ANTIOXIDANT AND ANTIMICROBIAL COMPOUNDS FROM THE MARINE ALGAE *PADINA ANTILLARUM*

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ANTIOXIDANT AND ANTIMICROBIAL COMPOUNDS FROM THE MARINE ALGAE *Padina antillarum*

By

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ABSTRACT

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Chia Pooi Yee

The antioxidant property and antimicrobial activity of extracts of the brown marine algae *Padina antillarum* were studied. Different extraction methods were applied in order to extract antioxidant and antimicrobial compounds. For the purpose of extracting antioxidant compounds, a 50% aqueous methanol extract of freeze-dried *P. antillarum* was subjected to liquid-liquid partitioning. The three extracts (chloroform, ethyl acetate and water) which were obtained from partitioning were subjected to total phenolic content and radical scavenging activity assays. The ethyl acetate extract showed the highest antioxidant activity and was subjected to activity-guided fractionation. Instrumental analyses (Gas Chromatography-Mass Spectrometer, ^{1}H-and ^{13}C-Nuclear Magnetic Resonance Spectrometer) revealed the presence of phloroglucinol (1,3,5-trihydroxybenzene) in this extract. The radical scavenging activity of phloroglucinol was compared to that of the known antioxidant plant compounds, quercetin, ascorbic acid and tannic acid, and was found to be weakest of the four in terms of free radical scavenging, ferric reducing antioxidant power and chelating ability. Phloroglucinol had weak activity against *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (ATCC 11778), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 13883), and *Escherichia coli* [both penicillin-sensitive (ATCC 25922) and penicillin-resistant strains (ATCC 35218)]. Phloroglucinol had no activity

towards leukemia cells (K562 cell line) and no detectable antifungal activity. For extraction of antimicrobial compound, freeze-dried *P. antillarum* sample was subjected to sequential extraction using organic solvents of increasing polarity (i.e. hexane followed by diethyl ether, chloroform, ethyl acetate, acetone, methanol and finally water). The hexane extract exhibited the highest and most consistent inhibitory activity against *P. aeruginosa* with a mean minimum inhibitory concentration (MIC, n=3) value of 0.625 mg/mL. Thus this extract was selected for further *in vitro* bioassay-guided fractionation. The structure of the partially-purified antimicrobial compound, which had a mean MIC of 0.031 mg/mL, was elucidated by GC-MS, and found to be diisooctyl phthalate.

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Big thanks to my partner, Ms. Lim Fei Shen for providing useful information to me. I would like to show my special thanks to my family and fellow friends for their support.

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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APPROVAL SHEET

This thesis entitled "**ANTIOXIDANT AND ANTIMICROBIAL COMPOUNDS FROM THE MARINE ALGAE *PADINA ANTILLARUM***" was prepared by CHIA POOI YEE and submitted as partial fulfillment of the requirements for the degree of Master of Science at Universiti Tunku Abdul Rahman.

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It is hereby certified that **CHIA POOI YEE** (ID No: **07UEM0877**8) has completed this thesis entitled " ANTIOXIDANT AND ANTIMICROBIAL COMPOUNDS FROM MARINE ALGAE *PADINA ANTILLARUM*" under supervision of Assoc. Prof. Dr. Khoo Kong Soo (Supervisor) from the Department of Chemical Science, Faculty of Science, and Assist. Prof. Dr. Sit Nam Weng (Co-supervisor) from Department of Biomedical Science, Faculty of Science.

I hereby give permission to the University to upload softcopy of my thesis in pdf format into UTAR Institutional Repository, which will be made accessible to UTAR community and public.

Yours truly,

_____
(CHIA POOI YEE)

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LIST OF ABBREVIATIONS

^{1}H-NMR	^{1}H-nuclear magnetic resonance
^{13}C-NMR	^{13}C-nuclear magnetic resonance
AIDS	Acquired immune deficiency syndrome
ATCC	American Type Culture Collection
BHA	Butyl hydroxyanisole
BHT	Butylated hydroxytoluene
CFU	Colony forming units
DBP	di-n-butyl phthalate
DEHP	Di-(2-ethylhexyl) phthalate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picylhydrazyl
FBS	Fetal bovine serum
FC	Folin Ciocalteu's
FIC	Ferrous ion chelating
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalent
GC-MS	Gas chromatography- mass spectrometer
HPLC	High performance liquid chromatography
INT	*p*-iodonitrotetrazolium violet
MBC	Minimum bactericidal concentration
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth

MIC	Minimum inhibitory concentration
MTT	3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide
NADH	Nicotinamide adenine dinucleotide, reduced form
PDA	Potato dextrose agar
PG	Propyl gallate
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
RPMI	Roswell Park Memorial Institute
ROS	Reactive oxygen species
TBHQ	Tertiary butylhydroquinone
TDH	Threonine dehydrogenase
TLC	Thin layer chromatography
TPC	Total phenolic content
UV-Vis	Ultraviolet-visible
WHO	World Health Organization

CHAPTER 1.0

INTRODUCTION

1.1 Natural Products

Natural products are compounds that are produced by living organisms or living systems (Hanson, 2003). They can be divided into three categories- primary and secondary metabolites, and high molecular weight polymeric materials. The secondary metabolites often attract the interest of researchers because they provided challenging synthetic targets and valuable medicines (Hanson, 2003). According to World Health Organization (WHO), approximately 80% of the world relies on natural sources for primary medicine while remaining 20% of the population relies on health care systems which also use natural sources (Farnsworth *et al*., 1985). The search for medicinal compounds has helped to drive the development of natural products chemistry.

1.2 Marine Natural Products

Natural products have been used as medicines since ancient times and plants have served as the major source of medicinally useful natural products due to the ease of accessibility. With the development of new diving techniques and machines, scientists had focused their research into the use of

marine natural products as pharmaceutical agent, in the belief that the diversity of natural products in marine environment may far exceed that of the terrestrial environment.

Marine natural products are organic compounds produced by microbes, sponges, seaweeds and other marine organisms (Faulkner, 2001). The host organism synthesizes these compounds as secondary metabolites to protect themselves and to maintain homeostasis by increasing water solubility, improving chemical stability and altering biological activity (Lipton, 2003). There is an increasing collaborative effort between marine natural products chemists and pharmacologists that led to the discovery of marine metabolites to enter preclinical studies and clinical trials. Furthermore, many marine compounds have been found to be useful as biomedical tools for exploring cellular processes at the molecular level (Carte, 1996).

Marine natural products encompass a wide variety of chemical classes, including terpenes, shikimates, polyketides, acetogenins, peptides, and alkaloids of varying structures and a multitude of compounds of mixed biosynthesis. Approximately 5000 compounds have been isolated from organisms living in shallow waters to 900 m depth of the sea (Sandell *et al.*, 2004). Two marine-derived natural products have advanced to the pharmaceutical drug market. They are analgesic ziconotides from the venom of fish-hunting cone snails and unusual sponge-derived nucleotides that served as model compounds for antiviral drugs such as virarabin (Fusetani, 2000).

1.3 Seaweeds

Seaweeds or marine algae are among the oldest members of the plant kingdom. They have little tissue differentiation, no true vascular tissue, no roots, stems or leaves and flowers (Dhargalkar & Pereira, 2005). Seaweeds are essential for maintaining the proper balance between chemical and biological environment of the oceans. They supply oxygen to the biosphere and they are also an important source of food (*ScienceDaily*, 2010).

Seaweeds produce secondary metabolites of interest (Faulkner, 1993). There are many reports of compounds derived from macroalgae with a broad range of biological activities, such as antibiotics, antivirals, antitumour, as well as neurotoxins (Ibtissam *et al*., 2009). Macroalgae are multicellular eukaryotic algae (Faulkner, 1993). These compounds include amino acids, guanidine derivatives, phenolic substances, bioluminescence, carotenoids, diterpenoids, indoles, and halogenated compounds.

Seaweeds are classified as green (Chlorophyta), brown (Phaeophyta), and red algae (Rhodophyta) and filamentous blue-green algae (Cyanobacteria). Brown algae belong to the phylum Phaeophyta of the kingdom Prostica. It contains chlorophyll a and c, as well as carotenes and xanthophylls, including the brown pigment fucoxanthin which masks the green colour of chlorophyll. Brown algae exist in varieties of forms and sizes, ranging from less than 1 mm long to some species that are among the largest photosynthetic organisms on Earth. Kelps are the brown seaweed which may grow to more than 60 m in

length (Raven *et al*., 1999). They are mostly not free-floating seaweeds but are attached to rocks, corals and other surfaces (Waaland *et al*., 1977).

1.4 Antioxidant

Seaweeds have been shown to have antioxidants such as phlorotannins, phenolic compounds and flavanoids (Heo *et al*., 2005). Eckol is a polyphenolic compound which was isolated from brown algae, *Ecklonia cava* and *demostrated* cytoprotective effect against oxidative stress (Zhang *et al*., 2006). Figure 1.1 shows the structure of eckol.

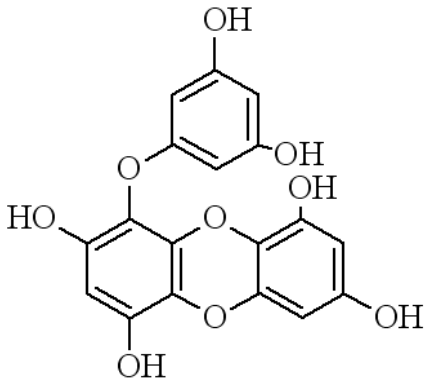


Figure 1.1: Structure of eckol which was isolated from *Ecklonia cava*

Antioxidants are compounds that prevent cell damage through oxidation by neutralizing free radicals. Free radicals are highly reactive and unstable molecules produced by cigarette smoke, toxic chemicals, excess sunlight and even metabolic processes. Reactive oxygen species (ROS) are

produced in the human body in the form of superoxide anion (O_2^-), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2) and nitric oxide (NO) (Ramamoorthy & Bono, 2007). These species may cause mutation in deoxyribonucleic acid (DNA), leading to cancer and other degenerative diseases such as cataracts and heart diseases.

The human body manufactures its own set of antioxidants called endogenous antioxidants. Endogenous antioxidants convert free radicals into harmless oxygen particles and water, after which they are changed into less active molecules and excreted. As endogenous antioxidants cannot totally neutralize excess free radicals, nutritionists suggest reinforcing them with a diet rich in antioxidants (Li *et al*., 2008). Antioxidants obtained through the diet or external sources are called exogenous antioxidants.

A relationship between the antioxidant equivalent intake and some chronic diseases has been suggested (Li *et al*., 2008). There is increasing evidence that antioxidants are active in preventing these degenerative diseases. According to reports of WHO, lung and breast cancers are the most common cancers in men and women respectively in year 2003 (WHO, 2003). Natural products have been used in the treatment of various chronic human pathologic conditions because they contain high antioxidative ingredients that could reduce the risk of cancers by neutralizing cell-mutating free radicals. Some studies have shown that antioxidants are able to prevent heart disease by inhibiting cholesterol deposits on blood vessel walls and by lowering overall cholesterol level (Li *et al*., 2008). Antioxidants have also been used as anti-

inflammatory agents in the treatment of arthritis and bronchitis (Aijith *et al*., 2007).

1.5 Antimicrobial Compounds

In the marine ecosystem, seaweeds are directly exposed to microorganisms. The ability of seaweeds to survive the infections caused by microorganisms has urged researchers to search for the antimicrobial agents in seaweeds. Many substances obtained from seaweeds such as alginate, carragenean and agar as phycocolloids have been used for medicinal purposes.

An antimicrobial compound is a substance that kills or slows the growth of microbes like bacteria (antibacterial activity), fungi (antifungal activity), or viruses (antiviral activity) (Febles *et al.*, 2009). Antimicrobial drugs are used to treat or prevent illness caused by microbes in humans or animals. However, the effectiveness of antimicrobial drugs has been decreasing due to the increase in microbial resistance. Many researchers have focused on the investigation of bioactive natural products to develop pharmaceutical drugs with fewer side effects.

1.6 Hypothesis

Seaweeds are known to produce a wide variety of secondary metabolites with unique biological activities (Konig *et al*., 1996). Some of these compounds have been reported to exhibit potent antibacterial, antifungal, antiviral, antitumor and antioxidant activities (Vairappan, 2003). Research on antimicrobial and antioxidant compounds from different geographical areas have indicated that seaweeds produce compounds with potent biological activities (Vairappan, 2003).

Due to the potential of seaweeds, *Padina antillarum* which is a type of brown algae was investigated for its antioxidant and antimicrobial properties. Previous research had shown that *P. antillarum* contain antioxidant and antimicrobial bioactive compounds (Chew *et al*., 2007). However, the structures of the compounds responsible for the antioxidant and antimicrobial activities remain unknown. This study was conducted to elucidate chemical structures of some of these compounds.

1.7 Objectives

The objectives of this study were to extract antioxidant and antimicrobial compounds from *P. antillarum.* Purification of antioxidant active extract was performed and the structure of the pure compound was elucidated by ^{1}H-nuclear magnetic resonance (^{1}H-NMR) and ^{13}C-nuclear magnetic

resonance (^{13}C-NMR). Characterization of the isolated antioxidant compound from *P. antillarum* was performed. Partial purification was performed on antimicrobial active extracts and the partially-purified active extract was further analyzed by high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometer (GC-MS).

CHAPTER 2.0

LITERATURE REVIEW

2.1 Bioactive Marine Natural Products

Bioactive marine natural products are chemical compounds produced by microbes, sponges, seaweeds, and other marine organisms (Faulkner, 2001). The host organism synthesizes these compounds as non-primary or secondary metabolites. These compounds may exert a biological effect on other organisms. Marine natural products exhibit a wide range of biological activities such as antibacterial, anticoagulant, antidiabetic, antitumuor, antifungal, and antiviral activities (Mayer & Hamann, 2005). Due to their potential for possible medical and pharmaceutical applications, compounds from many marine organisms have been studied (Baker *et al*., 1995).

Chemically bioactive compounds of marine organisms could be divided into phenols, terpenes, sterols, alkaloids and others. The chemistry and biological activities of the isolated compounds have been studied.

2.1.1 Phenols

Simple phenols are bioactive compounds consisting of a single substituted phenolic ring. Phenols extracted from seaweeds possess antibacterial properties against both Gram positive and Gram negative bacteria (Glombitza, 1974). 4, 5-disulphate potassium salt (Figure 2.1) and 5-bromo-3, 4-dihydroxybenzaldehyde (Figure 2.2) are brominated phenolic derivatives which were isolated from *Polysiphonia lanora* (red algae) which exhibited antibacterial activity (Hodgkin *et al*., 1966). The bromophenol compounds are synthesized through the shikimate pathway. Organic acids and phenolic compounds, especially polyphenols and tannins have also been shown to have antimicrobial activities (Glombitza, 1979; Chuyen *et al.*, 1982).

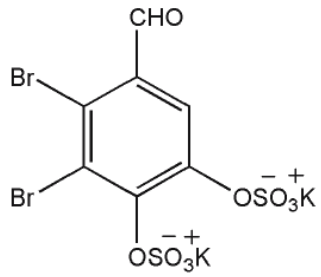


Figure 2.1: 2, 3-dibromabenzaldehyde-4, 5-disulphate potassium salt

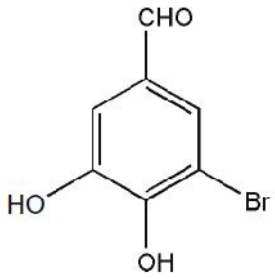


Figure 2.2: 5-bromo-3, 4-dihydroxybenzaldehyde

2.1.2 Terpenes and Terpenoids

Terpenes are formed based on an isoprene structure. They always occur as diterpenes, triterpenes and tetraterpenes, as well as hemiterpenes and sesquiterpenes. When the compounds contain additional elements, usually oxygen, they are termed terpenoids.

Zonarol (Figure 2.3) and isozonarol (Figure 2.4) are sesquiterpenes isolated from the brown algae, *Dictyopteris zonarioides* and exhibit strong growth inhibition towards pathogenic fungi which cause diseases in plants (Fenical *et al.*, 1972).

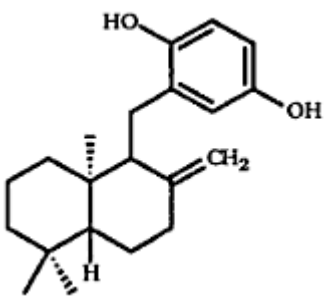


Figure 2.3: Zonarol

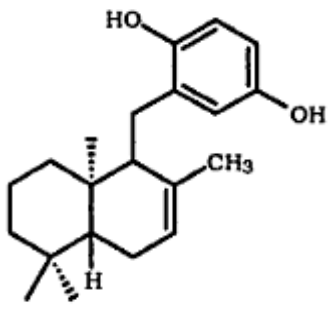


Figure 2.4: Isozonarol

Dictyol A (Figure 2.5) and Dictyol B (Figure 2.6) which are diterpenes isolated from the brown algae *Dictyota dichotoma* var. *implexa* possess antibacterial activity (Fattorusso *et al.*, 1976).

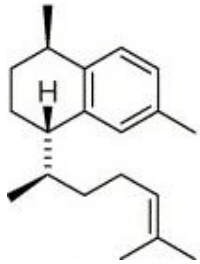


Figure 2.5: Dictyol A

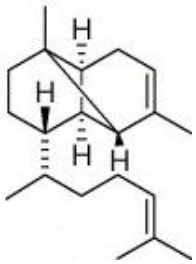


Figure 2.6: Dictyol B

Caulerpenyne (Figure 2.7), a sesquiterpene that was active only against Gram positive bacteria was isolated from the green algae *Caulerpa prolifera* (Amico *et al.*, 1978). Moreover, caulerpenyne is very potent towards human nasopharyngeal carcinoma cells (Amico *et al.*, 1978).

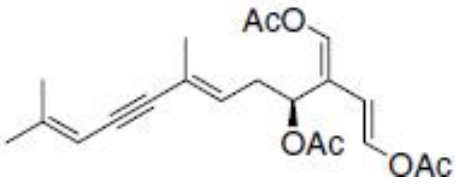


Figure 2.7: Caulerpenyne

The occurrence of terpenoids in sponges is widespread. Furanoid sesquiterpenoids have been isolated from *Dysidea*, *Euryspngia* and *Siphonodictyon* species. Nakafuran-8 (Figure 2.8) was isolated from *Dysidea fragilis* and exhibited antifeedant properties towards fish (Albericci *et al*., 1982).

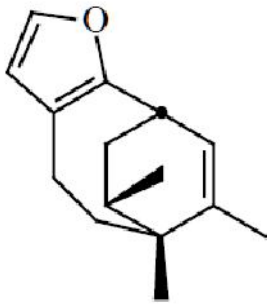


Figure 2.8: Nakafuran-8

2.1.3 Steroids

Many naturally-occurring substances are steroids, such as sterols, sex hormones, adrenal cortical hormones, cardiac glycosides, toad poisons and sapogenins. Marine organisms are a good source of unusual sterols. Halistanol (Figure 2.9) is a sulphated and alkaloidal sterol which was isolated from a marine sponge, *Halichondria moorei* (Kanazawa *et al*., 2001). Halistanol exhibits inhibitory activity towards *Staphylococcus aureus* and *Bacillus subtilis* (Kanazawa *et al*., 2001).

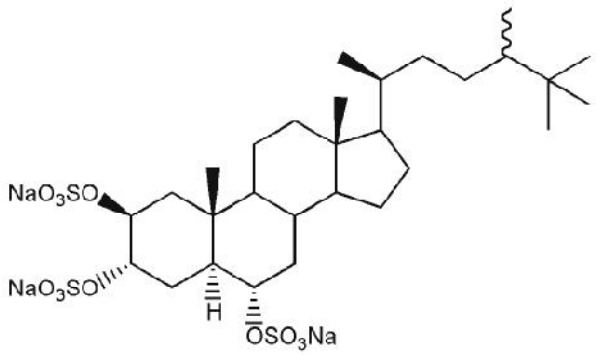


Figure 2.9: Halistanol

Laurinterol (Figure 2.10), isolated from the red algae *Laurencia intermedia* inhibited the growth of *Bacillus subtilis* (Irie *et al.*, 1970).

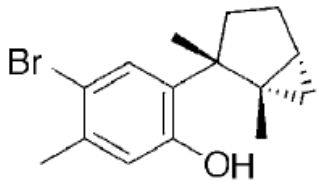


Figure 2.10: Laurinterol

Pacifenol (Figure 2.11) was isolated from the red algae *Laurencia* sp. It inhibited the growth of *Staphylococcus aureus* and *Mycobacterium segmantis* (Sims *et al.*, 1973).

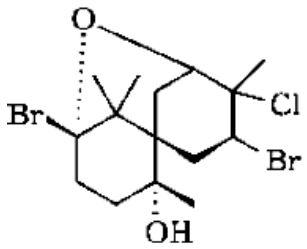


Figure 2.11: Pacifenol

Pachydictyol A (Figure 2.12) which was isolated from *Pachydictyon coriacerum* exhibited cytotoxic activity (Hirschfeld *et al*., 1973).

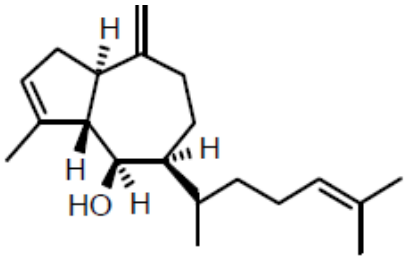


Figure 2.12: Pachydictyol A

2.1.4 Alkaloids

Alkaloids are chemical compounds that contain basic nitrogen atoms and are usually derived from amino acids. Most of the alkaloids are optically active, and nearly all of them are basic in nature. Alkaloids are mostly colourless and crystalline compounds.

Several alkaloids and other nitrogenous heterocyclic compounds have been obtained from marine sponges. Keramadine (Figure 2.13) is a bromine-

containing alkaloid, which is an antagonist of serotonergic receptor. It was isolated from the Okinawan sea sponge *Agelas* sp. (Nakamura *et al.*, 1984).

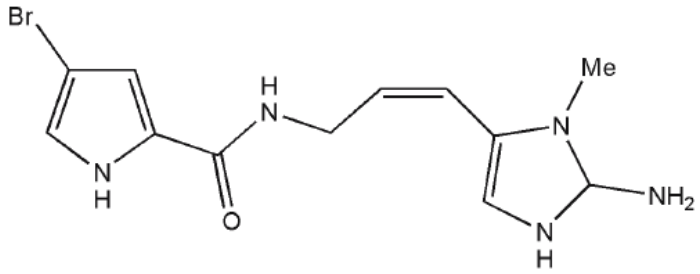


Figure 2.13: Keramadine

Xestospongin A (Figure 2.14) from *Xestospongia exigua* represents a new class of macrocyclic alkaloid incorporating 1-oxoquinolizidine rings and is a vasodilative compound which induces relaxation of blood vessels *in vivo* (Williams *et al*., 1998).

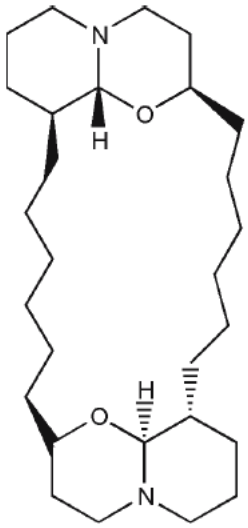


Figure 2.14: Xestospongin A

Aguilar-Santos and Doty (1968) isolated caulerpicin and caulerpin (Figure 2.15), from the marine algae *Caulerpa racemosa (*Aguilar-Santos & Doty, 1968*)*.

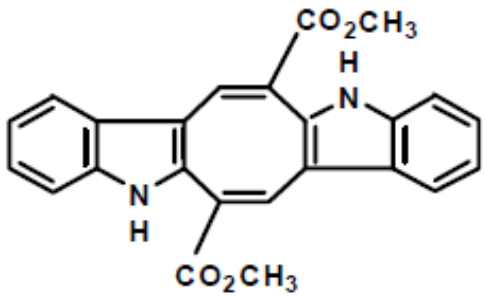


Figure 2.15: Caulerpin

2.2 Seaweeds

Seaweeds are the most abundant attached marine plants in the ocean (Bolton *et al.*, 2004). Most of them are green (Chlorophyta), brown (Phaeophyta), red algae (Rhodophyta) and cyanobacteria. Each group is characterized by specific combinations of photosynthetic pigments (Hunt, 1978). Seaweeds are structurally simpler than terrestrial plants. Being immersed in water, they can absorb nutrients, water, dissolved gases and sunlight through their entire surface of the plant. Unlike terrestrial plants, seaweeds have no roots, leaves nor a complex network to transport food and water around the plants (Hay, 2001).

Seaweeds greatly influence environmental conditions for other types of marine life by providing food, shade protection from waves and as substrate for attachment of other organisms. Seaweeds were one of the first groups of marine organisms whose natural product chemistry was studied extensively because of their abundance in shallow waters. During the past 30 years, marine natural product chemists have reported the discoveries of a large number of novel metabolites with useful pharmacological properties (Faulkner, 2000). The majority of the studies on seaweeds occurred after the development of many of the useful mechanism bioassays used today.

2.2.1 Classification of Seaweeds

Three major classes of photosynthetic pigments found in algae are chlorophylls, carotenoids (carotenes and xanthophylls), and phycobilins. Seaweeds are classified into three major groups according to their photosynthetic pigments which are green, brown and red algae.

The bright green colour characteristic of the green algae (Chlorophyta) is due to the predominance of chlorophyll over other accessory photosynthetic pigments. Although chemical studies of temperate species have not been productive, the tropical green algae are known to produce many interesting biologically active metabolites (Fenical & Paul, 1984).

In the brown algae (Phaeophyta), the green hue of chlorophyll is partially masked by the golden xantophylls accessory photosynthetic pigments. Many of the large, more familiar and dominant algae of temperate sea, such as kelps, belong to this division. Natural product studies of this division have been productive, with many reports of biologically active metabolites, predominantly diterpenes (Demetzos & Dimas, 2001).

The red algae (Rhodophyta) contain not only chlorophyll, but the accessory red phycoerythrin and blue phycocyanin pigments. They can display a wide range of colours, from bright green to various shades of red. They are as diverse in structure and habitats as they are in coloration. The chemistry of the red algae has been studied extensively because of their propensity to include halogens in their biosynthetic pathways. Indeed, the marine natural products literature was dominated by reports of halogenated metabolites derived from red algae (Carte, 1996).

2.2.2 Uses of Seaweeds

There are many uses of seaweeds since ancient time. One of the most important uses is its application in food industries. Seaweeds are the only sources of production for valuable polysaccharides such as alginates, agars and carrageenans. Agar is used as gel in foods and in bacterial growth media. Carrageenans are gel-forming compounds derived from red algae which belong to the genus *Euchema*. It has been used mostly as a preservative in meats and

fish products. Alginates are derived from large brown seaweeds growing in cold-water areas of the world. The main commercial sources of brown seaweeds are *Ascophyllum*, *Laminaria* and *Mycrocystis* (Patel, 2006).

Seaweeds also contain a wide spectrum of bioactive compounds valuable for human health. They are commonly used in Chinese medicine such as kunbu. Kunbu is a medicine for goitre, tumour, and swelling. Seaweeds also possess compounds exhibiting antimicrobial potential against the pathogenic microbes of medical, agricultural and environmental importance. Besides that, seaweeds had been reported to have pharmaceutical values. Their inflammatory and immunomodulating activities have made them useful in a few pharmacological assays used to discover potential therapeutics (Renn, 1993). More examples are shown in Table 2.1.

Seaweeds have been used as food particularly in Far Eastern countries due to their high content of polysaccharides, minerals and certain vitamins. Seaweeds contain more minerals than any other food due to the surface cell wall polysaccharides that freely and selectively absorb inorganic nutrient from the sea (Jacobsen, 2010). *Porphyra umbilicalis* (purple laver) is among the most nutritious seaweeds (Indergaard & Minsaas, 1991). Furthermore, seaweeds are also used as animal fodder. Algae usually contain single cell protein, which may supplement animal food.

Table 2.1: Examples of medicinal seaweeds and their applications

Types of seaweeds	Medicinal values
Green algae	
Caulerpa racemosa	Hypotensive and anaesthetic actions
Codium iyengarii	Inhibition of Gram positive and Gram negative bacteria
Ulva fasciata	*In vitro* antiviral activity
Ulva prolifera	Medicine for decreasing the level of cholesterol
Brown algae	
Durvillaea antartica	Treatment for scabies
Ecklonia kurome	Medicine to cure scrofula, goitre, tumour, oedema, testicular pain and swelling
Laminaria japonica	Medicine to dilate the cervix as the stipes swell to several times their original diameter when moistened
Padina boryana	Exhibited hypoglycaemic activity
Saccharina japonica	Medicine to cure scrofula, goitre, tumour, oedema, testicular pain and swelling
Sargassum confusum	Hypocholesterolaemic and hypoglycaemic agents
Sargassum fusiformis	Medicine for producing a cooling and blood cleansing effect for the treatment of glandular weakness
Zonaria diesingiana	Potential anticancer drug
Red algae	
Asparagopsis taxiformis	Medicine to cure goitre
Hypnea musciformis	Exhibited diuretic activity
Laurencia pannosa	Inhibition of marine bacteria
Porphyra atropurpurea	Medicine for dressing wounds and burns

(Stein & Borden, 1984; Naqvi *et al.,* 1980)

2.3 Oxidative Stress

For decades, oxidative stress has been suspected as a mechanism for some of the processes that lead to aging and diseases (*ScienceDaily*, 2009). Oxidative stress is defined as an imbalance between oxidants and antioxidants (Sies, 1985). Oxidative stress has been linked to the pathogenesis of many chronic diseases and has showed links to fatigue, muscle damage and reduced immune function (Bellinger *et al.*, 2008).

Oxidative stress contributes to more than one hundred diseases in humans including atherosclerosis, arthritis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer, acquired immune deficiency syndrome (AIDS) and also degenerative diseases associated with aging (Zima *et al.*, 2001). It can damage cells by chain reaction, such as lipid peroxidation or formation of DNA adducts that could cause cancer-promoting mutations or cell death.

Free radicals are generated during metabolism and energy production in the body (Parker & Cadenas, 2007). In order to counteract these free radicals, the body produces an armory of antioxidants such as radical scavenging enzymes (i.e. superoxide dismutase) and cellular antioxidant (i.e. vitamin E) to neutralize the free radicals. Antioxidants are able to protect living organisms from damage by inhibiting the initiation or propagation of oxidative chain reactions. However, the body's internal production of antioxidants is not able to neutralize all the free radicals generated. Furthermore, diseases, aging and chemical challenges such as drugs, pesticides, herbicides and various pollutants can disrupt this balance by inhibition of the cellular antioxidant defenses or by stimulation of the formation of free radicals (Finkel & Holbrook, 2000).

2.5 Free Radicals

A free radical is defined as any molecule or molecular fragment that contains one or more unpaired electrons (Gutteridge, 2003). The presence of

unpaired electrons makes free radicals more reactive than the corresponding non-radicals because free radicals strive to balance their unpaired electrons with electrons from other molecules. When a radical reacts with a non-radical another free radical is formed, creating a chain reaction. Depending on the free radical and the non-radical molecule involved, a chain reaction can give rise to wide array of free radicals, which potentially could be more or less reactive than the free radical that initiated the chain reaction. There are two major free radical groups which are oxygen-free radicals and nitrogen-free radicals.

Reactive oxygen species (ROS) is the term used to describe oxygen-derived free radicals and other oxygen-derived free radicals (Parker & Candenas, 2007). Oxygen generally exists in its diatomic ground state (O_2), which by definition is a biradical because it has two unpaired electrons spinning parallel (i.e. they both share the same spin quantum number) to one another in separate orbitals (Parker & Candenas, 2007). Thus oxygen is not very reactive towards non-radicals despite its strong oxidizing potential, as non-radical molecules have paired electrons spinning in opposite directions and does not fit the vacant orbital spaces of molecular oxygen, in accordance to Pauli's principle. Consequently, oxygen tends to accept one electron at a time with the potential to form highly reactive oxygen intermediates or ROS.

ROS are continuously produced since they are natural byproducts of cell metabolism. Toxic oxygen metabolites may cause tissue damage. Such damage develops whenever the balance between the rate of free radical production and the cell's ability to endogenously eliminate these is disrupted.

Nitric oxide is a free radical species which contains an unpaired electron in the outer orbital. It reacts relatively with O_2 producing nitrogen dioxide (NO_2), a very reactive species. Further reactions of $^{\cdot}NO$ with $^{\cdot}NO_2$ will eventually produce nitrite (NO_2^-), which is the major decomposition product of $^{\cdot}NO$.

ROS is a collective term that includes both oxygen radicals and certain non-radicals that are oxidizing agents and are easily converted into radicals (HOCl, O_3, $ONOO^-$, $^1O_2$, H_2O_2). Reactive nitrogen species (RNS) is also a collective term including nitric oxide and nitrogen dioxide radicals, as well as nonradicals such as HNO_2 and N_2O_4. $ONOO^-$ is often included in both categories. Peroxynitrite ($ONOO^-$) is a highly stable and toxic non-radical anion. It is able to react with DNA, ribonucleic acid (RNA), proteins and lipids.

2.5 Antioxidants

Antioxidants are chemicals that can react with radicals and prevent the oxidation of other molecules (Davles, 1995). In order for a chemical to be defined as an antioxidant, it must meet two conditions. When present in low concentrations it will delay or prevent the oxidation of another compound, and the radical formed from the resultant reaction must be relatively stable and must not promote oxidation (Sigh, 2002). A very common way to classify antioxidants is to divide them into mechanistically distinct groups-primary and

secondary antioxidants (Dapkevicius, 2002). Another classification of antioxidants is to divide them as (a) chain breaking (vitamin E, phenolics), (b) preventive (intracellular enzymes, such as catalase, superoxide dismutase and others) and (c) complementary (vitamin C, β-carotene, flavanoids) (William & Elliott, 1997).

Primary antioxidants delay or inhibit the initiation step and interrupt the propagation step of the radical chain reaction (Dapkevicius, 2002). Antioxidants act by transferring a hydrogen atom to a peroxy radical. The resulting radicals from the oxidized antioxidant are stabilized by resonance and are relatively unreactive and therefore are not capable of initiating or propagating the oxidative reaction (Figure 2.16) (Wong, 1989). Most of the antioxidants used in food protection are primary antioxidants. Basically they are different phenolic compounds with various ring substitutions: phenolics acids, catechins, flavanoids, anthocyanidins, lignins, tannins and coumarins. Synthetic antioxidants, like butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ) and propyl gallate (PG) also have phenolic structures. Another class of antioxidants is the secondary or preventive antioxidants. They include metal chelating agents, singlet oxygen quenchers, peroxide destructors and some others (Larson, 1997).

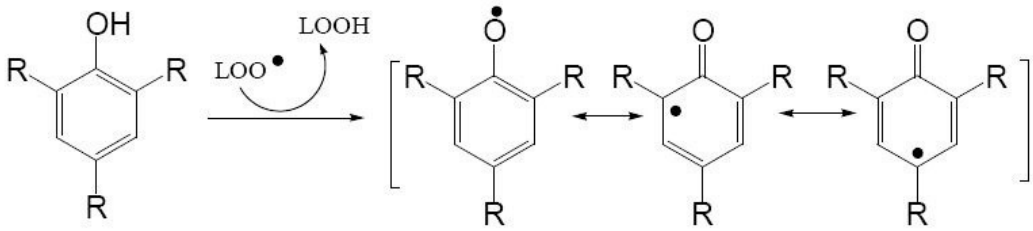


Figure 2.16: Resonance stabilization of phenoxy radical

Theories for antioxidant protective mechanisms in living systems include the donation of electrons or hydrogen atoms to oxidized molecules such as fatty acids, phospholipids, and proteins, which stabilizes them, scavenging radical compounds and atoms such as $OH^{\bullet}$, $O_2^{-\bullet}$, and $ROO^{\bullet}$, which become oxidized, and they coordinate transition metals such as copper and iron, which would prevent the interaction of the metal with an oxidization molecule thus preventing oxidation (Victor *et al*., 2006).

2.5.1 Natural and Synthetic Antioxidants

Antioxidants can be natural or synthetic. The best known synthetic antioxidants are butylhydroxyanisole (BHA), BHT, gallates and TBHQ. The biggest advantage of synthetic antioxidants is related to their low cost. Other advantages are their well-studied chemical and technological properties, which in most cases meet the demands of producers. However, synthetic antioxidants have been found to exhibit carcinogenic affect on experimental animals (Cakir *et al.*, 2003). Jun and Zhang (2007) also found that BHA have tumour-

initiating and tumour-promoting actions. Therefore, discovery of new natural antioxidants is important.

Among natural antioxidants, only a small percentage has been thoroughly analyzed and even fewer are actually being used. To date, only tocopherols, carotenoids, ascorbic acid and its derivatives, as well as extracts from rosemary and sage have been industrially applied in foods.

Tocopherols (Figure 2.17) are very important natural antioxidants. The antioxidant mechanism of tocopherol involves reactions with free radicals especially the peroxyl radical, resulting in the formation of a relatively stable phenoxy radical (Miliauskas, 2006).

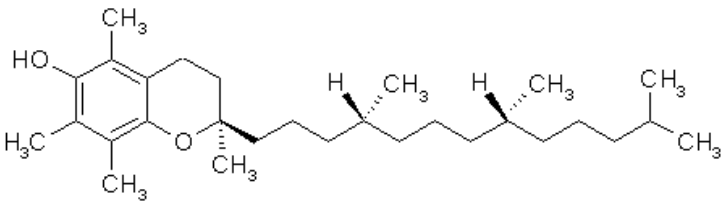


Figure 2.17: Tocopherol

Carotenoids also possess antioxidant activity. The radical trapping ability of carotenoids lies on the delocalization mechanism of unpaired electrons over the conjugated carotene system, making it less likely for the formed radical to take part in chain processes (Miliauskas, 2006).

Ascorbic acid acts as a multifunctional antioxidant and as a synergist for the primary antioxidants in which the original antioxidant molecules may be generated through reduction. In the presence of higher concentrations of metal ions, ascorbic acid shows pro-oxidant properties by reducing back oxidized metal ions after which they can initiate new free radical reactions (Stadtman, 1991).

Flavanoids represent a large and diverse group of phenolic compounds which can display a wide range of substitution patterns and oxidation states (Miliauskas, 2006). The compounds appear to possess variable mechanisms of action, which include radical scavenging and metal ion complexation. Quercetin (Figure 2.18) is a flavanoid which shows antioxidant activity.

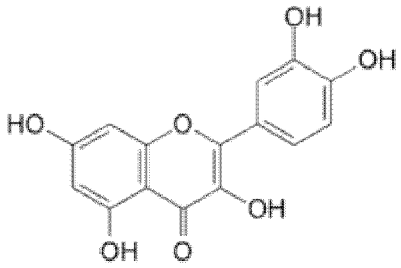


Figure 2.18: Quercetin

Another class of antioxidants are the phenolic acids which act as free radical terminators. Phenolic antioxidants act to inhibit lipid oxidation by trapping the peroxyl radical. This radical abstracts a hydrogen atom (or electron after prior loss of a proton) from the antioxidant to yield a phenoxyl radical (Marinova & Yanishlieva, 1992).

2.6 Classification of Medically Important Bacteria

Bacteria have been classified according to "Gram stain reaction", named after Christian Gram who devised the protocol for his staining process in 1884. On the basis of their reaction to the Gram stain, bacteria can be divided into two major groups which are Gram-positive and Gram-negative (Madigan & Martinko, 2006). Gram-positive bacteria appear in purple colour when treated with violet dye and iodine due to high amount (90%) of peptidoglycan (thicker layer) in the cell wall. In Gram-negative bacteria, the thin peptidoglycan layer in the periplasm does not retain the purple stain and the pink safranin counterstains the peptidoglycan layer.

The shape of bacteria is also used to classify them. Three basic shapes of bacteria are round (cocci), rod (bacilli) or spiral (spirilla) (Madigan & Martinko, 2006). Two types of Gram-positive and four types of Gram-negative bacteria were used in this research. The tested Gram-positive bacteria are *Staphylococcus aureus* (ATCC 6538) and *Bacillus cereus* (ATCC 11778). Gram-negative bacteria include *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* penicillin-sensitive strain (ATCC 25922) and *Escherichia coli* penicillin-resistant strain (ATCC 35218). These bacteria are explained in detail below.

S. aureus is a Gram positive coccus which appears in clumps like grapes when viewed under a microscope. It is a facultative anaerobic bacteria and forms large golden yellow colonies on rich medium (Todar, 2005). *S.*

aureus causes the majority of staph infections including skin infections, pneumonia, food poisoning, toxic shock syndrome and blood poisoning (bacteremia) (Madigan & Martinko, 2006).

Bacillus cereus is a Gram positive rod and facultative. It is endemic where the infection is maintained in the population without the need for external inputs (Madigan & Martinko, 2006). It is also a beta haemolytic bacterium that causes food borne diseases (Todar, 2006).

The genus *Klebsiella* belongs to the tribe Klebsiellae, a member of the family Enterobacteriaceae (Madigan & Martinko, 2006). Klebsiellae are nonmotile, rod-shaped, gram-negative bacteria with a prominent polysaccharide capsule. It has been a recognized pulmonary pathogen since its discovery over 100 years ago. It is also a common hospital-acquired pathogen that can cause urinary tract infections, nosocomial pneumonia and intra-abdominal infections (Madigan & Martinko, 2006).

E. coli is a Gram negative bacterium which belongs to the family of Enterobacteriaceae (Madigan & Martinko, 2006). It is a rod-shaped bacterium, facultatively anaerobic and unable to sporulate. *E. coli* is one of the most frequent causes of many common bacterial infections, including cholecystitis, bacteremia, cholangitis, urinary tract infections (UTI), and traveler's diarrhea (Nataro & Naper, 1998). As Gram negative bacteria, *E. coli* are resistant to many antibiotics which are effective against Gram positive organisms. Antibiotic such as amoxicillin, trimethoprim-sulfamethoxazole, ciprofloxacin,

nitrofurantoin are used to treat *E. coli* infection. Antibiotic resistance is a growing problem. In most bacteria examined, the cause of penicillin resistance has been attributed to the production of enzymes which inactivate penicillin, β-lactamase and amidase. These two enzymes are known to exist in *E. coli*.

P. aeruginosa is a Gram negative, aerobic rod belonging to the bacterial family Pseudomonadaceae. It is a free-living bacterium, commonly found in soil and water (Todar, 2004). However, it occurs regularly on the surfaces of plants and occasionally on the surfaces of animals. *P. aeruginosa* has become increasingly recognized as an emerging opportunistic pathogen of clinical relevance. It causes urinary tract infections, respiratory system infections, dermatitis, and a variety of systemic infections, particularly in patients hospitalized with cancer, cystic fibrosis, and burns (Qarah *et al.*, 2003).

2.7 The Action of Antimicrobial Drugs

Antimicrobial drugs are either bactericidal (they kill microbes directly) or bacteriostatic (they prevent microbes from growing) (Madigan & Martinko, 2006). Medically useful antimicrobial drugs exhibit selective toxicity, causing greater harm to the microorganism than the human host (Nester *et al.*, 2004). There are five mechanisms of action of antimicrobial drugs. These are inhibition of cell wall synthesis, inhibition of cell membrane function, inhibition of protein synthesis, inhibition of nucleic acid synthesis and inhibition of metabolic pathways (Nester *et al.*, 2004).

2.8 Previous Research on *Padina antillarum*

The seaweed used in this study is *Padina antillarum*. There are about 50 species of *Padina* worldwide, although most are poorly known and many would prove to be synonymous (Lee & Kamura, 1991). *P. antillarum* is traditionally known as *P. tetrastromatica* Hauck, which was recently designated as a synonym of this species (Wynne & DeClerke, 1999). *Padina antillarum* is distributed at Florida, East Asia, Hong Kong, Singapore, Thailand, Malaysia, Vietnam, Philippines, East Africa, India, Sri Lanka, Australia and New Zealand.

As few studies had been carried out on biomolecules of *P. antillarum*. A neutral laminaran like glucan and two new sulphated heteropolysaccharides comprising glucuronic acid, fucose, rhamnose, xylose, arabinose, galactose and glucose and half-ester sulphate were obtained (Prasado *et al*., 1984).

Five saturated fatty acids (isomyristic, palmitic, margaric, stearic and arachidonic) and three unsaturated fatty acids (palmitoleic, oleic and tetradecatrienoic) have been detected and identified. Two sterols, 24-methyl cholesterol and 24-methylone cholesterol were isolated from the genus *Padina* (Shakh *et al*., 1991).

Chew *et al.* (2007) found that antioxidant activity of *P. antillarum* was stronger than *Caulerpa racemosa* (green algae) and *Kappaphycus alverezzi* (red algae). Weak *in vitro* nematicidal activity was also found in *P. antillarum* by Rizvi and Shamel (2010). Vinayak *et al*. (2010) studied a few brown algae

for their cytotoxic and antioxidant activities. They found that *P. antillarum* showed strong free radical scavenging activity but weak cytotoxic activity (Vinayak *et al*., 2010).

A few of studies on *P. antillarum* had been carried out especially in Korea, India and Malaysia (Chew *et al*., 2007). However, the structure of the corresponding bioactive compounds has not determined. Therefore, this study is conducted to elucidate the structure of the bioactive compounds in *P. antillarum*.

CHAPTER 3.0

MATERIALS AND METHODS

3.1 Sample Collection and Preparation

P. antillarum (Figure 3.1) was collected from the shallow waters of the inter-tidal zone in Teluk Kemang, Port Dickson on five different days. The collection dates were 29th December 2005, 16th March 2006, 27th November 2007, 13th May 2008 and 28th April 2009. Upon collection, the samples were washed thoroughly with seawater to remove soil and debris, and were transferred to the laboratory in seawater for further cleaning. The samples were rinsed with distilled water to remove salt and epiphytes. The sample was freeze-dried using a freeze-dryer (Model: Martin Christ, Alpha 1-4, LD plus) and ground into powder using liquid nitrogen (Figure 3.2). The extraction was performed on the powdered samples to extract bioactive compounds.

Figure 3.1: *Padina antillarum*

Figure 3.2: Powdered *P. antillarum*

3.2 Isolation and Characterization of Antioxidants from *P. antillarum*

Bioassay-guided fractionation was used in order to isolate antioxidant compounds. The fractions that were collected in every stage of purification were subjected to antioxidant assays (total phenolic content and DPPH radical scavenging assays) to keep track of the bioactive compounds. The DPPH radical scavenging activity, ferric reducing antioxidant power (FRAP) and ferrous ion chelating activity of the isolated antioxidants were assessed and they were compared to the well-characterized antioxidants of plant origin (i.e. ascorbic acid, tannic acid and quercetin). The isolated antioxidants were also subjected to anticancer and antibacterial assays.

3.2.1 Selection of Extraction Solvents for the Extraction of Antioxidant Compounds from *P. antillarum*

The chemicals used were:

Ethyl acetate (Merck), hexane (Merck) and methanol (Merck)

Procedures:

Before extraction of *P. antillarum* samples in large quantities was carried out, extraction efficiency of various solvents for antioxidant compounds from five batches of *P. antillarum* were first tested. According to Chew *et al*. (2007), mixture of methanol and water will give the high extraction efficiency towards antioxidant compound as most of the antioxidants compounds are polar compounds (e.g. phenolic compounds, tannins, flavanols etc). To determine the reliability of the results tested by Chew *el al*. (2007), solvents which range from nonpolar to polar were tested to assess their extraction efficiencies. The selected solvents were hexane, ethyl acetate, 100% methanol and 50% methanol. Five grams of powdered *P. antillarum* of five batches were extracted by 50 mL of hexane, ethyl acetate, 100% methanol and 50% methanol respectively. These were agitated using an orbital shaker for 1 hour at 120 rpm, followed by gravity filtration using Whatman 542 filter paper. After that, 0.3 mL of each filtrate was transferred to a 10 mL glass sample vial and further dried using a vacuum concentrator (Model: Eppendorf). After filtrates had been dried, they were redissolved in 100% methanol and subjected to antioxidant assays.

3.2.2 Antioxidant Assays

Antioxidant properties of extracts were measured by two methods. There are total phenolic content (TPC) assay and DPPH radical scavenging assay.

3.2.2.1 Total Phenolic Content (TPC) Assay

The chemicals and reagents used were:
Folin-Ciocalteu's (FC) reagent (Fluka), gallic acid (Merck), methanol (Merck), sodium carbonate (Systerm)

Procedures:

Phenolic compounds are major category of natural products that play an important role as antioxidant. Thus it is important to conduct total phenolic content assay to screen phenolic compounds in seaweeds for antioxidant activities. The total phenolic contents were determined spectrophotometrically according to Folin-Ciocalteu's (FC) procedure used by Kahkonen *et al.* (1999). FC reagent will react with phenol and non-phenolic reducing substances to form blue chromogen. The colour development is due to the transfer of electrons at basic pH to reduce the phosphomolybdic/ phosphotungstic acid complexes to form chromogenin which the metals have lower valence (Bray & Thorpe, 1954).

Sample with a volume of 0.3 mL was mixed with 1.5 mL of FC reagent (diluted tenfold with distilled water). For the blank control, 0.3 mL of 100% methanol was added instead of sample. After 1 min, 1.2 mL of 7.5% (w/v) sodium carbonate solution was added. It is important to achieve enough though not excessive alkalinity (approximately pH 10) because of the relative ease of removing an electron from the phenolate ion under alkaline conditions. The mixtures were left to stand for 30 min for colour development in the dark as the FC reagent is light sensitive. After 30 min, the mixtures were shaken and absorbance was measured by single beam Ultraviolet-visible (UV-vis) spectrophotometer at 765 nm. A glass or plastic cuvette was used. All determinations were performed in triplicate.

Gallic acid was used as standard unit for total phenolic content determination because it covers a wide spectrum of phenolic compounds (Singleton *et al.*, 1999). A 100 mg/L gallic acid stock solution was prepared by dissolving 0.025 g of gallic acid in 250 mL of 100% methanol. The stock solution was wrapped with aluminium foil as it was light sensitive. A calibration curve was prepared using gallic acid standard solutions which ranged in concentration from 0 to 100 mg/L and the results were determined from regression equation of this calibration curve which was expressed as gallic acid equivalent (GAE) in mg/100g material. The preparation of gallic acid standard solutions is shown in Table 3.1.

Table 3.1: Preparation of Gallic acid standard solution

Concentration (mg/L)	Volume of 100% methanol (mL)	Volume of 100mg/mL Gallic acid stock (mL)
0.00	0.300	0.000
5.00	0.285	0.015
10.00	0.270	0.030
20.00	0.240	0.060
40.00	0.180	0.120
60.00	0.120	0.180
80.00	0.060	0.240
100.00	0.000	0.300

3.2.2.2 Determination of Free Radical-scavenging Activities of the Extracts

The chemical and reagents used were:

1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma Aldrich) and methanol (Merck)

Procedures:

1,1-diphenyl-2-picrylhydrazyl (DPPH) is used to evaluate the free radical scavenging activity of natural products. It is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH radical scavenging activity was measured using the method described by Leong and Shui (2002) with slight modifications. One milliliter of sample was added to 2 mL of DPPH (0.15 mM). One mililiter of 100% methanol was added to 2 mL of DPPH as blank control. The mixture was allowed to stand for 30 min in the dark at room temperature. After 30 min, absorbance was measured at 517 nm. All samples were tested in triplicate. The results were expressed in percentage of radical scavenging activity, calculated using the following formula:

$$\% \text{ radical scavenging activity} = \frac{(A_{Blank} - A_{Sample})}{A_{Blank}} \times 100\%$$

A_{Blank} refers to the absorbance of the blank control whereas A_{Sample} is the absorbance of the tested samples.

3.2.3 Extraction and Fractionation of Antioxidants from *P. antillarum*

The chemicals used were:

Anhydrous sodium sulphate (Systerm), chloroform (Merck), ethyl acetate (Merck) and methanol (Merck)

Procedures:

50% aqueous methanol was found to have the highest extraction efficiency towards antioxidants compared to the other solvents used. The powdered *P. antillarum* sample which was collected on 28th April 2009 (about 500 g) was immersed in 5 L of 50% aqueous methanol and shaken on an orbital shaker (Model: IKA) at 120 rpm for two days at room temperature. The extract was decanted and filtered using a filter paper. The seaweed residue was re-extracted a further four times. The extracts collected were concentrated under vacuum by rotary evaporator (Model: Büchi Rotavapor R-200) to remove the methanol. The total concentrated aqueous extract (approximately 5 L) was used for liquid-liquid extraction.

The aqueous extract was extracted with equal volume of chloroform. Before both of the layers were separated, the mixture was stirred vigorously using a magnetic stirrer. The mixture was poured into a separating funnel and two distinct layers were formed after 15 min. Both of the layers were separated and the aqueous layer was re-extracted a further two times. Anhydrous sodium sulphate was added into the separated chloroform layer (chloroform extract) to adsorb water and followed by filtration. The chloroform extract was evaporated to dryness using a rotary evaporator. The chloroform extract was further dried in a vacuum concentrator. The weight of the chloroform extract was recorded.

The aqueous layer was further extracted using ethyl acetate. Compounds of intermediate polarity were extracted into ethyl acetate layer (ethyl acetate extract). The weight of dried ethyl acetate extract was recorded.

The aqueous layer was dried using a vacuum concentrator. The weight of the aqueous extract was recorded. All dried extracts were re-dissolved in 100% methanol to a concentration of 1 mg/mL and subjected to antioxidant assays.

3.2.4 Isolation of Extract which Exhibited the Highest Antioxidant Activity

The chemicals used were:

Acetone (Merck), acetic acid (Systerm), C_{18} powder (15 µm, Merck), chloroform (Merck), dichloromethane (Merck), ethanol (Merck), methanol (Merck) and silica gel 60 (15 to 40 µm, Merck)

Procedures:

Fractionation of active extract was performed using column chromatography. Repeated separation through column chromatography using appropriate stationary phase and mobile phase solvents system was previously determined and optimized by thin layer chromatography (TLC). Two most common separation systems are normal phase chromatography and reversed phase chromatography. Normal phase chromatography use a polar stationary phase (i.e. silica gel) and a non-polar mobile phase with a gradually increasing concentration of the polar solvent. Thus hydrophobic compounds are eluted quicker than hydrophilic compounds. Reversed-phase chromatography using a nonpolar stationary phase and a polar mobile phase. The stationary phase consists of reversed phase material (i.e. C_8 and C_{18}). The more hydrophobic the matrix, the stronger the tendency of the column to retain hydrophobic compounds. Thus, hydrophilic compounds are eluted faster than hydrophobic compounds.

The active extract which exhibited the highest antioxidant activity was subjected to flash chromatography (Figure 3.3) to isolate the pure antioxidant compounds. Flash chromatography is a preparative column chromatography based on the basis of optimized prepacked columns (i.e normal phase or reversed-phase column) and an air pressure driven eluent at high flow rate. Before fractionation was carried out, the appropriate mobile phases were selected by referring to the results from thin layer chromatography (TLC) analysis. The mobile phases were selected based on the ability of the mobile phases to give good separation of the compounds in the extract.

Figure 3.3: Flash chromatography (Model: Supelco)

The active extract was subjected to silica gel flash chromatography with a dimension of silica cartridge at 40 x 150 mm to isolate antioxidant compound. The silica cartridge was packed with Merck silica gel 60 (15 to 40 µm particle size). A chloroform-acetone system was used as the mobile phase,

beginning with 100% chloroform and ending with a chloroform-acetone mixture of ratio 1:4. Ethanol was used to flush the remaining extract which was trapped in the silica gel cartridge. The selected mobile phases and volumes of each mobile phase are shown in Table 3.2. Ninety fractions with volumes of approximately 50 mL were collected at a flow rate of 8.5 mL/min. The fractions were evaporated to dryness under vacuum and were subjected to antioxidant screening assays (TPC assay and DPPH radical scavenging assay).

Table 3.2: Mobile phases for purification of extract which exhibited the highest antioxidant activity

No. of mobile phases	Mobile phases	Volume of each mobile phase, mL
1	100% chloroform	400
2	80% chloroform: 20% acetone	400
3	77% chloroform: 23% acetone	400
4	74% chloroform: 26% acetone	800
5	71% chloroform: 29% acetone	400
6	68% chloroform: 32% acetone	400
7	65% chloroform: 35% acetone	400
8	60% chloroform: 40% acetone	400
9	20% chloroform: 80% acetone	400
10	100% ethanol (flushing)	500

The active fractions which exhibited the highest antioxidant activity were further purified by C_{18} flash chromatography. A C_{18} cartridge with a dimension at 40 x 75 mm which was packed with Merck C_{18} (15 µm) was used. The initial mobile phase used was 100% acidified water, after which the methanol content was increased until it contained 100% methanol (Table 3.3). A total of sixteen fractions each with a volume approximately 50 mL were collected at a flow rate of 8.5 mL/min. The fractions were dried under vacuum

using a concentrator and antioxidant screening was carried out to keep track of the antioxidant compounds.

Table 3.3: Mobile phases which were used for purifications of active fractions from purification of extract which exhibited the highest antioxidant activity

No. of mobile phases	Mobile phases	Volume of each mobile phase, mL
1	100% acidified water	200
2	80% acidified water: 20% methanol	200
3	60% acidified water: 40% methanol	200
4	100% methanol	200

*Acidified water is a mixture of 1% acetic acid in distilled water.

The active fractions were subjected to silica gel flash chromatography using a 50 x 75 mm silica cartridge. Two mobile phases were selected which are shown in Table 3.4. A dichloromethane-acetone system was used as the mobile phase, beginning with dichloromethane-acetone mixture of ratio 4:1 and ending with a ratio of 2:3. A total of ten fractions each with a volume approximately 40 mL were collected at a flow rate of 8.5 mL/min. The fractions were screened for antioxidant activity.

Table 3.4: Mobile phases for purification of active fraction which was collected from C_{18} flash chromatography

No. of mobile phase	Mobile phases	Volume of each mobile phase, mL
1	80% dichloromethane: 20% acetone	200
2	40% dichloromethane: 60% acetone	200

Finally, the purity of the compounds isolated was analyzed using a Shimadzu high performance liquid chromatography (HPLC) (Model: PRC10A) fitted with a Merck LiChroCART® 75-4 C_{18} column and a Shimadzu gas chromatography-mass spectrometer (GC-MS) (Model: QP2010 Plus) fitted with a BP-1 (30 m X 0.25 mm, 0.25 μm film thickness) to assess the purity of the active fractions. The pure active fractions were subjected to ^{1}H-nuclear magnetic resonance (NMR) (Model: Bruker, JOEL, Japan, 400 MHz) and ^{13}C NMR (Model: Bruker, JOEL. Japan, 100 MHz) for structural elucidation.

The summary of the extraction and fractionation of antioxidant from *P. antillarum* are shown in Figures 3.4 and 3.5 respectively.

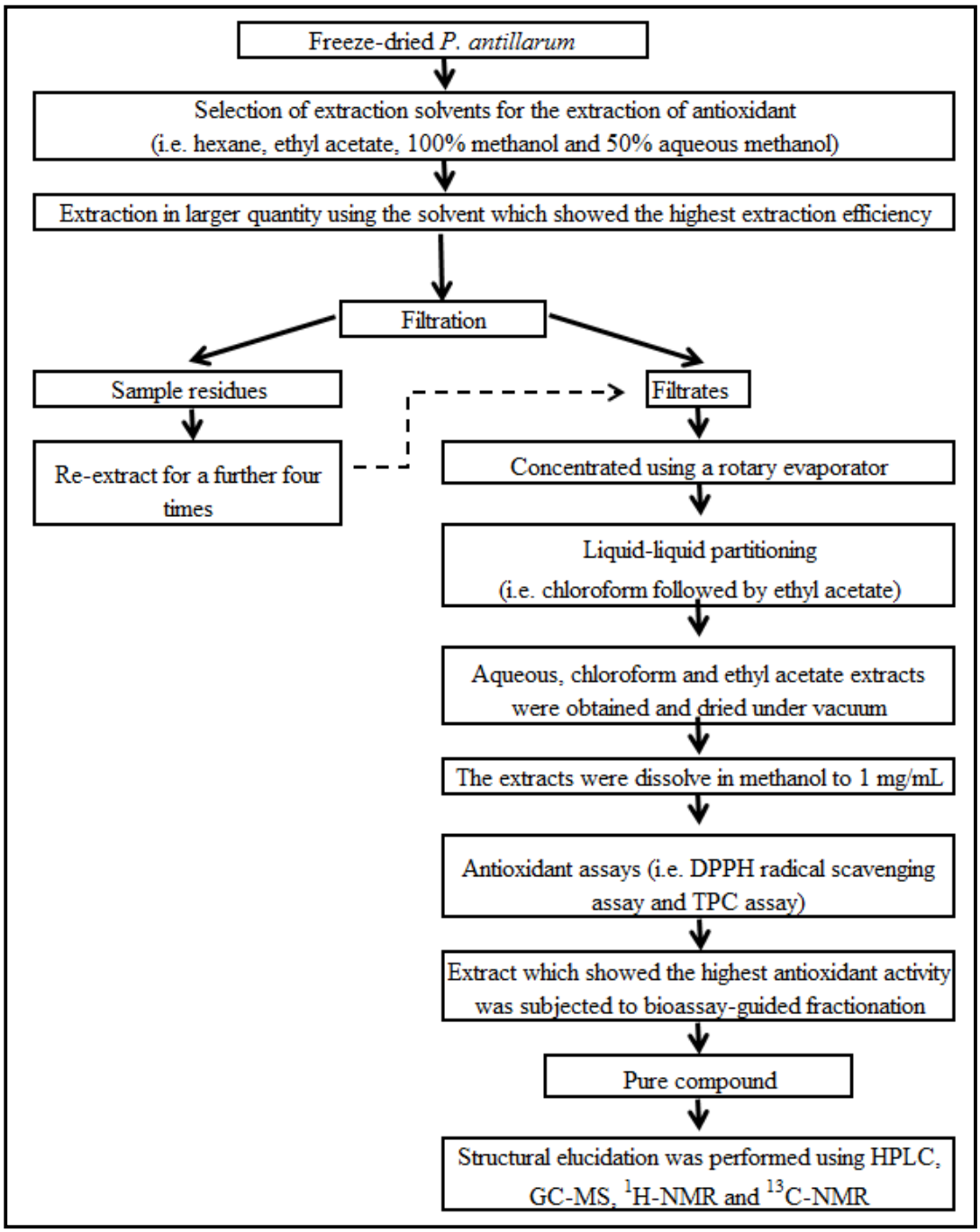


Figure 3.4: Flow chart of the extraction of antioxidant from *P. antillarum* and the chromatographic methods

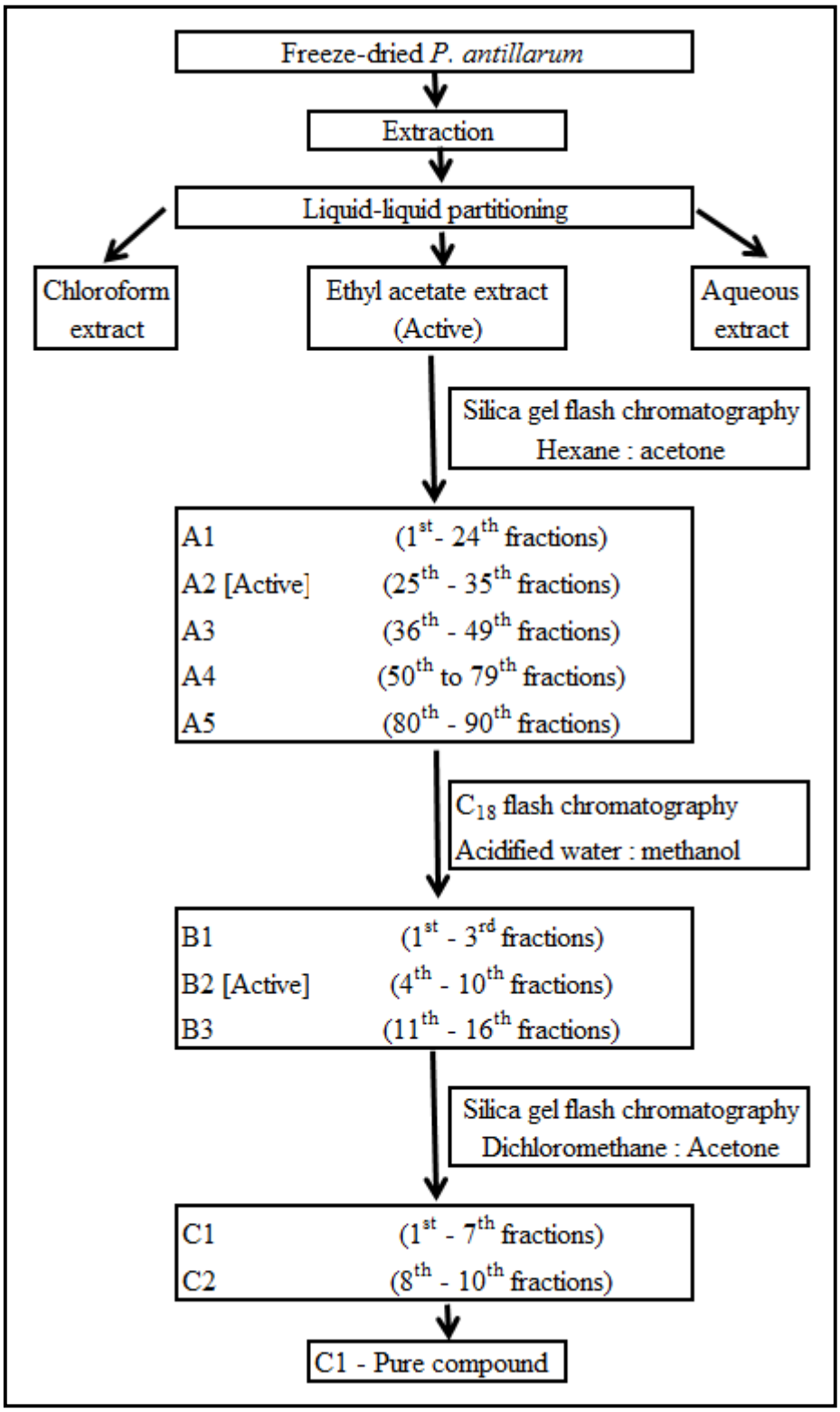


Figure 3.5: Flow chart of the progress to obtain the pure antioxidant from *P. antillarum*

3.2.5 High Performance Liquid Chromatographic (HPLC) Analysis on the Isolated Antioxidant from *P. antillarum*

The chemicals used:

1% acetic acid in distilled water (acidified water) and methanol (HPLC grade, Merck)

Procedures:

HPLC is a chromatographic technique that is used to identify, quantify and separate a mixture of compounds. The isolated antioxidant compound was subjected to Shimadzu HPLC (Model: PRC10A) fitted with a Merck LiChroCART® 75-4 C_{18} column. An aliquot of the filtrate (20 µL) was injected into the HPLC column and eluted with a linear gradient of mobile phase containing solvent A (0.1% acetic acid) and solvent B (methanol). The solvent gradient was programmed from 0% to 100% solvent B in 30 min with a flow rate of 1 mL/min. The peaks in the chromatogram were detected by a photodiode array detector (Model: SPD-M20A) at 254 nm.

3.2.6 Gas Chromatography-Mass Spectrometric Analysis on the Isolated Antioxidant from *P. antillarum*

The chemical used was:

Methanol (GC grade, Merck)

Procedures:

GC-MS is a chromatographic technique that can identify different volatile compounds within a test sample. The isolated antioxidant from *P. antillarum* was subjected to GC-MS to identify the antioxidant by the MS library (NIST 05). The isolated antioxidant was dissolved in methanol, after which it was filtered using a syringe filter (0.45 μm) prior to injection.

GC-MS analysis was carried out using a Shimadzu GCMS QP 2010 Plus instrument. The column used was BP-1 (30 m X 0.25 mm, 0.25 μm film thickness). The column oven temperature was programmed to increase gradually from 130^{o}C to 280^{o}C at 9^{o}C/min using helium as the carrier gas. The ion source temperature and interface temperature was set at 200^{o}C and 290^{o}C respectively. The pressure used was 118.3 kPa.

3.2.7 ^{1}H-Nuclear Magnetic Resonance and ^{13}C-Nuclear Magnetic Resonance Spectroscopic Analysis on the Isolated Antioxidant from *P. antillarum*

The chemical used was:

Deuterated methanol D4

Procedures:

The isolated antioxidant from *P. antillarum* was subjected to ^{1}H-nuclear magnetic resonance (NMR) (Model: Bruker, JOEL, Japan, 400 MHz) and ^{13}C

NMR (Model: Bruker, JOEL. Japan, 100 MHz) for structural elucidation. The isolated antioxidant was dried under vacuum, after which it was dissolved in deuterated methanol D4. The sample was transferred to a NMR tube (Norell, 400MHz) until it achieved a height of 4 cm in the NMR tube.

3.2.8 Thin Layer Chromatography (TLC)

The chemical used was:

Iodine (Merck)

Procedures:

TLC was used to assess the purity and number of compounds in the extracts or fractions. Crude extracts and fractions from every stage of purification and isolated compounds were analyzed on precoated 5 x 10 cm, 0.25 mm thickness silica gel 60 F_{254} aluminium plates which were purchased from Merck.

The extracts were spotted on TLC plate by using a fine glass capillary tube. The mobile phase was poured into a developing tank to a depth of 0.5 cm. A piece of filter paper was placed inside the developing tank to aid the saturation of the TLC chamber with solvent vapours. Different types of solvents or mixture of solvents were used to find out which solvents are able to isolate the compounds in the extract. The developed TLC was visualized under UV lamp. UV-active organic compounds appeared as purple spots on TLC.

The TLC was stained with iodine vapours to visualize UV-inactive organic compounds. These were visualized as brown spots.

3.2.9 Comparison of Antioxidant Activities between Isolated Antioxidants from *P. antillarum* and Standard Antioxidants

The chemicals used were:

Ascorbic acid (Merck), methanol (Merck), tannic acid (Sigma Aldrich) and quercetin (Merck)

Procedures:

The antioxidant activity of purified antioxidant that was isolated from *P. antillarum* was compared to well-characterized antioxidants derived from plants, ascorbic acid, tannic acid and quercetin. For comparison, these antioxidants were subjected to three antioxidant assays for comparison which were DPPH radical scavenging assay, ferric-reducing antioxidant power (FRAP) and ferrous ion chelating (FIC) assay. For the DPPH radical scavenging assay and FRAP, 100 ppm of antioxidant was prepared in 100% methanol. 5000 ppm of antioxidants was prepared in 100% methanol for FIC assay. Dilutions were performed to obtain a series of concentrations, as shown in Table 3.5.

Table 3.5 Series of concentration of antioxidants for different antioxidant assays

Antioxidant assays	Concentration, ppm					
DPPH radical scavenging assay	100	50	25	12.5	6.25	3.13
FRAP	100	50	25	12.5	6.25	3.13
FIC assay	5000	2500	1250	625	313	156.5

3.2.9.1 DPPH Radical Scavenging Assay

The chemicals used were:

1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich), ascorbic acid (Merck), tannic acid (Sigma Aldrich) and quercetin (Merck)

Procedures:

DPPH radical scavenging activity of the purified antioxidant that was isolated from *P. antillarum* was compared to ascorbic acid, tannic acid and quercetin. EC_{50} of the tested antioxidants were measured in order to compare their DPPH radical scavenging activities. The EC_{50} value expresses the effective concentration of antioxidant required to reduce DPPH radical concentration by 50%. The procedures were carried out as described in section 3.2.2.2.

3.2.9.2 Ferric-reducing Antioxidant Power (FRAP)

The chemicals used were:

Iron (III) chloride (R & M Chemicals), potassium ferricyanide (Systerm), potassium phosphate (Merck) and trichloroacetic acid (Merck)

Procedures:

The FRAP assay was determined based on the methods used by Chu *et al*. (2000). Potassium phosphate buffer (0.1 M, pH 6.6) with a volume of 2.5 mL and 2.5 mL of 1% w/v potassium ferricyanide were mixed with 1 mL of antioxidant of varying dilutions. The reaction mixture was incubated for 20 min at 50^{o}C, after which 2.5 mL of 10% w/v trichloroacetic acid was added in. After that, 2.5 mL of the mixture solution was transferred to 2.5 mL of water followed by addition of 0.5 mL of 0.1% w/v iron (III) chloride. The solution was incubated for 30 min at room temperature for colour development. The absorbance was measured at 700 nm. All assays were performed in triplicate.

3.2.9.3 Ferrous Ion Chelating (FIC) Assay

The chemicals and reagents used were:

Ferrozine (Fluka), iron (II) sulphate (Uni-Chem) and methanol (Merck)

Procedures:

Iron generates free radicals through the Fenton and Haber-Weiss reaction (Singh & Rajini, 2004). Iron can stimulate lipid peroxidation and accelerate peroxidation by decomposing lipid peroxides into peroxyl and hydroxyl radicals. Peroxyl and hydroxyl radicals are able to abstract hydrogen and perpetuate the chain reaction of lipid peroxidation (Büyükokuroglu, 2001). Antioxidants with FIC activity would prevent the Fenton-type reaction by reducing the concentration of the catalyzing transition metal in lipid peroxidation. The binding of the antioxidant components to metal ions was evaluated using FIC assay. It is reported that chelating agents that form σ-bonds with a metal are effective secondary antioxidant since they can reduce the redox potential. Thus, they stabilize the oxidized form of the metal ion (Zou *et al*., 2010).

The FIC assay was conducted based on Singh and Rajini (2004). Equal volumes of 0.1 mM iron (II) sulphate, antioxidants of various dilutions and 0.25 mM ferrozine were mixed. 100% methanol was added in instead of antioxidant for the blank control. The reaction mixtures were incubated for 10 min in dark at room temperature and the absorbance was measured at 562 nm. The test was performed in triplicate. The result was expressed in percentage of chelating ability using the following equation:

$$\text{Chelating ability (\%)} = [(A_{blank} - A_{sample})/A_{blank}] \times 100\%$$

A_{blank} refers to the absorbance of the blank control whereas A_{sample} is the absorbance of tested antioxidants.

3.2.10 Cytotoxicity Testing on Isolated Antioxidant from *P. antillarum*

The chemicals used were:

3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich), dimethyl sulfoxide (DMSO) (Merck) and K-562 leukemic cell (Sigma Aldrich)

Procedures:

The measurement of surviving or proliferating cells was achieved by MTT-cytotoxicity testing. Cytotoxicty testing is widely used in screening programme as cytotoxicity screening model provides important preliminary data for selecting potential extracts containing anticancer properties for future work (Cardellina II *et al.*, 1999). It is a colorimetric assay commonly used to assess viability of cell line.

Antioxidants that were isolated from *P. antillarum* were dissolved in dimethyl sulfoxide (DMSO) to achieve a concentration of 20 mg/mL. The stock solution was sonicated for 15 min before cytotoxicity test was carried out using 96-well plates. The cell line used for this study was K-562 (leukemic

cell) and tissue culture medium used comprises of Roswell Park Memorial Institute 1640 (RPMI) and fetal bovine serum (FBS).

The cytotoxicity test was performed in a laminar flow chamber. Stock solution with a concentration of 20 mg/mL was diluted to 100 µg/mL (working stock A) and 200 µg/mL (working stock B) with tissue culture medium respectively. The working stocks with a volume of 100 µL were added to the testing wells accordingly (Figure 3.6) except for the medium control and the cell control. Both working stocks were tested in triplicate. After that, 200 µL of tissue culture medium were added to medium control and 100 µL of tissue culture medium were added to cell control. Finally, 100 µL of cell suspension was added to all wells except for the medium control. The 96-well plate was incubated for 72 h at 37^{o}C with 5% carbon dioxide.

After incubation, 20 µL of 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) solution was then added to all wells. It was incubated for 3 h at 37^{o}C with 5% carbon dioxide. The plate was then centrifuged at 1500 rpm for 5 min and 150 µL of the supernatant from all wells was discarded, followed by addition of 150 µL of DMSO. The 96-well plate was shaken gently until all the precipitate was dissolved, after which the plate was placed in the ELISA microplate reader and the absorbance values of the wells were recorded at the wavelength of 550 nm. The precipitate (blue formazan crystal) was the reaction product of MTT solution and mitochondrial dehydrogenase which is present only in live cells. The formazan crystal

generated is directly proportional to the amount of live cells (Mossman, 1983). The percentage of cell viability was calculated using the formula below:

$$\text{Percentage of cell viability (\%)} = \frac{(A_{\text{sample}} - A_{\text{medium control}})}{(A_{\text{cell control}} - A_{\text{medium control}})}$$

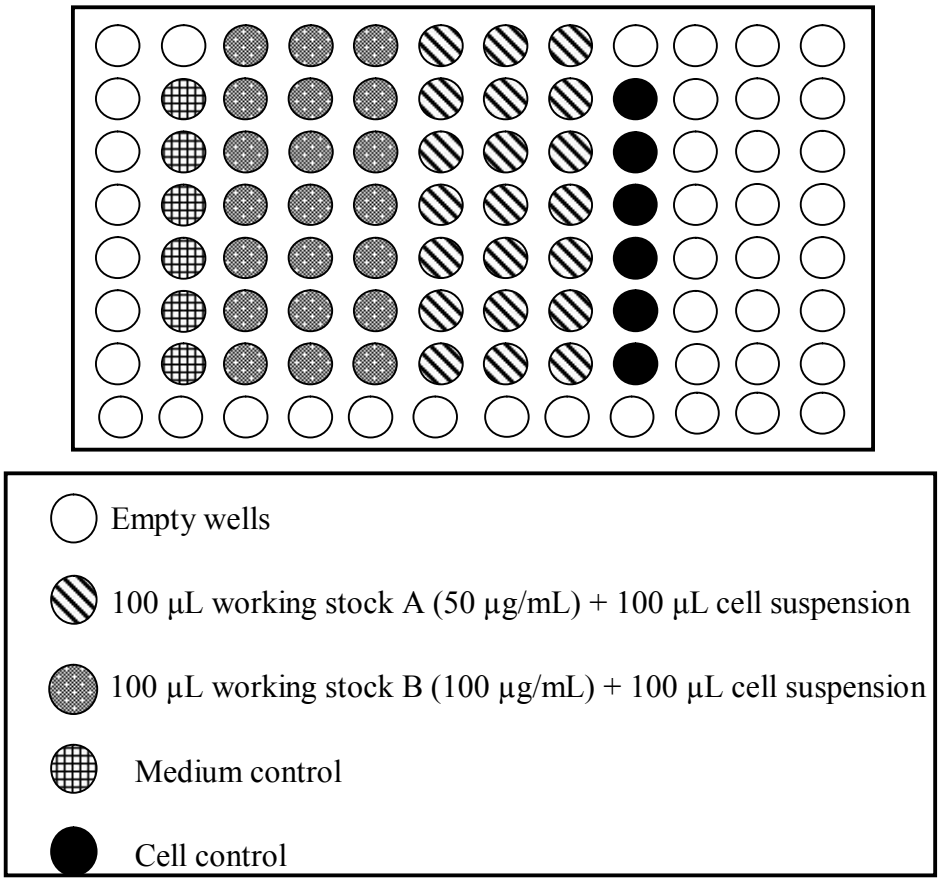


Figure 3.6: Design of the 96-well plate for cytotoxicity tests

3.2.11 Screening of Antibacterial Activity of Isolated Antioxidants from *P. antillarum*

The purified antioxidants that were isolated from *P. antillarum* were subjected to antibacterial and antifungal assays. The methods are described in section 3.3.2.

3.3 Partial Purification of Antimicrobial Compounds from *P. antillarum*

Bioassay-guided fractionation was used in order to isolate antimicrobial compounds from *P. antillarum*. The fractions that were collected in every stage of purification were subjected to antimicrobial assays to keep track of the bioactive compounds. Fractions with the lowest minimum inhibitory concentration (MIC) were subjected to further purification.

3.3.1 Extraction of Antimicrobial Compounds from *P. antillarum*

The chemicals used were:

Acetone (Merck), chloroform (Merck), diethyl ether (Merck), ethyl acetate (Merck), hexane (Merck) and methanol (Merck)

Procedures:

According to Siddiqui *et al*. (2009), some groups of antimicrobial compounds can be extracted by one solvent only. For examples, sterol can be extracted by ethanol and fatty acids can be extracted by ether only. Therefore, sequential extraction was used to extract 100 g of powdered *P. antillarum* which was collected on 13th May 2008. The solvents used ranged from nonpolar to polar (i.e. hexane, followed by diethyl ether, chloroform, ethyl acetate, acetone, methanol and finally distilled water). The extract for each solvent was agitated on an orbital shaker at 120 rpm for 24 h followed by gravity filtration using Whatman 542 filter paper. The seaweed residue was re-extracted for a further two times. The extracts were concentrated using a rotary evaporator and further dried using a vacuum concentrator. The weight of dried extracts were recorded and then stored at -20^{o}C until further analysis. Bioassay guided fractionation was applied in order to isolate antimicrobial compound from *P. antillarum*.

3.3.2 Microbial Susceptibility Testing

The inhibition of microbial growth was assessed by a colorimetric broth microdilution method, using *p*-iodonitrotetrazolium violet (INT) as an indicator. The reaction is based on the transfer of electrons from NADH, a product of the threonine dehydrogenase (TDH) catalyzed reaction, to the INT in viable microorganisms. During the active growth of bacteria or fungi, an electron is transferred from NADH (which is colourless) to p-

iodonitrotetrazolium violet resulting in the formation of a purple-colored formazan dye.

3.3.2.1 Preparation of Sample Extracts for Antimicrobial Screening

The seven extracts which were obtained from sequential extraction were redissolved in an appropriate solvent to a concentration of 20 mg/mL. The selected solvent must have no effect on the growth of the tested bacteria. Toxicity testing of the solvents was carried out to determine the inhibition of bacterial growth.

3.3.2.2 Bacterial Cultures

The bacteria used include both Gram positive and Gram negative bacteria. Gram positive bacteria were *Staphylococcus aureus* (ATCC 6538) and *Bacillus cereus* (ATCC 11778) whereas Gram negative bacteria were *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (penicillin-sensitive strain, ATCC 25922; penicillin-resistant strain, ATCC 35218).

3.3.2.3 Preparation of Bacterial Cultures

The chemicals used were:

Mueller-Hinton agar (Oxoid) and Mueller-Hinton broth (Oxoid)

Procedures:

The bacteria were subcultured on Mueller-Hinton agar (MHA) and incubated for 18-24 h at 37^{o}C before being used. Concentration of bacteria was adjusted spectrophotometrically by matching the turbidity of inocula with the turbidity of McFarland standard 0.5 (optical density: 0.08-0.10) to give a standardised number of colony forming unit (CFU) for all strains. This was carried out by transferring a few colonies of bacteria to Mueller-Hinton broth (MHB). Concentration of bacteria was 1 x 10^{8} CFU/mL within this range. The optical densities of the bacterial suspensions were measured using UV-Vis spectrophotometer at a wavelength of 625 nm. MHB was used as the blank. The dilution factor needed was calculated and dilution was carried out to obtain a final concentration of 5 x 10^{5} CFU/mL.

3.3.2.4 Fungal Strains

Fungi can be divided into yeasts and molds. Three species of yeasts were tested which were *Candida albicans* (ATCC 90028), *Candida parapsilosis* (ATCC 22019) and *Cryptococcus neoformans* (ATCC 90112). In

addition, two species of molds, *Aspergillus niger* (ATCC 16404) and *Trichophyton mentagrophytes* (ATCC 9533) were tested.

3.3.2.5 Preparation of Fungal Inocula

The chemicals used were:

3-(N-morpholino) propanesulfonic acid (Sigma-Aldrich), Roswell Park Memorial Institute (RPMI)-1640 medium broth with L-glutamine, without bicarbonate (Sigma-Aldrich) and potato dextrose agar (Merck)

Procedures:

All fungal strains were subcultured on potato dextrose agar (PDA). Different incubation time required for different strains. *C. albicans* and *C. parapsilosis* were incubated for 24 h at 37^{o}C while *C. neoformans* and *A. niger* were incubated for 48 h at 37^{o}C. A 120 h incubation period was needed for *T. mentagrophytes* at a temperature of 37^{o}C. A few colonies of fungus were transferred to the medium broth. The Roswell Park Memorial Institute (RPMI) - 1640 medium broth with L- glutamine, without bicarbonate and buffered at pH 7 with 3-(N-morpholino) propanesulfonic acid was used. For *C. albicans*, *C. parapsilosis* and *C. neoformans* suspensions, their optical density were adjusted to fall within the range of 0.12 to 0.15 at 530 nm. The optical densities of *A. niger* and *T. mentagrophtes* suspensions were adjusted to values of 0.09 to 0.11 at 530 nm. The concentration of the fungus within the respective range was 1 x 10^{6} - 5 x 10^{6} CFU/mL. Dilution was carried out to obtain the required

concentration of fungus. The final concentration of the *C. albicans* and *C. parapsilosis* suspensions were 0.5- 2.5 x 10^3 CFU/mL, that of *C. neoformans* was 0.5- 2.5 x 10^4 CFU/mL, and those of *A. niger* and *T. mentagrophtes* were 0.5-2.5 x 10^5 CFU/mL.

3.3.2.6 Toxicity of Solvents towards Tested Microorganisms

The chemicals used were:

Acetone (Merck), butanol (Merck), chloroform (Merck), dichloromethane (Merck), diethyl ether (Merck), dimethyl sulfoxide (Merck), ethanol (Merck), ethyl acetate (Merck), hexane (Merck), methanol (Merck) and *p*-iodonitrotetrazolium violet (BIO Basic INC.)

Procedures:

As the dried extracts contained large amounts of hydrophobic compounds, they were dissolved in organic solvents which increase the solubility of these compounds in water. Thus it was essential to determine the toxicity of the solvents on tested microorganisms (bacteria or fungi), because the solvent itself may be toxic to microorganisms.

96-well plates were used for this microbial susceptibility testing. The designs of the 96-well plates are shown in Figure 3.7. Broth medium with a volume of 100 µL was added to sterility control wells. A sterility control was used to ensure the sterility of the broth medium. Broth medium with a volume

of 75 µL was added to negative control wells followed by addition of 25 µL of selected solvents (i.e. hexane, diethyl ether, chloroform, ethyl acetate, methanol, dimethyl sulfoxide (DMSO), ethanol, dichloromethane, acetone and butanol) to the respective wells. The purpose of negative control was to ascertain sterility of tested solvents in broth medium. After that, 50 µL of broth medium and 50 µL of tested microorganism inocula were added to growth control of the 96-well plate. The growth control was used to assess the viability of the microorganisms. For the positive control and solvents testing, 50 µL of broth medium were added to the all wells from columns 2 to 11. Antibiotic (chloramphenicol) with a volume of 50 µL was added into A2 well while 50 µL of each solvent was added to the respective wells of B2 to H2. After that, 50 µL of the solution from wells of the 2nd column were transferred and mixed with the broth medium in the 3rd column. This was repeated for the other columns until the 11th column was reached, after which the last 50 µL of the solution was discarded. Microorganism inocula with a volume of 50 µL were added to positive control and solvent toxicity testing wells. The plate was covered with a lid and incubated at 37ºC for 24 h. After incubation, 20 µL of 0.4 mg/mL *p*-iodonitrotetrazolium violet (INT) was added to all wells. The MIC was defined as the lowest concentration of solvent in which no microbial growth occurred, indicated by an absence of colour change.

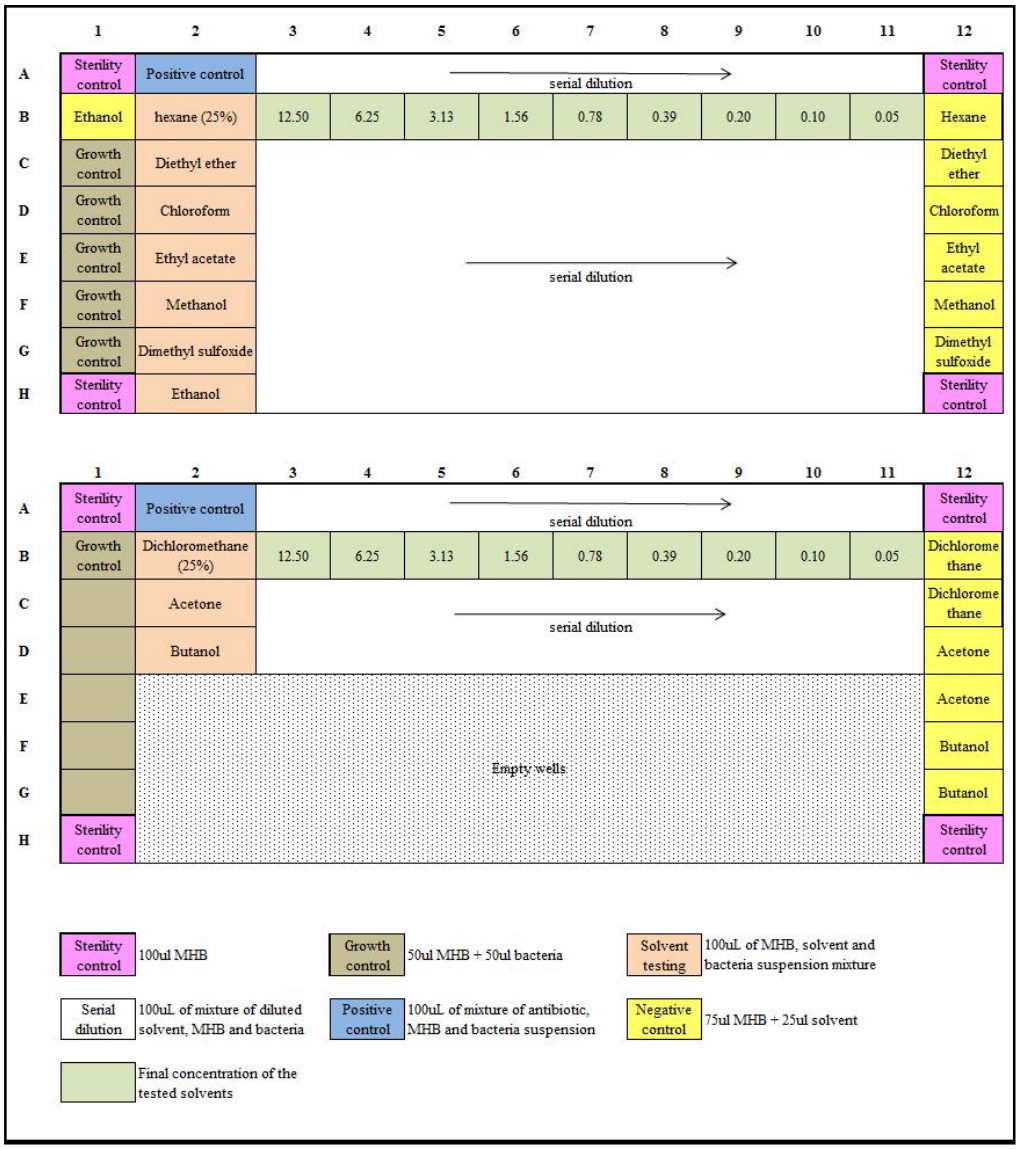


Figure 3.7: Design of the 96-well plate for solvent toxicity assessment

3.3.2.7 Microbial Susceptibility Testing on Crude Extracts

The dried crude extracts which were obtained from sequential extraction were dissolved in a solvent to a concentration of 20 mg/mL. The method that applied was same to the test for the assessment of solvent toxicity. The design of 96-well plate is shown in Figure 3.8.

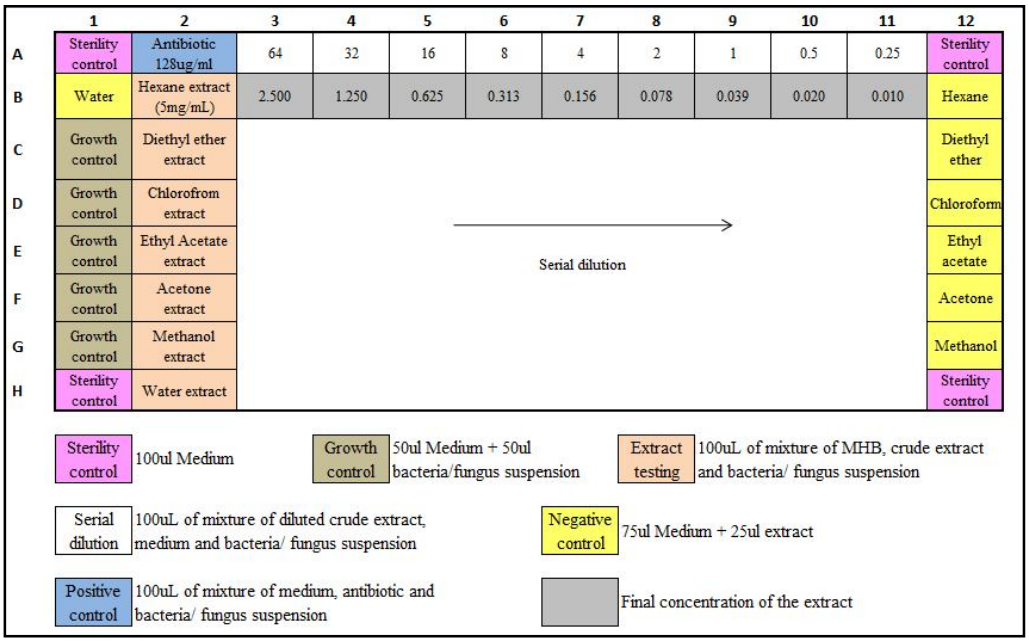


Figure 3.8: Design of the 96-well plate for colorimetric broth microdilution antimicrobial assay of crude extracts

3.3.3 Total Activities of Extracts

The total activities of the extracts were expressed as the total mass (mg/g) of each of the extract divided by its MIC value (mg/mL). Total activity value indicates the volume to which the extract derived from 1 g of sample material can be diluted while still inhibiting the growth of microorganism (Eloff, 2000).

3.3.5 Partial Purification of Antimicrobial Compound from *P. antillarum*

The chemicals used were:

Dichloromethane (Merck), diethyl ether (Merck), ethyl acetate (Merck), hexane (Merck), methanol (Merck), silica gel 60 (15 to 40 µm, Merck) and C_{18} (15 µm, Merck).

Procedures:

Activity-guided fractionation was performed on the active extract which exhibited the highest antimicrobial activity. Active extract was subjected to silica gel flash chromatography fitted with a 40 x 150 mm silica cartridge, in which the silica cartridge was packed with Merck silica gel 60 (15 to 40 µm). The initial mobile phase used was 100% hexane, after which the ethyl acetate content of the mobile phase was increased at every 5% until it contained 40% of ethyl acetate (Table 3.6). Finally, the column was eluted with 100% ethyl acetate. A total of ninety fractions with volumes of approximately 40 mL were collected at a flow rate of 8.5 mL/min. The fractions were concentrated using a rotary evaporator and subjected to TLC, after which the fractions were combined into twenty four samples ("V1" to "V24"). The samples were subjected to antimicrobial assay for keep track of the antimicrobial compounds which inhibited the growth of *P. aeruginosa*.

Table 3.6: Mobile phases for fractionation of hexane extract

No. of mobile phases	Mobile phases	Volume of each mobile phase, mL
1	100% hexane	300
2	95% hexane: 5% ethyl acetate	300
3	90% hexane: 10% ethyl acetate	300
4	85% hexane: 15% ethyl acetate	450
5	80% hexane: 20% ethyl acetate	300
6	75% hexane: 25% ethyl acetate	300
7	70% hexane: 30% ethyl acetate	300
8	65% hexane: 35% ethyl acetate	300
9	60% hexane: 40% ethyl acetate	300
10	100% ethyl acetate	400

Based on the MIC values and results from TLC analysis of the fractions, “V10” to “V17”, excluding “V12” were combined and further purification was performed using silica gel flash chromatography fitted with a 40 x 150 mm silica cartridge. The initial mobile phase was hexane-ethyl acetate system of increasing polarity (95:5 to 7:3 volume ratios) and ending with 100% ethyl acetate, as shown in Table 3.7. A total of fifty five fractions each with a volume of 100 mL were collected at a flow rate of 8.5 mL/min. The collected fractions were combined into eleven samples (“W1” to “W11”) based on the TLC analysis. The samples were subjected to antimicrobial assay.

Table 3.7: Mobile phases for fractionation of combined sample of "V10" to "V17", excluding "V12"

No. of mobile phases	Mobile phases	Volume of each mobile phase, mL
1	95% hexane: 5% ethyl acetate	1000
2	90% hexane: 10% ethyl acetate	2500
3	85% hexane: 15% ethyl acetate	500
4	80% hexane: 20% ethyl acetate	500
5	75% hexane: 25% ethyl acetate	500
6	70% hexane: 30% ethyl acetate	500
7	100% ethyl acetate	500

"V12" and "W5" were combined based on the MIC values and results from TLC analysis. Combined samples of "V12" and "W5" and "W6" were subjected to C_{18} reversed phase flash chromatography which was fitted with a 40 x 75 mm C_{18} cartridge respectively. The C_{18} cartridge was packed with Merck C_{18} (15 μm) particles. The initial mobile phase used was 100% acidified water, followed by increasing the methanol content in the mobile phase until it contained 100% methanol and finally eluted with 100% dichloromethane (Table 3.8). The fractions were subjected to antimicrobial assay. Four fractions with volumes of 100 mL were collected at a flow rate of 8.5 mL/min from both purifications respectively. Fractions "X1" to "X4" were collected from purification of combined samples of "V12" and "W5" whereas fractions "Y1" to "Y4" were collected from the purification of "W6". All of the eight fractions were subjected to antimicrobial assay.

Table 3.8: Mobile phases for fractionation of combined samples of "V12" and "W5" and "W6"

No. of mobile phases	Mobile phases	Volume of each mobile phase, mL
1	100% acidified water	100
2	50% acidified water: 50% methanol	100
3	100% methanol	100
4	100% dichloromethane	200

*Acidified water is a mixture of 1% acetic acid in distilled water

The active fractions ("X3" and "Y3") were combined and purified using silica gel flash chromatography which was fitted with a 40 x 75 mm silica cartridge. A hexane-diethyl ether system was used as the mobile phase, beginning with a hexane-diethyl ether mixture of volume ratios 7:3, followed by increasing the diethyl ether content in the mobile phase until it contained 70% diethyl ether and ending with 100% diethyl ether (Table 3.9). Thirty eight fractions with volumes of 30 mL were collected at a flow rate of 8.5 mL/min. The fractions were pooled into six samples ("Z1" to "Z6") based on the results of the TLC analysis, after which antimicrobial screening were carried out on these samples. The partial purified active fraction was subjected to GC-MS to identify of the active compounds in the fraction. It was also subjected to HPLC to assess its purity.

Table 3.9: Mobile phases for fractionation of combined samples of "X3"and "Y3"

No. of mobile phases	Mobile phases	Volume of each mobile phase, mL
1	70% hexane: 30% diethyl ether	300
2	50% hexane: 50% diethyl ether	300
3	30% hexane: 70% diethyl ether	300
4	100% diethyl ether	300

3.3.5 High Performance Liquid Chromatographic (HPLC) Analysis on the Partially Purified Antimicrobial Compound from *P. antillarum*

The chemicals used:

0.1% formic acid in distilled water (acidified water) and methanol (HPLC grade, Merck)

Procedure:

The partially purified antimicrobial compound was subjected to Shimadzu HPLC (Model: PRC10A) fitted with a Merck LiChroCART 75-4® C_{18} column. An aliquot of the filtrate (20 µL) was injected into the HPLC column and eluted with a linear gradient of mobile phase containing solvent A (0.1% formic acid) and solvent B (methanol). The solvent gradient was programmed from 0% to 100% solvent B in 30 min with a flow rate of 1 mL/min. The peaks in the chromatogram were detected by a photodiode array detector (Model: SPD-M20A) at 254 nm.

3.3.7 Gas Chromatography-Mass Spectrometric Analysis on the Partially Purified Antimicrobial Compound from *P. antillarum*

The chemical used was:

Methanol (GC grade, Merck)

Procedures:

GC-MS is a chromatographic technique that can identify different volatile compounds within a test sample. The isolated antimicrobial compounds from *P. antillarum* were subjected to GC-MS to identify the antimicrobial compounds by the MS library (NIST 05). The isolated antimicrobial compounds were dissolved in methanol, after which they were filtered using a syringe filter (0.45 μm) prior injected into GC-MS.

GC-MS analysis was carried out using a Shimadzu GCMS QP 2010 Plus instrument. The column used was BP-1 (30 m X 0.25 mm, 0.25 μm film thickness). The column oven temperature was programmed to increase gradually from 130^{o}C to 280^{o}C at 9^{o}C/min. The ion source temperature and interface temperature was set at 200^{o}C and 290^{o}C, respectively. The pressure used was 118.3 kPa. Helium gas was used as the carrier gas.

Figures 3.9 and 3.10 summarize the extraction and fractionation of antimicrobial compound from *P. antillarum* respectively.

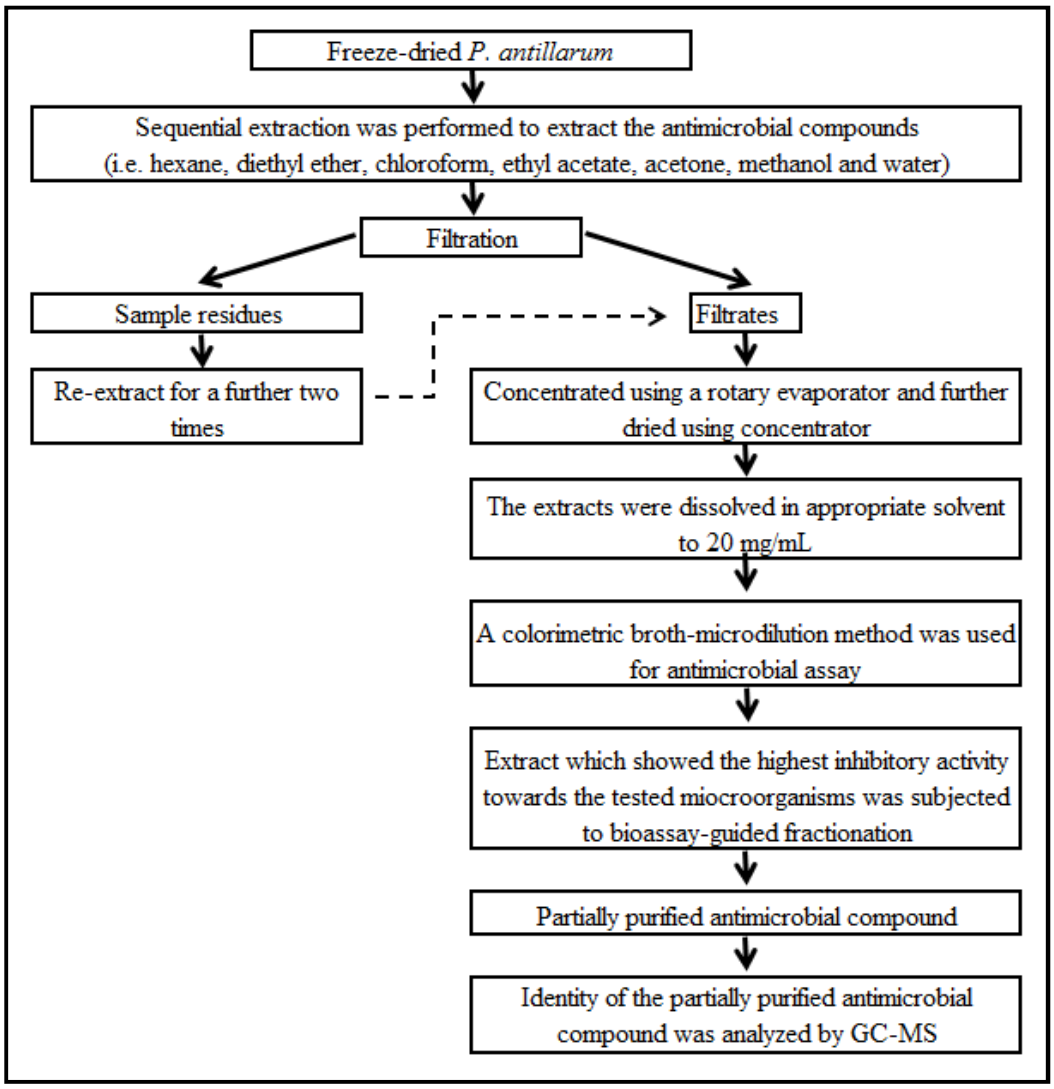
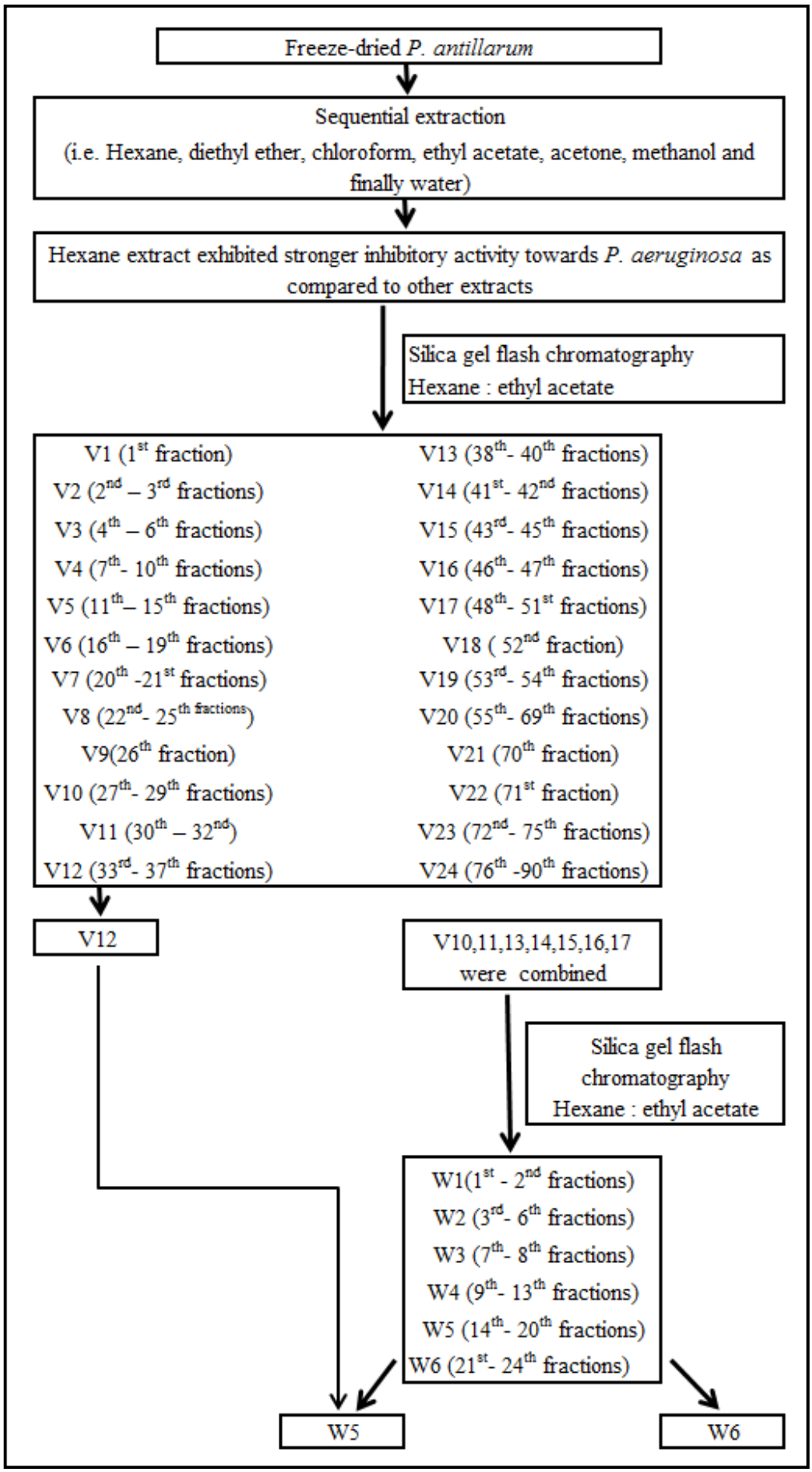


Figure 3.9: Flow chart of the extraction of antimicrobial compounds from *P. antillarum* and the chromatographic methods



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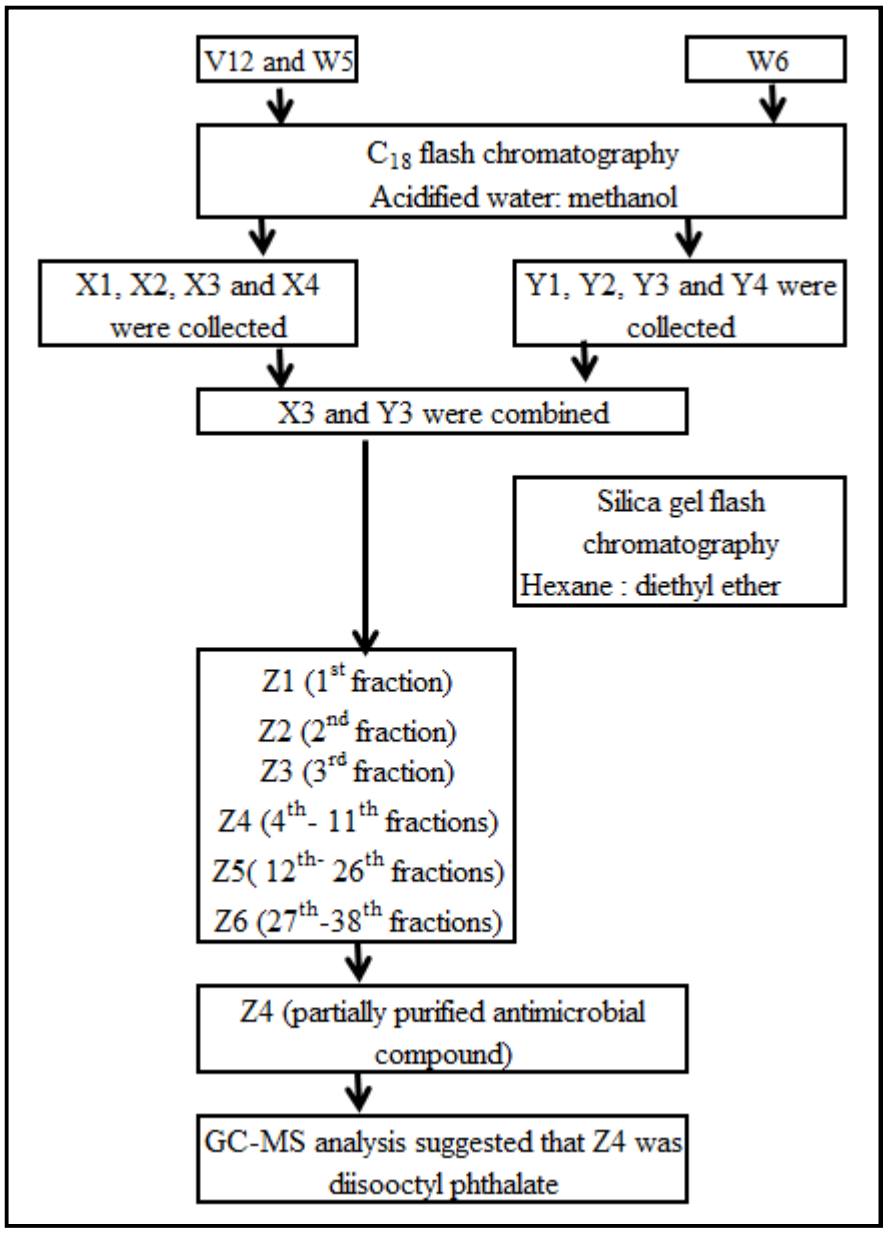

Figure 3.10: Flow chart of the progress to obtain the partially purified antimicrobial compound from *P. antillarum*

3.3.7 Determination of Minimum Bactericidal Concentration (MBC)

The chemicals used were:

Mueller-Hinton agar (Oxoid) and Mueller-Hinton broth (Oxoid)

Procedures:

Partially-purified active fractions were subjected to MBC assay. It was performed by transferring 20 μL of the solution from the wells of 96-well plate which showed inhibitory activity to a MHA agar plate. The solution was then spread evenly on a MHA agar plate using a glass spreader. Then, the plates were incubated for 18 to 24 h at 37^{o}C. MBC was defined as the lowest concentration yielding a negative subculture, i.e. less than 0.1% of the original bacterial population.

CHAPTER 4.0

RESULTS AND DISCUSSION

4.1 Collection of *Padina antillarum* from Teluk Kumang

The dry weights of *P. antillarum* which were collected on five different days are shown in Table 4.1. Collection of *P. antillarum* on 28th April 2009 gave the highest dry weight (550 g) while collection on 27th November 2007 showed the lowest dry weight (62 g).

Table 4.1: Dry weight of freeze-dried *P. antillarum* which were collected on five different days

Date of samples collection	Dry weight of freeze-dried samples
29th December 2005	76 g
16th March 2006	87 g
27th November 2007	62 g
13th May 2008	100 g
28th April 2009	550 g

4.2 Selection of Solvents for Extraction of Antioxidants from *P. antillarum*

In order to maximize the extraction of antioxidant compounds from *P. antillarum*, extraction efficiencies of four types of solvents (i.e. hexane, ethyl acetate, 100% methanol and 50% aqueous methanol) were assessed before

extraction in larger quantities was carried out. The Folin Ciocalteu's (FC) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assays were used to test how effectively each solvent extracted antioxidants. Five batches of freeze-dried *P. antillarum* were tested to appraise the stability of the antioxidant compounds.

4.2.1 Determination of Total phenolic Contents on the Five Batches of Solvent Extracts

FC assay was used to determine TPC of the solvent extracts. FC reagent will react with phenols and non-phenolic reducing substances to form blue chromogen that can be detected spectrophotometrically.

Gallic acid was used as the standard unit to determine TPC of the extracts because it covers a wide spectrum of phenolic compounds (Singleton *et al.*, 1999). The average of absorbance values (n=3) of the concentration of gallic acid are shown in Eppendix A.

A linear graph (r^2= 0.9986) was obtained from the gallic acid standard curve (Figure 4.1)

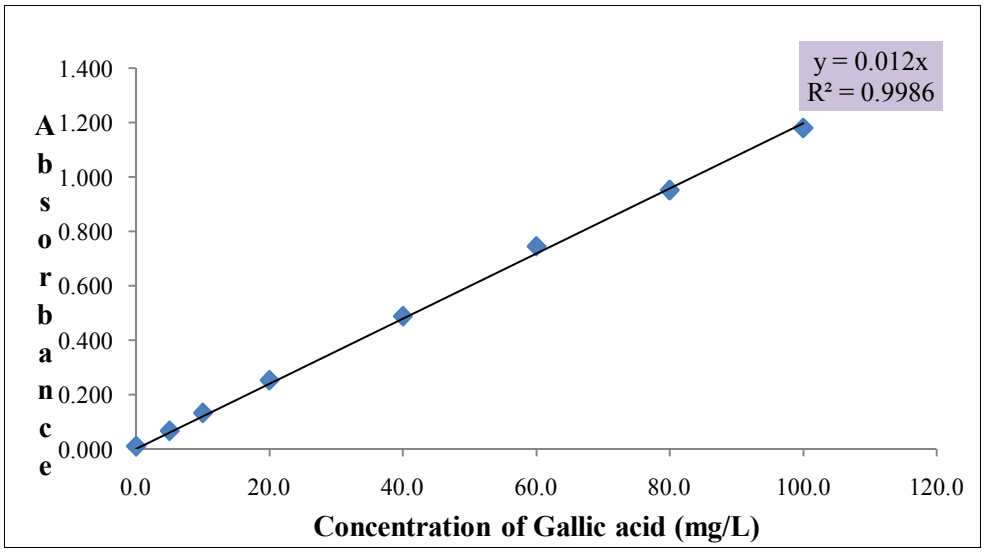

Figure 4.1: Graph of concentration of gallic acid versus absorbance

The TPC of the five batches of extracts were determined from the regression equation of gallic acid standard curve (y= 0.012 x) and expressed as gallic acid equivalents (GAE). An example of a calculation for this assay is shown in Appendix B. The TPC of five batches of extracts were analyzed and summarized in Appendix C. The comparisons of TPC on five batches of extracts are summarized in Table 4.2.

Table 4.2: Comparison of TPC of extracts from *P. antillarum* that were collected on five different dates

Dates	TPC of the Extracts			
	Hexane	Ethyl acetate	100% methanol	50% methanol
29/12/2005	12.00	76.17	187.33	387.33
16/3/2006	19.50	79.83	225.00	474.67
27/11/2007	22.67	76.00	232.00	484.67
13/5/2008	27.67	78.33	200.33	454.00
28/4/2009	8.00	47.67	184.00	518.00
Standard deviation	7.96	13.47	21.80	48.57

50% aqueous methanol gave the best extraction efficiency, based on the highest TPC values in the five batches of extracts. The TPC of 50% aqueous methanol extracts ranged from 387.33 mg GAE/g to 518.00 mg GAE/g of sample. Hexane showed the weakest extraction efficiency, shown by its lowest TPC value. The amount of phenolic compounds in hexane extract varied between 8.00 mg GAE/g to 27.67 mg GAE/g of sample. The antioxidant compounds of freeze-dried *P. antillarum* were relatively stable upon storage as the TPC of the extracts did not fluctuated drastically, as shown by their standard deviation (7.96 to 48.57). The TPC of 50% aqueous methanol extract from the batch collected on 29th December 2005 was the lowest and deviated the most compared to the 50% aqueous methanol extracts of different batches. The TPC of 50% aqueous methanol extracts from the batch collected on 16th March 2006, 27th November 2007 and 13th May 2008 were close to each other. The latest batch of *P. antillarum* which was collected on 28th April 2009 contained the highest amount of phenolic contents. Chew *et al*. (2007) also reported 50% aqueous methanol showed the highest extraction efficiency for extraction of antioxidant compounds from *P. antillarum*.

4.2.2 Determination of Percentage Radical Scavenging Activities on the Five Batches of Solvent Extracts

The percentage of DPPH radical scavenging activities of the five batches of extracts from *P. antillarum* that were collected on five different

dates is determined (Appendix D). An example of a calculation for this assay is shown in Appendix E.

Comparison of percentage of radical scavenging activities of extracts from *P. antillarum* that were collected on five different dates are summarized in Table 4.3.

Table 4.3: Comparison of percentage radical scavenging activities of *P. antillarum* extracts that were collected on five different dates

Dates	% of Radical Scavenging Activities of the Extracts			
	Hexane	Ethyl acetate	100% methanol	50% methanol
29/12/2005	20.50	36.53	64.71	88.80
16/3/2006	24.04	42.01	66.70	81.87
27/11/2007	18.93	36.45	62.20	80.37
13/5/2008	25.54	31.76	49.42	83.76
28/4/2009	6.23	15.71	68.72	89.75

50% aqueous methanol extracts exhibited the most effective DPPH radical scavengers, ranging from 80.37% to 89.75%. The DPPH radical scavenging activities in the 50% aqueous methanol extracts were correlated with their TPC (r^2= 0.9393), suggesting that phenolic compounds contribute to the DPPH radical scavenging activity of the extracts (Figure 4.2). 50% aqueous methanol extract of *P. antillarum* that was collected on 28th April 2009 showed the highest TPC value and percentage radical scavenging activity. Thus, 50% aqueous methanol was selected to use in large scale extraction of *P. antillarum* which were collected on 28th April 2009.

Correlation between average TPC and average DPPH radical scavenging activities of five batches of *P. antillarum* extracts is shown in Figure 4.2 (Appendix F).

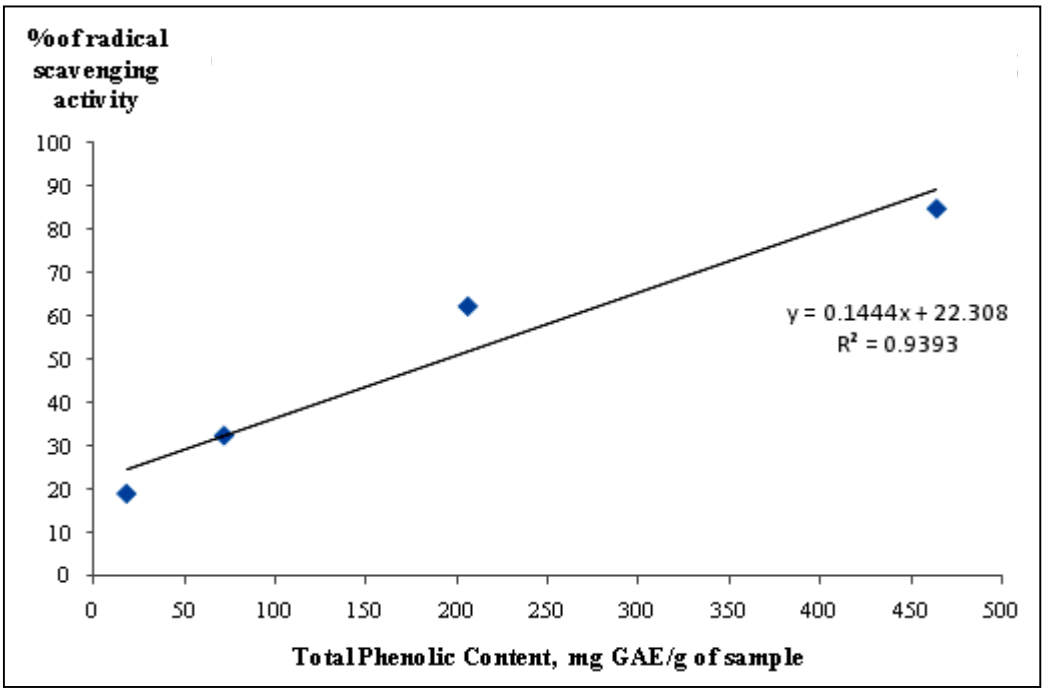

Figure 4.2: Correlation between average TPC and average DPPH radical scavenging activities of five batches of *P. antillarum* extracts

4.3 Extraction in Larger Quantities and Purification of Antioxidant Compound from *P. antillarum*

Five hundred grams of freeze-dried and powdered *P. antillarum* which was collected on 28th April 2009 was extracted with 50% aqueous methanol. The resulting extract was subjected to liquid-liquid partitioning using chloroform followed by ethyl acetate. Chloroform and ethyl acetate layers were

dried under vacuum using rotary evaporator and aqueous layer was subjected to freeze-drying.

The weights and the mass of the dried crude extracts from liquid-liquid partitioning are shown in the Table 4.4. The mass of the extract (mg/g powdered sample) was expressed as the amount of extract which can be extracted from 1 g of powdered *P. antillarum*.

Table 4.4: Weights and total mass extracted (mg/g powdered sample) of crude extracts

Crude extracts	Weight of crude extracts (mg)	Mass extracted (mg/g powdered sample)
Chloroform extract	388.8	0.78
Ethyl acetate extract	375.8	0.75
Aqueous extract	43431.0	86.86
Total	44195.6	88.39

The aqueous extract had the highest quantity of dried extract from *P. antillarum*, with a mass of 86.86 mg/g powdered sample. The ethyl acetate extract produced the least mass of extract at 0.75 mg/g powdered sample followed by chloroform extract at 0.78 mg/g powdered sample. Chloroform, ethyl acetate and aqueous extracts were subjected to antioxidant assays prior to purification.

4.4 Evaluation of Antioxidant Activity on the Extracts which were obtained from Liquid-liquid Partitioning

The crude extracts (1 mg/mL) were prepared in methanol and subjected to FC and DPPH assays. Methanol was used as the solvents to dissolve the extracts because it does not interfere with the reaction (Guo *et al.*, 2001). Other solvent systems, such as water or acetone, seem to result in low values for the extent of reduction (Guo *et al.*, 2001).

Chloroform, ethyl acetate and aqueous extracts from liquid-liquid partitioning were subjected to FC assay. The TPC values of the extracts are summarized in Table 4.5.

Table 4.5: The TPC of the crude extracts from liquid-liquid partitioning

Extracts (1 mg/mL)	Average of absorbance	Dilution factor	Average x dilution factor	TPC (GAE mg/g)
Chloroform extract	0.075	1	0.075	6.250
Ethyl acetate extract	0.936	2	1.873	156.083
Aqueous extract	0.488	1	0.488	40.667

denotes an active extract

Chloroform, ethyl acetate and aqueous extracts from liquid-liquid partitioning were subjected to DPPH radical scavenging assay. The percentages of radical scavenging activities of the extracts are summarized in Table 4.6.

Table 4.6: The percentages of radical scavenging activities of crude extracts that were obtained from liquid-liquid partitioning

Crude extract (1 mg/mL)	% of radical scavenging activities
Chloroform extract	2.82
Ethyl acetate extract	84.13
Aqueous extract	39.38

denotes an active extract

The highest amount of phenolic compounds was found in ethyl acetate extract at 156.083 mg GAE/g of sample. It was also the most effective DPPH radical scavenger (84.13% of radical scavenging activity) compared to chloroform (2.82% of radical scavenging activity) and aqueous (39.38% of radical scavenging activity). The results confirmed that phenolic compounds contribute to the DPPH radical scavenging activity of ethyl acetate extract. The antioxidant compounds in *P. antillarum* might be compounds of intermediate polarity as they were contained in the ethyl acetate layer.

4.5 Isolation of the Ethyl Acetate Extract

The ethyl acetate extract was purified by silica gel flash chromatography. A chloroform-acetone system was used as mobile phase, beginning with 100% chloroform and ending with a chloroform/acetone mixture of ratio 1:4. A total of 90 fractions were collected. The volume of the fractions collected was 50 mL. The fractions were subjected to FC and DPPH radical scavenging assays.

In the FC assay, fractions with high phenolic contents appeared in dark blue colour evidenced by high absorbance values. The fractions were combined as the graph of absorbance against the number of fractions (Appendix G), shown in Figure 4.3. The TPC of the fractions 25 to 35 produced a peak labeled as "A2", which contained high concentration of phenolic compounds. Fractions 25 to 35 were collected and pooled together to be concentrated by rotary evaporation before it was subjected to C_{18} flash chromatography.

Figure 4.3 below shows the graph of absorbance versus fractions numbers for TPC of the fractions which were collected from flash chromatography of the ethyl acetate extract.

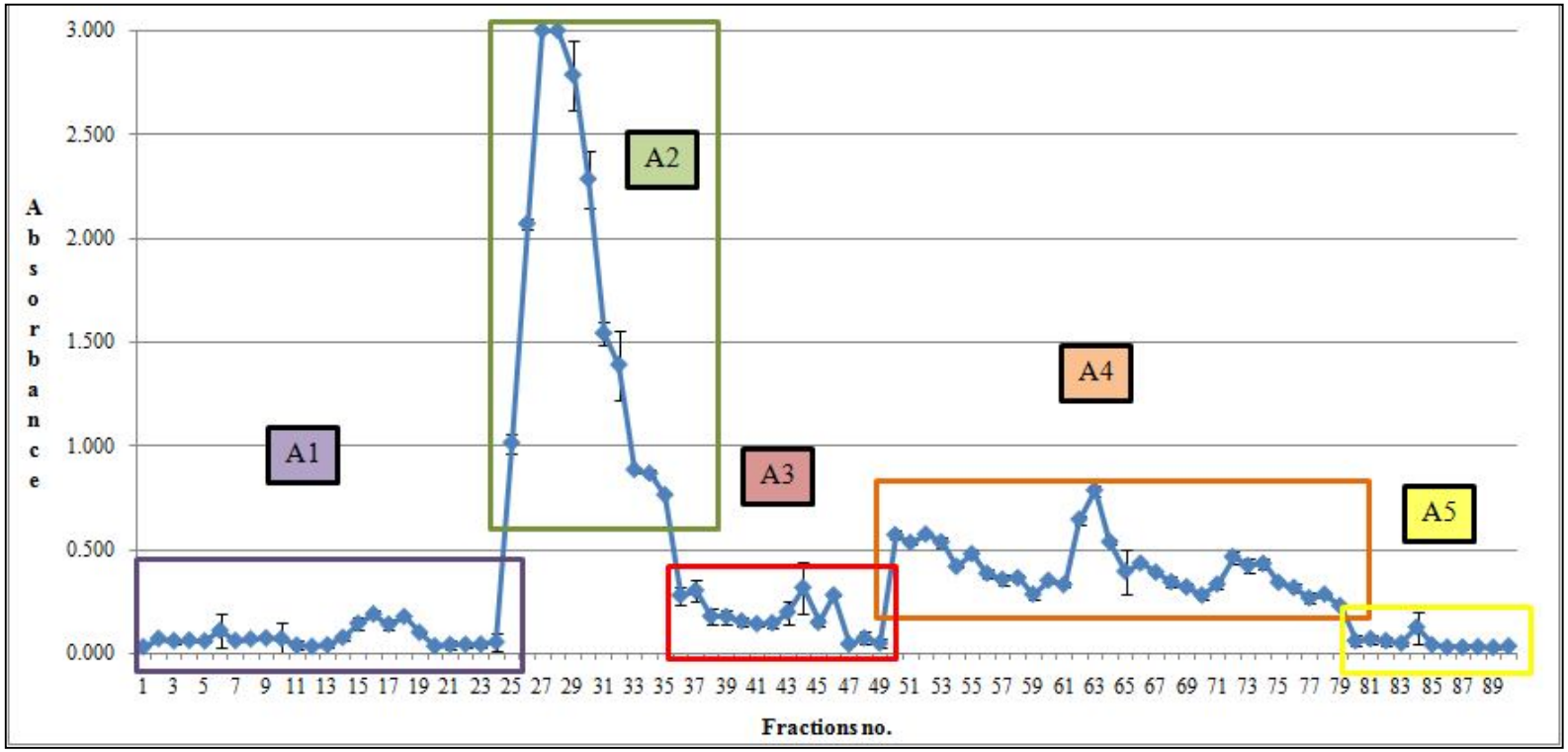

Figure 4.3: Graph of absorbance versus fractions number for TPC of the fractions which were collected from flash chromatography of the ethyl acetate extract

The DPPH radical scavenging assay also showed that “A2” (combined fractions of 25 to 35) possessed the highest radical scavenging activity (Appendix H). The highest DPPH radical scavenging activities were found in fractions 25 to 35 at the range of 62.84% to 89.43% (Figure 4.4). Fractions 25 to 35 were combined as “A2” and further purification was performed

Figure 4.4 below shows the graph of the percentages of radical scavenging activities versus fractions numbers of the fractions that were collected from flash chromatography of ethyl acetate extract.

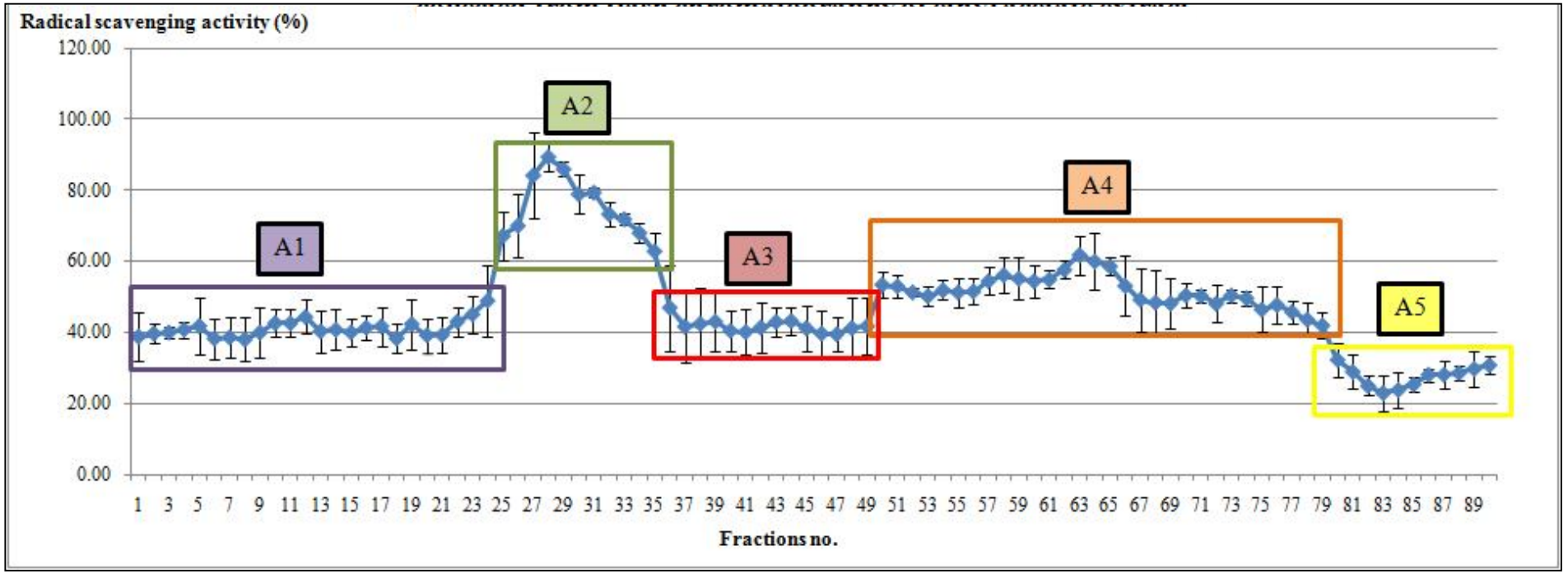

Figure 4.4: Graph of percentage of radical scavenging activities versus fractions number for fractions which were collected from the fractionation of ethyl acetate extract

The fractions were also combined based on the result of TLC analysis. Fractions with compounds of similar R_f values were combined. Figure 4.5 shows an example of how the fractions were combined based on TLC.

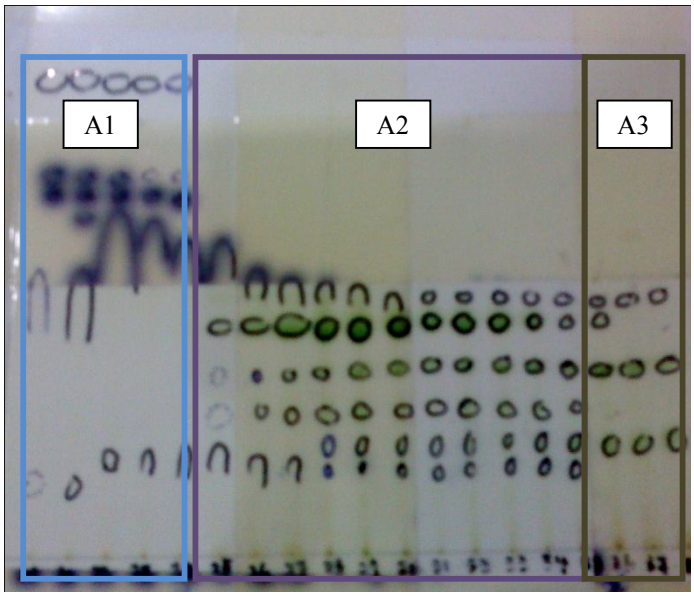

Figure 4.5: Thin layer chromatography on the fractions which were collected from fractionation of the ethyl acetate extract

Fractions 25 to 35 were combined and labeled as “A2”. “A2” gave the highest weight among the others at 220.6 mg (Table 4.7).

Table 4.7: Weight of the combined fractions of ethyl acetate extract of *P. antillarum*

Combined fractions	Weight of combined fractions (mg)
A1	48.7
A2	220.6
A3	22.5
A4	50.2
A5	13.8
Total	355.8

denotes an active fraction

4.5.1 Purification of "A2" Combined Fractions

"A2" was subjected to C_{18} reversed phase flash chromatography and 16 fractions were collected. The initial mobile phase used was 100% acidified water, after which the methanol content of the mobile phase was increased until it contained 100% methanol. A total of 16 fractions each with a volume of approximately 50 mL were collected. All fractions were subjected to TPC and DPPH radical scavenging activity assays to keep track of the fractions with antioxidant. The TPC of the fractions from the fractionation of "A2" are summarized in Appendix I. Fractions 4 to 10 were combined as "B2" due to their high contents of phenolic compounds which were indicated by high absorbance values. These fractions also showed high DPPH radical scavenging activities which ranged from 71.31 % to 90.97 % (Appendix J).

The TPC and DPPH radical scavenging assays (Figures 4.6 and 4.7) showed the presence of a peak that encompassed fractions 4 to 10, labeled as "B2", which represented fractions with the highest antioxidant activities.

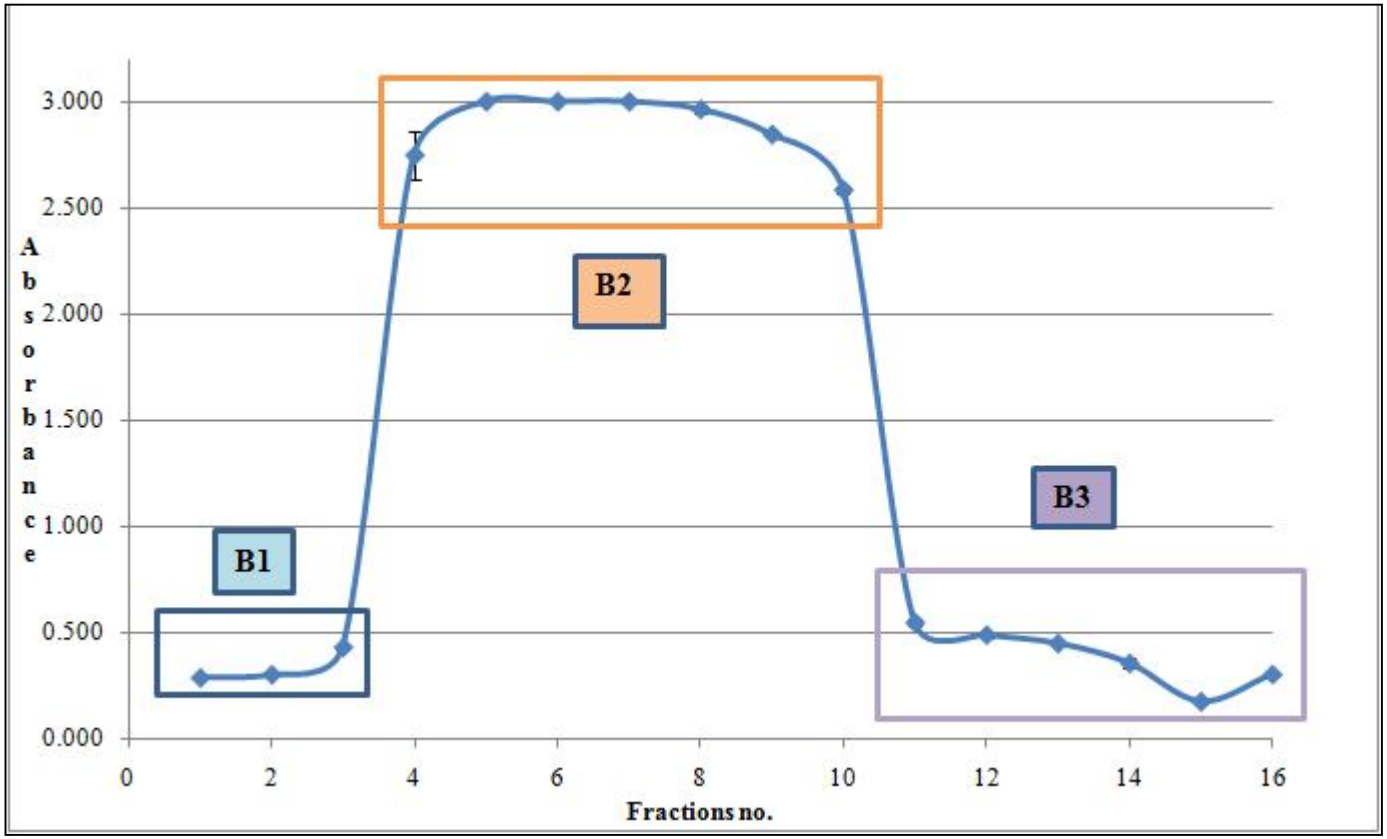

Figure 4.6: Graph of total phenolic contents of the fractions which were collected from fractionation of "A2"

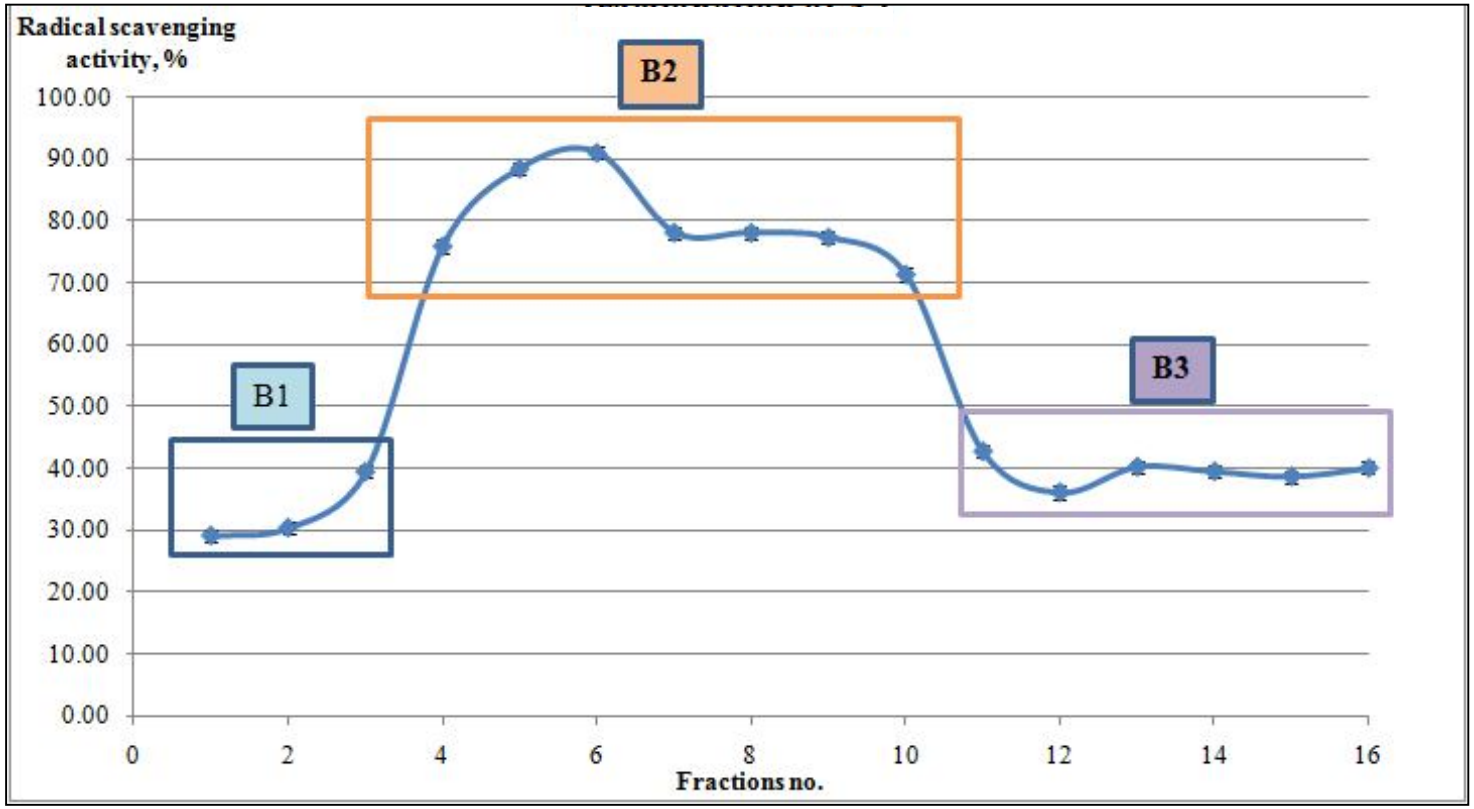

Figure 4.7: Percentage of Radical Scavenging Activities of the fractions which were collected from fractionation of "A2"

"B2" gave the highest weight among all at 171.8 mg (Table 4.8), followed by B3 (12.6 mg) and B1 (2.7 mg).

Table 4.8: Weight of the combined samples from fractions of "A2" purification

Combined fractions	Weight of combined fractions (mg)
B1	2.7
B2	171.8
B3	12.6
Total	187.1

denotes an active fraction

4.5.2 Purification of "B2" Sample

"B2" was purified by silica gel flash chromatography using a dichloromethane-acetone system as the mobile phase, with the polarity of which was increased by changing the ratios of dichloromethane to acetone from 4:1 to 2:3 ratio volumes. Ten fractions with volumes of approximately 30 mL were collected. These fractions were subjected to TPC and DPPH radical scavenging assays.

Fractions 1 to 7 were combined based on high content of phenolic compounds and DPPH radical scavenging activities (52.16% to 90.74%). The results of TPC and DPPH radical scavenging activities of the collected fractions are summarized in Appendix K and Appendix L respectively. Fractions 1 to 7 were combined and labeled as "C1".

Figure 4.8 and Figure 4.9 show the graph of TPC and DPPH radical scavenging activities of the fractions respectively. A single major peak, labeled as "C1", which covered fractions 1 to 7, was obtained.

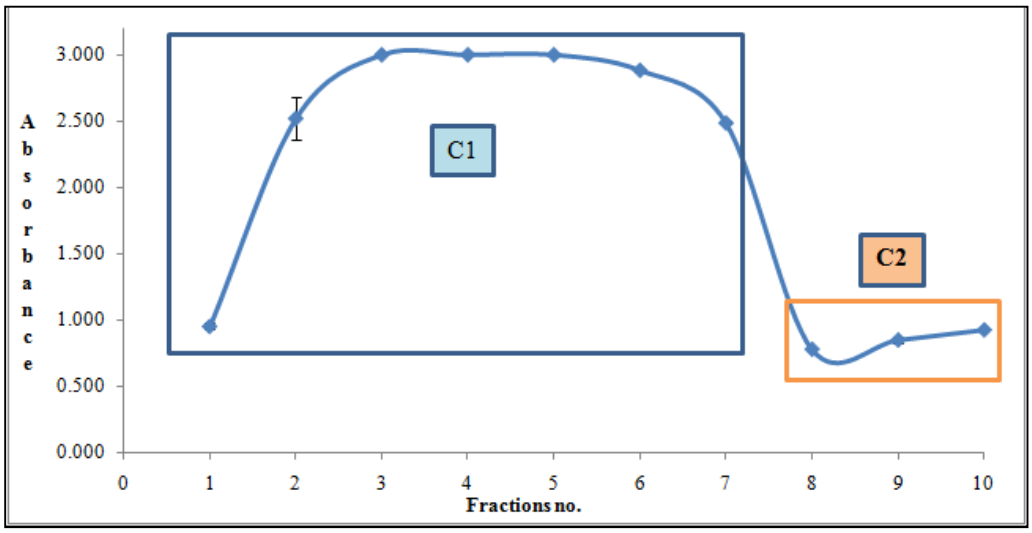

Figure 4.8: The TPC of fractions that were collected from fractionation of "B2"

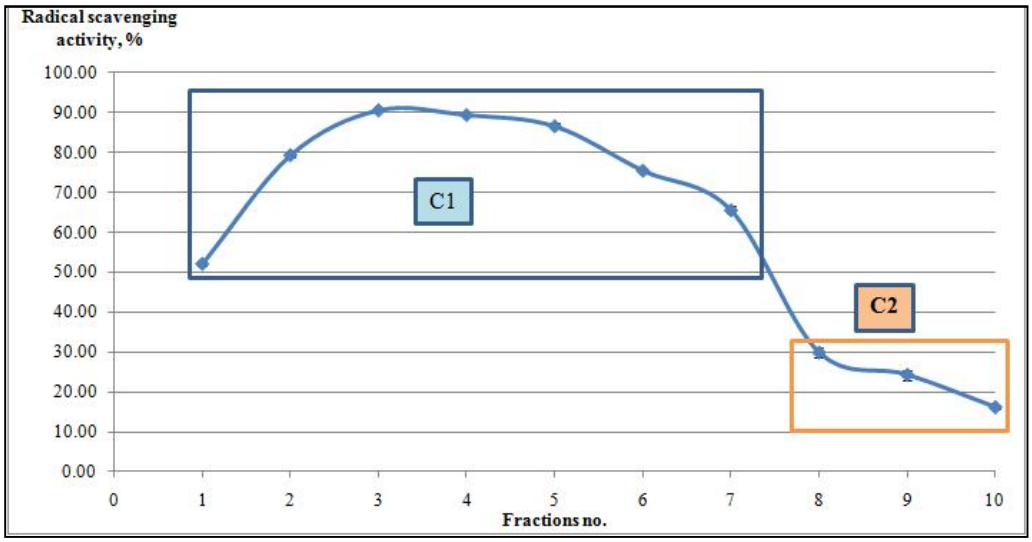

Figure 4.9: Percentages of radical scavenging activities of the fractions that were collected from fractionation of "B2"

Based on the TLC analysis, only a single spot was observed under UV light and iodine treatment, as shown in Figure 4.10. A solvent system of ethyl acetate-dichloromethane at 3 to 2 ratio volumes was used. This indicates that "C1" was quite pure.

Figure 4.10: TLC of "C1" using ethyl acetate-dichloromethane (3:2 ratio volumes) as mobile phase

The weight of "C1" was 135.9 mg, as shown in Table 4.9.

Table 4.9: Weights of "C1" and "C2" from fractionation of "B2"

Combined fractions	Weight of combined fractions (mg)
C1	135.9
C2	10.6
Total	146.5

denotes an active fraction

The purity of the isolated "C1" was analyzed using Shimadzu high performance liquid chromatography (HPLC). An aliquot of the filtrate (20 µL) was injected in to the HPLC column (Merck LiChoroCART® 75-4 C_{18} column) and eluted with a linear gradient of mobile phase containing solvent A (0.1% acetic acid) and solvent B (methanol). The solvent gradient was programmed from 0% to 10 % solvent B in 30 min with a flow rate of 1 mL/min. The peaks in the chromatogram were detected by a photodiode array detector (Model: SPD-M20A) at 254 nm.

In the chromatogram shown in Figure 4.11, a peak of retention time at 4.12 min was obtained, with a percentage peak area of over 97%.

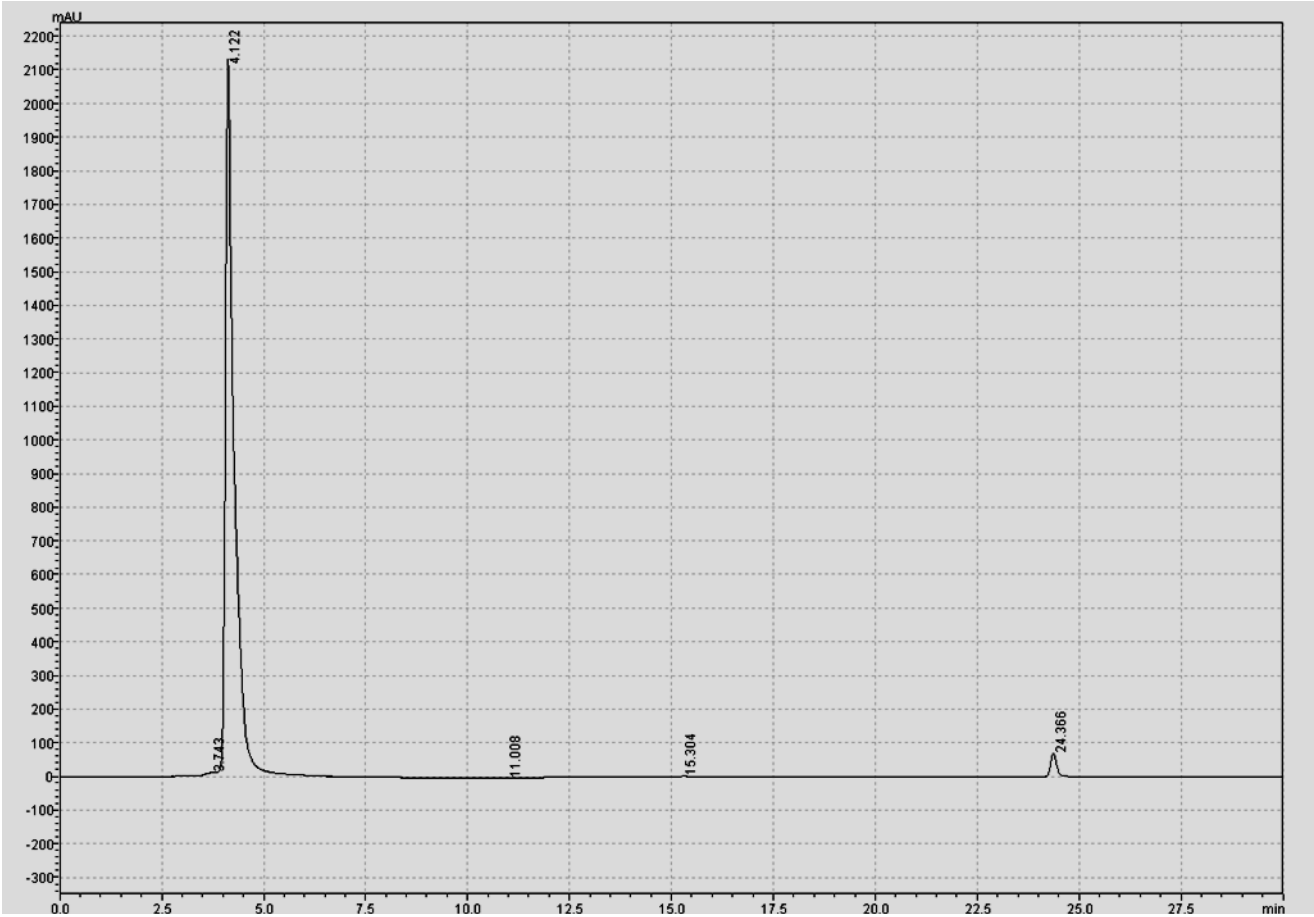

Figure 4.11: HPLC analysis of "C1" sample

The purity of "C1" was also confirmed by GC-MS fitted with a BP-1 (30 m X 0.25 mm, 0.25 μm film thickness) column. A peak with a retention time of 6.94 minutes was obtained, as shown in GC spectrum in Figure 4.12. Mass spectrum (Figure 4.13) of this peak was identified by the MS library as phloroglucinol with a molecular ion of 128 m/z. The mass spectrum of the isolated antioxidant had achieved 97.5% of similarity to the MS library.

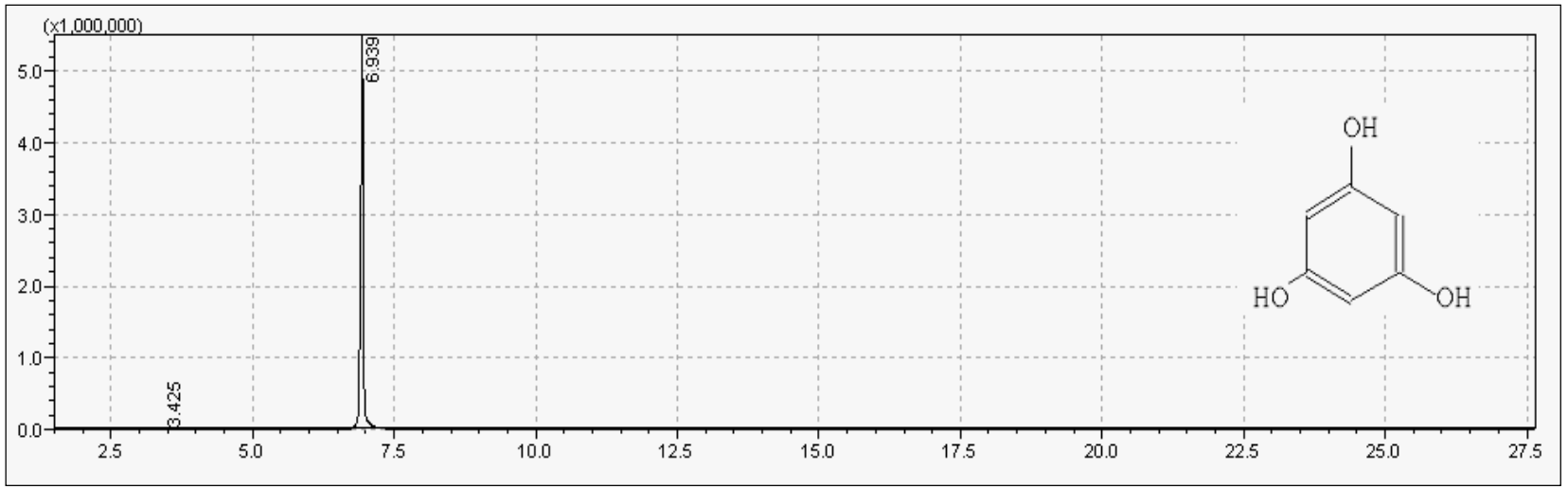

Figure 4.12: Gas chromatography of "C1" sample

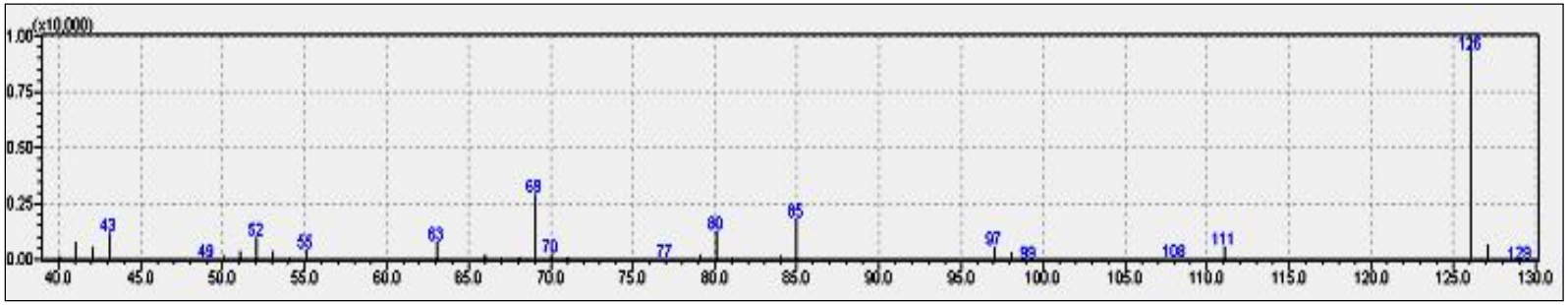

Figure 4.13: Mass spectrum of the component in the GC peak at retention time of 6.939min

4.5.3 Structure Elucidation of the Isolated "C1" Sample

"C1" was subjected to ^{1}H-nuclear magnetic resonance (^{1}H-NMR) and ^{13}C-nuclear magnetic resonance (^{13}C-NMR) for structural elucidation. ^{1}H and ^{13}C-NMR spectroscopy confirmed that "C1" is phloroglucinol (Figure 4.14). ^{1}H-NMR spectroscopy (Figure 4.15) results in a shift of hydroxyl protons at 5.676 ppm, and a shift of 8.906 ppm for benzylic protons. Benzylic protons were deshielded due to the hydroxyl groups which are electron-withdrawing groups. ^{13}C-NMR spectroscopy (Figure 4.16) results in carbon atoms 2, 4 and 6 assigned with a shift of 95.483 ppm and carbon 1, 3 and 5, which are equivalent, with a shift of 160.009 ppm.

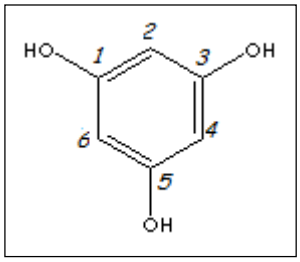

Figure 4.14: Structure of phloroglucinol

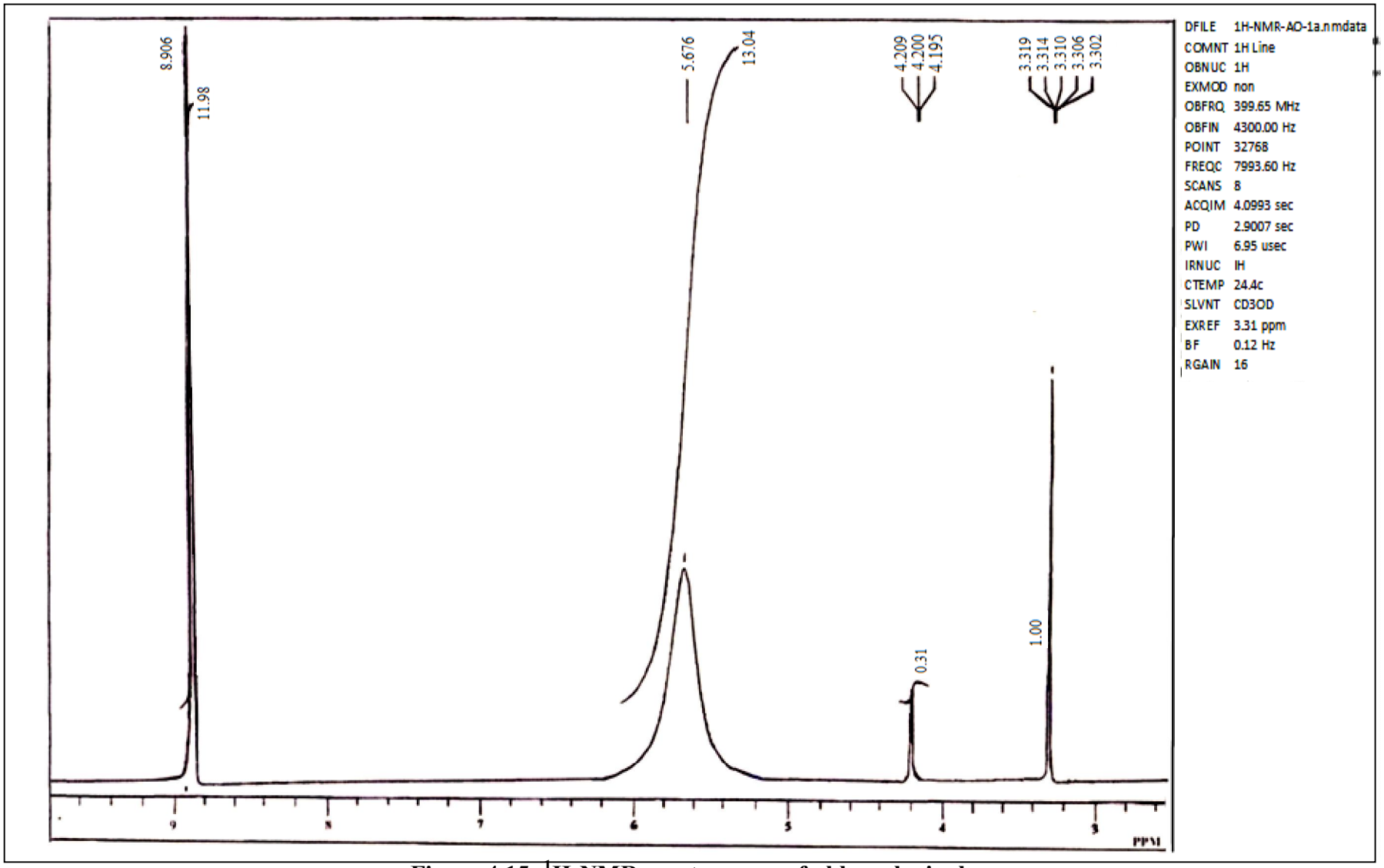

Figure 4.15: ^{1}H-NMR spectroscopy of phloroglucinol

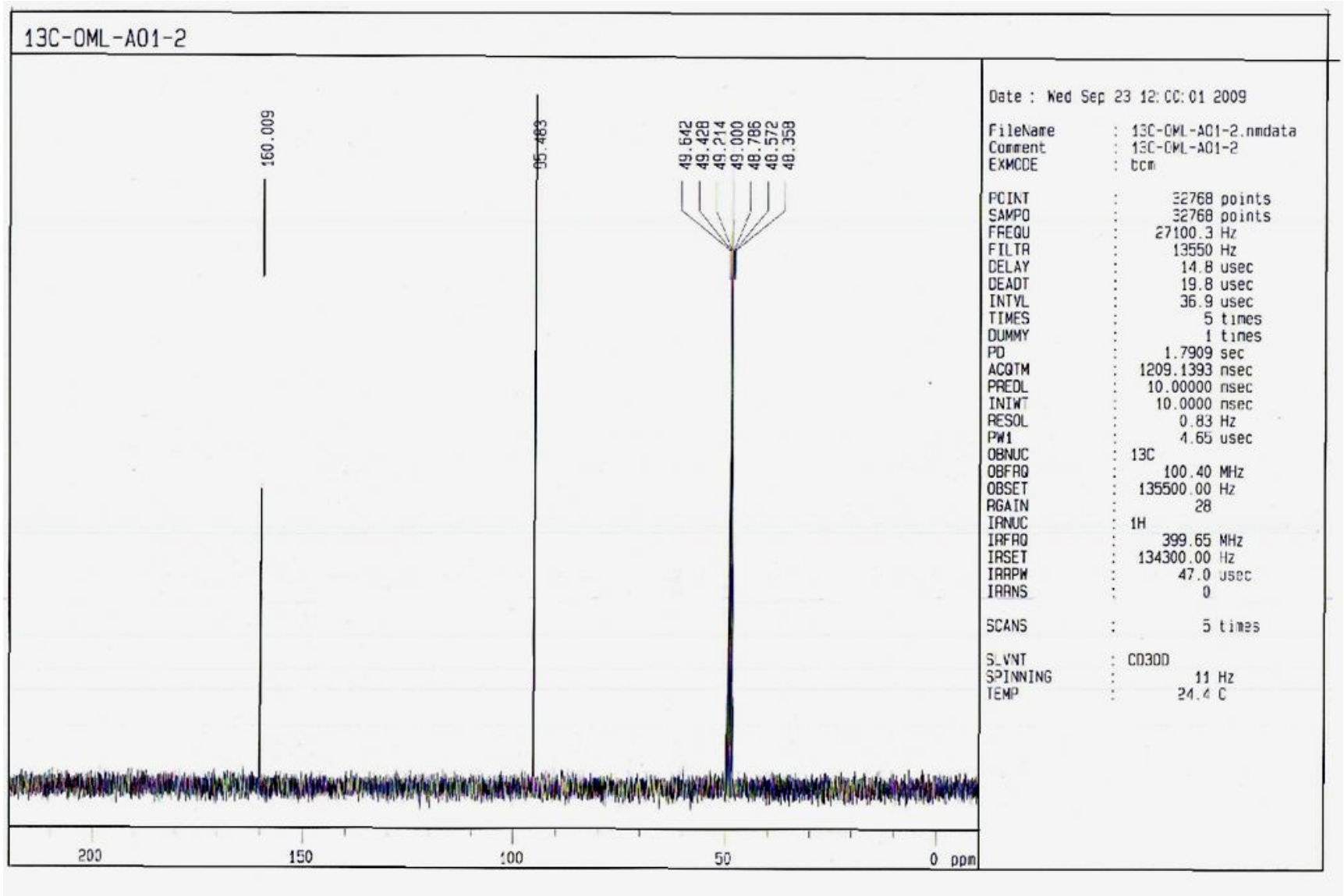

Figure 4.16: ^{13}C-NMR spectroscopy of phloroglucinol

4.6 Comparison of Antioxidant Activities between Isolated Antioxidants from *P. antillarum* and Standard Antioxidants

DPPH radical scavenging activities, ferric-reducing antioxidant power (FRAP) and ferrous ion chelating (FIC) of phloroglucinol which was isolated from *P. antillarum* was compared to well-characterized antioxidants such as ascorbic acid, tannic acid and quercetin.

4.6.1 Determination of DPPH Radical Scavenging Activity of Isolated Phloroglucinol and Standard Antioxidants

Different concentrations of isolated phloroglucinol and standard antioxidants (ascorbic acid, tannic acid and quercetin) were prepared and their DPPH radical scavenging activity was measured.

Table 4.10 summarized the percentage radical scavenging activities of the isolated phloroglucinol, ascorbic acid, tannic acid and quercetin.

Table 4.10: Percentages of radical scavenging activities of antioxidants at different concentration

Concentration, ppm	DPPH radical scavenging activity, %			
	Ascorbic acid	Tannic acid	Quercetin	Phloroglucinol
100.00	89.73	92.26	95.13	86.44
50.00	87.93	91.18	92.86	76.51
25.00	87.55	90.23	90.08	65.40
12.50	74.30	88.89	65.86	48.00
6.25	40.27	72.73	34.14	37.93
3.13	26.28	47.85	16.40	20.19

The purified phloroglucinol that was isolated from *P. antillarum* showed weaker DPPH radical scavenging activity with an EC_{50} value of 13.33 ppm, compared to well-characterized antioxidants derived from plants, such as quercetin (EC_{50}= 9.17 ppm), ascorbic acid (EC_{50}= 7.92 ppm) and tannic acid (EC_{50}= 3.33 ppm). The order of scavenging activity is related to substitution of hydroxyl groups in the aromatic rings of phenolics, thus, contributing to their hydrogen donating ability (Brand-William *et al*., 1995; Yen *et al*., 2005). Tannic acid has the most hydroxyl group attached to aromatic rings, followed by quercetin, ascorbic acid and phloroglucinol which were correlates to their DPPH radical scavenging activities.

Figure 4.17 shows the comparison of the radical scavenging activities for these antioxidants.

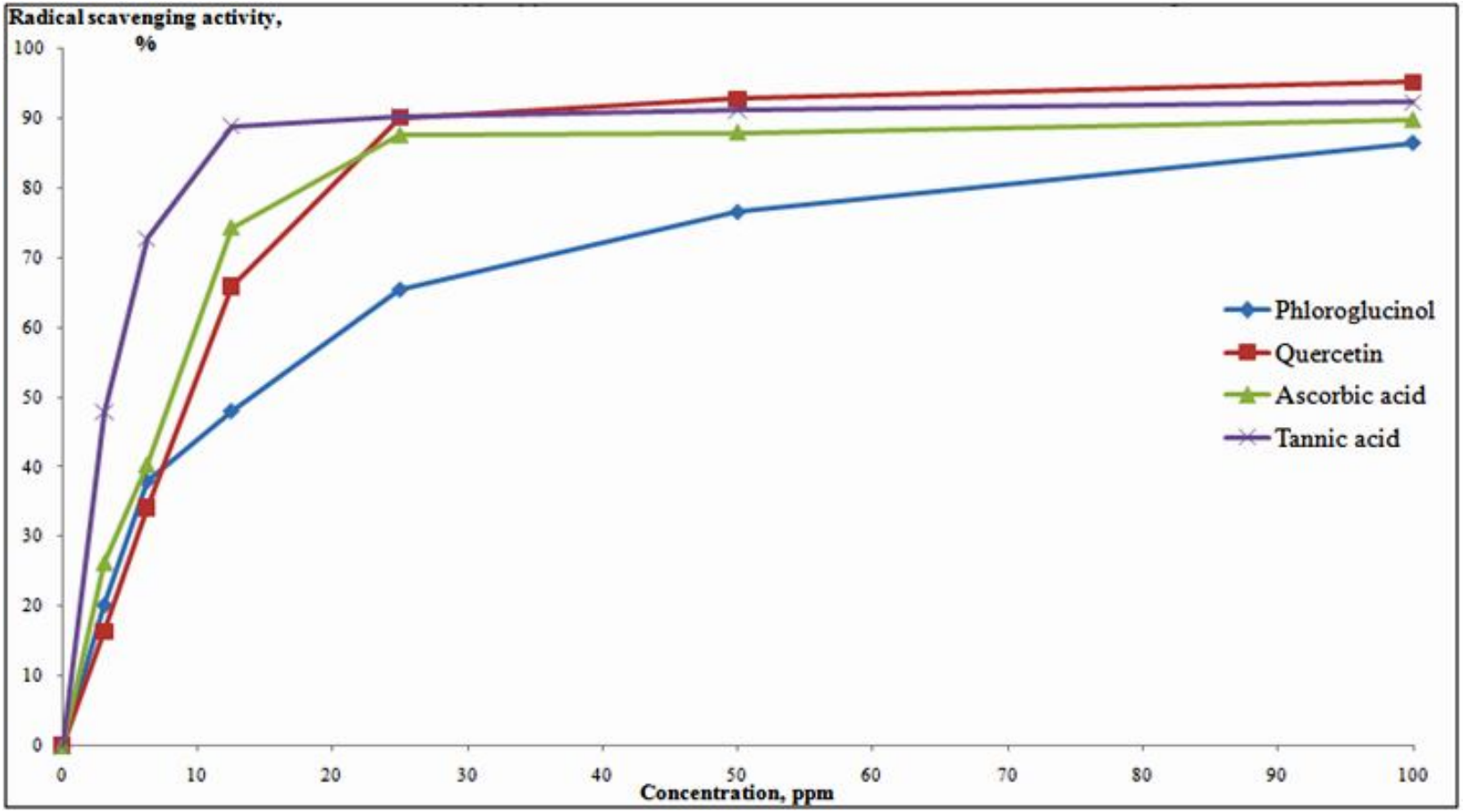

Figure 4.17: Radical scavenging activities of antioxidants at different concentration

The DPPH radical scavenging activities of the tested antioxidants were concentration-dependent. When the concentration of antioxidants increased, DPPH radical scavenging activities also increased. The DPPH radical scavenging activities of the well-characterized antioxidants achieved a plateau at an average of 90% DPPH radical scavenging activity from 25 ppm to 100 ppm. DPPH radical scavenging activity of isolated phloroglucinol did not increase as drastically as the well-characterized antioxidants. The DPPH radical scavenging activity increased gradually as its concentration increased.

4.6.2 Ferric-reducing Antioxidant Power (FRAP)

The measurement of the reductive ability of antioxidant is based on the ability of antioxidant to reduce ferric (III) to ferrous (II) in a redox-linked colorimetric reaction that involves single electron transfer. The change in absorbance is directly related to the reducing power of the electron donating antioxidants.

The FRAP value of isolated phloroglucinol was compared to those of ascorbic acid, tannic acid and quercetin. The ferric (III) to ferrous (II) transformation in the presence of phloroglucinol was weaker than ascorbic acid, quercetin and tannic acid, characterized by lower absorbance value. Tannic acid exhibited the highest FRAP, followed by quercetin, ascorbic acid and phloroglucinol, as shown in Figure 4.18. The FRAP values of these antioxidants were correlated with their DPPH radical scavenging activities.

Tannic acid has more hydroxyl groups attached to aromatic rings than quercetin, ascorbic acid and phloroglucinol which are thought to exert antioxidant action by terminating the free radical chain reaction by donating a hydrogen atom. The FRAP of tannic acid, ascorbic acid and quercetin was concentration dependent, as shown in Figure 4.18. When the concentration of antioxidants increased, FRAP values also increased.

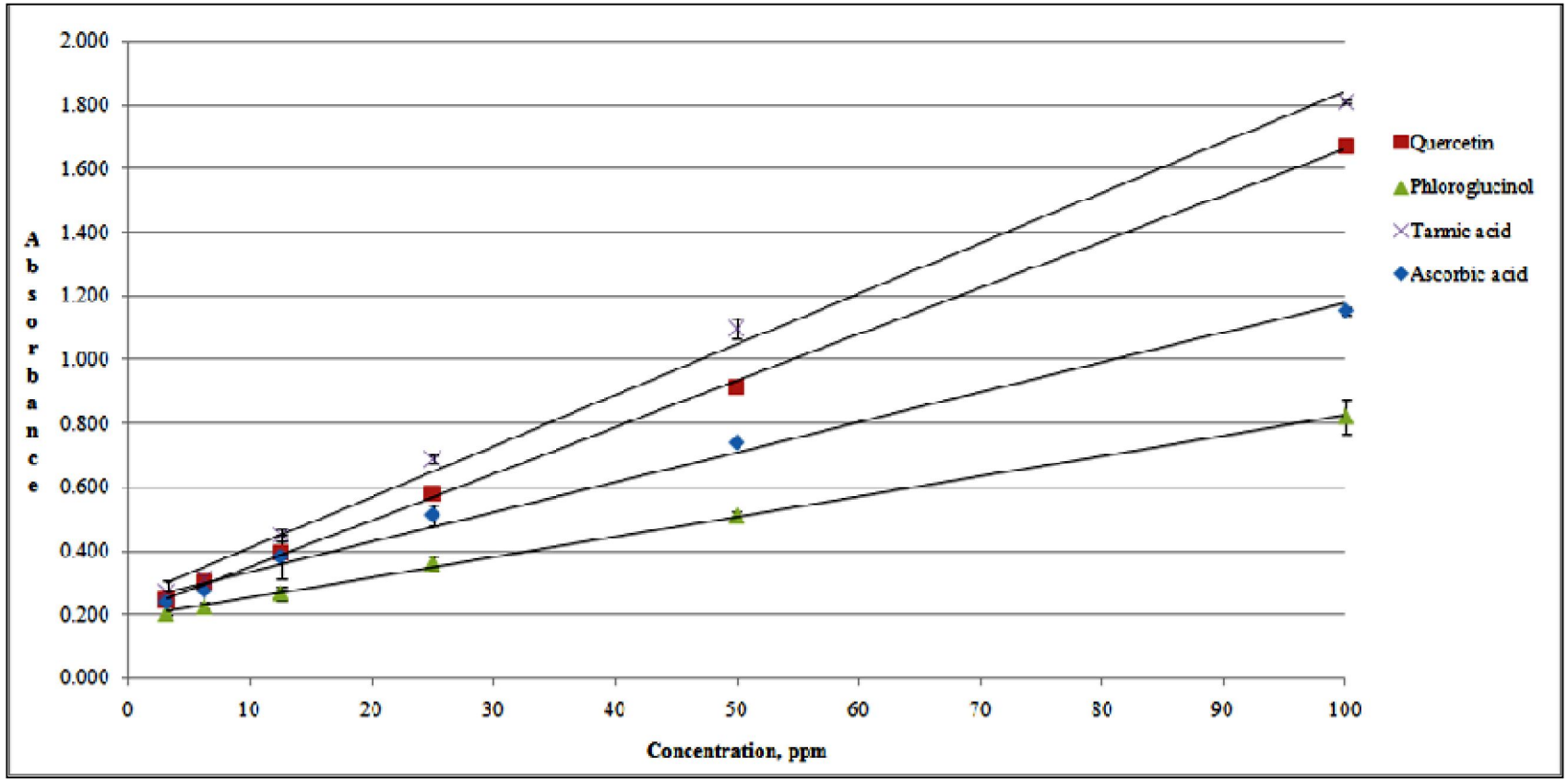

Figure 4.18: Ferric-reducing antioxidant powers of ascorbic acid, tannic acid, quercetin and isolated phloroglucinol

4.6.3 Ferrous Ion Chelating (FIC) Assay

Ferrozine was used in FIC assay. Ferrozine reacted with the divalent iron (ferrous ions) to form stable red coloured complex species. The formation of the red coloured complex were disrupted in the presence of chelating agent, thus the red colour of the complex was reduced. Therefore, measurement of colour reduction allows the estimation of the chelating activity of an antioxidant.

The chelating ability of purified phloroglucinol that was isolated from *P. antillarum* was compared to tannic acid, ascorbic acid and quercetin. The results showed that the chelating ability of phloroglucinol is much lower than that of the quercetin, tannic acid and ascorbic acid (Figure 4.19). This might be due to the weak binding ability of phloroglucinol which is unable to inhibit reactions that generate hydroxyl radicals.

Purification and characterization of phloroglucinol which was isolated from *P. antillarum* had been published in the proceeding of "The 2nd International Conference of Natural Products and Traditional Medicine" (Appendix M).

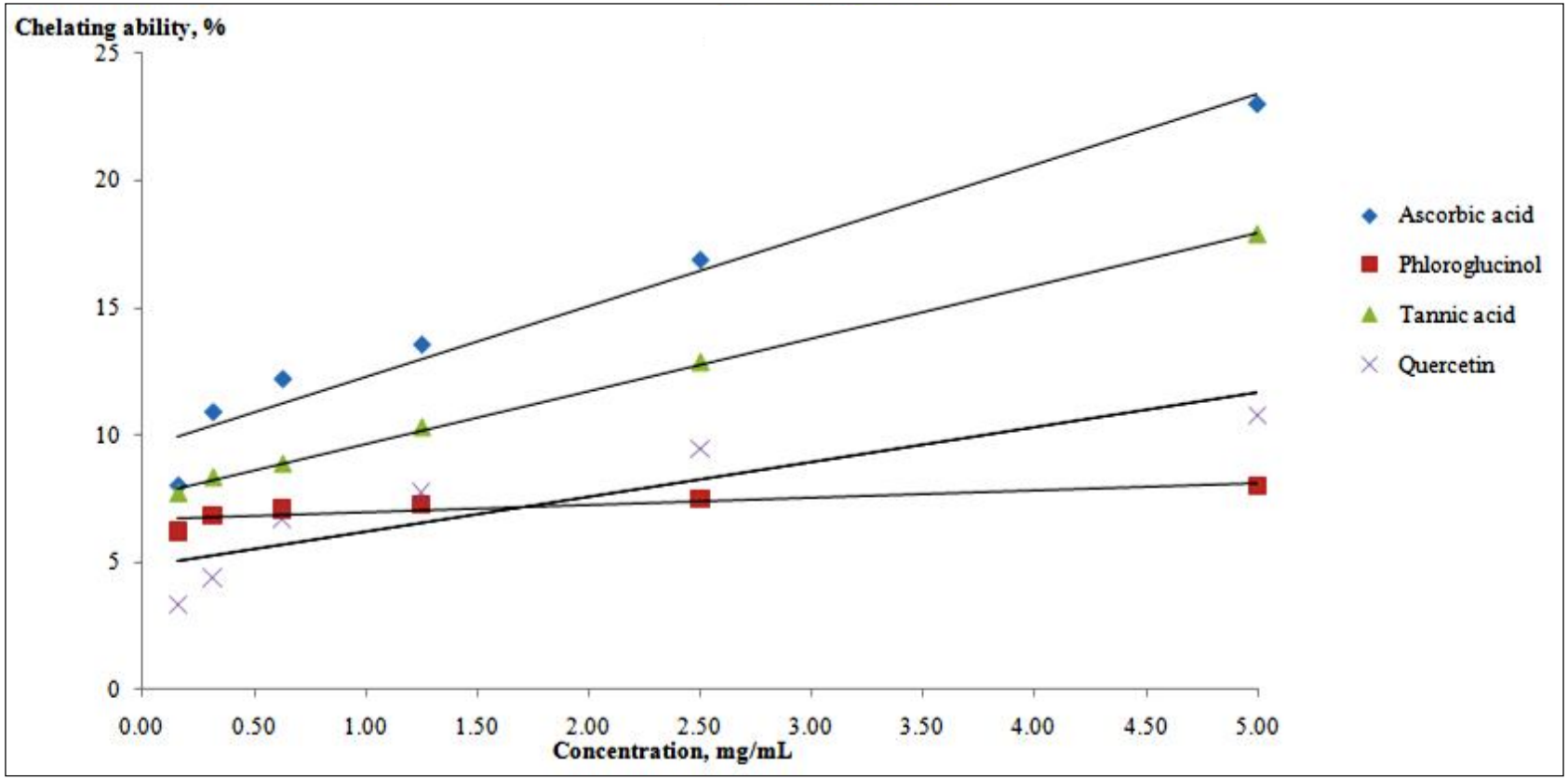

Figure 4.19: Ferrous ion chelating ability of isolated phloroglucinol, ascorbic acid, tannic acid and quercetin

4.7 MTT Cytotoxicity Testing on Purified Phloroglucinol which was Isolated from *P. antillarum*

A colorimetric cytotoxicity testing was carried out to determine the cytotoxic properties of phloroglucinol towards K 562 (leukemia cell) using 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) as an indicator. The presence of phloroglucinol has no effects on the growth of the K 562 cell line. A hundred percents cell viability was seen with phloroglucinol at concentration of 50 µg/mL (Table 4.11). The cell viability was only slightly decreased at 100 µg/mL of phloroglucinol. Increasing the concentration of phloroglucinol does not inhibit the cells growth (98.29% of cell viability).

Table 4.11: Average percentage viability of K 562 Cells as assessed by MTT cytotoxicity test

Concentration of phloroglucinol (µg/mL)	Average viability (%), n=3	Standard deviation (%)
50	112.22	7.43
100	98.29	8.25

4.8 Screening of Antimicrobial Activity on the Purified Phloroglucinol which was Isolated from *P. antillarum*

Isolated phloroglucinol was prepared at 4 mg/mL in methanol. Two-fold dilution was performed on the 96-well plate until the concentration of phloroglucinol achieved a concentration of 0.002 mg/mL. The MIC values of phloroglucinol towards the tested microorganisms are summarized in Table

4.12. Phloroglucinol inhibited growth of *S. aureus*, *P. aeruginosa* and *E. coli* (penicillin-sensitive strain) with a MIC value of 0.5 mg/mL. Growth of *K. pneumoniae* and *B. cereus* were inhibited by phloroglucinol with a MIC value of 1.0 mg/mL. Only *E. coli* (penicillin-resistant strain) was not inhibited by phloroglucinol. Phloroglucinol showed weak growth inhibition towards all bacteria compared to chloramphenicol which inhibited the bacteria with a MIC value ranging from 4 µg/mL to 8 µg/mL. Addition of phloroglucinol on all tested fungi showed no detectable antifungal activity.

Table 4.12: Antimicrobial activity of phloroglucinol

Tested microorganisms	Average minimum inhibitory concentration of phloroglucinol, mg/mL (n=3)	Average minimum inhibitory concentration of antibiotic, mg/mL (n=3)
Bacteria		
S. aureus	0.5 ± 0	0.008
B. cereus	1.0 ± 0	0.004
P. aeruginosa	0.5 ± 0	0.004
K. pneumoniae	1.0 ± 0	0.008
E. coli (penicillin-sensitive strain)	0.5 ± 0	0.004
E. coli (penicillin-resistant strain)	-	0.008
Fungi		
C. albicans	-	0.002
C. parapsilosis	-	0.002
C. neoformans	-	0.001
A. niger	-	0.004
T. mentagrophytes	-	0.001

"-" denotes no activity

A colorimetric broth microdilution assay was performed to assess the antimicrobial activity of phloroglucinol which was isolated from *P. antillarum*, using *p*-iodonitrotetrazolium violet (INT) as the indicator. Figure 4.20 shows an example of 96-well plate which contains *S. aureus*, *K. pneumoniae* and *E. coli* (penicillin-resistant strain). The growth of bacteria was indicated by the colour change from yellow to red.

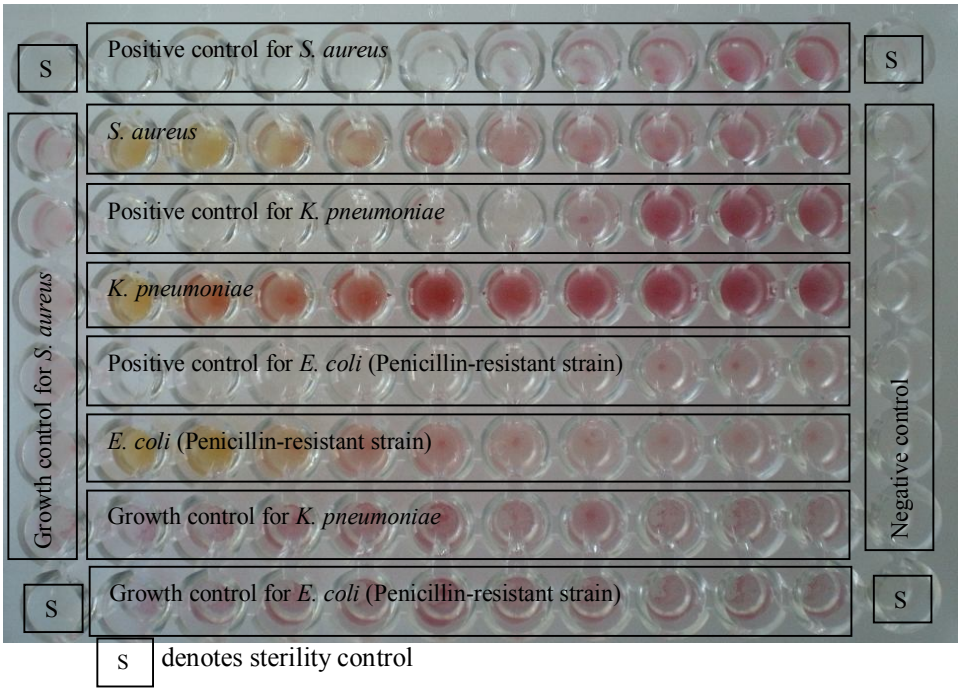

Figure 4.20: Inhibitory activity of isolated phloroglucinol towards *S. aureus* (ATCC 6538*), K. pneumonia*e (ATCC 13883) and *E coli* (penicillin-resistant strain, ATCC 35218)

The site and number of hydroxyl groups on the phenol group are thought to be related to the relative toxicity to the tested microorganisms, with evidence that increased hydroxylation results in increased toxicity (Geissman, 1963). More highly oxidized phenols had inhibitory effects (Scalbert, 1991;

Urs & Dunleavy, 1975). The mechanism thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by oxidized compounds, possibly through reaction with sulphydryl groups or through more non-specific interactions with the proteins (Mason & Wasserman, 1987).

4.9 Occurrence of Phloroglucinol

Monomeric phloroglucinol or 1, 3, 5- trihydroxybenzene has been isolated from terrestrial plants, such as *Eucalytus kino* and *Acacia arabica* (Singh & Bharate, 2006*)*. Monomeric phloroglucinol which was isolated from *E. kino* and *A. arabica* had been commercialized. It is also found in seaweeds of families Phaeophyceae and Fucaceae.

Phloroglucinol is more commonly found in derived form, and derivatives of phloroglucinol including glycosides and halogenated compounds have been reported (Singh & Bharate, 2006*)*. A long-chain unsaturated acyl phloroglucinol has been reported from the brown algae, *Zonaria tournefortii* (Amico *et al.*, 1982). The structure is shown in Figure 4.21.

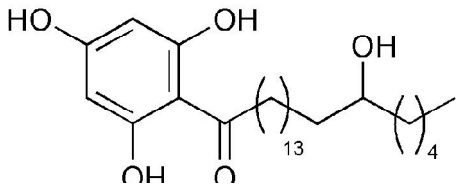

Figure 4.21: Long-chain phloroglucinol compound isolated from the brown algae *Zonaria tournefortii*

A variety of bromo, chloro and iodo derivatives of phloroglucinol are known to occur amongst marine organisms. For example, a bromo derivative of phloroglucinol was isolated from the red alga, *Rytiphea tinctorea,* as shown in Figure 4.22.

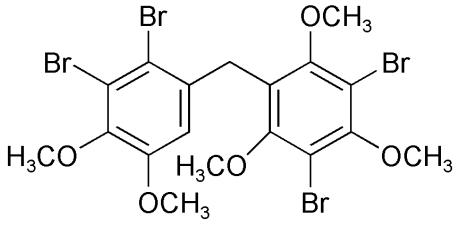

Figure 4.22: A halogenated compound isolated from the red alga, *Rytiphea tintorea*

Phloroglucinol has a number of chemical and industrial applications, e.g. as a reagent for the detection of aldehydes and for textile-dyeing. It may have pharmaceutical application (Singh & Bharate, 2006*)*. In this study, phloroglucinol was isolated from the brown algae *P. antillarum* and its antioxidant activity was assessed, as there have been few studies that have evaluated its potential as an antioxidant (Singh & Bharate, 2006).

4.10 Extraction of Antimicrobial Compounds from *P. antillarum*

Sequential extraction was used to extract antimicrobial compounds from *P. antillarum*. The solvents ranged from nonpolar to polar (i.e. hexane, diethyl ether, chloroform, ethyl acetate, acetone, methanol and finally water). The mass extracted of the extracts were calculated and summarized in Table 4.13. The mass extracted indicate the weights of the extracts which can be extracted from 1 g of freeze-dried *P. antillarum*. Water is the best extractant giving the highest mass of extract from *P. antillarum* with 81.8 mg/g of dried sample. Ethyl acetate gave the lowest mass of extract with a mass of 2.17 mg/g. Polar solvents (acetone, methanol and water) have higher extraction efficiencies on *P. antillarum* compare to non-polar solvents (hexane, diethyl ether, chloroform and ethyl acetate). The extracts, chromatographic fractions and pure compounds should be kept out of direct sunlight and preferably stored at -20^{o}C as a measure against the potential susceptibility of many marine secondary metabolites to oxidative degradation in air and against isomerization of double bonds in sunlight at room temperature (25^{o}C) (Ebada *et al*., 2008)

Table 4.13: Total Mass of Extracts which were extracted from *Padina antillarum* using different types of solvents

Extractants	Weight of extracts, mg	Mass extracted (mg/g)
Hexane	3000	30.00
Diethyl ether	478	4.78
Chloroform	1026	10.26
Ethyl acetate	217	2.17
Acetone	485	4.85
Methanol	6720	67.2
Water	8180	81.8

4.10.1 Toxicity of Solvents towards Bacteria and Fungi

The solvent itself may be toxic to the microorganisms, as some solvents may affect biological membranes of the microorganisms. Thus, the toxicity of ten solvents towards six types of bacteria and five types of fungi were assessed prior to antimicrobial testing on the crude extracts of *P. antillarum*. A colorimetric broth microdilution assay was performed, using *p*-iodonitrotetrazolium violet (INT) as the indicator. The growth of bacteria and fungi was indicated by a colour change to red.

Only dimethyl sulfoxide (DMSO) exerted inhibitory effects on the tested bacterial and fungal strains at concentration of 6.25% (v/v) to 25.00% (v/v). Among the bacterial strains, *K. pneumoniae* demonstrated the least growth inhibition by DMSO at concentration of 25% (v/v) while growth of *E. coli* (penicillin-resistant strain) was inhibited the most by DMSO at the concentration of 6.25% (v/v). The inhibition of growth of *S. aureus*, *B. cereus*, *P. aeruginosa* and *E. coli* (penicillin-sensitive strain) by DMSO was occurred at the same concentration of 12.50% (v/v). DMSO exhibited inhibitory activity towards *C. albicans* and *C. parapsilosis* at the concentration of 12.50% (v/v) while growth of *C. neoformans*, *A. niger* and *T. mentagrophytes* were inhibited by DMSO at a lower concentration which is at 6.25% (v/v).

The MIC value of solvents and positive control towards bacteria and fungi are summarized in Table 4.14.

Table 4.14: Minimum inhibitory concentration of various solvents towards bacterial and fungal strains

Microorganisms	Mean minimum inhibitory concentration (n=3) of solvents, % (v/v)										Mean MIC of positive control (n=3), µg/mL
	Hex	DE	$CHCl_3$	EA	DMSO	EtOH	MeOH	Ace	BuOH	DCM	
Gram positive bacteria											
S. aureus	-	-	-	-	12.50	-	-	-	-	-	8.00
B. cereus	-	-	-	-	12.50	-	-	-	-	-	4.00
Gram negative bacteria											
P. aeruginosa	-	-	-	-	12.50	-	-	-	-	-	4.00
K. pneumoniae	-	-	-	-	25.00	-	-	-	-	-	8.00
E. coli- penicillin sensitive	-	-	-	-	12.50	-	-	-	-	-	4.00
E. coli- penicillin resistant	-	-	-	-	6.25	-	-	-	-	-	8.00
Yeast											
C. albicans	-	-	-	-	12.50	-	-	-	-	-	2.00
C. parapsilosis	-	-	-	-	12.50	-	-	-	-	-	2.00
C. neoformans	-	-	-	-	6.25	-	-	-	-	-	1.00
Mould											
A. niger	-	-	-	-	6.25	-		-	-	-	4.00
T. mentagrophytes	-	-	-	-	6.25	-	-	-	-	-	1.00

Note: "-", no inhibition; Hex, hexane; DE, diethyl ether; CH3Cl, chloroform; EA, ethyl acetate; DMSO, dimethyl sulfoxide; EtOH, ethanol; MeOH, methanol; Ace, acetone; BuOH, butanol; DCM, dichloromethane.

Examples of toxicity of DMSO towards *P. aeruginosa* and *C. neoformans* are shown in Figure 4.23 and 4.24 respectively.

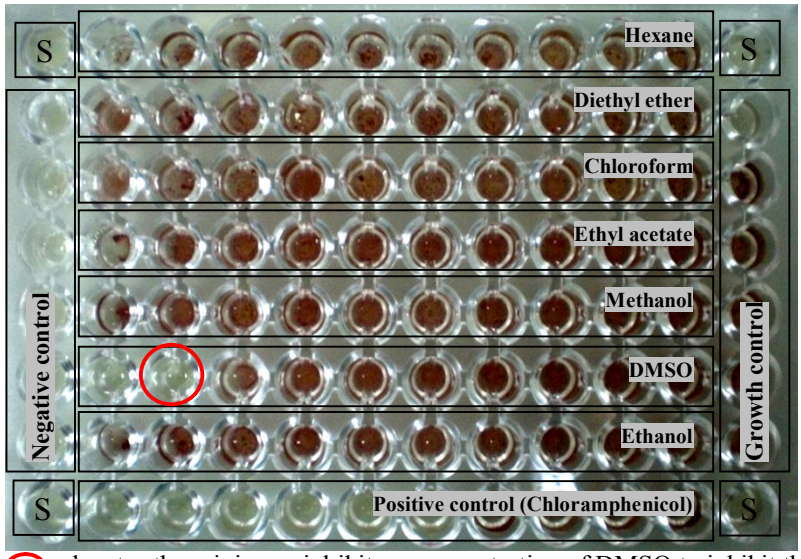

Figure 4.23: Inhibitory activity of DMSO towards *P. aeruginosa* (ATCC 27853)

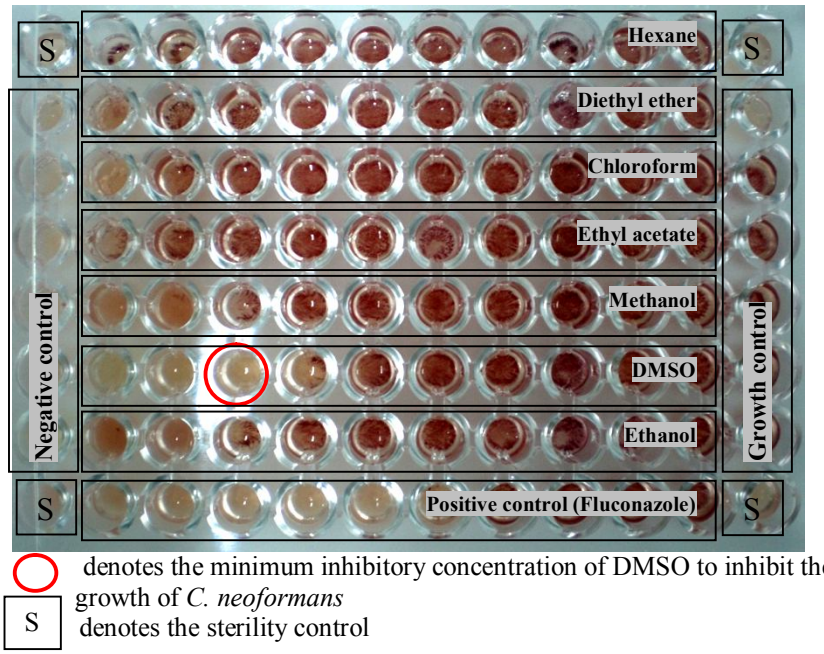

Figure 4.24: Inhibitory activity of DMSO towards *C. neoformans* (ATCC 90112)

DMSO exerted a marked inhibitory effect on a wide range of bacteria and fungi at concentrations likely to be encountered in antimicrobial testing programs in industries. It has been suggested that the bacteriostatic affects of DMSO might be due to the increased in oxygen uptake and the decrease in the rate of glycine transport (Ghajar & Harmon, 1968). Besides, inhibition of growth of the tested microorganisms might be due to the accumulation of solvents in the cytoplasmic membrane which would affect its structural properties, thus preventing the cell from performing essential functions such as dissipation of pH and electrical potentials and disruption of the normal flow of ions, proteins, lipids and endogenous metabolites and the effects on membrane protein (Shikkema *et al*., 1995). Higher concentration of DMSO will lead to an increase of pH, thus weakening the microorganisms (Basch & Gadebusch, 1968).

There is no standard method for defining the most suitable solvent or the optimal solvent concentration to be used. Hence, a suitable solvent must be employed to determine the conditions that result in the lowest degree of toxicity on the microorganisms used for bioassays. Miscibility between solvent and broth medium are also important. Since the broth medium is hydrophilic, only acetone, ethanol, butanol and methanol are suitable. From the study, it was observed that chloroform, diethyl ether, dichloromethane, ethyl acetate and acetone had some degree of corrosiveness to 96-well polystyrene plate used in the bioassay screening. In order to avoid false readings, those solvents would not be a good choice for dissolving the dry extracts for further bioassay screening.

Mixture of methanol and water with a ratio of 2:1 was selected to dissolve the crude extracts of *P. antillarum* prior to antimicrobial screening. This mixture of solvent was able to dissolve all the crude extracts, giving an exact concentration for bioassay screening. Moreover, the growth of bacterial and fungal strains are not affected.

4.10.2 Antibacterial Testing towards Six Types of Bacterial Strains

The extracts that were obtained from sequential extraction of *P. antillarum* were subjected to antimicrobial assay. The extracts were hexane, diethyl ether, chloroform, ethyl acetate, acetone, methanol and water extracts. All extracts except water extract demonstrated inhibition against *K.*

pneumoniae, B. cereus and *P. aeruginosa*. Mean MIC values ranged from 0.42 to 1.67 mg/mL. The hexane and diethyl ether extracts produced the most consistent inhibition of bacterial growth with a mean MIC value of 0.63 mg/mL. All the extracts excluding the water extract exhibited inhibition of growth on *E. coli* penicillin-resistant strains (range of mean MIC value= 1.67-2.50 mg/mL) but not *E .coli* penicillin-sensitive strain. Only the ethyl acetate extract (mean MIC= 4.17 mg/mL) and methanol extract (mean MIC= 3.33 mg/mL) inhibited the growth of *S. aureus*.

The mean MIC values and total activities of the extracts which were obtained from *P. antillarum* are summarized in Table 4.15. Chloramphenicol was used as the positive control for the antimicrobial tests.

Table 4.15: The mean MIC values (mg/mL) and total activity (mL/g) of extracts of *P. antillarum* towards six bacterial strains

Bacteria strains	Extracts of *P. antilarum*							Mean MIC of positive control (n=3), µg/mL
	Hexane	Diethyl ether	Chloroform	Ethyl acetate	Acetone	Methanol	Water	
Mean MIC values (n=3), mg/mL								
S. aureus	-	-	-	4.17±1.44	-	3.33±1.44	-	8.00±0
P. aeruginosa	0.63±0	0.63±0	0.52±0.18	0.52±0.18	0.63±0	1.04±0.36	-	4.00±0
B. cereus	0.63±0	0.63±0	0.63±0	0.42±0.18	0.83±0.36	1.25±1.08	-	4.00±0
K. pneumoniae	0.63±0	0.63±0	0.63±0	0.63±0	1.46±0.95	1.67±0.72	-	8.00±0
E. coli (penicillin-sensitive strain)	-	-	-	-	-	-	-	4.00±0
E. coli (penicillin-resistant strain)	1.67±0.72	1.67±0.72	1.67±0.72	1.92±0.72	2.50±0	2.50±0	-	8.00±0
Total activity, mL/g								
S. aureus	-	-	-	0.51	-	21.18	-	N/A
P. aeruginosa	48.00	7.65	19.69	4.17	7.76	64.49	-	N/A
B. cereus	48.00	7.65	16.42	5.19	5.82	53.76	-	N/A
K. pneumoniae	48.00	7.65	16.42	3.47	3.33	40.31	-	N/A
E. coli (penicillin-sensitive strain)	-	-	-	-	-	-	-	N/A
E. coli (penicillin-resistant strain)	18.00	2.88	6.15	1.13	1.94	26.88	-	N/A

"-" denotes no activity; "MIC" denotes minimum inhibitory concentration and "N/A" denotes not available

An example of antimicrobial screening on the extracts of *P. antillarum* towards *P. aeruginosa* using a 96-well plate is shown in Figure 4.25. Two-fold series dilution was performed on the extracts.

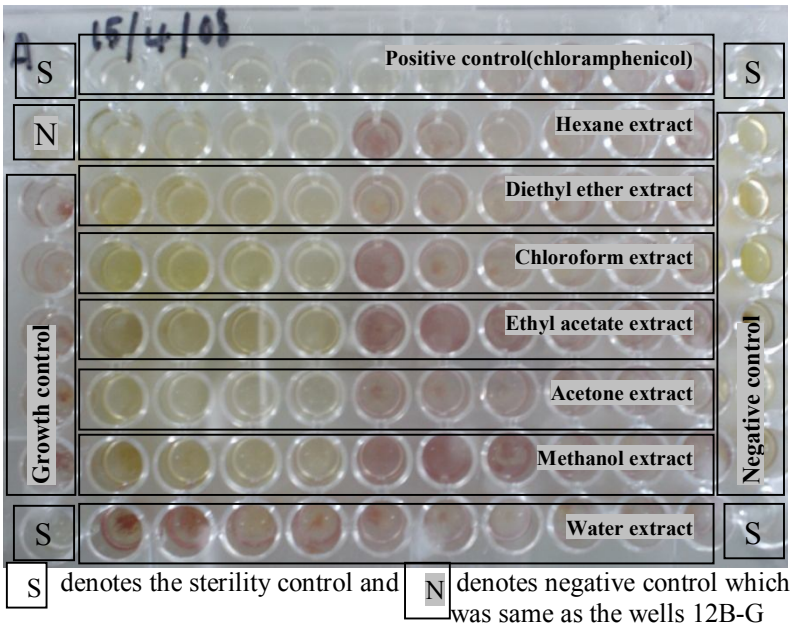

Figure 4.25: Inhibitory activity of the extracts of *P. antillarum* towards *P. aeruginosa* (ATCC 27853)

The total activity indicated that methanol extract had the most active antimicrobial activity towards *P. aeruginosa* and *B. cereus* as compared to other extracts. The total activity of methanol extract towards *P. aeruginosa* and *B. cereus* were 64.49 and 53.76 mL/g respectively. This implies that the methanol extract prepared from 1 g of freeze-dried *P. antillarum* could be diluted to a volume of 64.49 mL and still retain activity against *P. aeruginosa*. Hexane extracts also exhibited high total activity other than methanol extract

with a value of 48 mL/g towards *P. aeruginosa*, *B. cereus* and *S. aureus*. It gave a consistent MIC value over three tests.

P. antillarum is a potential source of antimicrobial agents because its extracts are able to inhibit both Gram positive and Gram negative bacteria. Both nonpolar and polar extracts showed inhibition on the growth of bacteria. *P. antillarum* might contain more than one antimicrobial. The hexane extract was selected for further purification due to its relatively high total activity and consistency of results. Due to insufficient amounts of extract, the fractions collected from subsequent stages of purification were only tested on *P. aeruginosa*.

4.10.3 Antifungal testing using Five types of Fungal Strains

The extracts of *P. antillarum* were subjected to antifungal testing. Table 4.26 summarizes the MIC values of the extract towards five types of fungal strains. All extracts exhibited inhibitory activitiy towards *C. neoformans* and *A. niger*. Mean MIC values ranged from 0.26 to 4.17 mg/mL. Ethyl acetate extract exhibited the strongest inhibitory activity towards *C. neoformans* with a mean MIC value of 0.26 mg/mL. However, the amount of ethyl acetate extract is the least compared to others and it was insufficient for further fractionation. Only the hexane extract showed inhibition towards *T. mentagrophytes* with a mean MIC value of 3.33 mg/mL. No growth inhibition was observed on all crude extracts towards *C. parapsilosis*. The growth of *C. albicans* was inhibited only

by hexane, diethyl ether and chloroform extracts with mean MIC values ranging from 2.50 to 5.00 mg/mL.

The total activity indicated that methanol extract had the most active antimicrobial activity towards *A. niger* and *C. neoformans* as compared to other extracts. The extract prepared using 1g of freeze-dried *P. antillarum* could be diluted to a volume of 53.76 mL and 81.00 mL and still retain inhibitory activity towards *A. niger* and *C. neoformans* respectively.

Table 4.16 shows the mean MIC values (mg/mL) and total activities (mL/g) of the extracts of *P. antillarum* towards five fungal strains. Fluconazole was used as the positive control in the antifungal tests.

Table 4.16: MIC values (mg/mL) and total activity values (mL/g) of extracts of *P. antillarum* towards five fungal strains

Crude Extracts	*Candida albicans*	*Candida parapsilosis*	*Cryptococcus neoformans*	*Trichophyton mentagrophytes*	*Aspergillus niger*
Mean MIC values (n=3), mg/mL					
Fluconazole	0.002± 0	0.002 ± 0	0.001 ± 0	0.001 ± 0	0.004 ± 0
Hexane	2.50 ± 0	-	0.63 ± 0	3.33 ± 1.44	4.17 ± 1.44
Diethyl ether	5.00 ± 0	-	1.04 ± 0.36	-	1.04 ± 0.36
Chloroform	4.17 ± 1.44	-	1.25 ± 0	-	0.52 ± 0.18
Ethyl acetate	-	-	0.26 ± 0.09	-	1.04 ± 0.36
Acetone	-	-	0.63 ± 0	-	1.25 ± 0
Methanol	-	-	0.83 ± 0.36	-	1.25 ± 0
Water	-	-	2.50 ± 0	-	3.33 ± 1.44
Total activity, mL/g					
Hexane	12	-	47.62	9.01	7.19
Diethyl ether	0.96	-	4.60	-	4.60
Chloroform	2.46	-	8.21	-	19.73
Ethyl acetate	-	-	8.35	-	2.09
Acetone	-	-	7.70	-	3.88
Methanol	-	-	81.00	-	53.76
Water	-	-	32.72	-	24.56

"-" denotes no inhibition and "MIC" denotes minimum inhibitory concentration

An example of antimicrobial screening on the extracts of *P. antillarum* towards *C. parapsilosis* using a 96-well plate is shown in Figure 4.26. Two-fold series dilution was performed on the extracts.

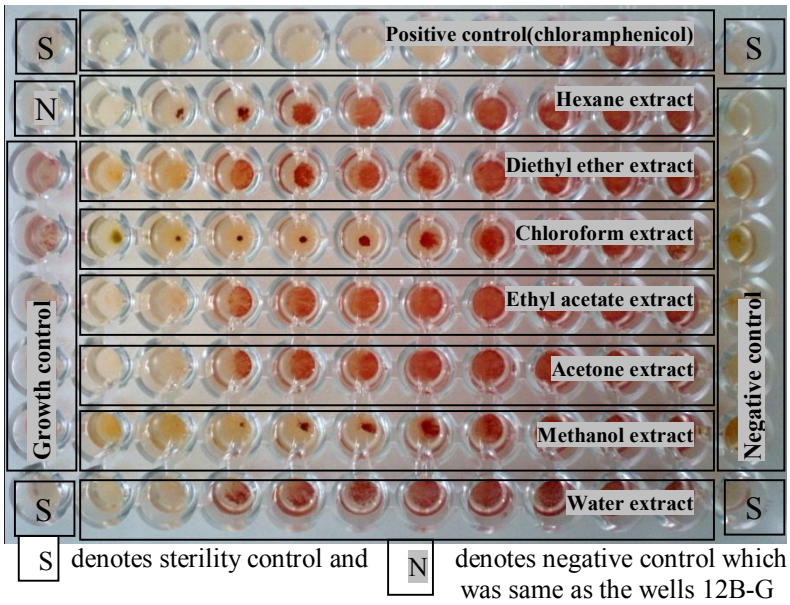

Figure 4.26: Inhibitory activity of the extracts of *P. antillarum* towards *C. parapsilosis* (ATCC 22019)

4.10.4 Purification of Hexane Extract

Activity-guided fractionation was performed on the hexane extract. Hexane extract was subjected to silica gel flash chromatography in which the initial mobile phase used was 100% hexane, after which the ethyl acetate content was increased until it contained 40% of ethyl acetate. Finally, the column was eluted with 100% ethyl acetate. A total of 90 fractions with each volume of approximately 40 mL were collected at a flow rate of 8.5 mL/min. The fractions were concentrated by using rotary evaporation and subjected to

TLC, after which the fractions were combined where appropriate. Figures 4.27 and 4.28 show the TLC of fractions 1 to 18 collected from the fractionation of the hexane extract of *P. antillarum*. The TLC was visualized after iodine treatment and under UV light. Fractions which contained of compounds at same R_f values were combined.

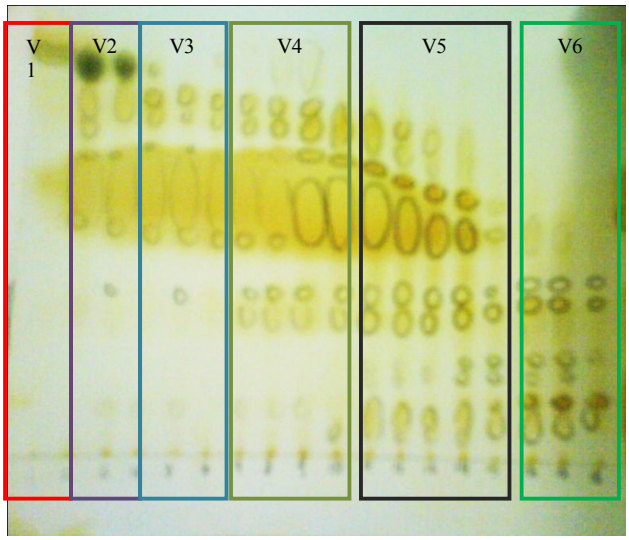

Figure 4.27: TLC, after iodine treatment, of fractions 1 to 18 collected from fractionation of the hexane extract of *P. antillarum*

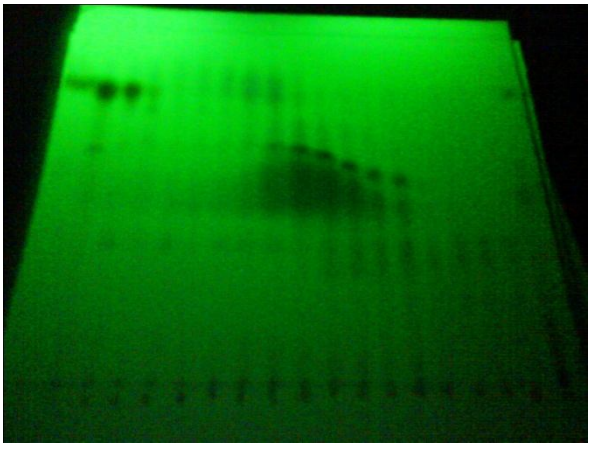

Figure 4.28: Visualization of TLC under UV light on the fractions 1 to 18 collected from fractionation of the hexane extract of *P. antillarum*

Ninety fractions from purification of hexane extract were pooled into twenty four samples ("V1" to "V24") which are summarized in Table 4.17.

Table 4.17: Ninety fractions which were collected from purification of hexane extract were pooled into 24 samples

Combined samples	Collected fractions	Weights, mg
V1	1	70.0
V2	2-3	129.1
V3	4-6	151.3
V4	7-10	131.1
V5	11-15	70.6
V6	16-19	10.7
V7	20-21	5.4
V8	22-25	14.2
V9	26	140.9
V10	27-29	330.9
V11	30-32	137.1
V12	33-37	10.6
V13	38-40	74.3
V14	41-42	30.5
V15	43-45	4.4
V16	46-47	22.4
V17	48-51	74.4
V18	52	6.8
V19	53-54	9.5
V20	55-69	16.8
V21	70	3.9
V22	71	6.8
V23	72-75	25.9
V24	76-90	20.2

"V1" to "V24" (10 mg/mL) were prepared in methanol, after which they were subjected to antimicrobial assay for keep track of the occurrence of the antimicrobial compounds which inhibited the growth of *P. aeruginosa*. Figure 4.29 shows an example of antimicrobial screening on the "V8" to

"V14" samples collected from fractionation of the hexane extract of *P. antillarum* towards *P. aeruginosa* using a 96-well plate.

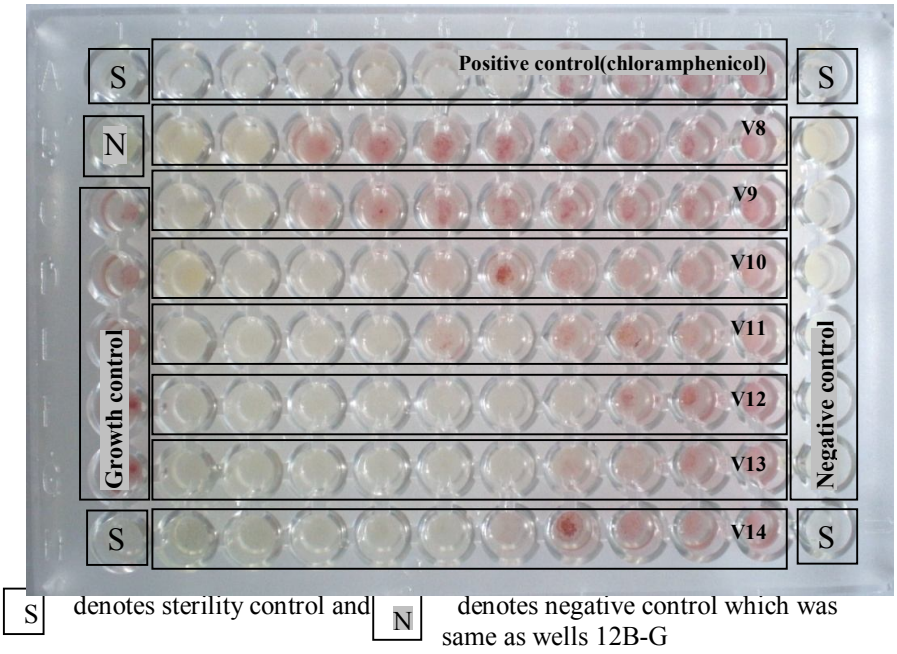

Figure 4.29: Inhibitory activity of "V8" to "V14" from the fractionation of the combined samples "V10" to "V17", excluding "V12" towards *P. aeruginosa* (ATCC 27853)

Table 4.18 summarizes the mean MIC values of "V1" to "V24" samples towards *P. aeruginosa.*

Table 4.18: Mean minimum inhibitory concentration of "V1" to "V24" towards *P. aeruginosa*

Combined samples	Mean minimum inhibitory concentration (n=2), mg/mL
V1	-
V2	2.50±0
V3	2.50±0
V4	2.50±0
V5	1.25±0
V6	1.25±0
V7	1.25±0
V8	1.88±0.88
V9	1.25±0
V10	0.23±0.11
V11	0.23±0.11
V12	0.059±0.03
V13	0.078±0
V14	0.078±0
V15	0.16±0
V16	0.12±0.06
V17	0.16±0
V18	0.63±0
V19	0.94±0.44
V20	0.63±0
V21	0.63±0
V22	1.25±0
V23	1.25±0
V24	1.25±0
Chloramphenicol (positive control)	0.004±0

"-" denotes no antimibrobial activity

"V12" (10.6 mg) exhibited the lowest mean MIC value at 0.059 mg/mL towards *P. aeruginosa*, indicating that the antibacterial compounds was the most abundant and purest in "V12". It was also confirmed by the TLC analysis. Samples "V10" to "V17", excluding "V12" also showed relatively low mean MIC values, ranging from 0.078 mg/mL to 0.23 mg/mL. It is possible that the same antibacterial compounds are also present in these samples at different

concentration. Samples “V10” to “V17”, excluding “V12” were combined and subjected to silica gel flash chromatography for further purification.

The combined “V10” to “V17”, excluding “V12” were purified using a hexane-ethyl acetate mobile phase of increasing polarity (95:5 to 7:3 volume ratios) and finally pure ethyl acetate. A total of fifty five fractions each with a volume of 100 mL were collected at a flow rate of 8.5 mL/min. The collected fractions were combined into eleven samples (“W1” to “W11”) after TLC analysis. Table 4.19 shows how the fractions were combined.

Table 4.19: Fifty five fractions which were collected from purification of “V10” to “V17”, excluding “V12” were combined into eleven samples

Combined samples	Collected fractions	Weights, mg
W1	1-2	52.0
W2	3-6	34.8
W3	7-8	4.9
W4	9-13	51.5
W5	14-20	207.2
W6	21-24	70.8
W7	25-38	103.3
W8	39-44	11.9
W9	45-49	10.9
W10	50-51	4.3
W11	52-55	60.0

Samples “W1” to “W11” were dissolved in methanol to a concentration of 4 mg/mL, after which they were subjected to antimicrobial assay. The mean MIC values of “W1” to “W11” are summarized in Table 4.20. “W5” and “W6” demonstrated the strongest inhibitory activity towards *P. aeruginosa* with a same mean MIC value of 0.063 mg/mL.

Table 4.20: Mean minimum inhibitory concentration of sample "W1" to "W11" towards *P. aeruginosa*

Combined samples	Mean inhibitory concentration (n=2), mg/mL
W1	1.00±0
W2	1.00±0
W3	0.50±0
W4	0.25±0
W5	0.063±0
W6	0.063±0
W7	0.13±0
W8	0.25±0
W9	0.25±0
W10	0.25±0
W11	0.25±0
Chloramphenicol	0.004±0

Figure 4.30 shows an example of 96-well plate which was used for antimicrobial screening on the "W1" to "W7" samples collected from the fractionation of the combined "V10" to "V17", excluding "V12" samples of *P. antillarum* towards *P. aeruginosa*.

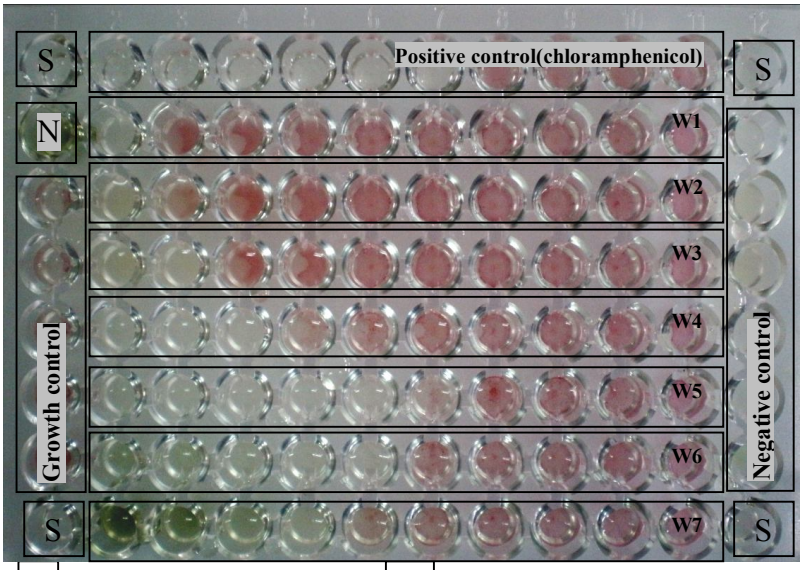

Figure 4.30: Inhibitory activity of "W1" to "W7" from the fractionatin of "V10" to "V17", excluding "V12" towards *P. aeruginosa* (ATCC 27853)

Based on the results of TLC, "V12" and "W5" contained similar compounds. "V12" and "W5" were combined to give a weight of 217.32 mg and further purification was performed. It is likely that "W6" (70.8 mg) also contained the same antibacterial compound as "V12" and "W5" samples. It was subjected to further purification.

Combined samples of "V12" and "W5" were subjected to C_{18} reversed phase flash chromatography in which the initial mobile phase used was pure acidified water, followed by increasing the methanol content in the mobile phase until it contained 100% methanol and finally eluted with 100% dichloromethane. Purification of "W6" also applied the same condition of purification. Four fractions with a volume of 50 mL were collected at a flow rate of 8.5 mL/min. Fractions "X1" to "X4" were collected from the

fractionation of combined samples of "V12" and "W5" whereas fractions "Y1" to "Y4" were collected from the fractionation of "W6". "X1" to "X4" fractions and "Y1" to "Y4" fractions were prepared at 1 mg/mL in methanol. Antimicrobial screening of fractions "X1" to "X4" and fractions "Y1" to "Y4" were performed using 96-well plates, as shown in Figure 4.31 and 4.32 respectively.

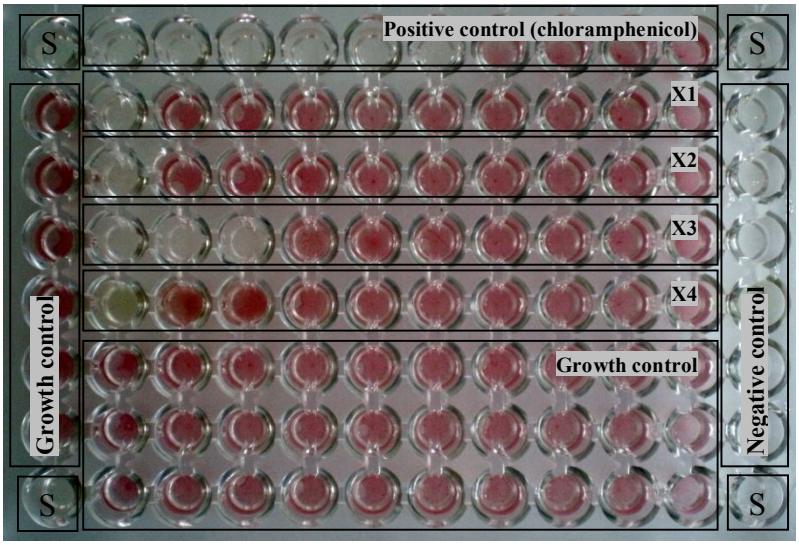

Figure 4.31: Inhibitory activities of "X1" to "X4" from the fractionation of the combined samples of "V12" and "W5" towards *P. aeruginosa* (ATCC 27853)

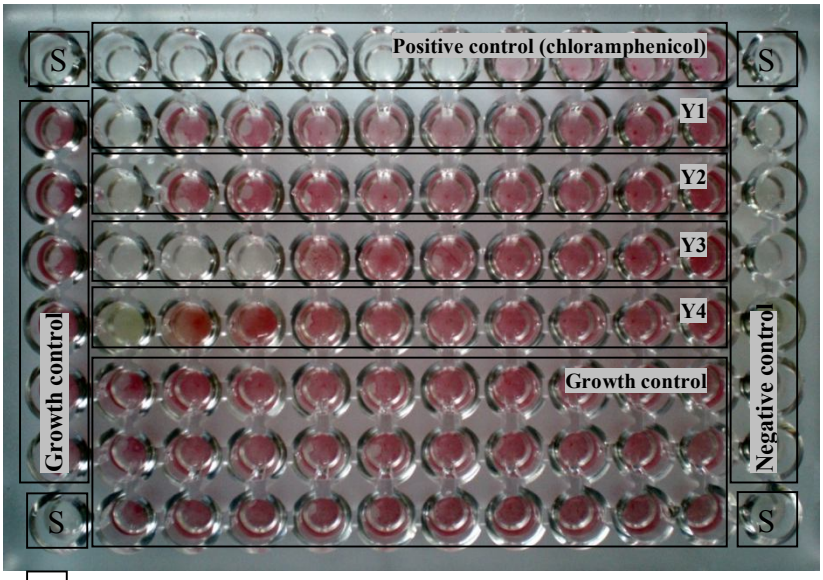

Figure 4.32: Inhibitory activities of "Y1" to "Y4" from the fractionation of the "W6" sample towards *P. aeruginosa* (ATCC 27853)

"X1" to "X4" and "Y1" to "Y4" fractions were subjected to antimicrobial assay and the results are summarized in Table 4.21 and Table 4.22 respectively.

Table 4.21: Mean minimum inhibitory concentration of the fractions "X1" to "X4" towards *P. aeruginosa*

Collected fractions	Mean minimum inhibitory concentration (n=2), mg/mL	Weights, mg
X1	0.25±0	1.2
X2	0.25±0	0.3
X3	0.063±0	18.8
X4	0.25±0	4.9
Chloramphenicol	0.004±0	N/A

"N/A" denotes no available

Table 4.22: Mean minimum inhibitory concentration of the fractions "Y1" to "Y4" towards *P. aeruginosa*

Collected fractions	Mean minimum inhibitory concentration (n=2), mg/mL	Weights, mg
Y1	0.25±0	0.4
Y2	0.25±0	1.0
Y3	0.063±0	28.3
Y4	0.25±0	1.2
Chloramphenicol	0.004±0	N/A

"N/A" denotes no available

"X3" (18.8 mg) and "Y3" (28.3 mg) had demonstrated the strongest growth inhibition towards *P. aeruginosa* among other fractions at a mean MIC value of 0.063 mg/mL. "X3" and "Y3" were combined after TLC was performed on both fractions. The fractions were purified using silica gel flash chromatography. A hexane-diethyl ether system was used as the mobile phase, beginning with a hexane-diethyl ether mixture of volume ratios 7:3, followed by increasing diethyl ether content in the mobile phase until it contained of 70% diethyl ether and ending with 100% diethyl ether. Thirty eight fractions each with a volume of 30 mL were collected at a flow rate of 8.5 mL/min. The fractions were pooled into six samples ("Z1" to "Z6") after TLC was performed. The combination of fractions is shown in Table 4.23.

Table 4.23: Combined samples of the fractions which were collected from purification of "X3" and "Y3"

Combined samples	Fractions collected	Weights, mg
Z1	1	3.7
Z2	2	4.5
Z3	3	1.5
Z4	4-11	28.6
Z5	12-26	2.1
Z6	27-38	6.2

Samples "Z1" to "Z6" (1 mg/mL) were prepared in methanol and subjected to antimicrobial assay. Table 4.24 summarizes the minimum inhibitory activity of sample "Z1" to "Z6". "Z4" (28.6 mg) exhibited the strongest inhibitory activity towards *P. aeruginosa* with a mean MIC value of 0.031 mg/mL, which is shown in Figure 4.33.

Table 4.24: Mean minimum inhibitory concentration of "Z1" to "Z6" towards *P. aeruginosa*

Combined samples	Mean minimum inhibitory concentration (n=2), mg/mL
Z1	-
Z2	-
Z3	2.50±0
Z4	0.031±0
Z5	2.50±0
Z6	-
Chloramphenicol	0.004±0

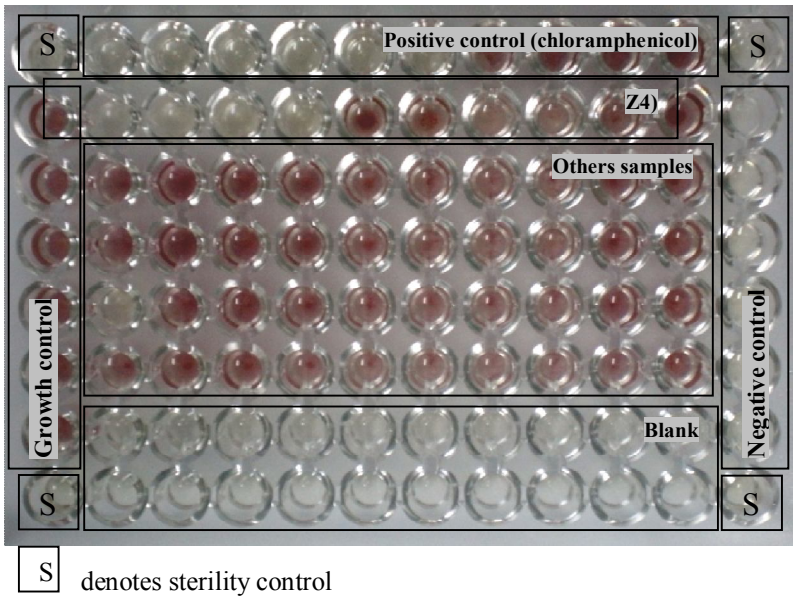

Figure 4.33: Inhibitory activity of "Z4" from the fractionation of combined samples "X3" and "Y3" towards *P. aeruginosa* (ATCC 27853)

4.10.5 Minimum Bactericidal Concentration (MBC) of "Z4"

The MBC of partially-purified "Z4" was determined. The MBC was defined as the lowest concentration that kills 99.9% of the bacteria (Nester *et al*., 2004). The MBC provide important quantitative information to assess the effectiveness of the antimicrobial agent. The MBC of "Z4" was 0.25 mg/mL in which 6 colonies of *P. aeruginosa* were observed on MHA, as shown in Figure 4.34. However, its amount was insufficient for further purification. It was subjected to HPLC to determine the purity of "Z4".

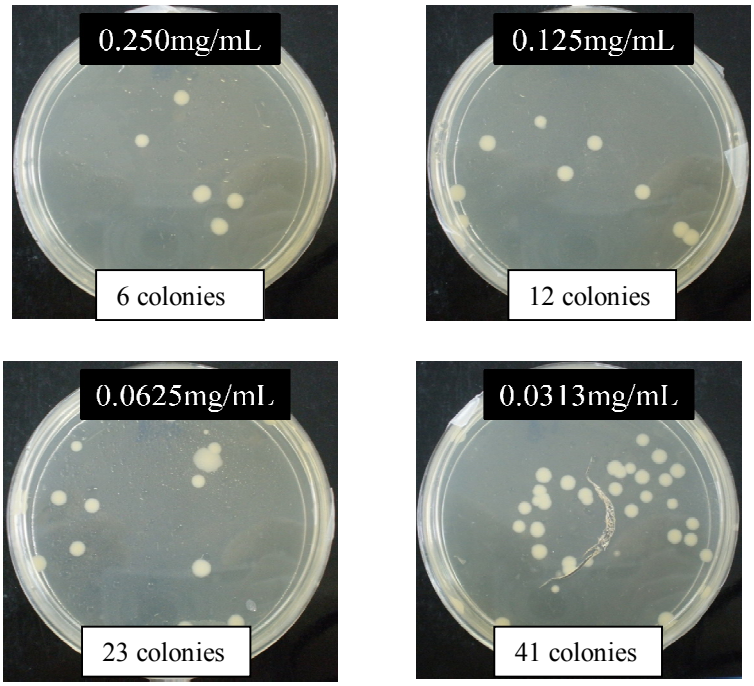

Figure 4.34: The minimum bactericidal concentration of "Z4" towards *P. aeruginosa*

4.10.6 High Performance Liquid Chromatography of "Z4"

The purity of "Z4" was analyzed using high performance liquid chromatography (HPLC). An aliquot of the filtrate (20 µL) was injected into the HPLC column (Merck LiChoroCART 75-4 C_{18} column) and eluted with a linear gradient of mobile phase containing solvent A (0.1% formic acid) and solvent B (methanol). The solvent gradient was programmed from 0% to 100% solvent B in 30 min with a flow rate of 1 mL/ min. Nine peaks were observed and the highest peak was found at retention time of 4.18 min, as shown in Figure 4.35.

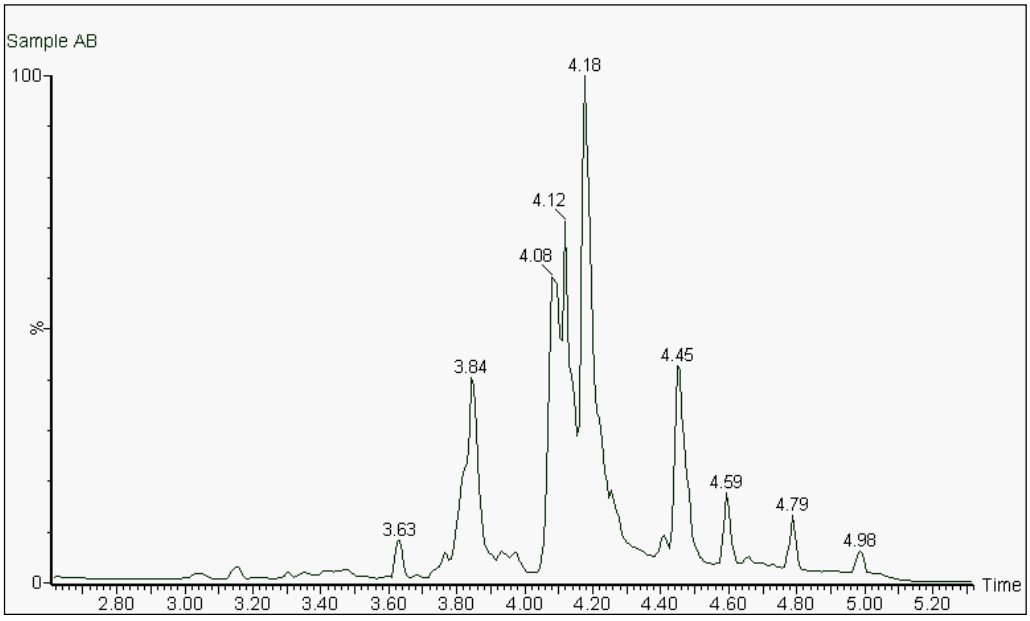

Figure 4.35: HPLC chromatogram of the partially purified "Z4" which was isolated from *P. antillarum*

4.10.7 Gas Chromatography-Mass Spectrometry of "Z4"

Partially-purified "Z4" was subjected to GC-MS analysis. A major peak with a retention time of 16.556 min was obtained (Figure 4.36). The mass spectrum of this component is shown in Figure 4.37 and it was identified by the MS library (NIST 05) with a percentage similarity of 96% as diisooctyl phthalate with a molecular ion of 390 m/z. Diisooctyl phthalate has a molecular weight of 390.56 g/mol. The structure of diisooctyl phthalate is shown in Figure 4.38.

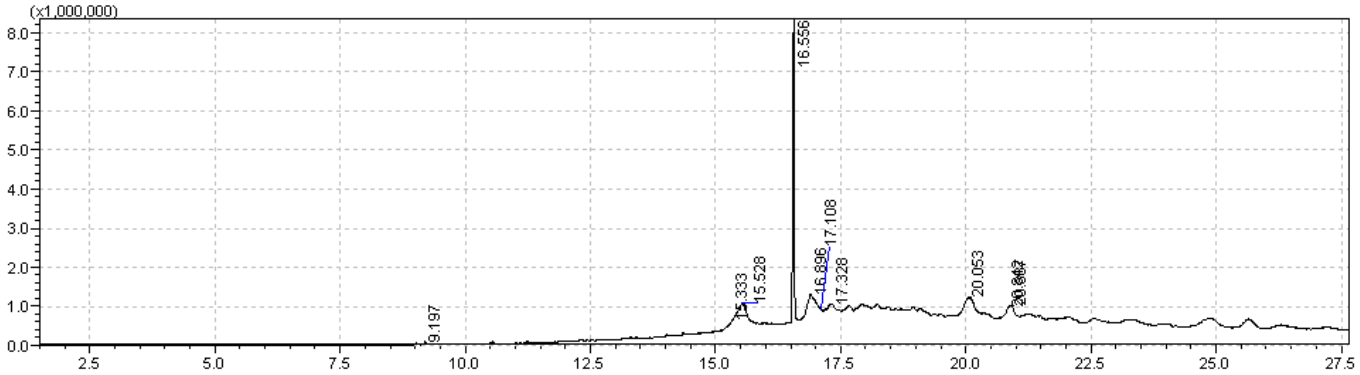

Figure 4.36: Gas chromatography of "Z4"

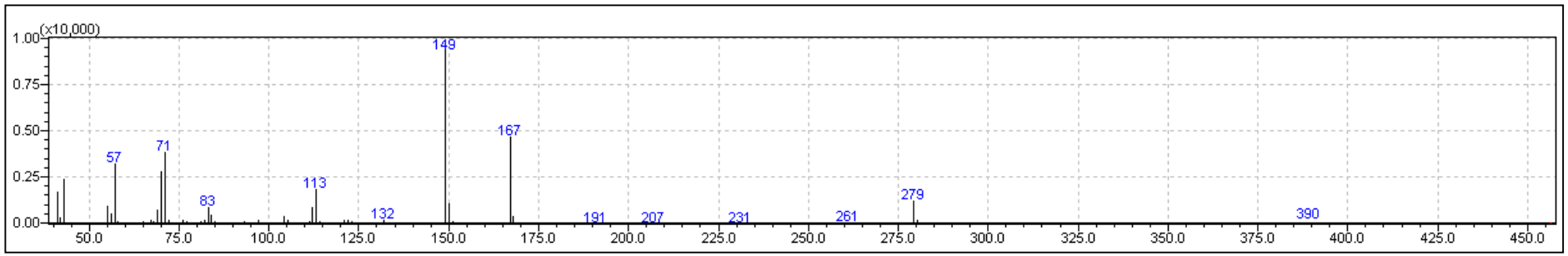

Figure 4.37: Mass spectrum of the component in the GC peak of retention time of 16.552 min, which was identified as diisooctyl phthalate by the MS library

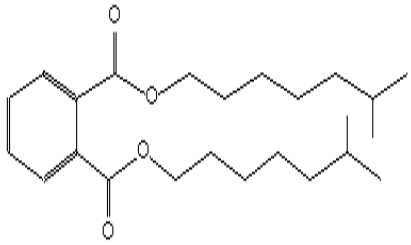

Figure 4.38: Structure of Diisooctyl phthalate

4.10.8 The Occurrence of Diisooctyl Phthalate in Seaweeds

Diisooctyl phthalate was detected in *P. antillarum* using GC-MS. Diisooctyl phthalate is a phthalate ester. Many findings of phthalate esters have been reported (Chen, 2004). For example, di-(2-ethylhexyl) phthalate (DEHP) has been isolated from terrestrial and marine organisms including plants, marine algae, fungal and bacterial culture broths (Chen, 2004). However, it is difficult to determine whether DEPH was produced by these organisms, the sample was contaminated during the separation process, or DEPH had accumulated in the organisms, because DEPH has been used abundantly in petrochemical phthalate ester (Gezgin *et al.*, 2001). It is not clear whether the isolated diisooctyl phthalate is a pollutant or a compound that naturally occurs in *P. antillarum* (Chen, 2004).

Although the effects of phthalate esters on biological systems have been known for more than 40 years, the biogenic origin has not been proven (Chen, 2004). Roles of phthalate esters are still under investigation. It was suggested

that phthalate esters might be stored in the cell membrane and help to maintain the flexibility of algal cells (Chen, 2004).

Chen (2004) investigated the origin of phthalate esters in seaweeds, proving that the red algae *Bangia atropuspurea* can synthesize DEPH and di-n-butyl phthalate (DBP) *de novo*. *B. atropurpurea*, *Porphyra angusta* and *Porphyra dentate* which were cultured under laboratory conditions showed different DEPH and DBP contents (Chen, 2004). Namikoshi *et al*. (2006) found that DBP was naturally synthesized by a green alga, *Ulva* sp. and two edible brown algae, *Undaria pinnatifida* and *Laminaria japonica*.

4.11 Summary

P. antillarum exhibited antioxidant and antimicrobial activities. The antioxidant responsible for the antioxidant activity is phloroglucinol. It is the first report to discover the occurrence of phloroglucinol in *P. antillarum*. Further investigation should be carried out to identify and characterized the antimicrobial compound. It is evident from the present study that *P. antillarum* could be utilized as a good natural source of antioxidant and a possible food supplement or as an antimicrobial agent in pharmaceutical industry.

4.12 Suggestions for Further Works

The isolated antioxidant from *P. antillarum*, phloroglucinol, is not as potent as standard antioxidants. Chemical modification of phloroglucinol should be carried out in order to obtain a new compound with higher potency. Collection of *P. antillarum* at different locations should be performed to assess the effects of environment towards the antioxidant activity of phloroglucinol.

Further isolation of antimicrobial compound should be carried out to elucidate the structure of the antimicrobial compound. Characterization of the isolated antimicrobial compound should be studied.

Toxicity of *P. antillarum* to normal human cell line (e.g. liver cell, lung cell, kidney cell etc) should be studied as *P. antillarum* could become a supplementary food which contain of antioxidant and antimicrobial compounds.

CHAPTER 5

CONCLUSION

The antioxidant compound that was isolated from ethyl acetate extract of *Padina antillarum* is phloroglucinol (1, 3, 5-trihydroxybenzene). Phloroglucinol exhibited relatively high radical scavenging activity and ferric reducing power, however, its antioxidant activity was lower than that of the well-characterized antioxidants of plant origin, quercetin, tannin acid and ascorbic acid. Phloroglucinol was also found to be a weak secondary antioxidant due to its low ferrous ion chelating activity. It did not appear to possess anticancer and antifungal activities. Phloroglucinol did not appear to kill leukemia cells and five types of fungi (*Aspergillus niger*, ATCC 16404; *Trichophyton mentagrophytes*, ATCC 9533; *Candida albicans*, ATCC 90028; *Candida parapsilosis*, ATCC 22019; and *Cryptococcus neoformans*, ATCC 90112).

The hexane extract of *P. antillarum* exhibited antimicrobial compounds towards *P. aeruginosa*, *K. pneumoniae* and *B. cereus*. Due to insufficient amount of the extract available, bioassay-guided fractionation was not performed. Instead, a partially-purified extract was tested on *P. aeruginosa*. The partially-purified antimicrobial compound which demonstrated inhibitory activity towards *P. aeruginosa* was subjected to GC-MS and it was identified as diisooctyl phthalate.

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Appendix A

Average absorbance values of gallic acid after reaction with Folin Ciocalteu's reagent

Table A: Average absorbance values of gallic acid after reaction with Folin Ciocalteu's reagent

Concentration of gallic acid (mg/L)	Average of absorbance, n=3	Standard deviation
0.0	0.013	0.001
5.0	0.069	0.010
10.0	0.135	0.010
20.0	0.255	0.004
40.0	0.490	0.029
60.0	0.746	0.009
80.0	0.952	0.021
100.0	1.180	0.015

Appendix B

An example of calculation to obtain TPC value

An example of a calculation for this assay is shown below. The hexane extract which was collected on 29th December 2005 was analyzed.

$$\text{Average of absorbance} = \frac{\text{Absorbance 1 + Absorbance 2 + Absorbance 3}}{3}$$

$$= \frac{0.072 + 0.076 + 0.069}{3}$$

$$= 0.072$$

Average of absorbance value was multiplied by the dilution factor.

$0.072 \times 1 = 0.072$

The value that was obtained (i.e. 0.072) was substituted for "y" in the equation.

$y = 0.012x$

$0.072 = 0.012x$

$$x = \frac{0.072}{0.012}$$

$= 6.0$ mg/L

Thus in 1 L of sample, there was 6.0 mg of gallic acid, and hence in 100 mL of hexane extract, there was 0.6 mg of gallic acid. Assuming that 6.0 mg of gallic acid was contained in 5 g of powdered *P. antillarum* which were used for extraction.

Therefore, in 100 g of powdered sample there is $\frac{0.6 \text{ mg} \times 100}{5}$

$= 12.0$ mg GAE/ g of sample

The TPC is expressed as 100 g of powdered *P. antillarum* contained an equivalent of 12.0 mg of gallic acid.

Appendix C

The TPC of five batches of extracts

Table B: Determination of TPC on five batches of *P. antillarum*

Extractants	Average of absorbance, n=3	Dilution factor	Average x dilution factor	TPC (mg GAE/g of sample)
Date of sample collection: 29/12/05				
Hexane	0.072	1	0.072	12.00
Ethyl acetate	0.457	1	0.457	76.17
100% methanol	0.562	2	1.124	187.33
50% methanol	0.581	4	2.324	387.33
Date of sample collection: 16/03/06				
Hexane	0.117	1	0.117	19.50
Ethyl acetate	0.479	1	0.479	79.83
100% methanol	0.675	2	1.350	225.00
50% methanol	0.712	4	2.848	474.67
Date of sample collection: 27/11/07				
Hexane	0.136	1	0.136	22.67
Ethyl acetate	0.456	1	0.456	76.00
100% methanol	0.696	2	1.392	232.00
50% methanol	0.727	4	2.908	484.67
Date of sample collection: 13/05/08				
Hexane	0.166	1	0.166	27.67
Ethyl acetate	0.470	1	0.470	78.33
100% methanol	0.601	2	1.202	200.33
50% methanol	0.681	4	2.724	454.00
Date of sample collection: 28/04/09				
Hexane	0.048	1	0.048	8.00
Ethyl acetate	0.286	1	0.286	47.67
100% methanol	0.552	2	1.104	184.00
50% methanol	0.777	4	3.108	518.00

Appendix D

The percentage of radical scavenging activities of five batches of extracts

Table C: Determination of radical scavenging activities of extracts from *P. antillarum* that were collected on five different dates

Extractants	Average of absorbance	% of radical scavenging activities
Date of sample collection: 29/12/05		
Hexane	0.921	20.50
Ethyl acetate	0.735	36.53
100% methanol	0.409	64.71
50% methanol	0.130	88.80
Blank	1.158	Not available
Date of sample collection: 16/03/06		
Hexane	0.880	24.04
Ethyl acetate	0.672	42.01
100% methanol	0.386	66.70
50% methanol	0.210	81.87
Blank	1.158	Not available
Date of sample collection: 27/11/07		
Hexane	0.965	18.93
Ethyl acetate	0.757	36.45
100% methanol	0.450	62.20
50% methanol	0.234	80.37
Blank	1.190	Not available
Date of sample collection: 13/05/08		
Hexane	0.955	25.54
Ethyl acetate	0.876	31.76
100% methanol	0.649	49.42
50% methanol	0.208	83.76
Blank	1.283	Not available
Date of sample collection: 28/04/09		
Hexane	0.893	6.23
Ethyl acetate	0.803	15.71
100% methanol	0.298	68.72
50% methanol	0.098	89.75
Blank	0.953	Not available

Appendix E

An example of calculation to determine the percentage of radical scavenging activity

Percentage of radical scavenging activity was measured using the following formula:

$$\% \text{ radical scavenging activity} = \frac{(A_{Blank} - A_{sample})}{A_{Blank}} \times 100\%$$

An example of a calculation for this assay is shown below. Hexane extract of *P. antillarum* that were collected on 29th December 2005 was calculated to have 20.50% of radical scavenging activity.

$$\% \text{ DPPH radical scavenging activity} = \frac{(1.158 - 0.921)}{1.158} \times 100\%$$

$$= 20.50\%$$

Appendix F

Average of TPC and radical scavenging activities of five batches of extracts

Table D: Average of TPC and average radical scavenging activities of five batches of *P. antillarum* extracts

Extract	Average TPC of 5 batches of extracts	Average percentage of radical scavenging activity of 5 batches of extracts
Hexane	17.97	19.05
Ethyl acetate	71.60	32.49
100% methanol	205.73	62.35
50% methanol	463.73	84.91

Appendix G

The TPC of the fractions from fractionation of the ethyl acetate extract

Table E: The TPC of the fractions from the fractionation of ethyl acetate extract

Fractions no.	Average of absorbance	Standard deviation (n=3)	Combined fractions
1	0.031	0.004	A1
2	0.071	0.008	
3	0.060	0.013	
4	0.063	0.002	
5	0.059	0.008	
6	0.110	0.083	
7	0.061	0.008	
8	0.069	0.005	
9	0.074	0.005	
10	0.071	0.077	
11	0.039	0.021	
12	0.033	0.006	
13	0.040	0.010	
14	0.076	0.013	
15	0.145	0.032	
16	0.190	0.015	
17	0.140	0.027	
18	0.177	0.009	
19	0.101	0.008	
20	0.035	0.007	
21	0.042	0.019	
22	0.043	0.016	
23	0.044	0.013	
24	0.055	0.039	
25	1.014	0.047	A2
26	2.069	0.027	
27	3.000	0.000	
28	3.000	0.000	
29	2.787	0.165	
30	2.285	0.137	
31	1.543	0.056	
32	1.391	0.167	
33	0.886	0.013	
34	0.867	0.018	
35	0.764	0.001	
36	0.279	0.042	A3
37	0.303	0.052	

Table E continued: The TPC of the fractions from the fractionation of ethyl acetate extract

Fractions no.	Average of absorbance	Standard deviation (n=3)	Combined fractions
38	0.179	0.036	A3
39	0.178	0.034	
40	0.155	0.023	
41	0.142	0.010	
42	0.147	0.022	
43	0.199	0.056	
44	0.317	0.125	
45	0.149	0.020	
46	0.280	0.001	
47	0.045	0.006	
48	0.074	0.030	
49	0.049	0.019	
50	0.571	0.020	A4
51	0.534	0.010	
52	0.574	0.015	
53	0.537	0.023	
54	0.419	0.004	
55	0.480	0.017	
56	0.385	0.023	
57	0.356	0.024	
58	0.364	0.015	
59	0.285	0.027	
60	0.351	0.012	
61	0.331	0.014	
62	0.646	0.021	
63	0.786	0.027	
64	0.538	0.014	
65	0.395	0.109	
66	0.435	0.009	
67	0.391	0.007	
68	0.345	0.027	
69	0.320	0.020	
70	0.280	0.020	
71	0.333	0.024	
72	0.465	0.030	
73	0.423	0.034	
74	0.433	0.027	
75	0.342	0.004	
76	0.317	0.018	
77	0.267	0.025	
78	0.285	0.012	
79	0.230	0.008	
80	0.060	0.026	A5
81	0.070	0.023	

Table E continued: The TPC of the fractions from the fractionation of ethyl acetate extract

Fractions no.	Average of absorbance	Standard deviation (n=3)	Combined fractions
82	0.060	0.022	A5
83	0.049	0.008	
84	0.127	0.076	
85	0.041	0.004	
86	0.030	0.003	
87	0.030	0.007	
88	0.032	0.001	
89	0.028	0.007	
90	0.035	0.001	

denotes an active fraction

Appendix H

The percentages of radical scavenging activities of the fractions from chromatography of ethyl acetate extract

Table F: The percentage of radical scavenging activities of the fractions from the fractionation of ethyl acetate extract

Fractions no.	Average % of radical scavenging activities	Standard deviation (n=3)	Combined fractions
1	38.82	6.743	A1
2	39.68	2.754	
3	39.97	1.721	
4	40.79	2.230	
5	41.82	7.862	
6	38.25	5.634	
7	38.61	5.850	
8	38.03	6.100	
9	40.00	7.002	
10	42.61	3.914	
11	42.60	3.914	
12	44.34	4.775	
13	40.32	5.974	
14	40.75	5.610	
15	40.01	3.808	
16	41.37	3.396	
17	41.72	5.513	
18	38.31	4.020	
19	42.31	7.203	
20	39.19	4.792	
21	39.29	5.145	
22	43.02	4.042	
23	45.08	5.282	
24	48.90	10.015	
25	67.16	6.787	A2
26	70.04	8.754	
27	84.21	12.013	
28	89.43	3.959	
29	85.95	2.124	
30	78.95	5.349	
31	79.37	1.214	
32	73.32	3.473	
33	71.87	1.598	
34	68.03	2.940	
35	62.84	5.186	
36	46.97	12.119	A3
37	41.65	9.924	

Table F continued: The percentage of radical scavenging activities of the fractions from the fractionation of ethyl acetate extract

Fractions no.	Average % of radical scavenging activities	Standard deviation (n=3)	Combined fractions
38	42.43	9.863	A3
39	43.05	8.339	
40	40.44	5.845	
41	40.10	6.488	
42	41.36	6.945	
43	42.92	4.164	
44	43.22	3.987	
45	41.19	6.377	
46	39.66	6.533	
47	39.47	4.654	
48	41.22	8.555	
49	41.75	7.811	
50	53.40	3.499	A4
51	52.97	3.085	
52	51.28	1.216	
53	50.21	2.743	
54	51.87	2.730	
55	51.26	4.106	
56	51.55	3.802	
57	54.44	3.915	
58	56.14	5.044	
59	55.15	5.895	
60	54.39	4.524	
61	54.87	2.549	
62	57.60	2.457	
63	61.79	5.492	
64	59.98	8.052	
65	58.58	2.432	
66	53.10	8.525	
67	49.17	8.888	
68	48.40	9.046	
69	48.18	7.285	
70	50.42	3.264	
71	50.26	1.907	
72	48.07	5.168	
73	50.40	1.791	
74	49.61	2.002	
75	46.39	6.418	
76	47.71	5.087	
77	45.73	3.202	
78	43.57	4.922	
79	41.94	3.531	
80	32.24	4.636	A5
81	28.90	4.831	
82	24.95	2.701	

Table F continued: The percentage of radical scavenging activities of the fractions from the fractionation of ethyl acetate extract

Fractions no.	Average % of radical scavenging activities	Standard deviation (n=3)	Combined fractions
83	22.90	4.875	A5
84	23.79	5.080	
85	25.27	1.988	
86	28.06	1.814	
87	28.08	3.762	
88	28.54	2.031	
89	29.72	5.080	
90	30.76	2.365	

denotes an active fraction

Appendix I

The TPC of the fractions from the fractionation of "A2"

Table G: The TPC of the fractions from the fractionation of "A2"

Fractions no.	Average absorbance	Standard deviation	Combined fractions
1	0.286	0.003	B1
2	0.301	0.003	
3	0.430	0.009	
4	2.748	0.110	B2
5	3.000	0.000	
6	3.000	0.000	
7	3.000	0.000	
8	2.964	0.017	
9	2.845	0.008	
10	2.584	0.010	
11	0.546	0.016	B3
12	0.488	0.009	
13	0.448	0.012	
14	0.355	0.022	
15	0.174	0.009	
16	0.302	0.002	

denotes an active fraction

Appendix J

The percentages of radical scavenging activities of fractions from the fractionation of "A2"

Table H: The percentages of radical scavenging activities of fractions from the fractionation of "A2"

Fractions no.	Average radical scavenging activities (%)	Standard deviation (n=3)	Combined fractions
1	29.11	0.008	B1
2	30.39	0.006	
3	39.42	0.007	
4	75.87	0.015	B2
5	88.39	0.010	
6	90.97	0.005	
7	78.08	0.012	
8	78.05	0.007	
9	77.29	0.018	
10	71.31	0.014	
11	42.74	0.010	B3
12	36.11	0.121	
13	40.25	0.009	
14	39.51	0.010	
15	38.69	0.011	
16	40.05	0.012	

denotes an active fraction

Appendix K

The TPC of fractions from the fractionation of "B2"

Table I: The TPC of fractions from the fractionation of "B2"

No. of fractions	Average absorbance	Standard deviation	Combined fractions
1	0.953	0.023	C1
2	2.520	0.161	(Pure
3	3.000	0.000	compound)
4	3.000	0.000	
5	3.000	0.000	
6	2.883	0.011	
7	2.486	0.012	
8	0.781	0.003	C2
9	0.851	0.020	
10	0.927	0.011	

denotes an active fraction

Appendix L

The percentages of radical scavenging activities of fractions from the fractionation of "B2"

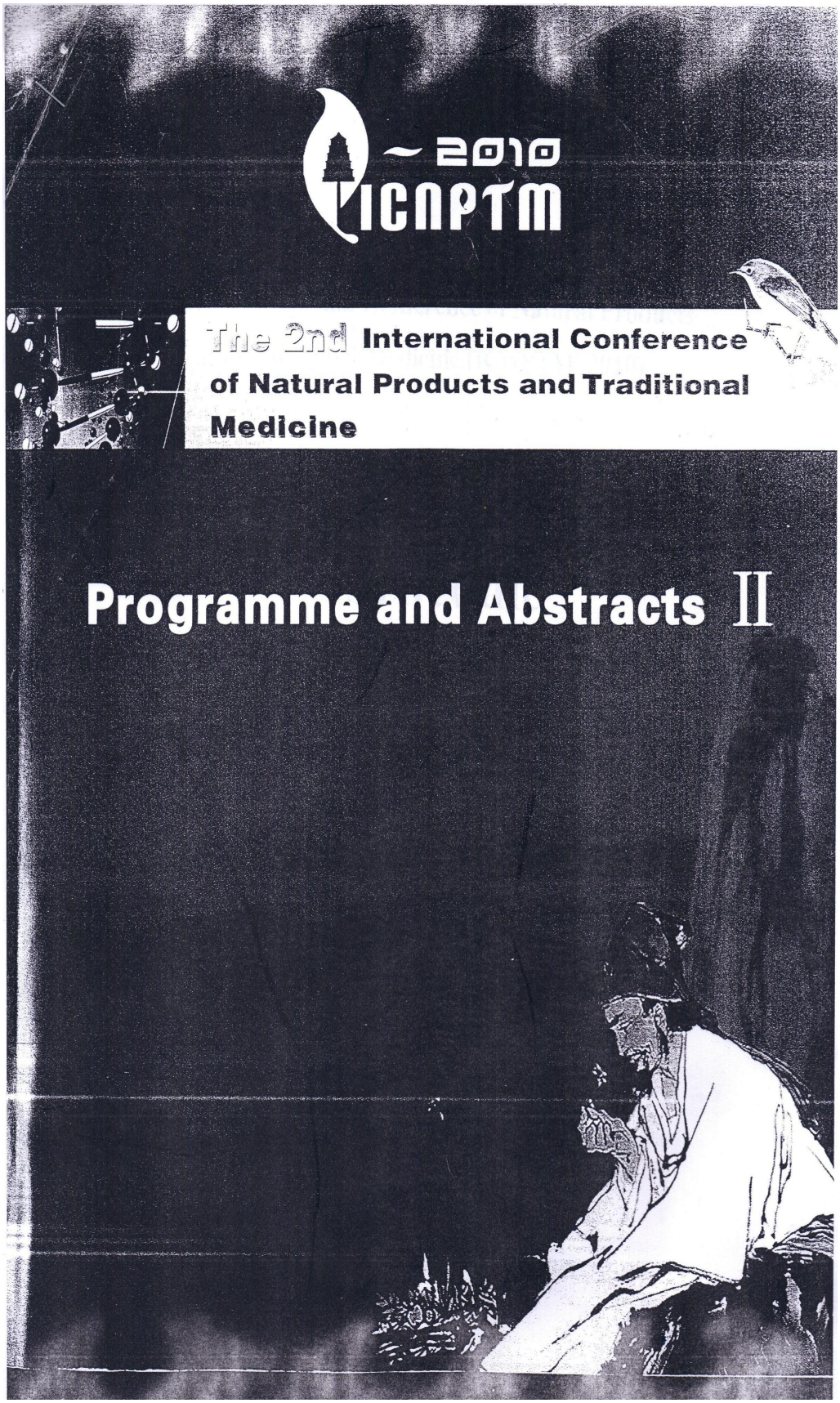
Table J: The percentages of radical scavenging activities of fractions from the fractionation of "B2"

No. of fractions	Average percentage of radical scavenging activities	Standard deviation	Combined fractions
1	52.16	0.641	C1 (Pure compound)
2	79.46	0.627	
3	90.74	0.248	
4	89.55	0.166	
5	86.72	0.561	
6	75.56	0.445	
7	65.62	0.942	
8	29.86	1.278	C2
9	24.26	1.195	
10	16.11	0.490	

denotes an active fraction

Appendix M

Purification and characterization of phloroglucinol which was isolated from *P. antillarum* had published in the proceeding of the 2nd International Conference of Natural Products and Traditional Medicine


~2010
ICNPTM
The 2nd International Conference of Natural Products and Traditional Medicine
Programme and Abstracts II

The 2nd International Conference of Natural Products and Traditional Medicine (ICNPTM' 2010)

Schedule

Hosting Organizations : Northwest University

Xi'an National Bio-pharmaceutical Incubator

Operation Organizations : The College of Life Sciences, Northwest University

Support Organizations : The Scientific Technical Development Group

eBioCenter Corporation

Yue Ji Hall Floor 2 月季厅

13:30-16:00 OS2: Natural Products Isolation and Bioactivity

Session Chairs:

Professor Hui Xu, Northwest A & F University

Dr KHOO, KS, *Department of Chemical Science and Department of Biomedical Science, Faculty of Science, Universiti Tunku Abdul Rahman, Malaysia*

1. Recent Advance of Podophyllotoxin Derivatives as Insecticidal Agents
   Professor Hui Xu, *Northwest A & F University, China(13:30-14:10)*

2. Purification and characterization of phloroglucinol, an antioxidant from the marine algae *Padina antillarum*
   Dr KHOO, KS, *Department of Chemical Science and Department of Biomedical Science, Faculty of Science, Universiti Tunku Abdul Rahman, Malaysia（14:10-14:40）*

3. Biotransformation of some important natural products and their bioactivity
   Prof. Jin-Ming Gao， *Research Centre for Natural Medicinal Chemistry，College of Science Northwest A & F University, China（14:40-15:20）*

4. Structure Elucidation and Antitumor Activities of Anthocyanins from Berry Fruits as Traditional Chinese Medicine.
   Prof. Qin Chuan-guang， *Faculty of Life Science, Northwestern Polytechnical University, Xi'an,. China（15:20-16:00）*

16:00-16:15 Coffee Break

16:15-18:30 OS4: Pharmacology and Biosynthesis of Natural Products

Session Chairs:

Professor Li Huang, Surgery Department, Duke University Medical Center

Professor Gao Wenyun, Northwest University, Xian China

1. I. Studies on the MEP pathway for the biosynthesis of terpenoids : synthesis of the key intermediates DX and DXP II. Crosstalk of the MVA and MEP pathways in higher plants
   Prof. Gao Wenyun, *Northwest University, Xi'an, China（16:15-16:45）*

2. Anti-HIV leads from natural products
   Prof. Li Huang, *Surgery Department, Duke University Medical Center, Durham, NC.（16:45-17:15）*

3. In vitro antibacterial activities of some local plants in Malaysia
   Dr Sit Nam Weng, *Faculty of Science, Universiti Tunku Abdul Rahman, Jalan Universiti, Bandar Barat, Malaysia (17:15-17:45)*

4. A Post-genomics Strategy for Drug Discovery and Design by Charactering Protein-Small Molecule Interactions
   Dr Jianxing Song, *Department of Biological Sciences, Faculty of Science, Department of Biochemistry, Yong Loo Lin School of Medicine and National University of Singapore (17:45-18:15)*

Purification and characterization of phloroglucinol, an antioxidant from the marine algae *Padina antillarum*

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Abstract: The antioxidant activity of extracts of the brown marine algae *Padina antillarum* was studied. A 50% aqueous methanol extract of powdered *P. antillarum* was subjected to liquid-liquid partitioning. The three extracts (chloroform, ethyl acetate and water) which were obtained from liquid-liquid portioning were subjected to total phenolic content and radical scavenging activity assays. The ethyl acetate extract showed the highest antioxidant properties, and was subjected to bioassay-guided fractionation. The ethyl acetate extract was purified using three stages of column chromatography, i.e. silica gel flash chromatography C_{18} flash chromatography and finally silica gel chromography. The purity of the purified component was assessed by HPLC, and structural elucidatiion was carried out using ^{1}H-nuclear magnetic resonance (^{1}H-NMR), ^{13}C-nuclear magnetic resonance (^{13}C-NMR), and gas chromatography-mass spectrometry (GC-MS) which revealed that the antioxidant compound is phloroglucinol (1,3,5-trihydroxybenzene). The antioxidant activity of phloroglucinol was compared to that of well-known antioxidants of plant origin, i.e. tannic acid, quercetin and ascorbic acid, which showed that its antioxidant activity is weaker than that of these compounds.

Keywords: marine algae, antioxidant, phloroglucinol, *Padina*

I. INTRODUCTION

The search for medicinal compounds has been a major endeavour in natural products chemistry. Plants have served as a major source of useful natural products. However, in recent decades, researchers have focused their research on marine natural products [1]. Marine natural products encompass a wide variety of chemical classes, including terpenes, shikimates, polyketides, acetogenins, peptides, alkaloids of varying structures and a multitude of compounds of widely different biosyntheses.

Seaweeds are a rich source of bioactive compounds. For this research, the antioxidant activities of extracts of the brown algae *Padina antillarum* was investigated. This species belongs to the family *Dictyotaceae* and is commonly found in intertidal zones, where it may often be exposed during low tides [1]. Its thalli is separated into segments of flat blades which are rolled into circles, creating a fan-like shape (Fig.1). The surface may be calcinated. *P. antillarum* is not widely used for medicinal purposes or as food, although in some Pacific islands it is used as a food seasoning in the dried flake form [2]

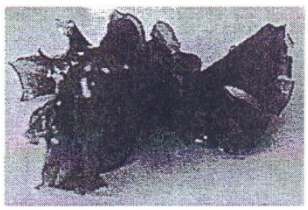


Figure 1: *Padina antillarum*

Extracts of *P. antillarum* have been demonstrated to have antibacterial (3), nematicidal (4), ichthyotoxicity (5) and antioxidant (6) activities. However, there is a scarcity of information regarding molecular structures of the bioactive compounds. This research focussed on isolating and characterizing antioxidant compounds from this seaweed.

II. METHODOLOGY

1. Chemicals and reagents

1,1-diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma Aldirch, and Folin-Ciocalteu's reagent was purchased from Fluka. Organic solvents were obtained from Merck. A VersaFlash Supelco chromatography system (which included a column holder, peristaltic pump and pre-packed chromatography columns), silica

cartridges of dimensions 40 X 100 mm and C_{18} cartridges of dimensions 40 X 75 mm were supplied by Supelco.

2. Extraction and evauation of antioxidant compounds

P. antillarum was collected from Teluk Kumang at Port Dickson, Malaysia. Upon collection, it was quickly rinsed with distilled water to remove sand, soil and debris. The samples were freeze-dried, then ground in liquid nitrogen. Extraction was performed on the powdered material. 5g of powdered material was used for each extract, and the first extraction solvent that was used for 50 ml of 50% aqueous methanol which is often used for the extraction of antioxidant compounds. Liquid-liquid partioning was performed on the 50% aqueous methanol extract using an equal volume of chloroform, followed partitioning using ethyl acetate. The extracts that were obtained from the liquid-liquid partitioning were dried using a vacuum concentrator.

2.1 *Total phenolic content and antioxidant activity assay*

The total phenolic content (TPC) and antioxidant activities (AOA) of the extracts were determined. The TPC was measured using Folin Ciocalteu's reagent [7]. Folin Ciocalteu's phenol reagent (1.5 ml) and 7.5% w/v Na_2CO_3 (1.2 ml) were added to 0.3 ml of each extract and the reaction mixture was incubated in the dark for 30 min. The absorbance of the reaction mixture was then measured at 765 nm. TPC was expressed in terms of mg gallic acid equivalents (GAE)/100 g dried samples. 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging was used to quantitate the antioxidant activity. The DPPH free-radical scavenging assay was carried out in triplicate, based on the methods described in [8] and [9] . Two ml of 0.15 mM DPPH was added to different dilutions of the extract (amounting to 1 ml) and the reaction mixture was incubated for 30 min after which ts absorbance was measured at 517 nm. The radical scavenging activity of the extract was calculated based on its concentration of extract/ standard antioxidant required to reduce DPPH radical concentration by 50% (IC_{50}).

2.2 *Ferrous ion-chelating (FIC) assay*

The FIC assay was conducted based on the method described in [9]. 1mL of 0.1mM iron (II) sulphate was added to extracts with different dilution respectively, followed by addition of 1mL of ferrozine solution. The reaction mixtures were allowed to react for 10min in the dark at room temperature after which the absorbance were measure at 562nm. The test was performed in triplicate. The results were expressed in percentageof chelating ability using the following equation:

Chelating ability (%) = $[A_{Blank} - A_{Sample}] / A_{Blank}$

3. Isolation of antioxidant compound by flash chromatography

Activity-guided fractionation was performed on the active extract. The ethyl acetate extract had the highest antioxidant activity, and was subjected to silica gel flash chromatography. A chloroform-acetone system was used as the mobile phase, beginning with 100% chloroform and ending with a chloroform/acetone mixture of ratio 1:4. 90 fractions with volumes of approximately 50 ml were collected at a flow rate of 8.5mL per min. The fractions that were collected by flash chromatography were subjected to antioxidant assays for tracking the occurrence of the antioxidants. The active fractions were further purified using C_{18} flash chromatography in which the initial mobile phase used was pure acidified water, after which the methanol content of the mobile phase was increased until it contained 100% methanol. A total of 16 fractions each with a volume of approximately 50 ml were collected at a flow rate of approximately 8.5mL/min. The fractions were a screened for antioxidant activity. Finally, the purity of the compounds isolated was analyzed using a Shimatzu high performance liquid chromatography (HPLC) (Model PRC 1019) fitted with a Merck LiChoroCART 75-4 C_{18} column, a Shimatzu gas chromatography-mass spectroscopy (GC-MS) (Model QP2010 Plus),. ^{1}H- nuclear magnetic resonance (NMR) and ^{13}C NMR spectroscopy were performed using a JOEL, Japan, ECA-400 (400 MHz) NMR spectrometer for structural elucidation.

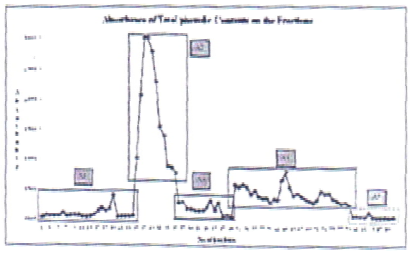
III. . RESULTS AND DISCUSSION

The total phenolic contents and radical scavenging activities of the extracts obtained by liquid- liquid partitioning are shown in Fig. 1. The chloroform extract gave a low TPC and radical scavenging activity, while the values obtained for the aqueous and ethyl acetate extracts were much higher. As the ethyl acetate extract gave the highest reading, it was selected for fraectionation by silica gel chromatography.

Fig. 1: Table showing the total phenolic content and radical-scavenging activity of the three extracts obtained by liquid-liquid paqrtioning.

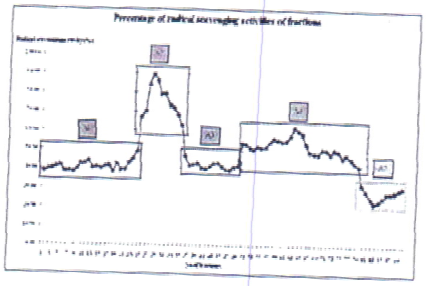
Extracts (1mg/mL)	TPC (mg GAE/ g)	Radical scavenging activities, %
Chloroform extract	6.25	2.82
Ethyl acetate extract	156.	84.1
Aqueous extract	40.7	39.4

Figure 2: Silica gel chromatography of the ethyl acetate extract. The fractions were monitored for Total Phenolic Content.



The TPC of fractions 25 to 35 produced a peak labeled as "A2", which were collected and pooled together to be concentrated by rotary evaporation before it was subjected to C_{18} chromatography. The radical scavenging assay also showed that these fractions possessed the highest activity (Fig. 3).

Figure 3: Silica gel chromatography of the ethyl acetate extract. The fractions were monitored for radical scavenging activity.



The fractions in peak "A2" were pooled together, concentrated by rotary evaporation and subjected to reversed-phase chromatography in a C_{18} column. Both the TPC and radical scavenging assays (Figures 4 and 5) showed the presence of a peak that encompassed fractions 4 to 10, labeled as "B2", which represented fractions with the highest antioxidant activities.

Figure 4: Reversed phase chromatography of components from peak A2 using a C_{18} column. The total pehnolic content of the fractions were monitored.

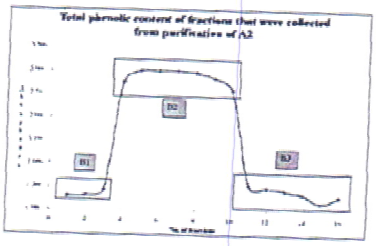
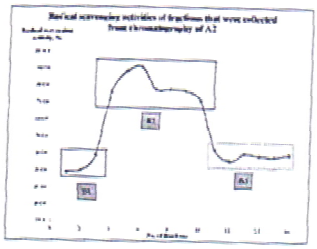


Figure 5: Reversed phase chromatography of components from peak A2 using a C_{18} column. The radical scavenging activity of the fractions was monitored



The components in peak "B2" were further fractionated by silica gel chromatography using a dichloromethane-acetone system, beginning with a dichloromethane: acetone (v/v) ratio of 4:1 and ending with a ratio of 2:3. 10 fractions each of volume 50 ml were collected, and the TPC and radical scavenging activities of the fractions were monitored (Figs. 6 and 7).

Figure 6: Silica gel chromatography components from peak "B2". The fractions were monitored for Total Phenolic Content.

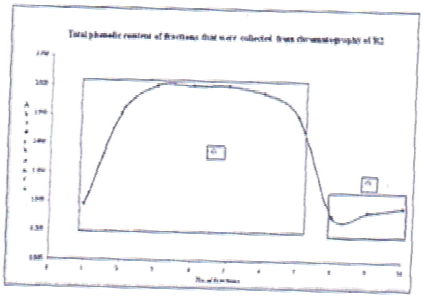
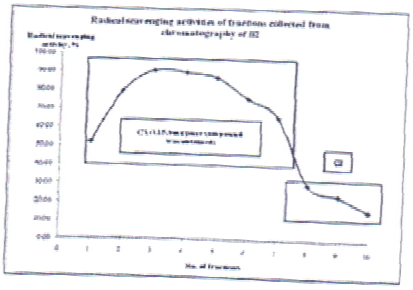
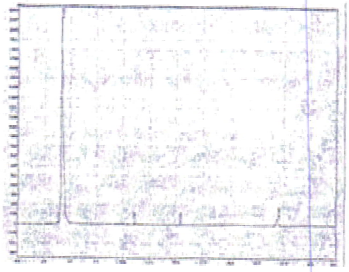


Figure 7: Silica gel chromatography components from peak 'B2". The fractions were monitored for radical scavenging activity.



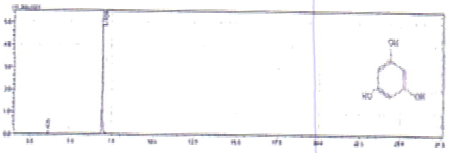
A single major peak, labeled as "C1", which covered fractions 1 to 7, was obtained. These fractions were pooled together, subjected to rotary evaporation and fractionated by HPLC using a C_{18} column to assess its purity. A major peak of retention time 4.12 minutes was obtained, with a percentage peak area of over 97% (Fig. 8).

Figure 8: HPLC analysis of fraction C1



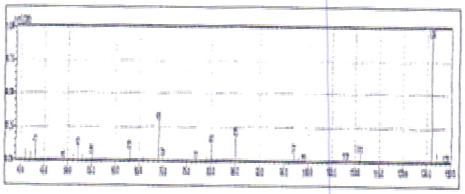
The purity of the fraction was also confirmed by GC (Fig. 9).

Figure . 9 Gas chromatography of fraction C1



The mass spectrum of the component in the main peak obtained, with a retention time of 6.939 mins, is shown in Fig. 10.

Figure 10: Mass spectrum of the component in the GC peak of retention time 6.939 mins, which is was identified by the MS library as phloroglucinol



^{1}H and ^{13}C NMR spectroscopy confirmed that the compound is phloroglucinol. ^{1}H NMR spectroscopy results in a shift of hydroxyl protons at 5.676ppm, and a shift of 8.906ppm for benzylic protons. ^{13}C NMR spectroscopy results in carbons 2,4, and 6 assigned with a shift of 95.483ppm and carbons 1,3 and 5 with a shift of 160.009 ppm (Figs. 11 and 12).

Figure 11: ^{1}H NMR spectroscopy of phloroglucinol

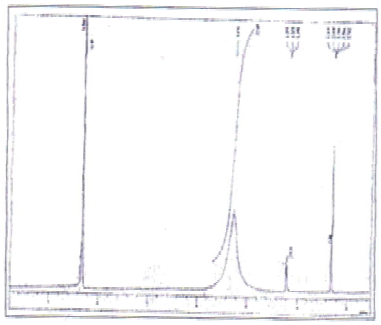
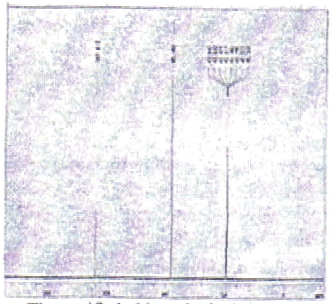
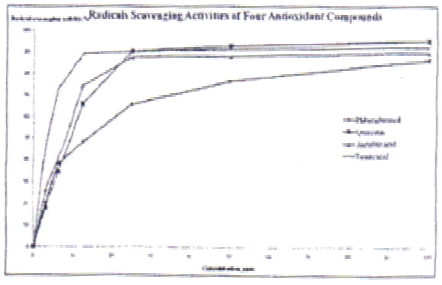


Figure 12: ^{13}C NMR spectroscopy of phloroglucinol



The purified phloroglucinol that was isolated from *P. antillarum* showed weaker free radical scavenging activity with an IC_{50} value of 13.33ppm, compared to well-known antioxidants derived from plants, such as quercetin (IC_{50}= 9.17ppm), ascorbic acid (IC_{50}= 7.92ppm) and tannic acid (IC_{50}= 3.33ppm) (Fig. 13)

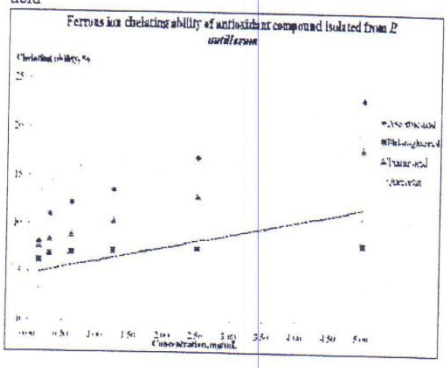
Figure 13: Radical scavenging assay of phloroglucinol, quercetin, tannic acid and ascorbic acid



A ferrous chelating assay was also performed to compare the chelating ability of phloroglucinol with that of the other three antioxidants. The results showed that

the chelating ability of phloroglucinol is much lower than that of the quercetin, tannic acid and ascorbic acid (Fig. 14). This might due to weak binding ability of phloroglucinol which is unable to inhibit reactions which generates hydroxyl radicals.

Figure 14: Comparison of ferrous ion-chelating abilities of phloroglucinol, quercetin, tannic acid and ascorbic acid



IV. CONCLUSION

Phloroglucinol has been isolated from terrestrial plants, such as *Eucalyptus kino* and *Acacia arabica* and also seaweeds of the families *Phaeophyceae* and *Fucacea* However, it is more commonly found in derived form, and derivatives of phloroglucinol, including glycosides, halogenated compounds and terpene adducts, have been reported. Phloroglucinol has a number of chemical and industrial applications, e.g. as a reagent for the detection of aldehydes and for textile-dyeing. [10]. Here we report its isolation from the seaweed *Padina antillarum* and an assessment of its antioxidant activity, as there has been few, if any, studies that have evaluated its potential as an antioxidant..

ACKNOWLEDGMENT

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