterial activity test, methanol leaves extracts were screened against five gram positive (*Staphylococcus aureus, Bacillus cereus, Streptococcus haemolytica, Bacillus subtilis* and *Sarcina lutea*) and five gram negative (*Escherichia coli-B, Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Shigella dysenteriae*) bacteria using agar disc diffusion method compared with antibiotics (tetracycline). Plants extracts were very efficient against all tested bacteria, whereas *C. tora* was more effective followed by *C. sophera* and *C. fistula*. Furthermore the extract of *C. tora* showed the highest zone of inhibition (15.67 mm) against gram positive *S. lutea* at 300 $mgml^{-1}$. In case of MIC and MBC, *C. sophera* extracts showed MIC values ranged from 100 (*S. lutea*) to 350 $mgml^{-1}$ (*S. haemolytica*) and MBC were ranged from 250 (*S. lutea*) to 450 $mgml^{-1}$ (*S. haemolytica* and *K. pneumoniae*) where *C. fistula* MIC were ranged from 150 (*S. lutea* and *S. dysenteriae*) to 350 $mgml^{-1}$ (*B. cereus*, *S. haemolytica* and *K. pneumoniae*) and MBC were ranged from 250 (*S. dysenteriae*) to 500 $mgml^{-1}$ (*K. pneumoniae*). Followed by *C. tora* MIC values were ranged from 25 (*S. lutea*) to 100 $mgml^{-1}$ (*B. cereus*, *S. haemolytica*, *E. coli-B* and *S. dysenteriae*) and MBC were ranged from 100 (*S. lutea*) to 250 $mgml^{-1}$ (*B. cereus E. coli* and *S. dysenteriae*) against all tested strains. The lowest MIC (25 $mgml^{-1}$) and MBC (100 $mgml^{-1}$) values were observed by *C. tora* against *S. lutea* is a good indication of high efficacy.

For antitumor activity test, studied three plants extracts were used by potato disc bioassay through *Agrobacterium tumefaciens* infection. Before this, antibacterial assay was performed on three *A. tumefaciens* strains (AtTa0112,

AtAc0114 and AtSl0105) to check whether extracts are lethal for bacteria or are inhibiting at any level that is necessary for the genetic transfer mechanism and finally induction of tumor. It confirmed that extracts has no effect on the viability of *Agrobacterium* strains. Although no significant differences were observed in tumor inhibition by three extracts but *C. tora* (57.14%) was more effective for tumor inhibition followed by *C. fistula* (47.70%) and *C. sophera* (47.66%) respectively at 1000ppm on AtSl0105. It was also observed that out of three concentrations (10, 100 and 1000 ppm), tumor inhibitions were only observed at 100 and 1000 ppm concentrations, while AtSl0105 was more prominent for producing tumor followed by AtAc0114 and AtTa0112.

Preliminary phytochemical studies showed that *C. tora* leaves contain maximum occurrence of phytoconstituents like alkaloids, flavonoids, saponins, tannins, glycosides and sterols, and absence of phenols and terpenoids. Followed by *C. fistula* showed presence of alkaloids (moderately), flavonoids, saponins, tannins, phenols and terpenoids, and absence of glycosides and sterols, whereas *C. sophera* showed presence of alkaloids, flavonoids, saponins, tannins, glycosides and steroids, and absence of phenols and tannins. For phytotoxicity test, radish seeds germination and root lengths were significantly inhibited by the studied extracts at 10000 ppm concentration. All extracts exhibited significant phytotoxicity on radish seeds and maximum seed germination inhibition (57%) was observed by methanol extract of *C. sophera* after 5th day of incubation followed by *C. tora* (23%) and *C. fistula* (16%) respectively. Also minimum root length (1.39 mm) was observed when radish seeds were grown in the presence of *C. sophera*.

In summary the above studies indicate that many of the species of *Cassia*, used in traditional medicine, are powerful inhibitors of both microbial and cancer cell growth, meaning there is high chance to develop plant based drugs for treating infectious and cancer type diseases from the studied plants.

ABBREVIATIONS

%	:	Percentage
&	:	Ampersand
μ	:	Micron
μg	:	Micro gram
°C	:	Degrees Celsius
ANOVA	**:**	**Analysis of variance**
BC	:	Before Christ
cfu	:	Colony forming units
DDW	:	Double distilled water
e.g.	:	Exempli gratia, for example
et al.	:	et alia, and others
EtOH	:	Ethanol
Fig.	:	Figure
g	:	Gram (s)
HCl	***:***	***Hydrochloric Acid***
$HgCl_2$	:	Marcuric Chloride
hr(s)	:	Hour (s)
LB	:	Luria-Bertani
MBC's	:	Minimum bactericidal concentration's
mg	:	Milligram
mg/l	:	Milligram/litre
MIC's	:	Minimum inhibitory concentration's
min(s)	:	Minute (s)
ml	:	Milliliter
ml^{-1}	:	Per milliliter
mm(s)	:	Millimeter (s)
NC	:	Negative control
nm	:	Nanometer
No.	:	Number
P value	:	Probability value
PBS	:	Phosphate Buffer Saline
PC	:	Positive control
pH	:	Negative logarithm of hydrogen
ppm	:	Parts-per-million
Sec	:	Second (s)
Sp./spp.	:	Species
T-DNA	:	Transfer DNA
Viz	:	Namely
w/v	:	Weight per volume

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Introduction

INTRODUCTION

Infectious diseases are the world's major threat to human health and account for almost 50,000 deaths every day (Ahmad and Beg 2001). Therefore, there is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action because there has been an alarming increase in the incidence of new and reemerging infectious diseases and development of resistance to the antibiotics in current clinical use (Parekh and Chanda 2008). Since the introduction of antibiotics there has been tremendous increase in the resistance of inverse bacterial pathogens (Cohen 1992, Gold and Moellering 1996). But due to various side effects, long duration of treatment and high cost of the drugs, treatments have not been successful in some cases. On the other hand, multiple drug resistance in pathogenic microorganisms have been frequently reported in current years throughout the world, mainly in developing countries, due to undiscriminating use of commercially available antibiotics in the treatment of infectious diseases. The rising incidence in multidrug resistance amongst pathogenic microbes has further necessitated the need to search for newer antibiotic sources (Cody et al. 2000, Veronika et al. 2006). Though, the resistance development by microbes cannot be clogged, suitable action will reduce the death and health care costs by using antibiotic resistant inhibitors of plant origin (Ahmad and Beg 2001).

Plants have benn utilized as medicines for thousands of years for curing various ailments and diseases (Samuelsson 2004). Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity (Benkeblia 2004). Nature continues to play an integral role in the discovery and isolation of novel molecules with medicinal properties, with more than 50% of chemotherapeutic agents currently in use having been

derived from natural products (Gurib-Fakim 2006). Floristic analysis showed that there are about 500,000 plant species on our planet. Out of these about 120,000 plant species can be used to create biologically active products, which are used in disease treatment (Tivy 1995). This attracted the attention of many scientists and encouraged them to screen plants to study the biological activity of their constituents from chemical and pharmacological investigations to therapeutic aspects (Panizzi et al. 1993). According to World Health Organization medicinal plants would be the best source to obtain a variety of drugs. About 80% of individuals from developing countries use traditional medicine, which has compounds derived from medicinal plants (Santos et al. 1995). Herbs and herbal medicine preparations have been used to treat ailments throughout the history of humanity (Iqbal et al. 2006). Plant derived medicines are generally better tolerated by patients and have fewer side effects. The development of drug resistance is also less documented in plant derived medicines (Vermani and Garg 2002).

Plants constitute about 25% of the total amount of natural agents which have yielded useful medicinal compounds (Rates 2001, Gurib-Fakim 2006). Eleven percent of the 252 drugs being considered by the World Health Organization as being basic and essential are of plant origin (Rates 2001). Many of the drugs currently used to treat bacterial and other infections were first isolated from natural sources including ethno medicinal plants (Coe and Anderson 1996). Nature has been a home of therapeutic agents for thousands of years, and an extraordinary number of recent drugs have been derived from natural sources, many based on their practice in traditional medicine. As Aristotle said: "Nature does nothing without purpose or uselessly" (McChesney et al. 2007).

Knowledge of medicinal plants was passed on from one generation to the next and was eventually recorded in herbal journals (Balunas and Kinghorn

2005). This knowledge is important and applicable to our times for various reasons. Firstly, it promotes the discovery of new alternatives to drugs currently being used. Secondly, it is important from a conservation point of view; if over exploitation of a medicinal plant species should occur, restrictive measures can and should be taken to ensure survival and sustainability of the specific species. From a cultural point of view, important knowledge regarding the traditional use of plants is lost as it is not being passed on from one generation to the next anymore. Thus it is important that this knowledge be documented to ensure that it is at the disposal of future generations who may benefit from it (Thring and Weitz 2006).

A large number of plants have been found to contain ingredients that have antibacterial, antifungal, and anticancer activities. Other plants are used in traditional medicine due to their antioxidant properties (Schinella et al. 2002, VanderJagt et al. 2002). Botanical drugs are derived from specific plant organs of a plant species. The following plant organs are the most important: aerial parts of the herb, leaf, flower, fruit, bark, root, rhizome and bulb. A large majority of botanical drugs are derived from leaves or aerial parts (Heinrich et al. 2004). Currently there are several initiatives taking place worldwide to support herb use in the maintenance of health and the prevention or treatment of disease. In fact, hundreds of thousands of scientists around the globe are publishing papers on new plant-derived compounds and their biological activity. Herbs hold a great promise for improving health, but growth will not be possible without continuing research and increasing awareness of sustainable trade (Kilham 2004).

According to Kong et al. (2003) among the recent 25 best-selling drugs in the world, 30% of the drugs originated from natural products. Morphine, codeine, quinine, aspirin and Taxol are examples of some well-known plant derived, standardized drugs (Van Wyk et al. 1997). Quinine, theophylline, penicillin G, morphine, paclitaxel, digoxin, vincristine, doxorubicin, cyclosporin, and

vitamin A all share two important characteristics: they are cornerstones of modern pharmaceutical care, and they are all natural products (Ebadi 2007).

Recently there has been a shift in universal trend from synthetic to herbal medicine, which can be said "Return to Nature". Medicinal plants have been known for millennia and are highly esteemed all over the world as a rich source of therapeutic agents for the prevention of diseases and ailments (Kumar et al. 2011).

Based on the above disscuss it is clear that we should look for to check various medicinal plants for their biological properties to develop new and new drug especially against multidrug resistant microbes. Therefore the study was under taken with the following aim and objectives:

Aim

The overall aim of the study was to investigate three plants extracts as a potential source of compounds possessing beneficial biological activities.

Objectives

The present work was undertaken with the following objectives

(i) To screen the antibacterial potential of *Cassia sophera*, *Cassia fistula* and *Cassia tora* plants extracts on the growth of ten human pathogenic bacteria using disc diffusion assay and subsequent determination of minimum inhibitory concentrations (MIC's) and minimum bactericidal concentrations (MBC's).

(ii) To study the antitumor activity of extracts using potato disc bioassay through *Agrobacterium tumefaciens* infection.

(iii) To study the qualitative phytochemical analysis of plants extracts using standard methods.

(iv) To determine the phytotoxic properties of extracts using radish seed bioassay.

Chapter-2
Review of Literature

REVIEW OF LITERATURE

2.1. Historical background of medicinal plants

In nature, plants of several variations are available which are responsible for various pharmacological actions. They termed as medicinal plants. On the other hand, some of them produce harmful effects on animal system; they are termed as toxic or poisonous plants (Dash 2016). Thus medicinal plants are defined as any plants which contain substances that can be used for the therapeutic purposes in one or more of its organ or substances which are precursors for the synthesis of useful drugs (Sofowora 1982). Furthermore, Elujoba (1997) noted that a plant become a medicinal plant only when its biological activity has been ethnobotanically reported or scientifically established.

The medicinal qualities of plants have been known to and exploited by man for centuries. Historic texts are rich in information regarding the medicinal applications of plants. The oldest written evidence of medicinal plants usage for preparation of drugs has been found on a Sumerian clay slab from Nagpur, approximately 5000 years old. It comprised 12 recipes for drug preparation referring to over 250 various plants, some of them alkaloid such as poppy, henbane and mandrake (Kelly 2009). The Chinese book on roots and grasses “Pen T’Sao”, written by Emperor Shen Nung circa 2500 BC, treats 365 drugs (dried parts of medicinal plants), many of which are used even nowadays such as the following: Rhei rhisoma, camphor, Theae folium, Podophyllum, the great yellow gentian, ginseng, jimson weed, cinnamon bark, and ephedra (Bottcher 1965, Wiart 2006).

The Indian holy books Vedas mention treatment with plants, which are abundant in that country. Numerous spice plants used even today originate from India: nutmeg, pepper, clove, etc (Tucakov 1971). The Ebers Papyrus, written circa 1550 BC, represents a collection of 800 proscriptions referring to

700 plant species and drugs used for therapy such as pomegranate, castor oil, aloe, senna, garlic, onion, fig, willow, coriander, juniper, common centaury, etc (Glesinger 1954, Tucakov 1964). The previously mentioned Ebers papyrus contains information on nearly a thousand different medicines, most of which are of plant origin. Of the hundreds of plants mentioned in the Bible, 25 to 30 have been identified as medicinal, while ancient Chinese scripts refer to thousands of phytomedicinal remedies. Hippocrates, who is widely considered to be the father of modern medicine, mentioned about 400 drugs, 91% of these originating from plants (Lev 2007).

On a global basis, at least 130 drugs all single chemical entities extracted from higher plants, or modified further synthetically, are currently in use, though some of them are now being made synthetically for economic reasons (Newman et al. 2000). Plants are used medicinally in different countries and are a source of many potent and powerful drugs (Srivastava et al. 1996). A wide range of medicinal plant parts such as root, stem, flower, fruit, twigs exudates and modified plant organs, are used for extract as raw drugs and they possess varied medicinal properties. Although hundreds of plant species have been tested for antimicrobial properties, the vast majority of have not been adequately evaluated (Balandrin et al. 1985).

2.2. Traditional medicine

Traditional medicine is the sum total of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness (WHO 2016). Some people especially in the rural communities rely completely on the healing properties of traditional medicinal plants provided by herbalism. Other people refer to herbalism only on certain occasions as alternatives to allopathic medicine or in combination with other

therapeutic regimes. The use, dosage and preparation methods of medicinal plants are usually provided by traditional herbalists in rural communities (Matsiliza and Barker 2001). Indigenous health care traditions are centred on the particular skills of an individual practitioner (Bodeker 2001). Traditional indigenous herbal knowledge serves as a source of many known and untapped contributions to an improved maintenance of health care (Rabe and van Staden 1997).

Plants are a traditional source of medicinal compounds; up to 40% of modern drugs may directly or indirectly be related to natural compounds (Reddy 2010). Al-Bakri and Afifi (2007) claim that the vast majority of modern drugs are derived from traditional herbal remedies. Traditional medicine based on folklore and anecdotal information has produced leads for new antitumor and antibiotic drugs (Wedge and Camper 1999).

2.3. Herbalism and Primary Health Care

Herbalism is not only practiced as an alternative health option but also to meet the growing urban demand for traditional medicine (Matsiliza and Barker 2001). Traditional herbal medicine is the major and in some cases the only source of health care available in many rural communities. Local people treat themselves with various medicinal plants at an early stage of disease, at a low cost and conveniently replacing the indiscriminate use of unprescribed drugs (Rabe and van Staden 1997). Traditional medicine systems have typically been the primary health care of the poorest levels of society. A large portion of the population still relies on traditional practitioners and local medicinal plants for satisfying their primary health care needs (Grierson and Afolayan 1999, Bodeker 2001).

The prices of pure isolated substances from plants are beyond the financial resources of most people in developing countries (George and van Staden 2000). Isolated compounds from medicinal plants might have the potential to

be promoted to such an extent that it may result in a similar outcome as some synthetic miracle compounds. The inappropriate uses of some synthetically produced drugs have facilitated the development of antimicrobial resistance, with its life threatening consequences (Lewis 1995). Therefore it is essential that the correct dosage, safety, stability, efficacy and other important factors pertaining to plants used indigenously are thoroughly researched and documented (Eloff 1998). It was recommended by the WHO in 1996 to test and document the different standards defining the identity, purity, and potency of plants used medicinally in the form of a plant monograph. Thereby the monograph serves as a revised carbon copy of traditional herbal knowledge for future generations. Herbal medicines, also called botanical medicines or phytomedicines, refer to herbs, herbal materials, herbal preparations, and finished herbal products that contain parts of plants or other plant materials as active ingredients (WHO Media Center 2008). The plant materials include seeds, berries, roots, leaves, bark or flowers (Ehrlich 2016).

2.4. Infectious Disease treatments

Infectious diseases are the leading cause of death worldwide. There has been an increasing interest in medicinal plants as a natural alternative to synthetic drugs because clinical efficacy of many existing antibiotics is being threatened by the emergence of multidrug-resistant pathogens (Bandow et al. 2003). About 50% of all deaths occur due to infectious diseases in tropical areas of world (Iwu et al. 1999). As par as causes of deaths are concerned, recent surveys prove it to be the second major cause of death worldwide and third major reason in developed countries (Nathan 2004).

Several members of enterobacteriaceae are responsible for causing severe infections (Fabio et al. 2007). Antibiotics are powerful medicines that fight bacterial infections and can save lives. The smart use of antibiotics is the key

to controlling the spread of resistance. Originally known as antiobiosis; antibiotics were drugs that had actions against bacteria. The term antibiosis which means ‘against life’ was introduced by the French bacteriologist Vuillemin as a descriptive name of the phenomenon exhibited by these drugs (Foster and Raoult 1974, Calderon and Sabundayo 2007). There are many types of antibiotics and each class inhibits a process that is different in the pathogen from that found in the host (Yonath and Bashan 2004). Antibiotics are used in treating human disease and uses may be contributing to the rapid development of antibiotic resistance in bacterial populations (Khachatourians 1998).

2.5. Cancer treatments

Cancer is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body (Cancer Fact sheet N°297 2014, Cancer 2014). Not all tumors are cancerous; benign tumors do not spread to other parts of the body (Cancer 2014). Possible signs and symptoms include a lump, abnormal bleeding, prolonged cough, unexplained weight loss and a change in bowel movements. While these symptoms may indicate cancer, they may have other causes (Cancer-Signs and symptoms 2014). Over 100 types of cancers affect humans (Cancer 2014). Many treatment options for cancer exist. The primary ones include surgery, chemotherapy, radiation therapy, hormonal therapy, targeted therapy and palliative care. Which treatments are used depends on the type, location and grade of the cancer as well as the patient's health and preferences. The treatment intent may or may not be curative (Cancer 2016).

2.6. Side effects of treatments

An antibiotic side effect is defined as an unwanted reaction that occurs in addition to the desirable therapeutic action of the antibiotic (Anderson 2017).

Antibiotics are major means of treating such infectious diseases but they are not effective in all cases and some microorganisms sometimes cannot be treated by them. So treatment methods are going to become fewer and fewer for them (Wenzel and Edmond 2000). One major limitation of efficacy of antibiotics is development of resistance in microbes and this resistance is spreading all over the world (Livemore 2003, Walsh and Amyes 2004). Although antibiotics are generally considered safe and well tolerated, they have been associated with a wide range of adverse effects (Slama et al. 2005). Side effects are many, varied and can be very serious depending on the antibiotics used and the microbial organisms targeted. The safety profiles of newer medications may not be as well established as those that have been in use for many years (Slama et al. 2005). One of the more common side effects is diarrhea, sometimes caused by the anaerobic bacterium *Clostridium difficile*, which results from the antibiotic disrupting the normal balance of the intestinal flora. Though all medications have side effects, antibiotic side effects can occur and may interfere with the patient's ability to tolerate and finish the course of medication (Anderson 2017).

Common side effects of antibiotics:

- Rash
- Upset stomach
- Fungal (yeast) infections like thrush
- Severe allergic reaction that results in difficulty breathing, facial swelling (lips, tongue, throat, face)
- Severe watery or bloody diarrhea or stomach cramps
- Mouth sores or white patches in your mouth or on your tongue.

These side effects are extremely variable from patient to patient and from antibiotic to antibiotic (Anderson 2017).

2.7. Natural compounds as a source drugs

As Aristotle said: "Nature does nothing without purpose or uselessly" (McChesney et al. 2007). Plants have formed the basis for the treatment of

diseases in traditional medicine systems for thousands of years, and continue to play a major role in the primary health care of about 80% of the world's population (Farnsworth et al. 1985). A huge variety of pharmacologically active compounds are just waiting to be discovered in nature. Some natural derived compounds used as therapeutic agents (da Rocha et al. 2001, Balunas and Kinghorn 2005). In general these compounds are secondary metabolites produced by organisms in response to some external stimuli such as infection, wounding, dryness or nutritional changes. Research into plant secondary compounds can be considered to have started when Friedrich Wilhelm Sertürner isolated morphine ("principium somniferum") from opium poppies in 1806 (Hartman 2007). This was followed by rapid advances in this field.

The formation of natural products represents a selected evolutionary advantage to the producing organism (Koehn and Carter 2005, Lam 2007). Over 100,000 secondary metabolites are known but only a few species have been studied for the presence of secondary metabolites (Vuorela et al. 2004).

2.7.1. Sources of antimicrobial agents

The alarming increase in the incidence of new and reoccurring infectious diseases, as well as the continued evolving of antimicrobial resistance amongst a wide array of pathogenic microbes, highlights the dire need for novel, effective antimicrobial agents (Eissa et al. 2016). Also the emergence of multiple-drug-resistant organisms necessitates the exploration of all available avenues by man in the search for novel antimicrobial agents, including plants and traditional phytomedicines (Feridberg 2009). The potential use of higher plants as a source of new drugs is still poorly explored. Of the estimated 250,000–500,000 plant species, only a small percentage has been investigated phytochemically and even a smaller percentage has been properly studied in terms of their pharmacological properties; in most cases, only pharmacological screening or preliminary studies have been carried out. It is

estimated that 5000 species have been studied for medical use (Payne et al. 1991). Indeed, more than 50% of drugs currently used are products of or derived from higher plants. Seventy eight percent of drugs of natural origin approved by the United States Food and Drug Administration between 1983 and 1994 were of antibacterial type (Toit et al. 2007). Higher plants produce thousands of chemical compounds with a wide array of functions, including acting as pollinator attractants and defending the plant against insects, herbivores and microbes. Antimicrobial activities of plants against multidrug resistant human pathogenic bacteria have been investigated (Rahman et al. 2011a, Akhtar et al. 2012, Sayeed et al. 2014a, Parvez et al. 2015, Nasrin et al. 2016a). Many phytochemicals acting as antimicrobial agents in the plant's defense are likewise active against human pathogenic organisms, and various studies report on the antimicrobial activities of crude plant extracts (Mannan et al. 2013, Rojas et al. 2003, Rana et al. 2015, Nasrin et al. 2016b). Plants with the potential of producing novel antimicrobial agents exhibit various forms of antibacterial, antifungal and antiviral activities (Janick 1999).

2.7.2. Sources of antitumor agents

For many years herbal medicines have been used and are still used in developing countries as the primary source of medical treatment. Thus, research has developed into investigating the potential properties and uses of plants extracts for the preparation of potential nanomaterial based drugs for diseases including cancer (Sivaraj et al. 2014). The discovery and development of efficacious anticancer agents, such as vinblastine and vincristine isolated from the *Catharanthus roseus* (L.) provided convincing evidence that plants could be a source of novel cancer chemotherapeutic agents (Cragg et al. 1996, Sayeed et al. 2014b). Several plant-derived compounds have been approved as anti-cancer drugs i.e. Vinblastine, vincristine, etoposide, teniposide, taxol, taxotere, topotecan and irinotecan, just to name a few (Ganesan 2010). Since

1961, nine plant-derived compounds have been approved for use as anticancer drugs in the United States; vinblastine (Velban), vincristine (Oncovin), navelbine (vinorelbine), etoposide (VP-16), teniposide (VM-26), taxol (paclitaxel), taxotere (docetaxel), topotecan (Hycamtin) and irinotecan (Camptosar) (Lee 1999). Many plant species are already being used to treat or prevent development of cancer. Multiple researchers have identified species of plants that have demonstrated anticancer properties with a lot of focus on those that have been used in herbal medicine in developing countries (Freiburghaus et al. 1996, Kamatou et al. 2008, Islam et al. 2013, Ochwang'I et al. 2014, Sayeed et al. 2014b). Now it is proven, conventional anticancer treatment has failed to meet its objectives: most agents have been revealed as mutagenic and/or carcinogenic (Fatber 1968) they are highly toxic, not only for cancer but also for normal cells. Apoptotic induction has been a target for innovative mechanism-based drug discovery. Resistance to programmed cell death (apoptosis) is an integral part of cancer cell development, and reestablishment of control of apoptosis is a known target mechanism for anticancer drugs (Gibb et al. 1997, Joshi et al. 2002). Certain products from plants are known to induce apoptosis in neoplastic cells, but not in normal cells, which would be the ideal characteristic of a successful anticancer drug (Hirano et al. 1995). Understanding the modes of action of plant derived anticancer compounds should provide useful information for their possible application in cancer prevention and perhaps also in cancer therapy. It is thus important to screen apoptotic inducers from plants, either in the form of crude extracts or as components isolated from them (Tharapadar et al. 2001).

2.8. Mode of antibacterial and anticancer action of phytochemicals

2.8.1. Antibacterial action

Although the antimicrobial properties of plants and their components have been reviewed in the past (Nychas 1995), the mechanism of action has not

been studied in great detail (Lambert et al. 2001). Considering the large number of different groups of chemical compounds present in plants, it is most likely that their antibacterial activity is not attributable to one specific mechanism but that there are several targets in the cell (Carson et al. 2002). The locations or mechanisms in the bacterial cell thought to be sites of action for plant components are indicated in Figure-2.1. Not all of these mechanisms are separate targets; some are affected as a consequence of another mechanism being targeted. An important characteristic of plants and their components is their hydrophobicity, which enables them to partition in the lipids of the bacterial cell membrane, disturbing the structures and rendering them more permeable (Sikkema et al. 1994). Leakage of ions and other cell contents can then occur (Cox et al. 2000, Lambert et al. 2001, Carson et al. 2002, Ultee et al. 2002). Although a certain amount of leakage from bacterial cells may be tolerated without loss of viability, extensive loss of cell contents or the exit of critical molecules and ions will lead to death (Denyer and Hugo 1991).

2.8.2. Anticancer action

The available anticancer drugs have distinctly different mechanisms of action which may vary at different drug concentrations and in their effects on different types of normal and neoplastic ells. While not selectively lethal to cancer cells, as such, in many instances these drugs produce more extensive injury and death to certain neoplastic cells than to the normal tissues, presumably because of quantitatively altered metabolic processes in the cancer cell. These selective anticancer effects, thus far, are difficult to anticipate in the individual patient, or to define in terms of demonstrable biochemical differences in the cancer cells (Karnofsky 1968). In the great majority of cases, also, initially responsive cancers recur in a form resistant to the previously effective agent. Despite the many unsolved problems, there is a

great deal of information on how anticancer drugs act at the cellular level to inhibit the growth of, or to destroy, susceptible cells. Information on mechanisms of action of various anticancer drugs is shown in the Figure-2.2 (Karnofsky 1968).

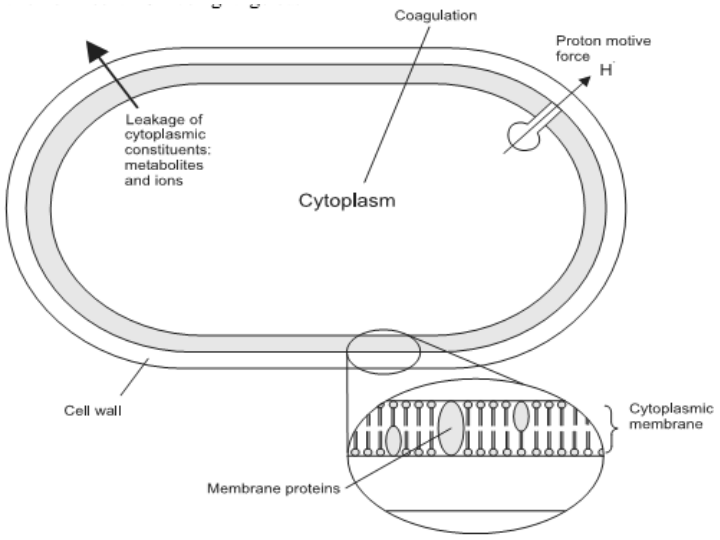


Figure-2.1: Locations and mechanisms in the bacterial cell thought to be sites of action for plants components: degradation of the cell wall (Helander et al. 1998); damage to cytoplasmic membrane (Ultee et al. 2002); damage to membrane proteins (Juven et al. 1994); leakage of cell contents (Cox et al. 2000); coagulation of cytoplasm (Gustafson et al. 1998) and depletion of the proton motive force (Ultee and Smid 2001).

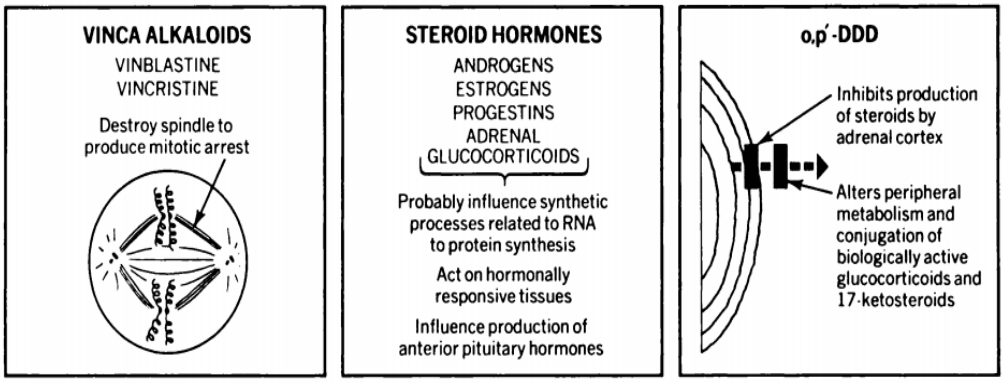


Figure-2.2: Mechanisms of action of various anticancer drugs.

2.9. Phytochemicals of Medicinal plants

Phytochemicals (from the Greek word phyto, meaning plant) are biologically active, naturally occurring chemical compounds found in plants, which provide health benefits for humans further than those attributed to macronutrients and micronutrients (Hasler and Blumberg 1999). They protect plants from disease and damage and contribute to the plant's color, aroma and flavor. In general, the plant chemicals that protect plant cells from environmental hazards such as pollution, stress, drought, UV exposure and pathogenic attack are called as phytochemicals (Gibson et al. 1998, Mathai 2000). These compounds are known as secondary plant metabolites and have biological properties such as antioxidant activity, antimicrobial effect, modulation of detoxification enzymes, stimulation of the immune system, decrease of platelet aggregation and modulation of hormone metabolism and anticancer property. Recently, it is clearly known that they have roles in the protection of human health, when their dietary intake is significant. More than 4,000 phytochemicals have been cataloged and are classified by protective function, physical characteristics and chemical characteristics (Meagher and Thomson 1999) and about 150 phytochemicals have been studied in detail. In wide-ranging dietary phytochemicals are found in fruits, vegetables, legumes, whole grains, nuts, seeds, fungi, herbs and spices (Mathai 2000). Phytochemicals accumulate in different parts of the plants, such as in the roots, stems, leaves, flowers, fruits or seeds (Costa et al. 1999). The exact classification of phytochemicals could have not been performed so far, because of the wide variety of them. There are two main classes of plant derived agents.

Phytoalexins: These are low molecular weight compounds produced in response to microbial, herbivorous, or environmental stimuli (VanEtten et al. 1994). Phytoalexins include simple phenylpropanoid derivatives, flavonoids, isoflavonoids, terpenes and polyketides (Grayer and Harborne 1994).

Phytoanticipins: These are produced in plants before infection or from pre-existing compounds after infection (VanEtten et al. 1994). Phytoanticipins include glycosides, glucosinolates and saponins that are normally stored in the vacuoles of plant cells (Osbourn 1996).

In resent year Phytochemicals are classified as primary or secondary constituents, depending on their role in plant metabolism. Primary constituents include the common sugars, amino acids, proteins, purines and pyrimidines of nucleic acids, chlorophyll's etc. Secondary constituents are the remaining plant chemicals such as alkaloids, terpenes, flavonoids, lignans, plant steroids, curcumines, saponins, phenolics, flavonoids, glucosides etc. (Figure-2.3) (Hahn 1998). Bioactive and disease preventing phytochemicals present in plant are shown in Table-2.1 (Saxena et al. 2013).

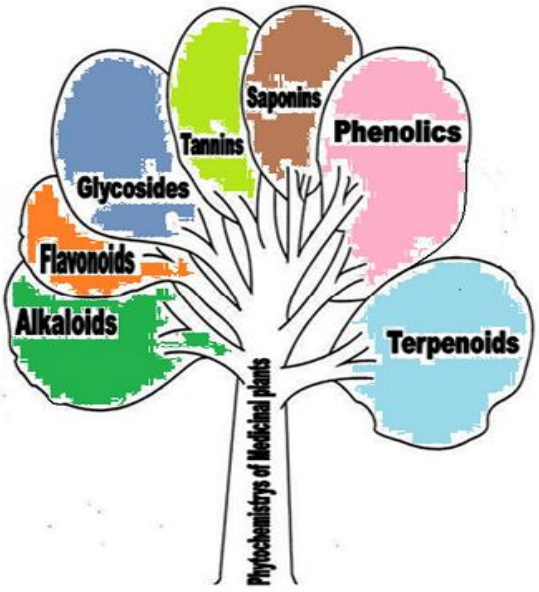


Figure-2.3: Phytochemistry of medicinal plants.

Table-2.1: Bioactive and disease preventing phytochemicals present in plant.

Classification	Main groups of compounds	Biological function
NSA (Non starch polysaccharides)	Cellulose, hemicellulose, gums, mucilages, pectins, lignins	Water holding capacity, delay in nutrient absorption, binding toxins and bile acids
Antibacterial & Antifungal	Terpenoids, alkaloids, phenolics	Inhibitors of microorganisms, reduce the risk of fungal infection
Antioxidants	Polyphenolic compounds, flavonoids, carotenoids, tocopherols, ascorbic acid	Oxygen free radical quenching, inhibition of lipid peroxidation
Anticancer	Carotenoids, polyphenols, curcumine, Flavonoids	Inhibitors of tumor, inhibited development of lung cancer, antimetastatic activity
Detoxifying Agents	Reductive acids, tocopherols, phenols, indoles, aromatic isothiocyanates, coumarins, flavones, carotenoids, retinoids, cyanates, phytosterols	Inhibitors of procarcinogen activation, inducers of drug binding of carcinogens, inhibitors of tumourogenesis
Other	Alkaloids, terpenoids, volatile flavor compounds, biogenic amines	Neuropharmacological agents, antioxidants, cancer chemoprevention

2.10. Recent Developments in Drug Discovery from Plants

Despite the large number of drugs derived from total synthesis, plant-derived natural products still contribute to the overall total number of new chemical entities (NCE) that continue to be launched to the market. Several reviews on drug discovery and development from natural sources (plants, marine fauna and microbes) have been published recently (Newman et al. 2003, Butler 2004, Butler 2005, Chin et al. 2006). A total of 122 biologically active compounds have been identified, derived only from 94 species of plants. A conservative estimate of the number of flowering plants occurring on the planet is 2, 50,000. Of these, only about 6% have been screened for

biological activity and a reported 15% have been evaluated phytochemically. Consistent findings should be carried out to discover a probable abundance of medicinal extracts in these plants (Turker and Usta 2008). Furthermore, an increasing reliance on the use of medicinal plants in the industrialized societies has been traced to the extraction and development of several drugs and chemotherapeutics from these plants as well as from traditionally used rural herbal remedies (UNESCO 1998).

A large proportion of such medicinal compounds have been discovered with the aid of ethnobotanical knowledge of their traditional uses. The rich knowledge base of countries like India and China in medicinal plants and health care has led to the keen interest by pharmaceutical companies to use this knowledge as a resource for research and development programs in the pursuit of discovering novel drugs (Krishnaraju et al. 2005). There has been an explosive growth of herbal drug industry recently. Data analysis has shown that more and more people are consulting the herbal medicine practitioners. World Health Organization has also identified the importance of herbal medicines. According to a study from U.S., 60-70% patients living in rural areas are dependent on herbal medicine for their day to day diseases.

2.11. Studied three medicinal plants

The plant kingdom is one of the attractive sources of novel antimicrobial compounds. Traditional medicine based on folklore and anecdotal information has produced leads for new antitumor and antibiotic drugs (Wedge and Camper 1999).

2.11.1. Cassia sophera (L.) Roxb.

Cassia sophera, Linn. known as 'Kasondi' is an important drug of Islamic System of Medicine (Unani Medicine). The plant is found throughout the world and in most tropical countries (Khare 2007).

Bangla name: Kolkasunda

Botanical Description

Classification

Botanical name: *Cassia sophera* (L.) Roxb.
Kingdom: Plantae
Phylum: Magnoliophyta
Class: Plantae
Order: Fabales
Family: Fabaceae
Sub family: Caesalpinioideae
Genus: *Cassia*
Species: *Cassia sophera* (L.) Roxb.

Using Information

Leaf juice is specific for ringworm; used in asthma, bronchitis and hiccup. Infusion of the leaves is useful in gonorrhoea and syphilitic sores. Bark, leaves and seeds are used as a cathartic; given in diabetes. Root bark ground into a paste is an appliaction for ringworm, pityriasis and psoriasis. A decoction of the whole plant is useful in diminishing urine and as an expectorant it gives relief in cases of acute bronchitis. In Khagrachari root paste along with black pepper is given to treat jaundice by the Marma; it is also given in bronchitis. EtOH (50%) extract of the plant is spasmolytic (Asolkar et al. 1992).

Chemical Constituents

Leaves contain a flavanol-C-glycoside and sennosides. Root bark contains anthraquinones, chrysophanol and physcion and ß-sitosterol. Heartwood contains chrysophanol physicon, chrysophanic acid, emodin, sopheranin, quercetin and ß-sitosterol. Flowers contain anthraquinone and flavanol glycosides, including chrysophanol, rhamnetin glucoside and campesterol, sitosterol and fucosterol (Ghani 2003).

2.11.2. Cassia fistula (L.)

Bangla name: Sonalu

Botanical Description

Classification

Botanical name: *Cassia fistula* (L.)
Kingdom: Plantae
Phylum: Magnoliophyta
Class: Plantae
Order: Fabales
Family: Fabaceae
Sub family: Caesalpinioideae
Genus: *Cassia*
Species: *Cassia fistula* (L.)

Using Information

The leaves are laxative and antiperiodic; useful in ulcers, inflammation and rheumatism; juice of the young leaves is used to cure ringworms. Pulp of the fruit is an agreable laxative, safe for children and pregnant women; given in liver disorder. The purgative properties are due to the presence of Sennoside B. Externally the pulp is considered good application for gout, rheumatism and ringworm. Seeds are given in jaundice. The fruit is reported to be used in Jaundice and diabetes in Khagrachari. Root, seeds and leaves also possess purgative properties. The bark and the wood are given in dysentery. Root is tonic; useful in fever and heart diseases. The Garo of Madhupur give bark juice against dysentery of cattle. Alcoholic extracts of the leaves and stems possess potential antibacterial properties. EtOH (50%) extract of pod and stem

bark showed hypoglycaemic, antivirous, anticancer; and antifertility activity in female albino rats (Asolkar et al. 1992).

Chemical Constituents

Leaves contain anthraquinone glycosides like rhein, sennosides A and B and flavones. Stem bark and wood contain leucoanthocyanidin, fistucacidin, flavones, anthraquinones, like barbalon, rhein and sennosides, lupeol, ß-sitosterol and hexacoasanol. Root bark contains tannin, phlobaphenes, oxyanthraquinone substances, flavonoid glycosides and hydroxyflavones. Flowers contain ceryl alcohol, kaempferol, fustulin and pro-anthocyanidins. Pods contain aloe-emodin, emodin, chrysophanol, rhein, fistulic acid and sennidin A and B. 5-Nonatetracontanone, 2- hentri-acontanone and ß-sitosterol have also been isolated from pods. Pulp contains rhein, glucose, sucrose and fructose; also contains essential oil, waxy and resinous sunbstances. Seeds contain galactomannan (Rastogi and Mehrotra 1990, Ghani 2003).

2.11.3. Cassia tora (L.) Roxb.

Cassia tora Linn is annual under shrub grows all over the tropical countries (throughout India, Pakistan, Bangladesh and west China) and grows well in wasteland as a rainy season weed 8.

Bangla name: Chacunda

Botanical Description

Classification

Botanical name: *Cassia tora* (L.) Roxb.
Kingdom: Plantae
Phylum: Magnoliophyta
Class: Plantae
Order: Fabales
Family: Fabaceae
Sub family: Caesalpinioideae
Genus: *Cassia*
Species: *Cassia tora* (L.) Roxb.

Using Information

Leaves are anthelmintic, laxative and antipyretic; cures bronchitis, asthma, leprosy and piles; used in feverish attacks of children while teething, pounded leaves in eczema, poultice in foul ulcers; hasten suppuration and form a warm remedy in gout, sciatica and rheumatism. Leaves are used for diabetes in Khagrachari. Decoction of the leaf is a mild purgative; used as a cure for coughs. Both leaves and seeds constitute a valuable remedy in skin diseases, for ringworm and itch. Seeds boiled with tea are taken for cold. Fruits and seeds are alexiteric, alterative, anthelmintic and astringent to the bowels; cures leprosy, tumours, skin diseases, scabies, cough, asthma, burning sensation and hemicrania; seeds ground with sour butter-milk or lime juice is beneficial against the irritation of itch or skin eruptions. Roots are specific for ringworm. EtOH (50%) extract of the plant is antivirus, spasmolytic and diuretic; alcoholic extract is active *in vivo* against P388 lympocytic leukemia in mice (Asolkar et al. 1992).

Chemical Constituents

Leaves and stems contain sennosides, D-mannitol, myricyl alcohol and ß-sitosterol. Leaves also contain emodin, a flavanol glycoside, triacontan-1-ol, stigmasterol, ß-sitosterol-ß-D-glucoside, friedelin, palmitic, stearic, succinic and d-tartaric acids, uridine, myo-inositol, d-ononitol, kaempferol, quercetin, juglanin, astragalin, quercitrin and isoquercitrin. Pods have been reported to contain sennosides. Seeds contain anthraquinones and anthraquinone glycosides, chrysophanic acid, rhein, emodin, gluco-obtusifolin, cascaroside, rubrofusarin, chrysophanol, torosachrysone, questin, naphthalenic lactones, isotoralactone, toralactone and cassialactone. Methanolic extract of the seeds yielded pure chrysophanol, chryso-obtusin, aurantio-obtusin, obtiosin, 2-glucosyl obtusifolin, cassiaside and rubro-fusarin-gentiobioside. Seeds also contain physcion, a new naphtha-a-pyrone-toralactone and an oxytocic principle. Roots contain anthraquinones and ß-sitosterol (Rastogi and Mehrotra 1990, Ghani 2003).

Chapter-3
Materials and Methods

MATERIALS AND METHODS

3.1. Materials

3.1.1. Plant materials

The following three medicinal plants and their parts were used in the present investigation.

Plant Descriptions				
Name of the Plants	**Local/Vernacular Name (Bengali)**	**Habitat**	**Family**	**Used parts**
Cassia sophera (L.) Roxb	Kolka sundha	Annual	Fabaceae	Leaf
Cassia fistula L.	Sonalu	Annual	Fabaceae	Leaf
*Cassia tora (*L.) Roxb.	Chakunda	Annual	Fabaceae	Leaf

3.1.2. Bacterial strains for antibacterial activity

In this present investigation following ten bacterial strains were used to study the antibacterial activity of crude extracts of three medicinal plants. All of the bacterial species were collected from the ICDDRB, Dhaka, Bangladesh.

Accession number	Gram positive strain	Accession number	Gram negative strain
1.BMLRU1002	*Staphylococcus aureus*	1.BMLRU1001	*Escherichia coli-B*
2.BMLRU1004	*Bacillus cereus*	2.BMLRU1005	*Klebsiella pneumoniae*
3.BMLRU1006	*Streptococcus haemolytica*	3.BMLRU1007	*Pseudomonas aeruginosa*
4.BMLRU1008	*Bacillus subtilis*	4.BMLRU1009	***Salmonella typhi***
5.BMLRU1012	*Sarcina lutea*	5.BMLRU1011	*Shigella dysenteriae*

3.1.3. Bacterial strains for antitumor activity

Three *Agrobacterium tumefaciens* strains namely AtTa0112, AtAc0114 and AtSl0105 were used to determine the antitumor activity of three medicinal plants extracts (Islam et al. 2009).

3.1.4. Bacterial culture medium

Luria-Bertani (LB) medium was used as nutrient agar medium for bacteria culture. Organisms were maintained by periodic subculture in LB medium. This medium was prepared according to Miller (1972) with slight modification.

Typical compositions of LB medium

Compositions	Amount/l
Yeast extract (Difco)	5.0 gm
Peptone (Difco)	10.0 gm
Sodium Chloride (Marck)	10.0 gm
Agar (Sigma)	15.0 gm

pH adjusted 7.0 ± 0.2

3.1.5. Antibiotics

The following antibiotics were used in different stages of the experiment.

Name of antibiotics	Manufacturers
Cefuroxime	Himedia Laboratories Pvt. Limited (India)
Rifampicin	Phytotechnology Laboratories (USA)
Kanamycin	Himedia Laboratories Pvt. Limited (India)
Camptothecin	Sigma, India
Tetracycline	Tetrax, Square, Bangladesh

3.1.6. Solvents

Traditional healers use primarily water as solvent, but it was found that the plant materials extracted in organic solvents showed profoundly distinct antimicrobial activity from aqueous extract. From this, methanol and ethanol were used in different stages of the experiment.

Solvent	Chemical Formula	Boiling point	Dielectric constant	Density
Methanol	CH_3-OH	65 °C	33	0.791 gml^{-1}
Ethanol	CH_3-CH_2-OH	79 °C	30	0.789 gml^{-1}

3.1.7. Equipments, chemicals and other materials

Relevant glassware, chemicals and equipments were used in different stages of the experiment.

3.2. Methods

The methods involved in the present investigation are described under the following heads:

3.2.1. Collection of plants materials

Naturally growing three medicinal plants were collected from different places of Rajshahi University campus and its vicinity. These plants were authenticated by A. H. M. Mahbubur Rahman, Associate Professor, Department of Botany, University of Rajshahi, Rajshahi 6205, Bangladesh.

3.2.2. Preparation of plants extracts

Preparation of plants extracts and antibiotic solution were carried out as described by Jigna and Sumitra (2006), Ekwenye and Elegalam (2005) with some modifications. Collected leaves were cleaned with tap water and crushed into small pieces and dried under shade. To continue seven days plant materials were dried until a constant weight was obtained. The dried materials were coarsely powdered using motor and pestle and electric blender (IR-091, China) was used to make a fine powder. Fifty-gram fine powder was dipped into 250 ml solvents (95% methanol) into a conical flask stoppered with rubber corks and left for 7 days with constant shaking using orbital shaker (IKA Labortechnik KS 250**,** Staufen, Germany). After seven days, the resulting mixture was than filtered into two stages. First, Teton cloth was used and second; Whatman no.1 filter paper was used for more delicate filtration. For quick evaporation extra solvent (95% methanol) from the extract, Water bath (4 holes analogue, Thermostatic water bath, China) was used under 60 °C. Semi solid filtrates were dissolved in solvents (methanol) and transferred into airtight screw cap tube and stored at 4 °C (Akueshi et al. 2002). Finally the extracts were used as mother extract for antibacterial, antitumor, phytochemical and phytotoxic studies.

3.2.3. Maintenance of bacterial strains

Luria-Bertani (LB) medium was used as nutrient agar medium for bacteria culture. Organisms were maintained by periodic subculture in Luria-Bertani (LB) medium.

3.2.4. Studies on Antibacterial activity

In vitro antibacterial activity of plants extracts were tested against ten studied bacteria using agar disc diffusion method described by Parekh and Chanda (2008).

3.2.4.1. Inoculums preparation from freshly cultured bacteria

All bacterial species were cultured on LB medium. This step was repeated to get a single colony. Single colony was then transferred into LB broth (liquid) medium and incubated at 37 °C for 24 h. Bacterial concentrations were adjusted to absorbance (600 nm) values of 0.969±0.02 which equivalent to 1.0×10^9 colony forming units of bacterial suspensions. These suspensions were used as inoculums.

3.2.4.2. Culture Plates preparation

After autoclave, sterilized Petri plates and medium was taken under running laminar flow as soon as possible. The medium was cooled to 50-55 °C. Then the sterilized medium was poured in equal amount (15 ml each) in each Petri plate and allowed to cool and solidify. Allow the plates to solidify and place them upside down to avoid excessive moisture on the surface of the medium. When the medium became solid the Petri plates marked with a glass marker pen according to their medium name and date. Then the plates were sealed by parafilm and were ready for use or store in 4 °C.

3.2.4.3. Preparation of discs impregnated with crude extracts and antibiotic

The filter paper (Whatman no.1) was punched with the punching machine and six-millimeter (6 mm) diameter in size discs were taken in to the screw

capped tube and sterilized in an autoclave. Discs were soaked with 10µl of particular plants extracts (100, 200 and 300 $mgml^{-1}$). Negative controls were prepared using only solvents (95% methanol) and antibiotics tetracycline (30 μgml^{-1}) was used as a positive control. The discs were placed at room temperature for air dry. Then dried discs were labeled according to particular extracts and antibiotic. Finally the labeled discs were taken into the vial and it was ready for antibacterial activity test.

3.2.4.4. Inoculation, discs placing, incubation and observation

Under aseptic conditions, sterilized discs (concentrations of plant extracts, positive and negative control) were placed on seeded LB agar plates. 30 µl of bacterial suspension (10^8 cfu/ml) was used for preparing seeded LB agar plates. The Petri plates were incubated at 37 °C for 24 h. After incubation, antibacterial activity was determined by measuring the zone of inhibition in millimeter and zone of inhibition above 7 mm was considered as positive result. Each assay was carried out in triplicate.

3.2.4.5. Determination of minimum inhibitory concentrations (MIC's) and minimum bactericidal concentrations (MBC's)

The minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial agent that prevents visible growth of a bacterium after 48 hr of incubation at 37 ^oC and the minimum bactericidal concentration (MBC) is the lowest concentration of an antimicrobial agent required to kill a particular bacterium. MIC's and MBC's of plant extracts were determined according to Doughari et al. (2007). For MIC determination, 0.5 ml of varying concentrations of the extracts (20, 50, 80, 100, 120, 150, 180, 200, 220, 250, 280, 300, 320, 350, 380 and 400 $mgml^{-1}$) were added with LB broth (2 ml) in test tubes, then a loop-full of the test bacteria (10^8 cfu/ml) was introduced. A tube containing LB broth was seeded only with the test bacteria, to serve as

control. The culture tubes were incubated at 37 °C for 24 h. After incubation, the tubes were examined for microbial growth by observing for turbidity.

To determine the MBC, for each set of test tubes in the MIC determination, a loop-full of broth was collected from those tubes that did not show any growth and inoculated onto sterile nutrient agar by streaking. All the plates were then incubated at 37 °C for 24 h. After incubation the concentration at which no visible growth was seen, noted as MBC.

3.2.5. Antitumor potato disc Bioassay

Three medicinal plants: *C. sophera*, *C. fistula* and *C. tora* were investigated for screening their antitumor potentiality using potato disc bioassay adopted standard procedure with some modifications (Galsky et al. 1980, Ferrigni et al. 1982, Turker and Camper 2002, Coker et al. 2003, Hussain et al. 2007, Islam et al. 2008, 2009, 2010a, 2010b, 2013). Three *A. tumefaciens* strains (AtTa0112, AtAc0114 and AtSl0105) were used as a tumor forming agent on potato disc.

3.2.5.1. Desired concentration preparation of extracts and control

For antitumor activity test, mother extracts (prepared earlier) were used to make desired concentrations (10, 100 and 1000 ppm) of the extracts and only solvent (95% methanol) was used as a negative control and camptothecin (30 μgml^{-1}) (tumor suppressant) served as a positive control using standard formula.

3.2.5.2. Bacterial (A. tumefaciens) culture preparation

Three *A. tumefaciens* strains namely AtTa0112, AtAc0114 and AtSl0105 were used for antitumor activity test. Bacterial strains were cultured on rifampicin (10 μgml^{-1}) containing LB agar. This step was repeated to get a single colony. Single colonies were then transferred into rifampicin containing LB broth and incubate at 30 °C for 48 h**.** After that, 6-7 loops bacterial suspensions were transferred into autoclaved phosphate buffer saline (PBS). Bacterial suspension containing PBS were used for inoculation on potato disc for

antitumor activity test**.** Bacterial concentrations were adjusted to absorbance (600 nm) values of 0.969±0.02 which equivalent to 1.0×10^9 colony forming units of bacterial suspensions.

3.2.5.3. Preparation of potato discs

Potato disc were used to assess the *in vitro* tumor induction capacity by *A. tumefaciens* and tumor inhibition by the plants extracts. Discs were prepared from red-skinned potatoes (*Solanum tuberosum* L.). Potatoes were collected from local market in Rajshahi, Bangladesh. To prepare discs, potatoes were rinsed thoroughly in tap water with detergent followed by commercial bleach (Savlon, ACI limited, Bangladesh) for 5 minutes, then washed with DDW. Inside the laminar air flow cabinet, potatoes were treated with 0.1% $HgCl_2$ for 5 minutes followed by washed with DDW two or more times**.** Surface sterilized potatoes were cut into 8mm diameter in size from the center of potato tissue (skin portion was discarded) by sterilize cork borer. Small segment of potato discs (like cylindrical) were kept into sterilize bottle with DDW to avoid moisture loss and rinsed twice more. Each potato segment was cut into 5mm (thickness) discs after excluding 1cm end pieces. Prepared 5mm × 8mm potato discs were used for inoculation.

3.2.5.4. Preparation of inoculants

The following design was followed for inoculums preparation. **For positive control**: 600 µl camptothecin (30 $\mu g ml^{-1}$) + 150 µl DDW + 750 µl *A. tumefactions* strains in PBS**. For negative control:** 600 µl methanol (95%) + 150 µl DDW + 750 µl *A. tumefactions* strains in PBS**. For test plants extracts:** 600 µl plants extracts (10, 100 and 1000 ppm) + 150 µl DDW + 750 µl *A. tumefactions* strains in PBS.

3.2.5.5. Inoculation, incubation and observation

Prepared 5mm × 8mm potato disc were transferred into water agar plates (1.5 gml^{-100}). Ten discs were placed in every Petri plate. Each disc was overlaid with 50µl of appropriate inoculums. After cutting the potato discs, inoculation was done within 30 minutes**.** Petri plates were sealed by parafilm and incubated at room temperature (27-30 °C) for 3 weeks. After 3 weeks, discs were stained with Lugol's iodine solution (10% KI and 5% I_2) for 30 minutes and tumors were observed under dissecting microscope, where the tumor cells lack starch. Data were statistically analyzed using MSTAT software. Each experiment was done in triplicate. Percentage of tumor inhibition was calculated (McLaughlin 1991, McLaughlin et al. 1993, McLaughlin and Rogers 1998). Twenty percent tumor inhibition was considered significant.

$$\% \text{ inhibition} = 100 - \frac{\text{Average number of tumors of sample}}{\text{Average number of tumors of control}} \times 100$$

3.2.6. Antibacterial assay (as a partial assay)

Before antitumor activity test, antibacterial assay was conducted on three *A. tumefaciens* strains to check their viability against plants extracts. The disc diffusion method was used to screen antibacterial activity describe by Parekh and Chanda (2008).

3.2.6.1. Inoculums preparation of bacteria and Culture Plates preparation

Inoculums and culture plates were prepared as described in 3.2.4.1. and 3.2.4.2.

3.2.6.2. Preparation of discs impregnated with crude extracts and antibiotics

Discs were soaked with 10 µl of particular plants extracts (250 $mgml^{-1}$) while antibiotics namely Kanamycin (30μgml^{-1}) and Cefuroxime (30 μgml^{-1}) were used as a positive controlwhereas negative controls were prepared using 95%

methanol. The discs were placed at room temperature for air dry. Then dried discs were labeled according to particular extracts and antibiotics. Finally the labeled discs were taken into the vial and it was ready for antibacterial activity test.

3.2.6.3. Inoculation, discs placing, incubation and observation

Under aseptic conditions, sterilized Whatman no.1 filter paper discs (6 mm in diameter) were placed on seeded rifampicin (10 $\mu g ml^{-1}$) containing LB agar plates. 30 µl of bacterial suspension (10^8 cfu/ml) was used for preparing seeded LB agar plates. The Petri plates were incubated at 37 °C for 24 h. After incubation, antibacterial activity was determined by measuring the zone of inhibition in millimeter and zone of inhibition above 7mm was considered as positive result. Each assay was carried out in triplicate.

3.2.7. Phytochemical Screening for different components

Standard procedures were carried out for qualitative test of plant extracts to find out the presence of certain bioactive compounds (Cromwell 1955, Harborne 1973, Trease and Evans 1989, Sofowora 1993, Kumar et al. 2013,).

3.2.7.1. Preparation of different reagents for phytochemical analysis

Dragendroff's reagent: Solution A: 1.7 g of basic bismuth nitrate and 20 g of tartaric acid were dissolved in 80 ml of distilled water. Solution B: 16 g of potassium iodide was dissolved in 40 ml of distilled water. Both solutions (A and B) were mixed in 1:1 ratio (Kumar et al. 2013).

Hager's reagent: 1 gm picric acid (2, 4, 6-trinitro phenol) was dissolved in 100ml distilled water up to saturation.

Mayer's reagent: 0.355 g of mercuric chloride was dissolved in 60 ml of distilled water. 5.0 g of potassium iodide was dissolved in 20 ml of distilled water. Both solutions were mixed and volume was raised to 100 ml with distilled water (Kumar et al. 2013).

Wagner's reagent: 2.27 g iodine and 2 g potassium iodide were dissolved in 5 ml distilled water and made up to 100 ml.

Tannic acid reagent: 10 gm tannic acid was dissolved in 100 ml distilled water. This reagent is very sensitive to most of the alkaloids and gave precipitates with most alkaloids.

1% ammonia: 1ml of ammonia dissolved in 99 ml of distilled water.

1% ammonium chloride: 1g of ammonium chloride was dissolved in 100ml distilled water.

Ferric Chloride (alcoholic): A 5% w/v solution of ferric chloride in 90% alcohol is used for the detection of phenols.

Lead acetate: A 25% basic lead acetate solution is used for the detection of flavonoid.

3.2.7.2. Procedure for qualitative test

3.2.7.2.1. Test for Alkaloids

5 g fresh finely chopped and pasted plant material was mixed up to moistered with 10 ml 2% HCL and heated in water at 60 ^{0}C for one hour. After cooling the extract was filtered through Whatmann No. 1 filter paper.

Two drops of extract were put on a microscopic groove slide with one drop of the alkaloid detecting reagent. The relative abundance of precipitate, if any, formed in the plant extract with the reagents was considered as an index of the quality of the presence of alkaloid and was expressed by + sings.

3.2.7.2.2. Test for Flavonoids

The aqueous extract (5 ml, corresponding to 1 g of plant material) was treated with a few drops of concentrated HCl and magnesium turnings (0.5 g). The presence of flavonoids was indicative if pink or magenta-red color developed within 3 min (Somolenski et al. 1972).

3.2.7.2.3. Test for Glycosides:

Five ml of aqueous extract were hydrolyzed separately with 5 ml each of conc. HCl and boiled for few hours on a water bath and hydrolysates were subjected to the following test: A small amount of alcoholic extract of samples was dissolved in 1ml water and then aqueous 10% sodium hydroxide was added. Formation of a yellow color indicated the presence of glycosides.

3.2.7.2.4. Test for Saponins

2.0 g of the powdered plant material was boiled in distilled water (10 ml) in a test tube in boiling water bath and filtered. Filtrate (10 ml) was mixed with 5 ml of distilled water and was shaken vigorously to the formation of stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously for the formation of emulsion thus a characteristic of saponins (Kumar et al. 2013).

3.2.7.2.5. Test for tannins

About 0.5 g of the extract was boiled in 10 ml of water in a test tube for 20-25 minutes and then filtered. A few drops of 0.1% ferric chloride was added and observed for blue-black, green, purple, blue-green or brownish coloration / precipitate (Kumar et al. 2013).

3.2.7.2.6. Test for terpenoids and sterols

The powder plant material (1g) will be macerated with 20 ml petroleum ether (60-80 ^{0}C) for 6 hour, filtered and the ether will be evaporated to dryness. The residue was dissolved in acetic anhydride (2ml), transferred to a test tube and cautiously, concentrated H_2S0_4 was poured along the side of the tube. Possible presence of sterols and/or triterpenes is indicated by the immediate appearance of violet color in case of triterpenes which changes to green on standing in case of sterol (Shayoub et al. 2015).

3.2.7.2.7. Test for phenols

Ferric Chloride Test: The extract (500 mg) was dissolved in 5ml of distilled water. To this, 3-4 drops of neutral 5% ferric chloride solution were added. A

blue-green or bluish black or dark green clour indicated the presence of phenolic compounds.

3.2.8. Radish seed phytotoxicity assay

The method of radish seed bioassay was followed as reported by Arzu and Camper (2002).

3.2.8.1. Desired concentration of the extracts and plate Preparation

Mother plants extracts (prepared earlier) were used to make 10,000 ppm concentration by 95% methanol for phytotoxicity test. The solution was poured in Petri plate containing sterilized Whatman no.1 filter paper and left in laminar flow till evaporation of the solvent. After evaporation 5 ml of distilled autoclaved water was poured. Negative controls were prepared using only methanol (95%) and double distilled water.

3.2.8.2. Seed placing, incubation and observation

Twenty five radish seeds (sterilized with 0.1% mercuric chloride solution) were placed in each plate. Petri plates were incubated in dim light at 25 °C. Number of seeds germinated and root length was measured at 5th days. The test was performed in triplicate and data were analyzed by ANOVA.

3.2.9. Statistical analysis

Statistical analysis (ANOVA) was performed using software SPSS (version 10.0; SPSS Inc., Chicago IL, USA) and MSTAT (version 2.10; Russell, D. Freed, Michigan State University, USA) and expressed as mean ± SEM. *P* values <0.05 were considered as significant.

Chapter-4
Results

RESULTS

4.1. Antibacterial activity test

4.1.1. Antibacterial activity of Cassia Sophera

The antibacterial activity of *C. sophera* extract against ten human pathogenic bacteria is shown in Table-4.1 and Figure-4.1. The methanol extract of *C. sophera* was effective against both gram negative and gram positive bacteria. The zone of inhibition was ranged from 7.33 to 12.00 mm against all tested bacteria. The highest (12.00 mm) and lowest (8.33 mm) zone of inhibition was found against *S. lutea* and *S. haemolytica* respectively at 300 $mgml^{-1}$. In case of positive control (PC), the zone of inhibition was ranged from 8.67 to 14.33 mm. Negative control (NC) exhibits no zone of inhibition against the entire tested bacteria.

Table-4.1: Antibacterial activity of methanol extract of *C. sophera* against ten human pathogenic bacteria. The activities were measure through zone of inhibition of bacterial strains.

Bacteria	Plant extracts ($mgml^{-1}$)			PC ($30\mu gml^{-1}$)	NC (95% methanol)
	100	200	300		
Gram positive	**Zone of inhibition in mm**				
Staphylococcus aureus	-	8.33±0.33	10.67±0.33	14.33±0.33	-
Bacillus cereus	-	7.67±0.33	9.33 ±0.33	12.00±0.58	-
Streptococcus haemolytica	-	-	8.33±0.33	9.67±0.33	-
Bacillus subtilis	-	8.33±0.33	10.67±0.33	10.67±0.33	-
Sarcina lutea	8.67±0.33	10.00±0.58	12.00±0.58	12.33±0.33	-
Gram Negative	**Zone of inhibition in mm**				
Escherichia coli-B	7.67±0.33	9.33±0.33	11.67±0.33	13.33±0.33	-
Klebsiella pneumoniae	-	7.33±0.33	9.33±0.33	10.67±0.33	-
Pseudomonas aeruginosa	-	8.67±0.33	10.67±0.33	11.33±0.33	-
Salmonella typhi	-	8.33±0.33	10.33±0.33	8.67±0.33	-
Shigella dysenteriae	-	7.67±0.33	9.33±0.33	10.33±0.33	-

4.1.2. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of C. sophera

Results of MIC and MBC of *C. sophera* are presented in Table-4.2. The results reveal that MIC values were ranged from 100 $mgml^{-1}$ (*S. lutea*) to 350 $mgml^{-1}$ (*S. haemolytica*) and MBC values were ranged from 250 $mgml^{-1}$ (*S. lutea*) to 450 $mgml^{-1}$ (*S. haemolytica* and *K. pneumoniae*) against all tested strains. The lowest MIC (100 $mgml^{-1}$) and MBC (250 $mgml^{-1}$) values were demonstrated against *S. lutea*.

Table-4.2: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of methanol extract of *C. sophera*.

Bacteria	MIC ($mgml^{-1}$)	MBC ($mgml^{-1}$)
Gram positive		
Staphylococcus aureus	200	300
Bacillus cereus	300	400
Streptococcus haemolytica	350	450
Bacillus subtilis	250	350
Sarcina lutea	100	250
Gram Negative		
Escherichia coli-B	200	300
Klebsiella pneumoniae	300	450
Pseudomonas aeruginosa	250	350
Salmonella typhi	300	400
Shigella dysenteriae	300	400

4.1.3. Antibacterial activity of Casssia fistula

The antibacterial activity of *C. fistula* extract against ten human pathogenic bacteria is shown in Table-4.3 and Figure-4.1. The methanol extract of *C. fistula* was effective against both gram negative and gram positive bacteria. The zone of inhibition was ranged from 7.33 to 12.67 mm against all tested bacteria. The highest (12.67 mm) and lowest (8.00 mm) zone of inhibition were found against *S. dysenteriae* and *S. haemolytica* respectively at 300 $mgml^{-1}$. In case of positive control, the zone of inhibition was ranged from 8.67 to 14.33 mm. Negative control exhibits no zone of inhibition against the entire tested bacteria.

Table-4.3.: Antibacterial activity of methanol extract of *C. fistula* against ten human pathogenic bacteria. The activities were measure through zone of inhibition of bacterial strains.

Bacteria	Plant extracts ($mgml^{-1}$)			PC ($30\mu gml^{-1}$)	NC (95% methanol)
	100	200	300		
Gram positive	**Zone of inhibition in mm**				
Staphylococcus aureus	-	7.33±0.33	9.33±0.33	14.33±0.33	-
Bacillus cereus	-	-	8.33 ±0.33	12.00±0.58	-
Streptococcus haemolytica	-	-	8.00±0.58	9.67±0.33	-
Bacillus subtilis	7.33±0.33	9.67±0.33	12.00±0.00	10.67±0.33	-
Sarcina lutea	8.67±0.33	10.00±0.58	12.33±0.33	12.33±0.33	-
Gram Negative	**Zone of inhibition in mm**				
Escherichia coli-B	-	8.00±0.58	9.67±0.33	13.33±0.33	-
Klebsiella pneumoniae	-	-	8.33±0.33	10.67±0.33	-
Pseudomonas aeruginosa	-	8.67±0.33	10.00±0.58	11.33±0.33	-
Salmonella typhi	-	7.33±0.33	9.33±0.33	8.67±0.33	-
Shigella dysenteriae	8.67±0.33	10.67±0.67	12.67±0.33	10.33±0.33	-

4.1.4. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of C. fistula

Results of MIC and MBC of *C. fistula* are presented in Table 4.4. The results reveal that MIC values were ranged from 150 $mgml^{-1}$ (*S. lutea* and *S. dysenteriae*) to 350 $mgml^{-1}$ (*B. cereus*, *S. haemolytica* and *K. pneumoniae*) and MBC values were ranged from 250 $mgml^{-1}$ (*S. dysenteriae*) to 500 $mgml^{-1}$ (*K. pneumoniae*) against all tested strains. The lowest MIC (150 $mgml^{-1}$) were demonstrated against both *S. lutea* and *S. dysenteriae* while, lowest MBC (250 $mgml^{-1}$) was demonstrated against *S. dysenteriae*.

Table-4.4.: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of methanol extract of *C. fistula*.

Bacteria	MIC ($mgml^{-1}$)	MBC ($mgml^{-1}$)
Gram positive		
Staphylococcus aureus	300	400
Bacillus cereus	350	450
Streptococcus haemolytica	350	450
Bacillus subtilis	200	350
Sarcina lutea	150	300
Gram Negative		
Escherichia coli-B	250	350
Klebsiella pneumoniae	350	500
Pseudomonas aeruginosa	250	350
Salmonella typhi	300	450
Shigella dysenteriae	150	250

4.1.5. Antibacterial activity of Cassia tora

The antibacterial activity of *C. tora* extract against ten human pathogenic bacteria is shown in Table 4.5 and Figure-4.1. The methanol extract of *C. tora* was effective against both gram negative and gram positive bacteria. The zone of inhibition was ranged from 9.33 mm (*S. haemolytica*) to 15.67 mm (*S. lutea*) against all tested bacteria. The highest (15.67 mm) and lowest (13.00 mm) zone of inhibition was found against *S. lutea* and *S. haemolytica* respectively at 300 $mgml^{-1}$. In case of positive control, the zone of inhibition was ranged from 8.67 to 14.33 mm. Negative control exhibits no zone of inhibition against the entire tested bacteria.

Table-4.5.: Antibacterial activity of methanol extract of *C. tora* against ten human pathogenic bacteria. The activities were measure through zone of inhibition of bacterial strains.

Bacteria	Plant extracts ($mgml^{-1}$)			PC ($30\mu gml^{-1}$)	NC (95% methanol)
	100	200	300		
Gram positive	**Zone of inhibition in mm**				
Staphylococcus aureus	10.67±0.33	12.33±0.33	14.67±0.33	14.33±0.33	-
Bacillus cereus	10.00±0.58	12.33±0.33	14.67±0.33	12.00±0.58	-
Streptococcus haemolytica	9.33±0.33	11.00±0.58	13.00±0.58	9.67±0.33	-
Bacillus subtilis	11.33±0.33	13.00±0.00	15.33±0.33	10.67±0.33	-
Sarcina lutea	12.00±0.58	13.33±0.33	15.67±0.33	12.33±0.33	-
Gram Negative	**Zone of inhibition in mm**				
Escherichia coli-B	10.00±0.58	13.33±0.33	15.33±0.33	13.33±0.33	-
Klebsiella pneumoniae	11.00±0.00	13.00±0.58	15.00±0.00	10.67±0.33	-
Pseudomonas aeruginosa	11.33±0.33	13.33±0.33	15.33±0.33	11.33±0.33	-
Salmonella typhi	11.33±0.33	13.00±0.58	15.00±0.58	8.67±0.33	-
Shigella dysenteriae	10.33±0.33	12.33±0.33	14.67±0.33	10.33±0.33	-

4.1.6. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of C. tora

Results of MIC and MBC of *C. tora* are presented in Table 4.6. The results reveal that MIC values were ranged from 25 $mgml^{-1}$ (*S. lutea*) to 100 $mgml^{-1}$ (*B. cereus*, *S. haemolytica*, *E. coli-B* and *S. dysenteriae*) and MBC values were ranged from 100 $mgml^{-1}$(*S. lutea*) to 250 $mgml^{-1}$ (*B. cereus E. coli* and *S. dysenteriae*) against all tested strains. The lowest MIC (25 $mgml^{-1}$) and MBC (100 $mgml^{-1}$) were demonstrated against *S. lutea*.

Table-4.6.: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of methanol extract of *C. tora*.

Name of Bacteria	MIC ($mgml^{-1}$)	MBC($mgml^{-1}$)
Gram positive		
Staphylococcus aureus	50	200
Bacillus cereus	100	250
Streptococcus haemolytica	100	200
Bacillus subtilis	50	150
Sarcina lutea	25	100
Gram Negative		
Escherichia coli-B	100	250
Klebsiella pneumoniae	50	200
Pseudomonas aeruginosa	50	150
Salmonella typhi	50	200
Shigella dysenteriae	100	250

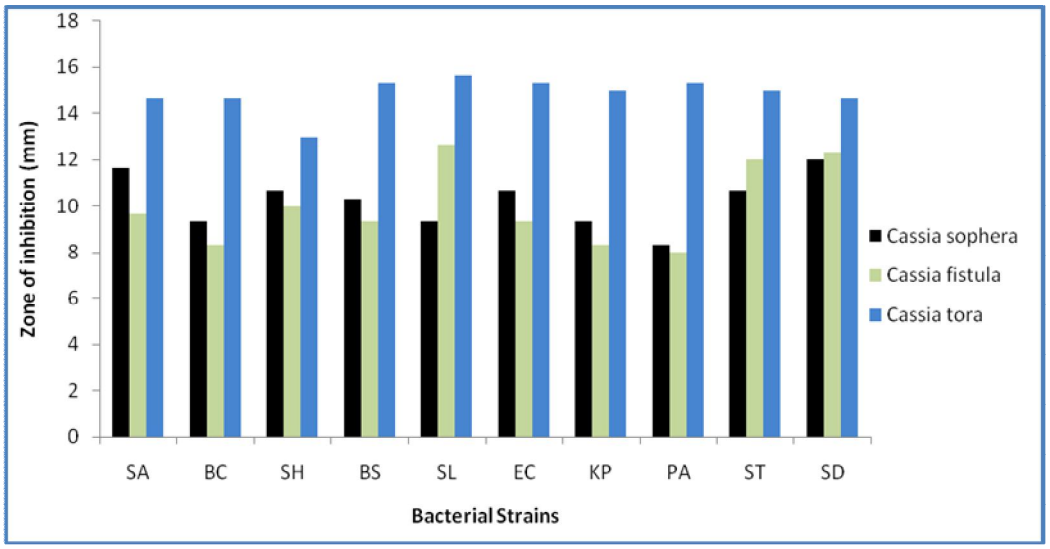


Figure-4.1. Comparative study for the zone of inhibition (mm) of three studied plants extracts at 300 mgml^{-1}. Here, SA = *S. aureus*, BC = *B. cereus*, SH = *S. haemolytica*, BS = *B. subtilis*, SL = *S. lutea*, EC = *E. coli*-B, KP = *K. pneumoniae*, PA = *P. aeruginosa*, ST = *S. typhi*, SD = *S. dysenteriae*.

4.1.7. Conclusion for antibacterial activity

- The overall result showed that *C. tora* was more effective against all tested bacteria followed by *C. sophera* and *C. fistula*.
- The methanol extract of *C. tora* was shown the highest antibacterial activity against *S. lutea* (15.67 mm).
- The lowest MIC (25 mg/ml) and MBC (100 mgml^{-1}) were observed by *C. tora* against *S. lutea*.

4.2. Antitumor activity test

Antitumor activity of three plants extracts were assessed using the potato disc bioassay. Three *Agrobacterium tumefaciens* strains (AtTa0112, AtAc0114 and AtSl0105) were used as a tumor forming agent on potato disc. Results are presented under the following heads.

4.2.1. Antibacterial assay against A. tumefaciens (as a partial assay)

Before antitumor activity test antibacterial assay was performed against all strains of *A. tumefaciens* to check whether extracts are lethal for bacteria or are inhibiting at any level that is necessary for the genetic transfer mechanism and finally induction of tumor. The effect of extracts on viability of *A. tumefaciens* was evaluated by using the agar disc diffusion method. Results of this assay confirmed extract has no effect on the viability of *Agrobacterium* strains because no zone of inhibition was recorded against all the studied *A. tumefaciens* strains. Whereas, inhibition zone were recorded for cefuroxime and kanamycin though negative control (95% methanol) did not show any visible zone of inhibition (Plate-4.1.). So tumor inhibition was observed only for the plant extract not for the other factors. This result indicates that there was no effect of plants extract on the viability of *A. tumefaciens*.

4.2.2. Antitumor potato disc assay

All Extracts exhibited tumor inhibition at the three concentrations (10 ppm, 100 ppm, and 1000 ppm) tested. Significant tumor inhibition was observed at 100 ppm and 1000 ppm but no significant tumor inhibition was observed at lower dose (10 ppm) against all the studied *A. tumefaciens* strains. Tumor inhibition was observed in a concentration-dependent mode. Statistical analysis using ANOVA showed that the effect of concentrations and particular plant extracts were highly significant (Table-4.7, 4.8 & 4.9).

PLATE-4.1

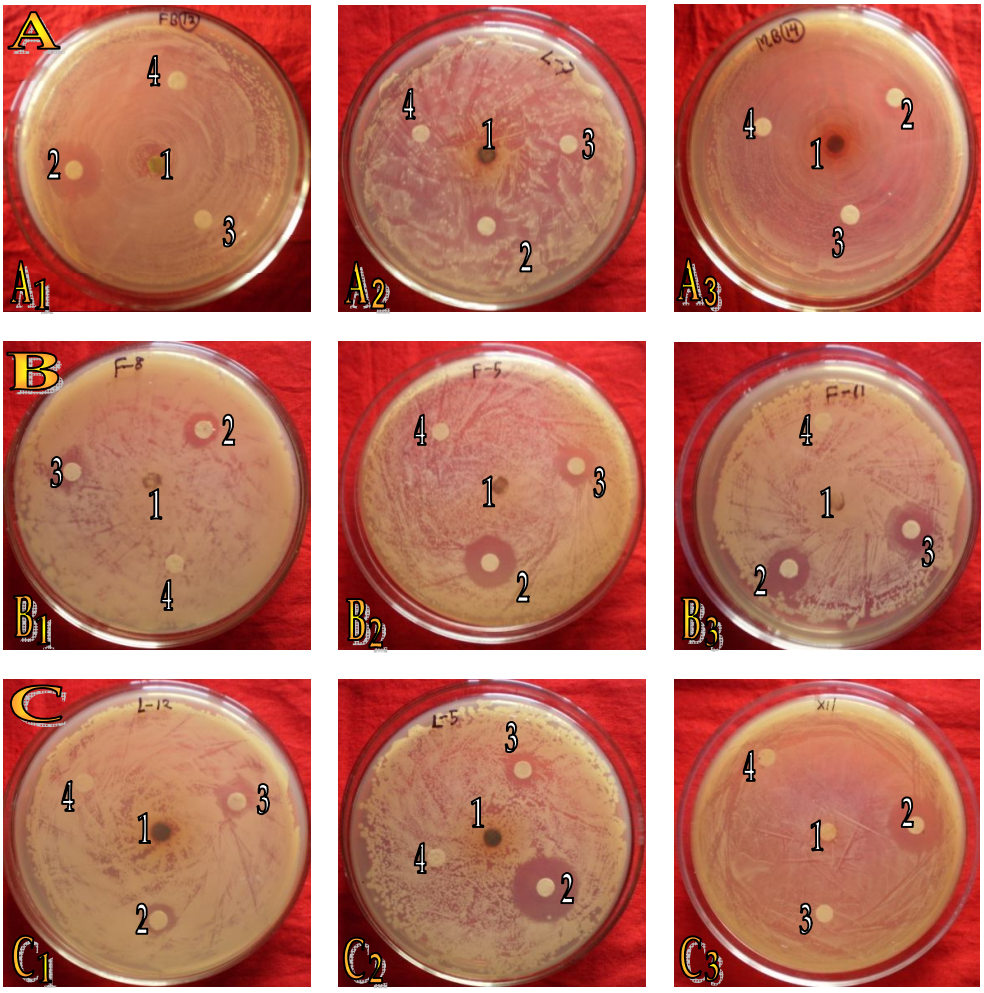


Plate-4.1: Effect of methanolic leaf extracts and antibiotics on three *A. tumefaciens* strains. Here, A = AtTa0112 [A_1: 1=*C. sophera*; A_2: 1= *C. fistula*; A_3: 1= *C. tora*]; B = AtAc0114 [B_1: 1=*C. sophera*; B_2: 1= *C. fistula*; B_3: 1= *C. tora*]; C = AtSl0105 [C_1: 1=*C. sophera*; C_2: 1= *C. fistula*; C_3: 1= *C. tora*]; 2 = Kanamycin; 3= Cefuroxime; 4 = Negative control (95% methanol).

4.2.2.1. Effect of methanol leaf extract of C. sophera on crown gall tumors produced by A. tumefaciens in the potato discs

Statistical analysis indicates that the methanol leaf extract of *C. sophera* reduce tumor formation is highly significant way in a concentration dependent manner across the strains (Table-4.7). Significant tumor inhibition was observed at 100 ppm and 1000 ppm plant extracts compared with the negative control, but 10 ppm had not shown any significant tumor inhibition against all the strains. Maximum 43.92%, 45.28% and 47.66% and minimum 15.42%, 16.03% and 19% tumor inhibition were recorded for AtTa0112, AtAc0114 and AtSl0105 *Agrobacterium* strains respectively (Figure-4.2). It was also observed that *A. tumefaciens* strain AtSl0105 was more prominent for producing tumor (8.5±0.31) followed by strains AtAc0114 (7.6±0.08) and AtTa0112 (7.13±0.14) suggests their differential sensitivity (Figure-4.2 and Plate-4.2), whereas 100% tumor inhibition was observed in positive control (Plate-4.2).

4.2.2.2. Effect of methanol leaf extract of C. fistula on crown gall tumors produced by A. tumefaciens in the potato discs

Statistical analysis indicates that the methanol leaf extract of *C. fistula* of reduce tumor formation in highly significant way in a concentration dependent manner across the strains (Table-4.8). Significant tumor inhibition was observed at 100 ppm and 1000 ppm plant extracts compared with the negative control, but 10 ppm had not shown any significant tumor inhibition against all the strains. Maximum 45.66%, 46.69% and 47.70% and minimum 16.63%, 19.75% and 21.47% tumor inhibition were recorded for AtTa0112, AtAc0114 and AtSl0105 *Agrobacterium* strains respectively (Figure-4.3). It was also observed that *A. tumefaciens* strain AtSl0105 was more prominent for producing tumor (7.8±0.69) followed by strains AtAc0114 (7.3±0.58) and AtTa0112 (6.1±0.31) suggests their differential sensitivity (Figure-4.3 and Plate 4.3), while 100% tumor inhibition was observed in positive control (Plate 4.3).

Table-4.7.: Statistical analysis of tumor inhibition by the methanol extract of *C. sophera* and tumor induction by *A. tumefaciens* strains on potato discs.

Source of variation	Degree of Freedom	Sum of squares	Mean Square	F Value
Strains (S)	2	0.09	0.04	<1
Concentration (C)	3	49.03	16.34	121.98
S × C	6	0.13	0.02	<1
Error	24	2.90	0.26	
Total	35	52.15		

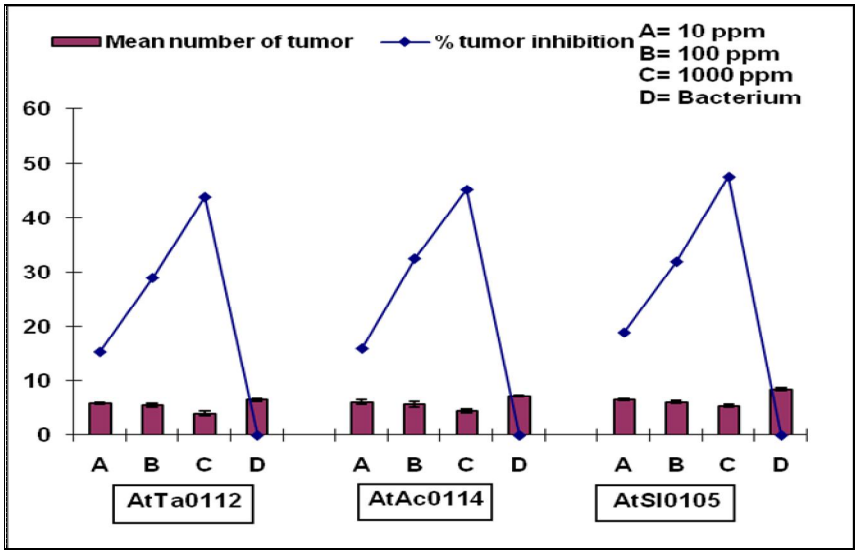


Figure-4.2: Effect of methanol leaf extract of *C. sophera* on crown gall tumors produced by *A. tumefaciens* strains on potato discs.

PLATE- 4.2

				
Bacterium (AtSI0105)	10ppm	100ppm	1000ppm	30ppm
				
Bacterium (AtTa0112)	10ppm	100ppm	1000ppm	30ppm
				
Bacterium (AtTa0114)	10ppm	100ppm	1000ppm	30ppm

Plate-4.2: Photographs show gradual tumor inhibition by the methanol leaf extract of *C. sophera* on potato discs in a concentration as well as strain dependent manner. A_1, B_1 and C_1 as negative control (methanol 95%); A_2, B_2 and C_2 as 10 ppm; A_3, B_3 and C_3 as 100 ppm, A_4, B_4 and C_4 as 1000 ppm plant extract and A_5, B_5 and C_5 as 30 ppm positive control (camptothecin).

Table-4.8: Statistical analysis of tumor inhibition by the methanol extract of *Cassia fistula* and tumor induction by *A. tumefaciens* strains on potato discs.

Source of variation	Degree of Freedom	Sum of squares	Mean Square	F Value
Strains (S)	2	0.18	0.09	2.52
Concentration (C)	3	55.46	18.48	47.59
S × C	6	0.12	0.02	<1
Error	24	2.90	0.41	
Total	35	58.66		

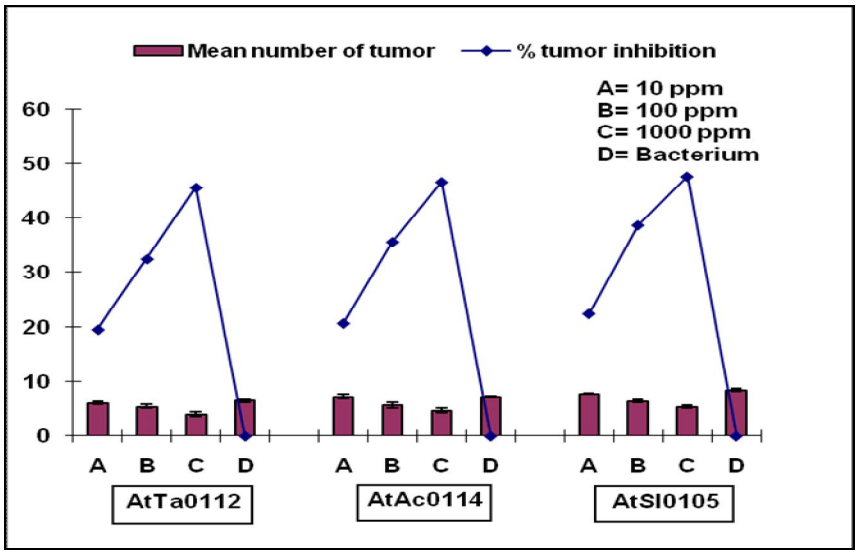


Figure-4.3: Effect of methanol extract of *C. fistula* on crown gall tumors produced by *A. tumefaciens* strains on potato discs.

PLATE-4.3

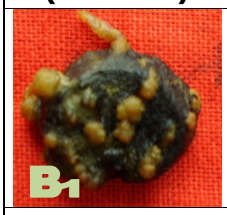
				
Bacterium (AtSI0105)	**10ppm**	**100ppm**	**1000ppm**	**30ppm**
				
Bacterium (AtTa0112)	**10ppm**	**100ppm**	**1000ppm**	**30ppm**
				
Bacterium (AtTa0114)	**10ppm**	**100ppm**	**1000ppm**	**30ppm**

Plate-4.3: Photographs show gradual tumor inhibition by the methanol extract of *C. fistula* on potato discs in a concentration as well as strain dependent manner. A_1, B_1 and C_1 as negative control (methanol 95%); A_2, B_2 and C_2 as 10 ppm; A_3, B_3 and C_3 as 100 ppm, A_4, B_4 and C_4 as 1000 ppm plant extract and A_5, B_5 and C_5 as 30 ppm positive control (camptothecin).

4.2.2.3. Effect of methanol leaf extract of C. tora on crown gall tumors produced by A. tumefaciens in the potato discs

Different concentrations of methanol leaf extract of *C. tora* show significant difference in respect of tumor inhibition. Tested three *A. tumefaciens* strains also differ significantly in respect of sensitivity against the plant extracts (Table-4.9).

Significant tumor inhibition was observed at 100 ppm and 1000 ppm plant extracts compared with the negative control, but 10 ppm had not shown any significant tumor inhibition against all the strains. Maximum 53.88, 55.18 and 57.14% and minimum, 21.46, 22.64 and 23.80% tumor inhibition were recorded for AtTa0112, AtAc0114 and AtSl0105 *Agrobacterium* strains respectively (Figure 4.4). It was also observed that *A. tumefaciens* strain AtSl0105 was more prominent for producing tumor (6.5±0.65) followed by strains AtAc0114 (6.1±0.55) and AtTa0112 (5.8±0.28) suggests their differential sensitivity (Figure 4.4 and Plate 4.4), while 100% tumor inhibition was observed in positive control (Plate 4.4).

4.2.3. Conclusion for antitumor activity

Result of the present study, three medicinal plants extracts presented a significant percentage of tumor inhibition in potato disc assay.

- This result showed that *C. tora* (57.14%) was more effective for inhibition of tumor than *C. fistula* and *C. sophera.*
- Significant tumor inhibitions were observed at 100 ppm and 1000 ppm concentrations.
- The *A. tumefaciens* AtSl0105 strain was more prominent for producing tumor than strains AtAc0114 and AtTa0112.
- So the present investigation showed that the result varied from strain to strain and concentrations of different plant extracts.

Table-4.9: Statistical analysis of tumor inhibition by the methanol extract of *Cassia tora* and tumor induction by *A. tumefaciens* strains on potato discs.

Source of variation	Degree of Freedom	Sum of squares	Mean Square	F Value
Strains (S)	2	0.97	0.48	6.51
Concentration (C)	3	73.82	24.60	188.64
S × C	6	0.04	0.08	<1
Error	24	1.97	0.20	
Total	35	76.80		

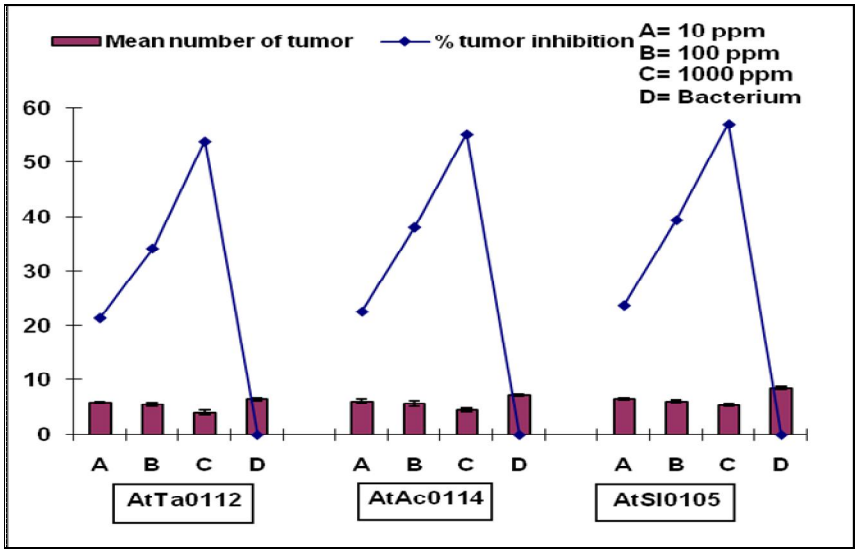


Figure-4.4: Effect of methanol extract of *C. tora* on crown gall tumors produced by *A. tumefaciens* strains on potato discs.

PLATE- 4.4

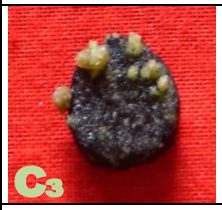
				
Bacterium (AtSI0105)	**10ppm**	**100ppm**	**1000ppm**	**30ppm**
				
Bacterium (AtTa0112)	**10ppm**	**100ppm**	**1000ppm**	**30ppm**
				
Bacterium (AtTa0114)	**10ppm**	**100ppm**	**1000ppm**	**30ppm**

Plate-4.4: Photographs show gradual tumor inhibition by the methanol extract of *C. tora* on potato discs in a concentration as well as strain dependent manner. A_1, B_1 and C_1 as negative control (methanol 95%); A_2, B_2 and C_2 as 10ppm; A_3, B_3 and C_3 as 100ppm, A_4, B_4 and C_4 as 1000ppm plant extract and A_5, B_5 and C_5 as 30ppm positive control (camptothecin).

4.3. Preliminary qualitative phytochemical screening

The preliminary qualitative analyses of bioactive compounds for the studied three plants extracts were analyzed in this study and wide range of phytochemical compounds were present in the three extracts shown in table-4.10. In this study, aqueous leaf extracts were examined qualitatively for their various secondary metabolites such as alkaloid, flavonoid, glycoside, terpenoid, sterol, saponin, tannin and phenol contents. The presence of secondary metabolites were denoted by "+" sign and absence of metabolites were denoted by "-'' sign. Out of three plants extracts, *C. tora* extract demonstrated the maximum occurrence of phytoconstituents (9/11) such as alkaloids, flavonoids, saponins, tannins, glycosides and sterol, and absence of phenols and terpenoids were observed. Followed by *C. fistula* extract showed presence of alkaloids (moderately), flavonoids, saponins, tannins, phenol and terpenoids and absence of glycosides and sterols, whereas *C. sophera* extract showed presence of alkaloid, flavonoids, saponins, tannins, glycosides and steroids and absence of phenol and tannins (Table-4.10).

From the preliminary test results, flavonoid and tannins test were positively shown by studied three plants extracts. Alkaloid and saponin were seen both in *C. fistula* and *C. tora* but absent in *C. sophera* extracts, whereas glycoside and sterol were found both in *C. sophera* and *C. tora*. Phenols and triterpinoids were observed only in *C. fistula* extract (Table-4.10.; Plate-4.5.). From results, various components were present, indicating their solubility in water while, the presence and absence of the phytoconstituents depends on the solvent medium used for extraction and the physiological property of individual taxa.

Table-4.10.: Phytochemical screening of leaf extract of three plants.

Secondary Metabolites	Test/Reagent	*C. sophera*	*C. fistula*	*C. tora*
Alkaloids	Dragendorff's (D)	-	+	+
	Mayer's (M)	+	+	+
	Wagner's (W)	-	-	+
	Hager's (H)	-	+	+
	Tannic acid (T)	-	+	+
Flavonoids	Con. HCl & magnesium	+	+	+
Saponins	Foam	-	+	+
Tannins	Ferric chloride	+	+	+
Phenols	Ferric chloride	-	+	-
Glycosides	conc. HCl& NaOH	+	-	+
Terpenoids	Acetic anhydride & con. H_2S0_4	-	+	-
Sterols		+	-	+

Note: + = Positive/ Present; - = Negative/Absent

PLATE-4.5

A B C

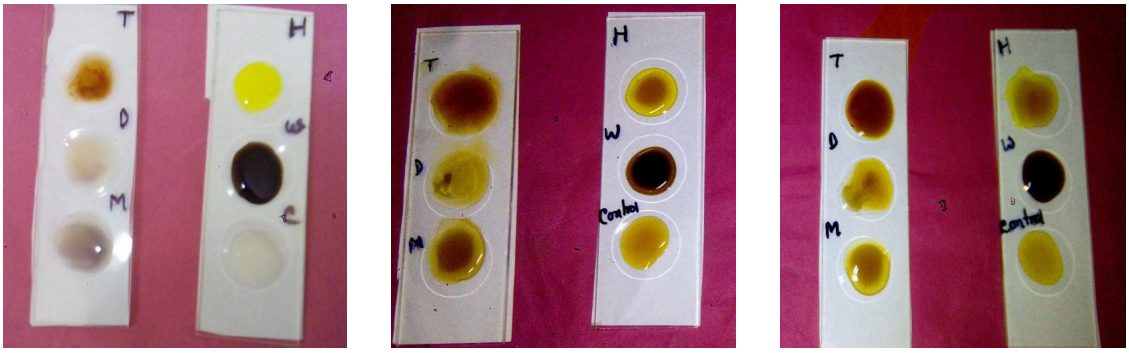


Figure: Alkaloids test A= *C. sophera;* B= *C. fistula*; C= *C. tora.*

A B C

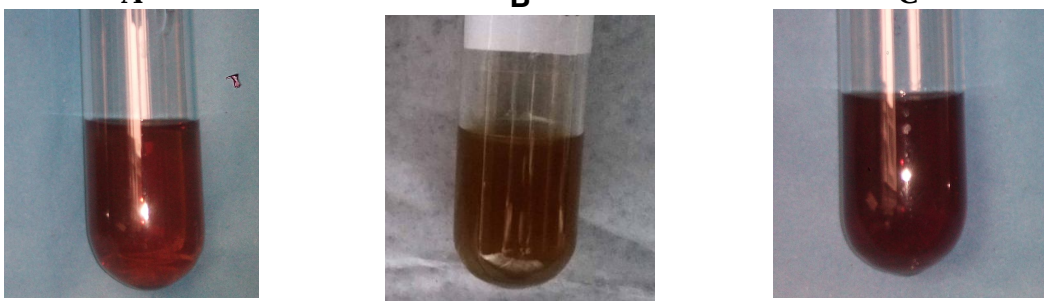


Figure: Flavonoids test A= *C. sophera;* B= *C. fistula*; C= *C. tora.*

A B C

Figure: Saponins test A= *C. sophera;* B= *C. fistula*; C= *C. tora.*

Plate- 4.5: Phytochemical screening of leaf extract of three plants extracts.

4.4. Radish seed phytotoxicity assay

Radish seed (*Raphanus sativus L.*) germination assay is important to define allelopathic potential of extracts. The activity involves the measurement of the average root length and number of seeds inhibition in which the water serves as negative control. Three plants: *C. sophera*, *C. fistula* and *C. tora* were investigated for phytotoxic analysis to evaluate their phytotoxic effects on seed germination and root length of radish seedling (Figure 4.5 & 4.6; Plate-4.6). A gradual increase in seed germination for all extracts was observed till 5th day of incubation. The seed germination inhibition was said to be significantly high when compared to control (Figure 4.5; Plate-4.6). Similarly, the root lengths of radish seeds germinated were significantly inhibited by the three extracts at 10,000 ppm concentrations (Figure-4.6). Maximum seed germination inhibition (57%) was observed after 5th day of incubation by methanol leaf extract of *C. sophera* than *C. tora* (23%) and *C. fistula* (16%) respectively, whereas negative and positive control did not show any inhibition (Figure 4.5 and Plate-4.6). Minimum root length (1.39 mm) was observed when radish seeds were grown in the presence of methanol leaf extract of *C. sophera*, while 3.56 mm and 6.93 mm were *C. tora* and *C. fitula* respectively (Figure-4.6). The order of phytotoxicity for the given extracts were *C. sophera* > *C. tora* > *C. fistula*.

4.4.1. Conclusion for phytotoxicity test

- Maximum seed germination inhibition (57%) was observed after 5th day of incubation by methanol leaf extract of *C. sophera*
- Minimum root length (1.39 mm) was observed when radish seeds were grown in the presence of *C. sophera.*

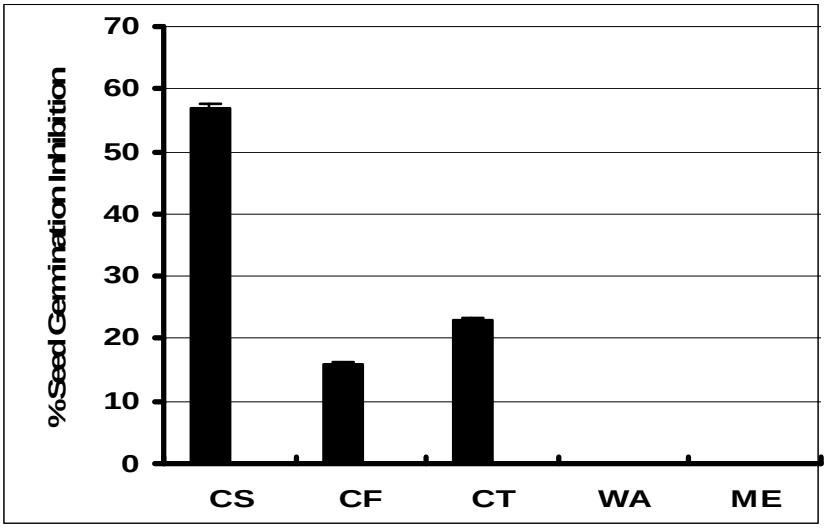


Figure-4.5. Percentage of radish seed germination inhibition by three plants extracts (at 10,000 ppm) after 5th day of incubation. Here CS= *C. sophera;* CF= *C. fistula*; CT= *C. tora*; WA= water; ME= 95% methanol.

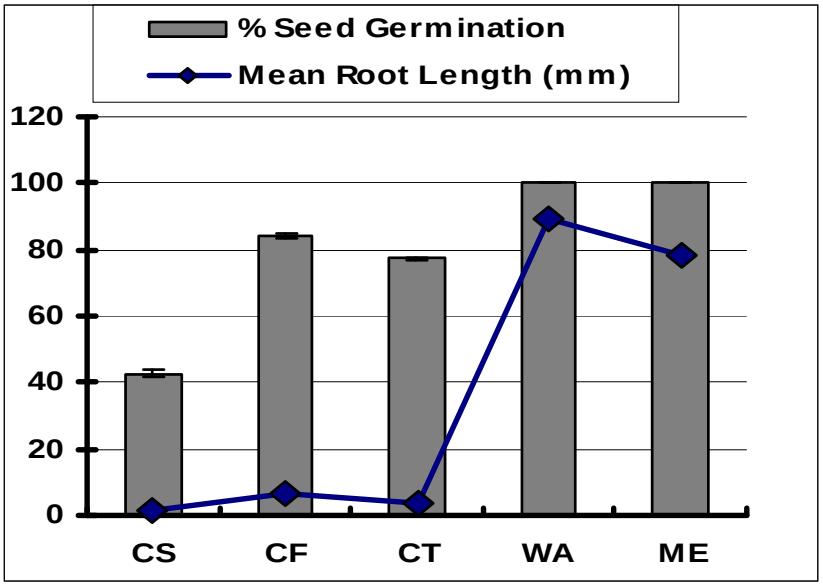


Figure-4.6. Effect of three plant extracts (at 10000 ppm) on radish seed germination and root length after 5th day of incubation. Here CS= *C. sophera;* CF= *C. fistula*; CT= *C. tora*; WA= water; ME= 95% methanol.

PLATE-4.6

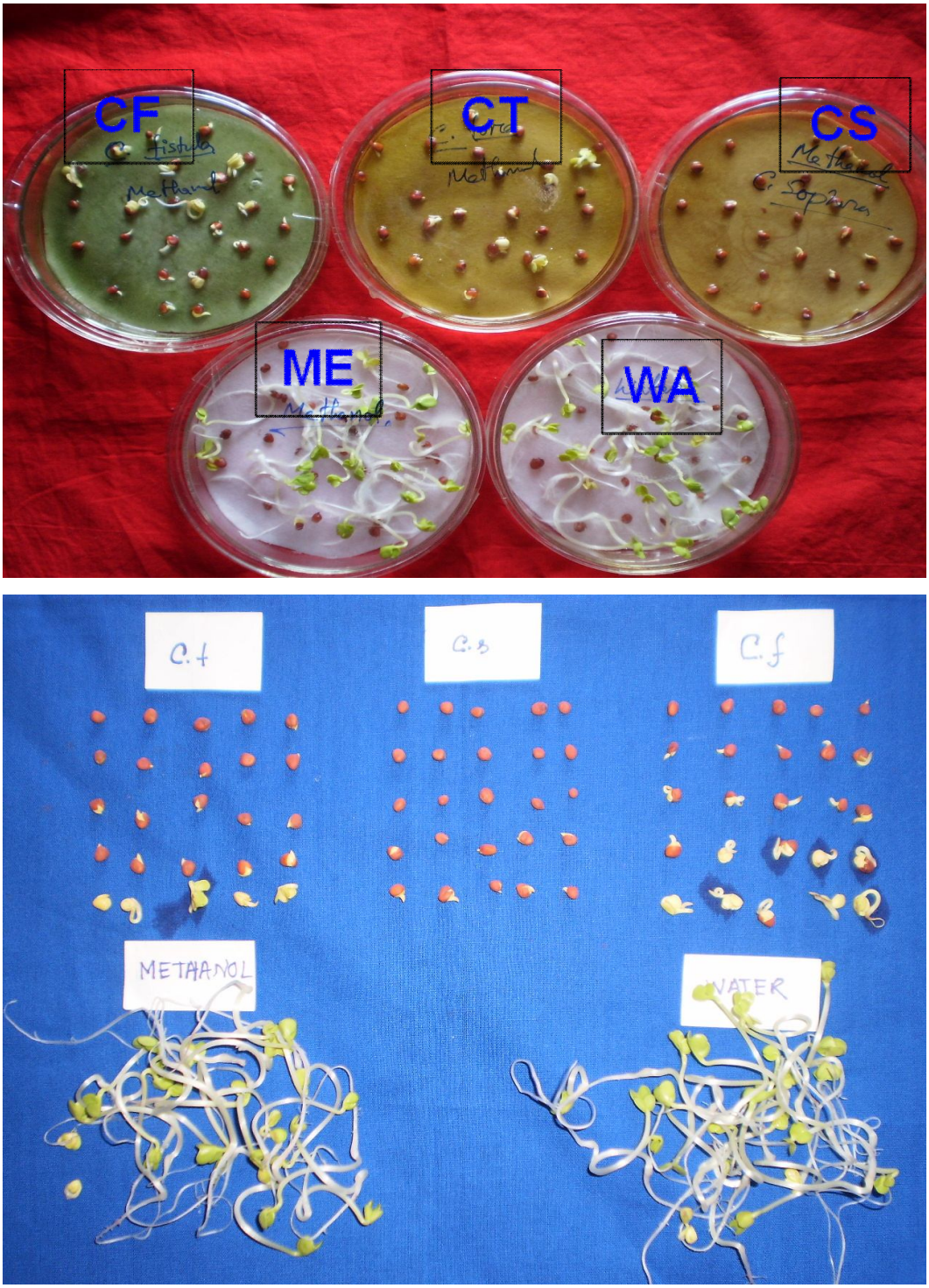


Plate-4.6: Photographs show radish seed germination inhibition by three plants extracts (at 10,000 ppm) after 5th day of incubation. Here CS= *C. sophera;* CF= *C. fistula*; CT= *C. tora*; WA= water; ME= 95% methanol.

Chapter-5
Discussion

DISCUSSION

5.1. Antibacterial activity test

Microorganisms are the concealed enemies to the mankind (Dash et al. 2011). To solve the drawbacks of chemical disinfectants, the agent which have the capacity to kill the microbes or arrest the multiplication, are called the antimicrobial agents or drugs. There are a lot of antibacterial agents of which some are discovered or established and some are hidden in the nature. Medicinal plants are rich sources of bioactive compounds and thus serve as important raw materials for drug production (Dash 2016). It is estimated that approximately one quarter of prescribe drugs contain plant extract or active ingredients obtained from or modeled of plant substances (Tripathi and Tripathi 2003).

Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. The presence of antibacterial substances in the higher plants is well established (Srinivasan et al. 2001, Rahman et al. 2011b, Nasrin et al. 2016b). Phytomedicine can be used for the treatment of diseases as is done in case of Unani and Ayurvedic system of medicines or it can be the base for the development of a medicine, a natural blueprint for the development of a drug (Parekh and Chanda 2007). The first step towards this goal is the *in vitro* antibacterial activity assay (Tona et al. 1998).

The *in vitro* antimicrobial activities from extracts by *Cassia* species have been reported from various parts of the world (Anushia et al. 2009). In the present investigation, the extracts of three plants namely *C. sophera, C. fistula and C. tora* were determined for antibacterial activities against ten pathogenic bacteria. From results, plants extracts were very effective against all tested bacteria, whereas various species of *Cassia* are reported to have antimicrobial

properties (Mazumder et al. 2008). Out of three plants extracts *C. tora* was more effective against all tested bacteria than *C. sophera* and *C. fistula*. The diameter of highest zone of inhibition was found 15.67 mm against gram positive *S. lutea*, which was previously studied by other plants (Kalyoncu et al. 2010, Begum et al. 2017). From the phytochemical results, this finding is probably due the alkaloid which was only present in this plant. Many researcher observed that alkaloid has a high capability of antimicrobial and antitumor properties (Cushnie et al. 2014, Kaur and Arora 2015, Mabhiza et al. 2016, de Almeida et al. 2017).

Extracts showed different degrees of growth inhibition depending upon the bacterial strains. Many researcher obtained similar results in other plants (Sheikh et al. 2010, Rahman et al. 2011a, 2011b, 2011c, Islam et al. 2013, Sayeed et al. 2014a, Nasrin et al. 2016a). These variations were found because strains are genetically different from each other, and this is probably due to the differences in chemical composition and structure of the cell wall of both types of microorganisms (Goyal et al. 2009), microbial growth, exposure of microorganisms to plant extracts, the solubility of extracts or extract components and the use and quantity of an emulsifier (Bansod and Rai 2008). Some investigators noted that sensitivity of microorganisms to chemotherapeutics differs according to type of strain (Çetin and Gürler 1989, Gücin et al. 1996). Studied three extracts consistently displayed superior potency when compared with typical antibiotics, because extracts were a mixture of various plant constituents and antibiotics were a refined and purified product (El-Mahmood and Doughari 2008). In most countries, chemical preservatives and antibiotics are not permitted in foods. There has been a great shift from the prescription of antibiotics to the use of medicinal plants (Ekwenye and Elegalam 2005).

From antibacterial activities of extracts, no significant differences were found between gram positive and gram negative bacteria, though most of the gram negative bacteria are more resistant than gram positive bacteria (Tomas-Barberan et al. 1988). It is an indication of the presence of broad spectrum antibiotic compounds or simply metabolic toxins in the plant (Parekh and Chanda 2007). However, according to zone of inhibition gram negative bacteria is more prominent than gram positive bacteria. Rizvi et al. (2009) observed that *Cassia* species had a significant activity against Gram positive microorganisms. This is in agreement with previous reports that plant extracts are more active against the gram positive bacteria than gram negative bacteria (Vlietinck et al. 1995, Rabe and van Staden 1997, Sheikh et al. 2010, Rahman et al. 2011a, 2011b, 2011c, Islam et al. 2013, Sayeed et al. 2014a, Nasrin et al. 2016a). This may be attributed to the fact that these two groups differ by its cell wall component and its thickness (Yao and Moellering 1995). As revealed in this study, *Cassia* extracts produces a wide-spectrum antibacterial effect on Gram-positive and Gram-negative microorganisms. It has determined that this antibacterial action is linked with the presence of complex phytochemicals (Nasrin et al. 2016a).

Increasing of the concentrations level of extracts had a significant inhibitory effect on all studied bacteria. Ekwenye and Elegalam (2005), Azu and Onyeagha (2007) reported that the efficacy of most plant extracts is concentration dependent. Also previous report said that active compounds may be present in insufficient quantities in the crude extracts to show activity with the dose levels employed and lack of activity can thus only be proven by using large dose and extracts may also be active against other bacterial species which were not tested (Farnsworth 1993, Jäger et al. 1996, Shale et al. 1999, Taylor et al. 2001).

It has been demonstrated that different phytoconstituents have different degree of solubility in different types of solvents depending on their polarity (El-Mahmood and Doughari 2008). Several authors have conducted antibacterial study using methanol as an extraction solvent and shown comparatively better activity than others (El-Mahmood and Ameh 2007, Nataraj et al. 2009, Prasannabalaji et al. 2012). The exact mechanism by which methanolic extract mediates its antibacterial effect is still to be elucidated. It is well documented that alcohols (ethanol or methanol) used as a solvent for plant extract preparation for their strongly extraction power. Many researchers have already been used methanol or ethanol as a solvent for evaluating cytotoxicity, phytotoxicity, antibacterial, antitumor activity in several plant species (Turker and Camper 2002, Hussain et al. 2007, Inayatullah et al. 2007, Nasrin et al. 2016b).

In this study the low MIC and MBC values (25 $mgml^{-1}$ and 100 $mgml^{-1}$) observed for *S. lutea* is a good indication of high efficacy against gram positive and gram negative bacteria and high MIC and MBC values are indication of low activity (El-Mahmood and Doughari 2008). These observations may be attributed to two reasons: firstly, the nature of biological active components whose activity can be enhanced in the presence of methanol. Secondly the strongly extraction capacity of methanol could have produced greater number of active constituents responsible for antibacterial activity (Bhattacharjee et al. 2006). One of the important findings of the study was that MIC values of extracts were lesser than MBC values which suggested that these extracts were bacteriostatic at lower concentration and bactericidal at higher concentration (Hassan et al. 2009).

5.2 Antitumor activity test

The areas of cancer and infectious diseases have a leading position in utilization of medicinal plants as a source of drug discovery. Among FDA approved anticancer and antiinfectious preparations drugs of natural origin have a share of 60% and 75% respectively (Newman et al. 2003). It is worthy to mention the vivid current interest in discovery of natural drugs for cancer treatment and chemoprevention (Kucuk 2002, Balunas and Kinghorn 2005). Huge number of plant species is screened and bioassayed for this purpose worldwide (Richardson 2001).

In recent years, plant derived bioactive substances that are capable of selectively arresting cell growth in tumor cells have received conciderable attention in cancer chemopreventive approaches (Jang et al. 2005). Many studies have been reported that the active phytochemicals with anti-cancer, anti-invasive and anti-metastatic activities in cancer cells (Ramos et al. 2005, Weng and Yen 2011, Rana et al. 2015, Nasrin et al. 2016b).

Plants continue to provide us new chemical entities (lead molecules) for the development of drugs against various pharmacological targets, including cancer (Jachak and Salani 2007). Some of the higher plant products i.e. vinblastine, vincristine, podophyllotoxin derivatives including etoposide and camptothecin and its derivative have already been marketed as very important anticancer drugs (Misawa 1994, Sayeed et al. 2014b). Based on this information, studied three plants extract was evaluated for their antitumor properties using potato disc bioassay. It was demonstrated that inhibition of crown gall tumor initiation on potato disc showed an apparent correlation with compounds and plant extracts known to be active in the 3PS (in vivo, murine leukemia) antitumor assay (Coker et al. 2003).

Antitumor potato disc assay is a valuable tool that indicates antitumor activity of test compound by their inhibition of formation of characteristic

crown galls induced in wounded potato tissues by *A. tumefaciens* (Inayatullah et al. 2007). The use of this bioassay has resulted in many short lists of plants with anticancer activity, and has helped with the discovery of novel compounds from plants (Galsky et al. 1981, Ferrigni et al. 1982, McLaughlin 1991, Lellau and Liebezeit 2003, Ahsan et al. 2007).

The members of *Cassia* species are rich sources of polyphenols, anthraquinone derivatives, flavonoids, and polysaccharides (Moriyama et al. 2003, Mohammed et al. 2013), and they have been found to exhibit anticancer activity (Prasanna et al. 2009, Yadav et al. 2010). Glycoside also plays major role in the cancer therapy (Hussain et al. 2008).

Before antitumor studies, antibacterial assay of plants extracts was performed to check viability of *Agrobacterium* and no inhibitory effect of plants extracts were recorded on viability of *Agrobacterium* growth. So results revealed that tumor formation was decreased only for the plants extracts not for the other factors. Similar phenomenon were found by Turker and Camper (2002), Hussain et al. (2007), Inayatullah et al. (2007), Islam et al. (2008, 2009, 2010a, 2010b), Sarker et al. (2011), Islam et al. (2013), Mannan et al. (2014) and Sayeed et al. (2014b). Hussain et al. (2007) also demonstrated antibacterial activity against *A. tumefaciens* to check whether extracts are lethal for bacteria or are inhibiting at any level that is necessary for the genetic transfer mechanism and finally induction of tumor. On the other side antibiotic resistance test showed that all *A. tumefaciens* strains were susceptible to kanamycin or cefuroxime which was supported by Koivunen et al. (2004), Karthy et al. (2009) and Islam et al. (2010).

Although no significant differences were found in tumor inhibition among three extracts but *C. tora* (57.14%) was more effective for inhibition of tumor followed by *C. fistula* (47.70%) and *C. sophera* (47.66%) at 1000 ppm on AtSl0105 respectively. This is due to presence of alkaloid in its leaves which

was previously observed by many research (Cushnie et al. 2014, Kaur and Arora 2015, Mabhiza et al. 2016, de Almeida et al. 2017). Rejiya et al. (2009) observed that *C. tora* leaf extracts possess potential synergistic cytotoxic effect with anticancer drugs, such as, cisplatin. Another research showed that emodine an anthraquinone present in *C. tora* possess anti-tumor activity. It shows inhibitory effect on angiogenic and metasis regulatory process. Because of its quinine like structure, emodine may interfere with electron transport process and in altering cellular redox status, which may account for its cytotoxic property (Choi et al. 1998). Medicinally *C. fistula* has been various pharmacological activities like antimicrobial and antifungal (Duraipandiyan and Ignacimuthu 2007), antipyretic, analgesic, larvicidal, anti-inflammatory, antioxidant, anti-tumor, hepatoprotective hypoglycemic activities (Gupta et al. 2000, Rajesh et al. 2014).

The mechanism by which *Agrobacterium* inserts materials into the host cell by a type IV secretion system, is very similar to mechanisms used by pathogens to insert materials (usually proteins) into human cells by type III secretion (Lai and Kado 2000). The Ti-plasmid causes the plant's cells to multiply rapidly without going through apoptosis, resulting in tumor formation similar in nucleic acid content and histology to human and animal cancers (Agrios 1997). According to Kempf et al. (2002), *Bartonella henselae*, a tumor causing bacteria in human shares a similar pathogenicity strategy to plant pathogen *A. tumefaciens*.

During antitumor activity test, tumor formation was observed when *Agrobacterium* strains were alive on living potato disc. A few potato discs were damaged due to the contamination and other physiological factors when there was no tumor formation was observed. This result indicates that living substrates (cell) with *Agrobacterium* is very much needed for finally induction tumor on potato disc. Such type results were observed by Islam et al. (2008,

2009, 2010a, 2010b), Sarker et al. (2011), Islam et al. (2013), Sayeed et al. (2014b) and Mannan et al. (2014). The attachment of the bacterium to a tumor-binding site is complete within 15 min following inoculation (Gologowski and Galsky 1978, McLaughlin et al. 1993). Turker and Camper (2002) described that inhibition of tumor formation in the potato disc assay could result from either anti-tumorigenesis or by affecting the viability of *A. tumefaciens*.

Based on the tumor induction capability on potato discs, studied three tested *A. tumefaciens* strains were significantly different which suggest their differential sensitivity. It was also observed that used three concentrations (10, 100 and 1000 ppm) of the plant extracts were also significantly different from each others in respect of tumor inhibition and inhibitions were only observed at 100 ppm and 1000 ppm concentrations. Hussain et al. (2007) have also shown that tumor inhibition rate on potato discs are depend on concentration of plant extract and also tumor producing *A. tumefaciens* strains. Overall results showed that *A. tumefaciens* strain AtSl0105 was more prominent strain for producing tumor followed by AtAc0114 and AtTa0112 strains suggests their different sensitivity. Islam et al. (2008, 2009, 2010a, 2010b), Sarker et al. (2011), Islam et al. (2013), Sayeed et al. (2014b) and Mannan et al. (2014) have also found similar results.

Results showed that all extracts of *Cassia* spp. significantly inhibited tumor formation on potato discs which indicates it could be a potential source of antitumor properties. Several workers conducted similar type of investigation and recommend large number of plant extracts as a potential source of anticancer agent (Turker and Camper 2002, Inayatullah et al. 2007, Hussain et al. 2007, Islam et al. 2008, 2009, 2010a, 2010b, Sarker et al. 2011, Islam et al. 2013, Sayeed et al. 2014b and Mannan et al. 2014)).

The exact mechanism by which methanol extract mediates its antitumor effect is still to be elucidated. Cytological changes indicate that methanol extract

might be having a direct tumorocidal effect on the tumour cells (Anushia et al. 2009). It is well documented that alcohols (ethanol or methanol) used as a solvent for plant extract preparation for their strongly extraction power. Many researchers have already been used methanol or ethanol as a solvent for evaluating cytotoxicity, phytotoxicity, antibacterial, antitumor activity in several plant species (Turker and Camper 2002, Hussain et al. 2007, Inayatullah et al. 2007). Camptothecin served as a positive control and 100% tumor inhibition was observed. Similar phenomenon was observed by Turker and Camper (2002) and Islam et al. (2009).

5.3 Phytochemical analysis

Drugs from the plants are easily available, less expensive, safe, and efficient and rarely have side effects. The plants which have been selected for medicinal use over thousands of years constitute the most obvious choice of examining the current search for therapeutically effective new drugs such as anticancer drugs (Dewick 1996), antimicrobial drugs (Phillipson and Wright 1996), antihepatotoxic compounds. Many metabolites have found to possess interesting biological activities such as bactericidal, fungicidal, hepatoprotective and muscle relaxant (Fabricant and Farnsworth 2001). It is reported that phytochemicals may act by inhibiting microbial growth, inducing cellular membrane perturbations, interference with certain microbial metabolic processes, modulation of signal transduction or gene expression pathways (Cushnie and Lam 2006, Godstime et al. 2014).

The therapeutic potentials, including antioxidant, antimicrobial and anticarcinogenic properties of higher plants are due to the presence of secondary metabolites (Kaur and Arora 2009). Phytochemicals are natural bioactive compounds found in plants, including the medicinal plants, fruits, vegetables, flowers, leaves, roots and fibres, and they act as a defense system against diseases, or more accurately protect plants against diseases

(Krishnaiah et al. 2009). Medicinal plants contain some organic compounds which provide definite physiological action on the human body and these bioactive substances include tannins, glycosides, alkaloids, carbohydrates, terpenoids, steroids, flavonoids, essential oils, phenolic compounds etc. (Edeoga et al. 2005). The phytochemical studies of the medicinal plants have provided some biochemical basis for their ethnopharmacological uses in the treatment and prevention of various diseases and disorders (Okigbo et al. 2009). Based on these information preliminary phytochemical studies showed that various secondary metabolites were present all the studied three plants and presence of bioactive constituents indicates that the *Cassia* can be used in a multitude of ways for the beneficiary of population (Jayanthi et al. 2011), showed considerable antimicrobial activity against gram positive microorganisms. Overall results of three plants, *C. tora* leaves were contain maximum occurrence of phytoconstituents. Such type of results was reported by Mannan et al. (2013), Sahadeo et al. (2014), Rana et al. (2015), Shaikh and Syed (2015), Supare and Patil (2015) Nasrin et al. (2016b) and Asba and Meeta (2017). Specially alkaloid was observed only in this plant and has a high capacity of antibacterial and antitumor properties. This findings are in agreement with other researchers (Cushnie et al. 2014, Kaur and Arora 2015, Mabhiza et al. 2016, de Almeida et al. 2017). Biological property of emodin molecule from *C. tora* is offering a broad therapeutic window, which in future may become a member of anticancer (Wu et al. 2001). Also Choi et al. (1994) observed that the phytochemicals of *C. tora* showed noticeable antibacterial effects on four strains of methicillin resistant *Staphylococcus aureus* with a minimum inhibitory concentration which is closely related to this study. On the other hand most of the antimicrobial effects of *C. fistula* are related to their components and secondary metabolites (Theeshan et al. 2004, Aneja et al. 2011). Results showed that this plant containing components like flavonoids,

saponin, tannins, phenols and triterpnoids that inhibit the growth of the tested bacterial strains (Draughon 2004, Rizvi et al. 2009).

In case of *C. sophera* flavonoids, tannins, glycosides and sterols were observed. Some researchers noted that *C. sophera* L. is an emerging alternative antimicrobial agent for human applications (Afolayan and Meyer 1995, Kuhnt et al. 1995, Kirtikar and Basu 2000, Rahman et al. 2009) and antibacterial activity of extract could be attributed to the presence of some bioactive phytochemicals (flavonoids, steroids, terpenoids, etc.) in leaves of *C. sophera* L. These findings are in agreement with the previous reports (Rahman et al. 2009, Chetan et al. 2011, Kharat et al. 2013, Krishna et al. 2015, Nandhini et al. 2016) from literatures that *C. sophera* L. has those compounds.

A large number of phytochemicals belonging to several chemical classes have been shown to have inhibitory effects on all types of microorganisms *in vitro* (Cowan 1999). Flavonoids are phenolic substances known to be synthesized by plants in response to microbial infection and they have been found to be antimicrobial substances against wide array of microorganisms *in-vitro*. They also are show strong anticancer activities (Okwu 2004). The extracts were also revealed to contain saponins which are known to produce inhibitory effect on inflammation (Just et al. 1998). Alkaloids, tannins and saponins have been reported to have medicinal properties. Terpenoids and steroids were detected which were reported to be active against antibacterial activity (Okwu 2004, Mannan et al. 2013, Rana et al. 2015, Nasrin et al. 2016b). Saponins and tannins also exhibit cytotoxic effects and growth inhibition making them suitable as tumor inhibiting agents (Akindahunsi and Salawu 2005, Asl and Hossein 2008). Glycosides are known to lower the blood pressure according to many reports (Nyarko and Addy 1990). Alkaloids have been associated with medicinal uses for centuries and one of their common biological properties is

their antimicrobial and cytotoxicity (Nobori et al. 1994, Cushnie et al. 2014, Kaur and Arora 2015, Mabhiza et al. 2016, de Almeida et al. 2017).

5.4 Phytotoxicity test

Phytotoxicity is an important attribute in determination of allelopathic potential of a plant species (Khan et al. 2011). It is a common tradition that easily grown, sensitive, reliable species (*Lemna minor*, *Lactuca sativa* and *Raphanus sativa*) seeds are used as test plants in allelopathic studies (Ramalakshmi and Muthuchelian 2012). In the presence study, radish seed phytotoxicity assay (Turker and Camper 2002, Islam et al. 2009) was used to evaluate growth stimulation or inhibition properties of studied plants extracts. This radish seed phytotoxicity assay has a wide range of application in research towards the discovery of active principles in plants (Ramalakshmi and Muthuchelian 2012). Also use of allelopathic behavior is one of the new options for sustainable weed management (Olofsdotter 1995). Seed germination and seedling growth has been widely accepted as main parameters to monitor growth responses owing to amendment (Anjum and Bajwa 2005). The radish seeds germination and root lengths were significantly inhibited by the leaves extracts at concentrations of 10000 ppm when compared to control. The extract exhibited significant phytotoxicity on radish seeds due to the presence of phytochemicals. Seed germination is considered to be the most critical stage of plant development and growth. The necessities of seed germination of any crop area are, i) water for reserves hydrolysis, ii) hydration of enzymes for operational iii) confirmation of cell membrane and organelles and iv) finally to provide the force for cell expansion induced by germination (Alam and Shaikh 2007). Delays in seed germination of any species can have important biological implications, because this will affect the establishment of seedlings in natural conditions (Chaves et al. 2001) and their chances of competing for resources with neighboring species (Xingxinag et al. 2009).

De Candolle (1832) was probably the first person to suggest the possibility that many plants may excrete something from their roots which may be injurious to other plants. The concept of allelopathy was further supported and further developed by Bonner (1950), Grummer and Beyer (1960), Evenari (1961), Whittaker (1970), Putman and Duke (1978), Fischer et al. (1978). Allelochemicals (inhibitors) are produced by plants as end products, by-products, and metabolites and are contained in the stem, leaves, roots, flowers, inflorescence, fruits and seeds of the plants. The plant part that had a strong effect on germination and seedling length was the leaves. This is corroborated by findings of Maharjan et al. (2007), where preliminary screening shown that leaf extract had the strongest allelopathic effect on seed germination, thus was selected for detail experiments. Tefera (2002) also found that the inhibitory allelopathic impact of leaf extract was more powerful than other vegetative parts. Of these plant parts, leaves seem to be the most consistent producers of these allelochemicals. The four ways in which allelochemicals escape from a plant are (Gulzar and Siddiqui 2014):

(i) Volatilization, during which the terpenes are released from the leaves of some plant species

(ii) Leaching (which has shown that living or dead leaves of many plants contain growth inhibitors)

(iii) Exudation in which case roots of several crop and non-crop species release large quantities of organic compounds that inhibit the growth of other plants

(iv) Decomposition, through which allelochemicals are released from the plant residue.

Maximum seed germination inhibition (57%) was observed after 5th day of incubation by methanol leaf extract of *C. sophera* followed by *C. tora* (23%) and *C. fistula* (16%) respectively, whereas negative control did not show any

inhibition. Gulzar et al. (2014) evaluate the allelopathic potentiality of *C. sophera* and *C. tora*, and recommended that allelochemicals extracted from extract of *Cassia* can be employed for the natural control of weeds, thus achieving the aim of environmental safety. Raoof and Siddiqui (2012), Gulzar and Siddiqui (2014) showed *C. tora* have a good allelopathic potential. Similarly, the allelopathic effect of *C. tora* on seed germination and growth of mustard has been elucidated by Sarkar et al. (2012). Some recent studies indicating the phytotoxic/allelopathic effect of aqueous extracts of weeds (Ismail and Kumar 1996, An et al. 1999, Quayyum et al. 2000). Root length inhibition was more obvious than shoot length, as root length is a more sensitive indicator of phytotoxic activity (Rinez et al. 2011). De Feo et al. (2003) through his studies has reported that radish seeds were the most sensitive to allelochemicals. Phytochemicals from *Cassia* are known for their allelopathic responses and great ecological significance with respect to invasion (Ghayal et al. 2007).

Methanol was used as a solvent for plant extract preparation. Many researchers have already been used ethanol or methanol as a solvent for evaluating phytotoxicity, cytotoxicity, antibacterial, antitumor activity in several plant species (Turker and Camper 2002, Inayatullah et al. 2007).

Allelochemicals inhibit germination and seedling growth probably by affecting cell division and elongation, processes that are very important at this stage, or by interfering with enzymes involved in the mobilization of nutrients necessary for germination (Batlang and Shushu 2007). The production of allelochemicals is widely influenced by genetics as well as environmental factors at different growth stages (Yu et al. 2003). Javaid and Anjum (2006) attributed the reduction in seedling length to reduced rate of cell division and cell elongation due to the presence of allelochemicals in the extracts. Minimum root length (1.39 mm) was observed when radish seeds were grown in the presence of *C. sophera.* Atoum et al. (2006) reported that allelochemicals are highly active in meristimatic tissue in the growing root.

Root growth is characterized by high metabolic rates and, for this reason, roots are highly susceptible to environmental stresses such as allelochemicals in soils (Cruz-Ortega et al. 1998).

Chapter-6
Conclusion and Recommendation

CONCLUSION AND RECOMMENDATION

In the present work, high antibacterial and antitumor capacities observed for *C. tora* than others, suggested that it may play a role in preventing human diseases, such as bacterial diseases and cancer. These *in vitro* assays indicate that these plants are a significant source of natural antimicrobial and anticancer agents, which might be helpful in pharmaceuticals drug development. The results indicate that finding medicinal plants with good biological activities is enhanced when plants are chosen on the basis of ethnomedical use and when a group of related plants are screened. Also this study gives an indication of the efficacy of the plants obtained from the traditional healers.

Among these, *C. tora* are more effective with the presence of phytocompounds in the extracts including alkaloids, flavonoids, saponins, tannins, glycosides and sterol as major constituents may be responsible for antibacterial and antitumor activities, and may be a source of new antibiotic compounds. According to the earlier reports as mentioned secondary metabolites have many therapeutic values, it can be said that studied three plants can play an important role in herbal medicinal purposes. This *in vitro* study demonstrated that folk medicine can be as effective as modern medicine to combat pathogenic microorganisms. It also may enhance the finding of new compounds/known compounds with new biological activities from these plant groups.

A standardized protocol for plant collection and preparation needs to be established in order to rule out any discrepancies in the activity of the plants due to internal and external factors and different analytical assays used. It is also possible that different antimicrobial and anticancer screening methods could produce different results, which should be taken into account when the current results are compared to previous findings.

The bioactive compounds responsible for antimicrobial and anticancer activities of the studied three plants need to be identified and isolated, and it should be taken into account that it might possibly be the synergistic activity of two or more compounds responsible for the medicinal properties of the studied three plants.

With regards to the antimicrobial activities of plant extracts, minimum inhibitory concentrations (MIC's) of the extracts which proved to have antimicrobial activity for the respective susceptible microbes need to be determined, the optimal dose concentrations established and the antimicrobial activity of the extracts for other pathogenic micro-organisms should be investigated.

With regard to the anticancer activity of the respective extracts, the optimal dosages of the extracts need to be determined, the anticancer activities of the respective extracts in malignant cells other than those which were screened for in this study needs to be investigated, and the toxicity profiles of the extracts for human cells should be determined.

After successfully obtained antibacterial and antitumor activity with phytotoxic analysis indicates that this result is positively correlated with each others and also indicates presence of bioactive compounds. Therefore, extracts from these plants could be seen as a good source for useful drugs. The traditional medicine practice is recommended strongly for these plants as well as it is suggested that further work should be carried out to isolate, purify, and characterize the active constituents responsible for the activity of these plants.

Finally plant extracts were found to be relatively safe for use as far as lethality is concerned however more studies on the toxicity of the plants are needed before declaring them completely safe for use in humans.

Based on the investigation, the followings are recommendation for targeted researcher and pharmaceuticals.

1. The *C. sophera*, *C. fistula* and *C. tora* were the most active medicinal plants and can be used for the treatment of various infectious deceases and cancer. However the amount use in crude form must be carefully studied.

2. Further studies are required in order to gain more clarity as to the specificity and biochemical mechanisms responsible for the antimicrobial and anticancer properties of theses studied three plants.

3. Studies should be performed to isolate, identify and characterized the active compounds responsible for antimicrobial, antitumor and phytotoxic activities.

4. C. tora contain strong antimicrobial, antitumor and phytotoxic compounds and therefore the plant is strongly recommended for further biological activities.

5. Since the methanol extract was only tested for antibacterial and antitumor activities, to obtain a complete profile the other solvents should be tested.

6. The alkaloids of *C. tora* are much active and therefore need a comprehensive study regarding its side effect.

7. Therefore, there is high chance to develop plant based drugs for treating infectious and cancer type diseases from the studied plants.

Chapter-7
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