Chapter 1: Introduction

1.1. Mangroves: An overview

Mangroves are assemblages of halophytic woody plants that inhibit tropic and sub tropic estuarine or brackish habitats. About 75% of the world's tropical coastline is covered with mangroves (Spalding et al., 1997). These estuarine environments are strongly dynamic in nature. Freshwater, from numerous channels and creeks, and tidal saline water alternatively washes these very special coastal wetlands. Being flashed by the saline water twice a day, the evergreen floral community occupying these environments is well adjusted to water stress, possesses mechanisms that allow water consumption versus salinity gradient, has broad and specified root systems (such as pneumatophores, to cope with the anaerobic muddy base and submerged conditions during the high tide), has marked tendency to vivipary and exhibits xerophytic adaptation techniques. Mangroves, as a matter of fact, are composed of a diverse collection of taxonomically uncorrelated plant species containing both shrubs as well as trees. However, despite of being taxonomically unrelated, these plants express resemblance in physical feature and structural adaption to their habitat as a result of confluent evolution (Yannew-Ewuise 1980).

Though all members of mangroves can grow well at minimum temperature average is 20˚C (Chapman V.J, 1976) each mangroves species may vary in their salinity tolerance, degree both in their salinity tolerance and the degree to which salinity may be necessary to maintain their growth and competitive dominance (Ellison A.M and Fransworth E.J, 2001). Due to their distribution along inter-tidal coastlines, mangroves are subjected to salt stress. To survive in such stressful habitat, mangroves possess salinity tolerant adaptive

mechanisms. Restriction of salt entrance into plants and salt secretion through special salt glands in leaves are common adaptive measures (Mizrachi et al., 1980; Tomlinson, 1986).

1.2. Mangrove in Malaysia:

Mangroves exist along 52 per cent of Malaysian coastline (about 60 per cent in Sabah and Sarawak and 40 per cent in Peninsular Malaysia). These swamp forests are the major type of coastal wetlands of the country covering over 637,164 ha that represent 2.5 per cent of the global resource of mangrove. Besides their ecological functions, mangroves are also economically beneficial to the country. Coastal wetlands, which are the huge source of commercial timber, play an important role in economically. The Matang Mangrove Forest Reserve that widely known as the best handled mangrove forest in the world has been sustainably managed for timber since the 1920s (WWAP, 2001).

With over sixty different species, Malaysian mangrove communities are extremely diverse and invaluable due to the hot and humid climate with high rainfall. Over half of Malaysia's mangrove forests are concentrated in Sabah while the rest are located around the central shores of Sarawak, mostly concentrated in the deltas of the Sarawak, Rajang and Trusan-Lawas. Smaller parcels inhabit the numerous lagoons and islands around Peninsula Malaysia (WWAP, 2001). Some areas show a relatively clear zonation pattern. Mangroves ascend out of the muck and mudflats of estuarine deltas. Mature stands usually comprise from 20 to 30 species of mangrove trees and are divided into three distinct vegetative zones where each area overlooked by specific key mangrove tree species (NE, 2008).

Avicennia-Sonneratia zone:

On the offshore edge, mangroves species inhume extensive root under the mud to endure the loosened the soil from the tidal flooding every day. *Sonneratia* fruits protruding projection for their regeneration and allow the fruits to drift on the current of water.

Bruguiera-Rhizophora zone:

Bruguiera-Rhizophora area is located on somewhat higher ground. This zone can tolerate only the flooding of high tide on more compacted soils. The area is discerned by the evenness of height and special root system which is eerie-looking. The seedlings of *Rhizophora mucronata* is elongated and hang like lithe cigars, which balanced to cliff into loose soil to begin a new life.

Back mangrove zone

In the back mangrove area the clay content is higher in the compressed soil. Mound-building crabs and lobsters arouse the ground level another meter or so, where a thickunderstorey emerges from clusters of large ferns. Beyond the tidal reach, nipa swamp forests prosper in brackish waters, where the predominant nipa palm, with its large feathery fronds, develops in contiguous thickets on riverbanks.

1.3. Environmental and economic importance of the mangroves:

Different kinds of productive and protective services have been provided by the mangrove wetland to coastal communities. A few major of them are listed below.

- An extremely important role of mangroves is that they serve as habitats for vast amount of filter-feeders that are fixed on the knot of roots such as barnacles, sponges, and shellfish. These filter feeders clean the water of nutrients and silt. As a result, clear water washes out into the sea, allowing the coral reef ecosystem to flourish (Field C., 1995).

- The mangrove wetlands are the nursery, feeding and breeding land to many economically important fish, prawns, crabs and mollusks.
- A huge amount of organic and inorganic nutrients are present at coastal water that enhances the potential of fishery.
- Mangroves are marine halophytic woody plants that serve as a nursing ground and a source of energy for detritus based coastal food chain, in addition to its uses in medicinal products, dyes, and tannins.
- In coastal areas, mangroves act as natural protection against the adverse impact of natural disasters such as storm surges, cyclones and Tsunami.
- Mangroves act as the final frontiers to protect the seashore against the adverse effect of rising sea (Srinivasa et al., 1998).
- They reduce coastal erosion as their specialized root systems help to stabilize the coastal soil. In addition, they play role in gaining land mass by accreting sea and adjacent coastal water bodies.
- They act as biological filters in polluted coastal areas.
- Mangroves have also some medicinal importance as well as commercial value except the ecological benefits. (Premanathan et al. 1996; Balu and Madhavan 1995).

1.4. Objectives of research:

In broader sense, the study aims to assess the bioactivity of *Sonneratia alba, Rhizophora mucronata and Bruguiera gymnorrhiza.* In view of this, the following specific objectives were undertaken:

- The study seeks to establish a protocol for seed germination of *S. alba*, considering the effects of hormone treatment on germination rate.

- Key objective is to assess the bioactivities (anti-oxidant activity and anti-microbial activities) of *S. alba* by following the bio-assay guideline. In addition to this, the study attempts to do the fraction of the active crude extract of *S. alba* into polar, semi-polar, and non-polar fraction, to assess the bioactivities, and to identify the numeric weight of active compound from most active fraction by using the liquid chromatography tandem mass spectrometry (LCMS/MS).
- Finally, to compare the antioxidant and antimicrobial activities of inbetween *in vitro* and *in vivo* explants of *S. alba* and with other two mangrove plants *Rhizophora mucronata* and *Bruguiera gymnorrhiza*.

Chapter 2: Literature review:

2.1. Medicinal plants:

"Let your food be your first medicine" (Hippocrates, 377 BC) was likely the beginning of the link among nutrition and welfare which emphasizes the importance of functional foods (Carbone 2005). In addition, the practice of medicinal plants is very well known for treating the diseases from ancient times. Even today because of the belief that medicinal plants are safe and effective most of the plant products are being used in local traditional systems of medicine (Dhawan 2003). In developing countries, a report of WHO survey indicates that about 80% of the populations depend on mostly conventional medication for their chief health care demands (Goyal, et al. 2007). Besides, scientific validations of medicinal plants have been ensured by various phytopharmacological studies which evaluate active plant constituents. So today, plants are the important raw materials for pharmacological research and drug developing (Mendonça 2006), and they are also being increasingly used as the complementary or alternative medicine in industrialized countries.

The practice of orthodox medicine is far-flung in China, Japan, India, Bangladesh, Thailand and Sri Lanka. The countries of the area such as China (30,000 species of higher plants), Indonesia (20,000), India (17,000), Myanmar (14,000), Malaysia (12,000) and Thailand (12,000) have large floras (Ics-Unido. 2006). In China, medicinal consumption mostly relies on traditional medicines. The legumes of the Fabaceae, the Caesalpiniaceae and the Mimosaceae use to make the herbal medicine in Thailand. The use of medicinal plants like Eupatorium perfoliatum in Central America medicinal plants have been widely used (Hoareau, et al. 1999).

2.1.1. Medicinal properties in plants:

Medicinal plants have been using as main reservoir of products used to maintain well being since nineteenth century, as Friedrich Wöhler of German chemist in 1828, trying to synthesize ammonium cyanate from silver cyanide and ammonium chloride, unexpectedly synthesized urea, which was the beginning of synthesis of organic compounds in history and revealed a new area of the synthetic compounds (Mendonça 2006). Step by step, those synthesized components replaced the uses of the plants as medicinal drugs. The breakthrough of antibiotic penicillin from *Penicillium notatum* mould then behaved as the marking point of the rediscovery of drugs that can derived from plant. Herbal remedies are back into prominence because of the ineffectiveness of conventional medicines such as antibiotics. Nowadays, plant derived commercial drugs at least 25%, such as atropine, vinblastine, quinine, aspirin, vincristine and morphine (Gilani and Atta-ur-Rahman, 2005). The history of modern psychopharmacology is short, and its current concepts are more "pharmaco-centric" than those of most other branches of modern medicine (Husain, et al. 2007). The recent story is that medicinal plants have involved isolating the active compounds, commencing with morphine isolation from opium. Drug finding from medicinal plants conducted to isolation of early drugs such as codeine, quinine, cocaine, and digitoxin, in addition to morphine, of which some are still in use (Balunas and Kinghorn 2005). In addition some synthetic medicines have been derived from medicinal herbs are silymarin, reserpine, digioxin, taxol, vincristine, ephedrine, vinblastine, artemisinin, aspirin, hypericin and quinine (Singh 2006).

Rediscovery of the connection between plants and health is responsible for launching a new generation of botanic therapeutics that admit plant-derived pharmaceutics, multi component botanical medicine, dietetic supplements, and operable foods. Many of these products will

soon accompaniment mainstream pharmaceuticals in the diagnosis, treatment and prevention of diseases, while concurrently contributing assess to agriculture (Raskin, et al. 2002).

Aspirin is the most consumed tablet, which is synthesized from *Salix alba*, consumed about 80 million only in US each year. Atropine, which is originated from *Atropa belladonna*, used to assuage pain and also treat paralysis that related to Parkinson's disease. Bark of *Chinchona pubescens* and *Chinchona officinalis* used to isolate Quinine to treat malaria. *Papaver somniferum* is used for Morphine isolation that is use as pain killer. Vincristine and vinblastine, which are developed from Catharanthus roseus, used to cure Hodgkin's disease, childhood leukemia, breast cancer and choriocarcinoma (Sumner et al., 2000).

2.1.2. Plants selection for medicinal properties discovery:

There are huge numbers of plant species detected on earth. However, amongst the figured 250,000-400,000 plant species, only about 6% have been examined for biological action, and approximately 15% have been studied phytochemically. This shows a need for phyto-pharmacological evaluation of herbal drugs (Goyal, et al. 2007). Some chemical substances of plants, which are generate a certain physiological activity on human body. These phytochemicals are the active constituents that exhibit some biolological activities concerning antioxidant, antimicrobial, antiinflammatory, and anticancer activities, ext. Exploration of the chemical constituents of the plants and pharmacological screening is of great importance which leads for development of novel agents (Goyal, et al. 2007).

Several screening approach paths can be chose and are contingent the mark diseases as well as on the usable information regarding the plants. Ethnobotanical backgrounds of plants are generally used to screen of specific compound by using the bioassay guideline (Atta-ur-

Rahman et al., 2001). The analysis of the application of plants, which is related with cultural practices and conventional beliefs, is called ethnobotany. The term admits the use of traditional medicinal plants that used to treat diseases (Heinrich et al., 2004). In Indian Ayurvedic practices, turmeric, scientific name is *Curcuma longa*, has been long known to have antimicrobial potential (Goel et al., 2008). Etnobotanical data revealed that curcumin has been screening from turmeric and have antimicrobial properties against *S. albus, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, B. subtilis* and *Bacillus cereus* (Jayaprakasha et al., 2005). The study of Biorational correlates the information from natural history with evolutional theory and ecological hypothesis (Scholes et al., 2005). Because of, plants are averted being pastured by herbivores much means that the plant comprises toxic. For instance is *Nerium oleander* with its oleandrin (a cardiac glycoside), which causes deadly if it is taken. Research carried on then found the utility of cardiac glycosides to treat cardiac arrhythmia and heart failure in human (Barbosa et al., 2008). The study of Chemotaxonomic of plants can also be carried since plant species among especial taxa are generally assumed to bear similar chemical properties (Bindseil et al., 2001). For examples, While Goren et al. (2002) examined biological action of the genus of Tanacetum. Braca et al. (2003) analyzed biological natural action of seven plants from Licania genus. Introductory knowledge on specific plant species got in literature that also can be applied as root of knowledge in breakthrough of plant derived medicines. Prior research carried on by Dat et al. (1992) involving the diuretic action of *Orthosiphon stamineus* has enlivened Olah et al. (2003) to isolate active compounds creditworthy for the diuretic action. The final method used in drug uncovering is by blind or random aggregation. This method is practicable when conducting with possible biodiversity in

uncontrived field. Farnsworth (1998) demonstrated that vinscristine and vinblastine discovered by method of random sampling.

2.2. Phenolic components in plants:

Among the various pytochemicals as the secondary metabolites of plants, phenolic compounds are the common ones and frequently present in the plant kingdom. Phenolic constituents exhibit several bioactivities such as antimicrobial, antioxidant, antiviral, antiinflammatory. Dietary phenolics that have being researched deeply in the last decades are divided into various subgroups and the major categories of phenolic compounds are flavonoids, phenolic acids, and tannins (King and Young 1999). Some of the other types of phenolics are stilbenes, lignans, coumarins and quinones (Chai, et al. 2002).

Flavonoids:

Flavonoids are the most important and most studied phenolic phytochemicals that are widely distributed in plants (Chai, et al. 2002). More than 6,400 flavonoid structures were determined in the performed studies (Silva, et al. 2006). Generally they include particular hydroxyl groups with the constitution of ring structures. They have a basic carbon skeleton (C6 + C3 + C6). Flavonoids are consist of several subclasses such as; flavones, flavonols, flavanones, flavanonols, chalcones, isoflavonoids, anthocyanins, biflavonoids (Chai, et al. 2002). Flavonoids are basically divided into two groups; anthocyanins and anthoxanthins. Anthocyanins have some colour pigments such as red, blue, and purple. Anthoxanthins possess colorless or yellow to white molecules (flavonols, flavones, isoflavones) (King and Young 1999).

Falvones, flanonols, flavanols (cathechins), anthocyanidins, isoflavonoids, and flavanones have been classified on the basis of generic structure of heterocycle C ring. Flavonols

(myricetin, kaempferol and quercetin), flavones (apigenin and luteolin), flavanols (epicatechin, epigallocatechin, epigallocatechin gallate, catechin, and epicatechin gallate), isoplavonoids (genistein), flavanones (naringenin) and anthocyanidins are common flavonoids in the diet (Liu 2004).

Flavonoids generally exist as glycosides, nevertheless some of them are found as aglycones. There is an insufficient knowledge about metabolism, extraction and absorption of dietary polyphenols in humans and recovery in the gastrointestinal surface. Furthermore, the hydrolysis of flavonoid glycosides and the reductive metabolism are performed by intestinal microorganisms (Rice-Evans, et al. 1997).

Phenolic acids:

Phenolic acids form another large class of phenolic compounds. Phenolic acids contain two main groups;

1. Hydroxybenzoic acids (e.g. vanillic acids, protocatechuic acids, p-hydroxybenzoic acid, gallic acid)
2. Hydroxycinnamic acids (e.g. caffeic acid, ferulic acid, cinnamic acids, coumaric acid, chlorogenic acids)

Tannins:

Phenolic polymers, commonly known as tannins and they are divided into two general classes:

1. Hydrolyzable tannins: They admit a central essence of polyhydric alcohol, for example hydroxyl and glucose groups. They are esterified partly or completely by hexahydroxy-diphenic acid or (ellagitannins)gallic acid (gallotannins).

2. Condensed tannins: They are more common and have more complex structures then the hydrolyzable tannins. They consist of oligomers and polymers of catechins.

In some cases hydrolyzable and condensed tannins are present together in plants, so this kind of tannins can be defined as complex tannins (Chai, et al. 2002). Polyphenolic phytochemicals are omnipresent in plants that they affair in several protective characters. An advocated human diet comprise significant amount of polyphenolics, as they have long been accepted to be antioxidants that scavenge exravagant, prejudicial, free radicals arising from normal metabolic processes (Stevenson, et al. 2007). Structural diversity of polyphenolics is a various class of plant secondary metabolites. Secondary metabolites have been characterized based on the presence of one or more six-carbon rings and two or more phenolic (associated with aromatic ring) hydroxyl groups. To be precise, mono-phenols for example p-coumaric acid are not polyphenolics, but they contribute lots of their characteristics and properties and are nearly usefully conceived as functional polyphenolics (Stevenson, et al. 2007).

2.3. Mangroves:

2.3.1. Mangroves as unconcerned genera:

Over the last two decades, mangroves have been studied extensively by marine scientists, ecologists and botanists (Lacerda, 2002; Kathiresan and Bingham, 2001; Tomlinson, 1986; Saenger et al., 1983; Chapman, 1976a; Macnae, 1968). Significant research attention was contributed to bear on the human fundamental interactions with these unparalleled forested wetlands was not until 1980s and early 1990s (Cormier-Salem, 1999; Hamilton et al., 1989). Earline's study was generally descriptive, recording the condition and uses of mangroves by coastal communities e.g., (Lacerda, 1993; Diop, 1993; Field and Dartnall, 1987; Kunstadter et al., 1986; Christensen, 1982; Taylor, 1982; Walsh, 1977). Most of the

studies on mangroves are limited in tissue culture technique due to contamination. Explants frequently turn brown or black and eventually die shortly after in vitro culturing (Kathiresan and Bingham, 2001). Mangrove species that have been successfully micropropagated are limited to *Sesuvium portulacastrum* (Kathiresan and Ravikumar, 1997), *Excoecaria agallocha* (Rao et al., 1998), *Avecennia officinalis* and *Acanthus ilicifolius* (Eganathen and Rao, 2001). Callus induction has been achieved from mangrove but the callus lacked the capacity to redifferentiate into adventitious buds or plantlets (Mimura et al., 1997; Kathiresan and Bingham, 2001).

2.3.2. Potentials of mangroves:

Mangrove forests not only play an essential role as the source of food for marine organisms (Nagelkerken and Velde, 2004) but these are a good source of food for human based on their nutrient potentiality (Kirui et al., 2006; Carvalho et al., 2007). Several mangrove plants are consumed as the medicinal plants in traditional medication for many years (Bandaranayake, 2002). Some recent studies confirmed the medicinal properties of some of mangrove plants which were consumed in folkloric medicine. For example, 3′, 4′, 5, 7-tetrahydroxyflavone isolated from *Sonneratia caseolaris*, a Bangladeshi mangrove plant, showed a significant exhibition activity against SMMC-7721 human hepatoma cells in an in vitro cytotoxic assay carried out by Minqing et al., (2009). Promising antibacterial activity of *Avicennia marina* mature leaves extract (Abeysinghe and Wanigatunge, 2006), methanol extract of *Excoecaria agallocha* leaves and shoots (Chandrasekaran et al., 2009) and antifungal activity of methanol extract of *Exoecaria agallocha* and *Bruguiera gymnorrhiza* trunks (Kazuhiko 2002) are some other examples of pharmaceutical potential of mangrove plants.

2.3.3. Medicinal potentials of mangroves:

Several mangrove plants are consumed as medicinal plants in traditional medicine for many years (Bandaranayake, 2002). Some recent studies confirmed the medicinal properties of some of mangrove plants which were consumed in folkloric medicine. For example, 3′,4′,5,7-tetrahydroxyflavone isolated from *Sonneratia caseolaris*, a Bangladeshi mangrove plant, that showed a significant exhibition activity against SMMC-7721 human hepatoma cells in an in vitro cytotoxic assay carried out by Minqing et al., (2009). Promising antibacterial activity of ethyl acetate extract of *Avicennia marina* mature leaves (Abeysinghe and Wanigatunge, 2006), methanol extract of *Excoecaria agallocha* leaves and shoots (Chandrasekaran et al., 2009) and antifungal activity of methanol extract of *Exoecaria agallocha* and *Bruguiera gymnorrhiza* trunks (Kazuhiko 2001) are some other examples of pharmaceutical potential of mangrove plants.

Rhizophora mucronata is one of the widespread mangrove plants. Reports show *R. mucronata* was traditionally used as both wood source and medicinal plant. The antibacterial (Lim et al., 2006; Ravikumar et al., 2010), antifungal (Mehdi et al., 2000), antiviral (Premanathan et al., 1999), insecticidal (Kabaru and Gichia, 2001), antidiarrhea (Das et al., 2009), antidiabetic (Alarcon-Aguilara et al., 1998), antiseptic (Fernandez et al., 2002) and antioxidant (Banerjee et al., 2008; Rahim et al., 2008) activities of R. mucronata have been reported.

Based on review of literature, bioactivity of different varieties/cultivars of a plant might show different values. Henríquez et al. (2009) reported the different antioxidant activities of five Chile apple cultivars. Different parts of a plant might also show different bioactivity potentials. For example, dissimilar antioxidant values from different parts of *Andrographis paniculata* were reported by Rafat et al. (2010a). The method of plant extraction can also

affect bioactivity potential. For example, Muthu et al. (2010) showed methanol extract of *Borreria hispida* had higher antibacterial activity than both ethyl acetate and petroleum ether extracts. The references also suggest the application of combination of different assays to indicate a specific bioactivity. As an example, the antioxidant capacity of *Oenanthe javanica* was higher than *Euodia redlevi* using DPPH free radical scavenging assay while the results of superoxide dismutase activity assay showed the higher antioxidant potential of *E. redlevi* compare to *O. javanica* from the same plant extracts (Rafat et al., 2010b).

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2.4. Taxonomy of mangroves:

The following table that is modified from Tomlinson (1986), give the species number of mangroves in plant family and genus.

Table 1.1: Taxonomy of mangrove

Common Name	Family	Genus, Number of Species
Black mangrove	Acanthaceae, vicenniaceae or Verbenaceae	*Avicennia*
Buttonwood, White Mangrove	Combretaceae	*Conocarpus*, *Laguncularia*, *Lumnitzera*
Mangrove palm	Arecaceae	*Nypa*
Red mangrove	Rhizophoraceae	*Bruguiera*, *Ceriops*, *Kandelia*, *Rhizophora*
Mangrove apple	Lythraceae	*Sonneratia*

2.5. *Sonneratia alba*:

2.5.1. Morphology of *S. alba*:

Sonneratia is a genus of the family Lythraceae. In Malay the *Sonneratia* is called 'berembang'. The others name of the *Sonneratia* are 'mangrove apple' in English, 'Mangrovenapfel', 'Pagatpat' in the Philippines, 'Perepat' in Singapore and 'Holzapfelmangrove' in German. *Sonneratia* trees are distributed in all tropical shores of the Eastern hemisphere from East Africa through Indo-Malaya to tropical Australia and into Micronesia and Melanesia, typically grown on the seaward fringe. The flowers of *Sonneratia* open at dusk emitting of sour milk or butter and last only one night. Flowers are pollinated by bats or hawk moths or honeybirds which drink the nectar in the calyx-cup.

The fruits are a large leathery berry seated on a star-like calyx with the seeds embedded in the pulp. Twenty species of *Sonneratia* genus has been identified. *S. alba, S. ovata, S. apetala* and *S. caseolaris* are the available *Sonneratia* species in Malaysia. According to Tomlinson, a species of *Eonycterus spelaea* is responsible for pollination of durian flower which get nectar from wide variety of mangroves and *Sonneratia* species especially *S. alba*.

The trees of *Sonnratia alba* are 3-15 miter tall, spreading, with broad, rather lax crown. The leaves are elliptic to ovate or obovate, 5-11 × 4-8cm, opposite, base rounded, apex broad, bounded, blade dull light green, broadly drop-shaped. Numerous white stamens and tiny, white petals are present in flowers. Flower tube shiny, 3-3.5cm at anthesis, smooth, often 6 ribbed. Fruits are pear-shaped, crowned by the persistent sepals whose tips bend back to the stalk. Sepals adaxially strong tinged red, 1.3-2cm, recurved in fruits. The fruits are globous, 2-4cm in diameter. Flowers are common in October to November. Chromosome no. of *Sonneratia alba* 2n = 22, 24. Seeds are white, flattened, buoyant and tiny. Special types of root called pneumatophores 30-100cm long, thick, blunts.

2.5.2. Traditional uses of *S. alba*:

Leaves are eaten raw or cooked, in Eastern Africa used a camel fodder. The ripe fruits are eaten and are said to taste like cheese by the people from Africa to the Malay and Javanese. *Sonneratia* is also used for firewood. The heavy timber is tolerant to shipworm and pests and is used for piling, building boats and posts for houses and bridges. The pneumatophores are used for fishing nets. The special type of harvest called coppicing is possible with *Sonneratia* pneumatophores. Along with *Avecennia* and *Rhizophora*, *Sonneratia* is used in replanting mangroves to protect castlines.

Sonneratia alba is also used as medicinal plant by the endogenous people. Different parts of the plant are used for different purpose by the local people in different country. Poultices used for cuts and bruises in Burma, it's also used for sprains and swellings. In Malaysia, ripe fruits are used to expel intestinal parasites and half-ripe fruits for cough.

2.6. *Rhizophora mucronata*:

2.6.1. Morphology of *R. mucronata*:

Rhizophora mucronata is a species of mangrove plants distributed in the coasts of the Indian Ocean, South and East Africa to Madagascar, Seychelles, Mauritius, Southeastern Africa to Southern China, Ryukyu throughout Malaysia to Northeastern Australia; Melanesia, Micronesia belongs to the family of Rhizophoraceae. The name comes from Greek word "rhiza" means root and "phora" means to bear, refers to the rooting system. Authority of the name of *Rhizhophora mucronata* goes to Poiret. The common names of Rhizophora mucronata in different countries are lengayong, pyoo, doeum prasak, bakbuan, bakau bakau hitam, bakau jangkar, mangoro, belukap, phangka and duoc bop in Brunei, Burmese, Cambodia, Filipino, Indonesia, Malaysia, Papua New Guinea, Singapore, Thailand and Vietnam respectively. The synonyms of the *Rhizophora mucronata* are *Rhizophora macrorrhiza* Griffith, *Rhizophora latifolia* Miq, *Rhizophora mucronata* var. typica A. Schimper.

The evergreen tree grows up to 25- 30m height and 70cm in diameter. The trees usually occur in a large zone, adjacent to the water. The heights of the trees gradually decrease towards landwards. Leaves are elliptical to oblong, opposite, 8-15cm long, 5-10cm wide, acute, entire, without visible veins, thick and leathery, glabrous, black-dotted beneath, yellowish. Presence of a long mucron at the end of the leaves up to 0.8cm, but usually broken off. Inter-petiolar stipules up to 100mm long, reddish, falling early. Leaves can be

identified on the forest floor by the presence of cork warts on the outside of the leaves which sometimes visible only in older or dried materials. Leaves of *Rhizophora mucronata* are a favorite of many crabs (Gillikin et al. 2004). *Rhizophora* is generally wind-pollinated. Flowers are self-compatible, bisexual and therefore may be able to self-pollinate (Hou D. 1992). Flowers occurs as axillary inflorescences and open within the leafy crown hanging downwards from a long peduncle, 2-3 times forked, with 3-8 flowers ca 15mm long. Flower opens with 4 white and hairy petals altemated with the calyx lobes. Flowers are bell-shaped hypanthium 4 pale yellow, pointed leathery sepals and 4 cream-colored petals 9mm long. Stamens are eight; stalk less, anther 6-8mm long, 4 opposite sepals and 4 opposite petals. Ovary is half-inferior, conical, 2-celled, with 2 ovules in each cell, 2-lobed style and berry ovoid or conical, 5-7cm long, brown, leathery. The seeds are all viviparous and the hypocotyl is very long. The fruits develop within persistent calyx, the sepals folding back, hypocotyl warty and green in color. The bark is almost smooth, grey, with vertical fissures. The taproot generally abortive; lateral roots are numerous, developed from base of the trunk, much branched, usually called stilt roots, hoop or pile like, supporting the tree; hanging air roots are sometimes also produced from the lower branches; stem in closed forest cylindrical or developing a straggling or semi-prostrate habit especially in unfavorable conditions (Hou D, 1992).

2.6.2. Traditional uses of *R. mucronata*:

Different parts of *Rhizophora mucronata* are used for different purposes. The wood of *Rhizophora mucronata* is used for fish traps, house frames, construction, piling and poles. Fruits can be eaten after scraping off the outside and boiling with wood ashes (Brukill, 1966). According to The Wealth of India, the fruits are sweet and edible, the juice made into a light wine. Young shoots are cooked and eaten as a vegetable and mangrove extract

is used for maintaining oil-well drilling muds within desired range of flow (C.S.I.R., 1948-1976). Bark used for tannin and dye, may be removed from stems for sale as firewood. Leaves are the source of black or chestnut dye (Burkill, 1966).

Leaves and roots also used by the local people as folk medicine in different countries. Asiatic mangrove is a folk remedy for angina, diabetes, diarrhea, dysentery, hematuria and hemorrhage (Duke and Wain, 1981). Leaves are pouticed onto armored fish injuries (Watt and Breyer-Brandwijk, 1962). Indochinese uses the roots for angina and hemorrhage. Malaysian use old leaves and/or roots for childbirth. Burmese use the bark for bloody urine, Chinese and Japanese for diarrhea, Indochinese for angina (Perry, 1980).

2.7. *Bruguiera gymnorrhiza*:

2.7.1. Morphology of *B. gymnorrhiza*:

The Malay name of *Bruguiera gymnorrhiza* is 'Tumu'. *Bruguiera gymnorrhiza* is species which is called 'black mangrove' belongs to the family Rhizophoraceae common in the middle and upper intertidal zone, rather than in the lower intertidal zone or along the seaward edge of mangrove stands. *B. gymnorrhiza* has the largest natural longitudinal range of all mangrove species. It is widely distributed trees in tropic, and occurs along the eastern coast of Africa from just north of East London (Eastern Cape) through Asia to the Ryukyu Islands of Southern Japan, into Micronesia and Polynesia (Samoa), and Southward to subtropical Australia (Queensland, New South Wales and Western Australia) and numerous tiny (and large) islands in the Pacific. The common names of *B. gymnorrhiza* are denges in Palau, jon in Marshall Islands, large-leafed mangrove in English, ong in Chunk, orange mangrove in northern Australia, sobmw in Pohnpei, sraol in Kosrae, and yangach in Yap.

Black mangrove is normally a single-stemmed tree with short buttresses and characteristic horizontal roots that occasionally form above-ground loops, presumably as an aid to gas exchange for the subsurface portions of the roots called 'knee roots'. It is a medium to tall tree range between 15 to 35m long. Leaves are elliptical, opposite, simple, dark green and coriaceous (leathery), aggregated at the tips of apical shoots in clusters of about 12 leaves, 8-22com long, and 5-8cm wide. *B. gymnorrhiza* trees can be distinguished from other *Rhizophora* species by the color, size and shape of leaves. Inflorescence has solitary flower buds, positioned at the first or rarely second node below the apical shoot, located in leaf axils. In the Southern hemisphere the flowering time is chiefly April to August, while in northern hemisphere from October to February. Special types of fruits, is called 'viviparous', which germinate on the parent plant produces by the large-leafed mangrove. Mature hypocotyl which is dispersal unit of seedlings with attached calyx bodies are located at the third to fifth nodes below the apical shot. Extended calyxes mostly remain attached with mature propagules when fall from parent trees. Fruiting started when the mature hypocotyls fall. Seed of the *B. gymnorrhiza* is viviparous, meaning that the species produces seeds hidden in the mature calyx (post-flowering). One hypocotyl is produced from each mature calyx, however on rare case twins may be observed. Mature trees have distinctive sturdy, above ground knee roots surrounding the stem base which anchor only shallowly in the sediments, to 1-2m depth.

2.7.2. Traditional uses of *B. gymnorrhiza*:

Propagules of large-leafed mangrove use as a source of food in India, Bangladesh and others parts of Southeast Asia. Peeled propagules and leaves have reportedly been boiled, soaked, and eaten as a staple in Papua New Guinea, though most probably only in times of severe shortage of other foods. Fruits are sold as a vegetable at the Honiara Market

[Solomon Islands] (Clark and Thaman, 1993). Seedlings are made into sweetmeat; they are sliced, soaked to leach out the tannins and then ground into paste. The seedlings also used to produce a dye that does not bleed in water (Peter K L Ng et. al., 1999). In Southeast Asia, the tree is one of the conventional dyes used in batik-making which develop an orange-red color. In the Marshall Islands, the bark is used to make rope for fishing nets. The bark may be used to flavor fish. The wood is widely used for structural constituents (e.g., beams, poles, and rafters) of traditional homes and other structures. It is also used for other purposes, ranging from traditional uses such as fishing stakes, spears, and coprahuskers to use as a source of chips for pulp production. The species has also been used for transmission and telephone poles in some regions and is likely durable in direct contact with the ground. *B. gymnorrhiza* is commercially planted in Indonesia, Sabah and Sarawak in Malaysia to produce wood chips that are turned into paper pulp or to produce rayon fabric. Leaves, barks and fruits are used as traditional medicine for different purposes by the endogenous people. The bark used to treat burns in the Solomon Islands, malaria in Cambodia, cure fish poisoning in Marshall Islands, treat diarrhea and fever in Indonesia. The fruits are used to treat eye problems, and scrapped skin of the fruit to stop bleeding. The fruits may also be chewed as a betel nut substitute. The leaves are used to control blood pressure in India (Peter K L Ng et. al., 1999).

2.8. Tissue culture and plant growth regulators:

2.8.1. Tissue culture in plant development:

While the basic tissue culture can be associated with the theory of cell has been submitted by Schleiden and Schwann in 1838. They think that each cell is an independent living unit and when the cells differentiate in an organism multicell still contain information that exists in the single cell of the first egg. According to this theory, cells have the ability to develop

into a perfect individual. This theory is known as Totipotency Concept. The German's botanist G. Haberlandt has been trying to culture the cells from *Lamium purpureum* and *Eichornia mollissima* leaves. He uses the Knop salt solution containing sucrose. Those cells can live for one month, however it failed to divide. According to White (1951), there are two problems that prevent the development of methods appropriate to the Culture of plants between the years 1902 to 1934: (a) problems selecting the appropriate plant material and (b) absence of a good nutrient medium formulation.

Three important discoveries in 1930 to 1940 gave a large contribution to the development of tissue culture: auxin as regulators for growth, identification of the importance of vitamin B-complex vitamins and growth, and identification of coconut water for tissue culture. In 1934, Gautheret has cultured three species of *Salix caprea* and *Populas nigra* in Knop solution containing glucose, vitamins B and indole-3-acetic acid (IAA). This formulation has been accelerating the growth of Salix's cambium tissue. In 1941, Van Overbeek, Conklin and Blakeslee have proven that coconut water can provide factors that promote the growth of Datura embryos.

2.8.2. Growth regulators in plant development:

Plant growth regulators can play an important role on plant physiology. Low concentration of plant growth regulators effect the physiological process of plants and usually the growth regulators determined as organic components, other than nutrients(Glemas, 1982). Bio-regulators also can be influential of exogenous application. The essence of a bio-regulator depends on the application rate, the time of treatment and the growth stage of the plant at the time of application (Dörffling, 1982). Cytokinins are significant in all phases of plant development, from cell division and cell enlargement up to formulation of flowers, fruit set and dry matter formation of plants (Mayeux, 1985; Cothren, 1994). Cytokinins may not

always be active unless other hormones are present (Hedin and McCarty, 1994b). However, cytokinins alone can often evoke a variety of physiological, metabolic, biochemical, and developmental processes when applied to plants (Elliott, 1982; Taiz and Zeiger, 1991). Since most plant growth and development processes are regulated by natural plant hormones, many of these processes may be manipulated either by altering the plant hormone level or enhancing the capacity of the plant to its natural hormones (Sawan et. al., 2000). Some studies have been done on seed germination of salt tolerance of *Acanthus ilicifolious*, *Aegiceras corniculatum* and *Avicennia marina* (Yong Ye. et al, 2005), poly ethylene glycol effect on *Avicennia schaueriana* and *Laguncularia racemosa* (Viviane F. Cavalcanti et al, 2007), lubricating oil effect on *Bruguiera gymnorrhiza* (Zhang et al, 2007).

2.8.3. Tissue culture in mangrove plants:

Mangroves are limited in tissue culture technique due to contamination. Explants frequently turn brown or black and eventually die shortly after in vitro culturing (Kathiresan and Bingham, 2001). Mangrove species that have been successfully micropropagated are limited to *Sesuvium portulacastrum* (Kathiresan and Ravikumar, 1997), *Excoecaria agallocha* (Rao et al., 1998), *Avecennia officinalis* and *Acanthus ilicifolius* (Eganathen and Rao, 2001). Callus induction has been achieved from mangrove but the callus lacked the capacity to redifferentiate into adventitious buds or plantlets (Mimura et al., 1997; Kathiresan and Bingham, 2001).

Based on review of literature, bioactivity of different varieties/cultivars of a plant might show different values. Henríquez et al. (2009) reported the different antioxidant activities of five Chile apple cultivars. Different parts of a plant might also show different bioactivity potentials. For example, dissimilar antioxidant values from different parts of *Andrographis*

paniculata reported by Rafat et al. (2010a). The method of plant extraction can also effect on any bioactivity potential. For example, Muthu et al. (2010) showed methanol extract of *Borreria hispida* has higher antibacterial activity than both ethyl acetate and petroleum ether extracts. The references also suggest the application of combination of different assays to indicate a specific bioactivity. As an example, the antioxidant capacity of *Oenanthe javanica* was higher than Euodia redlevi using DPPH free radical scavenging assay while the results of superoxide dismutase activity assay showed the higher antioxidant potential of *E. redlevi* compare to *O. javanica* from the same plant extracts (Rafat et al., 2010b).

2.9. Oxidative and oxidative stress:

Oxidant can be defined as either a chemical compound that readily transfers oxygen atoms or a substance that gains electrons in a redox chemical reaction. The oxidation number of a molecules change when an oxidant acts on a substrate. The production of reactive oxygen species is the destructive aspect of oxidative stress, which also includes free radicals and peroxides. Most of these oxygen-derived species are produced at low level by normal aerobic metabolism and the damage they cause to cells is constantly repaired. Superoxide anion (O_2-), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), organic hydroperoxide (ROOH), alkoxy radicals (RO·), peroxy radicals (ROO·), hypochlorous acid (HOCl) and peroxynitrine (ONOO-) are the examples of reactive oxygen species (Sies, 1985; Docampo, 1995; Rice-Evans et al., 1995). In humans, reactive oxygen can be produced by the leakage of activated oxygen form mitochondria during oxidative phosphorylation. Under normal conditions the multiple redox-active flavoproteins all contribute a small portion to the overall production of oxidants (Messner et al., 2002; Imlay, 2003). Xanthine oxidase, cytochromes P450 and NADPH oxidases are also capable of producing oxidants. Hydrogen

peroxide is most harmful oxidant which is produced by wide variety of enzymes. Reactive oxygen plays important role in redox signaling which is one kind of cell signaling. Reactive oxygen species injured the tissue and resulting the irradiation and hyperoxia. Scientist believes that it is responsible for Parkinson's disease, neurodegenerative disease, Alzheimer's disease, Lou Gehrig's disease and Huntington's disease. Oxidative stress is also thought to be responsible for cardiovascular disease, ischemic cascade due to oxygen reperfusion injury following hypoxia. The long term effect of reactive oxygen species occur when damage on DNA (Evans, 2004).

2.10. Antioxidants:

2.10.1. Definition:

'Anti' is a prefix which means against, in opposition to, or corrective in nature. Antioxidant means opposite of oxidant, commonly known as "free radicals". Antioxidants or anti-oxidation agents are molecules which are capable to bind the harmful molecules, reduce their destructive power and reduce the effect of dangerous oxidants (Sies, 1997).

2.10.2. Mechanism of antioxidants:

Free radicals introduced from sun or pollution as external sources, other sources includes stress, as well as things that unhealthy for body such as alcoholic beverages, unhealthy foods and cigarette smoke. Oxidation creates rust which causes a breakdown of cell. Free radicals produced by the breakdown of healthy cells, usually DNA as well as proteins and fats. Antioxidant can decrease the destructive power of oxidants or free radicals by binding the harmful molecules (Evans, 2004). Antioxidant can help to repair the damage cells.

Body has produced certain antioxidant enzymes such as superoxide dismutase, catalase and glutathione. These naturally occurring antioxidants mechanism are different in body

system. Superoxide dismutase changes the structure of free radicals and breaks them down into hydrogen peroxide, catalase break down the hydrogen peroxide into water and tiny oxygen particles or gases and glutathione binds with different toxic agents to change their form by which they are able to leave the body as waste (Zelko et al., 2002).

Foods such as dark green leafy vegetable and with strongest color fruits provide the antioxidant agents. Vitamin A, vitamin C, vitamin E and beta-carotene are most beneficial and common antioxidant agents found in fruits and vegetables (Jacob, 1996; Knight, 1998).

Antioxidants have enormous and endless benefits. It works as superstars in our bodies and cells. We feel great health, vitality and enthusiasm when the antioxidants are plentiful and we feel reverse physical experience while the antioxidants are few. It can guard our body 24 hours a day from free radicals.

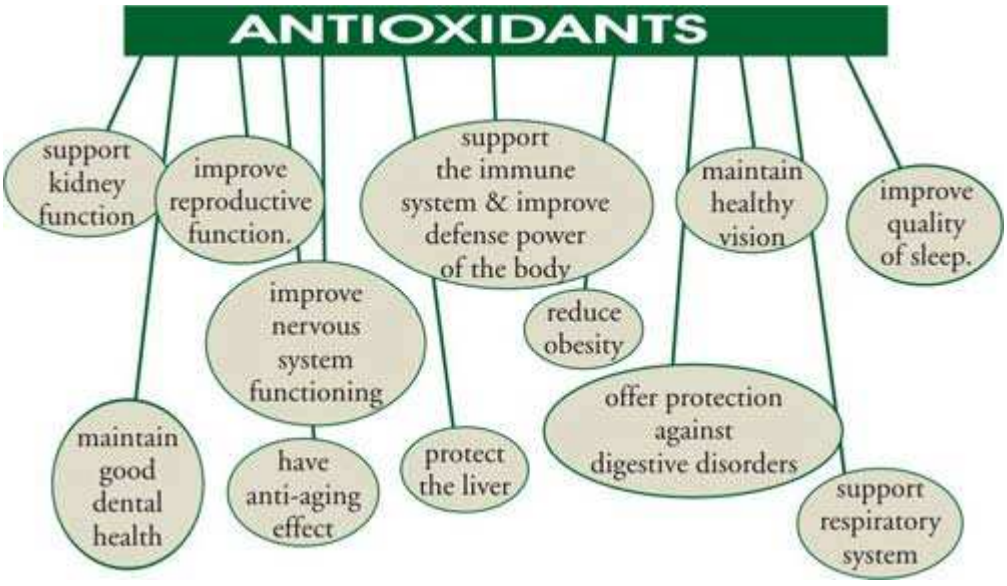

Figure 2.1: Benefit of antioxidants in our body system.

Source: http://www.amazing-glutathione.com/benefits-of-antioxidants.html

Antioxidants are mostly used as in dietary supplements and have been studied to find the prevention of diseases such as cancer, coronary heart disease and even altitude sickness. However, antioxidants are crucial for life, they can also be harmful. Insufficient or excess supplementation both can damage the cells and the body system.

The antioxidants acting in the body defense system are categorized into three defense line systems according to their functions (Deny, 2006; Noguchi and Niki, 1999). The first line of defense is the preventive antioxidants. These preventive-type antioxidants act to suppress free radical formation. The antioxidants will reduce hydroperoxide and hydrogen peroxide into alcohol and water without generating free radicals. The second line of defense is the radical-scavenging antioxidants. These radical scavenger-type antioxidants will hunt the radicals to inhibit chain initiation and break chain propagation. The third line of defense is the repair and de novo antioxidants. These type antioxidants will repair the oxidatively damaged phospholipids and their activities are accepted to be stimulated by oxidation. As is summarized in below, several sorts of antioxidants with different purposes play significant roles in these defense systems (Deny, 2006; Noguchi and Niki, 1999).

Defense System *In Vivo* Against Oxidative Damage
1. Preventive antioxidants: suppress the formation of free radicals
(a) Non-radical decomposition of hydroperoxides and hydrogen peroxide
Catalase, glutathione peroxidase, phospholipid hydroperoxide glutathione peroxidase, peroxidase, glutathione S-transferase
(b) Sequestration of metal by chelation
Transferrin, lactoferrin, haptoglobin, hemopexin, ceruloplasmin, albumin
(c) Quenching of active oxygen species
Superoxide dismutase, carotenoids, vitamin E
2. Radical-scavenging antioxidants: scavenge radicals to inhibit chain initiation and break chain propagation
Hydrophilic: Vitamin C, uris acid, bilirubin, albumin
Lipophilic: Vitamin E, ubiquinol, carotenoids, flavonoids
3. Repair and de novo enzymes: Repair the damage and reconstitute membranes
Lipase, protease, DNA repair enzymes, transferase

2.10.3. Plant derived antioxidants:

Various plant parts and plant origin has been used to discover the antioxidant potentials from plants. The hypothesis of antioxidant has claimed that antioxidant preclude oxidative

damages as well as cut out danger of chronic diseases (Stanner et al., 2004). Plant derived antioxidants which is called natural antioxidants are trying to discover with this hypothesis. Vitamin A, C, E and carotenoids, which are mainly diets find as plant derived antioxidants (Pietta, 2000). Other substances apart from vitamins also considered to contribute to the action of antioxidant that might be good for human health. The scientists have been studying widely due to the complex chemical structure of plant phenolic compounds or polyphenols and omnipresent concurrent in plant materials (Dimitrios, 2006). Simple molecules such as phenylpropanoids, phenolic acids and flavonoids to complex structure (lignins, melanins, tannins) are consider as phenolic compounds in where flavonoids are the most vastly distributed and common subgroup (Soobrttee et al., 2005; Bravo, 1998). The phenolics that are mostly found in diet, most remarkable fruits, vegetable and beverages (Luximon-Ramma et al., 2005; Bahorun et al., 2004; Luximon-Ramma et al., 2003).

Vegetables are huge source of phenolic compounds that were studied extensively and most commonly used vegetable reported as with highly antioxidant activity. Maisuthisakul et al. (2007) examined antioxidant activity with total phenolic content > 40mgGAE/g dry weight and EC_{50} values of DPPH free radical scavenging activity range from 0.23 to 0.55µg/µg of *Leucaena glauca*, *Cratoxylum formosum*, *Sesbania grandiflora* and *Syzygium gratum*. *Solanum lycopersicum* (tomatoes) and *Ipomoea reptans* also reported as high source of antioxidant activity (Dasgupta and De, 2007; Toor and Savage, 2005). *Brassica chinensis*, *Brassica oleracea*, *Atremisia vulgaris* and *Allium cepa* has been reported by Bahorun et al. (2004) as high antioxidant potentials.

Brassica oleracea, Brassica chinensis, Artemisia vulgaris and *Allium cepa* have been reported with high antioxidant activity by Bahorun et al., (2004). In where, kaempferol, quercetin and myrcetin are found as the main class of flavonoids. *Malus domestica* (apple)

reported by D'Abrosca et al. (2007) with high flovodoids components such as epicatechin, catechin, phloretin-2'- xyloglucoside and phlorodzin. *Morus alba* (Mulberry fruits) that was also reported as high antioxidant source because of the presence of anthocyanins (Bae and Suh, 2007).

Fruit juice, tea, wine and coffee that are beverages also known as important source of phenolic compound in human diet. Lakenbrink et al., (2000), Khokhar and Magnusdottir (2002) reported the Green tea, black tea, ground and instant coffee were as big source of phenolic contents (Balasundram et al., 2006; Luximon-Ramma et al., 2005; Khokhar and Magnusdottir, 2002; Lakenbrink et al., 2000).

Fraser et al., (2007) were reported that tea is rich in catechin including epicatechin 3-gallate, epigallocatechin-3-gallate, epicatechin and epigallocatechin, although compositions differ from each type of teas (green, black and oolong tea). Furulic acid, p-coumaric acid caffeic and caffeoylquinic acid are rich in coffee. The process of roastin is also increases the compounds of Maillard reaction product, which donate more antioxidant activity (Parras et al., 2007; Czerny et al., 1999). Gil et al., (2000) reported that pomegrante juice is a rich source of phenolics, anthocyanins and tannins. High anthocyanin contents are presents on wine that also know as potential of antioxidant agent (Rivero-Rerez et al., 2008).

Dried flower of *Hibiscus sabdariffa*, which is a source of anthocyanin and vitamin C, is drunk as red tea (Prenesti et al., 2007). Species, which also consider as a significant source of phenolic compounds, used as food flavor. The diet of South Asia, Asia and Mediterranean were believed to be healthier than western diet due to mostly uses of spices and herbs (Kaefer and Milner, 2008; Satia-Abouta et al., 2002).

The additional flavor and aroma of herbs and spices those are rich of volatile components of terpnes class also showed antioxidant potential. *Syzygium aromaticum* has correlation with phenolic content and prominent antioxidant activity. Volatile phenolic compounds were reported in this plant species (Ho et al., 2008). Cucurmin and curcuminoids are rich in *Curcuma longa* (turmeric) and sited as high free radical scavenger (Meghana et al., 2007; Fujisawa et al., 2004). *Coriandrum sativum* (coriander) is reported as known antioxidant such as geranoil, borneol, quercetin, cinnamic acid, eucalyptol, cineole, caffeic acid, rutin and ferrulic acid (Kaefer and Milner, 2008; Melo et al., 2005). Eugenol, myrcene, terpinen-4-ol, luaric acid and myristicin are rich in *Myristica fragrans* (nutmeg). Ascorbica acid, capsaicin, quercetin, beta-carotene, kaempferol, hesperidin and caffeic acid are rich in *Capsicum annuum* (chili pepper). Suhaj (2006) was reported *Zingiber officinale* with huge source of ascorbic acid, gamma-terpinene, betacarotene, isoeugenol and caffeic acid. *Piper guineense* and *Piper nigrum* were reported with high phenolic content by Agrob et al., (2006).

2.10.4. Antioxidant derived from mangrove plants:

Mangroves are shrubs and trees, which grow in saline coastal habitats in the tropics and subtropics. The mangrove dwellers get food and wide variety of traditional products and artifacts from mangroves. Extracts and chemicals from mangroves are used mainly in folkloric medicine (e.g. bush medicine), as insecticides and pesticides and these practices continue to this day (Bandaranayake et al., 2002). Promising antibacterial activity of ethyl acetate extract of *Avicennia marina* mature leaves, methanol extract of *Excoecaria agallocha* leaves and shoots and antifungal activity of methanol extract of *Exoecaria agallocha* and *Bruguiera gymnorrhiza* trunks are some other examples of pharmaceutical potential of mangrove plants. Sepal of *Sonneratia alba* was reported as

antioxidant and antilipid peroxidation (Abeysinghe et al., 2006; Chandrasekaran et al., 2009; Kazuhiko et al., 2002; Nuntavan et al., 2003). Leaves, trunks and barks also have been reported for its antioxidant properties (Wada et al., 2002). In China, fruits of *B. gymnorrhiza* have been used to treat diarrhea (Bamrungrugsa N, 1999). The medicinal use of fruits includes application of the treatment of shingles and eye diseases (Othman S, 1998). The bark has been used as an astringent treatment of diarrhea and malaria (Omu et al., 1998). The roots and leaves have been used to treat burn (Othman S, 1998).

2.10.5. Methods for determination of antioxidant activity:

DPPH Assay: DPPH radical is commercially available and does not have to be generated before assay like $ABTS^{\cdot}+$. This assay is established on the measurement of the reducing ability of antioxidants toward DPPH radical by measuring the decrease of its absorbance (Prior, et al. 2005).

The effects of the oxidative stress may be delayed or reduced by taking dietery supplements (Villeponteau, et al. 2000). It is crucial to find out the antioxidant capacities of dietary antioxidants. Various kinds of methods are being used for measuring antioxidant capacity of substances such as physical, chemical and biochemical generator systems. Most of these methods have quite time consuming procedures up to several hours for a single sample and many substances contain both lipid- soluble and water-soluble antioxidants. However most of the methods have a single measuring principle that determines only one of the two substance classes (Matthias, et al., 2001).

FRAP (Ferric Reducing Antioxidant Power): The reaction measures reduction of ferric to a colored product. The reaction detects compounds with redox potentials of <0.7 V, so

FRAP is a sensible test for the power to hold redox condition in cells or tissues. Reducing power seems to be associated to the level of hydroxylation.

2.11. Antimicrobial:

An antimicrobial that can inhibits or kills the development of microorganisms such as fungi, protozoans or bacteria. Antimicrobial drags can be use either kill microbes or prevent the growth of microbes. Pasteur and Joubert first discovered the antimicrobial activity; they observed one type of bacteria could prevent the growth of another. The mechanism of antimicrobial is that one bacterium produce antibiotic that's why other bacterium fails to grow. Scientifically antibiotics are the substance that kill or prevent the growth of other organism. Antibodies and synthetically formed compounds are included into antimicrobials. Penicillin and tetracycline invention was the milestone which providing better health for millions of people around the world. However, during the process of development of antimicrobials, microorganisms become resistant and adapted to previous antimicrobial agents because they may have kill the microbe completely, allowing them to change or survive or become resistant to the antimicrobial agents. A new technology called 'Antimicrobial nanotechnology' introduced by the scientist that may be some day a viable alternative against microbes.

2.11.1. Antimicrobial agent:

Microorganisms are responsible to make disease to human being. Bacterial Infectious diseases caused fifty thousands of premature deaths (Carey, 2004; Esterhuizen et al., 2006). Hence, the control of microorganisms is essential in curing and prevention of disease caused by their actions. Antimicrobials are substance, which can inhibit or kill the growth or prevents damage on account of the action of infectious microorganisms. There are a various number of antimicrobial agents currently available. When selecting for a particular

antimicrobial agent, its selective toxicity must be evaluated. Because of, the antimicrobial agent is desired to exhibit greater toxicity to the infecting pathogens then to the host organism (Atlas, et al. 1995). Terms of antimicrobial agents contains of antiviral, antifungal, antihelminthic, antiprotozoal and antibacterial agents (Baron et al., 1994).

Antibacterial agents are intimately related with antibiotics. Antibiotics are the biochemical produced by microorganisms from organic chemicals and many antibiotics in current medical use are chemically modified forms of microbial biosynthetic products (Atlas, et al. 1995). Antibiotics are substances that suppressed the development and killed other bacteria (Purohit et al., 2003). While antifungal agent is determined as substances, which inhibit or kill the growth of fungi (McDonnell, 2007)? Bacteria and fungi was the source of antibiotics but now most of the produced synthetically.

In the early 20th century, antibiotics were considered as one of the greatest breakthrough by which many lives saved against bacterial infection. However, the egression of resistance of bacteria to all acknowledged antibiotics class are globally accounted and begun to acquire the attention of lots of people for the demand of new antibacterial source (Ojala et al., 2000). Misapply over-prescription and abuses of antibiotics are conceived to be the causes of egress of resistance (Peterson and Dalhoff, 2004; Eloff, 2000). There are three mechanisms by which antibiotic resistance can happen: prevention of fundamental interaction between drug and target; effluence of the antibiotics from the cell; and direct devastation or change of the structure (Mendonca-Filho, 2006). New natural source of antimicrobial agents were then finally lead to intensive research on antimicrobial components from plants.

2.11.2. Plants as a natural source of antimicrobial components:

Since antibiotic were discovered, the research on antimicrobial compounds from plants were no longer carried on. The widespread and sometimes inappropriate use of antimicrobials is the main factors that give rise evolution of antimicrobial resistant bacteria species (Lowy, et al. 2003). The evolution of bacteria toward antimicrobial drug resistance increased rapidly in the last 60 years including the pathogenic species for humans (Courvalin, et al. 2005). So today it is a need to search and discover new, effective antimicrobials by supplying them with novel mechanisms of action and new targets to act on (Cloutier, et al. 1995). Plants are believed as a reliable natural source for the breakthrough of novel antimicrobial agents (Rangasamy et al., 2007). There are several reason for what research have been carried on plants as a source of antimicrobial agent: 1) lots of phyto-chemicals were prescribed by physicians as antimicrobial agents; of which various are already costing clinically examined; 2) the awareness has been increasing on the safety of present antibiotics; 3) the dominance of the transmission rate of human immunodeficiency virus (HIV) has promoted the intensive investigation of plant derivatives which is efficient but also cheap and approachable for use in underdeveloped countries with little entree to costly Western medicines; 4) chemist and microbiologist are eagerly interested due to rapid extinction of plant between past 20 years, whom conceive that collection of potentially beneficial phytochemical structures which could not be synthesized chemically is at risk of being lost irretrievably (Lewis and Elvin-Lewis, 1995; Borris, 1996; Cowan, 1999). Several scientific studies have also indicated the importance of new bioactive phytochemicals against multi-drug resistant bacteria (Mendonça, et al. 2006) which are pure molecules and also some of them exhibit much more effective

pharmacological activities than their synthetic alternatives (Iwu et al. 1999) Laboratories of the world have documented literally thousands of phytopharmaceutical agents that have important inhibitory effects on all types of microorganisms in vitro (Mendonça, et al. 2006).

In many different communities, the traditional therapeutic value has been recognized for treatment of bacterial infections. Researchers have also been carried to formalize the conventional therapeutic value of the plants as well as to discover its potential of antimicrobial activity. For example, Wiart et al. (2004) have described an extensive spectrum of antimicrobial activity of extracts of *Peristrophe tinctoria, Polyalthia laterifolia, Celosia argentea, Knema malayana, Eclipta prostrata, Dillenia suffruticosa, Rafflesia hasseltii, Piper stylosum* and *Solanum torvum*. Ibrahim and Osman (1995) also have reported the antimicrobial potential of *Cassia alata* against dermatophytic fungi *T. mentagrophytes* var. mentagorophytes, *Trichophyton mentagrophytes* var. interdigitale, *Microsporum gypseum* and *T. rubrum*. Grosvenor et al. (1995) have tested 124 plant species from Riau province, Indonesia and reported that 121 species exhibited inhibitory activity against *S. aureus* while 42 species inhibited the growth of *E. coli*.

2.11.3. Mangrove as a source of antimicrobial activity:

In different countries and communities mangroves are using as healing against some antimicrobial. The scientists has been used the different part of mangrove plant extract against some bacterial pathogen and they got the positive result from most of them. For example, ethyl acetate extract of *Avecennia marina* mature leaves showed promising antibacterial activity, leaves and shoots of methanol extract of *Excoecaria agallocha* exhibited antifungal activity (Chandrasekaran et al., 2009; Abeysinghe and Wanigatunge

2006). Kazuhiko (2001) also reported that methanol extract of *Exoecaria agallocha* and *Bruguiera gymnorrhiza* trunks has the antifungal activity.

Methanol, ethanol and chloroform extract of leaves of Malaysian *Rhizophora mucronata* reported as potential antimicrobial source against four bacterial pathogens namely *B. cereus, S. aureus, E. coli* and *P. aeruginosa* (Haq Imdadul et al., 2011).

2.12. Antimicrobial susceptibility test:

It is known that, now products from natural sources and derivatives hold about 50% of all the clinical drugs, one quarter arose from higher plants. Plant extracts are important materials that have potential antimicrobial agents that confer an antimicrobial defense against microbes in their own environment (Eldeen, et al. 2005).

However it is quite difficult to determine the appropriate plant species due to their enormous numbers in plant kingdom. So, screening methods for AST are of great important in order to detect phytochemical antimicrobial agents. The presently available testing methods to detect the potential of antimicrobial activity of natural products are classified in three general groups, including bio-autographic, dilution, and diffusion methods. The methods of bio-autographic and diffusion can be described as qualitative techniques by which only the absence or presence of contents with antimicrobial activity can be observed. On the contrary dilution methods are known as quantitative assays since they determine the minimal inhibitory concentration (Valgas, et al. 2007).

There has been much research interest in agar-based testing of disk-diffusion method, and commonly used because of its comparative simplicity and the deficiency demand for specialized instrumentation (Scorzoni, et al. 2007). Antimicrobial susceptibility discs are absorbent, paper discs that are impregnated with a certain antimicrobial agent and are

generally marked with the disk code and the drug concentration. The test process is established on the method of Kirby-Bauer. In that method discs have to place on surface of the agar plate, which is inoculated with the organism to be examined then incubated overnight. Clinically the *in vitro* antimicrobial susceptibility is used to determine antimicrobial agent of choice whenever the susceptibility of a bacterial pathogen is unpredictable (Hansen, et al. 1996). The liquid-dilution method also allows detecting of whether a compound or extract has antimicrobial potential at a certain concentration. The serial dilution test was described to afford the most consistent effects on the minimal inhibitory concentration (MIC) and was advocated as general standard methodology for the screening of natural products (Burrows, et al. 1993, Hansen, et al. 1996). The MIC usually is conceived one of the most introductory laboratory measurement of the potential of antimicrobial activity versus an organism. It is determined as the lowest concentration of an antimicrobial agent that is demanded to suppress the development of a particular organism in a well-standardized *in vitro* susceptibility screening. The traditional technique for evaluating the MIC needs disclosing the test organism to a series of two-fold dilutions of the antimicrobial agent in a desirable culture media (e.g. broth or agar formats) (Jorgensen, et al. 2004). MIC information is quantitative rather than qualitative; it may be useful for dosage determinations as well as antibiotic selection (Burrows, et al. 1993). Determinations of MIC are regulated by numerous variables, including the composition of the test medium, the size of the bacterial inoculums, and the duration of incubation. Also the test conditions of *in vitro* cannot cover all elements, which can have determined on *in vivo* antimicrobial activity (Burrows, et al., 1993).

2.13. Bio-assay guided fractionation:

A process where the biologically most active component is isolated from chromatographically fractionated and re-fractionated extract is called bioassay-guided fractionation. Throughout the process of fractionation produce a fraction, which assessed in a bioassay system and then only active fractions are fractionated (Atta-ur-Rahman and Basha, 1988). The method of Bioassay-guided fractionation is generally applied in drug discovery research because of its strength to directly link the examined extract and directed components using fractionation process that adopted with specific biological activity.

Bioassay-guided fractionation process have used by Cho et al (2003) to isolate active components from 23 medicinal plants as antioxidant research using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay and Silica gel chromatography. Consecutive fractionation proficiency adopted by DPPH assay was conducting to isolation of polyphenols such as gallic acid, quercetin-3-O-rutinose, (+)-catechin, quercetin, quercetin-3-O-β-D-glucoside, kaempferol, methyl gallate, procyanidin B-3 and quercetin-3-O-β-galactoside which possessed firm DPPH free radical scavenging activity.

2.14. LCMS/MS analysis:

There are different analytical techniques are used to do the qualitative identification of organic agents. The techniques of mass spectrometric offer the comparative advantages for productivity and speed of pharmaceutical analysis. In the liquid chromatography-mass spectrometry (LC-MS) techniques, components and molecular masses of sample of complex pharmaceutical research can be surveyed without pre-fractionation. Liquid chromatography tandem mass spectrometry (LCMS/MS) can provide the further structural details. The approach of LCMS/MS has been applied successfully in different field of

pharmaceutical research. The fields of the studies are natural products, degradants, impurities and metabolites. Structure clarification of metabolites of drug using the MSMS is established by the premise that metabolites retain substructures of parent drug molecule and, thus, produce MSMS product ions associated with those structures. Parent drug can use as substructural template, metabolite structures present in crude mixtures may be rapidly characterized and often identified without standards for each metabolite. Using the ionspray LCMS interface allows for the consistent analysis of labile and polar phase II metabolites at trace levels compared to earlier LCMS interfaces (e.g., thermospray) due to the low internal energy imparted to analyze and eliminates necessity of deconjugation prior to analysis.

Chapter-3: Materials and methods

3.1. Plant material:

Fruit, leaves and bark of *Sonneratia alba*, leaves and barks of *Rhizophora mucronata* and *Bruguiera gymnorrhiza* were used as plant materials in this experiment.

3.1.1. Plant sample collection:

All the plant materials were collected from Carey Island, Klang, Kuala Lumpur, Malaysia.

3.2. Plant sample preparation:

3.2.1. In vivo plant sample preparation:

The collected leaves and barks of *Sonneratia alba, Rhizophora mucronata* and *Bruguiera gymnorrhiza* were washed using the tap water and dried in an incubator at 40°C. Dried samples were ground to produce fine homogenous powders using an electric blender .

3.2.2. In vitro plant sample preparation:

3.2.2.1. Maturation of fruits:

All fruits were collected according to their size and tested their maturity by conventional floating methods. Maturation was tested based on described by M.S. Swaminathan Research Foundation in "Toolkit for establishing Coastal Bioshield". All fruits were tested with water in beaker, when they were floated considered as a mature fruits. Fruits were then kept by wrapping with thick cloth for 12 days.

3.2.2.2. Sterilization of seeds:

Sterilization was done not based on any specific methods. Several traditional techniques were used and failed. At the end, one modified technique worked well. Briefly, seeds were collected after blooming of the fruits and were immediately sterilized. Sterilization was performed by several steps. First the seeds were kept for 15 minutes with teepol, chlorox,

tween-20 and water and then washed the seeds under running water for 1 hour. Next all steps were conducted under laminar flow. First, the seeds were soaked in 70%ethanol for 1 minute then washed with sterile distill water. Seeds were then soaked in 0.1% marcuric chloride (HgCl2) for 3 minutes then washed with ethanol and then again put in $HgCl_2$ for three minutes and then soaked in 75% chlorox for 10 minutes. The seeds were washed with ethanol again and finally washed with sterile distill water for three to four times.

3.2.2.3. Culture of seeds:

Seeds were cultured *in vitro* and *in vivo* conditions. Under *in vivo* condition, seeds were grown on sand and soil mixture. Under *in vitro* culture, seeds were grown in MS and WPM media.

Three hormones were used, namely BAP, GA3 and Kinetin, with three concentration combination of 100, 500 and 1000ppm for each. Seeds were soaked for 24 h. Hormones solution were sterilized in autoclave (120°C for 20 min) before the seeds were soaked. Besides, control cultures were conducted for both *in vivo* and *in vitro* cultures without any hormone.

Seeds were cultured aseptically in 5mL of 2.8% phytagel medium with Murashige and Skoog (MS) and Woody Plant Medium (WPM) in 16mL culture tubes. They were cultured under 80μEm^2 sec light conditions at 23-24°C. Single seed was cultured in a culture tube covered with autoclaved translucent film. Both MS and WPM media contained 3% sucrose.

3.2.2.4. Sample preparation for bioactivity:

Hundreds of plant was germinated with same condition to get enough explants for bioactivity test. The roots were removed and the aerial parts of the germinated seedlings were collected for further study. The collected explants were dried in an incubator at 40 °C. Dried explants were ground to produce fine homogenous powders using an electric blender.

3.3. Extraction:

3.3.1. In vivo sample extraction:

The powder form plant samples were soaked in three selected solvents (95% ethanol, methanol and chloroform) at room temperature in the dark for three days. After three days all samples were filtered through Whatman® No. 1 filter paper (Whatman International, England) and the filtered solutions were then evaporated to dryness using evaporator. The plant extracts were dissolved in Dimethyl Sulphoxide (DMSO).

3.3.2. In vitro sample extraction:

Methanol, ethanol and chloroform were used as solvents to soak the powder form in vitro plant samples. The soaked solvent with samples were kept at room temperature in the dark for three days. All samples were filtered through Whatman® No. 1 filter paper (Whatman International, England) and the filtered solutions were then evaporated to dryness using evaporator. The plant extracts were dissolved in Dimethyl Sulphoxide (DMSO).

3.4. Determination of Total Phenolic Contents:

3.4.1. Preparation of reagent:

3.4.1.1. Galic Acid stock Solution:

In a 100ml volumetric flask, 0.5g of AR grade dry gallic acid (Sigma) was dissolved in 10ml of ethanol. The final volume was made to 100ml with methanol. The solution was stirred until gallic acid was completely dissolved.

3.4.1.2. Sodium Carbonate Solution:

100g of sodium carbonate (Sigma) were dissolved in 400ml of water and boiled.

3.4.2. Protocol:

The concentrations of phenolic compounds in all extracts of explants were expressed as gallic acid equivalents (GAEs), were determined according to the method by Cheung et.al (2003).

A 0.02ml of extracts at 5mg/ml and negative control were mixed 1.58ml of distilled water and 0.1ml of Folin-Ciocalteu's reagent. After 3 minutes, 0.3ml of saturated sodium carbonate (Na_2CO3) solution was added to the mixture. The contents were vortexes for 20 seconds and then left to stand at 40ºC for 30 minutes. Absorbance measurements ware recorded at 765 nm using a Hitachi U2000 spectrophotometer. A calibration curve, using gallic acid with a concentration range form 50-500mg/L gallic acid was prepared. Estimation of the phenolic compounds was carried out in triplicate. The results were mean value and were expressed as mg GAE (gallic acid equivalents)/L.

3.5. Antioxidant activities:

3.5.1. Superoxide Dismutase (SOD) Assay:

3.5.1.1. Equipment required:

a) Plate reader (450 nm filter)
b) 96-well micro plate
c) 10 ml & 100-200 ml pipettes and a multi-channel pipette
d) Incubator

3.5.1.2. Preparation of working solutions:

a) WST working solution: 1 ml of WST Solution was diluted with 19 ml of Buffer Solution.

b) Enzyme working solution: Centrifuge the Enzyme Solution was centrifuged for 5 sec. It was mixed by pipeting, and diluted 15µl of Enzyme Solution with 2.5 ml of Dilution Buffer.

3.5.1.3. General protocol:

Table 3.1 refer to the amount of solutions in each well.

a. 20 ml of sample solution were added to each sample and blank 2 well, and 20 µl of ddH2O (double distilled water) added to each blank 1 and blank 3 well.
b. 200 ml of WST Working Solution were added to each well, and mix.
c. 20 ml of Dilution Buffer were added to each blank 2 and blank 3 well.
d. 20 ml of Enzyme Working Solution were added to each sample and blank 1 well, and then mix thoroughly.
e. The plate was incubated at 37 °C for 20 min.
f. Reading and absorbance ware done at 450 nm using a micro plate reader.
g. The SOD activity (inhibition rate %) was calculated using the following equation:

SOD activity (inhibition rate %) = {[(Ablank 1 - Ablank 3) - Asample - Ablank 2)]/ (Ablank 1 - Ablank 3)} x 100

Table 3.1: Amount of each solution for sample, blank 1, 2, 3

	Sample	Blank 1	Blank2*	Blank3
Sample Solution	20µl	-	20µl	-
ddH_2O	-	20µl	-	20µl
WST Working Solution	200µl	200µl	200µl	200µl
Enzyme Working Solution	20µl	20µl	-	-
Dilution Buffer	-	-	20µl	20µl

3.5.2. Scavenging activity on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals:

3.5.2.1. Preparation of DPPH solution:

Stock Solution:

A stock solution of 1mg/ml of each extract was prepared and all extracts were dissolved in DMSO.

Ascorbic Acid:

A stock of ascorbic acid (Sigma) in DMSO was prepared at concentration of 400µg/ml. The stock solution was kept in flask wrapped with aluminum foil.

1, 1-diphenyl 1-2-picrylhydrazyl (DPPH):

A stock of DPPH (Sigma) in DMSO was prepared at concentration of 8mg/ml. The stock solution was kept in flask wrapped with aluminium foil.

Ascorbic acid as positive references standard:

Ascorbic acid was used as the positive reference standard in the DPPH assay. Reaction mixtures of ascorbic acid, DPPH and DMSO for the assay were prepared according to the Table 3.2.

Table-3.2: Reaction mixture of ascorbic acid, DPPH and DMSO for DPPH assay.

Concentration of Ascorbic Acid (µg/ml)	Volume of DMSO (µl)	Volume of Ascorbic Acid (µl)	Volume of DPPH solution (µl)
200.00	475.00	500.00	25.0
100.00	725.00	250.00	25.0
50.00	850.00	125.00	25.0
25.00	912.50	62.50	25.0
12..50	943.75	31.25	25.0
6.25	959.38	15.63	25.0
3.125	967.19	7.81	25.0
1.56	971.09	3.91	25.0
Control	975.00	-	25.0

The reaction mixture was incubated at room temperature and allowed to react for 30 minutes. The optical density was measured at 520 nm. DMSO was used blank. The DPPH radical without addition of ascorbic acid (as antioxidant) was used as the control.

Determination of percentage of inhibition:

The percentage of inhibition of each of the test samples was calculated according to the following formula:

Inhibition rate (%) = (OD control – OD sample)/ OD control X 100%

3.5.3. Determination of IC50 Value in DPPH assay:

3.5.3.1. Sample preparation:

OD control was the absorbance of the control and OD sample was the absorbance of extract/standard. IC_{50} for each extract was extrapolated from the graphs plotted using the OD value obtained. IC_{50} value (mg/ml) is the effective concentration at which DPPH radicals were scavenged by 50% and was obtained by interpolation from linear regression analysis.

The extracts were tested for antioxidant activity at concentration of 0.125mg/ml, 0.0625mg/ml, 0.03125mg/ml and 0.1625mg/ml to determine the IC_{50} value. Reaction mixtures containing positive extracts (1mg/ml), DPPH (8mg/ml) and DMSO were prepared according to Table 3.3. The assay procedures were repeated to the previous testing using ascorbic acid and the IC_{50} value for each extract was extrapolated from the graphs plotted using the OD values obtained.

Table 3.3: Reaction mixtures for positive extracts, DPPH and DMSO

Concentration of extracts (mg/ml)	Volume of Methanol (µl)	Volume of extracts (µl)	Volume of DPPH solution (µl)
0.125	850.00	125	25.0
0.0625	912.50	62.5	25.0
0.03125	943.75	31.25	25.0
0.01625	959.375	15.625	25.0
Control	975.00	-	25.0

All tests were run in triplicate and reading obtained was averaged.

3.5.4. Reducing Power Assay:

3.5.4.1. Chemical preparation:

Potassium ferricyanide 1%:

0.1g of potassium ferricyanide was dissolve with 10 ml of distill water inside blue cap centrifuge tube. The solution was vortexed to mix well.

Trichloroacetic acid (TCA) 10%:

50 g of TCA were dissolved with 500ml of distill water inside the scott bottle and wrapped with aluminium foil.

Ferric acid solution 0.1%:

0.1g of ferric chloride dissolved in 10ml of distill water inside blue cap centrifuge tube and vortex it to mix well.

0.2 Phosphate Buffer Solution at pH 6.6:

I. 0.2M monobasic stock: 27.8g of sodium phosphate monobasic (Na2H2PO4) were dissolved in 1000ml of distill water.
II. 0.2 M dibasic stock: 53.65g of sodium phosphate dibasic heptahydrate (Na2HPO4) were dissolved in 1000ml of distill water.
III. 0.2 M phosphate buffer: 62.5ml of monobasic stock, 37.5ml of dibasic stock and 200ml of distill water were added together to prepare the 0.2 M of phosphate buffer.

3.5.4.2. Protocol:

Reducing power of all extracts was determined based on the method described by Sorenson et al (1986). 0.25, 0.5, 1.0 and 2.0mg/ml of extract were tested to determine the reducing power. Each extract was dissolved in 1.0ml of DMSO inside the falcon tube. 2.5ml of 0.2 M phosphate buffer and 2.5ml of potassium ferricyonide then were added into the falcon

tube. Then the mixture was kept in water bath for 20 min at 50ºC. After incubation, 2.5ml of TCA solution was added to the mixture and then centrifuged the solution at 1000RPM for 10 minutes. 2.5ml supernatant were then transferred into a new tube and 2.5ml of distill water and 0.5ml of ferric chloride were added. The absorbances were taken at 700nm.

3.6. Antimicrobial Activity:

3.6.1. Bacterial pathogen:

Two gram positive and two gram negative bacterial pathogen were used to test the antimicrobial activities in this experiment. *Escherichia coli* and *Pseudomonas aeruginosa* were the gram negative pathogenic bacteria and *Staphylococcus aureus* and *Bacillus cereus* were the gram positive pathogenic bacteria which were obtained from Microbiology Division of Institute of Biological Sciences, University of Malaya (Phillip et al., 2007).

3.6.2. Sensitivity test:

3.6.2.1. Media preparation:

I. Bacto-agar preparation: 17g of bacto-agar were added in 1 liter distill water and mixed to dissolve and autoclaved the bacto-ager at 121ºC for 15 minutes.

II. Mueller-Hinton Broth preparation: 21g of Mueller-Hinton broth were added with 1 liter distill water and mixed to dissolve. Sterilize the mixture by autoclaving at 121°C for 15 minutes.

3.6.2.2. Extracts Preparation:

All the extracts were prepared at the concentration of 10mg/ml with DMSO.

3.6.2.3. Bacterial suspension preparation:

Bacteria were cultured in Bacto-agar for 24hr. The OD was measured with spectrophotometer at 620nm wavelength. The expected OD for this experiment was 0.08 to 0.1. NaCl was used to make the OD between 0.08 and 0.1.

3.6.2.4. Discs extract preparation:

Paper dicks were prepared by soaking into the extracts to test the antimicrobial activity.

3.6.2.5. Sensitivity test protocol:

I. The swab was dipped into the broth culture of the organism. The swab was gently squeezed against the inside of the tube to remove excess fluid. The swab used to streak a Mueller-Hinton agar plate for a lawn of growth. At first, the plate was made streaking in one direction, and then streaked at right angles to the first streaking, and finally streaked diagonally. End by using the swab to streak the outside diameter of the agar.

II. The plates were allowed to dry for about 5 minutes.

III. Extracts disks were placed on the surface of the agar by obtaining individual disks and placing them on the surface of the agar using flame sterilized forceps. Onto the surface of agar media the dicks were gently pressed by using sterilized forceps. Four dicks were placed in one agar media plate.

IV. The plates were inverted and incubated for 24hr at 37°C.

V. The inhibition zone was measured by using the metric ruler for each extract dick used.

3.7. LCMS/MS analysis of water fraction:

The analysis of LCMS/MS of water fraction of *Sonneratia alba* was performed using a Applied Biosystems 3200Q Trap LCMS/MS instrument. A Phenomenex Aqua C18 (50mm X 2.0mm X 5µm) column was used for separation. The column temperature was set at 30°C. The mobile phase consisted of water (A) and acetonitrile (B) with 0.2% formic acid and 2mM ammonium formate. The initial condition was set at 10% A to 90% B from 0.01 min to 5.0 min, remained at this condition for 2 min, then back to 10% A in 0.1 min and re-equilibrated for 3 min. The total run time was 10 minute at a flow rate of 0.25mL/minute.

Compatibility mode of LCMS/MS:

Acquisition information:

Sample Acq Duration:	10min0sec
Number of Scans:	0
Periods in File:	1
Batch Path:	D:\Analyst Data\Projects\Test\Batch\
Submitted by:	LCMSMS\Administrator
Logged-on User:	LCMSMS\Administrator
Synchronization Mode:	LC Sync
Auto-Equilibration:	Off
Comment:	
Software Version:	Analyst 1.4.2
Set Name:	Sample CW

Sample Name	SAW
Autosampler Vial:	21
Rack Code:	1.5mL Cooled
Rack Position:	1
Plate Code:	1.5mL Cooled
Plate Position	1

LC methods properties:

Shimadzu LC system Equlibration time = 0.50 min

Shimadzu LC system Injection Volume = 10.00 ul

Shimadzu LC Method Parameters

Pumps:

Pump A Model: LC-20AD

Pump B Model: LC-20AD

Pumping Mode: Binary Flow

Total Flow: 0.2500 mL/min.

Pump B Pct: 10.0 %

B Curve: 0

Pressure Range (Pump A/B): 0 - 4500 psi

Autosampler:

Model: SIL-20AC

Rinsing Volume: 500 µL

Needle Stroke: 52 mm.

Rinsing Speed: 35µL/sec.

Sampling Speed: 15.0µL/sec.

Purge Time: 25.0 min.

Rinse Dip Time: 0 sec.

Rinse Mode: Before and after aspiration

Cooler Enabled: No

Control Vial Needle Stroke: 52 mm

Oven:

Model: CTO-20AC

Temperature Control: Enabled

Temperature: 40°C

Max. Temperature: 85°C

System Controller:

Model: CBM-20A

Power: On

Event 1: Off

Event 2: Off

Event 3: Off

Event 4: Off

Time Program:

Time	Module	Events	Parameter
0.01	Pumps	Pump B Conc.	
5.00	Pumps	Pump B Conc.	
7.00	Pumps	Pump B Conc.	
7.10	Pumps	Pump B Conc.	
10.00	System Controller	Stop	

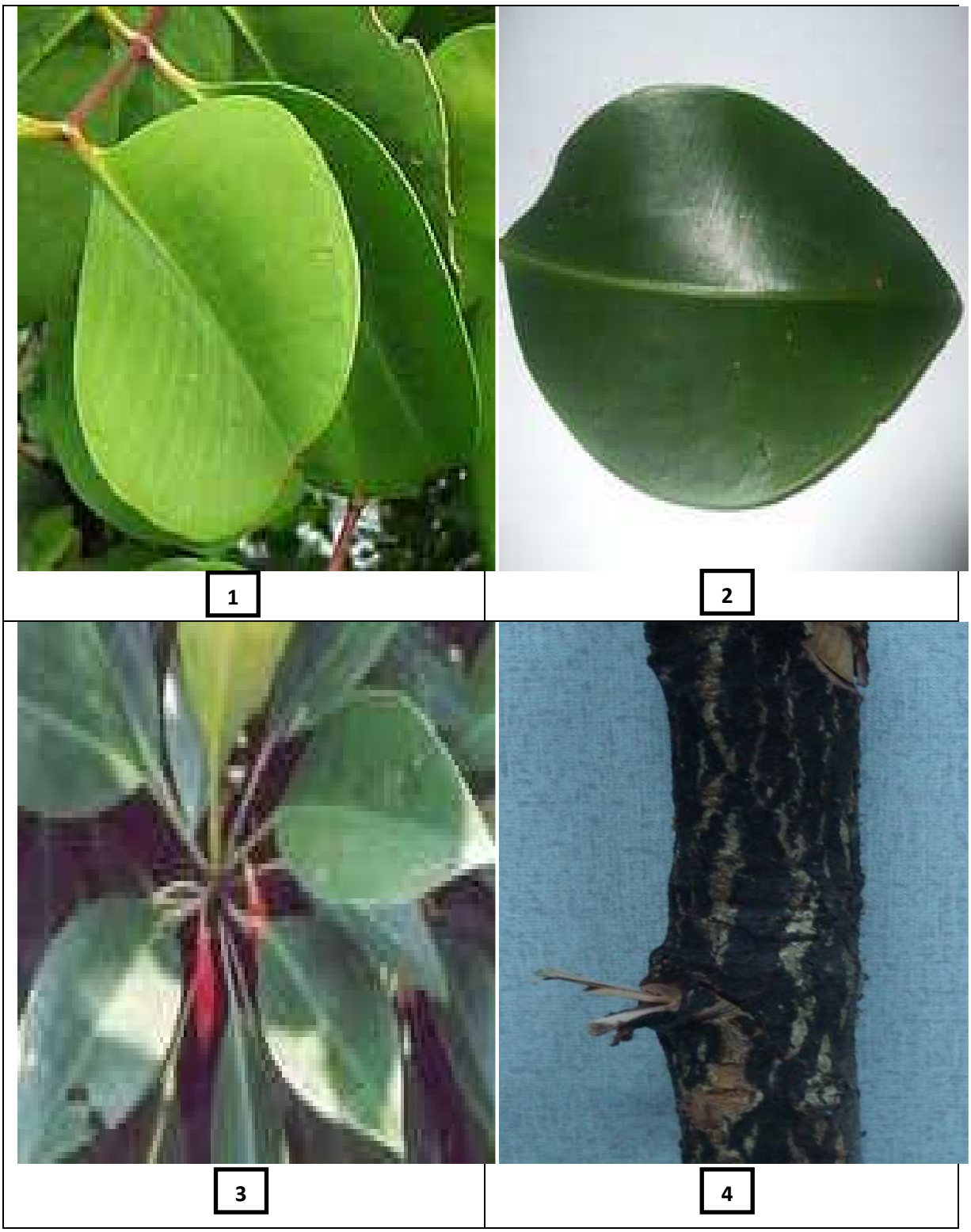

Figure 3.1: Picture showing- Leaves of *Sonneratia alba* (1), Leaf of *Rhizophora mucronata* (2), Leaves of *Bruguiera gymnorrhiza* (3) and Bark of *Sonneratia alba* (4).

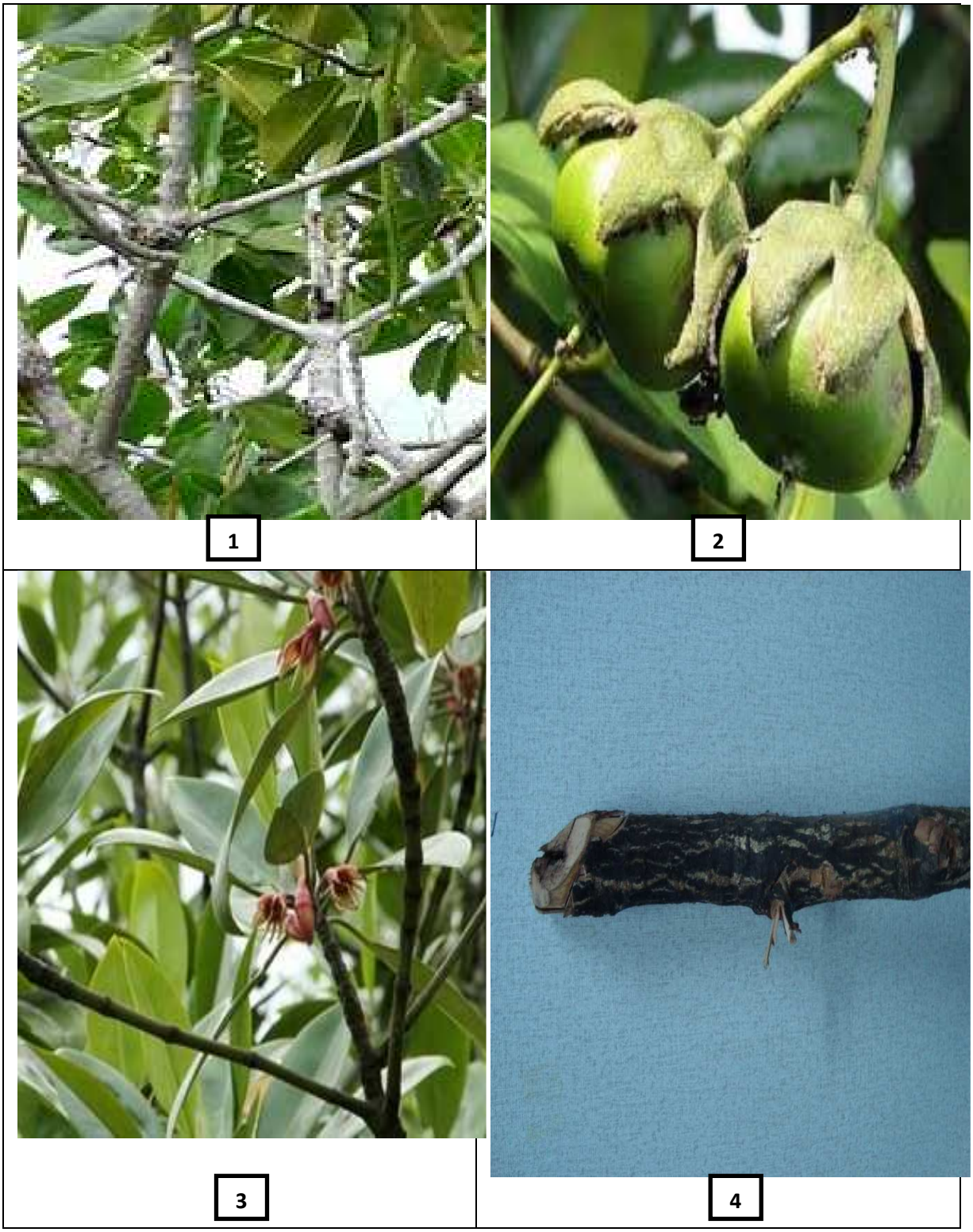

Figure 3.2: Picture showing-Stem of *Rhizophora mucronata* (1), Fruits of *Sonneratia alba* (2), Stem of *Bruguiera gymnorrhiza* (3), Stem of *Sonneratia alba* (4)

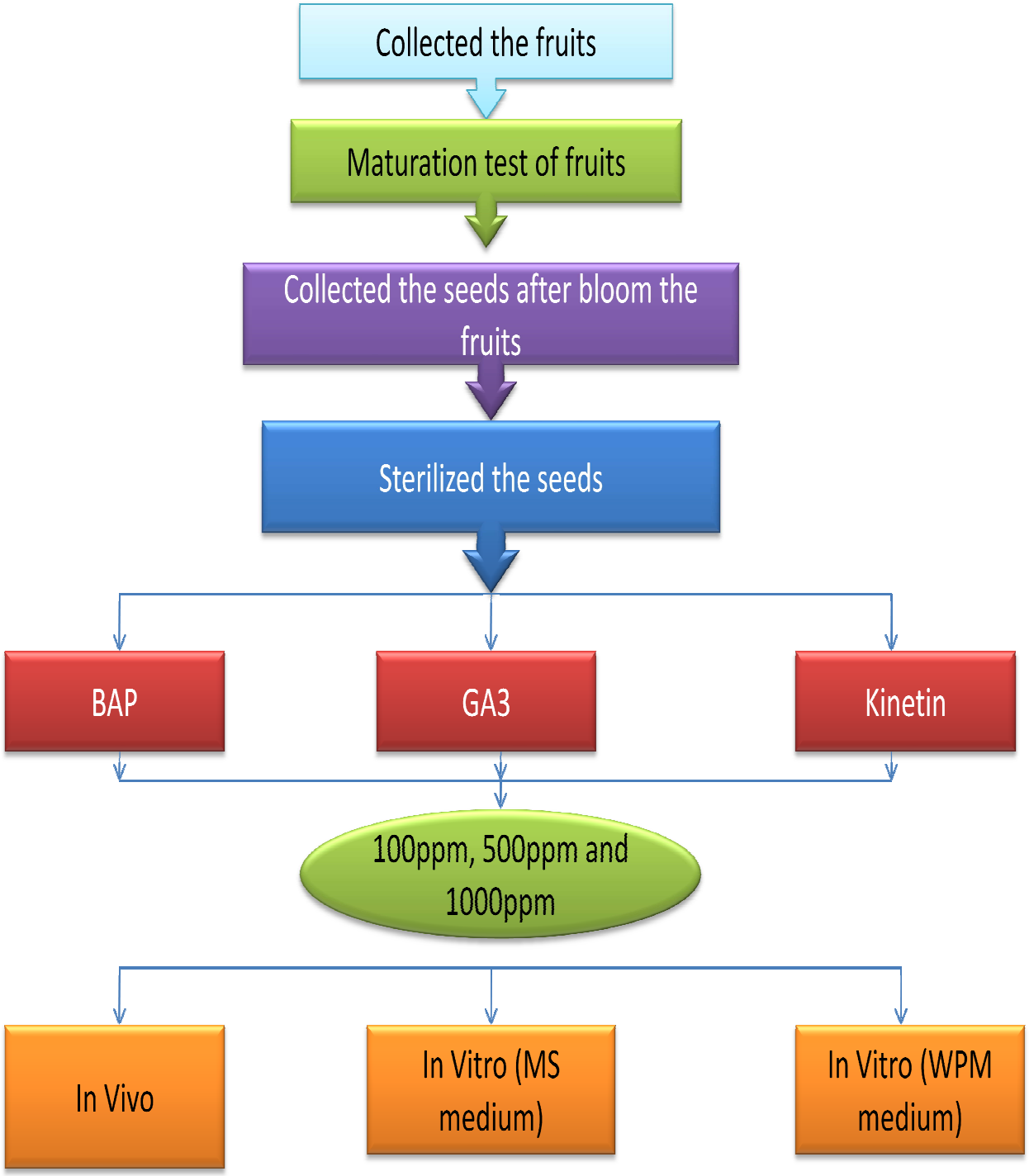

Figure 3.3: The flow chart of in vitro seed germination methods of *Sonneratia alba*.

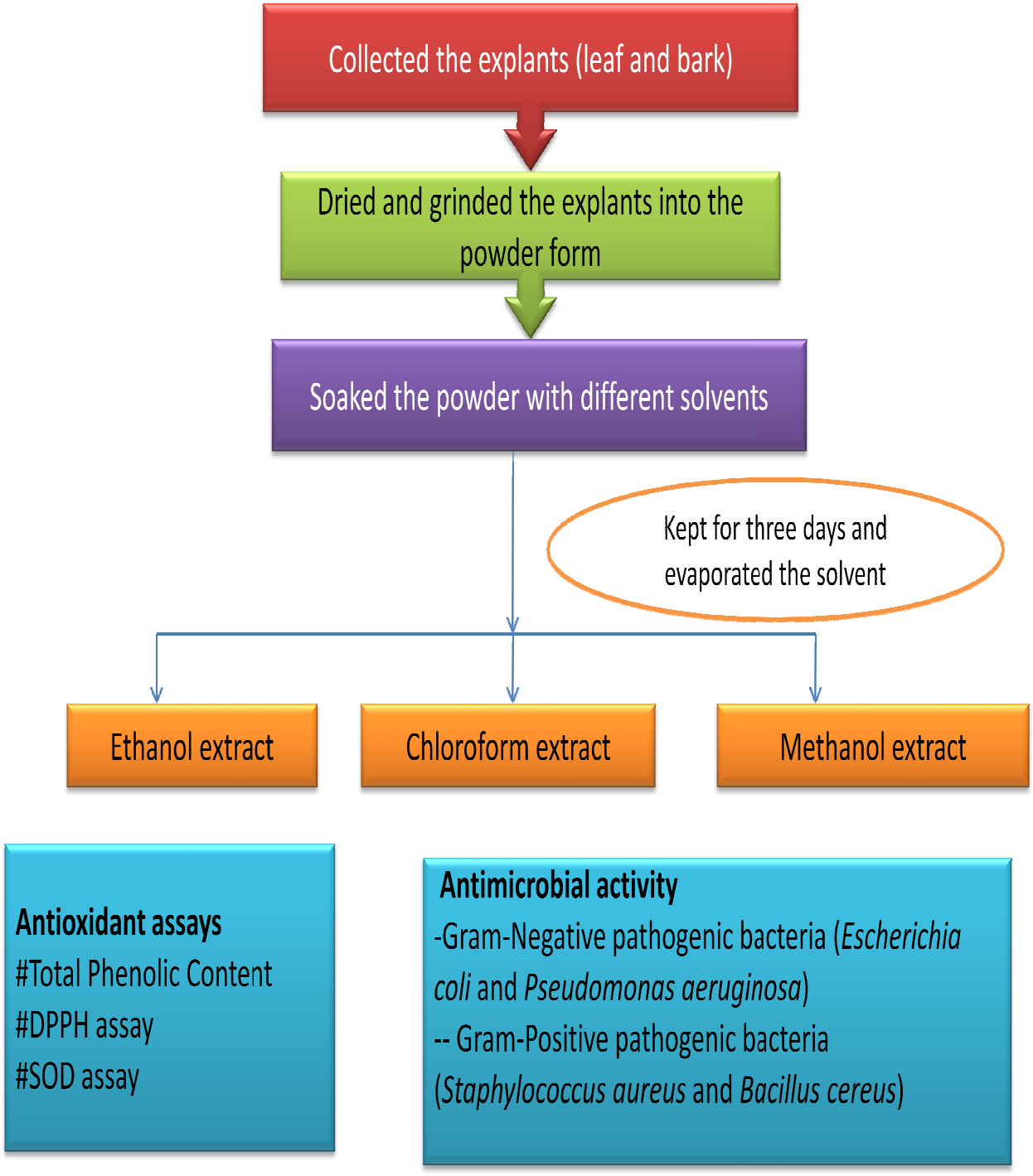

Figure 3.4: The flow chart of the methods of antioxidant and antimicrobial activities of crude extracts of *Sonneratia alba*, *Rhizophora mucronata* and *Bruguiera gymnorrhiza*.

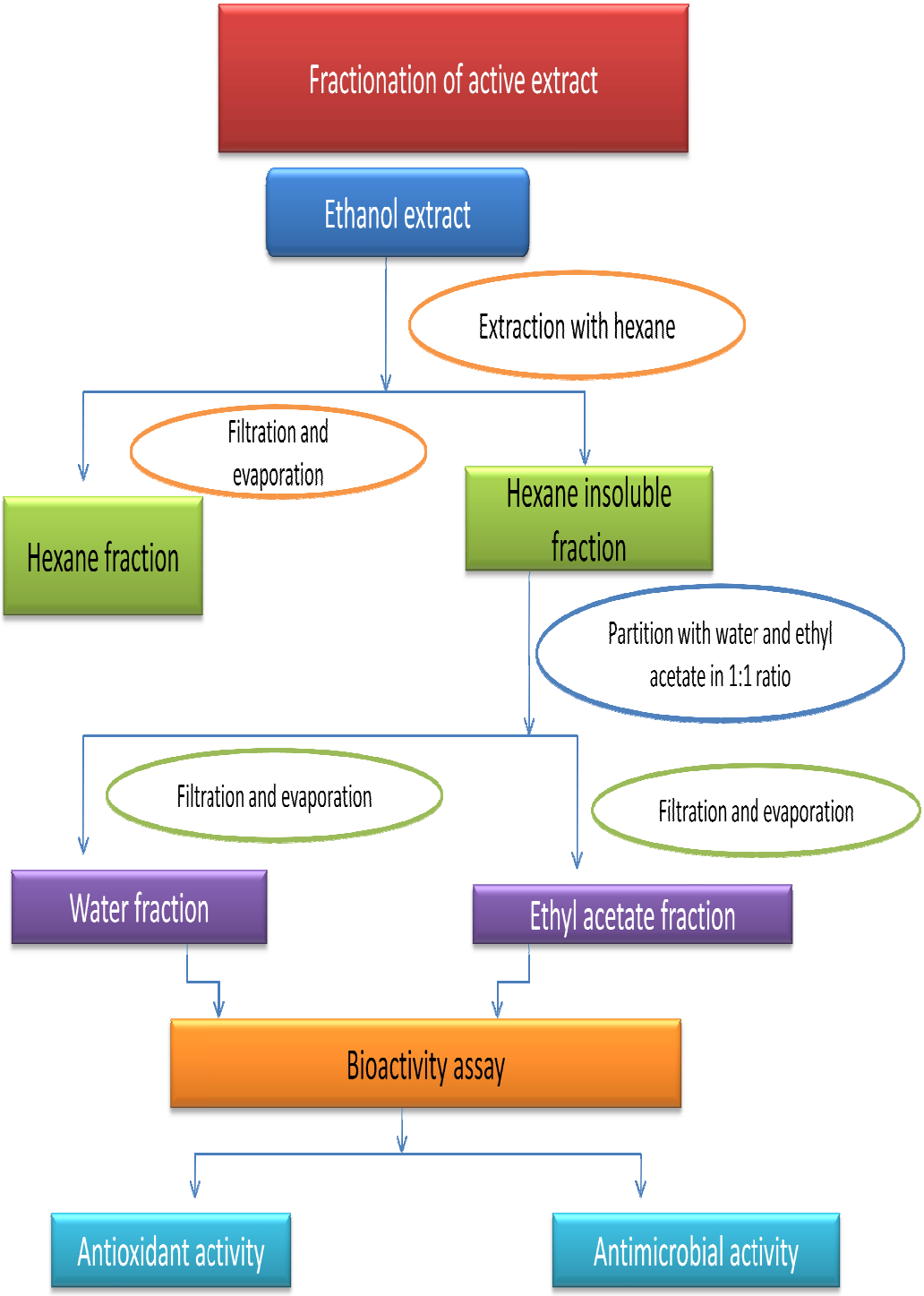

Figure 3.5: The flow chart of the methods of fractionation of the active crude extract into polar, semi-polar and non-polar fractions.

Chapter-4: Results

4.1. Effect of different hormones and medium on seed germination of *Sonneratia alba*:

Seeds of *Sonneratia alba* have been grown in different medium with different hormone concentration. It has been shown from Fig 4.1 the low concentration of BAP with WPM medium showed the highest germination rate among all concentrations and media. In vitro MS medium at the concentration of 500ppm showed the lowest germination at 40% rate.

Fig 4.2 showed that 100ppm GA_3 in WPM medium had the highest germination rate but the average germination rate was better in conventional *in vivo* medium. High and low concentrations of GA_3 *in vitro* medium showed a big difference in germination rate. Low rate of germination was observed at the concentration of 500ppm with *in vitro* MS medium.

High concentration of kinetin showed high rate of seed germination of *Sonneratia alba* (fig 4.3). 1000ppm with WPM medium showed better result compared with other medium and concentration. At the concentration of 500ppm showed the consistent germination rate in all medium. 100ppm WPM medium, 1000ppm MS medium and 100 and 1000ppm in vivo medium gave the 50% germination rate which was the lowest rate among all the medium and concentration.

In figure 4.4, all the medium and hormone concentration with control of seed germination of *Sonneratia alba* were compared. Among all the medium and concentration, BAP with in vitro WPM medium at the concentration of 100ppm showed the highest germination rate and GA_3 with MS and WPM media at the concentration of 500ppm showed the lowest germination rate which was also lower than control.

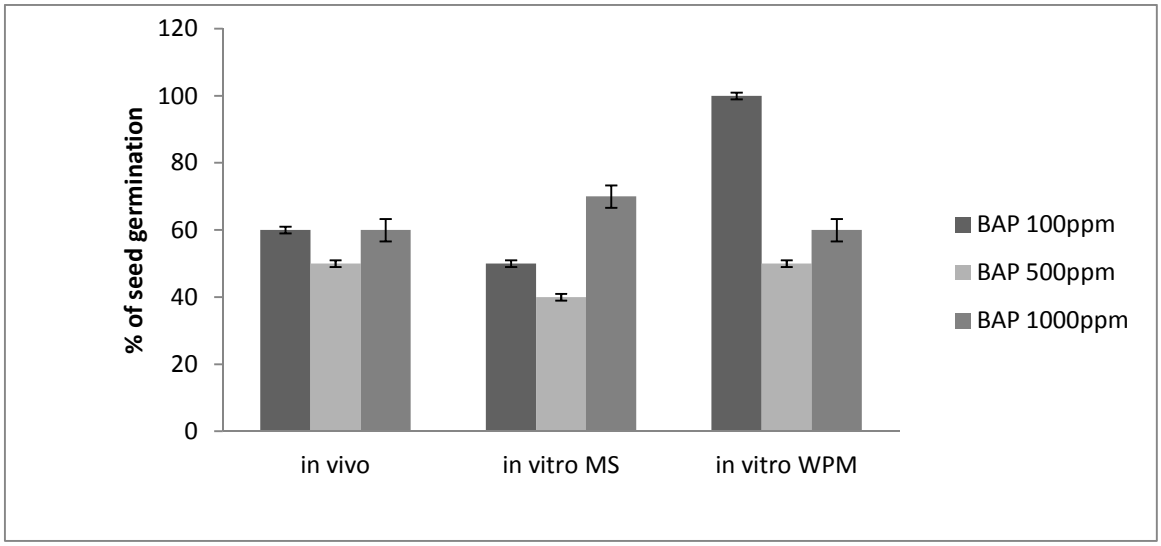

Figure 4.1: Seed germination of *Sonneratia alba* in different medium with BAP in concentration of 100ppm, 500ppm and 1000ppm. (Mean ± Sd, n=3)

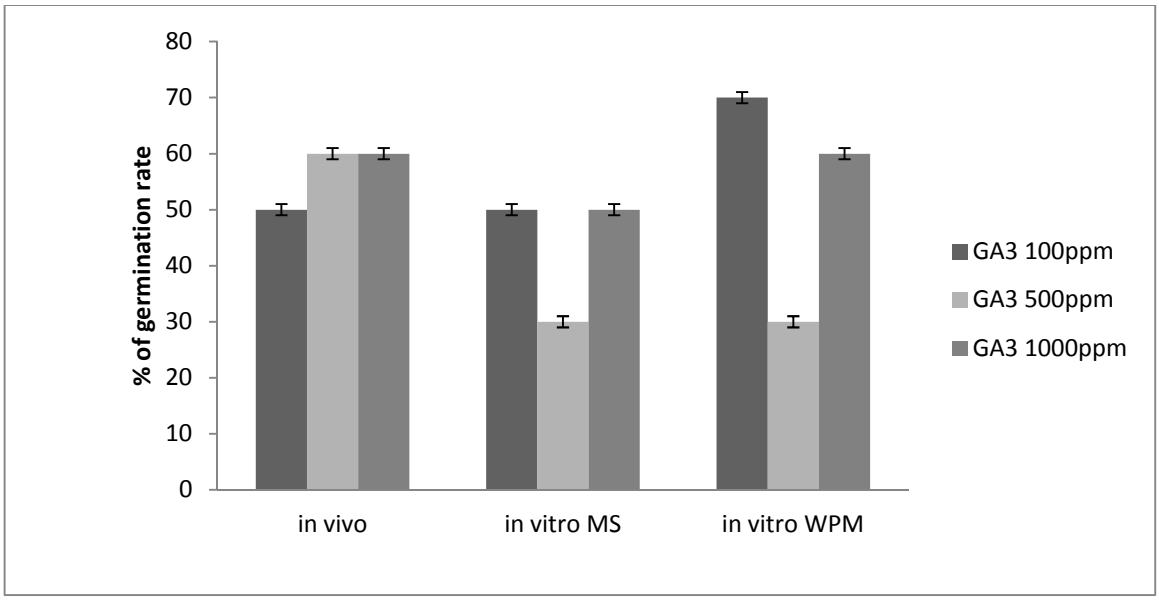

Figure 4.2: Seed germination of *Sonneratia alba* in different medium with GA_3 in concentration of 100ppm, 500ppm and 1000ppm. (Mean ± Sd, n=3)

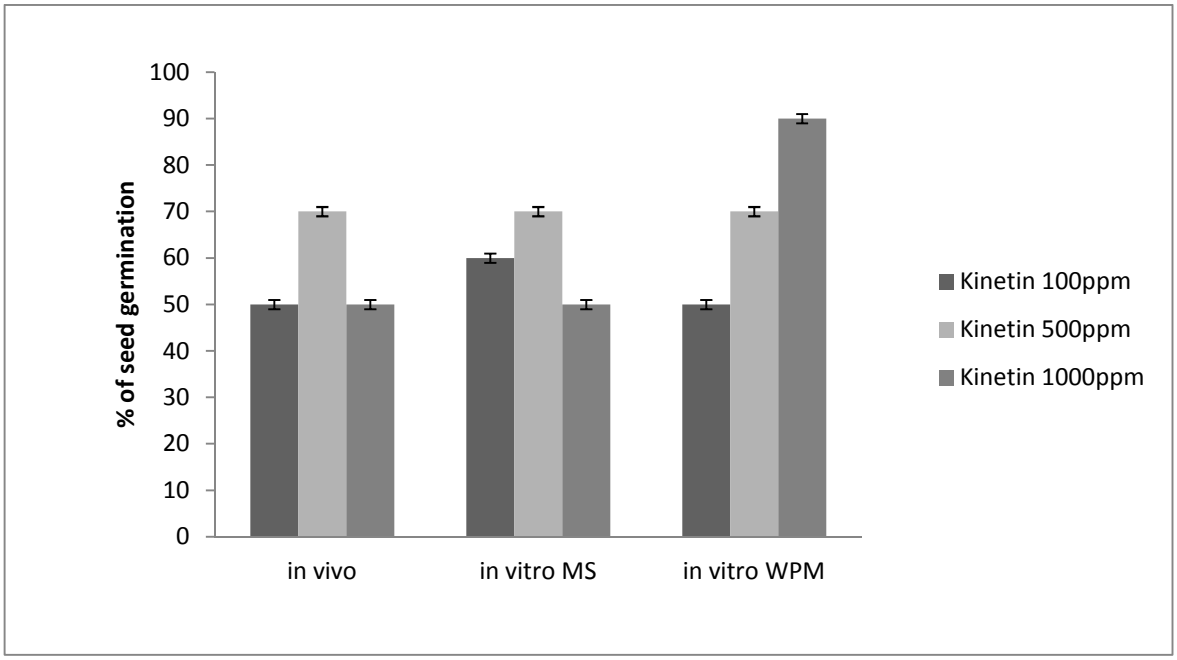

Figure 4.3: Seed germination of *Sonneratia alba* in different medium with Kinetin at the concentration of 100ppm, 500ppm and 1000ppm. (Mean ± Sd, n=3)

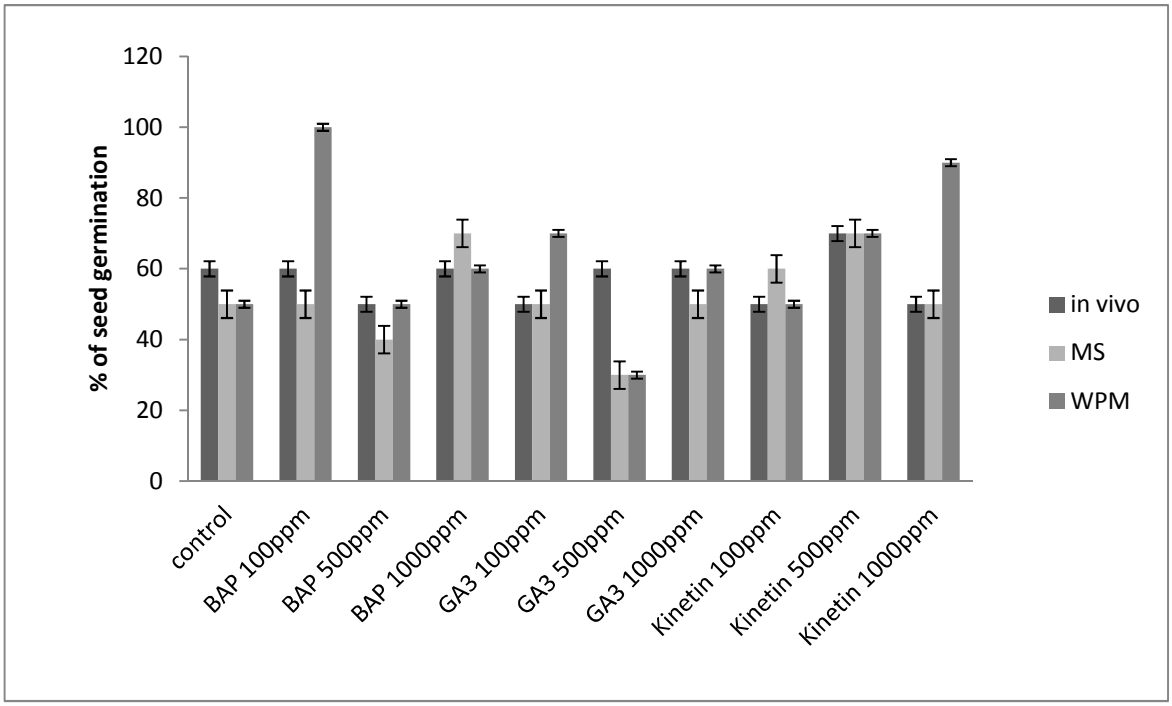

Figure 4.4: Seed germination of *Sonneratia alba* in different medium with different hormone at the concentration of 100ppm, 500ppm and 1000ppm. (Mean ± Sd, n=3)

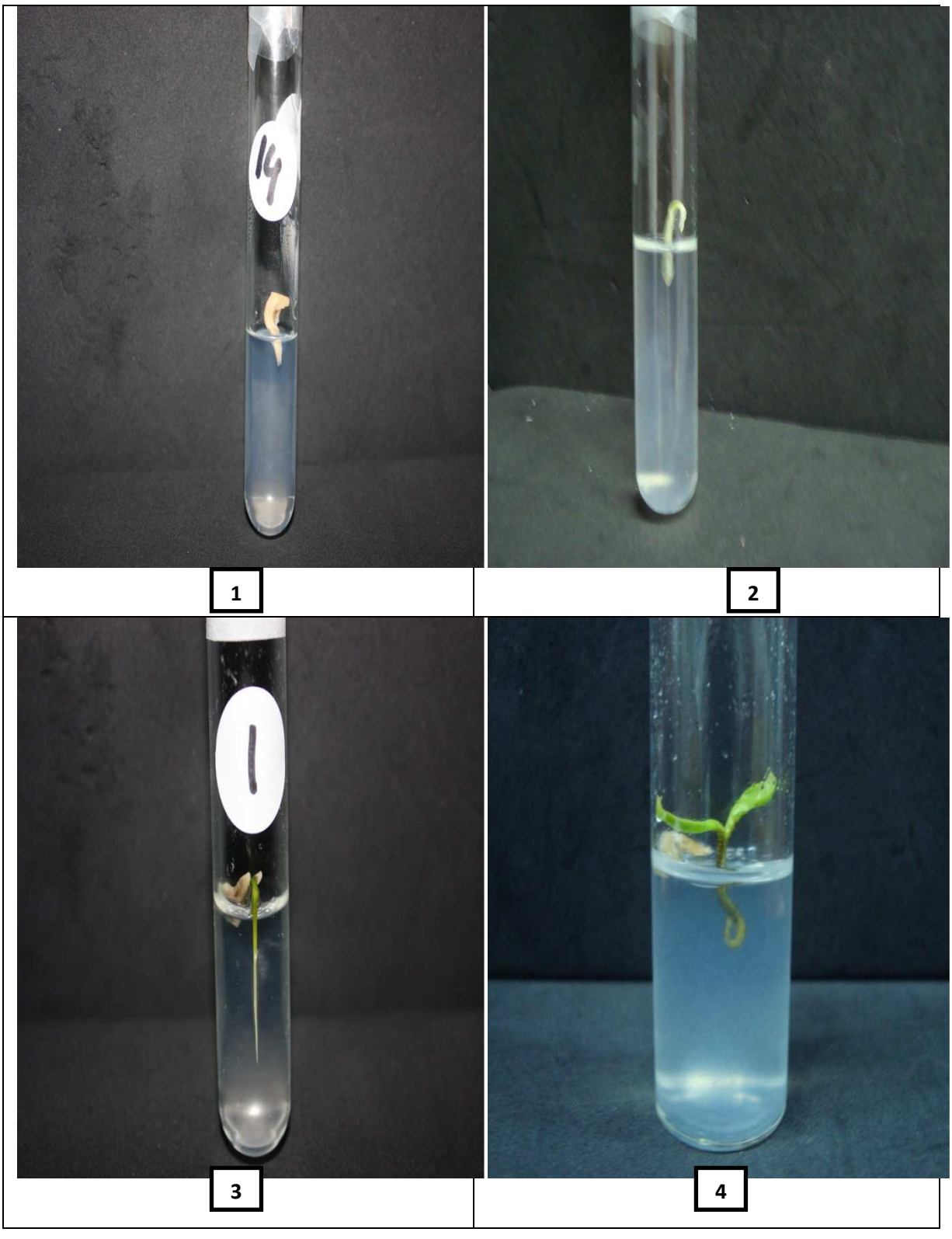

Figure 4.5: Different stage of Seed germination of Sonneratia alba with different hormones.

4.2. Total phenolic contents and the antioxidant activity of *Sonneratia alba*:

Antioxidant activities of methanol, ethanol and chloroform extracts of *in vivo* and *in vitro* explants of *Sonneratia alba* have been examined with SOD assay, DPPH assay and Reducing Power assay. Total phenolic content was also measured of all the extracts by using the Folin-Coicalteau method described by Slinkard and Singleton (1977).

It has been found from the table 4.1; all the methanol and ethanol extracts exhibited the high phenolic content whereas chloroform extracts exhibited low phenolic content. Ethanol extract of bark showed the highest phenolic content which was not significantly higher than methanol extract of bark but significantly higher than all other extracts. *In vitro* explants extracts showed comparatively lower phenolic contents than *in vivo* explants.

Ethanol extract of bark and chloroform extract of *in vitro* explants exhibited significantly highest and lowest antioxidant activity in SOD assay correspondingly. The ethanol extract of bark also showed the higher antioxidant activity than the positive control BHA.

Four concentrations have been used to evaluate the reducing power assay. The result of reducing power assay (Fig-4.6) showed that when the concentrations increased the reducing rates also increased. Among all the extracts, ethanol extract of barks showed the highest reducing rate and chloroform extract showed the lowest reducing rate. Low concentration of extracts showed low reducing rate and high concentration of extracts showed high reducing rate in this assay.

The result of IC_{50} value of methanol, ethanol and chloroform extracts of *in vivo* and *in vitro* explants has been presented in table-4.2. The result revealed that ethanol extract of bark showed the low IC_{50} value among all the crude extracts. However, ascorbic acid showed best IC_{50} value but there was no significant difference between ascorbic acid and ethanol

extract of bark. Chloroform extracts showed significantly high IC_{50} value compared with methanol and ethanol extracts.

Table 4.1: Total phenolic contents of *in vivo* and *in vitro* explants of ethanol, methanol and chloroform extracts of *Sonneratia alba.* Samples presented with different alphabetic letters are significantly different ($p<0.05$). (Mean ± SD, n=3)

Crude extracts of S. alba	Total phenolic contents (mg of GAE/g of dry explants
ML	**216.53 ± 3.09 c**
EL	**205.93 ± 4.278 cd**
CL	**23.60 ± 4.02 h**
MB	**299.53 ± 4.91 ab**
EB	**313.81 ± 5.54 a**
CB	**38.83 ± 2.95 g**
MV	**123.87 ± 3.24 e**
EV	**101.66 ± 2.58 f**
CV	**07.82 ± 1.49 i**

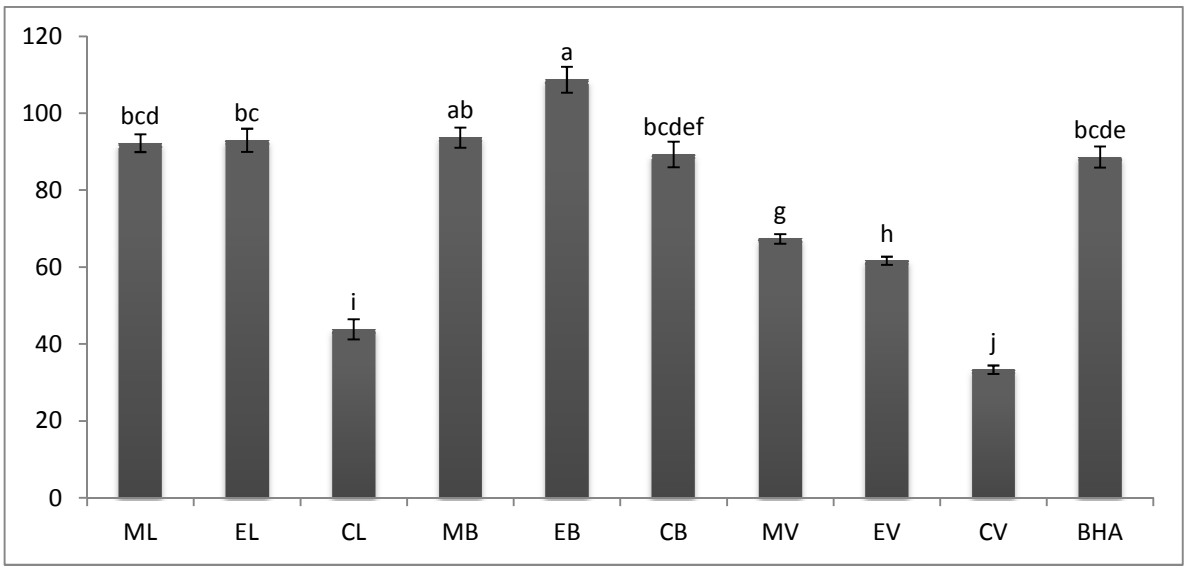

Figure 4.6: Antioxidant activity of ethanol, methanol and chloroform extracts of in vivo and in vitro explants of *Sonneratia alba* using SOD activity assay presented as inhibition rate. BHA was used as the positive control. Samples presented with different alphabetic letters are significantly different ($p<0.05$). (Mean ± SD, n=3)

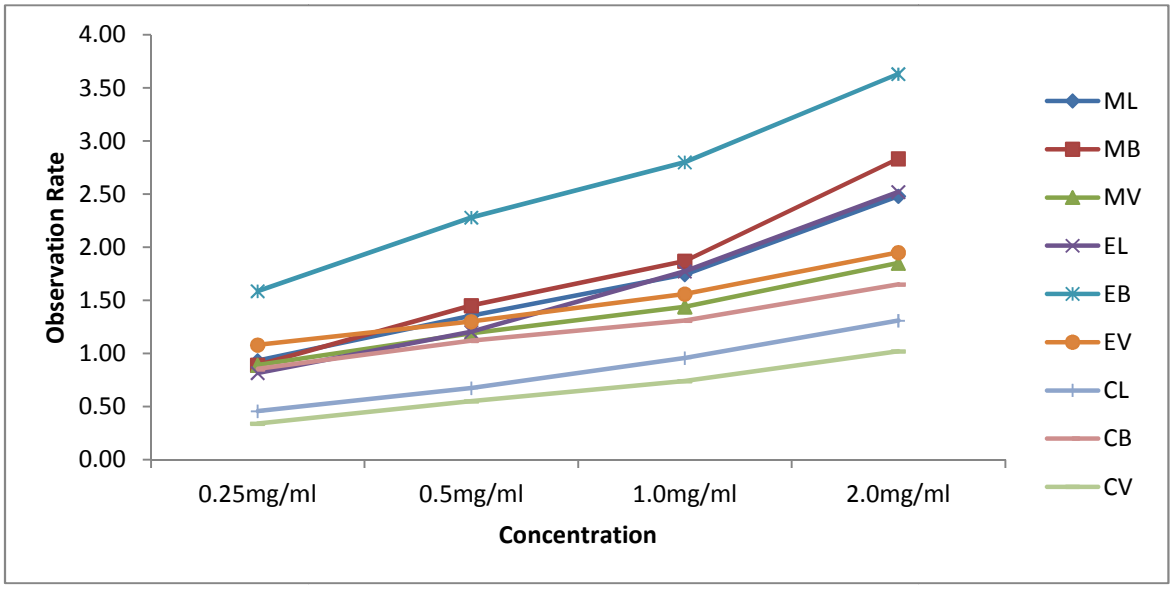

Figure 4.7: Antioxidant activity of ethanol, methanol and chloroform extracts of in vivo and in vitro explants of *Sonneratia alba* using reducing power assay presented. (Mean ± SD, n=3)

Table 4.2: IC_{50} value of in DPPH assay of methanol ethanol and chloroform extracts of in vivo and in vitro explants of *S. alba*. Samples presented with different alphabetic letters are significantly different ($p<0.05$). (Mean ± SD, n=3)

Crude extracts of S. alba	Value of IC_{50} (mg/ml)
ML	**0.038 ± 0.003e**
EL	**0.029 ± 0.004abcd**
CL	**0.27 ± 0.017hi**
MB	**0.025 ± 0.003abc**
EB	**0.0197 ± 0.001ab**
CB	**0.193 ± 0.015h**
MV	**0.073 ± 0.002f**
EV	**0.092 ± 0.003fg**
CV	**0.37 ± 0.004j**
Ascorbic Acid	**0.018 ± 0.003a**

4.3. Antimicrobial activity of *Sonneratia alba*:

The result of antimicrobial activity (table-3) showed that all the extracts except chloroform extracts of *in vitro* explants had the antimicrobial activity. Ethanol extracts exhibited higher antimicrobial activity among all the crude extracts. The ethanol extract of bark showed the highest inhibition zone against *S. aureus* 19.56cm. Chloroform extract of leaves had not shown any antimicrobial activity against *P. aeruginosa*. Meanwhile, in vitro explants also exhibited antioxidant activity but not as much as in explants. All the crude extracts and positive control showed high activity against *S. aureus* and low activity against *P. aureginosa*. Positive control (30 µg tetracycline) inhibited the growth of all tested bacteria significantly compared to the examined extracts.

Table 4.3: antimicrobial activity of methanol, ethanol and chloroform extracts of in vivo and in vitro explants of *Sonneratia alba.* (Mean ± SD, n=3)

Bacteria	ML	MB	MV	EL	EB	EV	CL	CB	CV	Positive control
B. cereus	**12.77 ±0.05**	**13.58± 0.04**	**9.46± 0.05**	**11.91 ±0.07**	**16.88± 0.09**	**8.74± 0.03**	**6.35± 0.01**	**7.55± 0.01**	**-**	**17.94±0 .04**
S. aureus	**14.82 ±0.05**	**16.86± 0.04**	**11.59 ±0.03**	**13.79 ±0.01**	**19.69± 0.12**	**9.03± 0.11**	**7.45± 0.05**	**8.78± 0.03**	**-**	**21.12±0 .03**
E. coli	**10.61 ±0.05**	**12.37± 0.03**	**8.44± 0.01**	**9.65± 0.05**	**13.78± 0.03**	**9.49± 0.04**	**5.81± 0.04**	**6.25± 0.05**	**-**	**14.35±0 .05**
P. aeruginosa	**9.85± 0.03**	**11.24± 0.01**	**6.45± 0.02**	**8.28± 0.03**	**12.12± 0.09**	**5.78± 0.02**	**-**	**5.48± 0.03**	**-**	**12.58±0 .03**

4.4. Total phenolic contents and antioxidant activity of *Rhizophora mucronata*:

Total phenolic content and the antioxidant activity of leaves and barks of *Rhizophora mucronata* have been studied in this experiment. SOD assay, DPPH assay and the reducing power assay used to evaluate the antioxidant potential of the samples.

The results of total phenolic content of leaves and barks extracts of *Rhizophora mucronata* have shown in table-4.4. It has been found from the table that ethanol extract of leaves had the highest phenolic component compare with all other extracts which was also significantly higher. Methanol and ethanol extracts showed better result compared with chloroform extracts. Chloroform extract of leaves showed significantly lower phenolic components.

Figure-4.7 has been shown that methanol extract of barks has the highest antioxidant potential in terms of enzymatic SOD assay. Methanol extract of leaves also showed better antioxidant activity which was even higher than the positive control BHA and there was no significant difference between the methanol extract of leaves and barks. The chloroform extract of barks showed the lowest activity which is significantly lower than other extracts.

Among all the extracts the ethanol extract of barks showed the better reducing rate in reducing power assay (fig-4.8). Whereas, chloroform extracts showed comparatively lower reducing rate than methanol and ethanol extracts. The reducing rate increased while the concentration of the sample increased. The lowest rate shown by the chloroform extract of leaves.

Scavenging activity of methanol, ethanol and chloroform extracts of leaves and barks of *Rhizophora mucronata* shown at figure-4.9. Among all the extract ethanol extract of leaves showed high scavenging activity which was not higher than the positive control ascorbic

acid but there was also no significant different between them. Chloroform extract of barks showed the significantly lower scavenging activity among all the tested extracts.

Table 4.4: Total phenolic contents of methanol, ethanol and chloroform extracts of leaves and barks of *Rhizophora mucronata*. Samples presented with different alphabetic letters are significantly different ($p<0.05$). (Mean ± SD, n=3)

Crude extracts of *Rhizophora mucronata*	**Total phenolic contents (mg of GAE/g of dry explants**
Methanol extract of leaves	**349.33 ± 0.61 b**
Ethanol extract of leaves	**358.6 ± 0.53 a**
Chloroform extract of leaves	**17.53 ± 0.12 e**
Methanol extract of barks	**271.33 ± 0.23 d**
Ethanol extract of barks	**300.27 ± 0.50 c**
Chloroform extract of barks	**12.67 ± 0.23 f**

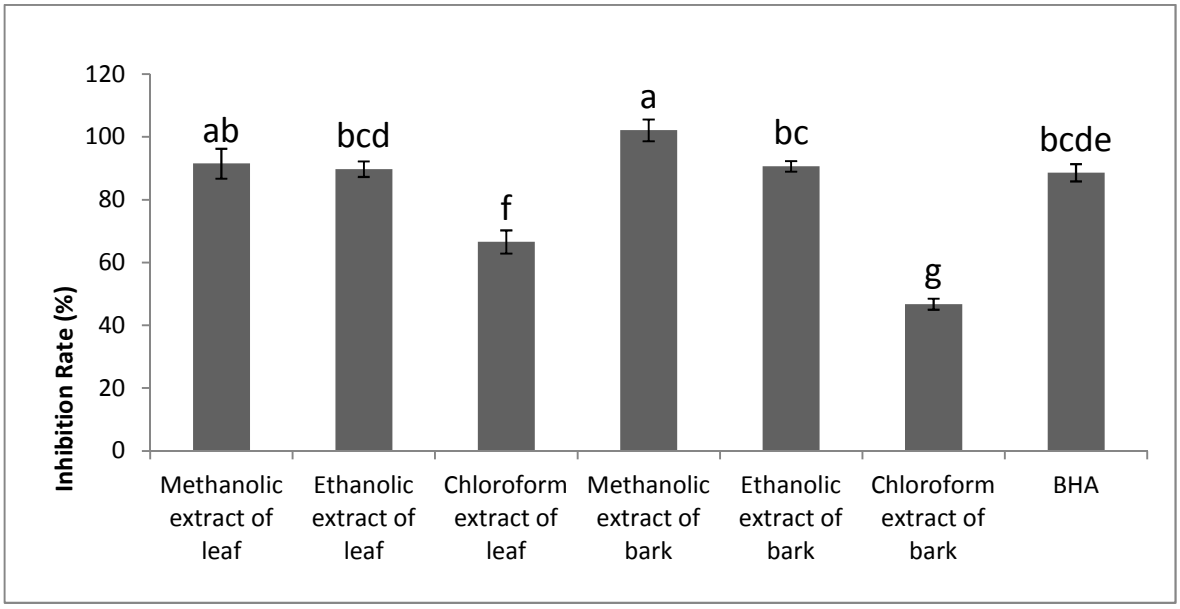

Figure 4.8: Antioxidant activity of methanol, ethanol and chloroform extracts of leaves and barks of *Rhizophora mucronata* using SOD activity assay presented as inhibition rate. BHA was used as the positive control. Samples presented with different alphabetic letters are significantly different ($p<0.05$). (Mean ± SD, n=3)

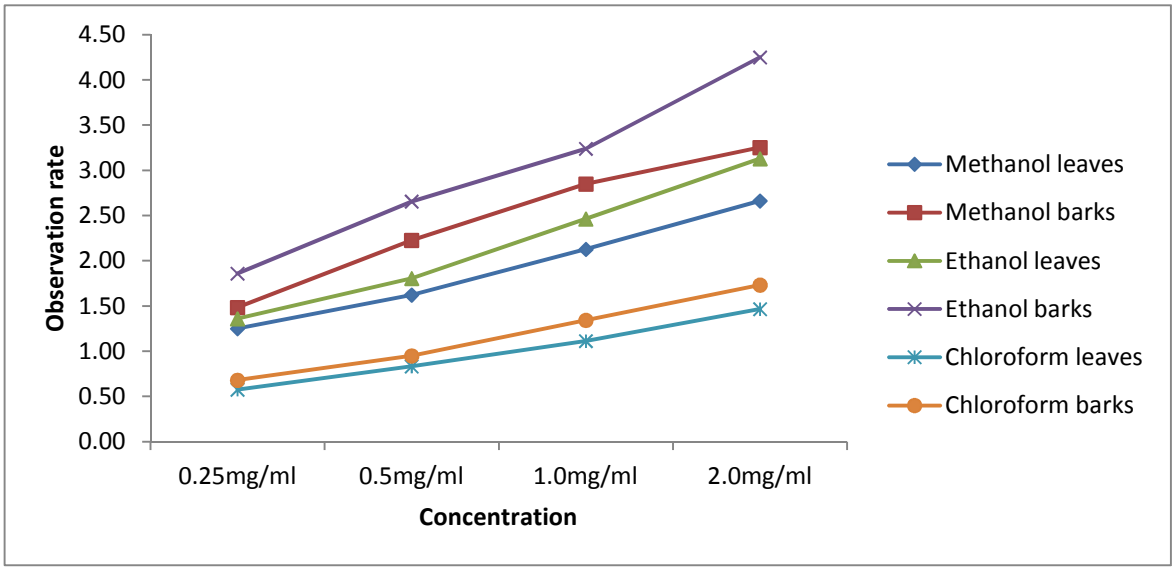

Figure 4.9: Antioxidant activity of methanol, ethanol and chloroform extracts of leaves and barks of *Rhizophora mucronata* using Reducing Power assay. (Mean ± SD, n=3)

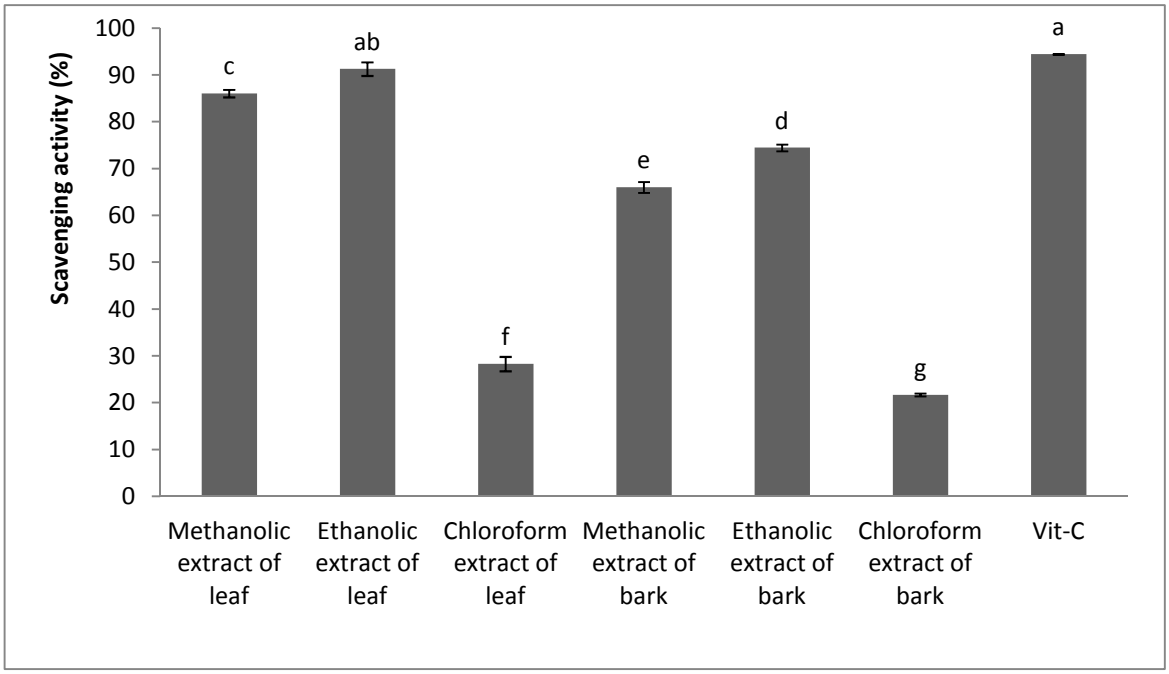

Figure 4.10: Antioxidant activity of methanol, ethanol and chloroform extracts of leaves and barks of *Rhizophora mucronata* using DPPH activity assay presented as inhibition rate. BHA was used as the positive control. Samples presented with different alphabetic letters are significantly different ($p<0.05$). (Mean ± SD, n=3)

4.5. Antimicrobial activity of *R. mucronata*:

Two gram positive and two gram negative bacteria have been tested to evaluate the antimicrobial activity of *Rhizophora mucronata* (table- 4.4). All the extracts except chloroform extract of barks showed high inhibition zone in gram-positive bacteria compare with gram-negative bacteria. Highest inhibition zone has shown the ethanol extract of leaves against *S. aureus* 20.78cm. Chloroform extracts showed low inhibition zone, moreover chloroform extract of barks didn't showed any antimicrobial activity against any bacteria. Ethanol extract of barks leaves exhibited the high antioxidant activity, while chloroform extract of barks showed no antimicrobial activity at all.

Table 4.5: The antimicrobial activity of methanol, ethanol and chloroform crude extracts of leaves of barks of *Rhizophora mucronata*. (Mean ± SD, n=3)

Bacteria	**Inhibition zone**						
	Methanol extract		**Ethanol extracts**		**Chloroform extracts**		**Positive control**
	Leaves	**Barks**	**Leaves**	**Barks**	**Leaves**	**Barks**	
B. cereus	**16.77±0.03**	**12.75±0.05**	**17.33±0.29**	**13.52±0.03**	**7.88±0.06**	**-**	**17.94±0.04**
S. aureus	**18.97±0.03**	**15.77±0.03**	**20.78±0.03**	**17.73±0.03**	**9.64±0.0**	**-**	**21.12±0.03**
E. coli	**13.88±0.03**	**10.58±0.03**	**14.33±0.29**	**12.63±0.03**	**-**	**-**	**14.35±0.05**
P. aeruginosa	**11.88±0.03**	**8.63±0.03**	**12.20±0.6**	**9.22±0.03**	**-**	**-**	**12.58±0.03**

4.6. Total phenolic contents and antioxidant activity of Bruguiera gymnorrhiza:

Table-4.6 represents the total phenolic contents of methanol, ethanol and chloroform extract of leaves and barks of *Bruguiera gymnorrhiza*. Ethanol extract of barks and chloroform extract of leaves showed the significantly highest and lowest total phenolics, respectively. Barks extracts exhibited the higher phenolics compared with leaves extracts.

Three antioxidant assays have been used to evaluate the potential of the antioxidant value of *Bruguiera gymnorrhiza*. The result of SOD assay has shown at figure-4.10. The results showed that ethanol extract of barks exhibited highest inhibition rate compared with all sample but not significantly higher than methanol and ethanol extracts of leaves.

Chloroform extract of leaves and barks showed low inhibition rate and there was no significant difference between them.

Result of reducing power assay shows on figure- 4.12. The result showed that the reducing rate is higher when the concentration is higher of the samples. Methanol and ethanol extracts exhibited the better antioxidant potential compared with chloroform extracts. Ethanol extract of barks and the chloroform extract of leaves had the highest and lowest reducing rate respectively.

Among all the crude extracts ethanol extract of barks showed the highest scavenging rate (87.24%), however there was no significant difference with methanol extract of barks and the significantly lowest rate showed on chloroform extract of leaves (30.53%) in terms of DPPH assay (figure- 4.11). Ascorbic acid showed the significantly higher antioxidant activity compared with all crude extracts.

Table 4.6: Total phenolic contents of methanol, ethanol and chloroform extracts of leaves and barks of *Burguiera gymnorrhiza*. Samples presented with different alphabetic letters are significantly different ($p<0.05$). (Mean ± SD, n=3)

Crude extracts of *Bruguiera gymnorrhiza*	**Total phenolic contents (mg of GAE/g of dry explants**
Methanol extract of leaves	**178.73 ± 0.23 d**
Ethanol extract of leaves	**189.4 ± 0.6 c**
Chloroform extract of leaves	**13.13 ± 0.23 f**
Methanol extract of barks	**268.47 ± 0.12 b**
Ethanol extract of barks	**284.93 ± 0.23 a**
Chloroform extract of barks	**23.6 ± 0.35 e**

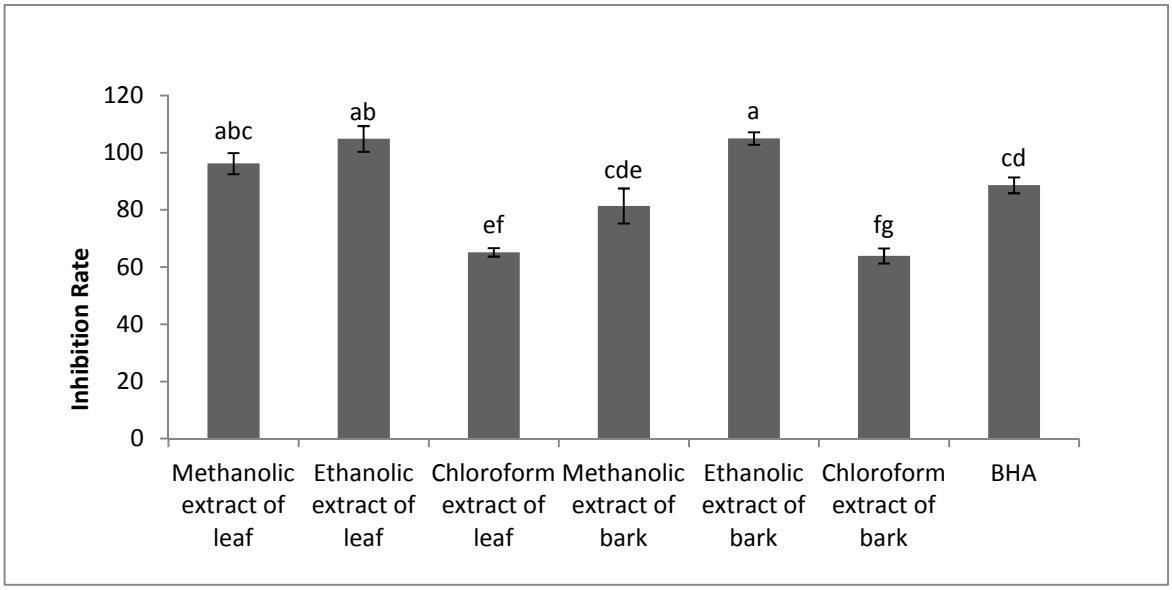

Figure 4.11: Antioxidant activity of methanol, ethanol and chloroform extracts of leaves and barks of *Bruguiera gymnorrhiza* using SOD activity assay presented as inhibition rate. BHA was used as the positive control. Samples presented with different alphabetic letters are significantly different ($p<0.05$). (Mean ± SD, n=3)

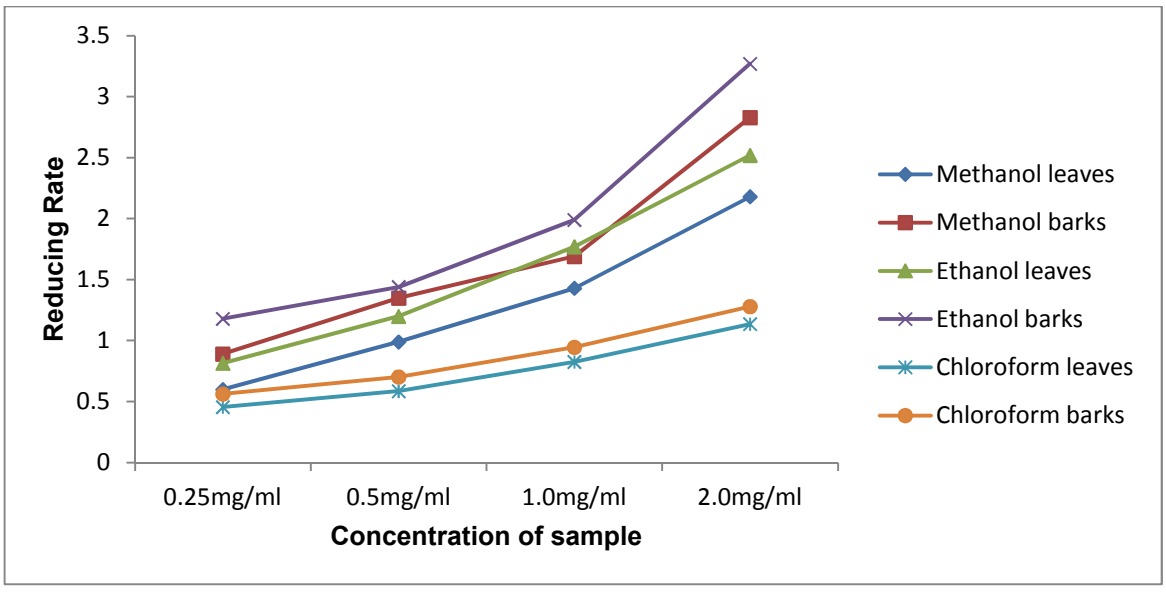

Figure 4.12: Antioxidant activity of methanol, ethanol and chloroform extracts of leaves and barks of *Bruguiera gymnorrhiza* using Reducing Power assay. (Mean ± SD, n=3)

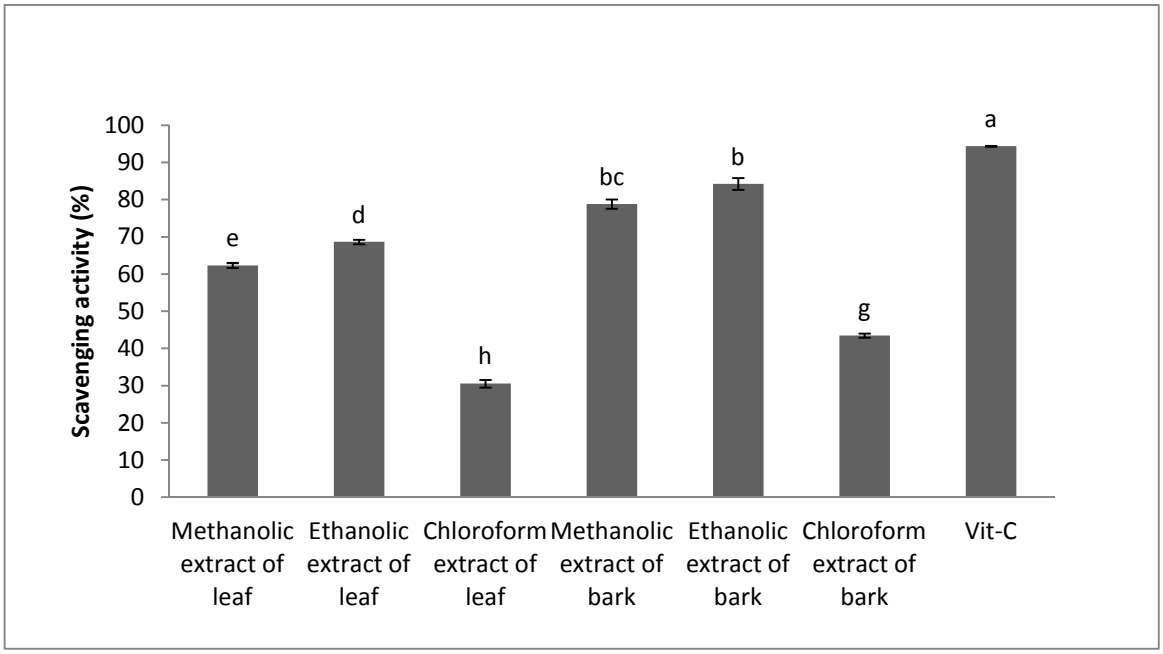

Figure 4.13: Antioxidant activity of methanol, ethanol and chloroform extracts of leaves and barks of *Bruguiera gymnorrhiza* using DPPH activity assay presented as inhibition rate. Ascorbic acid was used as the positive control. Samples presented with different alphabetic letters are significantly different ($p<0.05$). (Mean ± SD, n=3)

4.7. Antimicrobial activity of *Bruguiera gymnorrhiza*:

The results of antimicrobial activity of *Bruguiera gymnorrhiza* have shown on table-4.7. Methanol and ethanol extract of leaves and barks showed better antimicrobial activity compared with chloroform extracts of leaves and barks. The chloroform extract of leaves had shown no antimicrobial activity against any microbial whereas chloroform extract of barks had shown small inhibition zone against gram-positive bacteria and no inhibition zone against gram-negative bacteria. Between leaves and barks extracts, barks extracts showed better antimicrobial activity. All the extracts showed high inhibition zone against gram-positive bacteria compared with gram-negative bacteria.

Table 4.7: Antimicrobial activity of methanol, ethanol and chloroform crude extracts of leaves and barks of *Bruguiera gymnorrhiza.* (Mean ± SD, n=3)

Bacteria	**Inhibition zone**						
	Methanol extract		**Ethanol extracts**		**Chloroform extracts**		**Positive control**
	Leaves	**Barks**	**Leaves**	**Barks**	**Leaves**	**Barks**	
B. cereus	**12.68±0.03**	**15.88±0.03**	**14.87±0.03**	**16.79±0.04**	**-**	**8.76±.03**	**17.94±0.04**
S. aureus	**14.33±0.03**	**17.88±0.03**	**15.87±0.03**	**18.97±0.03**	**-**	**9.77±.03**	**21.12±0.03**
E. coli	**8.88±0.03**	**9.28±0.03**	**9.86±0.04**	**11.58±0.03**	**-**	**-**	**14.35±0.05**
P. aeruginosa	**7.87±0.03**	**8.85±0.05**	**8.88±0.03**	**9.82±0.03**	**-**	**-**	**12.57±0.03**

4.8. Bioactivity of fractionated ethanol extract of *Sonneratia alba*:

Ethanol extract of bark of *Sonneratia alba* was the best extract among all the extracts. The ethanol extract was then fractionated into polar, semi polar and non-polar fraction. The amount of hexane fraction was very little (0.3g out of 50g ethanol extract) that's why hexane fraction had not consider as further antioxidant and antimicrobial activities test. The water extract showed better antioxidant and antimicrobial activities in all the tests compare with ethyl acetate extract. In superoxide dismutase (SOD) antioxidant assay, water fraction showed higher antioxidant activity than ethyl acetate and BHA. But there is no significant difference between all the examined samples.

The IC_{50} value was calculated in DPPH assay. Ascorbic acid was used as positive control but there was no significant difference between the value of IC_{50} of water fraction and the ascorbic acid. The reducing power assay also showed that water fraction of *S. alba* is better fraction in antioxidant activity compare with ethyl acetate fraction. When the concentration increase the reducing rate also increase with the concentration. 2mg/ml showed the highest reducing rate both for water and ethyl acetate fraction.

Both water and ethyl acetate fraction showed positive result against all four bacterial pathogen. The inhibition rate was not as high as the positive control (tetracycline). The highest inhibition zone was found for water fraction against *S. aureus* (16.75cm) and the lowest against *P. aureginosa* (9.85cm). Ethyl acetate fraction showed less antimicrobial activity than water fraction. The lowest inhibition zone of ethyl acetate was 7.85cm where as 12.60 cm for tetracycline.

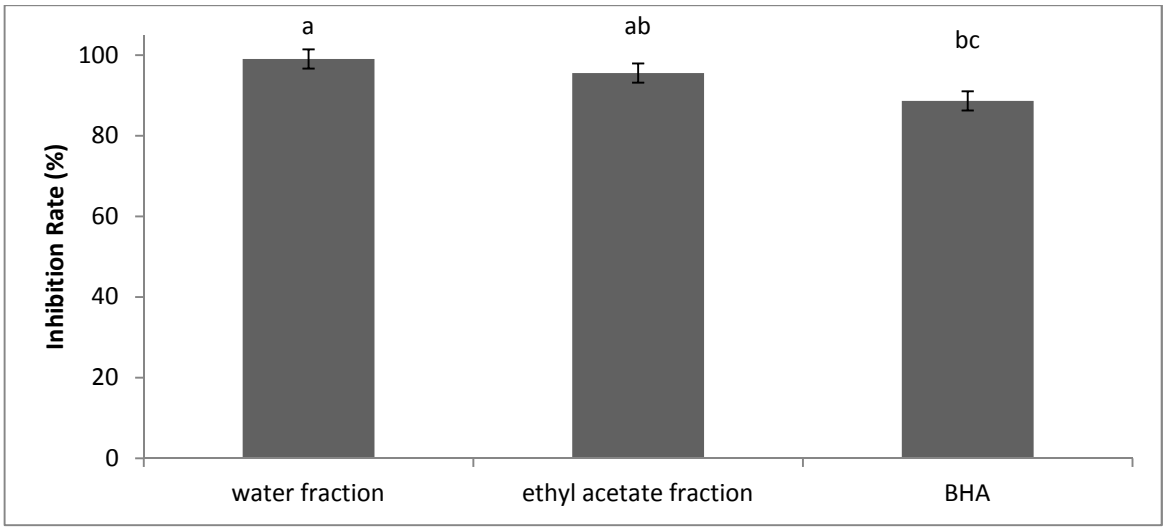

Figure 4.14: Antioxidant activity of water and ethyl acetate fraction of fractionated ethanol extract of *Sonneratia alba* using SOD activity assay presented as inhibition rate. BHA was used as the positive control. Samples presented with different alphabetic letters are significantly different ($p<0.05$). (Mean ± SD, n=3)

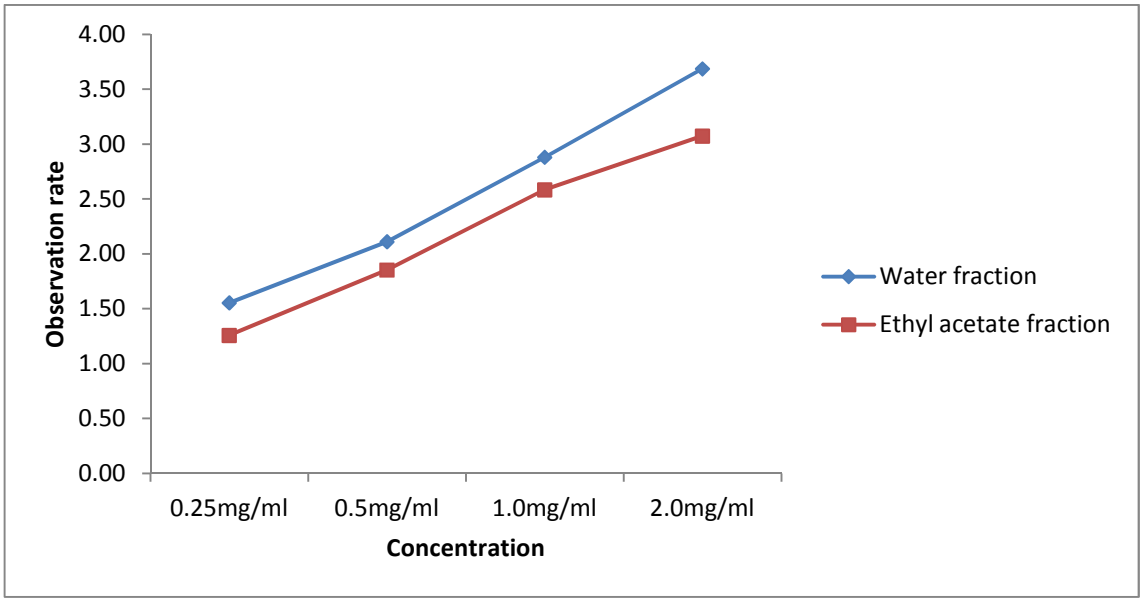

Figure 4.15: Antioxidant activity of water and ethyl acetate fraction of fractionated ethanol extract of *Sonneratia alba* using reducing power assay. (Mean ± SD, n=3)

Table 4.8: IC50 value of in DPPH assay of water and ethyl acetate fraction of fractionated ethanol extract of *Sonneratia alba.* Samples presented with different alphabetic letters are significantly different ($p<0.05$). (Mean ± SD, n=3)

Sample	Value of IC_{50} (mg/ml)
Water fraction	**0.024 ± 0.001 ab**
Ethyl acetate fraction	**0.124 ± 0.0012 c**
Ascorbic acid	**0.018 ± 0.004 a**

Table 4.9: antimicrobial activity of water and ethyl acetate fraction of fractionated ethanol fextract of *Sonneratia alba.* (Mean ± SD, n=3)

Bacteria	Water extract	Ethyl acetate extract	Positive control
B. cereus	**13.57 ± 0.03**	**9.62 ± 0.03**	**17.94 ± 0.04**
S. aureus	**16.75 ± 0.05**	**13.35 ± 0.05**	**21.12 ± 0.03**
E. coli	**10.48 ± 0.03**	**7.98 ± 0.03**	**14.35 ± 0.05**
P. aeruginosa	**9.88 ± 0.03**	**7.84 ± 0.04**	**12.58 ± 0.03**

4.9. The liquid chromatography tandem mass spectrometry (LCMS/MS) analysis of Water fraction:

The liquid chromatography tandem mass spectrometries (LCMS/MS) were used for detection of numeric mass of the active compound of water fraction of *Sonneratia alba*. We studied the nominal masses of the major active components in the water fraction, and we observed 8 such peaks at m/z values of 331, 345, 452, 456, 463, 473, 480 and 494. Each of the 8 major peaks observed were found to have rich fragmentation patterns from the MS/MS spectrum and this unique patterns (also known as fingerprint) corresponds to the high antioxidant activity of the extracted water fraction of the plant sample.

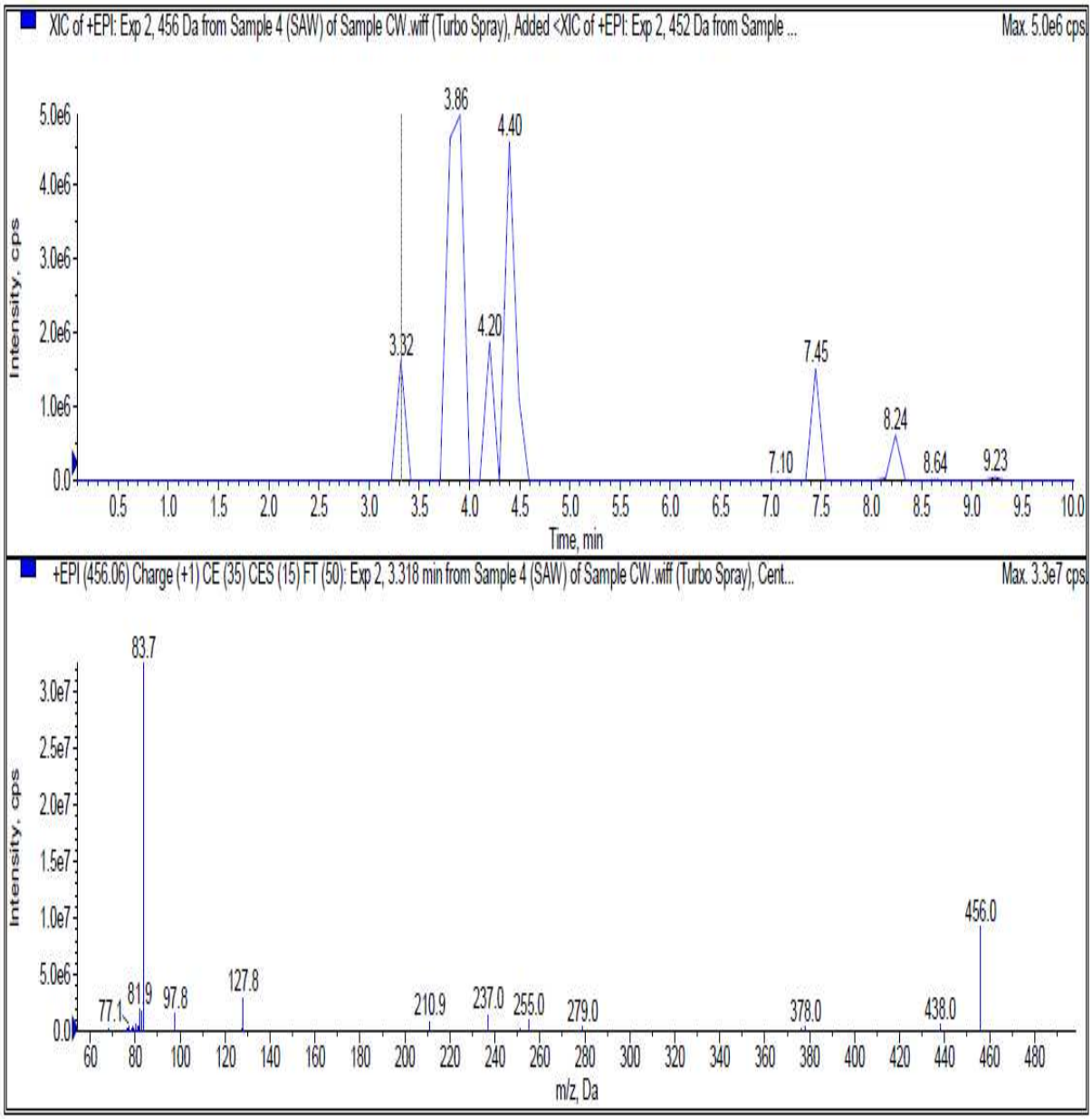

Figure 4.16: Fragmentation pattern from MS/MS spectrum of the major peak with m/z value 456.

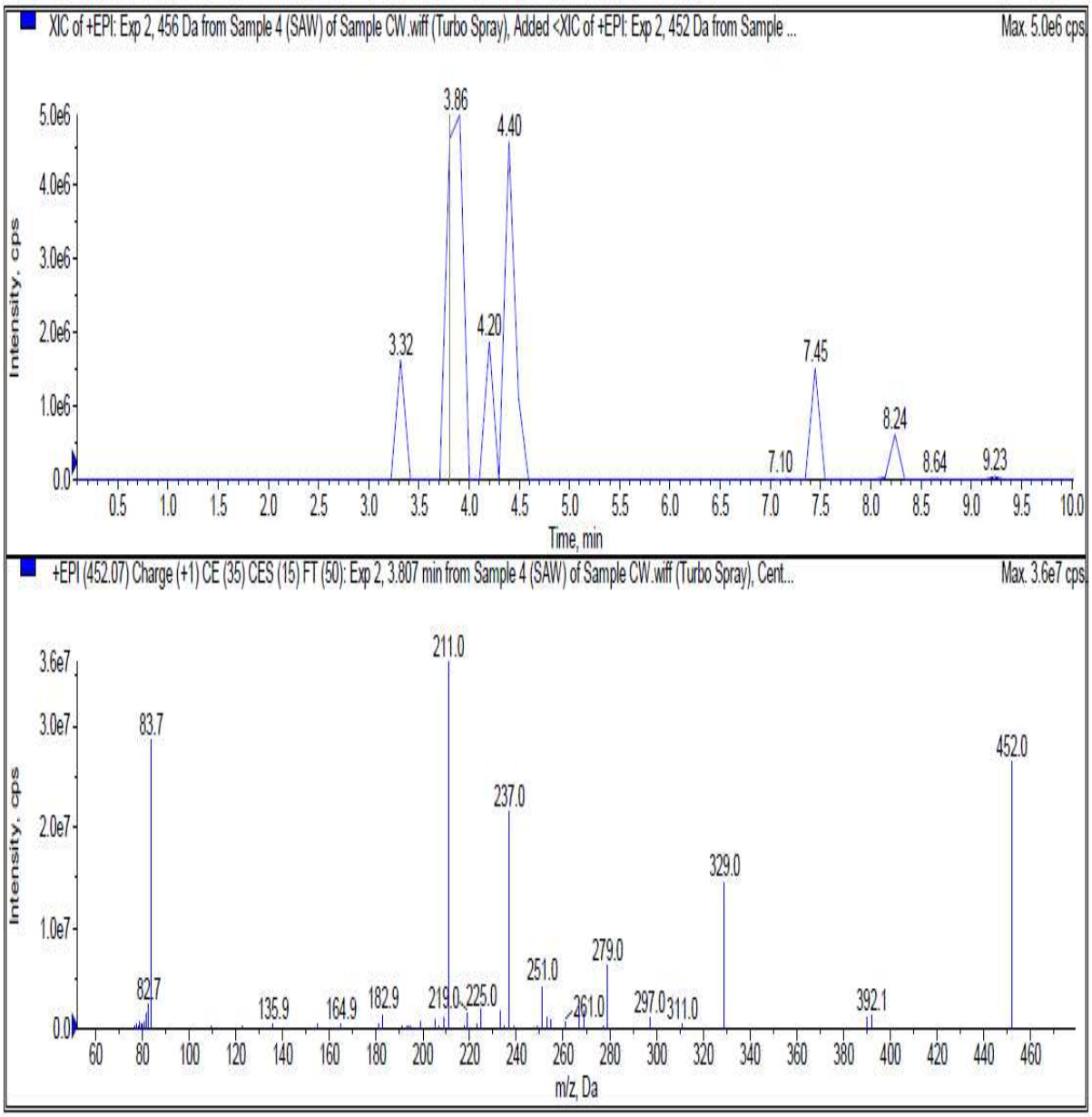

Figure 4.17: Fragmentation pattern from MS/MS spectrum of the major peak with m/z value 452.

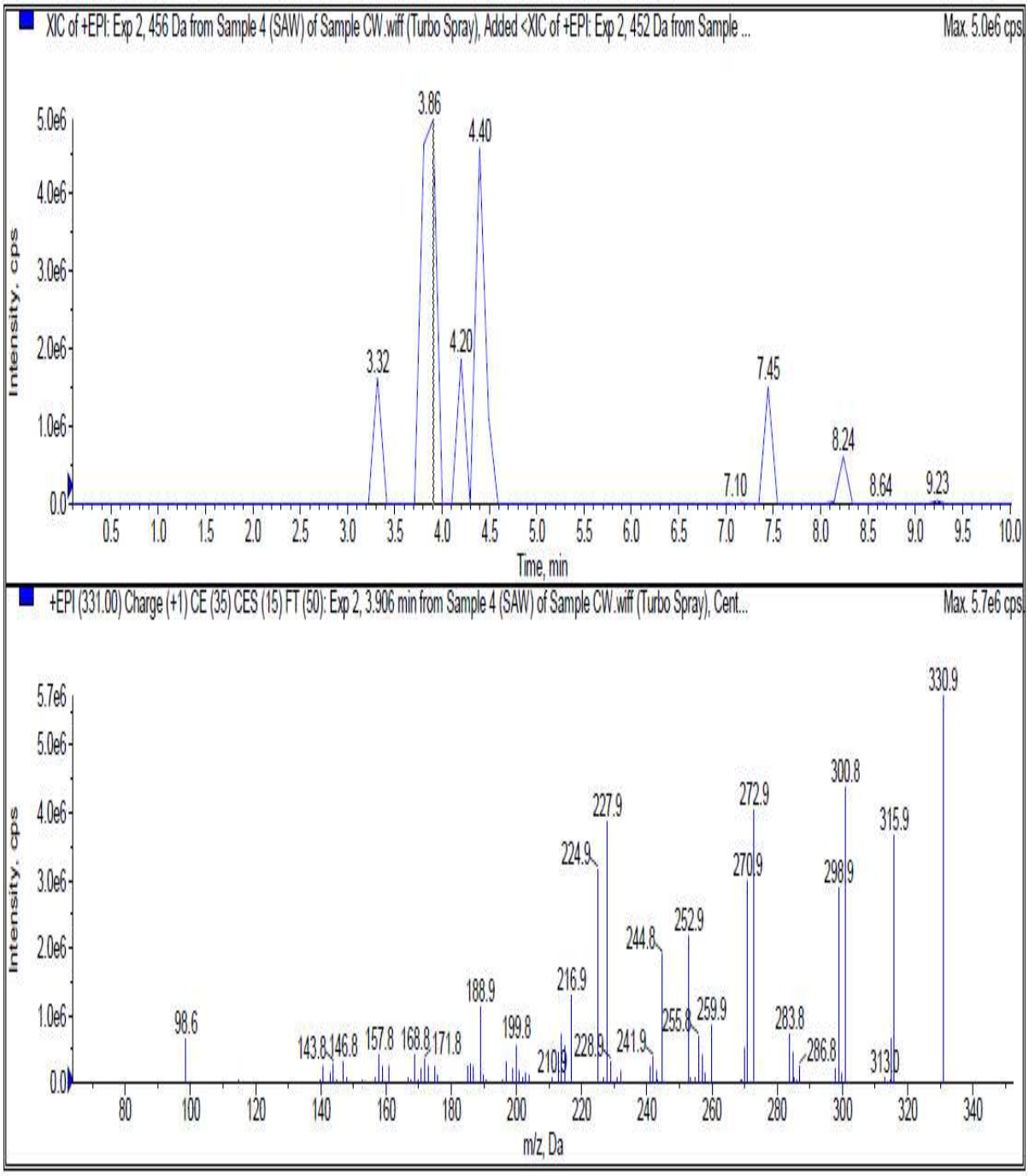

Figure 4.18: Fragmentation pattern from MS/MS spectrum of the major peak with m/z value 331.

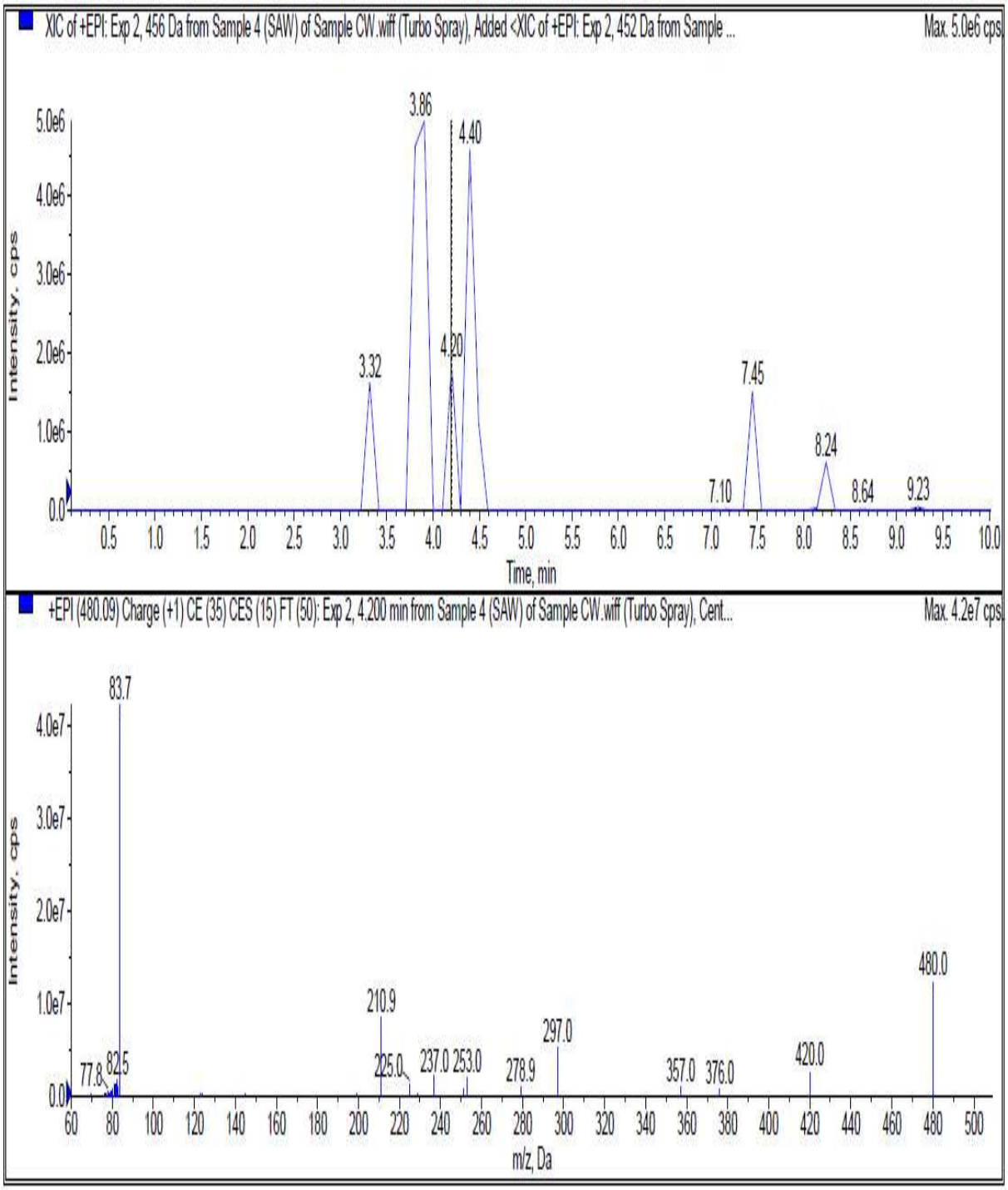

Figure 4.19: Fragmentation pattern from MS/MS spectrum of the major peak with m/z value 480.

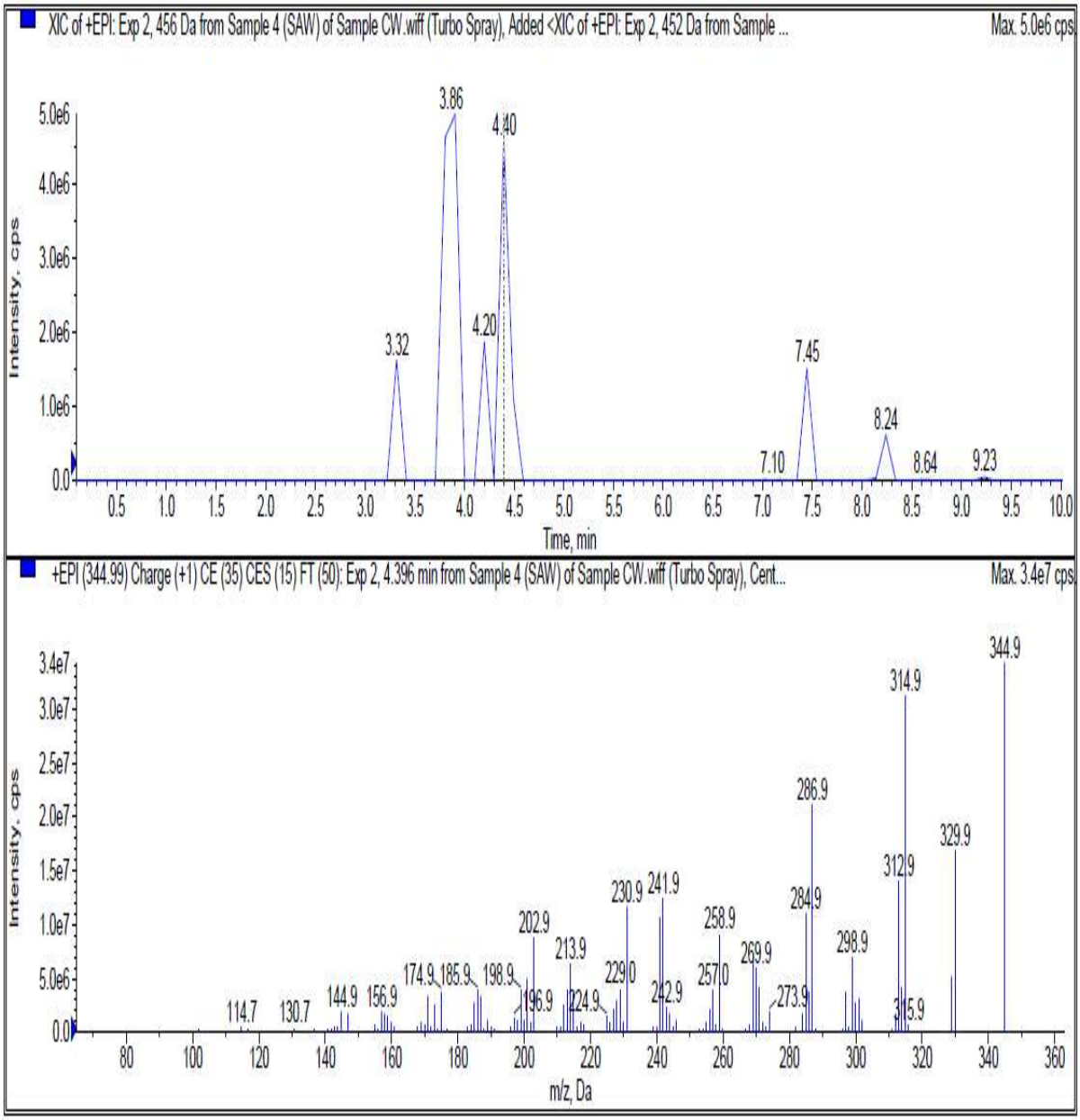

Figure 4.20: Fragmentation pattern from MS/MS spectrum of the major peak with m/z value 345.

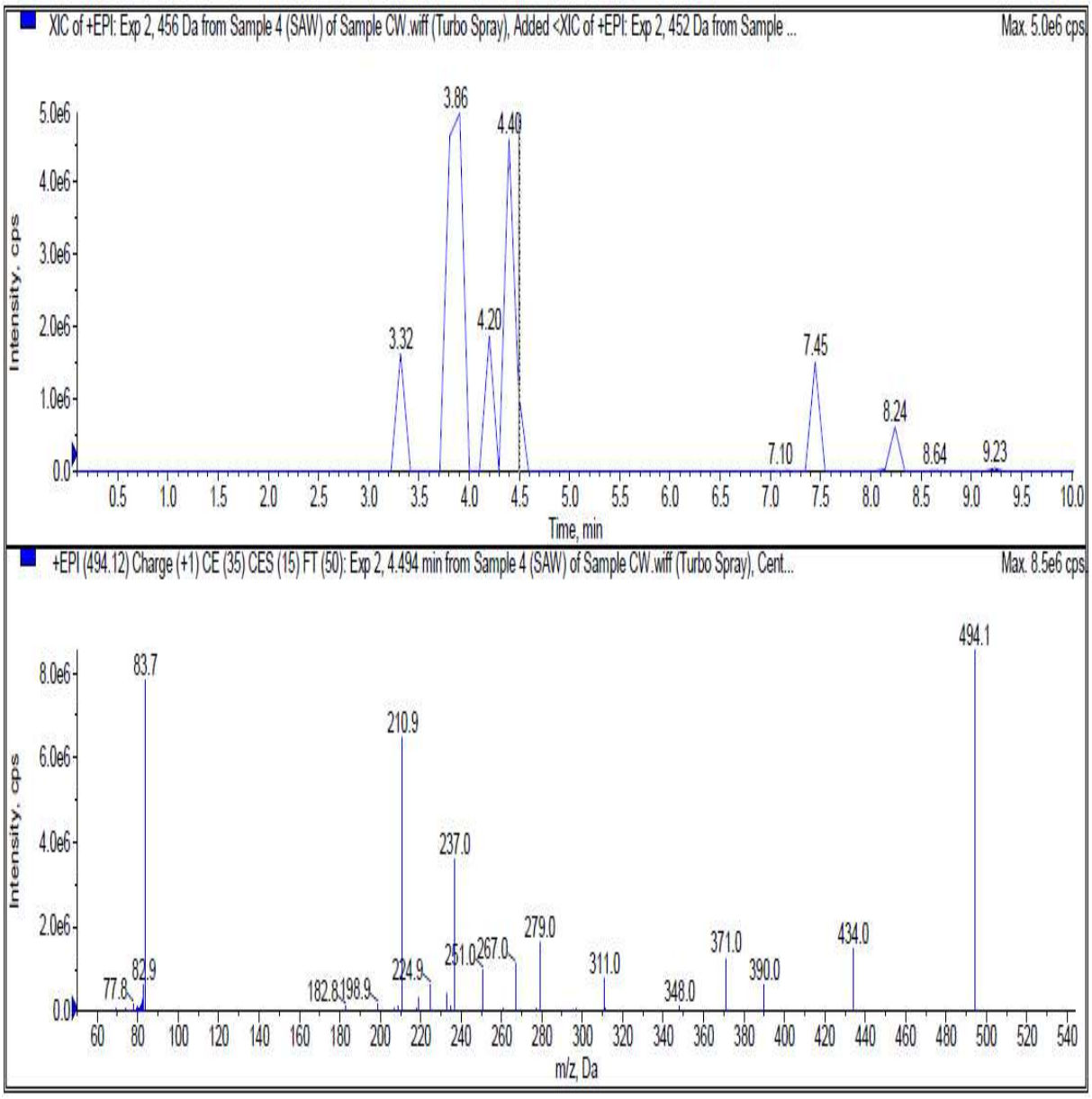

Figure 4.21: Fragmentation pattern from MS/MS spectrum of the major peak with m/z value 494.

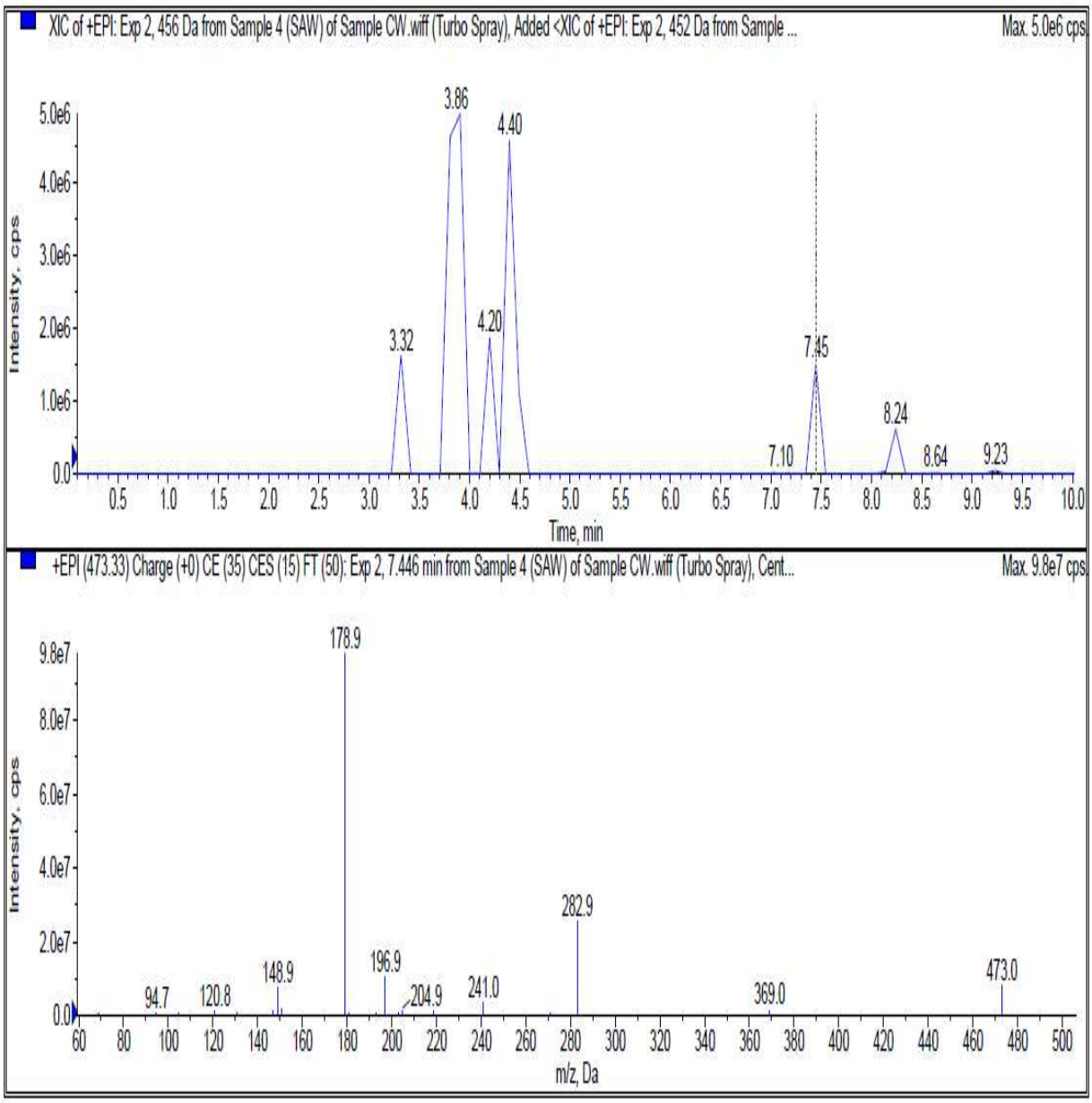

Figure 4.22: Fragmentation pattern from MS/MS spectrum of the major peak with m/z value 473.

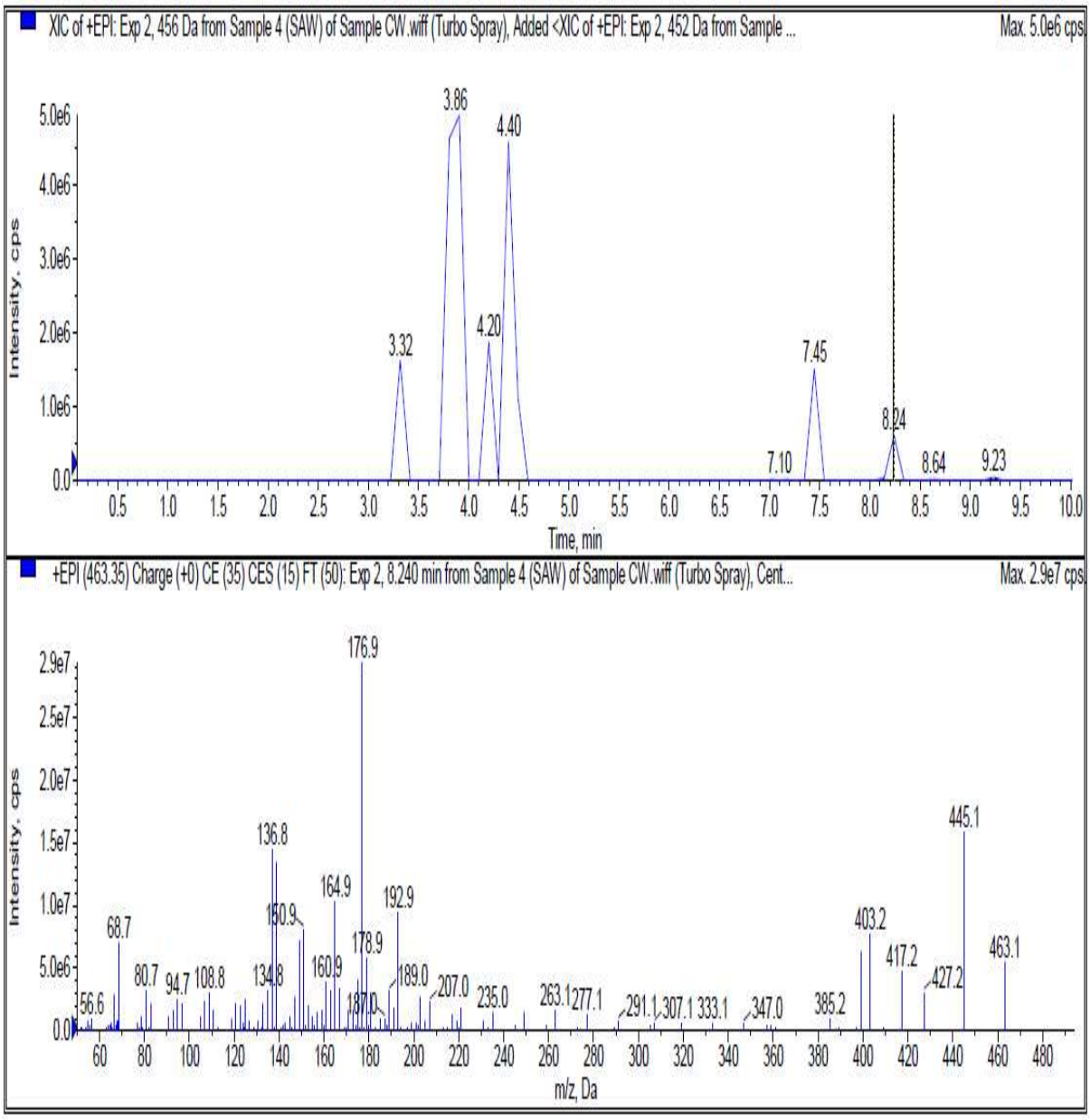

Figure 4.23: Fragmentation pattern from MS/MS spectrum of the major peak with m/z value 464.

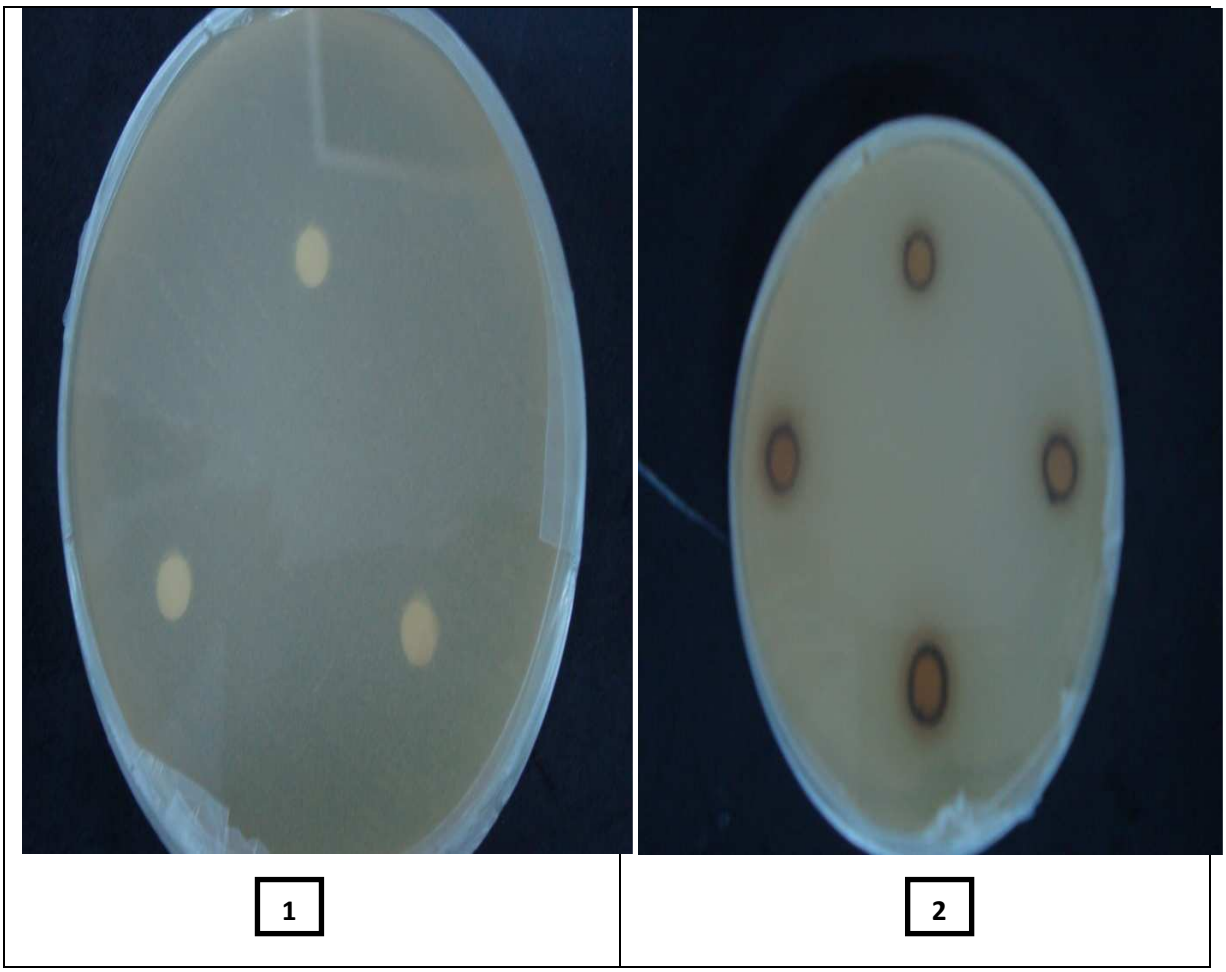

Figure 4.24: Pictures shows- no inhibition zone (1) and inhibition zone against bacterial pathogen

Chapter-5: Discussion

5.1. Effects of hormones on seed germination of *Sonneratia alba*:

In our study, we have used three different hormones and three different concentration as well as control to observe the germination rate of *Sonneratia alba* in three different medium.

As we know that cytokinins play an important role in cell proliferation as well as cell division, Otroshy M. *et al.*, (2009) have been reported that "medium supplemented with BAP can play important role in dormancy breaking and enhance the germination rate in seed of Asafoetida", in our study, we also have found that cytokinins such as BAP and kinetin played role in terms of seed germination of *Sonneratia alba*. We have found that WPM medium is better than MS media as well as in vitro medium for germination of seed. Tran Van Minh *et al* (2001) also found that WPM medium is better that MS medium for micropropagation of woody plants. Low concentration of BAP and high concentration of Kinetin with WPM medium showed the highest germination rate of *sonneratia alba*. Cytokinin can enhance the effect of GA_3 in the seed system of Celery and cause faster germination (Biddington and Thomas, 1978). However, GA_3 showed the poor germination rate compare with BAP and kinetin.

Top two of the highest germination rates (100 and 90%) were observed for *in vitro* cultures in WPM medium. 100% seeds were germinated in 100ppm BAP solutions, whereas under control treatment that is without any hormone only 50% seeds germinated in WPM medium. Among all the cultures with BAP solutions, *in vitro* MS culture (500ppm BAP) showed the lowest germination rate. The highest germination rate among all the GA3 hormone treatments were obtained for 100ppm solution in WPM medium. Whereas, the lowest rate (30%) was observed for *in vitro* cultures in both MS and WPM media with

500ppm GA3. This is also lower than that in the control culture. For the case of culture in Kinetin hormone, 70% seeds germinated at all the treatments with 500ppm solution. 90% seeds, the highest germination rate among all Kinetin treatments were achieved for cultures with 1000ppm solution in WPM medium.

5.2. Total phenolic contents:

Huge range of physiological properties has been exhibited by phenolic contents, such as antioxidant, anti-microbial, anti-inflammatory, anti-allergenic, anti-thrombotic, anti-artherogenic, vasodilatory and cardioprotective effects (Manach, Mazur, & Scalbert, 2005; Puupponen-Pimiä et al,. 2001; Middleton, Kandaswami, & Theoharides, 2000; Samman, Lyons Wall, & Cook, 1998; Benavente-Garcia, Castillo, Marin, Ortuno & Del Rio, 1997). Phenolic compounds which play a significant role in reproduction, growth and providing protection against predators and pathogens (Brovo, 1998). In fruits and vegetable, the phenolic content controls the color and sensory characteristics (Alasalvar, Grigor, Zhang, Quantick & Shahidi, 2001). These are derivatives of the pentose phosphate, shikimate and phenylpropanoid pathway in plants and consider as secondary metabolites (Randhir, Lin, & Shetty, 2004). These components are most widely happening phytochemicals groups that consider as physiological and morphological importance in plants. Medicinal herbs and trees as well as fruits and vegetable may contain a wide variety of phenolic compounds such as phenolic acid, tannins, stilbenes, coumarins, lignans, flavonoids, quinines etc which is called free radical scavenging molecules. Nitrogen compounds (betalains, amines and alkaloids), vitamins, terpenoids and endogenous metabolites are also known as free radical scavenging molecules which are rich in antioxidant activity (Larson, 1988; Shadidi and Naczk, 1995; Cotelle et al., 1996; Velioglu et al., 1998; Zheng and Wang, 2001; Cai et al., 2003). It has been found from epidemiological study that antioxidant compounds possess

antiatherosclerotic, antimutagenic, anti-inflammatory, antitumor, antibacterial, antiviral, or anticarcinogenic activities to a greater or lesser extent (Halliwell, 1994; Mitscher et al., 1996; Owen et al., 2000; Sala et al., 2002). Though there are some considerable controversy, the natural antioxidants has been related with reduced cardiovascular disease, diabetes, risks of cancer, and other ageing disease (Sun et al., 2002; Yang et al., 2001; Kuo, 1997; Mclarty, 1997; Hertog et al., 1995).

Phenols are very significant plant components on account of their radical scavenging power due to their hydroxyl groups (Hatano, Edamatsu, Mori, Fujita, & Yasuhara, 1989). The phenolic components may contribute instantly to the antioxidant action (Duh, Tu, & Yen, 1999). It is proposed that polyphenolic components have repressive impression on mutagenesis and carcinogenesis in human (Tanaka, Kuei, Nagashima, & Taguchi, 1998).

5.3. Bioactivity of mangrove plants:

Mangroves are halophytes which are by nature salt-tolerant plants that may be potentially practicable for economical applications as new reservoir of natural antioxidants in dietetic food (Meot-Duros, Le Floch, & Magne, 2008). Halophytes were come up to bear polyphenols and other bioactive content that were potentially useful for medicative uses. (Menzel & Leith, 1999). Vinga catjand, Vinga unguiculata and Oryza sativa have been reported with the effect of NaCl stress and the grandness of free radical O_2 and H_2O_2 in damage and injury of plants (Herna ndez, del Rio, & Sevilla, 1994; Singha & Choudhuri, 1990).

Several halophytes are fitted with mighty antioxidant systems, admitting enzymatic and non-enzymatic elements to defeat severe conditions like toxic ROS. (Ben Hamed,

Castagna, Elkahoui, Ranieri, & Abdelly, 2007). In this study, all three mangrove exhibited high phenolic content and bioactivities in terms of antioxidant and antimicrobial activities.

5.4. Total phenolic content of *Sonneratia alba*:

In this study, three different solvent has been used to evaluate the bioactivity of the mangrove plants. Different solvent showed different potential of bioactivity. The results clearly showed that extraction solvent significantly affected the total phenolic content of the prepared extracts. For example, methanol and ethanol extracts exhibited better activity compare with chloroform extracts. These results were agreement with previous studies which showed that methanol can extract the highest amount of phenolic compounds compare to petroleum ether, ethyl acetate and water from both their examined samples, *lentinus edodes* and *Volvariella volvacea* (Cheung et al., 2003). Based on the different extraction potential of solvent, it has been suggested that combination of several solvent is better than individual solvent. Alberto et al. (2006) applied combination of several solvents such as combination of acetate, methanol and water to extract the phenolic compounds of apple skins.

The results manifest distinctly the influence of the solvent on the extractability of antioxidant compound, particularly on the extractability of phenolics. Previous studies also showed some short of same results in which the scientist found that solvent exert a great power in phenolic extraction capacities in many species (Akowuah et al., 2005; Turkmen, Sari & Veliglu, 2006). *S. alba* can be introduced as a high value source of phenolic compound due to the high amount of this group of compounds. Comparison of amounts of phenolic compounds in different parts of Indian *Sonneratia* spp. was carried out previously by Banerjee et al. (2008) and it was shown that leaf explants of *S. apetala* contain the

highest amounts of phenolic compounds compare to the stem barks and roots. However, in our study we found that the phenolic compound is higher in barks than leaves.

5.5. Bioactivity of *Sonneratia alba*:

Primarily research on *Sonneratia* species focused on the morphological features (Baker & Van Steenis, 1951; Van Steenis, 1968; Duke & Jackes, 1987; Ko, 1993; Chen, 1996; Wang & Chen, 2002; Qin et al., 2007). A few reports have found concerning the phytochemistry and pharmacology of *Sonneratia* species. Two flavonoids and their antioxidant activity of leaves of *S. caseolaris* have been reported by Sadhu et al., (2006). Zheng and Pei (2008) accounted five triterpenoids with two sterols and studied the other chemical constituents of stems of *S. ovata*. The study showed a positive correlation between the total phenolic contents and the antioxidant activity of *in vivo* and *in vitro* explants of *Sonneratia alba*. The study also confirmed that the extraction solvent in preparation of sample has an important effect on the antioxidant activity.

Zhou and Yu (2004) attempted with four pure and solvent mixture extracts and they found that pure ethanol had the lowest polyphenols content in wheat bran grain sample. However, in this study demonstrate that ethanol extract exhibited the highest antioxidant activities in both leaves and barks samples.

5.5.1. IC50 value of *Sonneratia alba* in DPPH assay:

Anti-radical properties can be evaluated by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. The 50% inhibition of DPPH radical (IC_{50}) value was determined while ascorbic acid was used as positive control. The lowest IC_{50} value was with ethanol extract of bark (0.0197 ± 0.001mg/ml) and there was no significant difference between positive control ascorbic acid (0.018 ± 0.003mg/ml) and the ethanol extract of bark. Chloroform

extract of in vitro explants has the highest IC_{50} with value of 0.37 ± 0.004mg/ml which was significantly higher than among all extracts. Lowest IC_{50} value represents the highest antioxidant activity.

5.5.2. SOD assay of *Sonneratia alba*:

The results of SOD activity assay showed all samples including BHA with highest antioxidant potential obtained from ethanol extract of bark of *S. alba*. However, the ethanol extract of barks is significantly higher than all other extracts including positive control BHA, but not significantly higher than methanol extract of barks. The lowest antioxidant capacity was achieved by chloroform extract of *in vitro* explants but it was not statistically different from chloroform extract of leaves in this study. Among the *in vivo* explants, there were no significantly different between methanol and ethanol extracts of leaves and methanol and chloroform extracts of barks. In *in vitro* explants, methanol extract exhibited the higher activity that was not statically difference with methanol extract and followed by the chloroform extracts. All the *in vitro* explants extracts showed the lower activity than *in vivo* explants extracts except chloroform extract of leaves. Khorasani et al., (2010) was reported that *in vivo* explants showed higher SOD activity compared with *in vitro* explants in *Asparagus officinalis* which is an agreement of our study.

5.5.3. Reducing Power assay of *Sonneratia alba*:

The antioxidant activity of *S. alba* has evaluated by using reducing power assay. 2mg/ml and 0.25mg/ml of extract showed the highest and lowest reducing rate respectively in all *in vivo* and *in vitro* examined samples. *In vivo* explants showed better reducing rate than *in vitro* explants. Whereas ethanol extract of barks was the best sample among all *in vivo* and *in vitro* extracts. Chloroform extract of in vitro explants achieved the lowest reducing rate.

5.5.4. Antimicrobial activity of *Sonneratia alba*:

The results of antimicrobial activity of methanol, ethanol and methanol extracts of *S. alba* leaves and barks were presented in Table 3. No inhibition was observed for chloroform extract of leaves against the growth of any bacteria. Ethanol extracts of leaves and barks showed a higher antimicrobial activity against *E. coli*, *S. aureus, P. aeruginosa* and *B. cereus* compared to methanol extracts of leaves and barks. However, microbial growth inhibition by the positive control (30 µg tetracycline) of all tested bacteria was significant compare to the examined extracts.

5.6. Total phenolic content of *Rhizophora mucronata*:

The results of the study clearly showed that different part of the plant and extraction solvent significantly affected the total phenolic content of the prepared extracts. For example, phenolic compound present in methanol extract of leaves of *R. mucronata* were approximately 30 times more than the chloroform extracts of barks. Among all examined extracts ethanol extract of leaves with value of 358 ± 0.61 (mg of GAE/g of dry explants) showed the highest phenolic content that is significantly higher than other extracts. The amount of phenolic compound found in methanol extract of leaves in this study was higher than the total phenolic contents of *R. mucronata* leaves methanol extract reported by Banerjee et al., (2008). This difference might be related to the plants variety difference as several reports showed the total phenolic contents of plants varied with varieties/cultivars of the plants greatly. For example, total phenolic contents in peach influenced by cultivars (Kubota et al., 2000). Ahmed and Beigh (2009) reported that even subspecies of *Brassica oleracea* var. acephala showed variation of phenolic contents.

R. mucronata can be considered as a high value source of phenolic compound due to the high amount of this group of compounds. Comparison of amounts of phenolic compounds

in different parts of Indian *R. mucronata* was carried out previously by Banerjee et al. (2008) and it was showed that leaves explants of *R. mucronata* contain the highest amounts of phenolic compounds compare to the stem barks and roots which was a positive agreement of our study.

5.7. Bioactivity of *Rhizophora mucronata*:

Mangrove plants have been used as traditional medicine for various diseases in Indian subcontinent (Kirtikar & Basu, 1935). R. mangle is the most studied mangrove in Rhizoporaceae family. Sanchez et al., (2006) has been reported this plant with antiinfalmmatory, anti-ulcer, hypoglycemic, wound healing as well as antioxidant activity. *R. mucronata* has been reported by Premanathan et al., (1999) to have anti-HIV activity as well as HIV-induced cytopathic effects. The study confirmed that the extraction solvent in preparation of samples has an important effect on the antioxidant activities. Different parts of the plant also showed different bioactivity potentials. Combination of both enzymatic and non-enzymatic methods has been used in this study which was suggested by Hakiman and Maziah (2009) to get the more reliable results. All tested extracts achieved the highest antioxidant capacity in different examinations.

A number of synthetic antioxidants, such as 2- and 3-tert-butyl-4-methoxyphenol (butylated hydroxyanisole, BHA), and tert-butylhydroquinone (TBHQ) have been added to foodstuffs but because of toxicity issues, their use is being questioned (Valentao et al., 2002). Therefore, attention has been directed towards the development and isolation of natural antioxidants from plant sources.

Various plants are employed for medical function e.g. the bark of R. apiculata used for diarrhea and wound (School of Thai traditional medicine, 1981; Traditional Medicine

Association, 1981), *Avicennia alba* for wound (Yaadfon Association, 1981), *Acanthus ebracteatus* for chronic wound (Suchamuong, 1979), bark of *Rhizophora mucronata* for diarrhea (Pongboonrod, 1976), etc.

These results were in agreement with previous studies which showed that pure methanol was an effective solvent for antioxidant extraction, in particular on phenolic contents (Hertog et al., 1993; Siddhuraju & Becker, 2003).

5.7.1. SOD assay of *R. mucronata*:

The results of SOD activity assay (Figure 7) showed all samples including BHA with highest antioxidant potential obtained from *R. mucronata* methanol extract of barks. However the inhibition rates from methanol extract of barks is significantly higher than all extracts including positive control (88.64%), but not significantly higher than methanol extract of leaves (91.53%). The lowest antioxidant capacity was achieved by chloroform extract of barks in this study.

5.7.2. DPPH assay of *R. mucronata*:

DPPH is usually known as a stable free radical and become a stable diamagnetic molecule after assumes electron or hydrogen radical (Soares, Dins, Cunha, & Ameida, 1997). The results of free radical scavenging test (Figure 9) showed ethanol extract of leaves is not significantly comparable with positive controls ascorbic acid in terms of scavenging capacity. The best scavenging activity among the examined extracts was achieved from ethanol extract of leaves (91.23%) which was significantly different from all other extracts. Chloroform extract of leaves (28.82%) and barks (21.69%) both showed poor antioxidant activity in terms of scavenging capacity.

5.7.3. Reducing power assay of *R. mucronata*:

Reducing power assay has been used to evaluate the antioxidant activity of *Rhizophora mucronata*. The concentrations of 0.25, 0.50, 1.0 & 2.0mg/ml were used to find out the reduction of ferric to a colored product. The reducing rates increase while the concentrations of extracts were increased. Ethanol extract of barks and chloroform extract of leaves showed the highest and lowest reducing rate respectively. Methanol and ethanol extracts exhibited better reduction of ferric to colored product compared with chloroform extracts.

5.7.4. Antimicrobial activity of *R. mucronata*:

Two gram positive and two gram negative bacterial pathogen has been used to evaluate the antimicrobial activity of methanol, ethanol and chloroform extract of leaves and barks of *R. mucronata*. The highest inhibition zone observed by the ethanol extract of leaves against *S. aureus* (20.78 ± 0.03 cm). Chloroform extract of barks showed no inhibition zone against any bacterial pathogen. Whereas, chloroform extract of leaves exhibited no inhibition zone against gram negative pathogen (*E. coli* & *P. aeruginosa*). Overall leaves extracts showed better antimicrobial activity compared with barks extracts.

5.8. Total phenolic content of *Bruguiera gymnorrhiza*:

In this study, the evaluated phenolic content of the ethanol extracts of *B. gymnorrhiza* barks (284.93 mg/g of dry barks) was higher than other extracts. The amount of phenolic compound found in methanol and ethanol extracts of leaves and barks in this study was higher than the total phenolic contents of *B. gymnorrhiza* leaves and barks of aqueous methanol extract (134.16 mg/g of dry leaves and 131.90 mg/g of dry stem bark) reported by Banerjee et al. (2008). This difference might be related to the plants variety difference as

several reports showed the total phenolic contents of plants varied with varieties/cultivars of the plants greatly. For example, total phenolic contents in peach influenced by cultivars (Kubota et al., 2000). Ahmed and Beigh (2009) reported that even subspecies of *Brassica oleracea* var. acephala showed variation of phenolic contents.

B. gymnorrhiza can be introduced as a high value source of phenolic compound due to the high amount of this group of compounds. Comparison of amounts of phenolic compounds in different parts of Indian *B. gymnorrhiza* was carried out previously by Banerjee et al. (2008) and it was shown that leaf explants of *R. mucronata* contain the highest amounts of phenolic compounds compare to the stem barks and roots. However, in our study we found that the phenolic compound is higher in barks compared with leaves.

5.9. Bioactivity of *Bruguiera gymnorrhiza*:

The importance of combination of different methods to evaluate the antioxidant activity of the extracts is confirmed based on the results obtained from this study. Rafat et al., (2010a) suggested evaluating the antioxidant potential by applying the several assay of antioxidant. A number of synthetic antioxidants, such as 2- and 3-tert-butyl-4-methoxyphenol (butylated hydroxyanisole, BHA), and tert-butylhydroquinone (TBHQ) have been added to foodstuffs but because of toxicity issues, their use is being questioned (Valentao et al., 2002). Therefore, attention has been directed towards the development and isolation of natural antioxidants from plant sources.

Naturally occurring essence has been centering of interest to evaluate the antioxidant activity in recent years (Jayaprakasha, Jaganmohan Rao, & Sakariah, 2004). The scientists are trying to substitute the synthetic antioxidant by natural product. They are concerning about plant source and screening of raw materials for discovering new antioxidants. The

primary phenolics are consider as source of natural antioxidants, that may come about all division of the plants such as vegetable, nuts, fruits, seeds, barks, roots, and leaves (Pratt & Hudson, 1990).

5.9.1. SOD assay of *B. gymnorrhiza*:

The results of SOD activity assay (Figure-4.10) showed all samples including BHA (5 mg/ml) with highest antioxidant potential obtained from *B. gymnorrhiza* ethanol extract of barks followed by ethanol extract of leaves and methanol extract of leaves. However, the inhibition rate from ethanol extract of barks is significantly higher than chloroform extract of bark and leaves, methanol extract of barks and also form positive control (BHA), but not significantly higher than ethanol and methanol extract of leaves. The lowest antioxidant capacity was showed by chloroform extracts as well as it was statistically different from all other extracts in this study.

5.9.2. DPPH assay of *B. gymnorrhiza*:

The results of free radical scavenging test (Figure-4.12) showed none of the extracts were significantly comparable with positive control in terms of scavenging capacity. The best scavenging activity among the examined extracts was achieved from ethanol extracts of barks (84.28%) which were significantly higher than other extracts except methanol extracts of barks (78.85%). However, among all the extracts, chloroform extracts of leaves (30.53%) showed the lowest scavenging capacity.

5.9.3. Reducing power assay of *B. gymnorrhiza*:

The result of reducing power assay (Fig- 4.11) showed that when the concentrations increased the reducing rates also increased. Among all the extracts, ethanol extract of barks showed the highest reducing rate and chloroform extract showed the lowest reducing rate.

The second highest reducing rate found from the methanol extract of barks. As can be observed from graph (in Fig-12) barks extracts showed the better result compared with the leaves extract.

5.9.4. Antimicrobial activity of *B. gymnorrhiza*:

The results of antimicrobial activity of methanol, ethanol and chloroform extracts of *B. gynmorrhiza* leaves and barks were presented in Table 3. Chloroform extract of leaves showed no inhibition against the growth of any bacteria. Ethanol extracts of leaves and barks showed a higher antimicrobial activity against *E. coli*, *S. aureus, P. aeruginosa* and *B. cereus* compared with methanol extracts of leaves and barks. Positive control (30 µg tetracycline) inhibited the growth of all tested bacteria significantly compared to the examined extracts.

5.10. Bioactivities of fractionated ethanol extract of *Sonneratia alba*:

Ethanol extract of barks of *Sonneratia alba* has been fractionated into three fraction which are water fraction, ethyl acetate fraction and hexane fraction. The amount of hexane fraction was too little (0.3g from 50g of crude extract) that's why that was not consider for further bioactivity tests. Both water fraction and ethyl acetate fraction have been used to evaluate the antioxidant and antimicrobial assays. Water fraction showed the highest bioactivities compared with ethyl acetate fraction which was the positive agreement with Khokhar and Magnusdottir (2002) in where they found that water to be the best solvent for extraction tea catechins compared with 80% methanol and 70% ethanol.

5.10.1. SOD activity of the fractionated ethanol extract of *Sonneratia alba*:

Fig-13 showed the SOD antioxidant activity of water fraction and ethyl acetate fraction. Water fraction exhibited the better antioxidant activity than ethyl acetate fraction though there was no significant difference.

5.10.2. Reducing Power Assay of the Fractionated Ethanol Extract of *Sonneratia alba*:

Fig-14 represents the reducing power assay of water and ethyl acetate fraction of ethanol crude extract of bark of *Sonneratia alba*. Water fraction showed better reducing rate than ethyl acetate fraction and reducing rate increase while concentration increased.

5.10.3. IC50 Value in DPPH Assay of the Fractionated Ethanol Extract of *Sonneratia alba*:

IC_{50} value was measured in DPPH assay. Water fraction has the low IC_{50} value compared with ethyl acetate fraction (table-8) and had no significant difference with ascorbic acid.

5.10.4. Antimicrobial activity of the fractionated ethanol extract of *Sonneratia alba*:

Table-9 represents the antimicrobial activity of water and ethyl acetate fraction. Water fraction showed the better activity than ethyl acetate fraction. Though the activity was not as significant as the positive control but both fractions exhibited activity against all four gram positive and negative bacteria.

5.11. LCMS/MS analysis of active fraction of the fractionated ethanol extract of *Sonneratia alba*:

From our studies on the antioxidant activity on the water fraction of ethanol extract of leaves of *Sonneratia alba*. We have found that the water extract showed better antioxidant activity in term of both enzymatic and non-enzymatic assays. In order to understand more about the nature of the active compounds in the water fractions, we analyzed the fraction

using advanced liquid chromatography tandem mass spectrometry technique (LCMS/MS). We studied the nominal masses of the major active components in the water fraction, and we observed 8 such peaks at m/z values of 331, 345, 452, 463, 473, 480 and 494. Each of the 8 major peaks observed were found to have rich fragmentation patterns from the MS/MS spectrum and this unique patterns (also known as fingerprint) corresponds to the high antioxidant activity of the extracted water fraction of the plant sample. Further investigation and analytical studies are needed (techniques and data from instruments such as nuclear magnetic resonance, accurate masses, infrared absorption and others) to determine more details such as the correct structures for these active components.

Chapter 6: Conclusion and recommendation of research

6.1. Conclusion:

Medicinal plants play important role as natural raw material in important sectors such as cosmetics, food and pharmaceutical industries because of their antioxidant, antimicrobial, anticancer and anti-inflammatory activities. In the current study, three mangrove plant species were collected from Carey Island in Kuala Lumpur, Malaysia. Leaves and bark of all three plants were examined for various analyses in order to find out their antioxidant and antimicrobial potentials as well as for their total phenolic contents.

The first step of this study was the preparation and extraction of the collected plant explants. Different parts of the plants and solvents were used in this study. *In vivo* and *in vitro* explants were used for *Sonneratia alba* and for *Rhizophora mucronata* and *Bruguiera gymnorrhiza, in vivo* leaves and barks were used. Three different solvents namely methanol, ethanol and chloroform were used to prepare the sample extracts. In terms of *Sonneratia alba*, *in vivo* barks showed better activities with ethanol solvent compared to *in vitro* explants. Overall, for all three tested plants, methanol and ethanol solvent has proved as most efficient crude solvent than chloroform solvent. Our results confirmed that different extraction solvent have important effect on the antioxidant activities.

Biochemical analyses were done after the extraction process for each plant extract sample. Firstly, Folin-ciocalteu method was used to measure the total phenolic content of all samples and according to results, ethanol extract of leaves of *Rhizophora mucronata* exhibited the highest phenolic content as 358.6 ± 0.53GAEmg/g of dry explants. The other samples with high phenolic contents are methanol extract of leaves of *R. mucronata* (349.33 ± 0.61GAEmg/g of dry explants), ethanol extract of barks of *Sonneratia alba*

(313.81 ± 5.15GAEmg/g of dry explants) and ethanol extract of barks of *Bruguiera gymnorrhiza* (284.93 ± 0.23GAEmg/g of dry explants).

Secondly, DPPH assay, SOD assay and Reducing Power assay were used to evaluate the potential of antioxidant activities of all the prepared samples. According to the results ethanol extract of barks of *Sonneratia alba* exhibited highest antioxidant activity on SOD assay. IC50 value was measured of *S. alba* in vivo and in vitro explants by using the DPPH assay. Ethanol extract of barks showed lowest IC50 value as 0.0197 ± 0.001mg/ml. In reducing power assay, the antioxidant activities were increased while the concentration was increased. Results showed, in all antioxidant assay barks exhibited better antioxidant potential compared with leaves as well as chloroform solvent extracts showed lower activity than methanol and ethanol solvent extracts by which we can make a conclusion that different explants in different extraction solvent have and important effect on the antioxidant activities.

Antimicrobial activity of plant sample extracts were also evaluated using the disc diffusion methods as a part of bioactivity of all prepared samples. Two gram positive (*B. cereus* and *S. aureus*) and two gram negative (*E. coli* and *P. aeruginosa*) pathogenic bacteria were used in disc diffusion assay. Ethanol and methanol extracts of leaves and barks showed promising antimicrobial activities against all tested pathogenic bacteria. Chloroform extract of all plant exhibited comparatively lower antimicrobial activities.

In the next step, the ethanol extract of barks of *Sonneratia alba* was fractionated into polar, semi polar and non-polar fraction by following the bioassay guide line. Hexane fraction was not considered for further antioxidant and antimicrobial activities test because of the total amount of hexane fraction was too little (0.3g from 50g of crude extract). The water

and ethyl acetate fraction were tested with antioxidant assay as well as antimicrobial activities to find out the more active fraction between them. Water fraction showed better antioxidant activities and more inhibition zone in antimicrobial activities than ethyl acetate fraction.

In our last step, liquid chromatography tandem mass spectrometry (LCMS/MS) analysis revealed the nominal mass of the active compound of water fraction. The nominal masses of the major active components in the water fraction, and we observed 8 such peaks at m/z values of 331, 345, 452, 463, 473, 480 and 494. Each of the 8 major peaks observed were found to have rich fragmentation patterns from the MS/MS spectrum and this unique patterns (also known as fingerprint) corresponds to the high antioxidant activity of the extracted water fraction of the plant sample.

6.2. Recommendation of the research:

In our research, we have found that all three mangrove plants have high antioxidant and antimicrobial activities as well as high phenolic contents. The nominal masses of the active compound also have been found by using the LCMS/MS analysis. Further study can be done by isolating the active compounds from the active fraction. The isolated active compound can be used for pharmaceuticals industries to make medicine for some chronic diseases such as cancer, diabetes, and even AIDS.