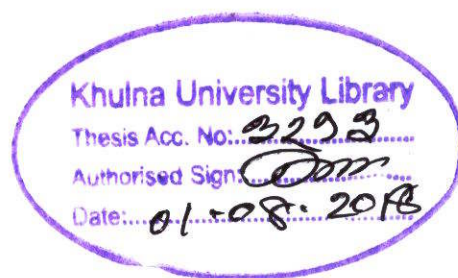


**Total Phenolic Content, Antioxidant and Antibacterial Activities of
Different Fractions of *Sonneratia apetala* (Buch.-Ham.) Fruit**



**A thesis
Submitted to**

**Biotechnology and Genetic Engineering Discipline, Life Science School,
Khulna University**

**For the partial fulfillment of requirement's for the degree of one and half years
Masters of Science in Biotechnology and Genetic Engineering**

Submitted by

MD. IFTEKHARUZZAMAN

Examination Roll No: MS-100704

Session # 2010-2011

**BIOTECHNOLOGY AND GENETIC ENGINEERING DISCIPLINE
LIFE SCIENCE SCHOOL, KHULNA UNIVERSITY
KHULNA-9208, BANGLADESH**

AUGUST, 2012

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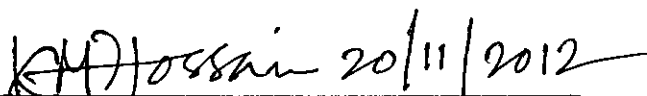
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**BIOTECHNOLOGY AND GENETIC ENGINEERING DISCIPLINE
LIFE SCIENCE SCHOOL, KHULNA UNIVERSITY
KHULNA-9208, BANGLADESH**

AUGUST, 2012

**DEDICATED TO MY
MOST RESPECTABLE PARENTS**

DECLARATION

This thesis report is the original research work of the author, except as otherwise stated. The material presented has not submitted either in whole or in part for a degree for this or any other university. The findings of the research has not been/will not be published anywhere without the written concurrence of the concerned supervisor. Legal action will be taken in any case of infringement regarding the declaration.



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The author

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ABSTRACT

Plants are the most important source of bioactive compounds providing health benefits. The mangrove fruit *Sonneratia apetala* (Buch.-Ham.) is extensively consumed by the people of coastal Bangladesh especially adjacent to the Sundarbans' forest. To seek out the potential source of bioactive compounds, free radical scavenging activity, antioxidant capacity and antibacterial activities, different fractions of *S. apetala* (Buch.-Ham.) fruit (seed and pericarp) were studied. The solvents used for the fractionation were n-hexane, di-ethylether, chloroform, ethylacetate and methanol. Among the fractions, methanol (MS) extract of seed had the highest polyphenols (221.95 mg GAE/g extract) content followed by di-ethylether (DS; 33.35 mg GAE/g) and ethylacetate (ES; 29.36 mg GAE/g) extracts of seed whether methanol (MP) extract of pericarp had the lowest polyphenols (10.31 mg GAE/g) content. From the experiment it was seen that MS showed the most potent DPPH radical scavenging activity with an IC₅₀ value 4.38 µg/mL followed by ES (30.37 µg/mL) and DS (38.23 µg/mL). MS showed 51.77 % NO radical scavenging activity at 800 µg/mL with an IC₅₀ value 415.20 µg/mL followed by DS and MP with IC₅₀ values of 1431 and 2030.46 µg/mL respectively. At 100 µg/mL, MS showed the highest absorbance (O.D. 1.672) followed by ES (O.D. 0.346) and DS (O.D. 0.321). At 200 µg/mL, chloroform (CS) extract of seed showed the highest metal (Fe²⁺) binding capacity (48.67 %) followed by MS (42.67 %) and hexane (HS; 40.13 %) extracts of seed. The IC₅₀ values for CS, MS and HS in chelating Fe²⁺ were 296.38, 498.01 and 396.83 µg/mL respectively. Moreover, MS showed the highest total antioxidant capacity (183.34 mg AAE/g; 121.42 mg GAE/g). This was followed by DS (177.11 mg AAE/g; 117.31 mg GAE/g) and CS (156.37 mg AAE/g; 103.56 mg GAE/g). Total antioxidant capacity was lowest in di-ethylether (DP) extract of pericarp (13.54 mg AAE/g; 8.96 mg GAE/g). In addition, MS inhibited the growth of *Escherichia coli*, *Salmonella paratyphi* A, *Salmonella typhi*, *Shigella dysenteriae* and CS inhibited the growth of *Vibrio cholera*, whereas both of them inhibited the growth of *Staphylococcus aureus*. The IC₅₀ values for MS against *E. coli*, *S. paratyphi* A, *S. typhi*, *S. dysenteriae* and *S. aureus* were 0.56 mg/mL, 0.2632 mg/mL, 1.8632 mg/mL, 0.847 mg/mL and 1.0186 mg/mL respectively whether that for CS against *S. aureus* and *V. cholera* were 1.33 mg/mL and 1.02 mg/mL respectively. MS completely inhibited the growth of *E. coli*, *S. paratyphi* A and *S. dysenteriae* at 2, 1 and 2 mg/mL respectively. In conclusion, the results showed that among the fractions MS, DS and CS are potential sources of bioactive compounds such as polyphenols, antioxidants, whereas MS and CS have potential antibacterial activities. Therefore the fruits of *S. apetala* have activities beneficial to health.

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CHAPTER 1

INTRODUCTION

INTRODUCTION

Human beings have been utilizing plants for basic preventive and curative health care since time immemorial (Farnsworth and Soejarto 1991). Plant or plant's part as a dietary provides not only essential nutrients needed for life but also other bioactive compounds for health promotion and disease prevention. Consumption of fruits and vegetables, grains as well as medicinal plant (for treatment against diseases) has been strongly associated with reduced risk of oxidative stress, cardiovascular disease, cancer, diabetes, Alzheimer's disease, cataracts, and age-related functional decline (Willett 1995; Temple 2000). Whereas, Allopathic medicine is known to have serious limitations in terms of untoward effects, drug resistance, high costs, technical expertise, etc. This convincing evidence suggests that a change in dietary behavior such as increasing consumption of fruits, vegetables and grains is a practical strategy for significantly reducing the incidence of chronic diseases. The protection that fruits and vegetables or medicinal plants provide against diseases has been attributed to the various antioxidant compounds (phytochemicals) such as phenolic compounds, flavonoids, anthocyanins, lignins, catechins, isocatechins, tannins, proanthocyanidins, vitamins C, and E, beta-carotene (Ames 1983; Steinberg et al. 1989; Gey 1990; Steinberg et al. 1991). Antioxidants are most important because they inhibit lipid peroxidation, scavenge free radicals, and chelate divalent cations. The antioxidant properties of these compounds are responsible for their anticancer, antiviral and anti-inflammatory properties (Cao et al. 1997). They can also prevent capillary fragility and platelet aggregation (Beavente-Garcia et al. 1997; Aviram, 1999). At present, there is overwhelming evidence to indicate that free radicals cause oxidative damage to lipids, proteins, and nucleic acids. Free radicals may lie at the heart of the etiology or natural history of a number of diseases including cancer and atherosclerosis. Therefore, antioxidants, which can neutralize free radicals, may be of central importance in the prevention of these disease states. (Stahelin et al 1991; Willett 1995).

Free radicals are electrically charged molecules i.e. they have an unpaired electron, which causes them to seek out and capture electrons from other substances in order to neutralize themselves. Generally, free radicals attack the nearest stable molecule, "stealing" its electron. When the "attacked" molecule loses its electron, it becomes a free radical itself, beginning a chain reaction and can be "thousand of events long" (Przedborski et al. 1998).

Once the process is started, it can cascade, finally resulting in the disruption of a living cell (Jesberger and Richardson 1991). The most dangerous free radicals are the atomic and molecular varieties of oxygen which are known as reactive oxygen species (ROS). The ROS produced in cells include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and free radicals such as the hydroxyl radical ($\cdot\text{OH}$) and the superoxide anion (O_2^-) (Valko et al. 2007). The hydroxyl radical is particularly unstable and will react rapidly and non-specifically with most biological molecules. This species is produced from hydrogen peroxide in metal-catalyzed redox reactions such as the Fenton reaction (Stohs and Bagchi 1995). These oxidants can damage cells by starting chemical chain reactions such as lipid peroxidation, or by oxidizing DNA or proteins (Sies 1997). Damage to DNA can cause mutations and possibly cancer, if not reversed by DNA repair mechanisms (Valko et al. 2004; Nakabeppu et al. 2006), while damage to proteins causes enzyme inhibition, denaturation and protein degradation (Stadtman 1992). In general, antioxidant systems either prevent these reactive species from being formed, or remove them before they can damage vital components of the cell (Sies 1997; Baillie et al. 2009). However, since reactive oxygen species do have useful functions in cells, such as redox signaling, the function of antioxidant systems is not to remove oxidants entirely, but instead to keep them at an optimum level (Rhee, 2006).

South Asian countries have a large number of valuable medicinal plants naturally growing mostly in fragile ecosystems that are predominantly inhabited by rural poor and indigenous community (Kharkil et al. 2003). Among them mangrove and mangrove associates, more specifically their fruits, leaves, stem, bark and roots are precious resource for folk medicine (Bandaranayake 1998). These plants possess diverse medicinal properties (Wangensteen and Alamgir 2009), since they are rich in biologically active antibacterial, antiviral and antifungal compounds viz. steroids, triterpenes, saponins, flavonoids, alkaloids, tannins (Bobbarala and Vadlpudi 2009). Thus extracts of particular parts of mangrove plants are widely used throughout the world (Abeyasinghe et al. 2006). So, it is worth to screen mangrove plants for the presence of new antibacterial compounds to combat pathogenic bacteria. However, the mangrove apple, *Sonneratia apetala* (Buch.-Ham.) belonging to the family of Lythraceae is grown abundantly in the largest mangrove forest 'Sundarbans' and commonly known as 'Keora' in Bangladesh (Das and Ghose 2003). According to Bandaranayake (1998) fruits and barks of the plants belonging to genus *Sonneratia* have remedial activity against asthma, febrifuge, ulcers,

swellings, sprains, bleeding, hemorrhages and piles. He also mentioned that leaves of *S. apetala* show anti-hepatitis activity. Seed is a very valuable part for any plant due to their unique capability to germinate and thus exclusive in chemical content among the other parts of a plant (Das and Ghose 2003). Ripe fruits are also used to expel intestinal parasites (Malay) and half-ripe fruit for coughs (Bandaranayake 1998). Crude methanolic extract of seed and pericarp of *S. apetala* fruit showed reported amount of antioxidant and antibacterial activities (Hossain et al. 2012). So, it is important to emphasize on the investigation processes to find out particular active components showing antioxidant and antibacterial potency of the fruit of *S. apetala*.

Objectives:

The objectives of the research work are mentioned below-

- Determination of the amount of polyphenols contents in the fractionated parts of *S. apetala* seed and pericarps
- Clarification of the potentiality of the fractions to scavenge DPPH and NO free radicals
- Determination of the reducing power of the fractionated parts of *S. apetala* seed and pericarps
- Grading of different fractions depending on their metal chelating activity
- To know the total antioxidant capacity of the fractions
- To know whether the fractionated extracts of the fruit of *S. apetala* possesses antibacterial compounds, and
- To know whether the extracts inhibit the growth of common pathogenic bacteria which cause diseases in humans.

CHAPTER 2

PLANT PREVIEW

PLANT PREVIEW

Sonneratia apetala (Seed and Pericarp)



Fig 1: Fruit of *Sonneratia apetala*

Sample Name: *Sonneratia apetala* Buchanan- Hamilton.

Systemic Classification:

Domain: Eukaryota

Kingdom: Plantae

Subkingdom: Viridaeplantae

Phylum: Tracheophyta

Class: Magnoliopsida

Order: Myrtales

Family: Lythraceae

Genus: *Sonneratia*

Specific epithet: *apetala* Buch.-Ham.

Botanical Name: *Sonneratia apetala* Buch.-Ham.

Local Name: Keora (Bengali), *Sonneratia* mangrove, Maram (Tamil), Kyalanki (Telugu), Khirwa (Oriya), {Kandal, Chipi} (Marathi).

English Name: Mangrove apple.

Distribution: Bangladesh, India, Sri Lanka, Myanmar, and some coastal regions of Asia, Africa and Australia.

Plant Description

The plant has been named *Sonneratia* in honor of Pierre Sonnerat (1749-1814), who travelled to New Guinea, the East Indies and China, and introduced many new plants to the botanists of Europe. *Sonneratia* mangrove is a small to medium size tree, which can attain a height of about 20m and a girth of about 2.5m. The tree occurs on newly accreted soil in moderately to strongly saline areas and is considered as a pioneer species in ecological succession. The leaves are simple opposite, entire and leathery. Bark thin, light brown, irregularly fissured. Flowers are apetalous, cream colored, and arranged in axillary 3-flowered or 7 flowered dichasial cyme. The flowers have no petals, but 4 prominent green sepals. Cream colored mass of stamens give the flowers a cream colored look. The style consists of a white, 2-3 cm long, curved, stigma, flattened like umbrella or mushroom. The upper portion is reddish in color. The fruit is globose, berrylike, leathery, indehiscent, and crowned by style base. Seeds are numerous, embedded in foul-smelling pulp, irregularly angular or falcate; seed coat is thickened, roughened, and somewhat corky.

Uses

The fruit of *S. apetala* is eaten in some area of Bangladesh and Africa. They are also processed to produce sour sauce and marketed in many regions. The leaves are mainly used as fodder. The timber of the plant is resistant to shipworm and pests and is used for building boats, piling and posts for bridges and houses. However, the wood corrodes metal, probably because of the timber's high mineral content. The pneumatophores are made into floats for fishing nets.

Traditional Medicinal Uses

Sonneratia is used in poultices for cuts, bruises, sprains and swellings. The wall of an old fruit is given as a vermifuge. Ripe fruit are used to expel intestinal parasites and half-ripe fruit for coughs. Fermented fruit juice is said to be useful in arresting haemorrhage.

CHAPTER 3

LITERATURE REVIEW



LITERATURE REVIEW

An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols (Sies 1997). Antioxidants are widely used as ingredients in dietary supplements in the hope of maintaining health and preventing diseases such as cancer, coronary heart disease and even altitude sickness. Although initial studies suggested that antioxidant supplements might promote health, later large clinical trials did not detect any benefit and suggested instead that excess supplementation may be harmful (Bjelakovic et al. 2007; Baillie et al. 2009). In addition to these uses of natural antioxidants in medicine, these compounds have many industrial uses, such as preservatives in food and cosmetics and preventing the degradation of rubber and gasoline.

A free radical is a compound with one or more unpaired electrons in its outer orbital (Jesberger and Richardson 1991). Electrons strongly prefer to go around in twos. So as a result of this lonely unpaired electron, free radicals have a tendency to steal back an electron from neighboring molecules (Sreejayan and Rao 1997). These neighboring molecules can be any of the foreign bodies or the body's own active cellular component.

When free radicals steal an electron from a surrounding compound or molecule, a new free radical is formed in its place. In turn, the newly formed radical then looks to return to its ground state by stealing electrons with antiparallel spins from cellular structures or molecules. Thus the chain reaction continues and can be "thousand of events long" (Goldfarb 1999). Free radicals produced *in vivo* include superoxide, the hydroxyl radical, nitric oxide, oxygen-centered organic radicals such as peroxy and alkoxy radicals, and sulfur-centered thiol radicals. Other oxygen-containing reactive species that are not radicals are also formed. These include hydrogen peroxide, peroxynitrite, and hypochlorous acid. While these are not radical species, they are actually or potentially damaging oxidants. The collective term ROS is often used to describe both radical and non radical species (Benzie and Strain 2005).

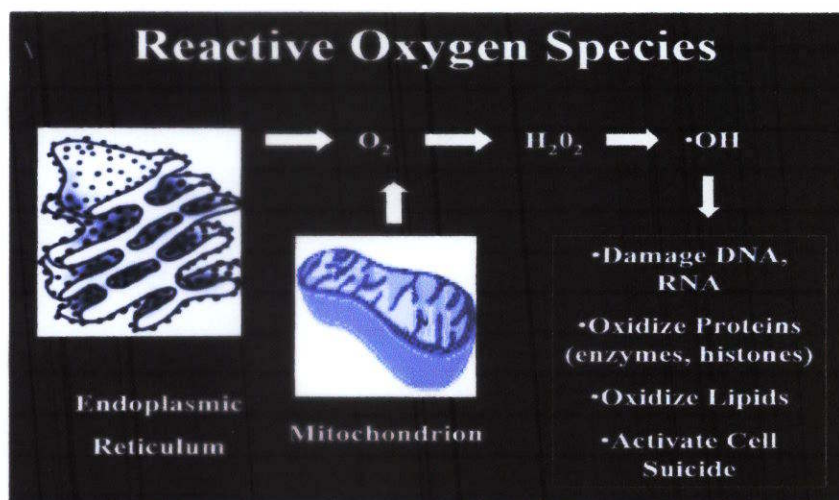


Fig 2: Formation of ROS in endoplasmic reticulum and mitochondrion.

Free radicals are extremely harmful and can damage important molecules within the body. Within the body there are many types of lipids, or fat molecules. One type is responsible for maintaining the structural integrity of the cell membrane. Free radicals can cause damage to the cell membrane by oxidation thereby making them leaky (Shigenaga et al. 1994). Oxidation of another type of lipid, the low-density-lipoprotein, is considered to play a major role in the development of atherosclerosis (the thickening of arterial walls) (Witztum 1994). Free radicals also cause damage to proteins and genes. Oxidative damage to DNA over time can cause changes to both the structure and function of chromosomes. These changes in the genetic code lead to cancer and other chronic diseases (Agarwal and Sohal 1994; Ames et al. 1995; Loft and Poulsen 1996; Musarrat et al. 1996). Free radicals also interfere with protein functions, leading to irregular and abnormal metabolism (Stadtman and Oliver 1991). Free radicals spoil foods and degrade materials such as rubber, gasoline and lubricating as well (Dexter et al. 1994).

To neutralize the free radicals, antioxidant plays an important role. In the late 19th and early 20th century, extensive study was devoted to the uses of antioxidants in important industrial processes, such as the prevention of metal corrosion, the vulcanization of rubber, and the polymerization of fuels in the fouling of internal combustion engines (Matill 1947). Early research on the role of antioxidants in biology focused on their use in preventing the oxidation of unsaturated fats, which is the cause of rancidity (German 1999). However, it was the identification of vitamins A, C, and E as antioxidants that

revolutionized the field and led to the realization of the importance of antioxidants in the biochemistry of living organisms (Jacob 1996; Knight 1998).

The possible mechanisms of action of antioxidants were first explored when it was recognized that a substance with anti-oxidative activity is likely to be one that is itself readily oxidized (Moreau and Dufraisse 1922). However in oxidized state, they are not reactive itself. The human body is generally well equipped with an array of 'antioxidative' strategies to protect against the damaging effects of free radicals. Our antioxidant system is complex, and consists of various intracellular and extracellular, endogenous and exogenous, and aqueous and lipid-soluble components that act in concert to prevent ROS formation (preventative antioxidants), destroy or inactivate ROS that are formed (scavenging and enzymatic antioxidants), and terminate chains of ROS-initiated peroxidation of biological substrates (chain-breaking antioxidants). In addition, metals and minerals (such as selenium, copper, and zinc) that are the key components of antioxidant enzymes are often referred to as antioxidants.

Antioxidants may counter the action of free radicals in three ways: increase in levels of enzymes that degrade free radicals (Rahimi et al. 2005), increase in levels of proteins such as transferrin that can bind the metals which stimulate the production of free radicals (Rahimi et al. 2005), chemical antioxidants such as vitamin C and E which act as direct free radical scavengers (Rahimi et al. 2005).

The importance of antioxidants in preventative medicine has been recognized for several years and polyphenols have become the focus of research interest due to the perceived health beneficial effects (Wollgast and Anklam 2000). Polyphenols have the ability to interfere with cell/tissue based assays in diabetes research by causing damage to cellular proteins, membrane lipids and nucleic acid. During the past decade the interest in polyphenols, including flavonoids has increased considerably with nutritionist, epidemiologist and food manufacturers taking a special interest in polyphenols this is mainly due to their various biological properties like their anti-oxidant effects. Polyphenols include other subclasses such as phenolic acids, stilbenes, lignans, tannins and oxidized polyphenols (George et al. 2005).

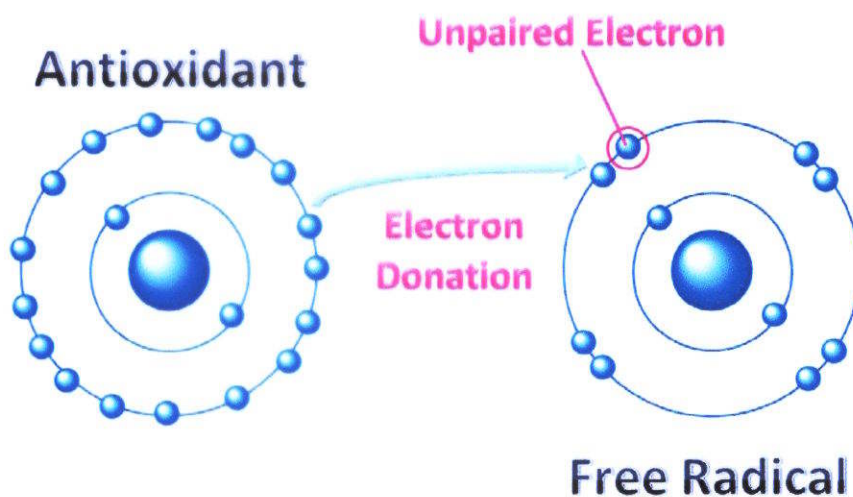


Fig 3: Role of antioxidants to neutralize free radicals.

Polyphenols constitute one of the most numerous and widely distributed groups of substances in the plant kingdom, with more than 8000 phenolic structures currently being identified (Wollgast and Anklam 2000). Polyphenols are products of the secondary metabolism of plants and originate from two main synthetic pathways; the shikimate pathway and the acetate pathway, both of which are derived or occur from glucose metabolism within plants (Wollgast and Anklam 2000). A group of polyphenolic compounds are also called tannins at times as they are powerful reducing agents, and have been known to inhibit the activity of digestive enzymes including that of trypsin and amylase (Thompson et al. 1984). Polyphenols may also exert their effect as chelators of divalent cations.

Table 1: Types of antioxidants within the body

Possible Antioxidative Activity	Example
Physical barriers preventing ROS generation or ROS access to important biological sites	Cell membranes
Chemical traps or sinks 'absorb' energy and electrons and quench ROS	Carotenoids, Anthocyanidins
Catalytic systems neutralize or divert ROS	Superoxide dismutase, Catalase, and Glutathione peroxidase
Binding and redox inactivation of metal ions prevent generation of ROS by inhibiting the Haber–Weiss reaction	Ferritin, Caeruloplasmin, Catechins
Sacrificial and chain-breaking antioxidants scavenge and destroy ROS	Ascorbic acid (Vitamin C), Tocopherol (Vitamin E), Uric acid, Glutathione, and Flavonoids

Source: Encyclopedia of Human Nutrition, Second Edition, 2005.

Table 2: Dietary antioxidants and their common sources

Antioxidants	Dietary Sources
Ascorbic acid (vitamin C)	Fruits and vegetables, particularly strawberries, citrus, kiwi, Brussels sprouts, and cauliflower
α -Tocopherol (vitamin E)	Green leafy vegetables, e.g., spinach, nuts, seeds, especially wheat germ, vegetable oils, especially sunflower
Carotenoids	Orange/red fruits and vegetables (carrot, tomato, apricot, melon, yam), green leafy vegetables
Flavonoids	Berries, apples, onions, tea, red wine, some herbs, citrus fruits, grapes, cherries

Source: Encyclopedia of Human Nutrition, Second Edition, 2005.

An overview of the antioxidant defense system within our body is following-

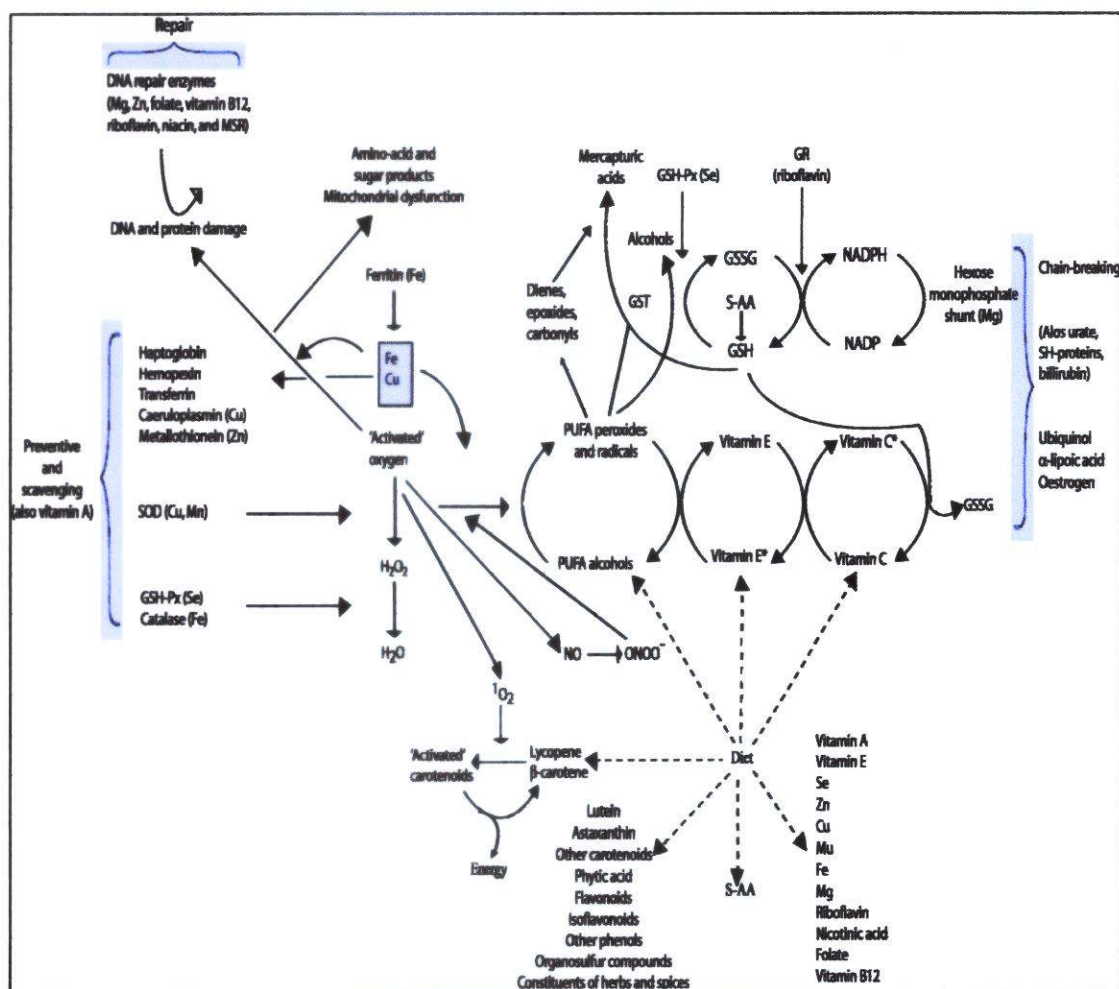


Fig 4: The integrated antioxidant defense system comprises both endogenous and dietary-derived antioxidants.

GR, glutathione reductase (EC1.6.4.2); GSH, reduced glutathione; GSH-Px, glutathione peroxidase (EC1.11.1.9); GSSG, oxidized glutathione; GST, glutathione-S-transferase (EC 2.5.1.18); MSR, methionine sulfoxide reductase (EC1.8.4.5); NADPH and, NADP, are, respectively, the reduced and oxidized forms of the co-factor nicotinamide adenine dinucleotide phosphate; PUFA, polyunsaturated fatty acid; S-AA, sulfur amino-acids; SH, sulfydryl; SOD, superoxide dismutase (EC1.15.1.1).

(Source: Encyclopedia of Human Nutrition, Second Edition, 2005)

In the book 'The Antioxidant Miracle', Packer and Carol Colman (1999) summarized the applications of antioxidants as following-

Table 3: Application of Antioxidants

Antioxidant	Strengthens	Helps Prevent	Other	Found In
Lipoic Acid	Memory, energy production, controls adult diabetes	Heart disease, cancer, stroke, cataracts	Recycles all five of the network antioxidants	Potatoes, spinach
Vitamin E	Increases life span, helps clear clogged arteries, protects skin, relieves arthritis	Heart disease, stroke, Alzheimer's, skin, prostate and breast cancer	Need all 4 tocopherols and 4 tocotrienols. "d-alpha" is natural, "dl-alpha" is synthetic	Vegetable oils, nuts, grain, tocotrienols in bran
Vitamin C	Immune system, recycles vitamin E	Cancer, heart disease, scurvy	More than 300 mg per dose is excreted	Red peppers, broccoli, cranberries, citrus
CoQ10	Rejuvenates brain cells	Heart disease, gum disease	Has been used for 20 yrs in Japan to treat and prevent heart disease	Seafood and organ meat
Glutathione	Detoxifies body, strengthens immune system, prolongs life	Prevents bronchitis and pneumonia	Takes 100 mg of lipoic acid for body to make it. It is the cell's primary antioxidant	Fresh fruits and vegetables
Flavonoids	Improve memory and concentration, lowers blood pressure	Blood clots, LDL cholesterol in arteries, reduces inflammation	Boosts effectiveness of vitamin C, found with it in nature	Citrus fruits, apples, onions, red grapes, berries, pine bark
Carotenoids	Converts to Vitamin A if needed, good for eyes and vision	Cancer, lycopene for prostate cancer, lutein for cataracts, heart attack	Give the color to yellow, orange, red, purple and dark green vegetables	The colorful fruits and vegetables: Alpha carotene is orange and in carrots, lutein is the green in spinach, lycopene in red tomatoes.
Selenium	Synergistic effect with vitamin E	Lung, prostate and colon cancer, stroke and heart disease	Trace mineral needed for formation of two antioxidants	Garlic, onion, broccoli, whole grains, red grapes, egg yolks

Antioxidant Activities of Mangrove Plants

The antioxidants from natural sources are given preference than synthetic sources and the DPPH method has been widely applied for estimating antioxidant activities recently (Molyneux, 2004). However, though several mangroves are extensively used in traditional medicine, only some of them were tested for biological activities and a very few were studied for antioxidant activity. According to Vadlapudi et al. (2009) mangrove plants have the potential in scavenging free radicals and can be a vital source of antioxidant phytochemicals. An intensive study found that *Thespesia populneoides*, *Sonneratia apetala*, *Acaryochloris marina*, and *Rhizophora mucronata* are good sources of natural antioxidants (Vadlapudi and Naidu 2009).

Patra et al. (2009) reported that *Excoecaria agallocha* possesses a promising antioxidant property and antifungal activity. Its therapeutic potential has already been demonstrated in case of epilepsy, ulcer, conjunctivitis, dermatitis, tooth ache, leprosy etc (Bandarnayake 2002). Previous phytochemical investigations on this species revealed the presence of diterpenoids (Kang et al. 2005; Wang and Guo 2005; Wang et al. 2006; Ji-Dong et al. 2007; Li et al. 2007), triterpenoids (Zou et al. 2006), flavonoid (Konishi et al. 2003) and phorbol esters (Nilsson and Ericson 1995).

Total polyphenol content and DPPH free radical scavenging assay of eight other mangrove plant leaves were studied by Agoramoorthy et al. (2008). Highest free radical scavenging activity was found in *E. agallocha* (30.3 µg/mL) whereas higher DPPH radical scavenging activity was also found in species such as *Bruguiera cylindrica* (42.9 µg/mL), *Ceriops decandra* (51.9 µg/mL), *Rhizophora apiculata* (64.9 µg/mL), *Aegiceras corniculatum* (74.3 µg/mL), *Rhizophora mucronata* (79.7 µg/mL) and *Ipomoea pes-caprae* (83.7 µg/mL). Banerjee et al. (2008) studied six mangrove and four mangrove associated plants and obtained best antioxidant result for *Ceriops decandra* stem bark extract. Calyces of *Sonneratia caseolaris* also exhibited strong antioxidant activity followed by stamens of *Sonneratia caseolaris*, calyces of *S. alba*, *Cynometra ramiflora* seeds, *Xylocarpus rumphii* fruit peel and branches (Bunyaphatsara et al. 2003). The antioxidant assay of another mangrove *Rhizophora mucronata* (100 µg/mL) showed IC50 values 43.17, 116, 60.06, and 46.76 µg/mL for DPPH, hydrogen peroxide, nitric oxide, and hydroxyl radical scavenging activity, respectively (Suganthi et al., 2009). Kokpal et al. (1990) had reported the bioactive compounds from mangrove plants. Wu et al. (1997)

reported the cytotoxic and antiplatelet aggregation activity of methanol extract of *Aglaia elliptifolia* because of containing certain compounds.

Reported antioxidant activity in *S. apetala*

Banerjee et al. (2008) reported the antioxidant activity of leaves, stem bark and root of *S. apetala*. The amount of total phenolic content of leaves, stem bark, and roots in GAE (Gallic Acid Equivalent) mg/g of dry material were 47.52 ± 2.22 , 42.68 ± 2.75 , and 42.75 ± 1.67 respectively. The reducing power was evaluated as AAE (Ascorbic Acid Equivalent) mg/g of dry material and that for leaves, stem bark, and roots were 5.71 ± 0.24 , 7.06 ± 0.07 , and 6.87 ± 0.10 respectively. The evaluation of anti-radical properties of mangrove plant materials was performed by DPPH radical scavenging assay. The 50% inhibition of DPPH radical, IC₅₀ value for leaves, stem bark, and roots were 163.49 ± 6.32 , 193.09 ± 14.35 , and 183.04 ± 1.74 respectively.

According to Hossain et al. (2012) seeds of *S. apetala* consist of very high content of polyphenols (300 ± 8.2 mgGAE/g extract), flavonoids (30.6 ± 0.7 CE/g extract), anthocyanins (2.3 ± 0.03 μ mol/g extract) and vitamin C (4.0 ± 0.08 mg/g extract). The IC₅₀ values for DPPH and NO free radical scavenging were 4.3 and 49.4 μ g/mL for seed extract and that for pericarp extract were 59.8 and 751.6 μ g/mL respectively. Seed extract also showed very high reducing power (O.D. 1.14 at 50 μ g/mL extract) and total antioxidant capacity (280.8 GAE or 310.24 AAE/g extract). In streptozotocin (STZ) induced type 2 diabetes rats, seed extract treated group showed serum glucose decreased from 13.75 ± 2.21 mmol/L (at 30 min) to 10.3 ± 1.75 mmol/L (at 135 min) and in the pericarp treated group from 14.36 ± 2.16 to 11.32 ± 1.74 mmol/L. The area under the glucose curve was more profoundly decreased in the seed treated group than the pericarp treated group (Hossain et al. 2012).

Antimicrobial Activities of Mangrove Plants

For centuries, mangroves have been traditionally used for food (fruits and nectar), feed and medicinal purposes in different parts of the world. They are well known to produce natural metabolites with diverse biological activities such as antibacterial (Abeyasinghe et al. 2006), antiviral (Zandi et al. 2008), antidiarrhoeal (Rouf et al. 2007), antifeedant (Wu et al. 2008), insecticidal (Calderon et al. 2001) and cytotoxic activities (Han et al. 2007). However, during the last decade screening of mangroves for bioactive compounds, has

received high interest as a potential bioresource for novel drug leads. Until now, more than 200 bioactive metabolites have been isolated from true mangroves of tropical and subtropical populations (Wu et al. 2008). According to their chemical structure, most of the isolated compounds belong to steroids, triterpenes, saponins, flavonoids, alkaloids, tannins and phenolics which having a wide range of therapeutic possibilities (Bandaranayake 1998).

Healing properties are attributed to *Rhizophora* trees in popular folk medicine. Root, leaf and stem extracts of *Rhizophora* trees have inhibitory properties, affecting the growth of various human pathogenic organisms. Among these are bacteria, fungi and viruses (Hernandez and Perez 1978). Mangrove plants belonging to the family Rhizophoraceae are highly active against viruses such as HIV and Hepatitis B virus (Premanathan et al. 1992, 1996, 1999, and Kathiresan et al. 1995). The antiviral compound was identified as a polysaccharide that is able to prevent HIV adsorption to the host cells. Another antiviral compound identified was lignin. Lignin protected mice against pathogenic *Escherichia coli*, and this antibacterial activity was attributed to the antioxidant property of lignin (Sakagami et al. 1998). *Pongamia pinnata* (L). an Indian mangrove medicinal plant was evaluated by Elanchezhian and Rajendran (1993) for antiviral properties against herpes simplex virus type- 1 (HSV- 1) and type- 2 (HSV-2) by *in-vitro* studies in Vero cells. A crude aqueous seed extract of *P. pinnata* completely inhibited the growth of HSV-1 and HSV-2 at concentrations of 1 and 20 mg/mL (w/v), respectively, as shown by complete absence of cytopathic effect.

Antibacterial activity is also shown by other different mangrove plants. *Salicornia brachiata* was studied by the agar diffusion and dilution methods. The methanolic extract of leaves was active against *Bacillus subtilis*, *Bacillus pumilus*, *Micrococcus luteus* and *Staphylococcus aureus* (Manikandan et al. 2009). The crude ethanol extract and other partially extracted fraction of the barks of *Xylocarpus granatum* possess antimicrobial activity against most of the test organisms depending upon the nature of their active ingredients in the extract and capacity of diffusion into the agar medium. Among the test organisms, the extract showed significant antimicrobial activity against *Staphylococcus epidermis*, *Staphylococcus aureus*, *Shigella boydii* and *Proteus spp.* and moderate activity against *Escherichia coli*, *Streptococcus pyogenes*, and no activity against *Shigella dysentery*, *Enterococci*, *Salmonella typhi* (Alam et al. 2006). Lauric, palmitic, linolenic,

linoleic, oleic, stearic and myristic acids are known to have potential antibacterial and antifungal agents (McGaw et al. 2002, Seidel and Tailor 2004). The study of Agoramoorthy et al. (2007), undoubtedly confirms that the leaves of *Excoecaria agallocha* contain higher relative percentage of the above mentioned fatty acids that has potential antibacterial and antifungal principle for clinical application. Bobbarala et al., (2009) also reported that methanol extracts of *Excoecaria agallocha* inhibited most bacterial and fungal growth. The methanol extract of *E. agallocha* L. showed considerably more activity than hexane and chloroform extracts.

Sastry and Rao (1994) found the benzene extract of *Gracilaria corticata* showed antibacterial activity only against *Salmonella typhi* and *Escherichia coli* whereas the methanol and chloroform extracts had activity against *P. aeruginosa*. The antimicrobial activity of the crude methanolic root extract of *Pluchea indica* (L.) and four isolated constituents were investigated using agar dilution method by Chatterjee et al. (2005). The Minimum Inhibitory Concentrations (MIC) ranging from 1000–2000 µg/mL, of the methanolic extract were recorded against 15 bacterial strains and maximum activity was exhibited against 12 strains. The petroleum ether, chloroform and ethyl acetate crude extracts (500 µg/disc) of the aerial part of *Marsilea quadrifolia* were screened against sixteen human pathogenic bacteria to check antibacterial activities by disc diffusion method. The petroleum ether extract showed no activity against the tested pathogenic organisms. On the other hand chloroform crude extracts showed excellent antibacterial activity with the average zone of inhibition of 11-20 mm by disc diffusion method, among the tested bacteria, the growth of *Pseudomonas aeruginosa* (20 mm) was highly inhibited. In case of ethyl acetate extract the highest activity was also seen against the growth of *P. aeruginosa* having the zone of inhibition of 18mm. Besides this, the chloroform and ethyl acetate crude extracts showed good activity against the growth of *Staphylococcus aureus* (18 mm), *Shigella dysenteriae* (16 mm) *Shigella shiga* (14 mm) and *Shigella boydii* (14 mm). These were the statistics from the study of Ripa et al. (2009). Bose and Bose (2008) suggested that the alcoholic and chloroform extracts of the leaves of *Acanthus ilicifolius* (L.) exhibited strong inhibitory action against *Bacillus subtilis*, *Staphylococcus aureus*, *Candida albicans*, *Aspergillus fumigates* and *Aspergillus niger* and moderate inhibitory action against *Pseudomonas aeruginosa* and *Proteus vulgaris*. A physician in Cali, Colombia, reported to cure throat cancer, with gargles of

mangrove bark (Garcia 1975). Bark of red mangrove trees have been used in folk remedy for a wide array of diseases (Duke and Wain 1981).

Punica granatum ethanolic peel extracts have shown antibacterial activity against *Escherichia coli* and methicillin-resistant *Staphylococcus aureus* bacteria. This antibacterial activity may be indicative of the presence of several metabolic toxins or broad-spectrum antibiotics (Choi et al. 2009). The antibacterial activity of *Acalypha indica* was investigated against three strains of human pathogenic bacteria viz., *Bacillus subtilis*, *Staphylococcus aureus* and *Klebsiella pneumonia* using ethylacetate, hexane and methanol extracts of leaves, stem and roots of *Acalypha indica*. The ethylacetate extracts of leaves and roots inhibited the growth of all the three selected bacterial species (Gangadevi et al. 2008). Antibacterial activity of mature leaves, tender leaves and bark extracts of *Avicennia marina*, *A. officinalis* and *Bruguiera sexangula* were evaluated by Abeysinghe et al. (2006) against pathogenic bacteria species of *Staphylococcus spp.*, *Proteus spp.*, *Escherichia coli*, *Shigella spp.* and *Pseudomonas spp.* All plant extracts in ethyl acetate showed strong inhibition compared to other extracts on all tested bacterial strains. Among all bacterial strains, *Pseudomonas sp.* and *E. coli* showed considerable growth inhibition against almost all plant extracts.

Reported Antimicrobial Activity of *S. apetala* (Buch.-Ham.)

Bobbarala et al. (2009) identified the antimicrobial activity of the mangrove plant extract of *S. apetala* on the various test microorganisms, including clinical multiple antibiotic resistant bacteria and phytopathogens. *In vitro* screening of *S. apetala* mangrove plant extracts showed species specific activity in inhibiting the growth of bacteria and fungi. Hexane, chloroform and methanol extracts showed good activity against all the pathogens, where as methanolic extracts were active against most of the pathogens.

According to Hossain et al. (2012) susceptibility test showed that methanolic seed extract of *S. apetala* inhibited the growth of both gram positive and gram-negative pathogenic bacteria. Methanolic Seed extract (300 µg/disc) inhibited the growth of all tested bacteria whereas methanolic pericarp extract showed no inhibition of *E. coli*, *E. faecalis*, *Pseudomonas sp.*, *S. flexneri* and *S. epidermidis*.

CHAPTER 4

MATERIALS AND METHODS

MATERIALS AND METHODS

MATERIALS

Collection of sample

Fruits of *S. apatala* (Buch.-Ham.) plants were collected from the Sundarbans, Khulna.

Preparation of samples

Used parts of the collected fruits were washed by water to remove undesirable materials. Then excess of water was drained off. The pericarps were then peeled off from the seeds and both the seeds and pericarps were cut into small pieces.

Preparation of extract

The sliced pieces of pericarp and seed were shed-dried until all the water evaporated. After getting biscuit like fragile form, the processed fruit parts were ground separately by a grinding machine into powder form. 10 grams of powder was extracted with 200 mL of 100 % hexane and kept in air tight bottle and after 30 minutes it was filtered by Whatman No. 1 filter paper to remove any fruit part or powder particle. The collected liquids were dried until a constant dry weight of each extract was obtained. Same procedure was repeated for the remaining dry residues using the other four solvents; diethyl ether, chloroform, ethyl acetate and methanol respectively. These were done separately for seed and pericarp of *S. apatala* (**Appendix 2: Fig 22**). After filtration the filtrates were air dried and the solid/crude extracts were kept in the refrigerator. Finally, stock solutions were prepared to 10 mg/mL or 20 mg/mL concentration by adding dimethylsulfoxide (DMSO) to conduct experiments and named HS, DS, CS, ES and MS to indicate hexane, di-ethylether, chloroform, ethyl acetate and methanol extracts of *S. apatala* seed respectively whereas HP, DP, CP, EP and MP to indicate hexane, di-ethylether, chloroform, ethyl acetate and methanol extracts of *S. apatala* pericarp respectively.

Chemicals used in the experiments

200mm Phosphate Buffer; P^H 6.6 (0.2 M Na_2HPO_4 , 0.2 M NaH_2PO_4), Potassium Ferricyanide, Trichloroacetic Acid, Ferric Chloride, Sulfuric Acid, Sodium Carbonate, 1M Naoh, L-Ascorbic Acid (AA), Gallic Acid (GA), DPPH (2, 2-Diphenyl-1-Picrylhydrazyl), Ammonium Molybdate (4mm), Ethanol, Folin Reagent (Ciocalteu's Phenol Reagent), Sulfanilamide, Naphthylethylene Diamine Dihydrochloride, Phosphoric Acid (Ortho), Sodium Nitroprusside, Methanol, Sprit, Sodium Di-Hydrogen Phosphate

(28mm), Sodium Acetate, Ferrous Chloride (2mm), Ethylene Diamine Tetra-Acetic Acid (EDTA), Ferrozine, Dimethyl Sulfoxide (DMSO), N-Hexane, Di-Ethylether, Chloroform, Ethylacetate, Methanol.

Materials used in the experiments

Laminar Air Flow (ESCO, EQU 03 EHTL, ESCO Micro Pvt Ltd., Singapore), Incubator (WTB Binder, Titling, Germany), Autoclave, Water Bath (Thermostatic Water Bath, Shanghai, China), One Drum Vial, Spirit Lamp, 500 Ml Volumetric Flask, Dry Heat Sterilizer (WTB Binder, Titling, Germany), Marker (Read Leaf, Japan), Nose Masks, Hand Gloves, Petri Dish, Refrigerator, Oven, Digital Balance, Inoculating Loop, Sterile Forceps, Sterile Scissor, Tissue Paper, Micropipettes, Test Tubes, Beaker, Ph Meter, Centrifuge, UV-Spectrophotometer, Vortex Mixture, Homogenizer, Grinder.

METHODS

Determination of total phenolic content (TPH)

The total concentration of phenolics (TPC) in the extracts was determined according to the Folin-Ciocalteu method (Ough and Amerine, 1988) with gallic acid as the standard and expressed as (mg) GA equivalents (GAE/g) (Aoshima and Ayabe, 2007; Hossain *et al.*, 2009).

Determination of DPPH radical scavenging activity

The determination of DPPH radical scavenging activity was determined according to the method of Blois 1958; Aoshima *et al.* 2004.

Determination of nitric oxide (NO) Scavenging Activity

Nitric oxide radical scavenging activity was determined according to the method reported by Marcocci *et al.* 1994.

Reducing power determination (RP)

The reducing power of the extract was determined according to the method of Oyaizu 1986.

Determination of ferrous (Fe^{2+}) ion chelating activity

Fe^{2+} ion chelating activity was determined according to the method reported by Dinis *et al.* 1994.

Total antioxidant capacity (TAC) measurement

The total antioxidant capacity of the extract was determined according to the method of Prieto et al. 1999.

Antibacterial assay**Bacterial strains and their maintenance**

In this screening test for antibacterial activity six pathogenic bacteria that cause common diseases in Bangladesh were collected from ICDDR'B as clinical isolates. They are listed below:

Table 4: List of bacteria with diseases caused by them

Gram Negative	Diseases	Gram Positive	Diseases
<i>Escherichia coli</i>	Gastroenteritis, urinary tract infections, and neonatal meningitis	<i>Staphylococcus aureus</i>	Scaled skin syndrome
<i>Salmonella paratyphi A</i>	Paratyphoid		
<i>Salmonella typhi</i>	Typhoid		
<i>Shigella dysenteriae</i>	Bacillary dysentery		
<i>Vibrio cholerae</i>	Cholera		

For routine culture and maintenance Trypton soy broth (TSB), McConkey agar and Nutrient agar (NA) media were used. However for antibacterial activity screening nutrient agar media was used.

Sub-culture of test organisms

The test microorganisms from the pure culture (preserved at 4°C) were streaked onto nutrient agar plates with the help of a sterile inoculating loop. For the growth of the test organism the inoculated plates were incubated at 37°C overnight (18 to 22 hrs).

Preparation of the discs

Sterilized blank paper discs were placed in sterilized petri dishes and were impregnated separately with the solution (20 mg/mL) of test materials so that each disc contain desired

volume of extract and left for a period of time in an aseptic condition for the complete evaporation of the solvents. Control discs were also prepared in order to determine whether remaining solvent (if any) in disc shows any antimicrobial activity. For the preparation of control disc equal volume of solvent without test material was used to soak the paper disc. Kanamycin was used as standard antibiotic discs for positive control.

Disc diffusion method for antibacterial activity

From the overnight culture plate, small portion of a fresh colony was transferred into test tube containing nutrient broth and incubated at 37°C until the growth reached the log phase (4-6 hrs; 5×10^6 CFU/mL). After optimum growth plates were seeded properly by pouring the culture broth with a Pasteur pipette to make bacterial lawn. The excess culture broth was withdrawn from the plates and allowed to dry for 5 minutes. Discs impregnated with solvent extracts were placed at proportionate distance from each other using a sterile needle. The plates were incubated overnight at 37°C and checked for inhibition zone. A transparent scale was used to measure the zone-diameter in mm.

Determination of inhibition of selected bacterial species by the potential extracts

Tube dilution method was used to determine percentage of inhibition of growth of *E. coli*, *S. paratyphi* A, *S. typhi*, *S. dysenteriae* and *S. aureus* in presence of MS and *S. aureus* and *V. cholerae* in presence of CS. In this method an increasing in concentration of the solvent extract were added to different test tubes. Sterilized screw-capped one drum vials each containing 2 mL of nutrient broth were taken. Then calculated amount of MS and CS was added from the stock solution (20 mg/mL) to the vials to obtain different concentrations (0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2 mg/mL) of the extracts. Then 10 µl of freshly grown inoculas (5×10^6 CFU/mL) was added to each vial. All the tubes were incubated at 37°C. After incubation, the optical densities (OD) were measured using spectrophotometer (Hitachi U-2910) at 700 nm.

CHAPTER 5

RESULTS

RESULTS

Total Polyphenols (TPH)

Total Phenolic Contents (TPH) of fractionated extracts of seed and pericarp of *S. apetala* are shown in **Fig 5**. The TPH content was expressed as gallic acid (GAE) equivalent. The TPH content ranged from 221.95 to 10.31 mg GAE/g of extract. The amount was highest in MS (221.95 mg GAE/g) followed by DS (33.35 mg GAE/g) and ES (29.36 mg GAE/g) whereas it was lowest in MP (10.31 mg GAE/g) followed by HP (10.40 mg GAE/g) and DP (14.82 mg GAE/g).

DPPH Radical Scavenging Activity

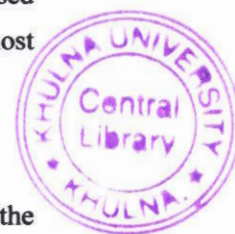
The DPPH radical scavenging activities of different fractions of seed and pericarp are shown in **Fig 6**. The level of free radical (DPPH) scavenging ranged from 93.42% to 0% at 100 µg/mL of extract. At this concentration, ES, DS and MS showed the highest DPPH radical scavenging activity (all are above 80%) whereas HS showed no activity whereas HP had the lowest (2.24%) activity followed by DP (8.28). Dose-dependent DPPH radical scavenging activities of DS, ES and MS are shown in **Fig 7** where ascorbic acid was used as positive control. The comparison of IC₅₀ values for DPPH scavenging of these most potent fractions are shown in **Fig 8**.

Nitric Oxide (NO) Scavenging Activity

NO scavenging assay was measured at 570 nm O.D. and shown in **Fig 9**. MS showed the highest activity (51.77 %) followed by DS (37.77 %) and MP (16.52 %) at a concentration of 800 µg/mL. CP showed the lowest NO scavenging activity (0.75 %) at the same concentration. Dose dependent activity of GA, DS and MS are shown in **Fig 10**. Comparison of IC₅₀ values for NO scavenging of DS, ES, MS and MP are shown in **Fig 11**.

Reducing Power

The more antioxidant compounds reduce more oxidized form of ferric iron (Fe⁺³) to ferrous iron (Fe⁺²). In the present study, reducing powers of the fractionated extracts of both seed and pericarp were measured at 700 nm OD. Reducing powers of the extracts determined using the potassium ferricyanide method is shown in **Fig 12**. At 100 µg/mL concentration, it was observed that MS had the highest absorbance (1.672) followed by



ES (0.346), DS (0.321) and CS (0.114). Dose-dependent reducing power of MS, ES and DS are shown in **Fig 13** where ascorbic acid was used as positive control.

Determination of Ferrous (Fe^{2+}) Ion Chelating Activity

Fe^{2+} ion chelating activity of the seed and pericarp fractions was measured at 562 nm wavelengths at a concentration of 200 $\mu\text{g/mL}$ and is shown in **Fig 14**, whereas, EDTA was used as the positive control. CS showed the highest ferrous ion chelating activity (48.67%). This was followed by MS (42.67%), HS (40.13%) and CP (32.67). EP showed the lowest Fe^{2+} chelating activity (17.23%). Dose-dependent Fe^{2+} ion chelating activity of HS, CS, MS and are shown in **Fig 15**. IC_{50} values are also shown in **Fig 16**.

Total Antioxidant Capacity (TAC)

Total antioxidant capacity (TAC) which was expressed as ascorbic acid equivalents (AAE) and gallic acid equivalents (GAE) of different fractions of seed and pericarp is shown in **Fig 17**. Methanol extract of seed showed the highest antioxidant capacity (183.34 mg AAE/g; 121.42 mg GAE/g). This was followed by di-ethylether (177.11 mg AAE/g; 117.31 mg GAE/g), chloroform (156.37 mg AAE/g; 103.36 mg GAE/g) and ethylacetate (110.54 mg AAE/g; 73.21 mg GAE/g) extracts of seed. Di-ethylether extract of pericarp showed the lowest antioxidant capacity (13.54 mg AAE/g; 8.96 mg GAE/g).

Disc Diffusion Test

Disc diffusion method was used to know the antibacterial activity of the fractionated extracts of the seed of *S. apetala*. MS (400 $\mu\text{g/mL}$) inhibited the growth of all tested bacteria except *V. cholerae* whereas CS inhibited the growth of *E. coli*, *S. aureus* and *V. cholerae*. ES inhibited the growth of *S. aureus* and *V. cholerae*. Results are shown in **Table 5**.

Determination of Inhibition of Growth (%) of Selected Bacterial Species by MS and CS

The percentage of inhibition of growth of selected bacteria by MS and CS was determined by tube dilution method and is shown in **Fig 18** and **Fig 19**. The study showed that at 2 mg/mL, MS inhibits *E. coli* and *S. dysenteriae* completely. MS also inhibits *S. paratyphi* A completely at 1 mg/mL. Comparison of IC_{50} values for MS against *E. coli*, *S. paratyphi* A, *S. typhi*, *S. dysenteriae* and *S. aureus* is shown in **Fig 20** whereas IC_{50} values for CS against *S. aureus* and *V. cholerae* is shown in **Fig 21**.

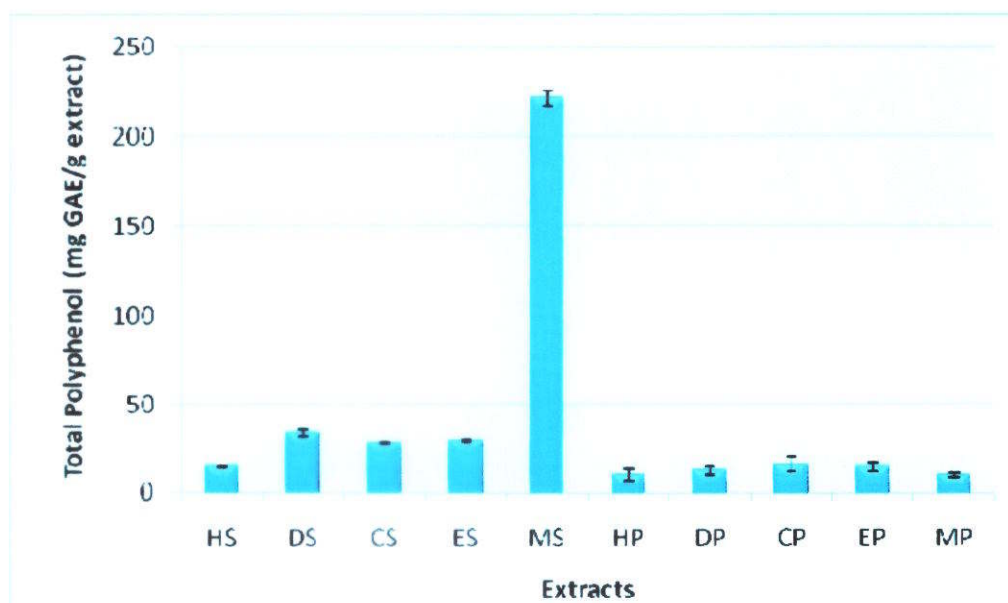


Fig 5: Total polyphenol contents (mg GAE/g extracts) in fractionated extracts of seed and pericarp of *S. apetala*. GAE= Gallic Acid Equivalent. Values are means $\pm$ SD (bars) from three experiments, n=3.

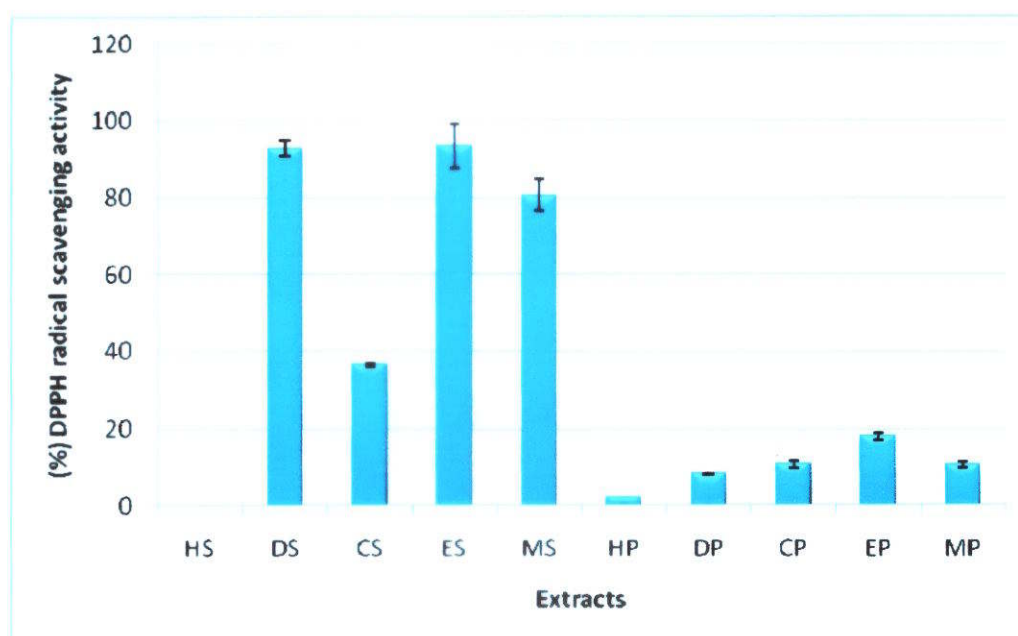


Fig 6: DPPH free radical scavenging activity of different fractions of *S. apetala* at 100 μ g/mL. The data are presented as mean $\pm$ SDs (bars), n=3.

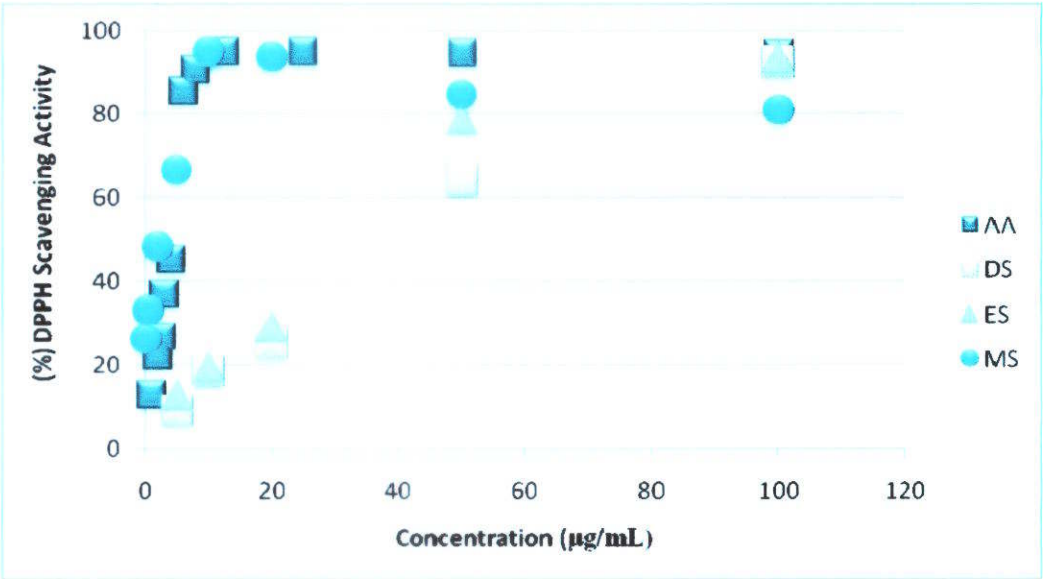


Fig 7: Dose-dependence of DPPH free radical scavenging activity of DS, ES and MS. Ascorbic acid (AA) was used as positive control. Data are mean $\pm$ SDs (bars), n=3.

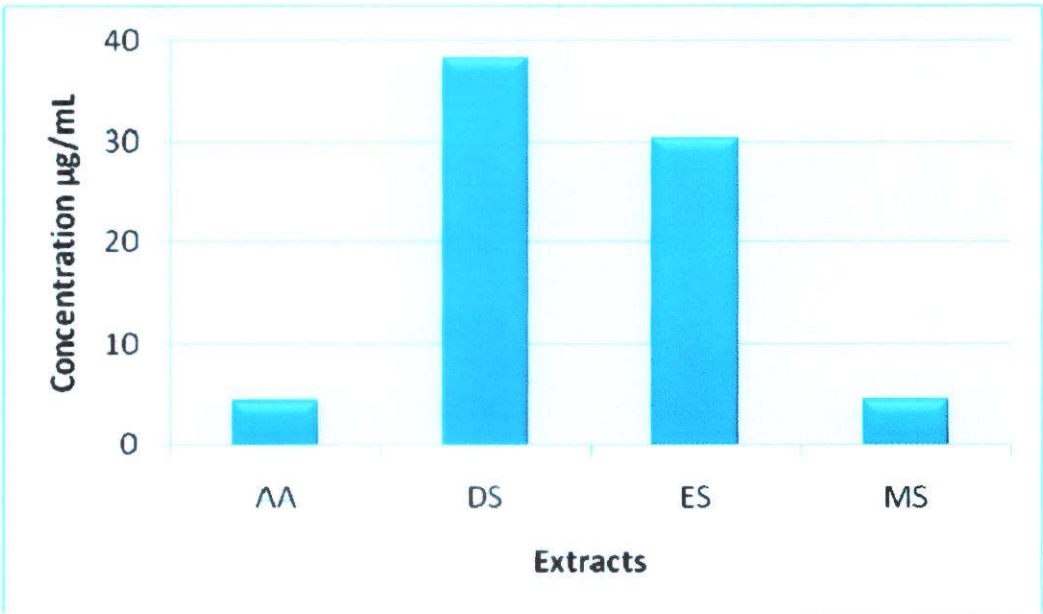


Fig 8: Comparison of IC₅₀ values of DPPH free radical scavenging activity of MS, ES and DS.

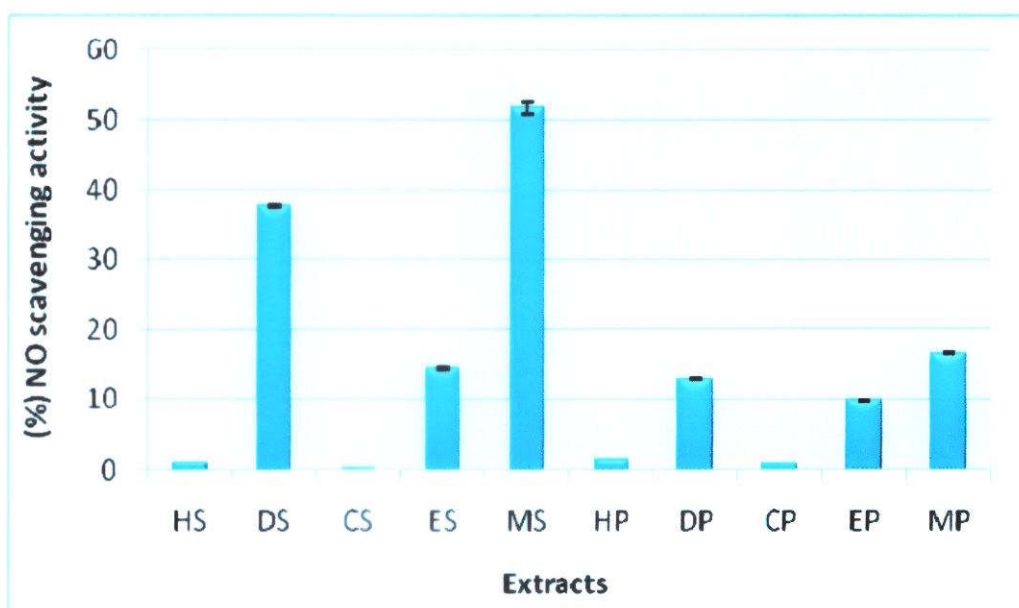


Fig 9: NO scavenging ability of different fractions of *S. apetala* at 800 µg/mL. Data are presented as mean $\pm$ SDs (bars), n=3-5.

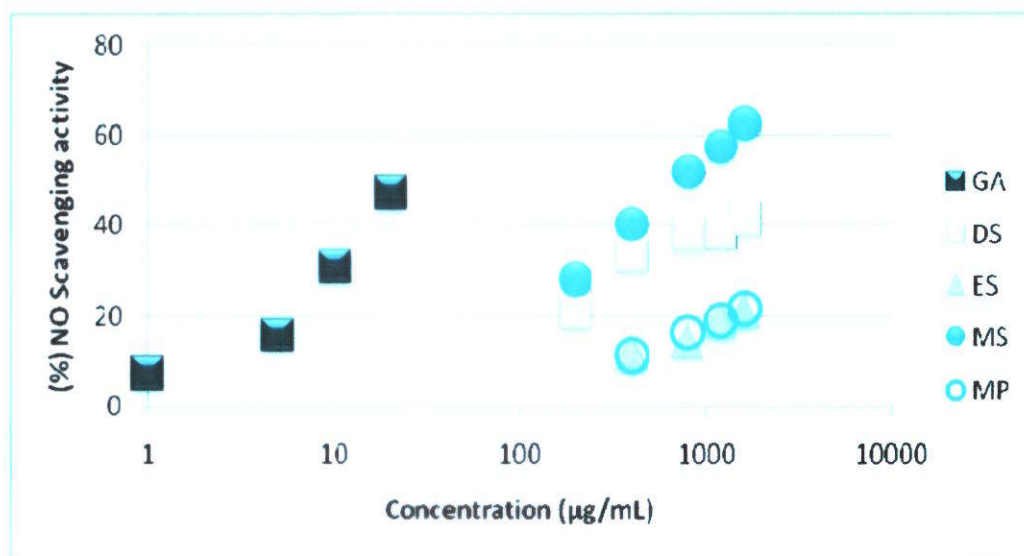


Fig 10: Dose dependence of NO scavenging activity of DS and MS. Gallic acid (GA) was used as positive control. Data are mean $\pm$ SDs (bars).

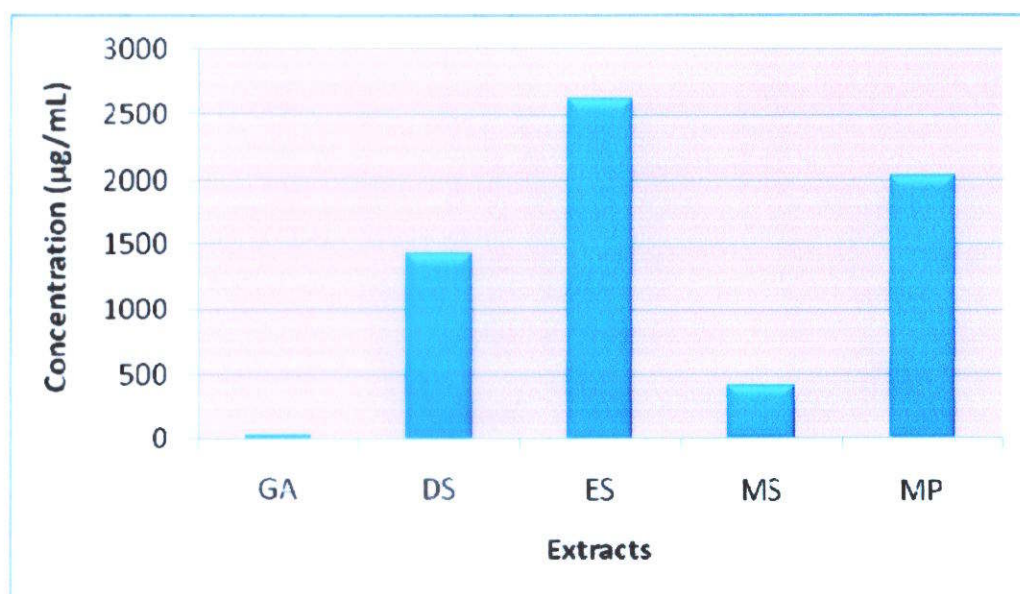


Fig 11: Comparison of IC_{50} values of NO free radical scavenging activity of DS, ES, MS and MP.

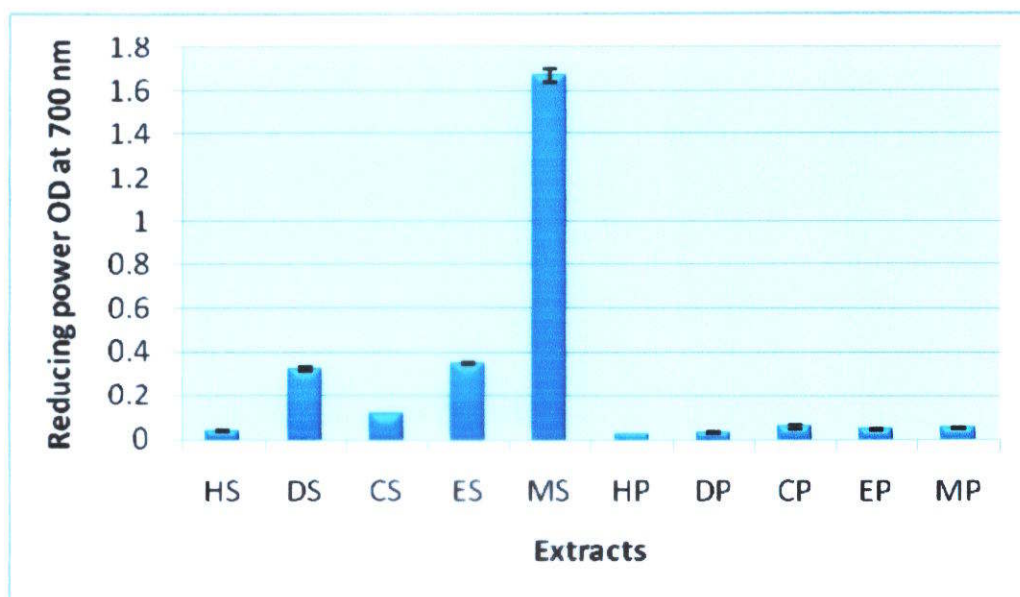


Fig 12: Reducing power of fractionated extracts of seed and pericarp of *S. apetala* at 100 µg/mL. The data are presented as mean $\pm$ SDs (bars), $n=3$.

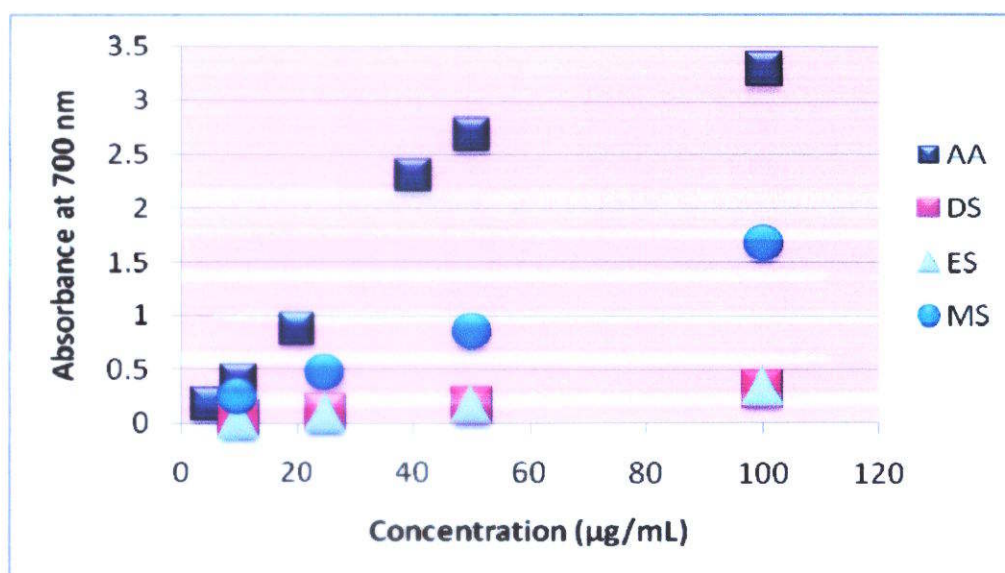


Fig 13: Dose-dependence of reducing power of DS, ES, MS and AA. AA indicates ascorbic acid and was used as positive control.

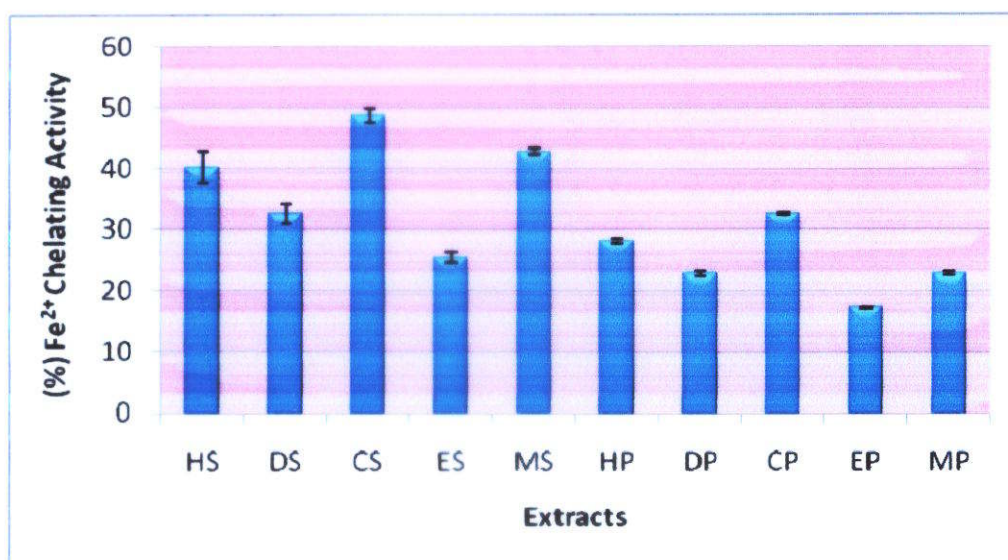


Fig 14: Ferrous (Fe²⁺) chelating activity of fractionated extracts of seed and pericarp of *S. apetala* at 200 µg/mL. Each data represents the mean ± SDs (bars), n=3.

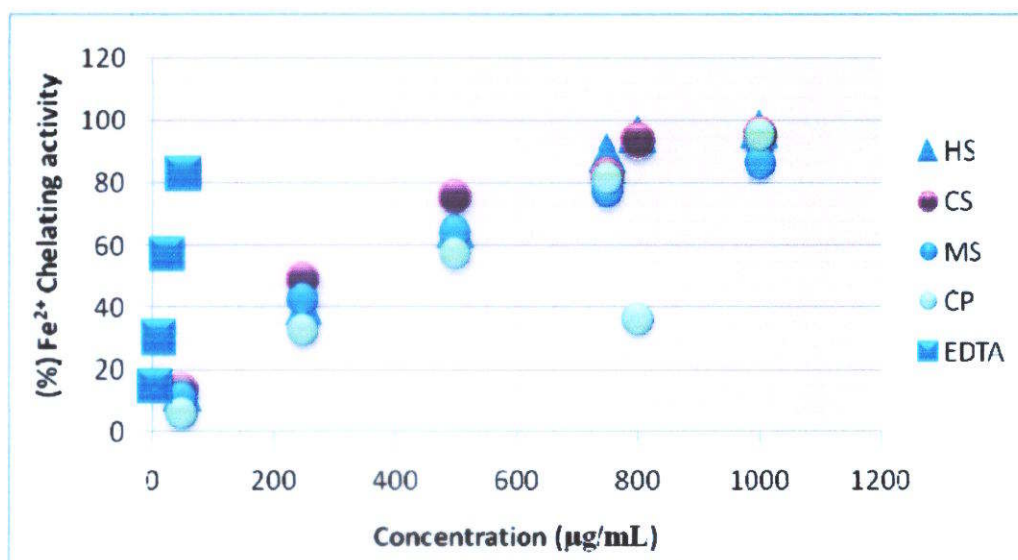


Fig 15: Dose-dependence of Fe²⁺ chelating activity of HS, CS, MS, CP and EDTA. EDTA served as positive control. Data are mean $\pm$ SDs (bars).

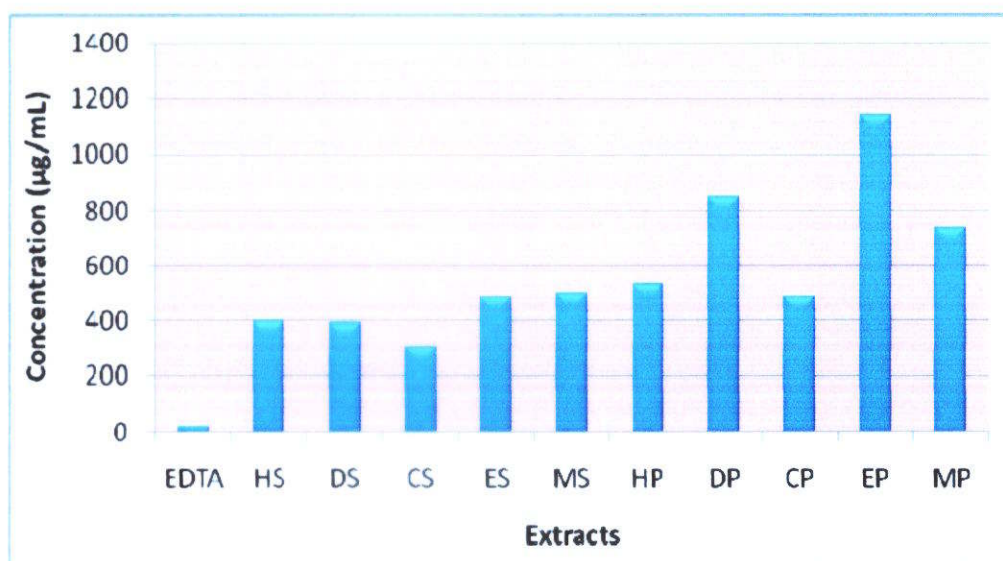


Fig 16: Comparison of IC₅₀ values of Fe²⁺ chelating activity.

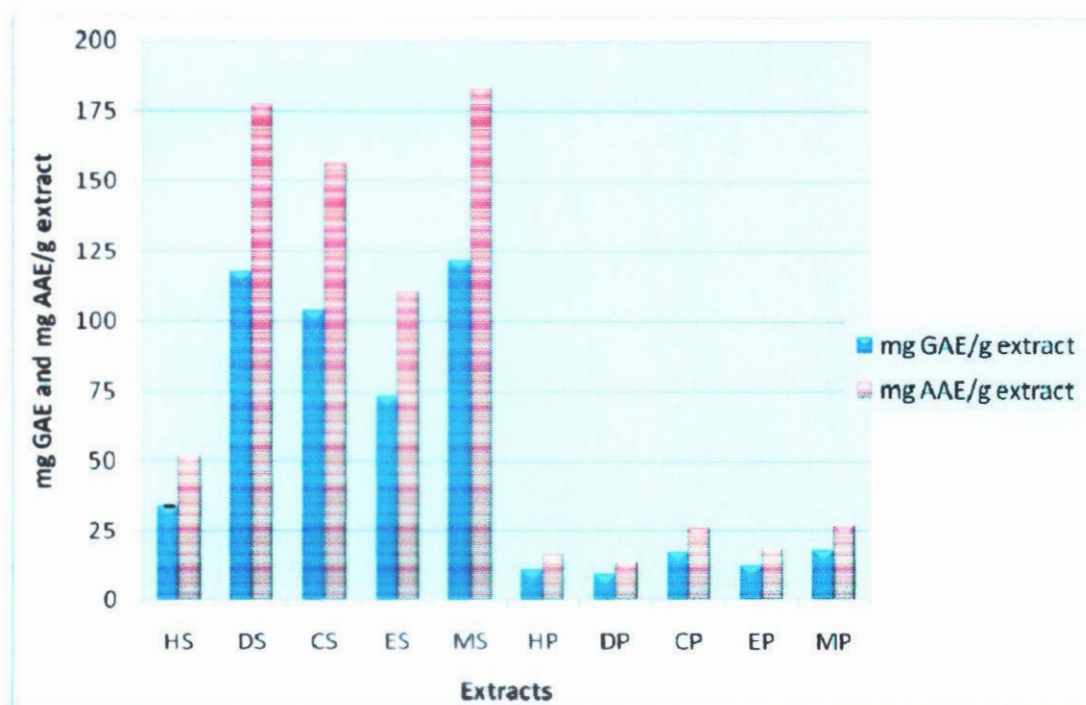


Fig 17: Total antioxidant capacity (mg/g extracts) of fractionated extracts of seed and pericarp of *S. apetala* respectively. (GAE: gallic acid equivalents; AAE: ascorbic acid equivalents). Each data represents the mean $\pm$ SDs (bars), $n=3$.

Table 5: Antibacterial activity of fractionated seed extracts of *S. apetala*

Bacterial Strains	Diameter of Zone of Inhibition (mm)						
	DMSO (20 µl/disc)	Kanamycin (30 µg/disc)	Different fractions of seed of <i>Sonneratia apetala</i> (400 µg/disc)				
			HS	DS	CS	ES	MS
<i>Escherichia coli</i>	-	15	6.5 ±0.0	8.75 ±0.35	8.5 ±0.0	-	13 ±0.0
<i>Salmonella paratyphi A</i>	-	23	-	-	-	-	14 ±0.71
<i>Salmonella typhi</i>	-	22	-	-	-	-	12.25 ±0.35
<i>Shigella dysenteriae</i>	-	22	-	-	-	-	12 ±0.0
<i>Staphylococcus aureus</i>	-	25	6.5 ±0.35	-	9.25 ±0.35	8.0 ±0.0	12 ±0.71
<i>Vibrio cholerae</i>	-	-	-	-	8.75 ±0.35	7.25 ±0.35	-

Numbers indicate the mean diameters of inhibition of triplicate experiments ± SD;
Control disc = 6 mm; “-” means no inhibition.

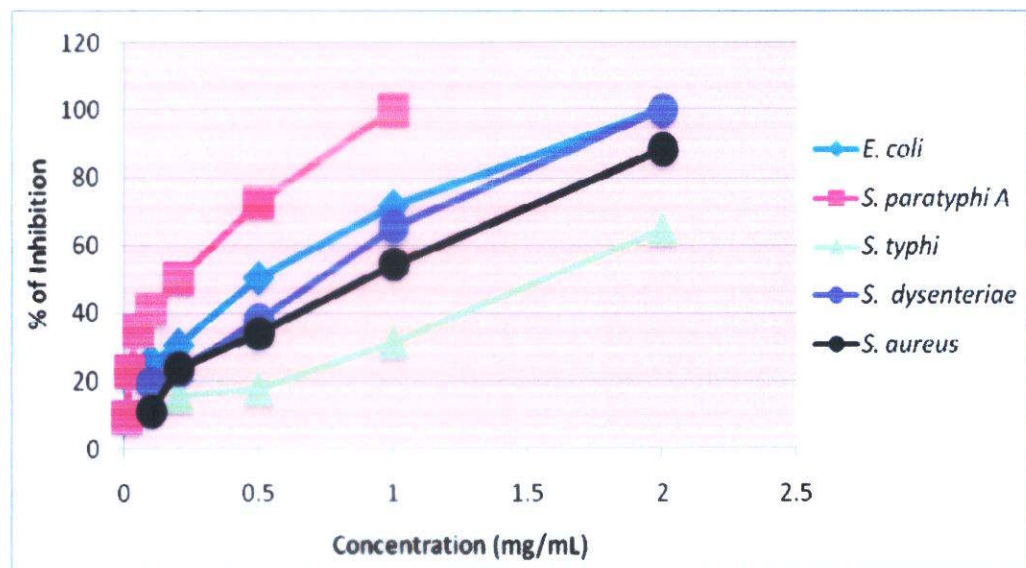


Fig 18: Dose-dependent inhibition (%) of the growth of selected bacterial species by MS.

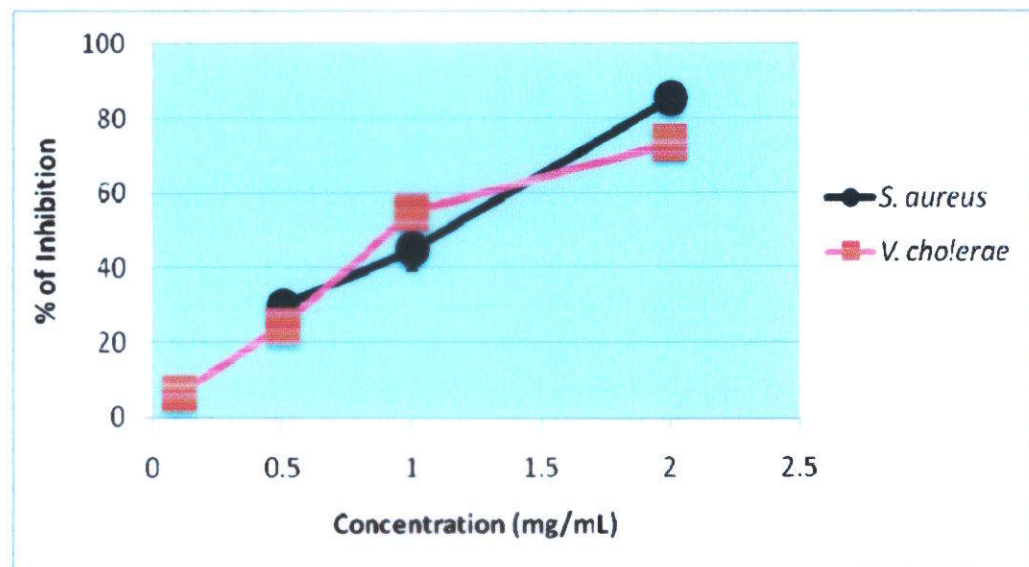


Fig 19: Dose-dependent inhibition (%) of the growth of selected bacterial species by CS.

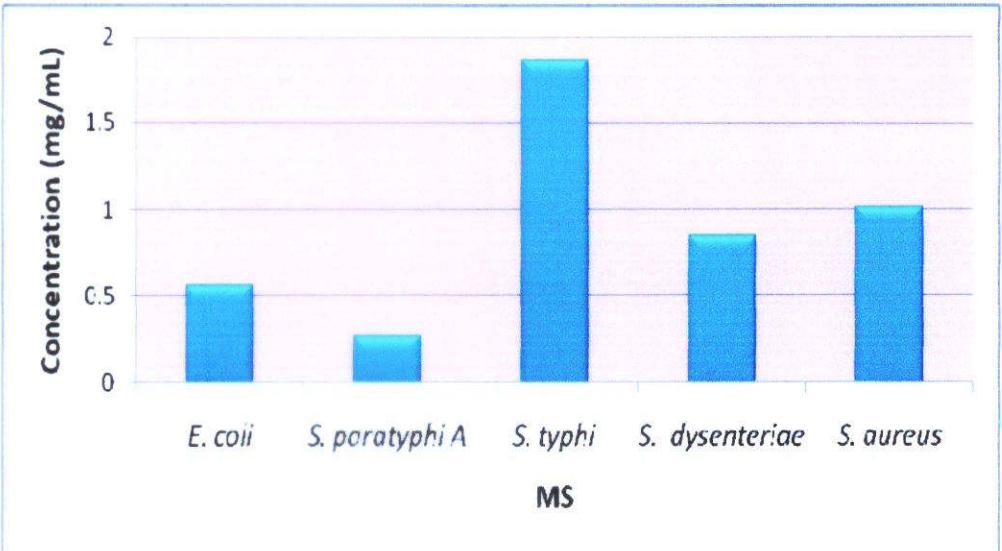


Fig 20: Comparison of IC_{50} values for MS against *E. coli*, *S. paratyphi A*, *S. typhi*, *S. dysenteriae* and *S. aureus*.

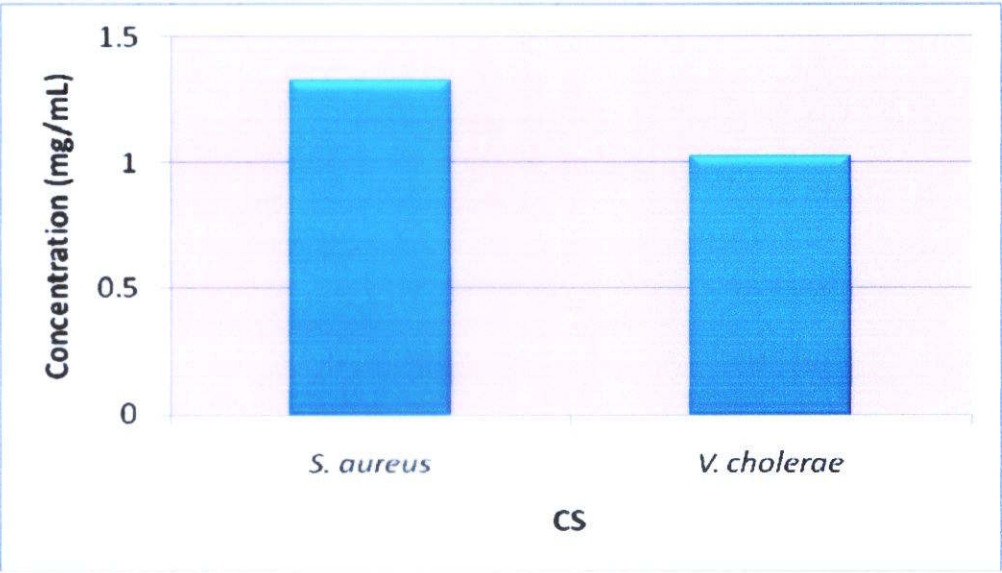


Fig 21: Comparison of IC_{50} values for CS against *S. aureus* and *V. cholerae*.

CHAPTER 6

DISCUSSION

produce toxic H_2O_2 *in vivo*. It has detrimental effect on cells for the production of hydroxyl radical ($OH^\bullet$), which oxidizes a number of molecules including DNA, RNA. Thus, removing H_2O_2 is crucial to protect cell components from oxidation (Hossain et al. 2009). From our experimental observation, it was revealed that methanol extract of *S. apetala* seed has very high reducing power, DPPH radical scavenging activity as well as NO scavenging activity. It's TAC (total anti-oxidant activity) was also high.

Nitric oxide (NO) is synthesized in many different mammalian cells types such as endothelial cells, vascular smooth muscular cells, neurons, platelets, macrophages and neutrophils. NO or some related reactive nitrogen species act as neurotransmitter, prevent platelet aggregation and they are one of the defense molecules of immune system against tumor cells, parasites and bacteria. However, large amounts of NO, peroxynitrite and other reactive nitrogen oxide species are considered to be potentially cytotoxic and capable of injuring the surrounding cells. This result shows that the extracts generated a dose-dependent inhibition of nitrite production. Similar reports were found for lotus seed extract (Yen et al. 2006) and leaves of *Pistacia atlantica* (Peksel et al. 2010). Nitric oxide is an essential bioregulatory molecule required for several physiological processes like neural signal transmission, immune response, control vaso-dilation and control of blood pressure (Palmer et al. 1987; Rees et al. 1989; Bredt and Snyder 1990; Gold et al. 1990) etc. However, the elevation of NO results in several pathological conditions, including cancer. NO is lipophilic in nature and can diffuse between cells very easily. NO is generated from the terminal guanido nitrogen atom of L-arginine by various NADPH-dependent enzymes called NO synthases (NOS), (Moncada et al. 1991). NO has an unpaired electron, hence is a free radical ($NO^\bullet$). NO becomes nitrosonium cation (NO^+) or nitroxyl anion (NO^-) by donating or accepting an electron, respectively (Nathan and Xie 1994). NOS is synthesized in a variety of cell types from multiple mammalian species and can produce consistent, high concentrations of NO upon induction with cytokines and or bacterial lipopolysaccharide (LPS) (Nathan and Xie 1994). The plant/plant products may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation *in vivo*. In the present study, methanolic fraction (MS) of *S. apetala* seed showed the highest NO scavenging activity followed by DS and therefore MS can be potent fraction to scavenge NO.

The transition metal ion, Fe^{2+} possess the ability to move single electrons by virtue of which it can allow the formation and propagation of many radical reactions, even starting with relatively non-reactive radicals (Aboul- Enein et al. 2003). The main strategy to avoid ROS generation that is associated with redox active metal catalysis involves chelating of the metal ions. HS, DS, CS, MS and CP displayed a remarkable capacity to bind iron, suggesting that they may protect against peroxidation. An affinity for ferrous ions minimizes the concentration of the catalyzing transition metal needed in a lipid peroxidation reaction (Hossain et al. 2009). Some extracts with low phenol contents shows good chelating activity but some extracts with high phenol contents shows good chelating activity. This lack of relationship is in agreement with other literature (Ghasemi et al. 2009; Heinonen et al. 1998; Assimopoulou et al. 2006; Nickavar et al. 2007). In this study, MS showed high phenolic content and high Fe^{2+} chelating activity and on the other hand, HS, DS, CS and CP showed high Fe^{2+} chelating activity but low phenolic content. It is known that only flavonoids with a certain structure and particularly hydroxyl position in the molecule can act as proton donating and show radical scavenging activity (Mensor et al. 2001; Hou et al. 2003). Furthermore, the extracts are very complex mixtures of many different compounds with distinct activities (Mensor et al. 2001; Hou et al. 2003). So the chelating activity of these extracts may be a function of these compounds and MS among the experimental extracts can be the most potential source for bioactive components for alleviating the deleterious effects of H_2O_2 , NO, HOCL, O_2^- , OH^- or other free radicals e.g. ROS, RNS by reducing it to H_2O . Owing to the complexity of the oxidation-anti-oxidation process, no single testing method is capable of providing a comprehensive view of the anti-oxidative profile of a sample (Parejo et al. 2002). Therefore, multi-method approach is necessary to assess anti-oxidative activity.

Extracts from different mangrove plants are reported to possess diverse medicinal properties (Bandaranayake 1998; Agoramoorthy 2007). For example, *A. illicifolius*, *A. marina* and *E. agallocha* showed significant analgesic activity (Kokpol 1984). Mangroves and mangrove associates possess novel agrochemical products, compounds of medicinal value, and biologically active compounds (Bandaranayake 2002). Extracts from different mangrove plants and mangrove associates are active against human and plant pathogens (Chandrasekaran 2009). Alkaloid, flavonoid, tannin and other phytochemicals are present in other plants as well (Barnabas 1988). Phytochemical screening showed that mangroves

contain these secondary metabolites which may show antibacterial activity. Therefore, the beneficial medicinal effects of plant materials may result from the combinations of secondary products present in the plant. Secondary products play a role in a plant's defense through cytotoxicity towards microbial pathogens and this could prove the usefulness of these secondary products as antimicrobial medicines for humans (Briskin 2000). MS showed antibacterial activity against *E. coli*, *S. paratyphi*, *S. typhi*, *S. dysenteriae* and *St. aureus* and CS showed activity against *St. aureus* and *V. cholerae*. Reportedly, phytochemical constituents such as polyphenols, flavonoids, anthocyanins, tannins, fatty acids etc. are bioactive and serve as defense molecules against various pathogens. Mixtures of active constituents showed a broad spectrum of biological and pharmacological activity (Robinson 1967; Coelho-de-Souza et al. 1998; Atindehou et al. 2002). Tannins (Ravikumar and Kathiresan 1993) form irreversible complexes with proline rich proteins, resulting in the inhibition of cell protein synthesis of bacteria (Scalbert 1991). Flavonoids are phenolic structure containing one carbonyl group complexes with extra cellular and soluble protein and with bacterial cell wall (Cowan 1999), thus exhibits antibacterial activity through these complexes. Generally the gram positive bacteria are believed to be more susceptible having only an outer peptidoglycan layer which is not an effective permeability barrier, whereas, the gram negative bacteria possesses an outer phospholipidic membrane carrying the structural lipopolysaccharide compound. This makes the cell wall impermeable to drug constituents because of the presence of multilayered peptidoglycan and phospholipidic bilayer. In spite of the barriers, the phytochemical constituents were effective in controlling the growth of pathogenic bacteria (Scherrer and Gerhardt 1971). Therefore, potential antibacterial activities of MS and CS are probably due to its high contents of these functional components. Most of the isolated compounds from mangrove plants belong to steroids, triterpenes, saponins, flavonoids, alkaloids, tannins and phenolics which have therapeutic properties (Bandaranayake 1998). Polysaccharide and lignin can serve as antiviral component (Sakagami et al. 1998). Lignin protected mice against pathogenic *Escherichia coli*, and this antibacterial activity was attributed to the antioxidant property of lignin (Sakagami et al. 1998). Fatty acids like lauric, palmitic, linolenic, linoleic, oleic, stearic and myristic acids are known to have potential antibacterial and antifungal agents (McGaw et al. 2002, Seidel and Tailor 2004). So, the antimicrobial activities of the fractions may be due to the components e.g., phenolics, steroids, triterpenes, saponins, flavonoids, alkaloids, fatty acids, polysaccharides, lignins etc.

Evidence is mounting for a role of dietary phytochemicals, including flavonoids, ascorbic acid, α -tocopherol, and carotenoids, in maintenance of health and protection from diseases. The epidemiological evidence for a protective effect of natural food and herb antioxidants against cancer, coronary heart disease, age-related macular degeneration, respiratory disease, cystic fibrosis, immune-disease, and Alzheimer's disease is impressive (Willett 1995), though numerous surveys have shown an inverse relationship between the intake of vegetables, fruits or other plants that have medicinal value with high polyphenols and the incidence of those disorders (Ing-chin chen et al. 2004).

CONCLUSION

CONCLUSION

Reportedly, with increasing the consumption/utilization of health promoting compound like polyphenol, flavonoids and anthocyanins, increased anti-oxidative activity *in vitro* or *in vivo* and thereby decreasing the intensity of cancer, ageing, neurological diseases, inflammation, allergic problem, diabetes and bacterial infections. Besides, a polyphenol may affect cell-to-cell signaling, receptor sensitivity, inflammatory enzyme activity or gene regulation (Virgili and Marino 2008). In this research work, fractionated extracts of seed and pericarp of *S. apetala* fruit were investigated to know the existence of beneficial functional bioactive components, antioxidant and antibacterial activities. Present study shows that *S. apetala* seed extract especially methanol fraction is potential source of polyphenols, antioxidants and antibacterial bioactive compounds and therefore have activities beneficial to human physiological health. However, further *in vivo* and *in vitro* studies are necessary to know their mechanism of action and potential active compound(s).

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APPENDIX 1

1. Arithmetic Mean ($\bar{X}$) = $\frac{\sum X}{n}$

Where, $\sum X$ = Summation of Observed Value

n = No. of Observation

2. Standard Deviation (SD) = $\sqrt{\frac{\sum (X - \bar{X})^2}{n}}$

Where, X = individual Value

$\bar{X}$ = Mean Value

n = No. of Observation

APPENDIX 2

Fractionation of Samples:

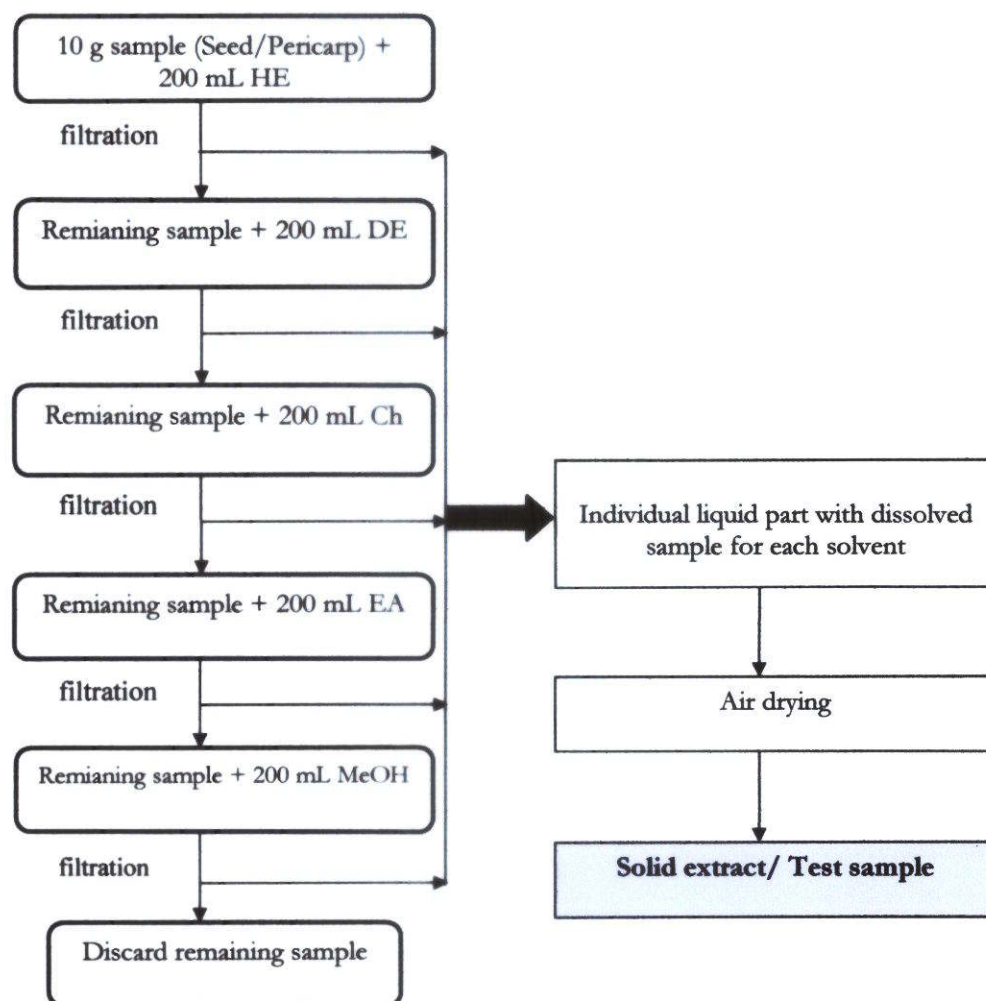


Fig 22: Schematic representation of the fractionation of samples with five different solvents (HE-Hexen, DE- Diethyl Ether, EA- Ethyl Acetate, Ch- Chloroform, MeOH- Methanol).

APPENDIX 3

Table 7: Dietary antioxidants: source, bioavailability, and concentrations in human plasma.

Dietary Antioxidants	Dietary source	Bioavailability	Concentration	Comment
Ascorbic acid (vitamin C)	Fruits and vegetables, particularly strawberries, citrus, kiwi, Brussels sprouts, and cauliflower	100% at low doses (<100 mg) decreasing to <15% at >10 g	25–80 mmol l ⁻¹	Unstable at neutral pH, concentrated in cells and the eye
Vitamin E (in humans mainly α -tocopherol)	Green leafy vegetables, e.g., spinach, nuts, seeds, especially wheat germ, vegetable oils, especially sunflower	10–95%, but limited hepatic uptake of absorbed tocopherol	15–40 mmol l ⁻¹ (depending on vitamin supply and lipid levels)	Major tocopherol in diet is γ form, but α form is preferentially taken up by human liver
Carotenoids (hundreds)	Orange/red fruits and vegetables (carrot, tomato, apricot, melon, yam), green leafy vegetables	Unclear, dose and form dependent, probably <15%	Very low (<1 mmol l ⁻¹)	Lutein and zeaxanthin are concentrated in macula region of the eye
Flavonoids (enormous range of different types)	Berries, apples, onions, tea, red wine, some herbs (parsley, thyme), citrus fruits, grapes, cherries.	Most poorly absorbed, quercetin absorption 20–50%, catechins <2%, dependent on form and dose	No data for most, likely <3 mmol l ⁻¹ in Total	Quercetin and catechins may be most relevant to humans health as intake is relatively high, there is some absorption, possible gastrointestinal-tract protection by unabsorbed flavonoids

Source: Encyclopedia of Human Nutrition, 2005.

Total phenolic *S. apetala* (Buch.-Ham.) fruit