Antioxidant, Antimicrobial and Preliminary Cytotoxic Activity of Two Mangrove Plants *Caesalpinia crista* and *Cynometra ramiflora*

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Declaration

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 The findings of the research have not been/ will not be published anywhere without the concurrence of the concerned supervisor.

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DEDICATED TO

MY BELOVED GRANDMOTHER

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The Author
December, 2014

Abstract

In this study, two mangrove shrubs *Caesalpinia crista* and *Cynometra ramiflora* from the same family (leguminosae) were selected to investigate the scientific basis for some of their traditional uses. For this purpose, bioactivities such as antioxidant, antibacterial and preliminary cytotoxic properties of these plants were evaluated using medium polar solvent (chloroform) extract and polar solvent (methanol) extract of different plant parts. In the present study, *in vitro* antioxidant potential was evaluated by DPPH free radical scavenging, Ferric chloride reducing power assay, Ferric reducing antioxidant power assay, Total phenolics, flavonoid and tannin content. Antimicrobial and cytotoxic activities were conducted by using disc diffusion and brine shrimp lethality bioassays respectively. Methanolic extract of *C. ramiflora* (stem) showed the highest free radical scavenging activity (IC_{50}: 32.65 µg/ml) and also had a capability to reduce Fe^{3+} to Fe^{2+} with reduction activity ranged from 82.697% to 326.392%. The total antioxidant capacity (84 ± 0.009 µM Fe (II)/mg) of methanolic extract of *C. ramiflora* (stem) was observed by FRAP assay and results suggested that methanolic extract possessed the highest antioxidant activity. Total phenolic content were estimated by the Folin-Ciocalteu colorimetric method; the total flavonoid content was estimated by aluminium chloride colorimetric method as the total tannin content was estimated by Folin-Denis method. The methanolic extract of *C. ramiflora* (stem) showed the highest concentration of phenolics (96.2 ± 0.010 GAE/mg of extract), flavonoid (166.4 ± 0.019 QE/mg of extract) and tannins (80.4 ± 0.009 GAE/mg of extract). The antibacterial activity of the plant *C. crista* extract exhibited excellent activity against Gram-positive bacteria (*S. aureus* and *P. aurigonisa*) and *C. ramiflora* showed good activity against both Gram-positive and Gram-negative bacteria (*B. megaterium, S. typhi, S. epidermis, E. coli, P. aurigonisa, S. aureus*). In case of preliminary cytotoxic activity, the significant 50% lethality concentration (LC_{50}) value was found in methanolic extract of *C. crista* and *C. ramiflora* (stem) extract. The experimental findings could be correlated with the traditional uses of these mangrove plants and showed the rational for further investigation which would be required for isolating the possible bioactive constituents responsible for such activities.

CONTENTS

Chapter 3: Materials and Methods

Chapter 4: Results and Discussion

Chapter 5: Conclusion

Chapter 6: References

LIST OF TABLES

LIST OF FIGURES

ABBREVIATION

CT:	Condensed tannins
$CHCl_3$:	Chloroform
DPPH:	2, 2-diphenyl-1-picrylhydrazyl
FRAP:	Ferric ion reducing antioxidant power
FC:	Folin-Ciocalteu
GAE:	Gallic Acid Equivalent
HT:	Hydrolyzable tannins
IC_{50}:	50% Inhibitory Concentration
LC_{50}:	50% Lethal Concentration
Mt-OH:	Methanol
mm:	millimeter
mcg:	microgram
nm:	nanometer
QE:	Quercetin Equivalent
SD:	Standard Deviation
TPTZ:	Tri-Pyridyl-Triazine
TSP:	Tryptic Soy Broth
UV:	Ultra Violate
µg:	microgram

1.0 Introduction

Natural sources are limitless inspiration for novel drug development because there are many promising drug candidates available [Newman et al. 2012]. About more than 50% of FDA-approved drugs were natural sources derivatives [Kingston 2011; Chin et al. 2006]. Among these sources "phytochemicals" (derived from plants) have been the single most productive source of leads for the development of drugs. Plants synthesize these chemicals for their particular needs, mainly in their defensive roles and they may become a base and natural blueprint for the development of new drugs. These biologically active compounds would provide selective ligands for disease-related targets and influence the disease-related pathways and eventually shift the biological network from disease status to the healthy status [Clardy et al. 2004; Jiangyong et al. 2013]. At least 12,000 such compounds have been isolated and a number estimated to be less than 10% of the total [Tapsell et al. 2006; Lai et al. 2004]. Chemical diversity of these chemicals may provide the core scaffolds for future drugs. The past few years, however, have experienced a renewed interest in the use of phytochemicals and more importantly their role as a basis for drug development. Approximately one-half of all licensed drugs that were registered worldwide in the last 25 years period were natural products or their synthetic derivatives [Newman et al. 2007].

There is no established record when plants were first used for medicinal purposes but if we date back over 5000 years to the Sumerians, who created clay tablets with recorded hundreds of medicinal plants for various illnesses. In India, this date back several thousand years to the Rig-Veda, there are some of the earliest available documents detailing the medical knowledge that formed the basis of the Ayurvedic system. Ancient China is also a source of information about the uses of plants as a medicine, China's legendary emperor who lived over 4500 years ago, is said to have written the first Chinese herbal, the *Pen Tsao* contained 365 medicinal plants and their uses [Sumner & Judith 2000]. Unani medicine with its Muslim and Greek roots is another widely practiced traditional medicine. Western medicine can be traced back to the Greek physician Hippocrates (460-377 BC), who believed that a disease had a natural cause and

used various natural remedies in his treatments [Loudon et al. 2002]. The 15, 16 and 17th centuries were the great age of traditional medicine because much information is available in English and other languages rather than Latin or Greek [Gimmel et al. 2013]. In the nineteenth century, scientists began purifying the active extracts from medicinal plants. Continuing this progress for many accounts such population rise, inadequate supply of drugs, prohibitive cost of treatments, side effects of several allopathic drugs and development of resistance to currently used drugs for infectious diseases.

Today there is a renewed interest in investigating plants for medically useful compounds, with some of the leading pharmaceutical and research institutions involved in this search. According to WHO, to reduce the financial burden on developing countries 70% to 80% of the population relies on traditional plant based medicines for their primary health care needs [WHO, 2008]. More than two thirds of the world's plant species - at least 35,000 of which are estimated to have medicinal value come from the developing countries. At least 7,000 medical compounds in the modern pharmacopoeia are derived from plants. Researchers identified 122 compounds and widely used in modern medicine today [Interactive European Network, 2002-2005]. There are various examples of development of new drugs from the plant sources. Acetyldigoxin (*Digitalis lanata*) used as cardiotonic, Vinblastine and Vincristine (*Catharanthus roseus*) both used as antitumor and antileukemic agent, Glasiovine (*Octea glaziovii*) as antidepressant, Nordihydroguaiaretic acid (*Larrea divaricata*) as antioxidant and so many. During the years 2005–2007, 13 natural product related drugs were approved [Butler 2008], such as Elliptinium for cancer, Galantamine and Huperzine-A for Alzheimer's disease. In 2008, clinical trials are ongoing on more than 100 natural product derived drugs [Harvey, 2008].

Medicinal plants are an important element of indigenous medical systems. For example, in the northwestern Amazon, indigenous people use at least 1300 plant species to create "wildness drugs." In Southeast Asia, traditional healers use 6500 different plants to treat many diseases [Kong et al. 2003] and mangrove forests are one of the unique and important sources of these medicinal plants. The remaining mangrove forest areas of the world in 2000 was 53,190 square miles (137,760 km^2) spanning 118 countries and

territories [Giri et al. 2011; Status and distribution of mangrove forests, 2008]. Approximately 75% of world's mangroves are found in just 15 countries and Asia has the largest amount (42 %) of the world's mangroves [Giri et al. 2011]. The Sundarbans is the largest single block of tidal halophytic mangrove forest in the world [Ramsar List 2013], located in the Ganges river delta and the southern region in Bangladesh [Giri et al. 2011]. In the Sundarbans various kinds of trees up to medium height and shrubs grow in saline coastal sediment habitats. These plants require a number of physiological adaptations to overcome the problems of anoxia, high salinity and frequent tidal inundation. In this harsh environment, plants have evolved a special mechanism to help their offspring survive. Living in an extreme environment, the plants of mangrove origin produces unique and valuable chemical classes of compounds that may not be found in terrestrial plants. However, the people inhabiting areas near mangrove forests highly depend on these forests to meet their needs including their healthcare. Many mangrove plants have ethnopharmacological relevance and have also been exploited by the local people in the search for remedies for various ailments. Traditional wisdom and records indicate that Shundarban plants represent an exciting resource for possible lead structures in drug design. So the Sundarbans has substantial ecological and economic importance at local, national and global scales.

In the present study, two candidate species of the Sundarbans namely *Caesalpinia crista* and *Cynometra ramiflora* have been chosen to validate scientifically the usage of these plants in traditional medicine or ethnomedicine. The mangrove plant *C. crista (Caesalpiniaceae)* is widely distributed throughout the tropical and subtropical regions of Southeast Asia [Kalauni et al. 2005] and it is a large straggling, very thorny shrub. It is also called Kutum Kata by local people in the Sundarbans of Bangladesh and generally all parts of the plant are used for medicinal purpose but leaves and seeds are significant for its medicinal value. Powder from the seeds are used by local people for various ailments such, it relieves abdominal pain during gynecological disorders, is beneficial for diarrhea, dysentery, colitis and best medication for malarial fever and anthelmintic. Leaves are used for relieve constipation and piles, oils from leaves are valuable for nervine tonic [Nadkarni et al. 1976]. Roots have been used for the treatment of

rheumatism and backache [Medicinal Herb Index in Indonesia, 1995]. This plant is also used as mental stress relaxation health drink by forest dwellers [Ramesh et al. 2010].

C. ramiflora is distributed throughout the regions of Oceania and Southeast Asia. It is a small to medium-sized shrub locally known as Shingra [http://en.wikipedia.org/wiki/Sundarbans]. Local people used its roots for purgative and laprosy and leaves are used as anti-herpetic and skin diseases [http://www.stuartxchange.org/Oringen.html].

Therefore, the broad objective of this present study is to investigate the scientific basis of the medicinal uses and subsequently evaluating biological potential of these plants. The following objectives were considered:

- Determination of antioxidant properties of *C. crista* and *C. ramiflora* using chloroformic and methanolic extracts.
- Investigation of antimicrobial activity of the selected plant species against gram positive and gram negative bacteria by Disc diffusion method.
- Assessment of preliminary cytotoxic activities of *C. crista* and *C. ramiflora* using brine shrimp lethality method.

CHAPTER 2
Literature Review

2.1 Literature Review of *C. crista*

2.1.1 General Information

C. crista is a prickly climber and a well-known medicinal plant. In Bangladesh it is commonly known as "Kutum Kata" [www. trees of sundarban khulnaview.mht]. This plant is a member of the genus *Caesalpinia* and a rich source of diterpenes. It has some scientific synonyms: *Caesalpinia paniculata, Caesalpinia paniculata, Guilandina paniculata, Guilandina semina Lour.*

2.1.1.1 Taxonomy of *C. crista*

Kingdom: Plantae

Subkingdom: Viridaeplantae

Infrakingdom: Streptophyta

Division: Tracheophyta

Subdivision: Spermatophytina

Infradivision: Angiospermae

Class: Magnoliopsida

Superorder: Rosanae

Order: Fabales

Family: *Leguminosae*

Genus: Caesalpinia L.

Species: Caesalpinia crista L

2.1.1.2 Common Name of *C. crista*

Latin name: *Caesalpinia crista*

Bengali name: Kutum Kata, Natakaranja

English name: Fever nut

Hindi name: Latakaranja, Kantkarej, Kantikaranja, Sagar gota

Sanskrit name: Kantaki karanja, Kuberakshi, Lata karanja, Vitapa karanja

Tamil name: Mut-konrai

2.1.1.3 Geographical Distribution of *C. crista*:

C. crista is a popular medicinal plant widely distributed throughout the tropical and subtropical regions of Southeast Asia [Williamson 2002]. It is found throughout the hot and humid part of India, China, Myanmar, Sri Lanka, Cambodia, Vietnam and Taiwan. It has also been found in the America, Australia and the Pacific Ocean islands. It is widely distributed in the Shudarban of Bangladesh [Khatun et al. 2006].

2.1.1.4 Description of the Plant:

C. crista (caesalpiniaceae) is a large straggling, very thorny shrub. The branches are armed with hooks and straight, hard, yellow prickles. This plant has profound medicinal use and is proved to have adaptogenic, althelmintic, anti-inflmmatory, antipyretic, analgesic, anti-amyloidogenic, antibacterial, antidiabetic, antifilarial, antioxidant, nootropic, immunomodulatory, hypoglycemic and hepatoprotective activity [Suryawanshi et al. 2011].

Figure 2.1: Pictorial view of the plant *C. crista.*

2.1.1.5 Different parts of plant:

2.1.1.5.1 The Leaves:

Leaves are bipinnate, 20 to 30 centimeters long, petioles prickly, pinnae 6-8 pairs, 5-7.5 cm. long, with a pair of hook stipulary spines at the base. Main leaf axis armed with stout, sharp, recurved spines, divided into 4-8 pairs of secondary branches [Khatun et al. 2006]. Leaflets 6-9 pairs, 2-3.8 by 1.3-2.2 cm., membranous, elliptic-oblong, obtuse, strongly mucronate, glabrous above, more or less puberulous beneath; petioloules very short; stipels of short hooked spines [Suryawanshi et al. 2011].

Figure 2.2: Leaves of *C. crista.*

2.1.1.5.2 The Flowers:

The flowers are yellow, pleasantly perfumed, bisexual, simple and panicle racemes and about 1 cm long. Calyx lobes are about 5-8 mm long. Petals are variable, about 5-9 mm long, the upper petal with reddish markings on the inner surface towards the base. Flowers have 10 stamens with woolly green filaments, about 9-10 mm long, clothed in long white hairs about 3 mm long particularly on the lower half . Anthers are about 1.5 mm long. Pollens are orange and ovary shortly stalked, stigma not much wider than the style. Style is about 7 mm long and ovules one per ovary [www.keys.trin.org.au]. Flowering starts in mid August and continues till the second week of April. Maximum flowering takes place between last week of August to September, while it is moderate between January to mid April. During the period of last week of April to first week of August, the plant is without flowers [Kirthikar et al. 1987; Chauhan et al. 2010].

Figure 2.3: Flowers of *C. crista*

2.1.1.5.3 The fruits and seeds:

The fruits are pods, 4 to 5 centimeters long, 2.5 to 3 centimeters wide, inflated and covered with slender spines and contain one or two seeds [Sala 2002]. Seeds are hot and dry, globes or rounded. Seed coat is hard, glossy, and greenish to ash grey in color. The kernel surface is furrowed and ridged, hard, pale yellowish to white, circular to oval, flattened and about 1.23- 1.75 cm. in diameter. A scar of the micropyle lies at one end of the kernel, from where arises a prominent ridge demarking the two cotyledons of the embryo. Plumule radical axis is thick, cylindrical and straight. Taste is very bitter and odor is nauseating and unpleasant. 100 seeds weigh from 225 to 250 g [Kirthikar et al. 1987; Deore et al. 2010].

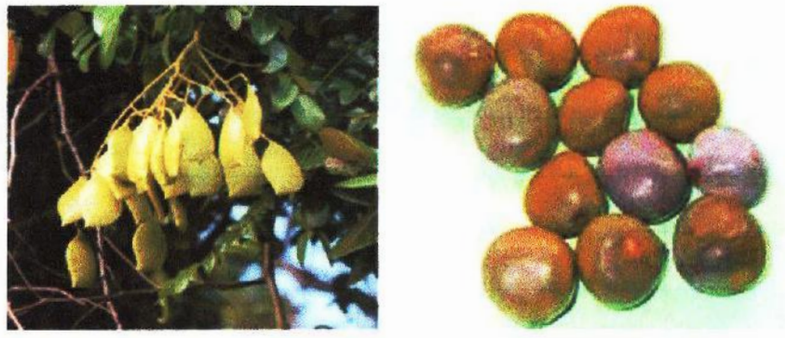


Figure 2.4: Fruits and Seeds of *C. crista.*

2.1.1.6 Use of the Plant

2.1.1.6. 1 Medicinal uses

Almost all parts of the plant *C. crista* have medicinal value. This plant is used, both externally and internally. The medical uses of the plant are described below according to various parts of it:

Use of leaves:

Externally leaves are used for dispersing inflammatory or traumatic swelling and internally, used for abdominal pain, diarrhea, dysentery and colitis. The leaves are used to treat Hepatitis A .The tender leaves are given along with the honey to ward off the mucous secretions and also used for disorder of the liver. The leaves fried in ghee, eliminate vata and relieve constipation, hence valuable in piles. The juice of leaves used for worm infections in children.

Finely powdered leaves used as uterine tonic for women immediately after delivery. The oil prepared from the leaves, is a valuable nervine. Paste of leaves used for pain and edema. In Malaya, young leaves used for intermittent fevers and as vermifuge. In Indo-China, leaves used as deobstruent and emmenagogue. In Ceylon young leaves applied to toothaches and decoction of leaves used as gargle for sore throat.

Use of seeds:

Externally seeds are used as ointment, mixed with castor oil, applied to hydrocoeles, acute orchitis and glandular swellings. The seeds are stimulant to the uterus, improve the menstrual discharge in oligomenorrhea and reduce the pain in lower abdominal region. The skin of the seed is extremely beneficial in the treatment of leucorrhea, diarrhea, dysentery and colitis. The seeds also render contraceptive activity. Seeds are used as three forms like powder, oil and paste.

Powdered form:

Internally roasted and powdered seeds administered for hydrocoele and leprosy. Powdered seeds also used as febrifuge and regarded as tonic. Decoction of roasted seeds used for consumption and asthma. The powder of its roasted seeds with ghee mitigates the condition and relieves the pain. During postpartum period, the abdominal pain is eliminated with the roasted seed powder, asafetida, ghee and little amount of salt. The seeds powder, given with milk, controls the diarrhea.

Oil form:

Oil expressed from seeds used for convulsions, palsy and a variety of nervous complaints. Fixed oil from the seeds used as emollient and as embrocation to remove freckles and to stop ear discharges. The massage with its seed-oil particularly helps in rheumatic disorders and arthritis.

Paste form:

The wounds and ulcers should be dressed with the paste of its seed mashed in water. In abdominal flatulence, the paste of its seeds mashed in water, eases the problem.

In country based:

In the Philippines, seeds are used for stomach troubles and as a mild purgative and decoctionn from crushed seeds used as emetic and for dysentery.In some part of Vidharbha region, seed kernels used as crude learning and memory enhancer. In Assam, seeds are used in the treatment of diabetes. According to Giesen, the seeds are used to treat malaria and parasitic worms.

Use of roots

Root juice used orally and externally as application for ophthalmia. In India, roots employed as diuretic and used for cases with stone or gravel in the bladder. In Ayurveda, sprouts and root bark used to treat tumors. In French Guiana root decoction used as febrifuge. Roots also used for gonorrhea. Externally and internally the juice of the stem is used for eye diseases. Roasted fruit also used for the same purpose.

2.1.2 Biological and Chemical Investigation of *C. crista*

2.1.2.1 Bioassay related work

Antioxidant activity

Previously two papers are published based on antioxidant potentiality of *C.crista* plant extract. Mandal et al. (2011) reported that 70% methanol extract of *C. crista* leaves acts as an antioxidant and ROS scavenger; which may be due to the presence of phenolic and flavonoid compounds. In this study, total antioxidant activity was evaluated as trolox equivalent antioxidant capacity value of 0.546 ± 0.014. The extract was investigated for different ROS scavenging activities and IC_{50} values were found to be 0.44 ± 0.1 mg/ml, 24.9 ± 0.98μg/ml, 33.72 ± 0.85μg/ml, 61.13 ± 3.24μg/mL and 170.51 ± 4.68μg/mL for

hydroxyl, superoxide, nitric oxide, singlet oxygen and hypochlorous acid, respectively and no significant results were obtained in scavenging of hydrogen peroxide and peroxynitrite anion. The extract was also found to be a potent iron chelator with IC_{50} = 279.85 ± 4.72μg/mL. The plant extract (100mg) yielded 50.23 ± 0.003mg/mL gallic acid equivalent phenolic content and 106.83 ± 0.0003mg/mL quercetin equivalent flavonoid content. I*n vivo* experiments, the extract treatment showed significant increase in the level of superoxide dismutase, catalase, glutathione-*S*-transferase and reduced glutathione.

Cytotoxic activity

A major portion of the current pharmacological research is devoted to anticancer drug design. Chemoprevention is an extremely promising strategy for cancer prevention and some plants have been used in traditional medicine which currently accepted as one of the main sources of cancer chemopreventive drug discovery. Three papers were found where cytotoxic activity of *C.crista* studied. Bodakhe et al. (2011) established that *C. crista* root bark extract posses anticancer activity and increase the life span. This study was evaluated against the Ehrlich ascites carcinoma (EAC) tumor model. The activity was assessed by using tumor volume, packed cell volume, mean survival time, increased in life span and hematological studies. When the cell line was induced in the animal, the weight of animal was increased. Their experiment showed that the extract at 150 mg/kg dose decreased the body weight indicating the antitumor activity. Ascites fluid is the nutritional requirement of tumor cells and this study showed that the *C. crista* extract decreasing the nutritional fluid volume and arresting the tumor growth. They also showed that this extract brought back the hemoglobin content, RBC and WBC count more or less the normal levels which indicate that this extract possess protective action on the hemopoitic system. Doxorubucin was taken as standard. So the *C. crista* extract significantly inhibited the tumor volume, packed cell volume, tumor cell count and hematological parameters which were altered by tumor inoculation were restored.

Sarkar et al. (2011) showed that the extract from *C. crista* leaves have significant cytotoxic activity (IC_{50}: 188.42±18.74 µg/ml). In this experiment, the cytotoxic effect of hydroalcoholic extract of *C. crista* was studied on both murine tumor cell Ehrlich's

Ascites Carcinoma (EAC) and normal spleenocyte cell. In vitro the cytotoxic activity was determined by the tetrazolium cell proliferation reagent (WST-1) assay. The result showed that the plant extract was non toxic for the normal spleenocyte cell, whereas it showed toxicity for EAC cells. This study provides an important basis for further investigation into the isolation, characterisation and mechanism of cytotoxic compounds from this plant.

Das et al. (2010) reported two new diterpenoids, 6beta-cinnamoyloxy-7beta-acetoxyvouacapen-5alpha-ol and 6beta, 7beta-dibenzoyloxyvouacapen-5alpha with several known constituents. Furthermore, the compounds were tested for in vitro cytotoxic activity against two cancer cell lines, HL-60 (human promyelocytic leukemia) and HeLa (human cervical carcinoma) using the MTT assay. Compounds showed significant decrease in cell viability in the test cell lines in a concentration dependent manner. IC_{50} value was measured with each cell line after four individual observations was found to be, compound 1 (17.37±0.88), (33.41±0.75); compound 2(19.77±1.54), (33.90±0.66); Camptothecin (0.59±0.04), (0.94±0.07); Etoposide (1.81±0.20), (8.93±0.69) on both HL-60 and HeLa cells.

Antimalarial activity

Malaria is caused by the protozoan parasite of the genus *Plasmodium* and transmitted by mosquitoes of the genus *Anopheles* and it remains the greatest human killer among parasitic infections. It is a prevalent disease in many parts of Asia, Africa, Central and South America, the Australian Continents and some Caribbean islands. The appearance of drug-resistance *P. falciparum* has made the treatment of malaria is problematic, so it need to search the new alternatives of current drugs. In this regard scientists in 2004 and 2006, two experiments were conducted for showing antimalarial activity of *C. crista* plant extract.

Linn et al. (2004) showed their one of isolated diterpene from CH_2Cl_2 extract of the seed kernels of *C. crista* have a dose-dependent inhibitory effects on *P. falciparum* FCR-3/A2 growth in vitro. Firstly they tested in vivo and showed significant inhibition of parasitemia level (98.6%) at the in dose of 10 mg/kg in mice infected with *P. berghei.* Furthermore, seven new furanocassane-type diterpenes [caesalpinins C-G (1-5) and

norcaesalpinins D and E (6, 7)] together with norcaesalpinins A-C (8-10) and 11 known compounds (norcaesalpinins A-C, 2-acetoxy-3-deacetoxycaesaldekarin e, caesalmin B, caesaldekarin e, caesalpin F, 14(17)-dehydrocaesalpin F, 2-acetoxycaesaldekarin e, 7-acetoxybonducellpin C, and caesalmin G) were isolated on the basis of spectroscopic analysis. The isolated diterpenes showed significant dose-dependent inhibitory effects on *P. falciparum* FCR-3/A2 growth in vitro but norcaesalpinin E (7) showed the most potent inhibitory activity (IC_{50}, 90 nM).

Kalauni et al. (2006) observed that the norcaesalpinin E (28) plays the most important role for the anti-malarial activity. In this study, 44 cassane- and norcassane-type diterpenes isolated from $CHCl_3$ extract of *C. crista* collected from Myanmar and Indonesia were evaluated for their antimalarial activity against the malaria parasite *P. falciparum* FCR-3/A2 clone in vitro. Among the diterpenes tested, norcaesalpinin E (28), a C-17-norcassane-type diterpene with an acetoxyl group at C-1 and a hydroxyl group at C-7, showed the most potent activity (IC_{50}, 0.090 μM) which is stronger than the well-known anti-malarial drug, chloroquine (IC_{50}, 0.29 μM).

Anthelmintic activity

Gastrointestinal parasites pose a serious threat to the productivity of livestock in developing nations. Many farmers in the developing countries are unable to afford synthetic anthelmintics for their livestock. In this scenario, the farmers depend on time-honored, centuries-old, affordable and accessible treatments for parasites. This concept has led to an interest of scientists in ethnoveterinary medicine (EVM). In EVM, medicinal plants are used to treat parasite infections. A number of plants have been listed to treat parasitic infections and a few of them have been scientifically validated.

Jabbar et al. (2007) showed *C. crista* posses more potent anthelmintic activity than *Chenopodium album* in egg hatch test. In vitro anthelmintic activity of crude aqueous methanolic extract (AME) of the *C. crista* (L.) seed kernel was determined using mature Haemonchus contortus and their eggs in adult motility assay and egg hatch test and which exhibited dose- and time-dependent anthelmintic effects by causing mortality of worms and inhibition of egg hatching. In egg hatch test, the LC_{50} value of *C. crista* was 0.134μg/mL and positive control oxfendazole was 1.9μg/mL. The ovicidal activity

of extract was low ($p \leq 0.05$) as compared to positive control. In vivo anthelmintic activity was evaluated in sheep naturally infected with mixed species of gastrointestinal nematodes by administering crude powder and AME in increasing doses (1.0-3.0 g/kg). Maximum reduction in eggs per gram (EPG) of faeces was recorded as 93.9% with *C. crista* and Levamisole (7.5 mg/kg) used as standard, showed 95.1-95.6% reduction in EPG.

Hepatoprotective potential

Hepatotoxicity in patients with iron overloading as liver is mainly the active storage site of iron in our body. Iron is the most important transition element of the body, although an optimum level of iron is always maintained by the cells to balance between essentiality and toxicity, in some situations it is disrupted, resulting in iron overload which is associated to the oxidative stress induced disorders. In all iron-overload-induced diseases, iron removal by iron chelation therapy is an effective life-saving strategy. The currently available iron-chelating agents show several side effects and limitations that direct towards the finding of a more effective and safe drug.

Sarkar et al. (2012) concluded that the *C. crista* Linn. (CCME) extract has protective effect against iron-overload-induced liver toxicity as evidenced by biochemical and histopathological studies and iron overload was induced by intraperitoneal administration of iron dextran into mice. Oral administration of CCME markedly reduced the elevated levels of serum enzymes and billirubin of iron-overloaded mice to approach the normal control values. The increased level of ferritin is generally noticed in iron-overload-induced liver toxicity which substantially reduced as treated with CCME dose-dependently. They also showed that CCME significantly increased the $[Fe(ferrozine)_3]^{2+}$ complex formation with time. So all findings suggest its benefit in pathological sequence of iron-overload-linked liver disease and can be used as promising hepatoprotective agent.

Nootropics activity (enhance the cognitive skill)

Dementia is a kind of neurodegenerative disorder and the stressful condition is one of the major causes of memory loss and other cognitive dysfunctions. Traditionally

herbal drugs have been used to enhance cognitive function. In this regard, some work has been done. Kshirsagar (2011) established that the aqueous extract of dried seed kernels of *C. crista* significantly beneficial to improve cognition in disorders like dementia and various neurodegenerative disorders. In this work, aqueous extract ameliorated the amnesic effect of scopolamine in mice. Redial arm maze and Morries water maze paradigm served as the exteroceptive behavioral models. Aqueous extract of dried seed kernels of *C. crista* was compered with standard drug piracetam in scopolamine induced amnesia in mice. Among the test extract, tested at 150mg/kg p.o. showed the value near to standard. Ramesh et al. (2010) showed that the *C. crista* aqueous extract could able to inhibit the Aβ aggregation from monomers and oligomers and also able to dis-aggregate the pre-formed fibrils. Beta-amyloid (βA) deposition is the fundamental cause Alzheimer's disease (AD). It is degenerative disease because Aβ can self-assemble to form oligomers, protofibrils and diffuse plaques through multistep-nucleated polymerization. Their studies aimed to decrease Aβ levels or prevention of Aβ aggregation is of therapeutic significance for AD drug discovery. Natural products as alternatives for AD drug discovery are a current trend. They showed that the *Caesalpinia crista* leaf aqueous extract or pure compounds having anti-amyloidogenic potential. This study focused on ability of *C. crista* leaf aqueous extract on the prevention of (i) the formation of oligomers and aggregates from monomers (Phase I: Abeta (42) +extract co-incubation); (ii) the formation of fibrils from oligomers (Phase II: extract added after oligomers formation); and (iii) dis-aggregation of pre-formed fibrils (Phase III: aqueous extract added to matured fibrils and incubated for 9 days). The aggregation kinetics was monitored using thioflavin-T assay and transmission electron microscopy (TEM). This study provides an insight on finding new natural products for AD therapeutics.

Bioassay related work on *C. crista* has been compiled into the table 2.1.

Table 2.1 Summarize the previous bioassay related works on *Caesalpinia crista.*

Activity	Used plant portion	Solvent extract used	Assay used	Comments	References
Antioxidant activity	Leaves	70% methanolic extract	***In Vitro***		Mandal et al. 2011
			Trolox Equivalent Antioxidant Capacity.	The TEAC value of was 0.546 ± 0.014 as a potent antioxidant.	
			Hydroxyl Radical Scavenging Activity	Plant extract (IC_{50} : 0.44 ± 0.1mg/mL) is a better hydroxyl radical scavenger than standard mannitol (0.85 ± 0.02mg/mL).	
			Scavenging of Superoxide Radicals	No significant result.	
			Assay of Nitric Oxide Radical Scavenging	Nitric oxide (IC_{50} :33.72 ± 0.85μg/mL) superbly inhibits nitric oxide and is better than the standard curcumin (97.12 ± 3.09 μg/mL).	
			Scavenging Activity of Hydrogen Peroxide	No significant result.	

			Determination of the Effects on Peroxynitrite	No significant result.	
			Reaction with Singlet Oxygen	No significant result.	
			Reaction with Hypochlorous Acid	The extract scavenged hypochlorous acid more efficiently (IC_{50} : 170.51 ± 4.68μg/mL) than ascorbic acid (IC_{50} :198.42 ± 10.71μg/mL).	
			Fe^{2+} Chelation	Extract shows less activity.	
			Measurement of Reducing Power	Extract has some reducing capacity.	
			Determination of Total Phenolic and Flavonoids Content	*C. crista* plant extract shows significant amount of phenolic and flavonoid content.	
			In Vivo Assay of Antioxidant Enzymes	The plant extract as evidenced by the increased level of these antioxidant systems (SOD, CAT, GST and GSH) in extract treated mice.	

	Leaves	Methanol: Water (7 : 1)	**<u>In Vitro</u>** DPPH radical scavenging And protection against Fe^{2+}-mediated oxidative DNA damage	This extract (IC_{50} : 14.2 ± 0.63 μg/ml) is an effective DPPH free radical scavenger. Ascorbic acid (IC_{50} : 5.27 ± 0.27 μg/ml) as a standard. The significant reduction in the formation of nicked DNA and increase in supercoiled DNA in the presence of the extract reveals its excellent iron-chelating activity.	Sarkar et al. 2012

Cytotoxic activity	Root bark	Ethanolic extract	**In Vivo** Male Swiss albino mice	Possess significant antitumor activities in the Ehrlich ascites carcinoma (EAC) bearing mice at the dose of 150 mg/kg.	Bodakhe et al. 2011
	Leaves	Ethanol:Water (6 : 4)	***In Vivo*** Spleenocyte cells from mice	Showed the highest cytotoxicity (IC_{50}: 188.42±18.74µg/ml) against EAc cell lines.	Sarkar et al. 2011
		Furano cassane diterpenoid	MTT assay used HL-60 & HELA cell line	Showed significant decrease in cell viability in test cell lines.	Das et al. 2010
Antimalarial activity	Seed kernels	Chloroformic extract {Norcaesalpinin E (28) }	***In Vivo*** Used parasite *P. falciparum*	Showed most potent activity with an IC_{50}: 0.090µM	Kalauni et al. 2006
	Seed kernels	Chloroformic extract {Norcaesalpinin E (7) }	***In Vivo*** Used parasite *P. falciparum*	Showed most potent inhibitory activity (IC_{50}: 90nM)	Linn et al. 2004
Anthelmintic activity	Seed kernels	Aqueous methanolic extract	***In Vivo*** Adult motility assay and egg hatch test; using mature *Haemonchus contortus*	Exhibited dose-and time-dependent anthelmintic effects (LC_{50}: 0.134mg/ml) in egg hatch test.	Jabbar et al. 2007
Hepatoprotective		Methanol:Wat			

potential	Leaves	er (7 : 3)	***In Vivo*** Used hepatotoxicant iron overload albino mice model.	Confirmed the hepatoprotective effect against the model hepatotoxicant iron overload mice.	Sarkar et al. 2012
Nootropics activity (enhance the cognitive skill)	Seed kernels	Aqueous extract	***In Vivo*** Used scopolamine induced amnesia in mice (Redial arm maze and Morries water maze model).	Significantly increased the learning memory performance.	Kshirsagar 2011
	Leaves	Aqueous extract	Used Thioflavin T-assay and Transmission electron microscopy	Found that extract could not only prevent Aβ fibril formation but also dis-aggregated Aβ fibrils.	Ramesh et al. 2010

2.1.2.2 Compounds Isolation related work

Studying all the literature of compounds isolation on *C. crista*, we found that all compounds are diterpenes group. Diterpenes are a structurally diverse class of C_{20} natural compounds constitute the second largest class of terpenes and widely distributed in nature [Rakshit et al 2010]. Diterpenes have attracted growing attention because of their interesting biological and pharmacological activities. Although thousands of diterpene compounds have been described in nature from terrestrial and marine organisms, only few of them became clinically effective. Overall, the anticancer drug taxol, used in therapy against ovarian, breast, and lung cancer, is an example of structure discovered from nature and used as medicine. Also used in therapy is the diterpene resiniferatoxin, an uitrapotent vanilloid, isoiated from the *Euphorbia resinifera* latex, in clinical trials for bladder hyperiflexia and diabetic neuropathy [Lanzotti et al. 2013].

Kalauni et al. (2004) firstly observed that the chloroformic extract of *C. crista* seed kernels possess Cassane- and Norcassane-Type Diterpenes. Five new cassane-type diterpenes and three new norcassane-type diterpenes have been isolated together with 12 known cassane-type diterpenes, 14(17)-dehydrocaesalmin F, caesaldekarin e, caesalmin B, caesalmin C, caesalmin E, 2-acetoxy-3-deacetoxycaesaldekarin e, 2-acetoxycaesaldekarin e, caesalpinin C, 7-acetoxybonducellpin C, caesalpinin E, norcaesalpinin B, and 6-acetoxy-3-deacetoxycaesaldekarin e. Linn et al. (2005) isolated seven new furanocassane-type diterpenes [caesalpinins C-G (**1-5**) and norcaesalpinins D and E (**6**, **7**)] together with norcaesalpinins A-C (**8-10**) and 11 known compounds (norcaesalpinins A-C, 2-acetoxy-3-deacetoxycaesaldekarin e, caesalmin B, caesaldekarin e, caesalpin F, 14(17)-dehydrocaesalpin F, 2-acetoxycaesaldekarin e, 7 acetoxybonducellpin C, and caesalmin G) from the chloroformic extract of *C. crista* seed kernels. They also observed that the isolated diterpenes showed significant dose-dependent inhibitory effects on *Plasmodium falciparum* FCR-3/A2 growth in vitro. Kinoshita et al. (2005) isolated new cassane diterpene-acids, neocaesalpins H and I from the leaves of *C. crista.* These compounds were characterized as having an a,b -butenolide hemiacetal ring that is rare in nature. Kalauni et al. (2006) again observed that the 44 cassane- and norcassane-type diterpenes isolated from *C. crista* of Myanmar and

Indonesia were evaluated for their antimalarial activity against the malaria parasite *P. falciparum* FCR-3/A2 clone *in vitro*.

Awale et al. 2006 found ten new furanocassane-type diterpenes named, caesalpinins H—P (1—9) and norcaesalpinin F (10) were isolated from the CH2Cl2 extract of the seed kernels of *C. crista*. Das et al. (2010) found two new diterpenoids, 6beta-cinnamoyloxy-7beta-acetoxyvouacapen-5alpha-ol and 6beta, 7beta-dibenzoyloxyvouacapen-5alpha with several known constituents. Furthermore, the compounds were tested for in vitro cytotoxic activity against two cancer cell lines, HL-60 (Human promyelocytic leukemia) and HeLa (Human cervical carcinoma) using the MTT assay. Compounds showed significant decrease in cell viability in the test cell lines in a concentration dependent manner. Tian et al. (2012) found 1α-acetoxy-5α, 7β-dihydroxycassa-11,13 (15)-diene-16,12-lactone, a new cassane-type diterpene was isolated from Caesalpinia crista. The new compound was evaluated for antitumour activity against T47D, DU145 and showed significant inhibitory activities.

Table 2.2 lists the compounds, their plant parts they are isolated from *C. crista* and the respective assays.

Table 2.2 Compound isolation based works on *C. crista*

Compound Name	Used plant portion	Solvent extract used	Assay used	Comments	References
New Caesalpinins MA (1), ME-MJ (2-7), ML (8), MO (9), C-F (12-150), H-K (16-19), M-P (20-23) and norcaesalpinins MC (10), MD (11) and A-F (24-29) with known compounds caesalmins B (30), C (31), E (32) and G (33); caesaldekarin e (34); 2-acetoxycaesaldekarin e (35); 2-acetoxy-3-deacetoxycaesaldekarin e (36); 6-acetoxy-3-deacetoxycaesaldekarin e (37), 14(17)-dehydrocaesalmin F (38); bounducellpins B (39) and C (40); 7-acetoxybonducellpin C (41); 1-deactoxy-1-oxocaesalmin C (42); ζ-caesalpin (43); and 1-deacetylcaesalmin C (44).	Seed kernels	Chloroformic extract	TLC and H-NMR spectrum	Norcaesalpinin E (28), a C-17-norcassane-type diterpene with an acetoxyl group at C-1 and a hydroxyl group at C-7, showed the most potent activity (IC_{50}, 0.090 μM) which is stronger than the well-known anti-malarial drug, chloroquine (IC_{50}, 0.29 μM).	Kalauni et al. 2006
Seven new furanocassane-type diterpenes [caesalpinins C-G (1-5) and norcaesalpinins D and E (6, 7)] together with	Seed kernels	Chloroformic extract	TLC and H-NMR spectrum	norcaesalpinin E (7) showed the most potent inhibitory activity (IC_{50}, 90	Linn et al. 2005

norcaesalpinins A-C (8-10) and 11 known compounds (norcaesalpinins A-C, 2-acetoxy-3-deacetoxycaesaldekarin e, caesalmin B, caesaldekarin e, caesalpin F, 14(17)-dehydrocaesalpin F, 2-acetoxycaesaldekarin e, 7-acetoxybonducellpin C, and caesalmin G).				nM) on *Plasmodium falciparum* FCR-3/A2 growth in vitro.	
Five new cassane-type diterpenes, caesalpinins MA-ME (1-5), and three new norcassane-type diterpenes, norcaesalpinins MA-MC (6-8), have been isolated, together with 12 known cassane-type diterpenes, 14(17)-dehydrocaesalmin F, caesaldekarin e, caesalmin B, caesalmin C, caesalmin E, 2-acetoxy-3-deacetoxycaesaldekarin e, 2-acetoxycaesaldekarin e, caesalpinin C,7-acetoxybonducellpin C, caesalpinin E, norcaesalpinin B, and 6-acetoxy-3-	Seed kernels	Chloroformic extract	TLC and H-NMR spectrum	Structures elucidated by analysis of their spectroscopic data.	Kalauni et al. 2004

deacetoxycaesaldekarin e.					
Ten new furanocassane-type diterpenes named, caesalpinins H-P (1-9) and norcaesalpinin F (10)	Seed kernels	Chloroformic extract	Spectroscopic analysis	caesalpinin N (7) represents the first example of furanocassane-type diterpene possessing an aldehyde group at C-14.	Awale et al 2006
Two cassane-type furanoditerpenes [caesalpinins MM (1) and MN (2)], two cassane-type furanoditerpenes [caesalpinins MO (3) and MP (4)], together with eight known cassane-type diterpenes, 1-deacetoxy-1-oxocaesalmin C (5), 1-deacetylcaesalmin C (6), caesalmin C (7), bonducellpin C (8), caesaldekarin e (9), 2-acetoxycaesaldekarin e (10), 2-acetoxy-3-deacetoxycaesaldekarin e (11), and norcaesalpinin E (12).	Seed kernels	Chloroformic extract	Spectroscopic techniques.	Compounds 5 and 6 were for the first time isolated from a natural source.	Kalauni et al. 2005

Two new diterpenoids, 6beta-cinnamoyloxy-7beta-acetoxyvouacapen-5alpha-ol and 6beta,7beta-dibenzoyloxyvouacapen-5alpha-ol			NMR spectrum	Compounds showed significant cytotoxic activity against two cancer cell lines	Das et al. 2010
Cassane diterpene-acids, neocaesalpins H and I.	Leaves		NMR spectrum	These compounds were characterized as having an alpha, beta-butenolide hemiacetal ring that is rare in nature.	

Structure of bioactive compounds isolated from C. crista

Several well studied compounds isolated from *C. crista* and the respective bioactivities related to these compounds are shown in Figure 2.5, 2.6 and 2.7.

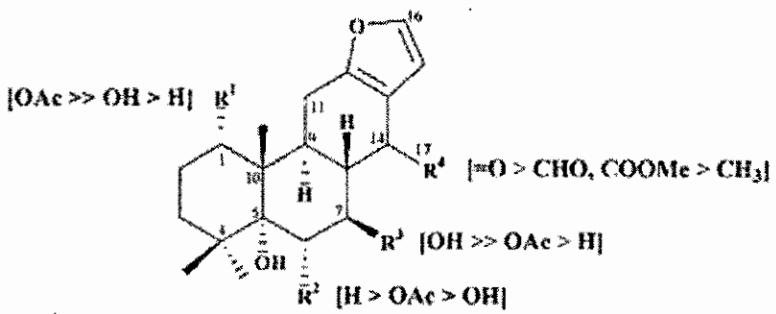


Figure 2.5: Structure–Activity Relationship of cassane- and norcassane-type diterpenes on antimalarial Activity [Kalauni et al. 2006].

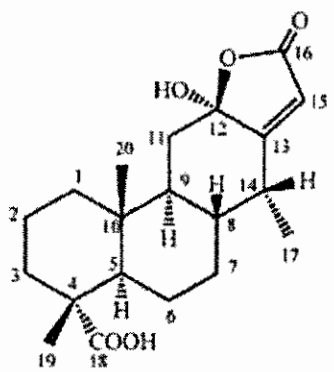


Figure 2.6: The structure of Neocaesalpin H [Kinoshita et al. 2005].

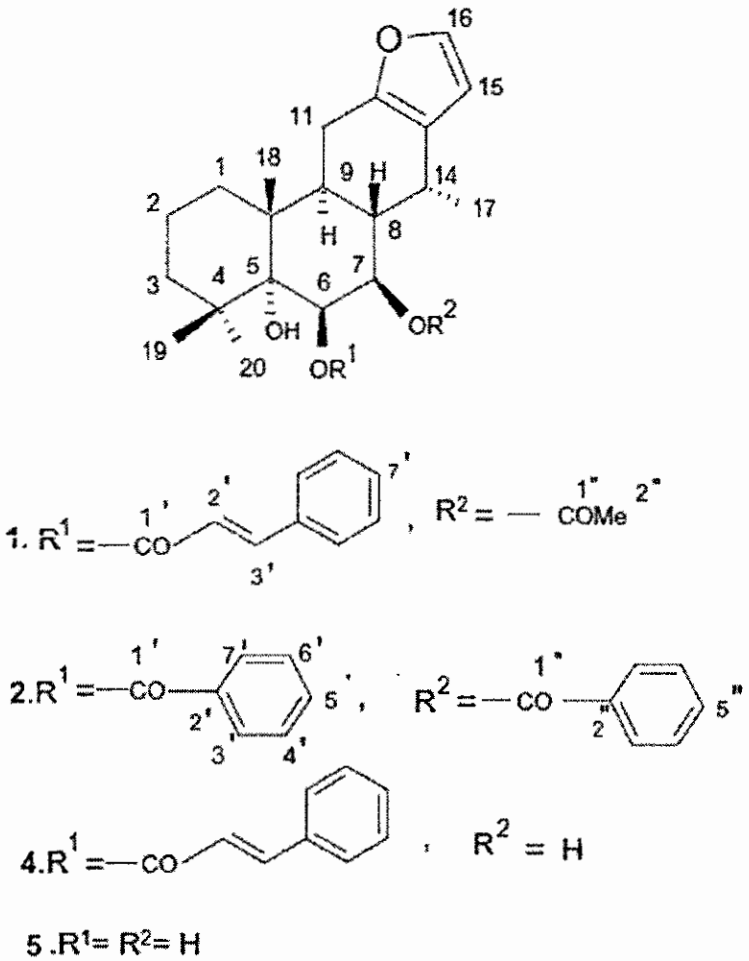


Figure 2.7: The structure of 6b-cinnamoyloxy-7b-hydroxy-vouacapen- 5a-ol [Das et al. 2010].

2.2 Literature Review of *C. ramiflora*

2.2.1 General Information

C. ramiflora was described by Carl Linnaeus in 1753. The name is considered as validly published. *C. ramiflora* is a species in the genus *Cynometra* which contains approximately 86 to 107 species and belongs to the family of the Fabaceae (Legume Family). *C. ramiflora* is small to medium sized tree, commonly known as Shingra in Bangladesh [www.en.hortipedia.com].

2.2.1.1 Taxonomy of *C. ramiflora*

Domain: *Eukaryota*

Kingdom: *Plantae*

Subkingdom: *Viridaeplantae*

Phylum: *Tracheophyta*

Subphylum: *Euphyllophytina*

Class: *Magnoliopsida*

Subclass: *Rosidae*

Superorder: *Rosanae*

Order: *Fabales*

Family: *Leguminosae*

Subfamily: *Caesalpinioideae*

Tribe: *Detarieae*

Genus: *Cynometra*

Specific epithet: *ramiflora* - L.

2.2.1.2 Common Name

Botanical name: *Cynometra ramiflora* L.

Latin name: *Cynometra ramiflora*

Other vernacular names:

Philipines: Balitbitan

Indonesia: Kateng, Kepel, Wunut.

Malaysia: Katong laut

Cambodia: Chom prinh

Thailand: Phang kha, Ma khak, Maeng kha.

Vietnam: C[aa]y tr[aa]m ngh[eej], C[aa]y m[os]t.

India: Ula, Ulad

Bangladesh: Shingar, Shingra.

2.2.1.4 Geographical Distribution:

C. ramiflora is distributed from India, throughout Southeast Asia, Malaysia, Sri Lanka, Indo-china, Southern China, Thailand, Philippines and New Guinea to the Pacific and often erroneously reported from Australia. It is also found in the Sudarbans of Bangladesh.

2.2.1.5 Description of the Plant:

C. ramiflora is a small to medium-sized tree. The trees grow to a height of approximately 10 meters, stem to 30 cm in diameter. The outer bark is smooth but with numerous lenticels. It is dark grey to brown. The inner bark is whitish or light brown to red with pale pinkish-brown sapwood and cream to reddish-brown heartwood. *C. ramiflora* is a characteristics constituent of the inner finger of mangrove forests but it is also found in land, reverie and even savanna where vegetation cover up to 525 m altitude. The density of the wood is 720-1115 kg/m3 at 15% moist content.

2.2.1.6 Different parts of *C. ramiflora:*

2.2.1.6.1 The leaves:

The leaves are alternate, smooth, imparipinnate, 30-38 cm long. Leaflets 2-4 pairs, ovate, elliptic or lanceolate, sob falcate, sub acute, glabrous on both surfaces, base rounded very inequilateral except that of the terminal leaflet (Figure 2.8). Elliptic or lanceolate is 2.5 x 7 cm and petiole is 1.5 cm long. Mature leaves deep green while emerging or new young leaves flaccid, drooping, glossy white. The lower pair is usually smaller than upper one.

Figure 2.8: Leaves of *C. ramiflora.*

2.2.1.6.2 The flower:

Flowers yellowish-white, in axillary panicles or short racemes; petals and sepals similar, subequal, 4-8 mm long, Petals separate, lanceolate or linear oblong (Figure 2.9). Flowers are actinomorphic or somewhat irregular. Calyx is 4-5 lobed, glabrous and calyx lobes exceeding or about equal to corolla. Stamens are 9-10, completely free, separate and long excreted. Filaments are glabrous and style is terete. Ovary densely clothed in hairs and ovules 1-2 per ovary.

Figure 2.9: Inflorescence of *C. ramiflora.*

2.2.1.6.3 The fruits and seeds:

Fruits (pods) are broad-ovoid or ellipsoid, brain-like surface, thick-woody, scurfy brown, 2-5 x 1.3-4 cm, indehiscent. Fruit are exerted from calyx and becoming woody (Figure 2.10). Fruits are 1or 2 seeded. Seeds ovoid to round in outline, surface smooth, olive, brown, or black (Figure 2.11). Matured fruits seen in December.

Figure 2.10: Fruits of *C. ramiflora.*

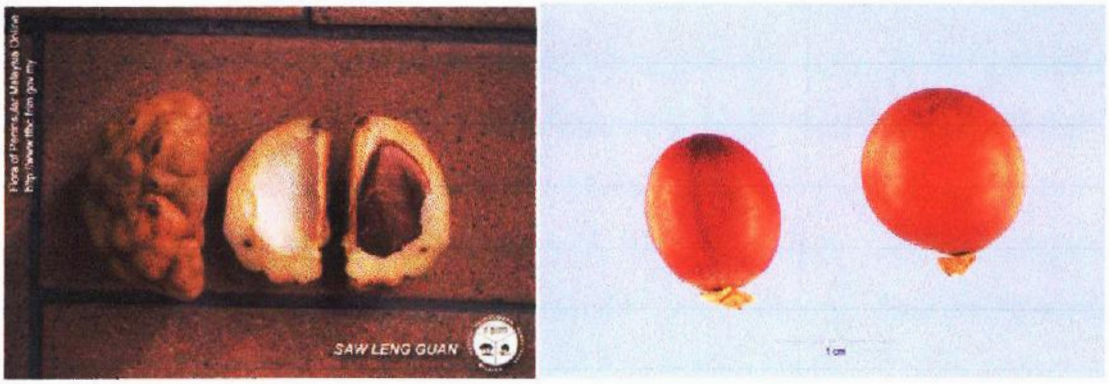


Figure 2.11: Seeds of *C. ramiflora.*

2.2.1.7 Use of the Plant

Medicinal uses

- Roots are purgative (an agent for removing the bowels).
- Seeds and leaves are used as anti-herpetic (any of several viral diseases).
- In Malabar, leaves used to make a lotion for skin diseases.
- Oil drawn from the seeds also used for skin infection and used for laprosy.

Others

Timber: Wood used for interior and light construction, plywood, decorative veneers. Also used for firewood and charcoal.

2.2.2 Biological and Chemical Investigation of *C. ramiflora*

2.2.2.1 Bioassay related work

Phytochemical analysis related works of *C. ramiflora* are presented below:

Natural products and related drugs are used to treat many diseases including bacterial infection, cancer and immunological disorders. Bangladesh has a rich and prestigious heritage of herbal medicines among the South Asian countries. For this consideration, Uddin et al. (2009) investigate the cytotoxic effect of some medicinal plant extracts. Among this extracts, the methanolic extracts of *C. ramiflora* were screened for cytotoxic activity against healthy mouse fibroblasts (NIH3T3) and three human cancer-cell lines (gastric: AGS; colon: HT-29; and breast: MDA-MB-435S) using the MTT

assay. It showed low toxicity (IC_{50} > 2.5 mg mL^{-1}) against mouse fibroblasts but selective cytotoxicity (IC_{50} 0.2–2.3 mg mL^{-1}) against different cancer cell lines.

There are million of people worldwide suffering from diabetes and still now in modern medicine no satisfactory effective therapy is available to cure diabetes. So new treatments are needed to save and improve human lives. In present day diabetic research evaluation and identification of some new natural molecules with antidiabetic property have become one of the major objectives. In this regard, Tiwari et al. (2008) was coducted to screening new natural antidiabetic agents using experimentally validated animal models of diabetes. Methanolic extract of *C. ramiflora* was found to lower the postprandial blood glucose level of the sucrose loaded rats significantly. Quantitative glucose tolerance of each animal was calculated by area under curve (AUC) method using Prism Software. The area under curve of the control group and experimental group was compared to determine the percentage antihyperglycaemic activity. The extract of *C. ramiflora* which showed improvement of around 21.6% on glucose tolerance of sucrose loaded rats as compared to the AUC of sham control group at 250 mg/kg dose level.

The mangrove plants are hugely used for medicinal purposes by local people. The purpose of this study Bunyapraphatsara et al. (2003) evaluated the biological potential of the plants in the mangrove forest using antioxidant, lipid peroxidation inhibition and cancer chemoprevention tests. *C. ramiflora* L. seeds showed antioxidant activity with EC_{50} value was found to be 3.33µg/ml using DPPH method. The inhibition of lipid peroxidation in mice brain homogenate was determined using thiobarbituric acid method. This plant showed strong lipid peroxide formation inhibition, IC_{50} value was 0.8992µg/ml. In this experiment, cancer chemoprotective activity was screened by using quinine reductase induction activity and this plant showed moderate activity.

Mirca et al. (1989) was exhibited the highest lectin activity from *C. ramiflora*. The lectin was isolated from the seeds of *C. ramiflora* by extraction with normal saline solution (NSS) and purified by high-pressure gel permeation chromatography using the Protein Analysis Column I-125. Ammonium sulfate fractionation and gel filtration on Sephadex G-100 failed as purification methods for the lectin extract. The lectin isolate was found to be non-specific. It agglutinated human erythrocytes of blood groups A, O, and B including erythrocytes from cow, carabao, goat, and swine. It failed to agglutinate

rabbit erythrocytes. SDS-gel electrophoresis showed that the lectin has a molecular weight of about 14,400-16,200. It is highly acidic protein with isoelectric pH below 3.5.

CHAPTER 3
Materials and Methods

3.0 Materials and Methods

3.1 Plant materials

3.1.1 Collection of the sample:

C. crista and *C. ramiflora* plants were selected for present research work. These plants were collected from Dhangmaree, Chadpai range, East zone of the Sundarbans, Khulna, Bangladesh on 16th December, 2011.

3.1.2 Preparation of Extract

3.1.2.1 Drying and Grinding

The plants were carefully cleaned and separated from undesired materials. Leaves and stems of *C. ramiflora* were separated and cut into small pieces. The whole part (leaves and stems) of *C. crista* together cut into small pieces. The sliced plants materials were dried under sunshade and air for several weeks. After completion of drying the plants materials were ground into powder form with a grinder. The powder of plants were weighted by an electric balance and stored in airtight plastic bags separately in a dark cool and dry place for further use.

3.1.2.2 Extraction with solvent

The powder of *C.crista* and *C.ramiflora* were taken into different clean, flat-bottomed glass jars and soaked into chloroform. They were then sealed and kept for a period of 5 days in a dark room accompanying occasional shaking and stirring. The mixtures filtered coarsely using white cotton material. As chloroform is a medium polar solvent so medium polar compounds were filtrate. After filtration the residues were kept 4 day and then dissolved into methanol for a period of 6 days. Polar compounds were filtrate because of polar solvent.

3.1.2.3 Evaporation and storage

Filtrates obtained from different solvent systems were evaporated at room temperature with an electric fan until they were completely dried. The crude extracts were weighed and kept in refrigerator for further experiments.

3.2 Evaluation of Antioxidant Activity

Anti-oxidant

Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals and are highly active to react with other molecules which can start chain reactions [Sies & Helmut 1997]. These radicals are important part of life, which are produced during cellular metabolism and functional activities and have important roles in cell signaling, apoptosis, gene expression and ion transportation. Although oxidation reactions are crucial for life, they can also be damaging [Boonchoong et al. 2004]. Excessive free radicals attack bases in nucleic acids, amino acid side chains in proteins and double bonds in unsaturated fatty acids, cause oxidative stress, which can damage DNA, RNA, proteins and lipids resulting in an increased risk for cardiovascular disease, cancer, autism and other diseases. Antioxidant is a molecule that inhibits the oxidation of other molecules. Cells are normally able to defend themselves against free radicals damage through the use of intracellular enzymes to keep the homeostasis of free radicals at a low level (Figure 3.1). However, during times of environmental stress and cell dysfunction, free radicals levels can increase dramatically, causes significant cellular damage in the body. In order to prevent or reduce the free radicals-induced oxidative damage, the human body and other organisms have developed an antioxidant defense system that includes enzymatic, metal-chelating and free radical-scavenging activities to neutralize these radicals after they have formed. In addition, intake of dietary antioxidants may help to maintain an adequate antioxidant status in the body [Giles et al. 2002].

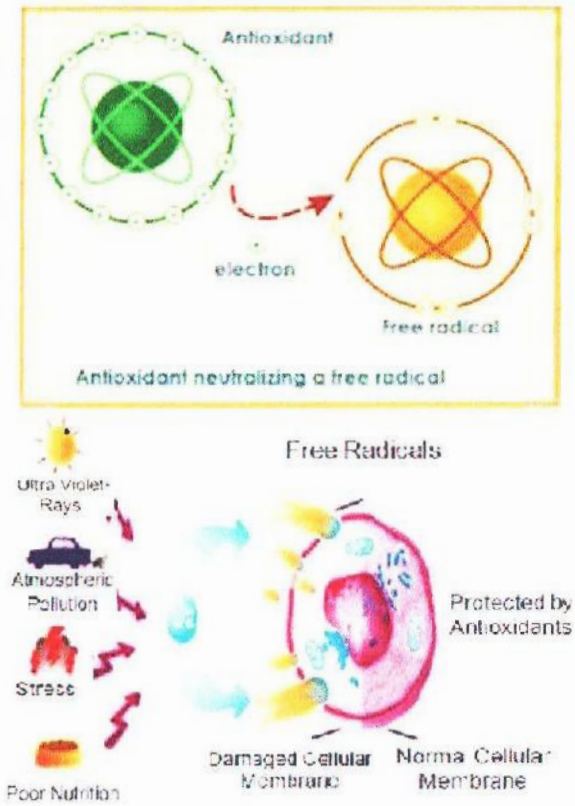
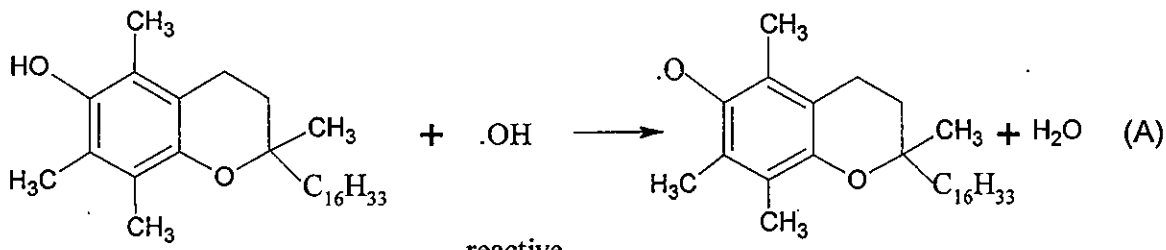
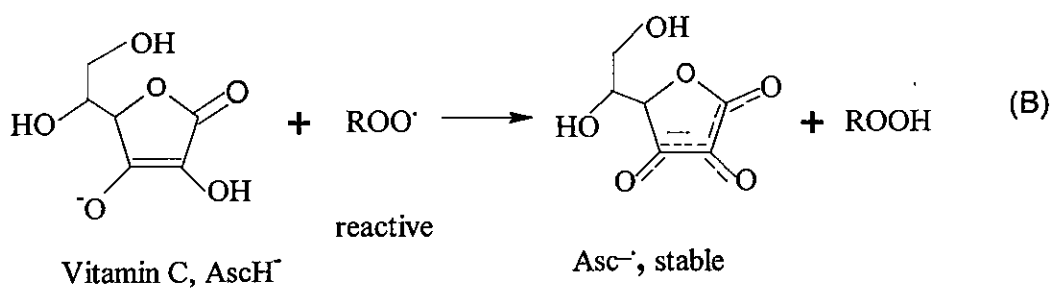


Figure 3.1: Mechanism and action of antioxidants.

Antioxidants can neutralize free radicals by accepting or donating electrons to eliminate the unpaired condition of the radical. The antioxidant molecules may directly react with the reactive radicals and destroy them, while they may become new free radicals which are less active, longer-lived and less dangerous than those radicals they have neutralized. They may be neutralized by other antioxidants or other mechanisms to terminate their radical status. Antioxidants are classified into two broad divisions, depending on whether they are soluble in water (hydrophilic) or in lipids (hydrophobic). In general, water-soluble antioxidants react with oxidants in the cell cytosol and the blood plasma, while lipid-soluble antioxidants protect cell membranes from lipid peroxidation. Many antioxidants have aromatic ring structures and are able to delocalize the unpaired electron. For example, Vitamin C ($AscH^-$) in the aqueous phase and vitamin E (TOH) in the lipid phase will directly react with or neutralize hydroxyl, alkoxyl ($LO\cdot$) and lipid peroxyl ($ROO\cdot$) radicals and form H_2O, alcohol and lipid hydroperoxides, (Figure 3.2) respectively. Vitamin E itself becomes a phenyl radical ($TO\cdot$) and vitamin C turns to a

very stable radical ($Asc^{-\bullet}$), due to its delocalized structure. Furthermore, vitamin C can also neutralize the radical form of other antioxidants such as glutathione radical and vitamin E radical and regenerate these antioxidants. Vitamin C itself is readily regenerated from $Asc^{-\bullet}$ with NADH or NADPH-dependent reeducates [Hossain et al. 1985]. Many antioxidants may directly react with free radical intermediates induced by reactive oxygen species and terminate the chain reaction, thereby stopping the free radical-induced damage [DeFeudis et al. 2003].





$$AscH^- + TO^\cdot \longrightarrow TOH + Asc^{-\cdot} \quad (C)$$

Figure 3.2: Direct reactions of vitamin E (TOH) with hydroxyl radical (A) and vitamin C ($AscH^-$) with lipid peroxyl radical ($ROO^\cdot$) (B), and regeneration of vitamin E from vitamin C (C).

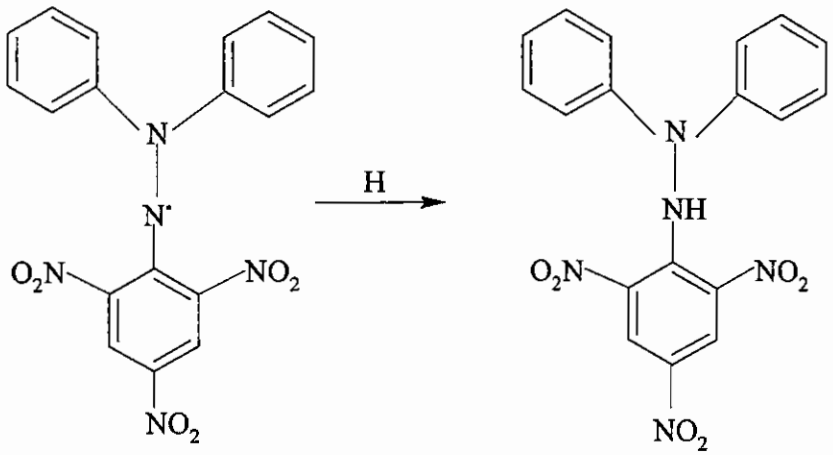
Small molecules such as vitamin C, vitamin E, uric acid and glutathione play important roles as cellular antioxidants. Synthetic antioxidants such as tert-butylhydroxyl-toluene, tert-butylhydroxyanisole and *tert*-butylhydroquinone have been

widely used in the food industry to retard lipid oxidation. However, such synthetic antioxidants are not preferred for pharmacologic use due to toxicological concerns. Thus, more and more interests have focused on identifying plant extracts to use as dietary antioxidant supplements [Rababah et al. 2004]. Many research models have been established in chemical or biological systems for the studies of antioxidant activity as well as identification of new antioxidants especially from natural substances. Generally, the antioxidant ability was measured and presented as 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH) scavenging activity, total antioxidant activity, total antioxidant potentials, ferric chloride reducing power, trolox equivalent antioxidant capacity, total radical absorption potentials and oxygen radical absorption capacity. These methods are based on either a single-electron transfer reaction or a hydrogen atom transfer reaction from an antioxidant or oxidant to a free radical. The change of optical absorbance of either antioxidant or oxidant is measured for the antioxidant capability.

3.2.1 DPPH Free Radical Scavenging Assay

3.2.1.1 Principle

A simple, rapid and inexpensive method to measure antioxidant capacity of sample involves the use of free radical, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) [Sharma & Bhat 2009]. It is a dark-colored crystalline powder composed of stable free-radical molecules and a trap ("scavenger") for other radicals. The DPPH method can be used for solid or liquid samples and is not specific to any particular antioxidant component, but applies to the overall antioxidant capacity of the sample. Antioxidants reduce 2, 2-diphenyl-1-picrylhydrazyl (purple color) to 2, 2-diphenyl-1-picrylhydrazine (colorless compound). The odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm reduces from 9660 to 1640 when the odd electron of DPPH radical becomes paired with hydrogen from free radical scavenging antioxidant to form the reduced DPPH-H. The resulting decolorization is stoichiometric with respect to number of electrons captured (Figure 3.3) [Cuvelier et al. 1992].



2,2-diphenyl-1-picrylhydrazyl 2,2 diphenyl-1-picrylhydrazine

Figure 3.3: Reaction between DPPH and antioxidant.

3.2.1.2 Chemical Requirements

The chemicals were ethanol, distilled water, 0.004% DPPH ethanolic solution, Quercetin (as positive control).

3.2.1.3 Equipments

The equipments used for this experiment were test tubes, beakers, digital balance, vortex machine, refrigerator, marker, Micropipette (20µl, 200µl and 1000µl), pipettes (5ml, 10ml), spoon, test tube stand, aluminum foil and UV spectrophotometer.

3.2.1.4 Methodology of DPPH free radical scavenging assay:

1. At first 9 test tubes were taken to make aliquots of 9 concentration (1.57, 3.13, 6.25, 12.50, 25, 50, 100, 200 and 400 µg/ml) by serial dilution technique for plant extract and 9 test tubes were taken to make aliquots of 9 concentration (1.57, 3.13, 6.25, 12.50, 25, 50, 100,200 and 400 µg/ml) by serial dilution technique for Quercetin which was taken as positive control.
2. Sample extract and quercetin were weighed and dissolved in ethanol to make homogeneously stock solution with the highest concentration i.e. 400 µg/ml, with the help of vortex and sonicator.
3. DPPH was also weighed and dissolved in ethanol to make 0.004% (w/v) solution.

4. After making the desired concentrations, 2ml of 0.004% DPPH solution was applied on each test tube containing 1ml of solution by pipette. The final volume of the solution was 3ml. This was done for both the quercetin and sample extract.
5. Then the test tubes were kept for 30 minutes in dark to complete the reaction.
6. DPPH was also applied on the blank test tubes at the same time where only ethanol was taken as blank.
7. After 30 minutes, absorbance of each test tube was determined by UV sepectrophotometer at 517 nm.
8. Percentage of inhibition was calculated as-

   $\%\ \text{Inhibition} = [(\text{Absorbance}_{\text{Blank}} - \text{Absorbance}_{\text{Sample}}) / \text{Absorbance}_{\text{Blank}}] \times 100$
9. IC_{50} value is the concentration of sample required to scavenge 50% of DPPH free radical and was calculated from the plot of % inhibition against the log concentration of sample extract.

3.2.2 Reducing Power Assay

3.2.2.1 Principle

Reducing power assay measures the electron-donating capacity of an antioxidant [Yen & Chen 1995]. In this assay, the yellow color of the test solution changes to various shades of green color depending on the reducing power of each compound. Reducing power assay method is based on the principle that substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then react with ferric chloride to form ferric ferrous complex. Therefore, measuring the formation of Perl's Prussian blue at 700 nm can monitor the Fe^{2+} concentration [Chen et al. 2010].

Potassium ferricyanide + Ferric chloride $\xrightarrow{\text{Antioxidant}}$ Potassium ferrocyanide + Ferrous chloride.

The reducing power was determined according to the method of Oyaizu (1986) with some modification [Oyaizu 1986].

The percent increase in reducing power was calculated using the following equation:

$$\text{Increase in reducing power (\%)} = \frac{A\ test - A\ blank}{A\ blank} \times 100$$

[Where A test is absorbance of test solution; A blank is absorbance of blank].

3.2.3 FRAP (Ferric ion Reducing Antioxidant Power) Assay

Ferric ion reducing antioxidant power (FRAP, also Ferric reducing ability of plasma) is a simple test to determine the total antioxidant power of sample. The FRAP assay was first performed by Iris Benzie and J. J. Strain of the Human Nutrition Research Group at the University of Ulster, Coleraine. It is often used to measure the antioxidant capacity of plant extract, foods, beverages and nutritional supplements containing polyphenols [Benzie et al. 1996].

3.2.3.1 Principle

The FRAP assay (ferric ion reducing antioxidant power) evaluates total antioxidant power and is chosen to assess the presumable effects of medicinal plants [Szollosi et al. 2002]. FRAP assay depends upon the ferric tripyridyltriazine (Fe(III)-TPTZ) complex to the ferrous tripyridyltriazine (Fe(II)-TPTZ) by the action of electron donating antioxidants at low pH. Fe(II)-TPTZ has an intensive blue color and can be monitored at 593 nm. The FRAP assay was determined according to the method of Benzie and Strain (1996) [Benzie et al. 1996].

3.2.3.2 Chemicals required

The chemicals were acetate buffer (300 mM; pH 3.6), TPTZ (2, 4, 6-tripyridyl-s-triazine), ferric chloride ($FeCl_3$. $6H_2O$).

3.2.3.3 Equipments

Test tubes, beakers, digital balance, vortex machine, water bath, micropipette (20µl, 200µl and 1000µl), pipettes (5ml), UV spectrophotometer, marker and aluminum foil.

3.2.3.4 Acetate buffer (300 mM; pH 3.6) preparation

3.1gm sodium acetate trihydrate was weighed and added into 16 ml of glacial acetic acid and make the volume to 1L with distilled water.

3.2.3.5 TPTZ (2, 4, 6-tripyridyl-s- triazine) preparation

0.031gm TPTZ dissolve in 10ml of 40mM HCl and kept at 50°C in water bath for 20minutes.

3.2.3.6 Ferric Chloride $FeCl_3.6H_2O$ (20 mM) preparation

0.054gm $FeCl_3.6H_2O$ dissolves in 10ml distilled water.

3.2.3.7 FRAP reagent preparation

The working FRAP reagent was prepared by mixing 25ml acetate buffer, 2.5ml TPTZ solution and 2.5ml $FeCl_3.6H_2O$ solution, then warmed at 37°C before using.

3.2.3.8 Protocol for FRAP assay

1. Sample extract and Quercetin were weighed and dissolved in distilled water to make homogeneously stock solution with the highest concentration i.e. 400 µg/ml, with the help of vortex and sonicator.
2. Five test tubes were taken to make aliquots of 5 concentrations (25, 50, 100,200 and 400 µg/ml) by serial dilution technique for plant extract and 5 test tubes were taken to make aliquots of 5 concentrations (25, 50, 100,200 and 400 µg/ml) by serial dilution technique for Quercetin which was taken as positive control.
3. Another five test tubes were taken with 0.2ml extract and quercetin solution.
4. Then 3ml FRAP reagent was applied on each test tube.
5. The mixture was kept at 37°C in water bath for 30minutes.
6. After cooling, the absorbance was measured at 593nm.

3.2.4 Total Phenolic Content Assay

Phenolic compounds are among the most widely occurring secondary metabolites in the plant kingdom, acting mainly as phytoalexins, attractants for pollinators and plant pigmentation, antioxidants and protective agents against UV light [Gottlieb et al. 2000]. Studies have indicated that the phenolic compounds are a major source of natural

antioxidants because their structure allows them to donate hydrogen atoms or electrons for free radical scavenging.

Some of the commonly used methods to determine the content of phenolics are approximately 100 years old, initially were developed by Folin and Colleagues at Harvard Medicinal School to study the metabolism of proteins in humans. Then Folin and Denis (1915) reported a method which applied to the determination of phenolics in sample. Due to the limitation of this method Folin and Ciocalteu (1927) was reported a modified method, mainly the modification was occurred on reagent, which referred to as the Folin-Ciocalteu reagent, was used to determine the content of phenolics from a wide range of sources. The Folin–Ciocalteu reagent (FCR) is a mixture of phosphomolybdate and phosphotungstate, clear bright yellow solution. Total phenolic content was estimated by the Folin-Ciocalteu method described by Singleton and Rossi [Singleton & Rossi 1965].

3.2.4.1 Principle

Polyphenols in plant extracts react with redox reagents (Folin-Ciocalteu reagent) to form blue complex chromogens that can be detected by visible-light spectrophotometry. The color development is due to the transfer of electrons at basic pH to reduce the phosphomolybdic/phosphotungstic acid complexes to form chromogens [Katzung et al. 2009].

3.2.4.2 Chemicals requirement

Sodium Carbonate (7% w/v), Folin-Ciocalteu (FC) reagent (diluted 10-fold with distilled water), Gallic acid (standard).

3.2.4.3 Equipments

Test tubes, beakers, digital balance, vortex machine, micropipette (20µl, 200µl and 1000µl), pipettes (5ml, 10ml), UV spectrophotometer, marker and aluminum foil.

3.2.4.4 Methodology for Total Phenolic Content

1. Gallic acid was used as standard. 250µg/ml stock standard solution of gallic acid was prepared by dissolving 0.0025 gm in 10 ml distilled water and then diluted to five concentration (50, 100, 150, 200 and 250 µg/ml).
2. The extract was prepared at concentration of 100µg/ml.

3. 9 ml distilled water and 1 ml FC reagent was added to each diluted gallic acid and extract solution.
4. The mixture was allowed to stand at room temperature for 5 minutes.
5. Then, 10 ml of 7% sodium carbonate was added to the mixture and mixed gently.
6. 25 ml adjust with distilled water.
7. After standing at room temperature for 60 minutes, the absorbance was measured at 750nm using spectrophotometer.

The standard calibration curve of gallic acid was plotted.

3.2.5 Total Flavonoid Content

Flavonoids are bioactive polyphenols which play a vital role in photosynthesising cells [Cushnie et al. 2005]. They are secondary metabolites characterised by flavan nucleus and C6-C8-C6 carbon-skeleton (Figure 3.4) [Heim et al. 2002; Peterson et al. 1998; Tsuchiya et al. 2010]. The basic structural feature of flavonoid is 2-phenyl-benzo-γ-pyrane nucleus consisting of two benzene rings (A and B) linked through a heterocyclic pyran ring (C) as shown in following figure [Cushnie et al. 2005]:

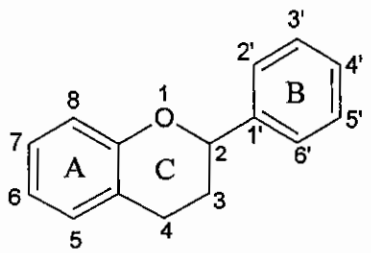


Figure 3.4: Basic structure of flavonoids.

Flavonoids differ in their arrangement of hydroxyl, methoxy and glycosidic side groups and in the conjunction between A and B rings [Heim et al. 2002]. A variation in C ring provides division of subclasses. According to their molecular structure, they are divided into eight classes (Figure 3.5) [Tsuchiya et al. 2010]:

7, whereas, C-glycosides possess sugar groups bound to Carbon of aglycone usually 6-C or 8-C [Buhler & Miranda 2000].

Flavonoids are phenolic substances isolated from a wide range of plants, with over 8000 individual compounds known. They act in plants as photoreceptors, visual attractors, feeding repellants and for light screening [Pietta 2000]. The flavonoids have aroused considerable interest recently because of their potential beneficial effects on human health. Many studies have suggested that flavonoids exhibit biological activities, including antibacterial, antiallergenic, antiviral, anti-inflammatory [Cushnie et al. 2005, Cook et al. 1996, Murray 1998], cytotoxic, antitumour, treatment of neurodegenerative diseases and vasodilating actions [Murray 1998, Williams et al. 2004, Tsuchiya et al. 2010, Chebil et al. 2006]. In addition flavonoids are known to inhibit lipid-peroxidation, platelet aggregation, capillary permeability and fragility, cyclo-oxygenase and lipoxygenase enzyme activities. They exert these effects as antioxidants, free radical scavengers, chelators of divalent cation [Cook et al. 1996, Chebil et al. 2006, Middleton et al. 2000]. These are also reported to inhibit variety of enzymes like hydrolases, hyalouronidase, alkaline phosphatise, arylsulphatase, cAMP phosphodiesterase, lipase, α-glucosidase, kinase [Narayana et al. 2001]. However, most interest has been devoted to the antioxidant activity of flavonoids, which is due to their ability to reduce free radical formation and to scavenge free radicals. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free radical scavenging activities. Flavonoids are powerful antioxidants against free radicals and are described as free-radical scavengers [Pal et al. 2009]. This activity is attributed to their hydrogen-donating ability. Indeed, the phenolic groups of flavonoids serve as a source of a readily available "H" atoms such that the subsequent radicals produced can be delocalized over the flavonoid structure [Tripoli et al. 2007]. Free radical scavenging capacity is primarily attributed to high reactivities of hydroxyl substituents that participate in the reaction [Heim et al. 2002] as shown in fig. 3.6:

$$F\text{-}OH + R^{\cdot} \longrightarrow F\text{-}O^{\cdot} + RH$$

Figure 3.6: Scavenging capacity of free radical (R.)

10% Aluminum chloride (10% AlCl3): 10gm in 100ml distilled water.

1 M Sodium hydroxide (1 M NaOH): 4gm NaOH in 100ml distilled water.

3.2.5.3 Equipments

Test tubes, beakers, volumetric flask, digital balance, vortex machine, micropipette (200µl and 1000µl), pipettes (5ml, 10ml), UV spectrophotometer, marker and aluminum foil.

3.2.5.4 Methodology for Total Flavonoid Content

1. Quercetin was used as standard and were weighed and dissolved in distilled water to make homogeneously stock solution with the highest concentration i.e. 400 µg/ml, with the help of vortex and sonicator.
2. Five test tubes were taken to make aliquots of 5 concentrations (25, 50, 100,200 and 400 µg/ml) by serial dilution technique for Quercetin.
3. The extract was prepared at concentration of 100µg/ml.
4. 5 ml distilled water and 0.3 ml of 5% $NaNO_2$ was added to each diluted Quercetin and 1ml of extract solution.
5. After 5 minutes 0.6 ml of 10% $AlCl_3$ was added.
6. After 5 minutes 2 ml of 1M NaOH.
7. Then the mixture was allowed to stand at room temperature for 5 minutes.
8. After standing at room temperature, the absorbance was measured at 510 nm using spectrophotometer.

The standard calibration curve of Quercetin was plotted.

3.2.6 Total Tannin Content

Tannins are a complex group of polyphenolic compounds found in a wide range of plant species as secondary metabolites which have been known and used by man for centuries. Their name comes from the French '*tan*' meaning the bark of the holm oak and other trees used in tanning [Harborne, 1999; Schofield, 2001]. Tannins are a heterogeneous group of high molecular weight phenolic compounds with the capacity to form reversible and irreversible complexes with proteins (mainly), polysaccharides (cellulose, hemicellulose, pectin, etc.), alkaloids, nucleic acids and minerals etc (Figure 3.10 and 3.11) [Mcleod, 1974; Mole et al. 1987, Mangan, 1988; Mueller et al. 1992; Van

soest, 1994; Giner, 1996; Schofield, 2001]. Tannins play an important role in plant function and evolution [Cates & Rhoades, 1977; Zucker, 1983] . Tannins found in plants are divided into two major classes: the condensed and the hydrolysable tannins.

Hydrolyzable tannins (HT) are further grouped into gallotannins and ellagitannins that are composed of gallic acid or hexahydroxydiphenic acid esters, respectively, linked to a sugar moiety. More complex hydrolyzable tannins can form by oxidative transformations which can yield macrocylic ellagitannins.

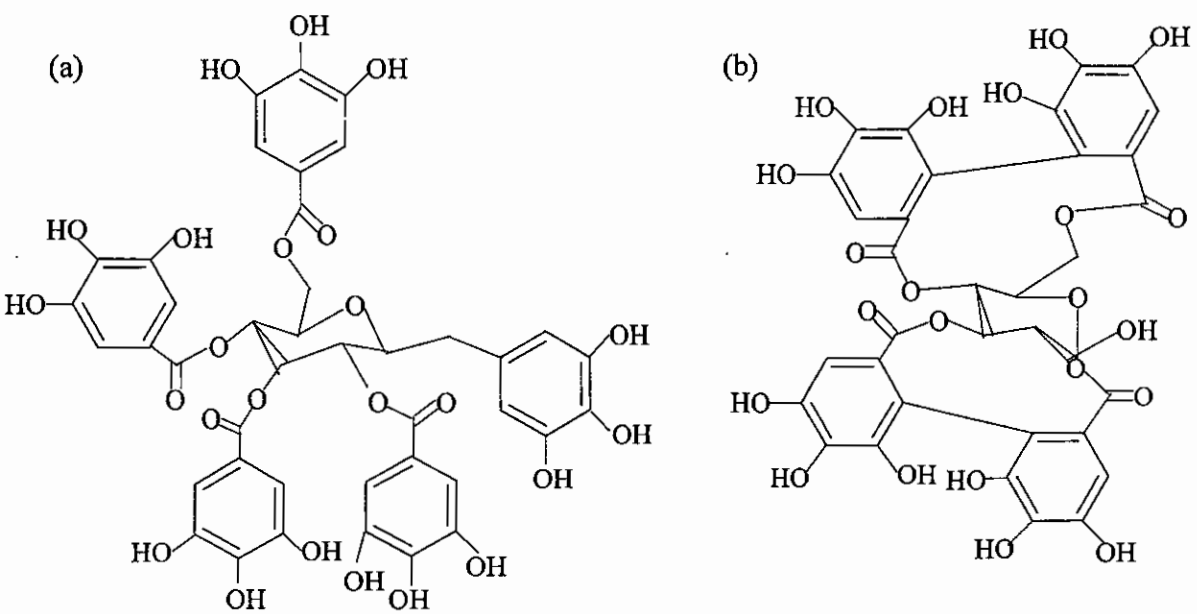


Figure 3.10: Hydrolyzable tannins (a) a simple gallotannin and (b) a simple ellagitannin.

Condensed tannins (CT) also referred to as proanthocyanidins are polymers of three-ring flavanols joined with C-C bonds. The monomer units that make up CT are distinguished by the number of OH groups on the B-ring: procyanidins (PC) have a di-hydroxy B-ring while prodelphinidins (PD) have a tri-hydroxy B-ring. The monomer units may also have either *cis* or *trans* C2–C3 stereochemistry [Okuda et al. 2000].

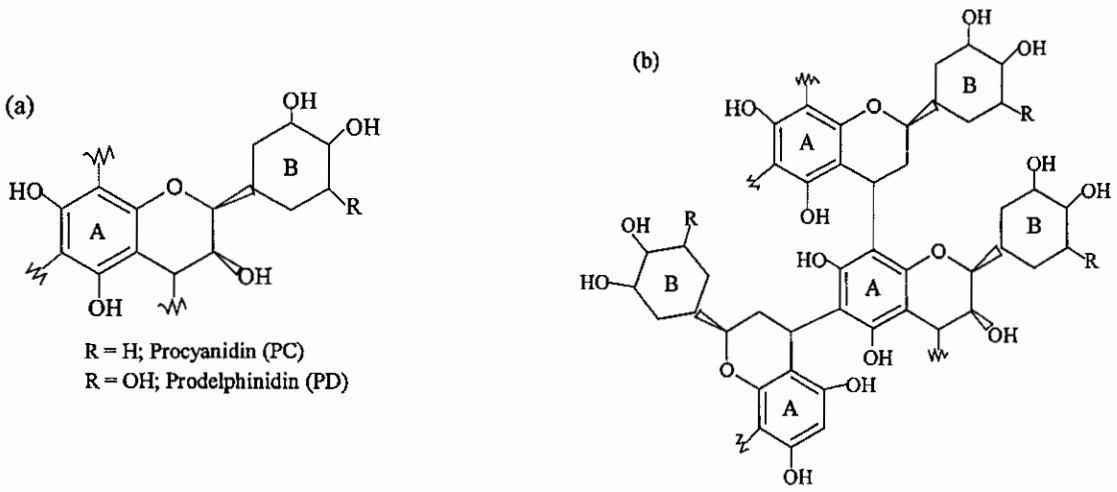


Figure 3.11: (a) a condensed tannin trans monomer unit, (b) a condensed tannin trimer showing different intermonomer linkages (C4–C8 and C4–C6) and different *cis/trans* monomers.

The tannins of different plant species have different physical and chemical properties [Mangan, 1988] and therefore they have very diverse biological properties [Zucker, 1983]. Gymnosperms and monocots produce only CT, dicots can produce either CT or HT or a mixture of both tannin types [Bate-Smith, 1977; Haslam, 1988]. It is important to note that even within CT and HT there is a wide range of structural variation [Okuda et al. 2000; Porter 1989; Porter 1992]. Differences between individual tannins arise from variations in chain length, number of OH groups, position of intermonomer linkages (e.g., C4–C6 or C4–C8 linkages in CT, C-C or ether bonds in HT), stereochemistry, branching extent, glycosylation and substitutions with aliphatic or carbohydrate moieties. Tannins composed of both procyanidins and ellagitannins are also known [Okuda et al. 2000; Porter, 1992]. Thus there is an enormous diversity in tannins, and the larger the polymer the greater the probability of variation [Behrens, 2003]. Most plants produce a variety of tannin structures [Polshettiwar, 2007], suggesting that within individual plants there are distinctive roles for specific tannin compounds [Hagerman, 1998; Zucker, 1983].

Content of tannins in plant extract was determined by Folin-Denis method [Polshettiwar et al. 2007].

3.2.6.1 Principle

The method is based on the oxidation of molecule containing a –OH groups. The tannin and tannin like compound reduce Phosphotungustomolybdic acid in alkaline solution to produce a highly blue colored solution [Cetkovic et al. 2008, Jain & Dixit, 2004].

3.2.6.2 Chemicals requirement

Sodium Carbonate (35% w/v), Folin-Ciocalteu (FC) reagent (diluted 10-fold with distilled water), Gallic acid (standard).

3.2.6.3 Equipments

Test tubes, beakers, digital balance, vortex machine, micropipette (20µl, 200µl and 1000µl), pipettes (5ml, 10ml), UV spectrophotometer, marker and aluminum foil.

3.2.6.4 Methodology for Total Tannin Content

1. Gallic acid was used as standard. 100µg/ml stock standard solution of gallic acid was prepared by dissolving 0.001 gm in 10 ml distilled water and then diluted to five concentrations (6.25, 12.5, 25, 50 and 100 µg/ml).
2. The extract was prepared at concentration of 100µg/ml.
3. 7.5 ml distilled water and 0.5 ml FC reagent was added to each diluted gallic acid and extract solution.
4. The mixture was allowed to stand at room temperature for 5 minutes.
5. Then, 1 ml of 35% sodium carbonate was added to the mixture and mixed gently.
6. 10 ml adjust with distilled water.
7. After standing at room temperature for 30 minutes, the absorbance was measured at 725nm using spectrophotometer.

The standard calibration curve of gallic acid was plotted.

3.3 Evaluation of Antibacterial Activity

There is constant interaction between microbes and humans in all walk of human life. The vast majority of the microbes in the body are rendered harmless by the protective effects of the immune system and few are health beneficial. Microbes have been the causes of many diseases in the human being and other animals. Infectious disease development involves the complex interactions between the microbe and the host. Microbes produce disease by killing host cells or by liberating toxins that can cause tissue damage and functional derangements even without extensive colonization of host tissues. So, we need to protect ourselves from these to lead a healthy life. The capacity of the substances to kill or inhibit microbial growth is called antimicrobial activity and the substances that possess this activity are called antimicrobials. The history of antimicrobials began with the observation of Pasteur and Joubert, who discovered that one type of bacteria could prevent the growth of another. In the age of time various types of antimicrobials like antibiotics, antivirals, antifungals, anti-parasitics have been developed against diseases caused by corresponding microorganisms.

Advances of modern medicine, many infectious diseases once considered incurable and lethal are now amenable to treatment with antimicrobial agents. The remarkably powerful and specific activity of antimicrobial drugs is due to their selectively for highly specific targets that are either unique to microorganisms. Among these targets are specific bacterial and fungal cell wall-synthesizing enzymes that required for nucleotide synthesis and DNA replication of microbes [Bertram & Susan 2009]. Nowadays multiple drug resistance has developed due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious disease. For this reason, antibiotics are sometimes associated with adverse effects on the host including hypersensitivity, immune-suppression and allergic reactions. This situation forced scientist to search for new antimicrobial substances. Given the alarming incidence of antibiotic resistance in bacteria of medical importance, there is a constant need for new and effective therapeutic agents. Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases. Antimicrobial properties of medicinal plants are being increasingly reported from different part of the world. They are effective in the treatment of infectious diseases while simultaneously mitigating many

of the side effects that are often associated with synthetic antimicrobials. The beneficial medicinal effects of plant material effects of plant materials typically results from the combination of secondary products present in the plant which are capable of producing definite physiological action on body.

Mangrove plant extracts have been used for centuries as a popular method for treating several health disorders. Plant-derived substances have recently become of great interest owing to their versatile applications. Mangroves are biochemically unique, producing a wide array of novel natural products. Mangrove and mangrove associates contain biologically active antibacterial, antiviral and antifungal compounds. In this point of view, in my research I have selected two mangrove plants and evaluate antibacterial activity of crude chloroformic and methanolic extracts of leaf and stem of *C.ramiflora* and *C.crista.*

In vitro antimicrobial of plants can be detected by observing the growth response of various microorganisms to those plant extracts or their solvent fractions that are placed in contact with them. This can be done in three ways (diffusion, dilution and bioautographic methods) and a number of modifications have been made in the technique in order to obtain better results. Since some factors (culture medium composition, microorganisms tested, extractive method, pH, solubility of the sample in the culture medium etc.) can change results, it is difficult to standardize a procedure for the study of antimicrobial plants. Diffusion and dilution methods have been employed to study the antimicrobial activity of medicinal plants. Bioautography is another method for studying antimicrobial activity [Myran & Mendoza 1998]. Disc diffusion technique is widely acceptable for the preliminary screening of antimicrobial activity. If the test material has any antimicrobial activity, it will inhibit the growth of microorganism giving a clear distinct zone called "zone of inhibition". The antimicrobial activity of the test agent is determined by measuring the diameter of the zone of inhibition in term of millimeter. Antimicrobial activity of the crude extract was determined by disc diffusion method. However, no distinction between bacteriostatic and bacteriocidal activity can be demonstrated by this method [Reiner 1982].

3.3.1 Test of Antimicrobial Activity by Disc Diffusion Assay

3.3.1.1 Principle

In this method measured amount of test sample were dissolved in definite amount of volatile solvent to get a solution of known concentration (µg/ml). Then sterile filter paper discs were impregnated with known amount of test substances using micropipette and dried. Standard antibiotic discs were used as positive control and disc impregnated with solvent was used as negative control. These discs were then placed in Petri dishes containing agar medium seeded with the test organisms of log phase. The test material diffuses from the discs to the surrounding medium. The seeded Petri dishes containing test and control discs were then placed into the incubator for 12 to 18 hours at 37°C to allow the growth of microorganisms. If the test material had any antimicrobial activity, it would inhibit the growth of microorganism giving a clear distinct zone called "Zone of Inhibition". The antimicrobial activity of the sample was determined by measuring the diameter of the zone of inhibition in terms of millimeter.

3.3.1.2 Materials for test

3.3.1.2.1 Test material:

Crude chloroformic and methanolic extract of the leaves and stems of *C.ramiflora* and *C.crista.*

3.3.1.2.2 Positive control:

Antibiotic disc Ciprofloxacin 5 mcg/disc.

3.3.1.2.3 Equipment:

Aluminum foil, autoclave, cotton, digital slid calipers, electronic balance, filter paper discs, laminar air flow cabinet, marker, micropipette, nose mask, apron and gloves, Petri dishes, pipette, punch machine, refrigerator, spirit lamp, sterile forceps, sterile inoculating loop and test tubes.

3.3.1.2.4 Reagents:

Distilled water, ethanol, nutrient agar medium and nutrient broth.

3.3.1.3 Bacteria used for the activity test

Both Gram-positive and Gram-negative bacterial strains were taken for the test (Table 3.1). The bacterial strains used for this investigation are listed in following table. These organisms are preserved in Microbiology Laboratory of Biotechnology and Genetic Engineering Discipline, Khulna University, Bangladesh.

Table 3.1: List of bacteria used for the evaluation of antimicrobial activity

Gram-positive	Diseases	Gram-negative	Diseases
Staphylococcus aureus	Skin infections, respiratory disease and food poisoning.	*Escherichia coli*	Food poisoning
Staphylococcus epidermis	Skin infections	*Vibrio cholerae*	Cholera
Bacillus megaterium	Non-pathogenic	*Salmonella typhi*	Typhoid fever
		Pseudomona aurigonisa	Infection in pulmonary tract, urinary tract, burns and wounds.

3.3.1.4 Culture Media

A mixture of nutrient used in the laboratory to support growth and multiplication of a culture (a population of microorganisms) is called culture medium [Pelczar et al. 1996]. The nutritional requirements of bacteria vary widely so there are great differences in the chemical compositions of the media used in the laboratory. Some microorganism can grow in medium containing only inorganic compounds, whereas others require a medium containing organic compounds (amino acids, sugars, purines or pyrimidines, vitamins or coenzymes). Some require complex natural substances (peptone, autolysate, blood cells or blood serum) and some cannot as yet be grown in an artificial laboratory

medium and can be propagated only in a living host or living cells. Each kind of organism also requires specific physical conditions for growth. For example, some bacteria cannot grow below 40°C, some cannot grow above 20°C and some require a temperature close to that of the human body (i.e., 37°C). Light may be another important physical condition. The successful cultivation of bacteria requires an awareness of all of these factors. Each kind of microorganism grows in a characteristic manner. For example, growth in a liquid medium may be abundant or sparse; it may be evenly dispersed throughout the medium or it may occur only as sediment at the bottom or only as a thin film or pellicle at the top. On solid media, microbes grow as colonies distinct, compact masses of cells that are macroscopically visible. Colonies are characterized by their size, shape, texture, consistency, color and other notable features [Pelczar et al. 1993]. Chemically defined media are needed for the cultivation of autotrophs and are also useful for defining the nutritional requirements of heterotrophs. However, for the routine cultivation of heterotrophs, chemically defined media are not generally used. Instead, certain complex raw materials such as peptones, beef extract and yeast extract are used and the resulting media support the growth of a wide variety of heterotrophic bacteria. Agar is included as a nonnutritive solidifying agent when a solid medium is desired [Pelczar et al. 1993]. The following media are used normally to demonstrate the antibacterial activity and to make subculture of the test organisms:

- Nutrient Agar Media
- Nutrient Broth Media
- Muellar-Hinton Agar Media
- Tryptic Soy Broth (TSP)

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

3.3.1.5 Media Composition:

For 100ml

Ingredients	Amount
Distilled water	100ml
Peptone	0.5gm
Beef extract	0.3gm
Agar	1.5gm

3.3.1.6 Methodology:

3.3.1.6.1 Preparation of media:

3.3.1.6.2 Nutrient agar media:

Nutrient agar media was prepared by mixing commercially available ingredients in powdered form with recommended amount of water. Following steps were done to do this:

1) For 100ml nutrient agar media, 0.5gm peptone, 0.3gm beef extract and 1.5gm agar were taken in the screw capped conical flask.
2) So 100ml distill water was added to that amount of powder.
3) Then the media was subjected to sterilization in the autoclave.

3.3.1.6.3 Nutrient broth media:

Nutrient agar media was prepared by mixing commercially available ingredients in powdered form with recommended amount of water. Following steps were done to do this:

1) For 100ml nutrient agar media, 0.5gm peptone and 0.3gm beef extract were taken in the screw capped conical flask.
2) So 100ml distill water was added to that amount of powder.
3) Then the mixture was shaken well and then it was subjected to sterilization in the autoclave.

3.3.1.6.4 Sterilization:

Both the culture media, Petri dishes, micropipette, test tubes, forceps, screw capped vial, screw capped conical flasks, pipette were sterilized by autoclaving at a temperature of 121°C and a pressure of 15Ibs/sq inch for 15 minutes. Blank disc were subjected to dry heat sterilization at 110°C for 20 minutes. The Laminar air flow cabinet was disinfected by ethanol with spray bottle. The UV light in the laminar air flow cabinet was switched on before one hour to avoid accidental contamination.

3.3.1.6.5 Preparation of Subculture:

This was done by culturing pure microbial population in the agar media. To obtain better growth of bacterial colony tow generation of subculture were prepared. Following steps was performed for preparation of first generation of subculture:

1) At first prepared culture media was poured into Petri dishes which were marked for desired strains of microorganisms.
2) At least two subculture plates were made for each strain.
3) The Petri dishes were allowed to cool in refrigerator for 15 minutes.
4) Then the inoculating loop (sterilized by spirit lamp) was dipped into the pure culture of bacteria and streaking was done in the Petri dishes marked for those strains of bacteria.
5) Then the Petri dishes were allowed to incubate for 12-18 hours at 37°C in the incubator.
6) After 12-18 hours bacterial colony was observed in the culture media.
7) Then the prepared plates of first generation subculture were kept in refrigerator which can be used for 2 months.

3.3.1.6.6 Preparation of Seeded Test Plates:

For the evaluation of antimicrobial activity by disc diffusion assay seeded test plate of microbes is necessary to made. It was done by two steps:

In the first step-

- 2 ml of nutrient broth was taken in each screw capped vial where all of them were marked according to their strains. Two vials were taken for each strain.
- Then each vials containing nutrient broth were inoculated by the inoculating loop which looped single bacterial colony from the sub culture. These tasks were done in the laminar air flow cabinet.

- Then all the vials were allowed to incubate at 3-4 hours at 37°C. This was done to get the bacteria in their log phase at which the growth rate is very high.

In the second step-

- The sterilized agar medium was poured into sterilized Petri dishes in such a way as to give a uniform layer of depth of approximately 4 mm. After the medium became cooled at room temperature, it was stored in a refrigerator (4°C).
- Incubated vials log phase were removed from the incubation.
- Test tubes were shaken by rotation to get a uniform suspension of bacteria.
- Then 5µl nutrient broth containing bacteria was taken into corresponding solidified medium containing Petri dishes.
- The Petri dishes were rotated several times, first clock wise then anticlockwise to assure homogenous distribution of the test organisms. Thus the seeded plates were ready for disc diffusion assay. These tasks were done in the laminar air flow cabinet.

All tasks were done in the laminar air flow cabinet.

3.3.1.6.7 Preparation of Test Sample

0.04 gm each of chloroformic and methanolic extract of *C. ramoflora* and *C. crista* were accurately measured by using the electronic balance and taken into screw capped vials (vials were nullified on the electronic balance by auto zero). Then 10 ml of ethanol was added in the vial to get the sample of 4mg/ml.

3.3.1.6.8 Preparation of Discs:

Three types of discs were used for the antimicrobial screening:

a) Sample discs
b) Standard discs and
c) Blank discs

3.3.1.6.9 Sample discs:

Filter papers previously sterilized were perforated by punch machine thus tiny discs of 5 mm diameter were obtained. Then the discs were taken in a blank Petri dish. 20µl and 40µl of sample solution were applied on the discs with the help of micropipette in an aseptic condition from the stock solution. Then the discs were allowed to dry for a

few minutes under the laminar to remove the solvent ethanol as it has some antimicrobial activity which can bias the evaluation.

3.3.1.6.10 Standard discs:

These were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antibacterial agent with that produced by test samples. In this investigation Ciprofloxacin (5 mcg/disc) standard disc were used as the reference.

3.3.1.6.11 Blank discs:

Blank discs were used a negative control. They ensure that the residual solvents (left over the discs even after air drying) and the filter paper were not active themselves.

3.3.1.6.12 Application of Discs

Sample impregnated discs, standard antibiotic discs and negative control discs were placed gently on the solidified agar plates, freshly seeded with the test organisms with the help of a sterile forceps to assure complete contact with medium surface. The spatial arrangement of the discs was such that the discs were not closer than 15 mm to the edge of the plate and far enough apart to prevent overlapping the zones of inhibition. Finally the plates were incubated upside down at 37°C for 12-18 hours.

3.3.1.6.13 Determination of Antimicrobial Activity:

After proper incubation, the antimicrobial activity of the test agent was determined by measuring the diameter of zone of inhibition in term of millimeter with a transparent scale.

3.4 Evaluation of Cytotoxic Activity

3.4.1 Evaluation of Brine Shrimp Lethality Assay for toxicity test

The brine shrimp bioassay is a simplest, less expensive and easily achievable method replacing cell lines bioassay in order to determine the toxicity of plants extracts by the estimation of their medium lethality concentration LC_{50} [Meyer et al. 1982]. This bioassay was proposed by Michael *et al.* in 1956 and modified by others. This method is normally conducted to draw inferences on the safety of the plant extracts and to further depict trends of their biological activities and considered as a useful tool for the preliminary assessment toxicity [Solis et al. 1993]. Nauplii of the brine shrimp *Artemia salina* are invertebrate of the fauna of saline aquatic and marine ecosystem [Lewan et al. 1992] and are most convenient test organisms for toxicity studies. *Artemia* cysts can indeed be stored for years without losing their viability and upon immersion in sea water; nauplii will hatch within a period of 1 to 2 days. As such, *Artemia* has a unique advantage over all other zooplankters as a bioassay organism since it does not require any maintenance or stock culturing. In the first larval stage (instar I) the digestive tract of the nauplius is not in contact yet with the external medium and the larva only consumes its yolk. After a certain period of time, the nauplius molts into the second larval stage (Instar II) and starts feeding on particular matter. The molting into the further larval stages is dependent on both temperature and quality and quantity of food. When not fed, *Artemia* larvae will die during the third or fourth instar stage. It is demonstrated that second and third instars are significantly more sensitive to toxicants than first instar larvae [Reeve 1963]. In most of the literature, brine shrimp bioassay used *Artemia* cysts those were hatched within 15 to 18 hr before the first instar larvae molted to the second stage. So the factors which should be kept under strict control are the exact origin of the strain, the temperature during incubation and hatching, the moment of harvesting of the larvae, the period of time between the harvest and the start of the bioassay, the temperature and the medium during the test. The *Artemia* nauplii have in the past 3 decades been used to test general toxicity in teratology screens and in ecotoxicology. Furthermore, from the pharmacological perspective, a good correlation has been found with brine shrimp lethality test to detect anticancer compounds in plant extracts. However, the present study

percentage of lethality of brine shrimp nauplii was calculated at each concentration for each sample.

$$\%\ Mortality = \frac{Avg.no.of\ alive\ srimp\ of\ control - Avg.no.of\ alive\ shrimp\ of\ sample}{Avg.no.of\ s\square rimp\ of\ control} \times 100$$

CHAPTER 4

Results and Discussion

4.0 Results and Discussion

4.1 Antioxidant Related Assay

4.1.1 DPPH Free Radical Scavenging Assay

The DPPH antioxidant assay is a rapid, direct and reliable method for the antioxidant screening of plant extracts. A number of methods are available for the determination of free radical scavenging activity but the assay employing the stable 2, 2-diphenyl-1-picryl-hydrazyl radical (DPPH) has received the maximum attention owing to its ease of use and its convenience. This test is based on the ability of DPPH, a stable nitrogen-centered free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 515-517 nm and also for a visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the color of DPPH changes from violet to yellow, which can be quantitatively measured from the changes in absorbance. The purple color of DPPH fades due to conversion of it to 2, 2-diphenyl-1-picryl hydrazine resulting in decrease in absorbance. The DPPH radical scavenging activities of sample increased gradually as the concentration increased. Decrease in absorbance of DPPH solution depends on intrinsic antioxidant activity of samples as well as on speed of reaction between DPPH and antioxidant. Quercetin was chosen as the reference antioxidant for this test.

In this study, antioxidant activity of chloroformic and methanolic extracts of *C. crista* and *C. ramiflora* were examined and analyze their graphical representation.

4.1.1.1Chloroformic extract of *C. crista*

Graphical analysis of Absorbance vs. Log Concentration, the absorbance of the extract decreased gradually as the log concentration increased. In figure 4.1 showed the absorbance of chloroformic extract of *C. crista* and standard quercetin at different log concentration.

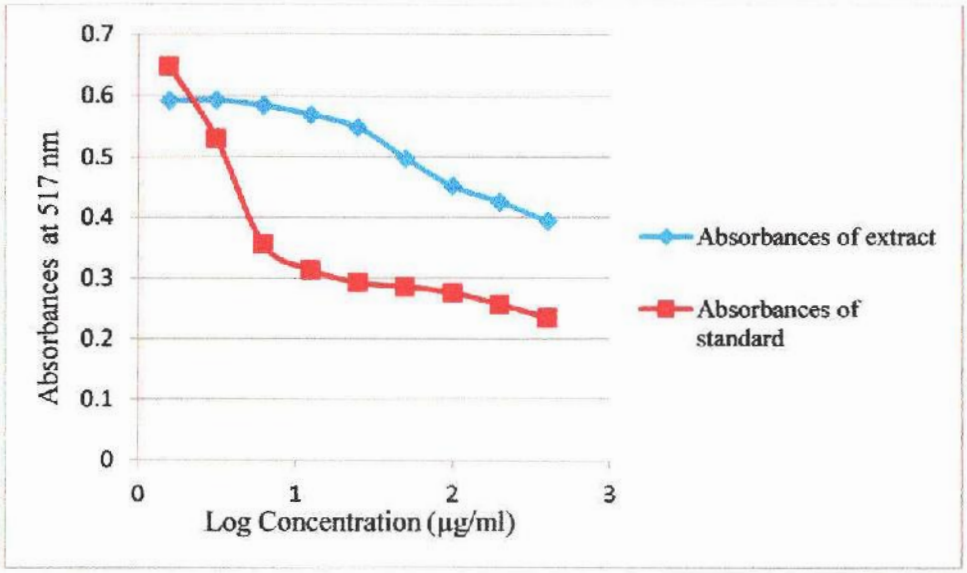


Figure 4.1: DPPH Scavenging assay of *C.crista* chloroform extract (Absorbance vs Log Concenitration).

Figure 4.2 showed that the comparison of percentage inhibition of chloroformic extract of *C. crista* and standard quercetin at different concentrations. The concentration at which 50% inhibition (IC_{50}) of DPPH occurred were obtained by extrapolation. By using the equation y = mx + c (where 'c' is intercept and 'm' is slope); IC_{50} value of extract was found at the concentration of 225.9 µg/ml and standard quercetin was 7.62 µg/ml. Here the concentration of the extract required for 50% inhibition is much greater than that of control.

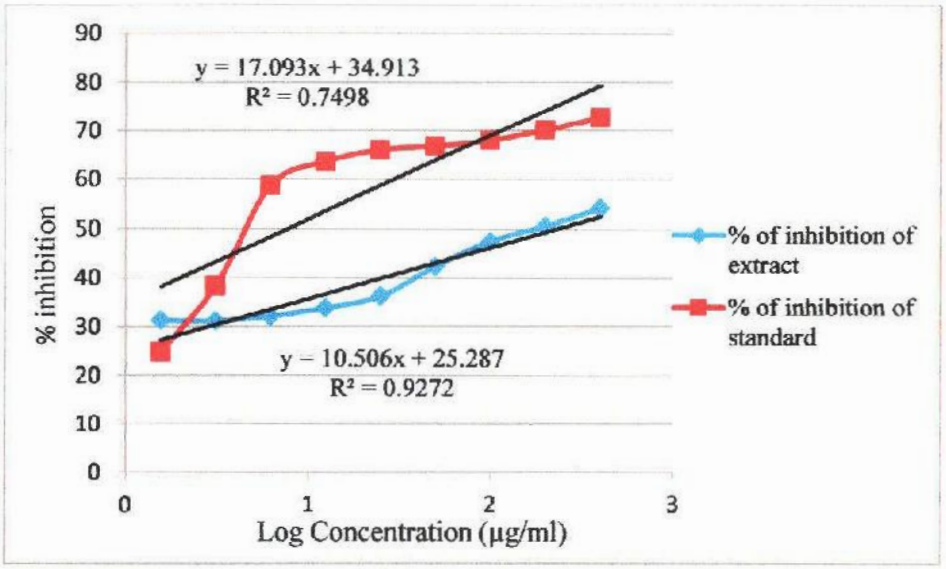


Figure 4.2: DPPH Scavenging assay of *C.crista* chloroform extract (% inhibition vs Log Concentration).

4.1.1.2 Methanolic extract of *C. crista*

Analysis of *C. crista* methanolic extract, the absorbance of the extract decreased gradually as the log concentration increased. Absorbance at different log concentration of extract and standard quercetin is given in figure 4.3.

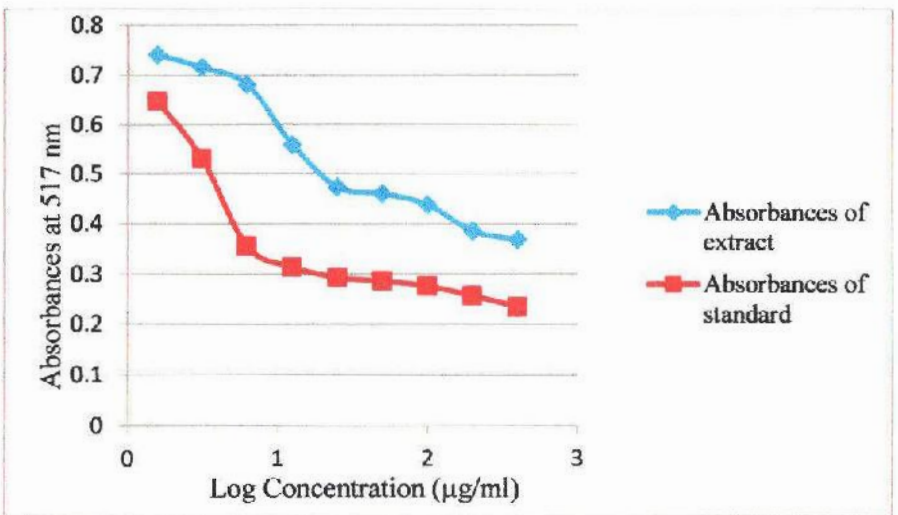


Figure 4.3: DPPH Scavenging assay of *C.crista* methanolic extract (Absorbance vs Log Concenitration).

Comparison of percentage inhibition of methanolic extract of *C. crista* and standard quercetin at different log concentrations is given in fig. 4.4. The concentration at which 50% inhibition (IC_{50}) of DPPH occurred were obtained by using the equation y = mx + c. IC_{50} value of extract was found at the concentration of 103.7 µg/ml and standard quercetin was 7.62 µg/ml. So we observed that the concentration of the extract required for 50% inhibition is greater than that of control.

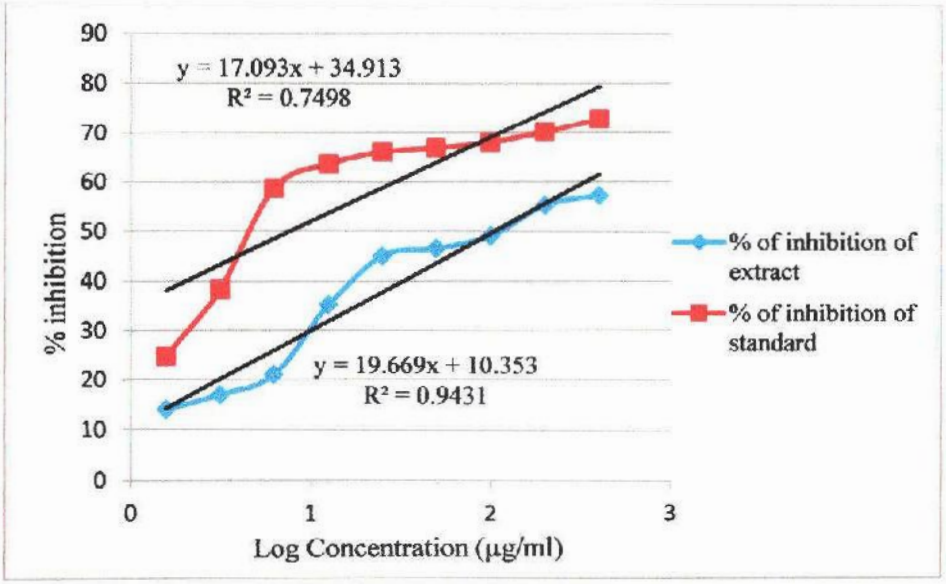


Figure 4.4: DPPH Scavenging assay of *C.crista* methanol extract (% inhibition vs Log Concentration).

4.1.1.3 Chloroformic extract of *C. ramiflora* leaf

Graphical analysis of Absorbance vs. Log Concentration, the absorbance of the extract decreased gradually as the log concentration increased. In figure 4.5 showed the absorbance of chloroformic extract of *C. ramiflora* leaf and standard quercetin at different log concentration.

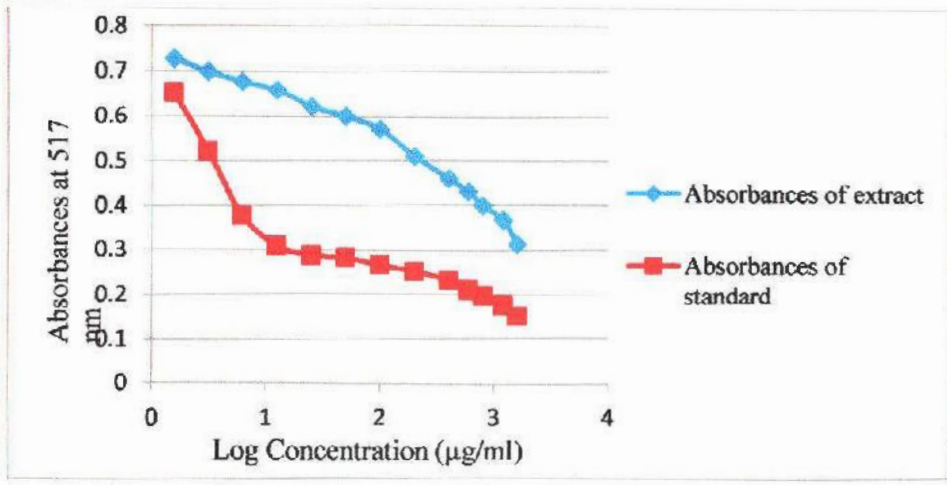


Figure 4.5: DPPH Scavenging assay of *C. ramiflora* leaf chloroform extract (Absorbance vs Log Concentration).

Comparison of percentage inhibition of chloroformic extract of *C. ramiflora* leaf and standard quercetin at different log concentrations is given in fig. 4.6. The concentration at which 50% inhibition (IC_{50}) of DPPH occurred were obtained by using the equation y = mx + c. IC_{50} value of extract was found at the concentration of 537.03 µg/ml and standard quercetin was 8.95 µg/ml. So we observed that the concentration of the extract required for 50% inhibition is much greater than that of control.

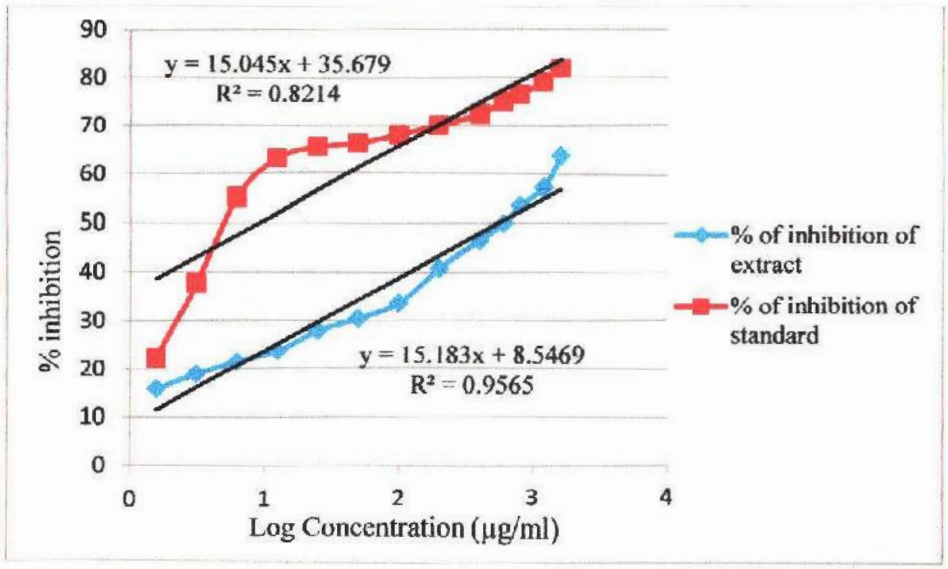


Figure 4.6: DPPH Scavenging assay of *C.ramiflora* leaf chloroform extract (% inhibition vs Log Concentration).

Figure 4.10 showed that the comparison of percentage inhibition of chloroformic extract of *C. ramiflora* stem and standard quercetin at different concentrations. The concentration at which 50% inhibition (IC_{50}) of DPPH occurred were obtained by extrapolation. By using the equation y = mx + c (where 'c' is intercept and 'm' is slope); IC_{50} value of extract was found at the concentration of 606.7 µg/ml and standard quercetin was 8.95 µg/ml. Here the concentration of the extract required for 50% inhibition is much greater than that of control.

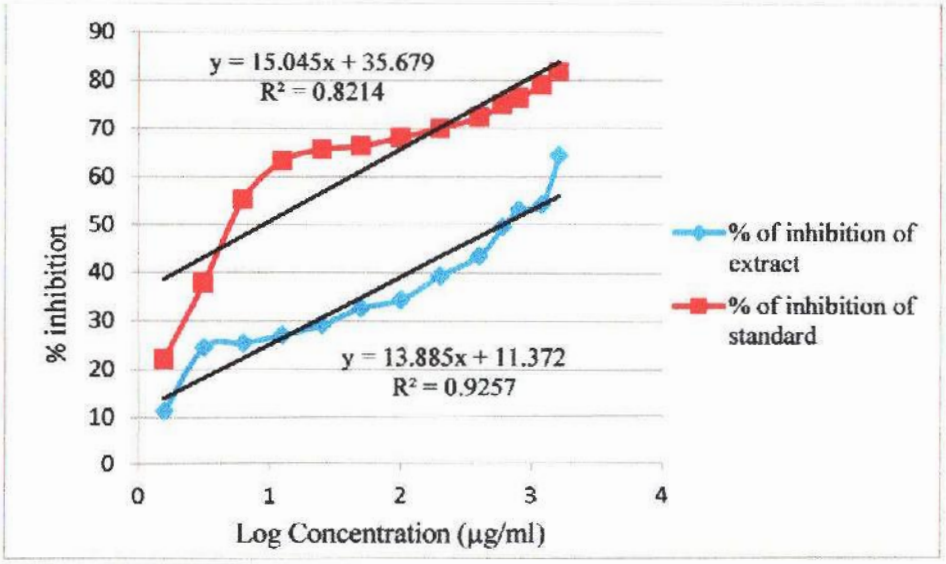


Figure 4.10: DPPH Scavenging assay of *C.ramiflora* stem chloroform extract (% inhibition vs Log Concentration).

4.1.1.6 Methanolic extract of *C. ramiflora* stem

Graphical analysis of Absorbance vs. Log Concentration, the absorbance of the extract decreased gradually as the log concentration increased. In figure 4.11 showed the absorbance of methanolic extract of *C. ramiflora* stem and standard quercetin at different log concentration.

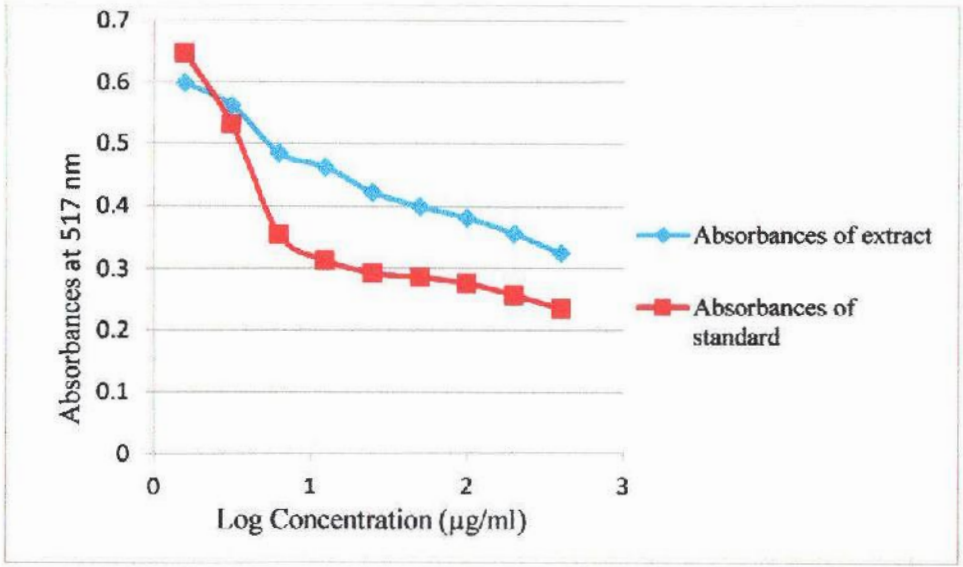


Figure 4.11: DPPH Scavenging assay of *C. ramiflora* stem methanolic extract (Absorbance vs Log Concenitration).

Figure 4.12 showed that the comparison of percentage inhibition of methanolic extract of *C. ramiflora* stem and standard quercetin at different concentrations. The concentration at which 50% inhibition (IC_{50}) of DPPH occurred were obtained by extrapolation. By using the equation $y = mx + c$ (where 'c' is intercept and 'm' is slope); IC_{50} value of extract was found at the concentration of 32.65 µg/ml and standard quercetin was 7.62 µg/ml. Here the concentration of the extract required for 50% inhibition is much nearer than that of control. It indicates that methanolic extract of *C. ramiflora* stem possess potential antioxidant properties.

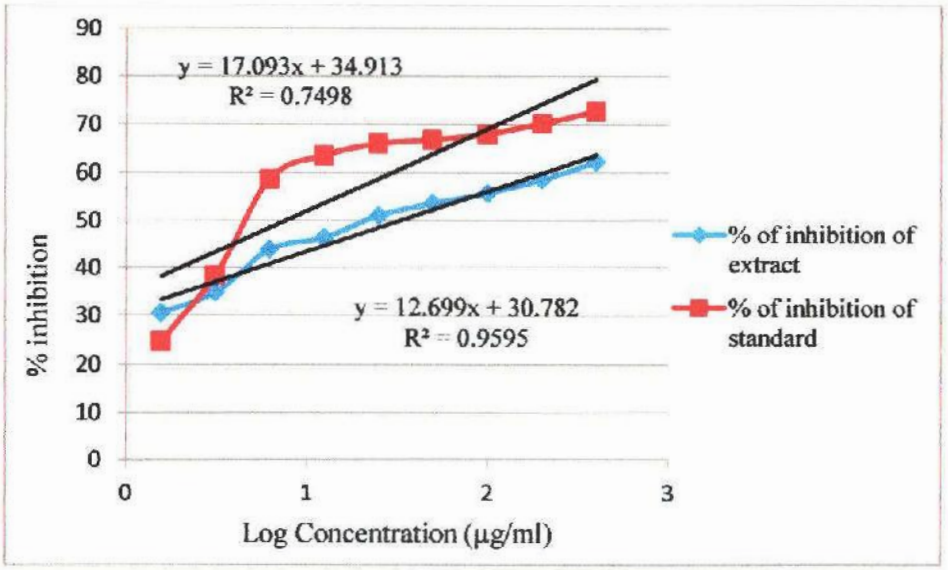


Figure 4.12: DPPH Scavenging assay of *C.ramiflora* stem methanolic extract (% inhibition vs Log Concentration).

4.1.1.7 Discussion

Antioxidant activity of plants extract were examined by DPPH free radical scavenging assay where plant extracts exhibited radical scavenging effect of DPPH in concentration dependent manner. In this study, two mangrove plants *C. crista* and *C. ramiflora* from the same family were selected for knowing their medicinal properties. On the basis of their ethnobotanical information we emphasized to analyze their antioxidant properties in this research.

Chloroformic extract of *C. crista* exhibited 50% radical inhibitions at 225.94 µg/ml concentration and methanolic extract showed at the concentration of 103.75 µg/ml. Whereas, standard quercetin exhibited 50% inhibition at a concentration of 7.620 µg/ml. Figure 4.2 shows the comparison of percentage inhibition between chloroformic extract of *C. crista* and standard quercetin. This figure represents that the scavenging activity of extract did not increased likely to the standard. So chloroformic extract of *C. crista* have a smaller antioxidant potentiality, may be due to the presence of non polar or less polar compounds. Figure 4.4 shows the comparison between percentage inhibition of methanolic extract of *C. crista* and standard quercetin. This figure represents that the scavenging activity of extract nearer to the standard. It indicates moderate antioxidant

property of methanolic extract of *C. crista*. This is in agreement with the findings of Mandal et al. 2009, who investigated that methanolic extract of *C. crista* leaves acts as an antioxidant and ROS (reactive oxygen species) scavenger. This moderate activity may be due to the presence of cassane and norcassane type diterpenes [Kalauni et al. 2004; Awale et al. 2006; Kalauni et al. 2005; Kinoshita et al. 2005]. Literature suggests that presence of such compounds exert potent cytotoxic properties [Das et al. 2010; Tian et al. 2012] and antimalarial properties [Kalauni et al. 2006; Linn et al. 2005].

Chloroformic extract of *C. ramiflora* leaves exhibited 50% radical inhibitions at 537.03 µg/ml concentration whereas standard quercetin exhibited 50% inhibition at the concentration of 8.953µg/ml. Figure 4.6 shows the comparison of percentage inhibition between chloroformic extract of *C. ramiflora* leaves and standard quercetin. This figure represents that the scavenging activity of extract was very poor. Methanolic extract of *C. ramiflora* leaves exhibited 50% radical inhibitions at 102.801 µg/ml concentration. Whereas, standard quercetin exhibited 50% inhibition at the concentration of 7.602 µg/ml. Figure 4.8 shows the comparison between percentage inhibition of methanolic extract of *C. ramiflora* leaves and standard quercetin. This figure represents that the scavenging activity of extract nearer to the standard. It indicates moderate antioxidant property of methanolic extract of *C. ramiflora* leaves.

Chloroformic extract of *C. ramiflora* stem exhibited 50% radical inhibitions at 606.73 µg/ml concentration whereas standard quercetin exhibited 50% inhibition at the concentration of 8.953µg/ml. Figure 4.10 shows the comparison of percentage inhibition between chloroformic extract of *C. ramiflora* stem and standard quercetin. This figure represents that the scavenging activity of extract was very much poor. Methanolic extract of *C. ramiflora* leaves exhibited 50% radical inhibitions at 32.65 µg/ml concentration. Whereas, standard quercetin exhibited 50% inhibition at the concentration of 7.602 µg/ml. Figure 4.12 shows the comparison between percentage inhibition of methanolic extract of *C. ramiflora* stem and standard quercetin. This figure represents that the scavenging activity of methanolic extract increased sharply like standard quercetin. Smallest IC_{50} values of methanolic extract indicate this extract as potent radical scavenger.

So analyzing the all results of DPPH free radical scavenging activity of plant extracts, it is noticed that two different solvent extracts contained different antioxidant capacities in term of radical scavenging power. The results showed that methanolic extracts have a greater antioxidant potentiality than the chloroformic extract. Chloroformic extract contain non-polar or less polar compounds and methanolic extract contain polar compounds. This low free radical scavenging activity of chloroformic extract in may be due to the presence of non-polar or less polar compounds. Methanolic extracts might contain phenolics compounds, e.g. flavonoids, coumarins or phenylpropanoids, tannins, saponins were obtained using solvents of high polarity; the methanolic extract manifested greater power antioxidant. So we can say that yield of the extract were better when extraction was done with methanolic solvents. This indicates that the methanolic solvent system is more efficient for the recovery of antioxidant components, thus offering higher extract yields. However, further research is needed to identify individual components forming antioxidative system and develop their application for food and pharmaceutical industries.

4.1.2 Reducing power assay

In this assay, the reducing power of the extracts was determined by direct electron donation in the reduction of ferri cyanide $[Fe(CN)_6]^{3-}$ to ferro cyanide $[Fe(CN)_6]^{4-}$. The product was visualized by addition of free Fe^{3+} ions after the reduction reaction, by forming the intense Prussian blue color complex, $(Fe^{3+})_4[Fe^{2+}(CN\text{-})_6]^3$ and quantified by absorbance measurement at 700 nm. The reducing power of the extracts increases with the increase in amount of sample. Increased absorbance of the reaction mixture indicates increased reducing power.

4.1.2.1 Chloroformic extract of *C. crista*

The reducing power of all the extracts increased with increase in concentration. Table 4.1 shows that the chloroformic extract of *C. crista* range of 26.285 to 84.857 percent increase in reducing power whereas standard quercetin exhibited the ranges from 191.688 to 334.545. Figure 4.13 shows the comparison between chloroformic extract of *C. crista* and standard quercetin.

Table 4.1: Reducing power assay of chloroform extract of *C.crista*

Concentration (µg/ml)	Absorbance ± SD	% Increase in reducing power
Blank	0.35	
25	0.442 ± 0.007	26.285
50	0.469 ± 0.018	34
100	0.518 ± 0.022	48
200	0.58 ± 0.012	65.714
400	0.647 ± 0.021	84.851

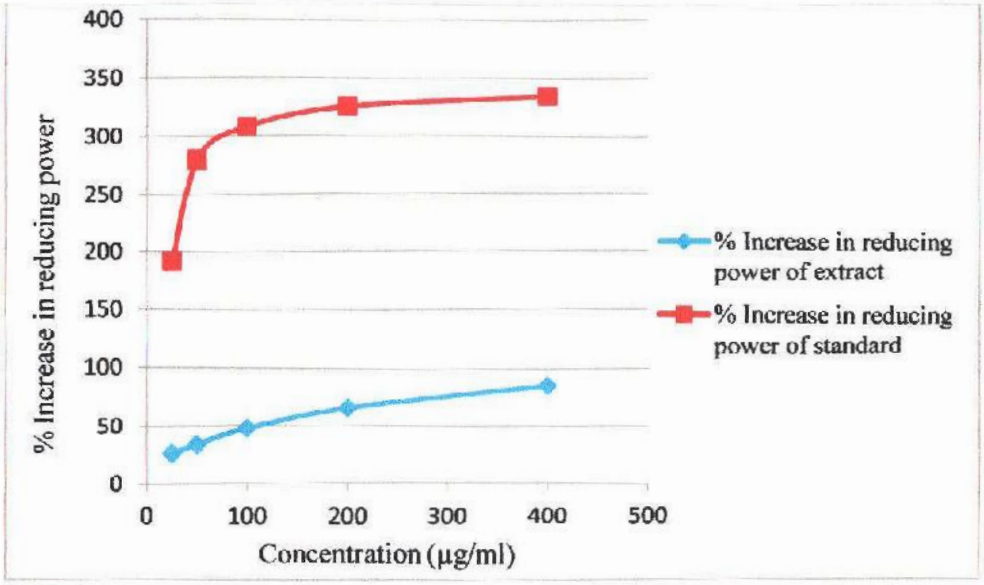


Figure 4.13: Reducing ability of chloroformic extract of *C.crista* and standard Quercetin at various concentrations.

4.1.2.2 Methanolic extract of *C. crista*

In the case of *C. crista* methanolic extract, table 4.2 shows that the methanolic extract exhibited the ranges from 51.399 to 248.510 whereas standard quercetin exhibited the ranges from 191.688 to 334.545. Figure 4.14 shows the comparison between methanolic extract of *C. crista* and standard quercetin.

Table 4.5: Reducing power assay of chloroform extract of *C.ramiflora* (stem)

Concentration (µg/ml)	Absorbance ± SD	% Increase in reducing power
Blank	0.48	
25	0.515 ± 0.003	7.291
50	0.524 ± 0.017	9.166
100	0.553 ± 0.019	15.208
200	0.594 ± 0.004	23.75
400	0.685 ± 0.005	42.812

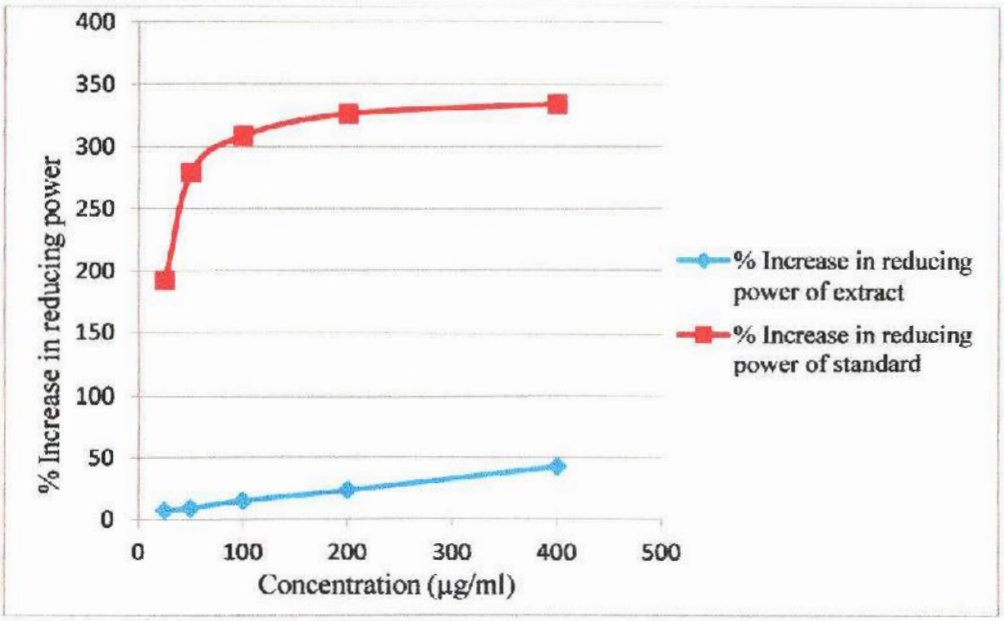


Figure 4.17: Reducing ability of chloroformic extract of *C.ramiflora* (stem) and standard Quercetin at various concentrations.

4.1.2.6 Methanolic extract of *C. ramiflora* stem

In the case of *C. ramiflora* stem methanolic extract, table 4.6 shows that the methanolic extract exhibited the ranges from 82.697 to 326.392 whereas standard quercetin exhibited the ranges from 191.688 to 334.545 percent increase in reducing power. Among the extracts this extract shows the highest percent increase in reducing power. Figure 4.18 shows the comparison between methanolic extract of *C. ramiflora* stem and standard quercetin.

Table 4.6: Reducing power assay of methanol extract of *C.ramiflora* (stem)

Concentration (µg/ml)	Absorbance ± SD	% Increase in reducing power
Blank	0.341	
25	0.623 ± 0.059	82.697
50	0.695 ± 0.062	103.812
100	0.833 ± 0.833	144.281
200	1.14 ± 0.056	234.31
400	1.454 ± 0.386	326.392

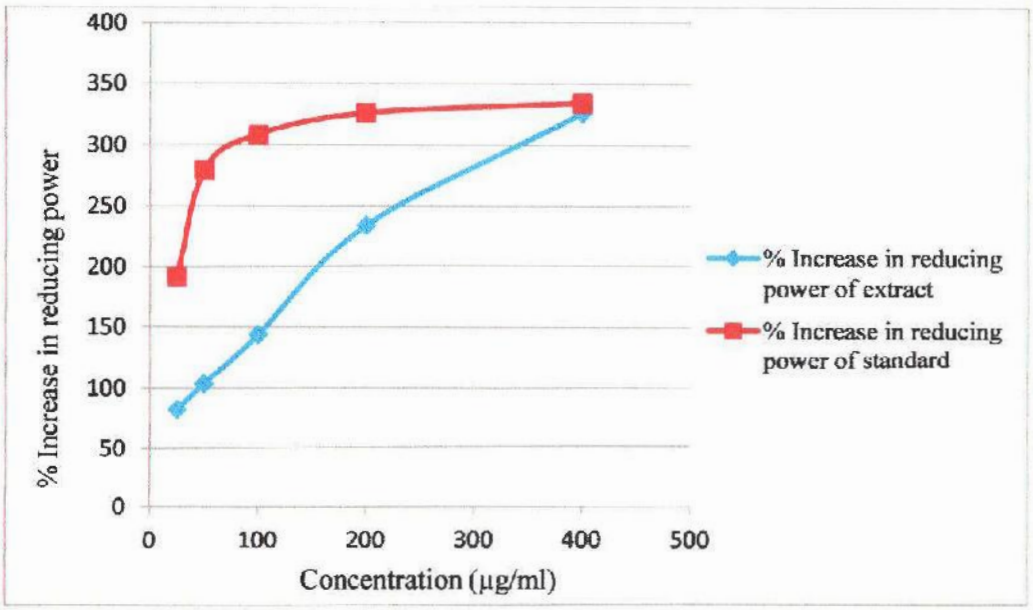


Figure 4.18: Reducing ability of methanolic extract of *C.ramiflora* (stem) and standard Quercetin at various concentrations.

Table 4.7: Reducing power assay of standard Quercetin

Concentration (µg/ml)	Absorbance ± SD	% Increase in reducing power
Blank	0.385	
25	1.123 ± 0.013	191.688
50	1.461 ± 0.044	279.48
100	1.574 ± 0.022	308.831
200	1.642 ± 0.029	326.493
400	1.673 ± 0.012	334.545

4.1.2.7 Discussion

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants [Jung et al. 2008]. This assay has proven the effectiveness of various extracts compared to the standard antioxidant, Quercetin. Literature reports are evident that the reducing power of bioactive compounds is associated with antioxidant activity. The presence of reductants (i.e. antioxidants) in extracts causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, the Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. In the concentration range investigated, all the extracts demonstrated reducing power that increased linearly with concentration. In case of chloroformic extract of *C. crista*, the maximum absorbance was found (0.647 ± 0.021) at 400 µg/ml concentration; whereas (1.673 ± 0.012) was found for standard quercetin. Figure 4.13 showed percentage increase in reducing power of extract in comparison to the standard. Analyzing this figure, chloroformic extract of *C. crista* had not much reductive capability with compare to the standard.

Methanolic extract of *C. crista* possess moderate reducing potential (1.279 ± 0.084) in compared to standard quercetin (1.673 ± 0.012). Figure 4.14 showed the comparison between percentage increase in reducing power of methanolic extract of *C. crista* and standard quercetin. This figure represents that the reductive capability of extract quite nearer to the standard. This is in agreement with the findings of Mandal et al. 2009, who investigated that methanolic extract of *C. crista* leaves showed moderate reducing capability.

In case of chloroformic extract of *C. ramiflora* leaves, the maximum absorbance was found (0.592 ± 0.006) where standard quercetin showed (1.673 ± 0.012) at concentration of 400µg/ml. Analyzing figure 4.15 chloroformic extract of *C. ramiflora* leaves had much poor reductive capability with compare to the standard.

In table 4.4, the maximum absorbance of methanolic extract of *C. ramiflora* leaves was found (1.238 ± 0.089) where standard quercetin showed (1.673 ± 0.012) at concentration of 400µg/ml. Figure 4.16 showed the comparison between percentage increase in

reducing power of methanolic extract of *C. ramiflora* leaves and standard quercetin, which represents the moderate reductive capability of extract.

In case of chloroformic extract of *C. ramiflora* stem, the maximum absorbance was found (0.685 ± 0.005) where standard quercetin showed (1.673 ± 0.012) at concentration of 400µg/ml. Analyzing figure 4.17 chloroformic extract of *C. ramiflora* stem had poor reductive capability with compare to the standard.

Among all the extracts methanolic extract of *C. ramiflora* stem possess highest reducing potential (1.454 ± 0.386) in compared to standard quercetin (1.673 ± 0.012). Figure 4.18 showed the comparison between percentage increase in reducing power of methanolic extract of *C. ramiflora* stem and standard quercetin. It indicates potential reductive capability of methanolic extract of *C. ramiflora* stem.

In the present investigation, methanolic extract of *C. ramiflora* stem possess highest reducing potentiality and highest scavenging activity in DPPH assay in compared to all extracts. The reducing power of the extract might be due to their hydrogen donating ability. Possibly, methanolic extract of *C. ramiflora* stem contain high amounts of reductive, which could react with radicals to stabilize and terminate radical chain reactions.

4.1.3 FRAP (ferric reducing antioxidant power) Assay

The total antioxidant potential of sample was determined using FRAP assay as a measure of antioxidant power. FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue colored FeII-tripyridyltriazine compound from colorless oxidized FeIII form by the action of electron donating antioxidants. In this research work, the total antioxidant potential of *C. crista* and *C. ramiflora* was determined by using FRAP reagent with standard Quercetin. Standard curve was prepared using different concentrations (100- 6.25) µg/ml. In the FRAP assay the antioxidant efficiency of the antioxidant under the test was calculated with reference to the reaction signal given by an Fe^{2+} solution of known concentration, this representing a one-electron exchange reaction. The results were expressed in µM Fe (II)/100g dried plant material using the equation $y = 0.005x + 0.042$, coefficient $R^2 = 0.991$, where y is absorbance at 593 nm and x is the total

antioxidant potential of different extracts. Absorbance of quercetin in different concentration is given in table 4.8 and from which calibration curve plotted in figure 4.19.

Table 4.8: Absorbance of stadard quercetin at different concentration

Concentration (µg/ml)	Avg. of Absorbance (593 nm)	Absorbance ± SD
Blank	0.0955	
100	0.603	0.603 ± 0.012
50	0.358	0.358 ± 0.024
25	0.1965	0.1965 ± 0.018
12.5	0.11475	0.11475 ± 0.030
6.25	0.05625	0.05625 ± 0.011

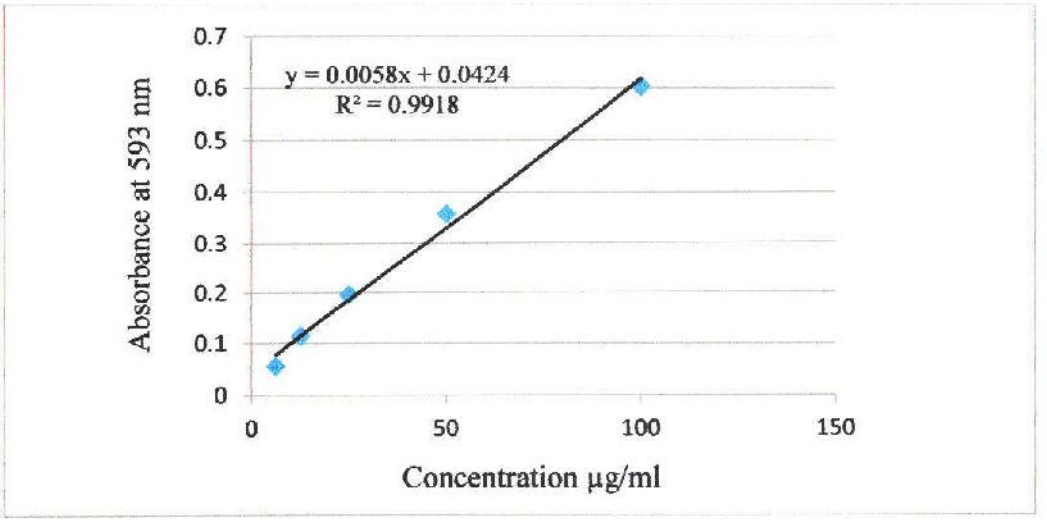


Figure 4.19: Calibration curve of standard Quercetin for determination of FRAP assay.

4.1.3.1 Total antioxidant capacity of *C. crista*

In this research; using standard calibration curve, total antioxidant power of chloroformic and methanolic extract of *C. crista* was determined. Table 4.9 shows the absorbance and antioxidant power of chloroformic and methanolic extract of *C. crista*. Total antioxidant power of chloroformic extract exhibited 35.4 ± 0.023 µM Fe (II)/100g

of dried plant material and methanolic extract showed 70.4 ± 0.031 µM Fe (II)/100g of dried plant material.

Table 4.9: Antioxidant power of *C. crista*

Extract	Conc. (µg/ml)	Absorbance	Ferric reducing antioxidant power (µM Fe (II)/g) ± SD
$CHCl_3$	100	0.219	35.4 ± 0.023
MtOH	100	0.394	70.4 ± 0.031

4.1.3.2 Total antioxidant capacity of *C. rmiflora* (leaf)

From quantitive estimation of total antioxidant capacity of chloroformic extract of *C. ramiflora* leaves showed 22.2 ± 0.013 µM Fe (II)/100g of dried plant material and methanolic extract showed 57.8 ± 0.015 µM Fe (II)/100g of dried plant material respectively. Absorbance and ferric reducing antioxidant power of *C. ramiflora* leaves extract is furnished in table 4.10.

Extract	Conc. (µg/ml)	Absorbance	Ferric reducing antioxidant power (µM Fe (II)/g) ± SD
$CHCl_3$	100	0.153	22.2 ± 0.013
MtOH	100	0.331	57.8 ± 0.015

Table 4.10: Antioxidant power of *C. ramiflora* (leaf)

4.1.3.3 Total antioxidant capacity of *C. rmiflora* (stem)

From quantitive estimation of total antioxidant capacity of chloroformic extract of *C. ramiflora* stem showed 50 ± 0.007 µM Fe (II)/100g of dried plant material and methanolic extract showed 84 ± 0.009 µM Fe (II)/100g of dried plant material. Absorbance and ferric reducing antioxidant power of *C. ramiflora* stem extract is furnished in table 4.11. Methanolic extract of *C. ramiflora* stem had highest antioxidant capacity.

Table 4.11: Antioxidant power of *C. ramiflora* (stem)

Extract	Conc. (µg/ml)	Absorbance	Ferric reducing antioxidant power (µM Fe (II)/g) ± SD
$CHCl_3$	100	0.292	50 ± 0.007
MtOH	100	0.462	84 ± 0.009

4.1.3.4 Discussion

FRAP assay measure the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine [Fe^{3+}-TPTZ] complex and producing a colored ferrous tripyridyltriazine [Fe^{2+}-TPTZ] [Guo et al. 2003]. Generally, the reducing properties are associated with the presence of compounds which exert their action by breaking the free radical chain by donating a hydrogen atom. FRAP assay treats the antioxidants in the sample as a reductant in a redox-linked colorimetric reaction [Ercan et al. 2011].

The reducing capacity of an extracts may serve as a significant indicator of its potential antioxidant activity. FRAP assay showed variability between various extracts. In this research using FRAP assay the range of antioxidant capacity of extracts was 22.2 to 84.0 μM Fe (II)/ 100g of dried plant material. From the quantitative estimation of total antioxidant potential of *C. crista*, choloroformic and methanolic extract of *C. crista* exhibited 35.4 ± 0.02 and 70.4 ± 0.03 μM Fe (II)/ 100g of dried plant material respectively. Methanolic extract of *C. crista* showed significant antioxidant power. Investigating the results of the present study, *C. ramiflora* leaves showed the slightest antioxidant capacity. Choloroformic and methanolic extract of *C. ramiflora* leaves exhibited 22.2 ± 0.013 and 57.8 ± 0.015 μM Fe (II)/ 100g of dried plant material respectively. Among the entire extracts *C. ramiflora* stem showed highest antioxidant capacity, specially its methanolic extract. Choloroformic and methanolic extract of *C. ramiflora* stem exhibited 50 ± 0.007 and 84 ± 0.009 μM Fe (II)/ 100g of dried plant material respectively.

In this research work, previous assays also showed that the methanolic extract had a greater antioxidant potentiality than the chloroformic extract. Amazingly, methanolic extract of *C. ramiflora* stem found as the strongest scavenger (fig. 4.12) in DPPH assay and also strong reducing power capability (fig. 4.18) in reducing power assay. Methanolic *C. crista* showed second highest capacity in these tests.

4.1.4 Total Phenolic Content

The amount of total phenol was determined with the Folin-Ciocalteu assay using Gallic acid as a standard phenolic compound. This method is a fast and simple method and can be useful in characterizing and standardizing botanical samples. Folin-Ciocalteu method is based on oxidation of phenolics by a molybdotungstate in Folin-Ciocalteu reagent to yield a colored product with λmax 745 – 750 nm [Javanmardi et al .2003].

A linear calibration curve of Gallic acid, in the range of 25–250 µg/ml with coefficient of determination (R^2) value of 0.968 was obtained. Absorbace of gallic acid in different concentration is given in table 4.12 and from which calibration curve plotted in figure 4.20. Using this calibration curve, the total amount of phenolic content of plant extract was determined using the equation $y = 0.002x + 0.036$, where y is the absorbance at 750 nm and x is total phenolic content in the different plant extracts.

Table 4.12: Absorbance of standard gallic acid at different concentration

Concentration (µg/ml)	Avg. of Absorbance (750 nm)	Absorbance ± SD
Blank	0.0656	
250	0.4876	0.4876 ± 0.012
200	0.444	0.444 ± 0.016
150	0.3693	0.3693 ± 0.005
100	0.253	0.253 ± 0.006
50	0.133	0.133 ± 0.011
25	0.0526	0.0526 ± 0.008

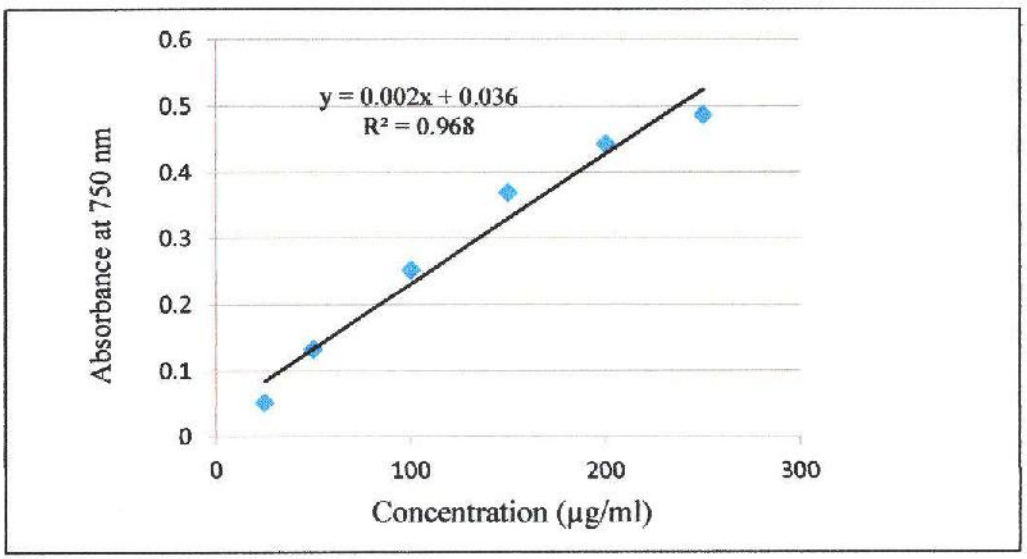


Figure 4.20: Calibration curve of standard Gallic acid for determination of total phenolics content.

4.1.4.1 Total phenolic content of *C. crista*

In this work, total phenolic content of different plant extract of *C. crista* was determined using the standard calibration curve. Table 4.13 represents the amount of total phenolic content in chloroformic and methanolic extract of *C. crista*. Chloroformic extract shows 7.7 ± 0.013 mg GAE/mg of extract and methanolic extract shows 52.5 ± 0.012 mg GAE/mg of extract respectively.

Table 4.13: Total phenolic content of *C. crista*

Extract	Conc. (µg/ml)	Absorbance (750 nm)	Total Phenolic Content (GAE/mg of extract) ± SD
$CHCl_3$	100	0.0514	7.7 ± 0.013
MtOH	100	0.141	52.5 ± 0.012

4.1.4.2 Total phenolic content of *C. ramiflora* (leaf)

From quantitive estimation of total phenolic content of chloroformic extract of *C. ramiflora* leaves showed 6.3 ± 0.002 GAE/mg of extract and methanolic extract shows 84.8 ± 0.023 mg GAE/mg of extract respectively in table 4.15.

Table 4.14: Total phenolic content of *C. ramiflora* (leaf)

Extract	Conc. (µg/ml)	Absorbance (750 nm)	Total Phenolic Content (GAE/mg of extract) ± SD
$CHCl_3$	100	0.0486	6.3 ± 0.002
MtOH	100	0.2056	84.8 ± 0.023

4.1.4.3 Total phenolic content of *C. ramiflora* (stem)

In this work, total phenolic content of different plant extract of *C. ramiflora* stem was determined using the standard calibration curve. Table 4.15 represents the amount of total phenolic content in chloroformic and methanolic extract of *C. ramiflora* stem. Chloroformic extract shows 17.2 ± 0.019 mg GAE/mg of extract and methanolic extract shows 96.2 ± 0.010 mg GAE/mg of extract respectively.

Table 4.15: Total phenolic content of *C. ramiflora* (stem)

Extract	Conc. (µg/ml)	Absorbance (750 nm)	Total Phenolic Content (GAE/mg of extract) ± SD
$CHCl_3$	100	0.0704	17.2 ± 0.019
MtOH	100	0.2284	96.2 ± 0.010

4.1.4.4 Discussion

Phenolics or polyphenols are secondary plant metabolites that are ubiquitously present in plants and plant products. Many of the phenolic compounds have been shown to contain high levels of antioxidant activities [Razali et al. 2008]. The antioxidant properties of phenolic compounds originate from their properties of proton loss, chelate formation and dismutation of radicals. In fact, in some studies, theoretical methods have been proposed to estimate the antioxidant activities of phenolic substances. Their structure activity relationships are examined for this purpose. Phenols are compounds that have the ability to destroy radicals because they contain hydroxyl groups [Das and Pereira 1990; Younes 1981]. These important plant components give up hydrogen atoms from their hydroxyl groups to radicals and from stable phenoxyl radicals; hence, they play an important role in antioxidant activity. Therefore, determination of the quantity of

phenolic compounds is very important in order to determine the antioxidant capacity of plant extracts [De Gaulejac et al. 1999; Hatano et al. 1989].

Investigating the results of the present study, the quantitative estimation of total phenolic content of chloroformic extract of *C. crista* exhibited 7.7 ± 0.013 mg GAE/mg of extract. Methanolic extract of *C. crista* showed the amount of total phenolic content is 52.5 ± 0.012 mg GAE/mg of extract. So this poor amount of phenolic content in *C. crista* extract could be related to the higher IC_{50} values (225.9 µg/ml and 103.7 µg/ml respectively) means weaker scavengers. Mandel et al. (2009) also showed that the 70% methanolic extract of *C. crista* leaves contain total phenol was 50.23 ± 0.003 mg GAE/mg of extract. According to the literature, polyphenols are an important group of pharmacologicaliy active compounds that are considered tc be the most active antioxidant derivatives in plants [Okawa et al. 2001].

From the quantitative estimation of total phenolic content of chloroformic extract of *C. ramiflora* leaves shows that the amount of total phenolic content is 6.3 ± 0.002 mg GAE/mg of extract. This lowest amount of phenolic content could be relevant to the higher IC_{50} value (537 µg/ml) in DPPH assay, lowest reducing capability in reducing power assay and smallest antioxidant power (22.2 ± 0.013 µM Fe (II)/g) in FRAP assay. Methanolic extract of *C. ramiflora* leaves showed the amount of total phenolic content is 84.8 ± 0.023 mg GAE/mg of extract. In this research work, we found a quite good amount of polyphenolic content, but that did not seem to be contributed towards a good antioxidant property as we found the IC_{50} value at a concentration of 102.801µg/ml in DPPH assay. This phenolic amount is not also relevant to the reducing capability and total antioxidant power. This could be due to the fact that the free radical scavenging assay is not only specific to the presence of polyphenols [Singleton et al. 1965].

Chloroformic extract of *C. ramiflora* stem shows that the amount of total phenolic content is 17.2 ± 0.019 mg GAE/mg of extract. Methanolic extract of *C. ramiflora* stem shows that the amount of total phenolic content is 96.2 ± 0.010 mg GAE/mg of extract. The amount of total phenol in methanolic extract of *C. ramiflora* stem was the highest compared to all the other extracts in this research work. Amazingly, methanolic extract of *C. ramiflora* stem was found as the strongest scavengers (IC_{50} 32.658µg/ml) in DPPH assay, strongest reductant capability in reducing power assay and also possess highest

antioxidant capacity in FRAP assay. According to the literature, polyphenols are an important group of pharmacologically active compounds that are considered to be the most active antioxidant derivatives in plants [Okawa et al. 2001].

4.1.5 Total Flavonoid Content

Flavonoids are the most common and widely distributed group of plant phenolic compounds, characterized by a benzo-γ-pyrone structure. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties. Total flavonoid contents can be determined in the sample extracts by reaction with sodium nitrite, followed by the development of colored flavonoid-aluminum complex formation using aluminum chloride in alkaline condition which can be monitored spectrophotometrically at maximum wavelength of 510 nm [Bakar et al. 2009]. A linear calibration curve of Quercetin, in the range of 25–400 µg/ml with coefficient of determination (R^2) value of 0.984 was obtained. Absorbace of quercetin in different concentration is given in table 4.16 and from which calibration curve plotted in figure 4.21. Using this calibration curve, the total amount of flavonoid content of plant extract was determined using the equation y = 0.001x + 0.023, where y is the absorbance at 510 nm and x is total flvonoid content in the different plant extracts.

Table 4.16: Absorbance of standard quercetin at different concentration

Concentration (µg/ml)	Avg. of Absorbance (510 nm)	Absorbance ± SD
Blank	0.076	
400	0.718	0.718 ± 0.001
200	0.369	0.369 ± 0.013
100	0.258	0.258 ± 0.002
50	0.093	0.093 ± 0.003
25	0.04	0.04 ± 0.006

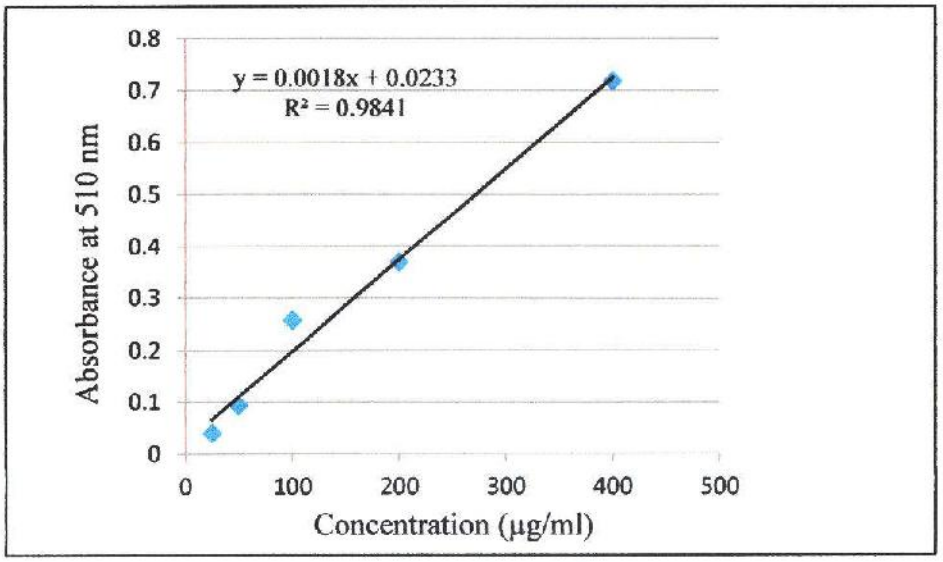


Figure 4.21: Calibration curve of standard Quercetin for determination of total flavonoid content.

4.1.5.1 Total flavonoid content of *C. crista*

In this work, total flavonoid content of different plant extract of *C. crista* was determined using the standard calibration curve. Table 4.17 represents the amount of total flavonoid content in chloroformic and methanolic extract of *C. crista*. Chloroformic extract shows 41 ± 0.009 QE/mg of extract and methanolic extract shows 100.4 ± 0.008 QE/mg of extract respectively.

Table 4.17: Total flavonoid content of *C. crista*

Extract	Conc. (µg/ml)	Absorbance (510 nm)	Total Flavonoid Content (QE/mg of extract) ± SD
$CHCl_3$	100	0.064	41 ± 0.009
MtOH	100	0.123	100.4 ± 0.008

4.1.5.2 Total flavonoid content of *C. ramiflora* (leaf)

From quantitive estimation of total flavonoid content of chloroformic extract of *C. ramiflora* leaves showed 68.4 ± 0.016 QE/mg of extract and methanolic extract shows 86.2 ± 0.028 QE/mg of extract respectively in table 4.18.

Table 4.18: Total flavonoid content of *C. ramiflora* (leaf)

Extract	Conc. (µg/ml)	Absorbance (510 nm)	Total Flavonoid Content (QE/mg of extract) ± SD
$CHCl_3$	100	0.0914	68.4 ± 0.016
MtOH	100	0.1092	86.2 ± 0.028

4.1.5.3 Total flavonoid content of *C. ramiflora* (stem)

In this work, total flavonoid content of different plant extract of *C. ramiflora* stem was determined using the standard calibration curve. Table 4.19 represents the amount of total flavonoid content in chloroformic and methanolic extract of *C. ramiflora* stem. Chloroformic extract shows 85.4 ± 0.026 QE/mg of extract and methanolic extract shows 166.4 ± 0.019 QE/mg of extract respectively.

Table 4.19: Total flavonoid content of *C. ramiflora* (stem)

Extract	Conc. (µg/ml)	Absorbance (510 nm)	Total Flavonoid Content (QE/g of extract) ± SD
$CHCl_3$	100	0.1084	85.4 ± 0.026
MtOH	100	0.1894	166.4 ± 0.019

4.1.5.4 Discussion

Flavonoids constitute a wide array of biological active compounds that are found abundantly in plant kingdom and dietary intake [Cushnie et al. 2005]. They are gaining interest due to their wide variants and number of members. These are reported to be effective in pathogenesis of majority of diseases. Antioxidant activity is the foundation of many actions which lead to its beneficial effects in majority of the diseases. Flavonoids are powerful antioxidants against free radicals and are described as free-radical scavengers [Pal et al. 2009]. This activity is attributed to their hydrogen-donating ability. Indeed, the phenolic groups of flavonoids serve as a source of a readily available "H" atoms such that the subsequent radicals produced can be delocalized over the flavonoid

structure [Tripoli et al. 2007]. Flavonoids inhibit lipid peroxidation in vitro at an early stage by acting as scavengers of superoxide anion and hydroxyl radicals. They terminate chain radical reaction by donating hydrogen atom to a peroxy radical, thus, forming flavonoids radical, which, further reacts with free radicals thus terminating propagating chain [Cook et al. 1996; Ferreira et al. 2010]. The flavonols having ortho or para hydroxyl group in the 2-phenyl ring are known to have strong antioxidant properties, while free hydroxyl at the 5, 7- positions proved to have a pro-oxidant effect [Heim et al. 2002]. A positive correlation between the content of flavonoids and the antioxidant capacity in plant extract has been found [Nagarajan et al. 2008]. For that reason, determination of the quantity of flavonoids compounds is very important in order to determine the antioxidant capacity of plant extracts.

Investigating the results of the present study, the quantitative estimation of total flavonoid content of chloroformic extract of *C. crista* exhibited 41 ± 0.009 QE/mg of extract. This poor amount of flavonoid content in *C. crista* extract could be related to the weaker scavenger in DPPH assay (fig. 4.2). Methanolic extract of *C. crista* showed the amount of total flavonoid content is 100.4 ± 0.008 QE/mg of extract. In this research work, we found methanolic extract of *C. crista* showed a quite good amount of flavonoid content, moderate reducing capability in reducing power assay and also showed a good antioxidant potentiality in FRAP assay, but that did not seem to be contributed towards a good antioxidant property as we found the IC_{50} value at a concentration of 103.7 µg/ml in DPPH assay along with poor phenol content. This could be due to the fact that the free radical scavenging assay is not only specific to the presence of polyphenols or flavonoids [Singleton et al. 1965]. But we also found in the literature, polyphenols or flavonoids are an important group of pharmacologically active compounds that are considered to be the most active antioxidant derivatives in plants [Okawa et al. 2001].

From the quantitative estimation of total flavonoid content of chloroformic extract of *C. ramiflora* leaves shows that the amount of total flavonoid content is 68.4 ± 0.016 QE/mg of extact. Methanolic extract of *C. ramiflora* leaves showed the amount of total flavonoid content is 86.2 ± 0.028 QE/mg of extract. In this research, we found that, this lowest amount of flavonoid content could be relevant to the higher IC_{50} values in DPPH

assay, lowest reducing capability in reducing power assay and smallest antioxidant power in FRAP assay.

Chloroformic extract of *C. ramiflora* stem shows that the amount of total flavonoid content is 85.4 ± 0.026 QE/mg of extract. Methanolic extract of *C. ramiflora* stem shows that the amount of total flavonoid content is 166.4 ± 0.019 QE/mg of extract. The amount of total flavonoid in methanolic extract of *C. ramiflora* stem was the highest compared to all the other extracts in this research work. Remarkably, in this research, methanolic extract of *C. ramiflora* stem was found as the strongest scavengers (IC_{50} 32.658µg/ml) in DPPH assay, strongest reductant capability in reducing power assay and also possess highest antioxidant capacity (84 ± 0.009 µM Fe (II)/g) in FRAP assay. According to the literature, polyphenols or flavonoids are an important group of pharmacologically active compounds that are considered to be the most active antioxidant derivatives in plants [Okawa et al. 2001].

4.1.6 Total Tannin Content

Tannin compounds are polyphenolic compounds that are very complex and widely distributed in almost all plant species [Okuda 2005; Makkar and Becker, 1998]. These complex polyphenolic compounds are soluble in water and organic solvents [Haslam 1996]. In this research work, Tannin content in each sample was determined using Folin-Denis method and Gallic acid as a standard [Polshettiwar et al. 2007]. The tannin and tannin like compound reduce Phosphotungustomolybdic acid in alkaline solution to produce a highly blue colored solution with λmax 725 nm [Cetkovic et al. 2008, Jain & Dixit 2004]. A linear calibration curve of Gallic acid, in the range of 6.25–100 µg/ml with coefficient of determination (R^2) value of 0.988 was obtained. Absorbance of gallic acid in different concentration is given in table 4.20 and from which calibration curve plotted in figure 4.22. Using this calibration curve, the total amount of tannin content of plant extract was determined using the equation $y = 0.005x + 0.037$, where y is the absorbance at 725 nm and x is total tannin content in the different plant extracts.

Table 4.20: Absorbance of standard gallic acid at different concentration

Concentration (µg/ml)	Avg. of Absorbance (725 nm)	Absorbance ± SD
Blank	0.058	
100	0.586	0.586 ± 0.016
50	0.354	0.354 ± 0.038
25	0.196	0.196 ± 0.002
12.5	0.095	0.095 ± 0.004
6.25	0.057	0.057 ± 0.002

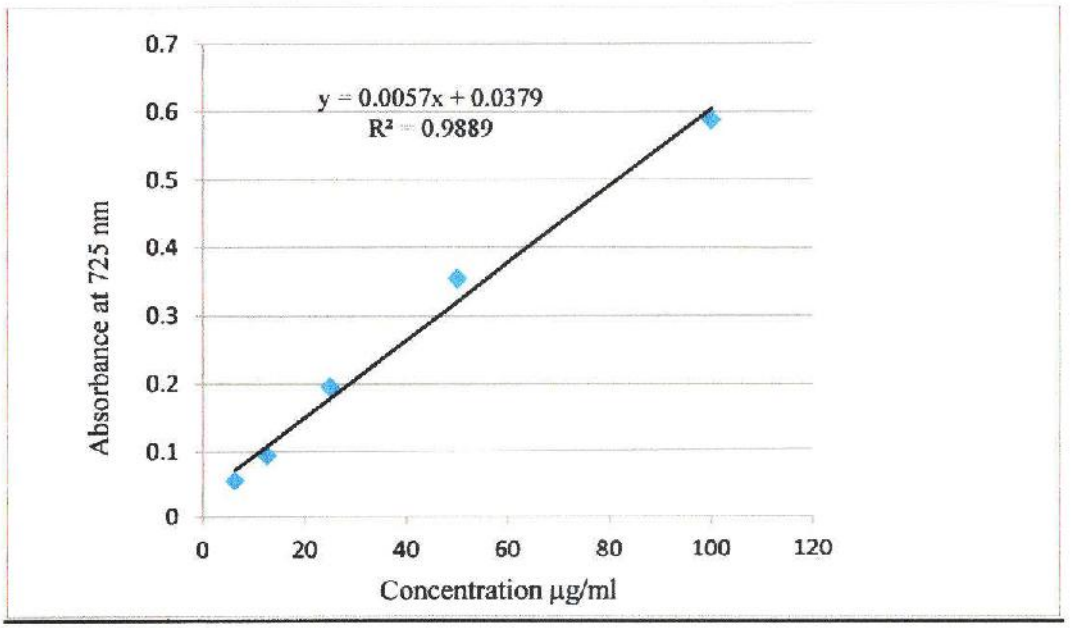


Figure 4.22: Calibration curve of standard Gallic acid for determination of total tannin content.

4.1.6.1 Total tannin content of *C. crista*

In this work, total tannin content of different plant extract of *C. crista* was determined using the standard calibration curve. Table 4.21 represents the amount of total tannin content in chloroformic and methanolic extract of *C. crista*. Chloroformic extract shows 9 ± 0.006 GAE/mg of extract and methanolic extract shows 76.6 ± 0.011 GAE/mg of extract respectively.

Table 4.21: Total tannin content of *C. crista*

Extract	Conc. (µg/ml)	Absorbance (725 nm)	Total Tannin Content (GAE/g of extract) ± SD
CHCl	100	0.082	9 ± 0.006
MtOH	100	0.42	76.6 ± 0.011

4.1.6.2 Total tannin content of *C. ramiflora* (leaf)

From quantitive estimation of total tannin content of chloroformic extract of *C. ramiflora* leaves showed 12.2 ± 0.010 GAE/mg of extract and methanolic extract shows 65 ± 0.011 GAE/mg of extract respectively in table 4.22.

Table 4.22: Total phenolic content of *C. ramiflora* (leaf)

Extract	Conc. (µg/ml)	Absorbance (725 nm)	Total Tannin Content (GAE/g of extract) ± SD
CHCl	100	0.098	12.2 ± 0.010
MtOH	100	0.36	65 ± 0.011

4.1.6.3 Total tannin content of *C. ramiflora* (stem)

In this work, total tannin content of different plant extract of *C. ramiflora* stem was determined using the standard calibration curve. Table 4.23 represents the amount of total tannin content in chloroformic and methanolic extract of *C. ramiflora* stem. Chloroformic extract shows 23.2 ± 0.009 GAE/mg of extract and methanolic extract shows 80.4 ± 0.009 GAE/mg of extract respectively.

Table 4.23: Total phenolic content of *C. ramiflora* (stem)

Extract	Conc. (µg/ml)	Absorbance (725 nm)	Total Phenolic Content (GAE/g of extract) ± SD
CHCl	100	0.153	23.2 ± 0.009
MtOH	100	0.439	80.4 ± 0.009

4.1.6.4 Discussion

Tannins are very complex polyphenolic compounds and are widely distributed in almost all plant species [Tamilselvi et al. 2012]. Tannins have been reported to exhibit a wide range of activities and prevent the attack of free radicals on the human body [Narasimhan et al. 2006]. Therefore, determination of the quantity of tannin compounds is very important in order to determine the antioxidant capacity of plant extracts.

Investigating the results of the present study, the quantitative estimation of total tannin content of chloroformic extract of *C. crista* exhibited 9 ± 0.006 GAE/mg of extract. This poor amount of tannin content in *C. crista* extract could be related to the weaker scavenger in DPPH assay (fig. 4.2). Methanolic extract of *C. crista* showed the amount of total tannin content is 76.6 ± 0.011 GAE/mg of extract. In this research work, we found methanolic extract of *C. crista* showed a quite good amount of tannin content, moderate reducing capability in reducing power assay, a good antioxidant potentiality in FRAP assay and quite good flavonoid content, but that did not seem to be contributed towards a good antioxidant property as we found the IC_{50} value at a concentration of 103.7 µg/ml in DPPH assay along with poor phenol content. This could be due to the fact that the free radical scavenging assay is not only specific to the presence of polyphenols or tannins [Singleton et al. 1965]. But we also found in the literature, polyphenols or tannins are an important group of pharmacologically active compounds that are considered to be the most active antioxidant derivatives in plants [Okawa et al. 2001].

From the quantitative estimation of total tannin content of chloroformic extract of *C. ramiflora* leaves shows that the amount of total tannin content is 12.2 ± 0.010 GAE/mg of extract. Methanolic extract of *C. ramiflora* leaves showed the amount of total tannin content is 65 ± 0.011 GAE/mg of extract. In this research, we found that, among the extract *C. ramiflora* leaves showed the lowest amount of tannin content which could be relevant to the higher IC_{50} values in DPPH assay, lowest reducing capability in reducing power assay, smallest antioxidant power in FRAP assay and poor flavonoid content. Chloroformic extract of *C. ramiflora* stem shows that the amount of total tannin content is 23.2 ± 0.009 GAE/mg of extract. Methanolic extract of *C. ramiflora* stem shows that the amount of total tannin content is 80.4 ± 0.009 GAE/mg of extract. The amount of total tannin in methanolic extract of *C. ramiflora* stem was the highest

compared to all the other extracts in this research work. Remarkably, in this research, methanolic extract of *C. ramiflora* stem was found as the strongest scavengers (IC_{50} 32.658µg/ml) in DPPH assay, strongest reductant capability in reducing power assay, possess highest antioxidant capacity (84 ± 0.009 µM Fe (II)/g) in FRAP assay along with good amount of phenol and flavonoid content. According to the literature, polyphenols or tannins are an important group of pharmacologically active compounds that are considered to be the most active antioxidant derivatives in plants [Okawa et al. 2001].

4.2 Antibacterial activity Related Assay

The type and level of biological activity exhibited by any plant material depends on many factors, including the plant part, geographical source, soil conditions, harvest time, moisture content, drying method, storage conditions, and post-harvest processing. For example, the relatively high temperatures that can be generated during tissue grinding can denature chemical constituents and the extraction solvent, time period, and temperature can affect the level and composition of secondary metabolites extracted from plant tissues. Petroleum ether, chloroform, methanol, ethanol and acetone separately or mixed with water are commonly used to extract bioactive compounds from plant materials, depending on the intended use of the extract. In this study chloroform and methanol were chosen as extraction solvents because of their availability. In the present study an attempt has been made to screen two mangrove plants from same family collected from Sundarban, known for their medicinal properties, especially antimicrobial activity against a number of both gram positive and gram negative bacteria such as *S. aureus* (skin flora), *S. epidermis* (skin infection), *B. megaterium* (Non-pathogenic), *E. coli* (Food poisonous), *P. aurigonisa* (pulmonary infection), *V. cholera* (Cholera) and *S. typhi* (Typhoid fever). Disc diffusion method was used to investigate antimicrobial activity of choloroformic and methanolic extract of *C. crista* and *C. ramiflora*. Standard antibiotic discs of Ciprofloxacin (5 mcg/disc) were used for comparison purpose and it was found that the extracts showed responses in varying levels. The result of the

antibacterial activity of the chloroformic and methanolic extracts was measured from the diameters from the zone of inhibition expressed as mm. The extracts exhibited varying levels of activities against the bacteria.

4.2.1 Disc Diffusion assay

4.2.1.1 Chloroformic extract of *C. crista*

Chloroformic extract of *C. crista* showed its maximum inhibition against *S. aureus* with average zone of inhibition (22 ± 1.2 mm) and *P. aurigonisa* with average zone of inhibition (19. 25 ± 3.4 mm) for 400µg/disc at concentration in compared to standard Ciprofloxacin (5µg/disc) as well. The diameters of zone of inhibition of Chloroformic extract of *C. crista* against ali tested microorganisms are shown in table 4.24 and illustrative appearance is represented in fig. 4.23.

Table 4.24: Antimicrobial activity of chloroformic extract of *C. crista* and positive control Ciprofloxacin

Test microorganisms	Diameter of zone of inhibition (mm ± SD) (n = 4)			
	$CHCl_3$		Positive control Ciprofloxacin	Negative control
	(200µg/disc)	(400µg/disc)	(5µg/disc)	
Gram-positive bacteria				
Bacillus megaterium	12 ± 1.4	22 ± 2.3	26.25 ± 0.9	n.d
Staphylococcus aureus	17.75 ± 1.5	22 ± 1.2	24.75 ± 0.5	n.d
Staphylococcus epidermis	13 ± 2.5	16.5 ± 3.1	24.5 ± 6.4	n.d
Gram-negative bacteria				
Escherichia coli	15.75 ± 1.8	17.75 ± 0.9	21.5 ± 1	n.d
Pseudomona aurigonisa	13 ± 2.4	19. 25 ± 3.4	22.5 ± 4.9	n.d
Salmonella typhi	11.75 ± 2.6	15.75 ± 2.8	26 ± 1.1	n.d
Vibrio cholerae	10.25 ± 0.5	13.5 ± 1.9	27.5 ± 1	n.d

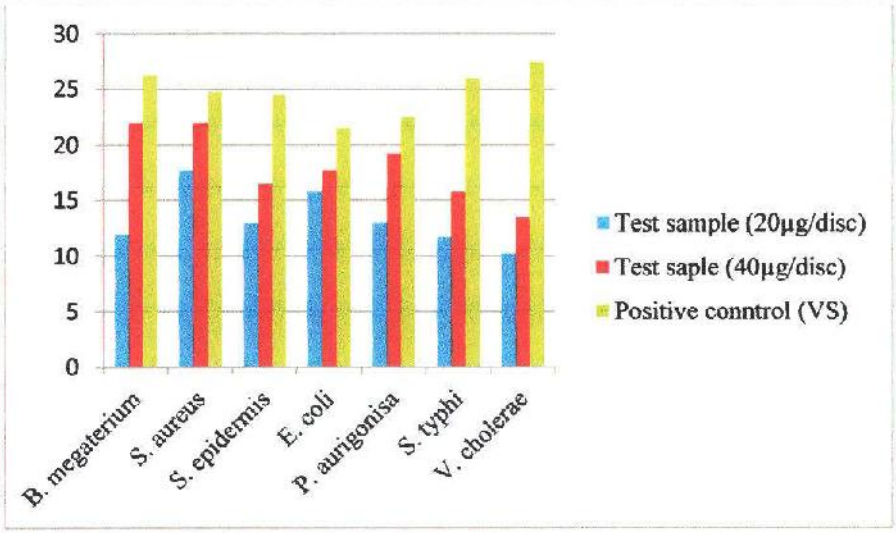


Figure 4.23: Evaluation of antimicrobial activity of *C.crista* chloroformic extract (Microorganisms vs Zone of inhibition).

4.2.1.2 Methanolic extract of *C. crista*

Methanolic extract of *C. crista* showed its maximum inhibition against *Staphylococcus epidermis* with average zone of inhibition (23.25 ± 1.2 mm) for 400µg/disc at concentration in compared to standard Ciprofloxacin (5µg/disc) with average zone of inhibition (20.75 ± 0.9 mm) as well. The diameters of zone of inhibition of Chloroformic extract of *C. crista* against all tested microorganisms are shown in table 4.25 and illustrative appearance is represented in fig. 4.24.

Table 4.25: Antimicrobial activity of methanolic extract of *C. crista* and positive control Ciprofloxacin

Test microorganisms	Diameter of zone of inhibition (mm ± SD) (n = 4)			
	MtOH		Positive control Ciprofloxacin	Negative control
	(200µg/disc)	(400µg/disc)	(5µg/disc)	
Gram-positive bacteria				
Bacillus megaterium	10.25 ± 2.21	15 ± 1.4	25 ± 1.4	n.d
Staphylococcus aureus	16.5 ± 2.64	19.25 ± 2.2	25.5 ± 2.3	n.d
Staphylococcus epidermis	19 ± 0.8	23.25 ± 1.2	20.75 ± 0.9	n.d
Gram-negative bacteria				
Escherichia coli	12 ± 1.6	17.25 ± 1.5	24 ± 0	n.d
Pseudomonas aurigonisa	12 ± 2.4	14.25 ± 1.5	29.25 ± 1.5	n.d
Salmonella typhi	14.5 ± 1	17.5 ± 1	26.25 ± 1.5	n.d
Vibrio cholerae	17.5 ± 2.88	21.5 ± 3	28 ± 2.4	n.d

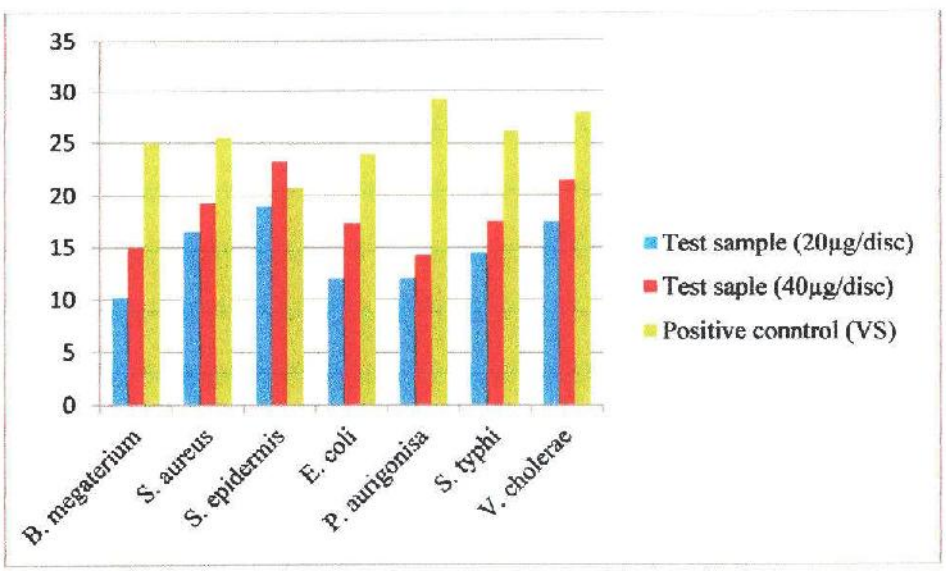


Figure 4.24: Evaluation of antimicrobial activity of *C.crista* methanolic extract (Microorganisms vs Zone of inhibition).

4.2.1.3 Chloroformic extract of *C. ramiflora* leaf

Chloroformic extract of *C. ramiflora* leaves showed its maximum inhibition against *Bacillus megaterium* with average zone of inhibition (26.75 ± 2.6 mm) and *Salmonella typhi* with average zone of inhibition (20 ± 0 mm) for 400µg/disc at concentration in compared to standard Ciprofloxacin (5µg/disc) with average zone of inhibition (25.75 ± 0.9 mm) and (22 ± 0.8 mm) respectively as well. The diameters of zone of inhibition of Chloroformic extract of C. ramiflora leaves against all tested microorganisms are shown in table 4.26 and illustrative appearance is represented in fig. 4.25.

Table 4.26: Antimicrobial activity of chloroformic extract of *C. ramiflora* (leaf) and

Test microorganisms	Diameter of zone of inhibition (mm ± SD) (n = 4)			
	CHCl		Positive control	Negative control
	(200µg/disc)	(400µg/disc)	Ciprofloxacin (5µg/disc)	
Gram-positive bacteria				
Bacillus megaterium	19.5 ± 1.7	26.75 ± 2.6	25.75 ± 0.9	n.d
Staphylococcus aureus	19.5 ± 0.5	21.75 ± 2.5	32 ± 0	n.d
Staphylococcus epidermis	17.5 ± 1	21 ± 1.1	31 ± 1.4	n.d
Gram-negative bacteria				
Escherichia coli	16 ± 1.1	21.25 ± 1.5	26.25 ± 1.5	n.d
Pseudomona aurigonisa	17.75 ± 0.5	24.75 ± 0.5	27 ± 1.1	n.d
Salmonella typhi	13.5 ± 1	20 ± 0	22 ± 0.8	n.d
Vibrio cholerae	16.5 ± 1	24 ± 1.1	30 ± 0	n.d

positive control Ciprofloxacin

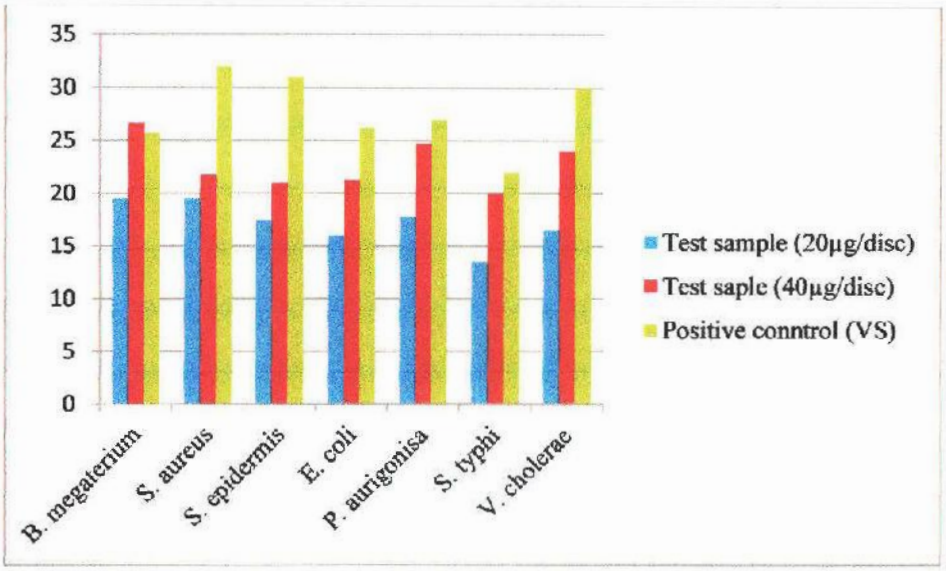


Figure 4.25: Evaluation of antimicrobial activity of *C.ramiflora* (leaf) chloroformic extract (Microorganisms vs Zone of inhibition).

4.2.1.4 Methanolic extract of *C. ramiflora* leaf

Methanolic extract of *C. ramiflora* leaves showed its maximum inhibition against *Staphylococcus epidermis* with average zone of inhibition (22 ± 2.9 mm) for 400µg/disc at concentration in compared to standard Ciprofloxacin (5µg/disc) with average zone of inhibition (30.75 ± 0.5 mm) as well. The diameters of zone of inhibition of methanolic extract of *C. ramiflora* leaves against all tested microorganisms are shown in table 4.27 and illustrative appearance is represented in fig. 4.26.

Table 4.27: Antimicrobial activity of methanolic extract of *C. ramiflora* (leaf) and positive control Ciprofloxacin

Test microorganisms	Diameter of zone of inhibition (mm ± SD) (n = 4)			
	MtOH		Positive control	Negative control
	(200µg/disc)	(400µg/disc)	Ciprofloxacin (5µg/disc)	
Gram-positive bacteria				
Bacillus megaterium	12.5 ± 0.5	17 ± 0.8	26.75 ± 1.5	n.d
Staphylococcus aureus	12.75 ± 0.9	17.5 ± 1	26.75 ± 1.2	n.d
Staphylococcus epidermis	11 ± 0.8	22 ± 2.9	30.75 ± 0.5	n.d
Gram-negative bacteria				
Escherichia coli	10.5 ± 0.5	13.25 ± 1.2	19 ± 1.1	n.d
Pseudomona aurigonisa	12.75 ± 2.2	15.5 ± 1.7	28.5 ± 1	n.d
Salmonella typhi	9.5 ± 0.5	14.75 ± 0.5	29.5 ± 0.5	n.d
Vibrio cholerae	13.5 ± 1.7	18.5 ± 1.9	30.25 ± 0.5	n.d

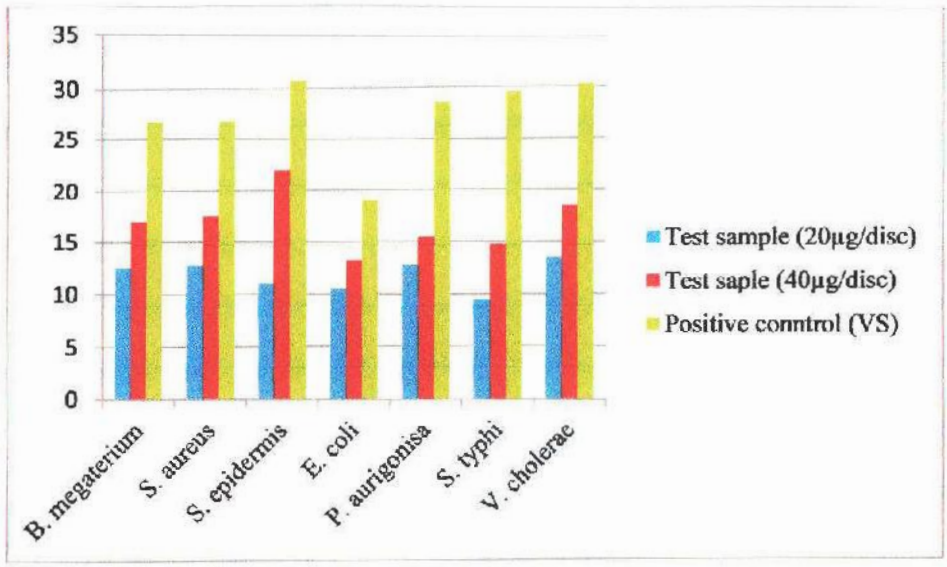


Figure 4.26: Evaluation of antimicrobial activity of *C.ramiflora* (leaf) methanolic extract (Microorganisms vs Zone of inhibition).

4.2.1.5 Chloroformic extract of *C. ramiflora* stem

Chloroformic extract of *C. ramiflora* stem showed its significant inhibition against *Bacillus megaterium* with average zone of inhibition (26 ± 1.1 mm), *Escherichia coli* with zone of inhibition (23.25 ± 2.2 mm), *Pseudomona aurigonisa* with average zone of inhibition (23.5 ± 1 nm) and *Salmonella typhi* (25.75 ± 1.5 mm) for 400µg/disc at concentration in compared to standard Ciprofloxacin (5µg/disc) with average zone of inhibition (19.75 ± 3.7 mm, 25.5 ± 1mm, 26.25 ± 0.9 mm and 23.5 ± 1.7 mm) respectively as well. The diameters of zone of inhibition of Chloroformic extract of *C. ramiflora* stem against all tested microorganisms are shown in table 4.28 and illustrative appearance is represented in fig. 4.27.

Table 4.28: Antimicrobial activity of chloroformic extract of *C. ramiflora* (stem) and positive control Ciprofloxacin

Test microorganisms	Diameter of zone of inhibition (mm ± SD) (n = 4)			
	$CHCl_3$ (200µg/disc)	$CHCl_3$ (400µg/disc)	Positive control Ciprofloxacin (5µg/disc)	Negative control
Gram-positive bacteria				
Bacillus megaterium	18.5 ± 0.5	26 ± 1.1	19.75 ± 3.7	n.d
Staphylococcus aureus	16.75 ± 0.5	21.5 ± 0.5	30.5 ± 1	n.d
Staphylococcus epidermis	18.25 ± 2.3	24. 5 ± 0.5	31.25 ± 2.5	n.d
Gram-negative bacteria				
Escherichia coli	17 ± 1.8	23.25 ± 2.2	25.5 ± 1	n.d
Pseudomona aurigonisa	18.25 ± 2.3	23.5 ± 1	26.25 ± 0.9	n.d
Salmonella typhi	18 ± 1.4	25.75 ± 1.5	23.5 ± 1.7	n.d
Vibrio cholerae	17.5 ± 0.5	24.75 ± 2.0	32.5 ± 1.2	n.d

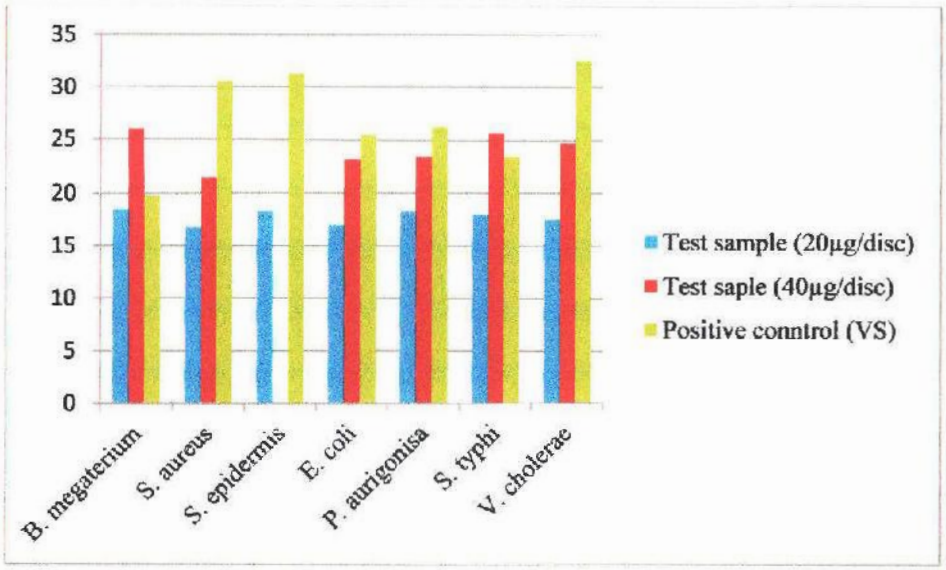


Figure 4.27: Evaluation of antimicrobial activity of *C.ramiflora* (stem) chloroformic extract (Microorganisms vs Zone of inhibition).

4.2.1.6 Methanolic extract of *C. ramiflora* stem

Methanolic extract of *C. ramiflora* stem showed its maximum inhibition against *Staphylococcus aureus* with average zone of inhibition (19.5 ± 1.9 mm) and *Escherichia coli* with average zone of inhibition (20 ± 0 mm) for 400µg/disc at concentration in compared to standard Ciprofloxacin (5µg/disc) with average zone of inhibition (21.75 ± 3.9 mm and 22.25 ± 1.5 mm) respectively as well. The diameters of zone of inhibition of methanolic extract of *C. ramiflora* stem against all tested microorganisms are shown in table 4.29 and illustrative appearance is represented in fig. 4.28.

Table 4.29: Antimicrobial activity of methanolic extract of *C. ramiflora* (stem) and positive control Ciprofloxacin

Test microorganisms	Diameter of zone of inhibition (mm ± SD) (n = 4)			
	MtOH (200µg/disc)	(400µg/disc)	Positive control Ciprofloxacin (5µg/disc)	Negative control
Gram-positive bacteria				
Bacillus megaterium	13.5 ± 1.7	15.25 ± 1.5	25.75 ± 1.2	n.d
Staphylococcus aureus	13 ± 1.1	19.5 ± 1.9	21.75 ± 3.9	n.d
Staphylococcus epidermis	13.75 ± 0.5	17.75 ± 2.5	30 ± 0	n.d
Gram-negative bacteria				
Escherichia coli	15.5 ± 1	20 ± 0	22.25 ± 1.5	n.d
Pseudomonas aurigonisa	12 ± 3.4	17.75 ± 2.5	25.5 ± 1	n.d
Salmonella typhi	11.5 ± 2.5	19.75 ± 3.3	24.25 ± 0.9	n.d
Vibrio cholerae	14.25 ± 0.5	18 ± 0.8	29.5 ± 1	n.d

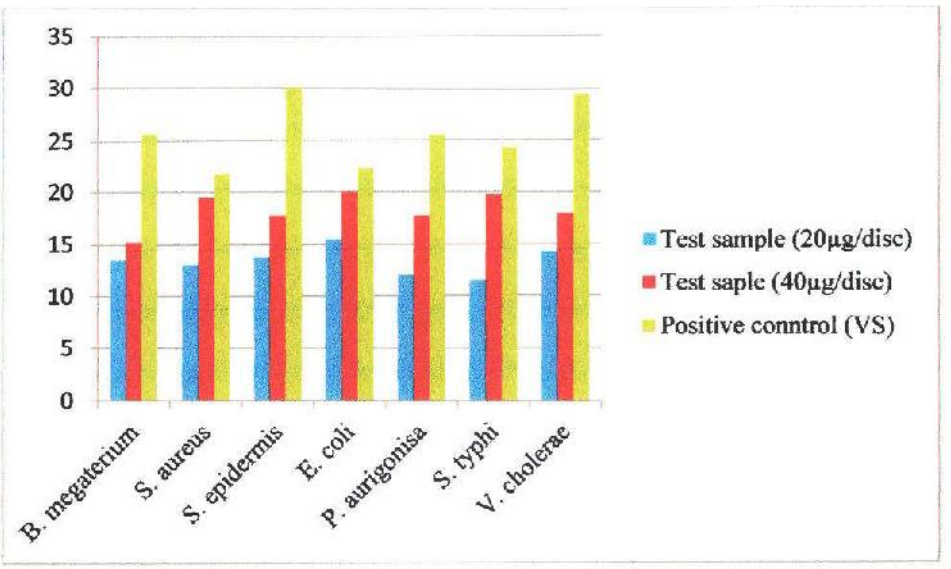


Figure 4.28: Evaluation of antimicrobial activity of *C.ramiflora* (stem) methanolic extract (Microorganisms vs Zone of inhibition).

4.2.1.7 Discussion

Advances of modern medicine, many infectious diseases once considered incurable and lethal are now amenable to treatment with antimicrobial agents. The remarkably powerful and specific activity of antimicrobial drugs is due to their selectively for highly specific targets that are either unique to microorganisms [Bertram & Susan 2009]. Nowadays multiple drug resistance has developed due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious disease. Given the alarming incidence of antibiotic resistance in bacteria of medical importance, there is a constant need for new and effective therapeutic agents. Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases. Antimicrobial properties of medicinal plants are being increasingly reported from different part of the world [Recio 1995]. The beneficial medicinal effects of plant materials typically results from the combination of secondary products present in the plant. Mangrove plant extracts have been used for centuries as a popular method for treating several health disorders. Mangrove and mangrove associates contain biologically active antibacterial, antiviral and antifungal compounds. In this point of view, in this research we have selected two mangrove plants and evaluate antibacterial activity of crude chloroformic and methanolic extracts of *C.crista* and *C.ramiflora* (leaf and stem) by using disc diffusion method. A number of gram positive bacteria (*B.megaterium, S. aureus, S. epidermis*) and a number of gram negative bacteria (*E. coli, P. aurigonisa, S. typhi and V. cholera*) were used in this test. The results of the present study clearly indicated that all the extract inhibited the growth of all the tested pathogens. The antimicrobial activities of the crude extracts were increased with increasing the concentration. Now here we discuss about the activity of all plant extract against individual microorganism.

Chloroformic extract of *C. crista* showed significant zone of inhibition against *S. aureus* (22 ± 1.2 mm) and *P. aurigonisa* (19. 25 ± 3.4 mm) for 400µg/disc at concentration compared to standard Ciprofloxacin. Methanolic extract of *C. crista* showed its maximum inhibition against *S. epidermis* with average zone of inhibition (23.25 ± 1.2 mm) for 400µg/disc at concentration in compared to standard Ciprofloxacin (5µg/disc) with average zone of inhibition (20.75 ± 0.9 mm) as well. So, different solvent extract of

C. crista showed maximum activity against gram positive bacteria. Both bacteria are responsible for skin diseases. In case of *C. crista*, lower activity was observed against Gram-negative bacteria. This can be explained because the outer membrane of Gram-negative bacteria is known to present a barrier to the penetration of numerous antibiotic molecules and the periplasmic space contains enzymes which are able of breaking down foreign molecules introduced from the outside [Duffy and Power 2001; Poole 1994; Schaechter et al. 1999].

Analyzing the results of the antimicrobial activity of *C. ramiflora* leaves, we found that the chloroformic extract showed maximum inhibition against *B. megaterium* (26.75 ± 2.6 mm) and *S. typhi* (20 ± 0 mm) for 400µg/disc at concentration in compared to standard Ciprofloxacin (5µg/disc) (25.75 ± 0.9 mm) and (22 ± 0.8 mm) respectively. *B. megaterium* is a nonpathogenic bacterium. *Salmonella typhi* is a gram negative bacteria and which is a main culprit for typhoid fever. But there is no information in ethnobotanically that the *C. ramiflora* leaves used as typhoid fever. Methanolic extract of *C. ramiflora* leaves showed its maximum inhibition against *S. epidermis* with average zone of inhibition (22 ± 2.9 mm) for 400µg/disc at concentration in compared to standard Ciprofloxacin (5µg/disc) with average zone of inhibition (30.75 ± 0.5 mm) as well. *S. epidermis* is a gram positive bacteria and responsible for skin diseases. As ethnobotanically *C. ramiflora* leaves has long been used as a traditional folk remedy for skin diseases. Chloroformic extract of *C. ramiflora* stem showed its significant inhibition against *B. megaterium* with average zone of inhibition (26 ± 1.1 mm), *E. coli* with zone of inhibition (23.25 ± 2.2 mm), *P. aurigonisa* with average zone of inhibition (23.5 ± 1 nm) and *S. typhi* (25.75 ± 1.5 mm) for 400µg/disc at concentration in compared to standard Ciprofloxacin (5µg/disc) with average zone of inhibition (19.75 ± 3.7 mm, 25.5 ± 1mm, 26.25 ± 0.9 mm and 23.5 ± 1.7 mm) respectively. Methanolic extract of *C. ramiflora* stem showed its significant inhibition against *S. aureus* with average zone of inhibition (19.5 ± 1.9 mm) and *E. coli* with average zone of inhibition (20 ± 0 mm) for 400µg/disc at concentration in compared to standard Ciprofloxacin (5µg/disc) with average zone of inhibition (21.75 ± 3.9 mm and 22.25 ± 1.5 mm) respectively.

So, different solvent extract of *C. ramiflora* stem showed maximum activity against both gram positive and gram negative bacteria. *E. coli* responsible for food poisoning, *P.*

aurigonisa is a pulmonary infection causing bacteria and *S. typhi* responsible for typhoid fever.

The reason for the different sensitivity between gram-positive and gram-negative bacteria could be ascribed to the morphological differences between these micro-organisms, Gram-negative bacteria having an outer phospholipidic membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes, while porins constitute a selective barrier to the hydrophilic solutes with an exclusion limit of about 600 Da [Nikaido and Vaara 1985]. The Gram-positive bacteria should be more susceptible having only an outer peptidoglycan layer which is not an effective permeability barrier [Scherrer and Gerhardt 1971]. Investigating all the results of antimicrobial activity, we found that the plant extract of *C. crista* exhibited excellent activity against gram positive bacteria *C. ramiflora* showed good activity against both gram positive and gram negative bacteria.

4.3 Cytotoxic activity Related Assay

4.3.1 Brine Shrimp Lethality Bioassay

Brine shrimp *Artemia salina* are most convenient test organisms for toxicity studies. Brine shrimp lethality bioassay is predictive of cytotoxicity activity of samples. The technique is easily manageable and requires small amount of test material. The aim of this method is to provide a front-line screen that can be backed up by more specific and more expensive bioassays once the active compounds have been isolated. This simple bioassay used for testing plant extracts bioactivity which in most cases correlates reasonably well with cytotoxic and anti-tumor properties.

The lethality of the chloroformic and methanolic extracts to brine shrimp were determined on *A. salina* after 24 hours of exposure the samples. Test samples showed different mortality rate at different concentrations and the mortality rate of brine shrimp was found to be increased with increasing concentration of the sample. The lethal concentration (LC_{50}) values of the plant extracts were obtained by a plot of percentage of the shrimp nauplii killed against the concentrations of the extracts. Vincristine sulphate was used as positive control.

4.3.1.1 *C. crista* (whole plant)

The LC_{50} values of chloroformic and methanolic extract of *C. crista* obtained from brine shrimp lethality Bioassay were 5.794 µg/ml and 2.972 µg/ml compared to positive control (vincristine sulphate, VS, LC_{50} 0.128 µg/ml). These results are furnished in table [4.30 and 4.31]. From this data, the percent of lethality of the brine shrimp nauplii for each concentration and control was calculated. An approximate linear correlation was observed when logarithm of concentration versus percentage of mortality was plotted on the graph paper and the values of LC_{50} were calculated using Microsoft Excel 2007 [Figure 4.29].

Table 4.30: Effect of Chloroformic and Methanolic extract of *C. crista* and positive control Vincristine sulphate on Brine shrimp

Concentration (µg/ml)	Log C	% Mortality		LC_{50} (µg/ml)		
		CHCl	MtOH	CHCl	MtOH	Std. (VS)
5	0.69897	45.313	54	5.794	2.972	0.128
10	1	54.062	62			
20	1.30103	69.375	72			
40	1.60206	82.5	86			
80	1.90309	89.062	90			
160	2.20412	95.625	96			
320	2.50515	100	100			

Table 4.31: The result of Cytotoxicity of Chloroformic and Methanolic extract *C. crista* and positive control on Brine shrimp

Sample	LC_{50} (µg/ml)	Regression equation	R^2
CHCl	5.794	$y = 31.66x + 25.83$	0.964
MtOH	2.972	$y = 26.57x + 37.42$	0.965
VS	0.128	$y = 18.38x + 64.64$	0.85

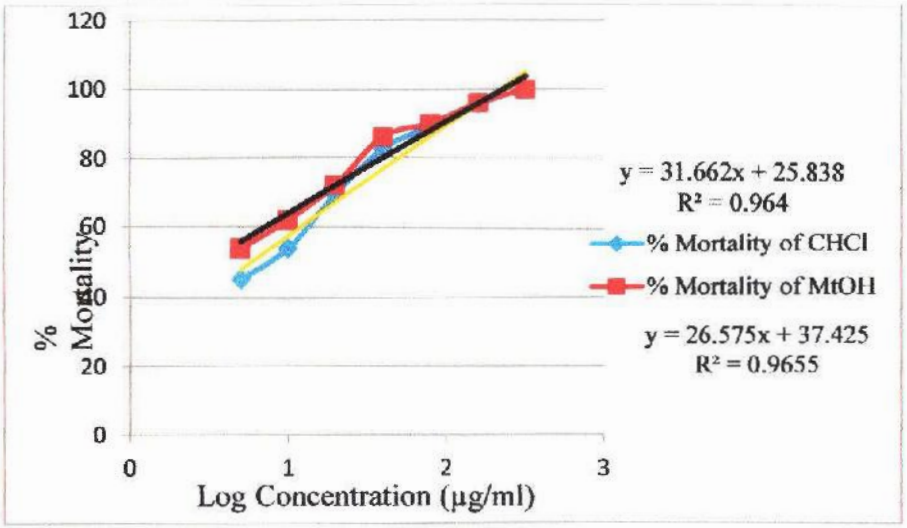


Figure 4.29: Plot of log concentration of chloroformic and methanolic extract of *C. crista* versus percent shrimp mortality after 24h of exposure.

4.3.1.2 *C. ramiflora* leaf

The LC_{50} values of chloroformic and methanolic extract of *C. ramiflora* leaves obtained from brine shrimp lethality bioassay [tables 4.32 and 4.33] were 8.279µg/ml and 4.613µg/ml respectively compared to positive control (vincristine sulphate, VS, LC_{50} 0.128 µg/ml). From this data, the percent of lethality of the brine shrimp nauplii for each concentration and control was calculated. An approximate linear correlation was observed when logarithm of concentration versus percentage of mortality was plotted on the graph paper and the values of LC_{50} were calculated using Microsoft Excel 2007 [Figure 4.30].

Table 4.32: Effect of Chloroformic and Methanolic extract of *C. ramiflora* (leaf) and positive control Vincristine sulphate on Brine shrimp

Concentration (µg/ml)	Log C	% Mortality		LC_{50} (µg/ml)		
		CHCl	MtOH	CHCl	MtOH	Std. (VS)
5	0.69897	39.25	47.5	8.279	4.613	0.128
10	1	50.5	60.625			
20	1.30103	64	71.562			
40	1.60206	79.75	78.125			
80	1.90309	91	86.875			
160	2.20412	95.5	97.812			
320	2.50515	100	100			

Table 4.34: Effect of Chloroformic and Methanolic extract of *C. ramiflora* (stem) and positive control Vincristine sulphate on Brine shrimp

Concentration (µg/ml)	Log C	% Mortality		LC_{50} (µg/ml)		
		CHCl	MtOH	CHCl	MtOH	Std. (VS)
5	0.69897	44.75	55.375	5.297	1.596	0.128
10	1	57.5	70.25			
20	1.30103	72.375	83			
40	1.60206	80.125	85.125			
80	1.90309	89.375	95.75			
160	2.20412	97.875	100			
320	2.50515	100	100			

Table 4.35: The result of Cytotoxicity of Chloroformic and Methanolic extract of *C. ramiflora* (leaf) and positive control on Brine shrimp

Sample	LC_{50} (µg/ml)	Regression equation	R^2
CHCl	5.297	y = 31.26x + 27.34	0.963
MtOH	1.596	y = 24.45x + 45.03	0.979
VS	0.128	y = 18.38x + 64.64	0.85

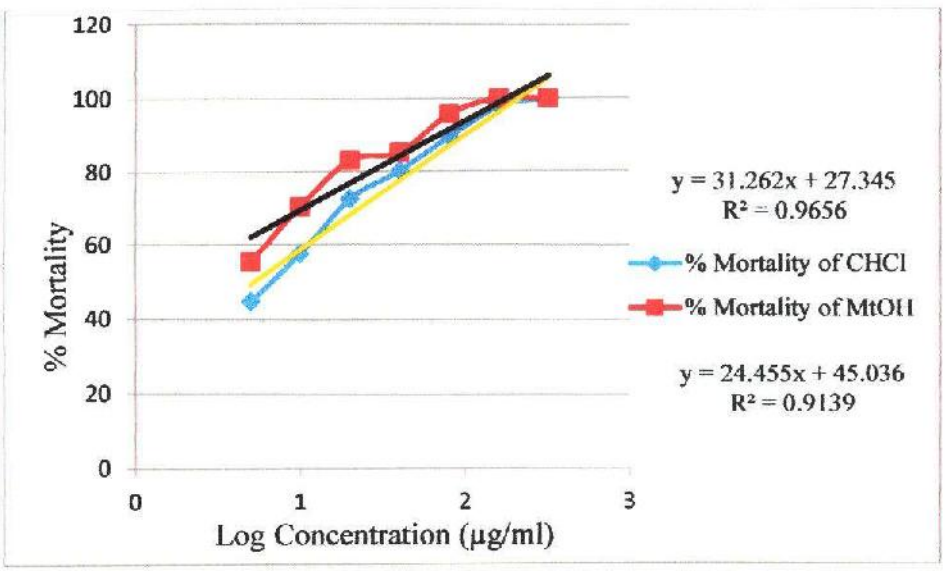


Figure 4.31: Plot of log concentration of chloroformic and methanolic extract of *C. ramiflora* (S) versus percent shrimp mortality after 24h of exposure.

4.3.1.4 Discussion

The brine shrimp lethality bioassay is normally conducted to draw inferences on the safety of the plant extracts and to further depict trends of their biological activities and considered as a useful tool for the preliminary assessment toxicity [Solis et al. 1993]. It can be extrapolated for cell-line toxicity and ant-tumor activity [Anderson et al. 1982]. In this research two mangrove plants *C. crista* and *C. ramiflora* were selected, mainly based on their traditional uses and availability. Ethnobotanical information provided that these plants were used for dispersing swelling and inflamation. The extracts studied in this work showed significant lethality against brine shrimp, which has been successfully used as a simple biological test to guide the fractionation process of plant extracts in order to detect anti-tumour compounds. However, the present study aimed at determining the toxicity effect of plant *C. ramiflora* and *C. crista* using brine shrimp. Test samples showed different mortality rate at different concentrations and the mortality rate of brine shrimp was found to be increased with increasing concentration of the sample. The lethal concentration (LC_{50}) values of the plant extracts were obtained by a plot of percentage of the shrimp nauplii killed against the concentrations of the extracts.

Chloroformic and methanolic extract of *C. crista* revealed LC_{50} values at the concentration of 5.794 µg/ml and 2.972 µg/ml compared to positive control (vincristine sulphate, VS, LC_{50} 0.128 µg/ml). Percent mortality of methanolic extract of *C. crista* is nearer to the standard, which indicates this extract could be a potential anti-cancering agent. This is in also agreement with the previous studies. Bodakhe et al. 2011, established that ethanolic extract of root-bark of *C. crista* inhibited the tumor volume, packed cell volume, tumor cell count and hematological parameters which were altered by tumor inoculation were restored. According to Sarkar et al. 2011, hydroalcoholic extract of *C.crista* showed toxicity for murine tumor cell Ehrlich's Ascites Carcinoma. Das et al. 2010, isolated new diterpenoids, 6beta-cinnamoyloxy-7beta-acetoxyvouacapen-5alpha-ol and 6beta, 7beta-dibenzoyloxyvouacapen-5alpha. Furthermore, the compounds were tested for in vitro cytotoxic activity against two cancer cell lines, HL-60 (Human promyelocytic leukemia) and HeLa (Human cervical carcinoma), showed significant decrease in cell viability in the test cell lines in a concentration dependent manner.

The LC_{50} values of chloroformic and methanolic extract of *C. ramiflora* leaves obtained from brine shrimp lethality bioassay were 8.279µg/ml and 4.613µg/ml respectively compared to positive control (vincristine sulphate, VS, LC_{50} 0.128 µg/ml). The LC_{50} values of chloroformic and methanolic extract of *C. ramiflora* stem obtained from brine shrimp lethality Bioassay were 5.297 µg/ml and 1.596 µg/ml compared to positive control (vincristine sulphate, VS, LC_{50} 0.128 µg/ml). Percent mortality of methanolic extract of *C. ramiflora* stem is much nearer to the standard, which indicates this extract could be a potential anti-cancering agent. This is in also agreement with the previous studies. By previous studies analyzing it revealed that, methanolic extracts of *Cynometra ramiflora had* selective cytotoxicity against three human cancer-cell lines (gastric: AGS; colon: HT-29; and breast: MDA-MB-435S) [Uddin et al. 2009].

Thus, the results on *C. crista* support its use in traditional medicine and the significant result of *C. ramiflora* is an indicative of the presence of potent cytotoxic components which warrants further investigation.

CHAPTER 5

Conclusion

5.0 Conclusion

Mangroves are potentially of great commercial value but this is often not recognized. Mangrove plants have the ability to grow where no other vascular plants can, as shown by their existence in calm, nutrient-rich environments. They thrive under stressful and extreme tropical environmental conditions such as high concentration of moisture and high temperature. They exist in muddy, shifting, saline and anaerobic conditions, soil acidity and high-low tides of brackish water. Hence, the plants that can survive in these extreme habitats have evolved special methods to survive. Production of unique chemicals may be one such strategy. It is therefore reasonable to assume that the mangrove plants produce metabolites which in turn are unique to them and are of interest to the scientist.

In this study, an exploratory extraction was performed on an adequate amount of two mangrove plants *C. crista* and *C. ramiflora* (stem and leaf) to obtain an extract of varying polarity using chloroform and methanol to gain medium-polar and polar compounds extract. Furthermore, the determination of antioxidant capacity, total phenolics, flavonoid and tannin content along with the antimicrobial and cytotoxic activity of both plants. *C. crista* and *C. ramiflora* have a significant role to play in the health care system of the people who lived near the mangrove forests. Our *in vitro* studies provide sound scientific footing to enhance confidence on the traditional claims of *C. crista* and *C. ramiflora.* The present research study deals with the evaluation of antioxidant, antimicrobial and cytotoxic activity of chloroformic and methanolic extract of *C. crista* and *C. ramiflora* (stem and leaf).

Oxidative stress is a condition in which free radicals are generated extra- or intracellularly, which can exert their toxic effects to the cells. An increase in the antioxidant reserves of the organism can reduce oxidative stress and some of the plant-derived agents may help to reduce it. Currently, there has been an increased interest globally to identify antioxidant compounds from plant sources which are pharmacologically potent and have low or no side effects for use in protective medicine. Mangrove plants inhabiting a very hostile environment conditions are exposed to enhance antioxidant production and consequently the concentration and activity of the

antioxidative enzymes are high in these species to neutralize free radicals. Research findings revealed that almost all mangrove plants are endowed with rich source of antioxidant compounds. In this research study, phytochemical screening of the plants revealed that methanolic extracts of both plant exhibited significant antioxidant activity as measured by various antioxidant assays. Among the extracts methanolic extract of *C. ramiflora* showed highest antioxidant activity and contain considerable amounts of phenols, flavonoids and tannins. Since previous and present studies suggest that methanolic extract of *C. crista* showed significant antioxidant activity and contain sizeable amounts of phenols, flavonoids and tannins.

Plants represent an unlimited source of phytochemicals. In the present study an attempt has been made to screen two mangrove plants from same family collected from Sundarban, known for their medicinal properties. These two plants are used in folklore medicine and have found applications as anti-diarrheal, dysentery, colitis and skin diseases. Basis on their traditional uses several common pathogenic bacteria like *S. aureus*, *S. epidermis*, *E. coli*, *V. cholera*, *S. typhi* and *P. aurigonisa* were used in the present investigation. In antimicrobial activity, all plant extracts exhibited inhibition against both gram positive and gram negative bacteria. Investigating all the results of antimicrobial activity, we found that the plant extract of *C. crista* exhibited excellent activity against gram positive bacteria *C. ramiflora* showed good activity against gram negative bacteria.

Further studies are required to fractionate the extract, to identify the bioactive compounds and should be directed to carry out in vivo studies of its medicinal active components in order to determine their extract mechanism of action and to improve nutritional profile and health benefits as well as to prepare natural pharmaceutical products of high value.

CHAPTER 6
References

6. References

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