BIOLOGICAL ACTIVITIES
OF
SELECTED MALAYSIAN SEAWEEDS

By

SHUBA A/P SALVAM

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ABSTRACT

Antioxidant activity of nine seaweeds, two microalgae, two teas, two dried fruit skins and one dried flower were determined. Among the seaweeds extracts, *Sargassum binderi* showed the highest AOx (antioxidant activity) for its boiled water extract in both AEAC (ascorbic acid equivalence antioxidant capacity) and DPPH (Diphenyl-1-picryl-hydrate) assays. Methanolic extracts of *Sargassum binderi* and *Padina tetrastromatica* showed the highest AOx in DPPH and AEAC assays respectively. *Gracilaria changii* showed the highest AOx for its methanolic extract in Galvinoxyl assay. Boiled water extract of *Padina tetrastromatica* showed the highest total phenolic content in Total Phenolic assay. The two microalgae tested were *Spirulina platensis* and *Chlorella vulgaris* where the former showed highest AOx for its boiled water extract in AEAC and Total phenolic assays while the latter showed higher AOx in AEAC, Galvinoxyl and Total Phenolic assays for its methanol extract. The other samples studied also showed AOx in all the assays tested for their boiled water and methanolic extracts. It was determined in this study that storage, drying and grinding method, as well as the solvent used on samples influenced AOx. Ethyl acetate (PT Ea) and ethanol (PT Et) extracts of *Padina tetrastromatica* and *Kappaphycus alvarezii* ethanol (KA Et) extracts chosen for animal studies showed that they significantly reduced inflammation, pain and ulcer lesions (except for PT Ea that was not used in the ulcer study) but were concentration dependent. PT Ea showed the highest LD_{50} (1845 mg/kg) followed by PT Et (1596 mg/kg) and KA Et (1799 mg/kg). The seaweeds studied showed antioxidant, anti-inflammatory, antinociceptive and anti-ulcerogenic activities. These seaweeds could be useful for their biological activities.

ABSTRAK

Aktiviti antioksidan bagi sembilan jenis rumpai laut, dua mikroalga, dua jenis teh, dua kulit buah yang telah dikeringkan dan satu bunga kering telah ditentukan dalam kajian ini. Di antara ekstrak rumpai laut yang telah dikaji, *Sargassum binderi* menunjukkan aktiviti antioksidan yang paling tinggi untuk ekstrak air panas dengan ujian analisis AEAC (muatan antioksidan keseimbangan asid askorbik) dan DPPH (Difenil-1-pikril-hidrat). Pengekstrakan metanol *Sargassum binderi* dan *Padina tetrastromatica* turut menunjukkan aktiviti antioksidan yang paling tinggi dengan ujian DPPH dan AEAC. Manakala pengekstrakan metanol *Gracilaria changii* menunjukkan aktiviti antioksidan yang paling tinggi dengan ujian Galvinoksil. Ekstrak air panas *Padina tetrastromatica* menunjukkan kandungan fenol yang paling tinggi dengan ujian penjumlahan fenol. Dua jenis mikroalga yang dikaji ialah *Spirulina platensis* dan *Chlorella vulgaris* di mana, ekstrak air panas *Spirulina platensis* menunjukkan aktiviti antioksidan yang tinggi dengan ujian AEAC dan penjumlahan fenol. yang tinggi bagi ekstrak air panas. Mikroalga *Chlorella vulgaris* pula menunjukkan aktiviti antioksidan yang tinggi dengan ujian AEAC, Galvinoksil dan ujian penjumlahan fenol bagi pengekstrakan metanolnya. Sampel lain yang dikaji turut menunjukkan aktiviti antioksidan yang tinggi di dalam semua ujian bagi ekstrak methanol dan ekstrak air panas. Kajian ini menunjukkan kaedah simpanan, pengeringan dan pengisaran serta pemilihan pelarut mempengaruhi aktiviti antioksidan. Pemilihan ekstrak etil asetat (PT Ea) dan etanol (PT Et) *Padina tetrastromatica* dan *Kappaphycus alvarezii* ethanol (KA Et) untuk kajian terhadap haiwan menunjukkan pengurangan kesakitan, ulser dan keradangan (kecuali bagi PT Ea yang tidak digunakan dalam kajian ulser) tetapi bergantung kepada kepekatan. PT Ea menunjukkan LD_{50} (1845 mg/kg) yang paling tinggi diikuti oleh PT Et (1596 mg/kg) dan KA Et (1799 mg/kg). Rumpai laut menunjukkan

aktiviti antioksidan, anti-keradangan, antinociceptive dan anti-ulser. Rumpai laut ini boleh digunakan berdasarkan aktiviti biologinya.

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ABBREVIATIONS

%	percentage
˚C	degree celcius
˙OH	hydroxyl radical
µM	micromolar
$^1O_2$	singlet oxygen
1^{st}	first
2^{nd}	second
3^{rd}	third
4^{th}	fourth
5^{th}	fifth
AA	Arachidonic acid
$ABTS^{\cdot-}$	2,2'-azino-bis(3-ethylbenzo tiazoline-6-sulfonic-acid) radical cation
$ABTS^{2-}$	2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)
AEAC	Ascorbic Acid Equivalent Capacity
ANOVA	Analysis of variance
AOx	Antioxidant activity
ATP	adenosine triphosphate
BBM	Bold's Basal Medium
Ca^{2+}	Calcium ion
CNS	central nervous system
COX	Cyclooxygenase
dH_2O	distilled water
DNA	Deoxyribonucleic acid
DPPH	Diphenyl-1-picryl-hydrate

E	East
et al	and others
EtOH	ethanol
FCR	Folin-Ciocalteu Reagent
Fw	forward
g	gram
g/mol	gram per mol
GABA	gamma-aminobutyric acid
GAE	Gallic acid equivalents
GFRQ	Galvinoxyl Free Radical Quenching
GI	gastrointestinal
GSH	glutathione
h	hour
H. pylori	*Helicobacter pylori*
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HO^-	free hydroxyl radicals
i.p	intra peritoneal
K+	potassium ion
KA Et	*Kappaphycus alvarezii* ethanol
Kg	kilogram
KH_2PO_4	potassium dihydrogen phosphate
LD_{50}	Lethal Dose 50%
LDL	low-density lipoproteins

LKIM	Lembaga Kemajuan Ikan Malaysia
LOX	lipoxygenase
mg/kg	milligram per kilogram
mg/ml	miligram per milliliter
min	minute
ml	milliliter
mM	millimolar
mm^2	millimeter square root
mRNA	messenger ribonucleic acid
N	North
Na_2CO_3	sodium Carbonate
NaCl	sodium chloride
NADPH	nicotinamide adenosine dinucleotide phosphate
nm	nano meter
NMDA-	N-methyl-d-aspartate
NO	Nitric oxide
$NO^{\bullet}$	nitric oxide radical
NOS	nitric oxide synthase
NSAID	nonsteroidal anti-inflammatory drugs
O_2	oxygen
$O_2^{\bullet -}$	Superoxide
OD	optical density
OH^+	Hydroxide ion cation
OH^-	Hydroxide ion anion

ONOO	peroxynitrite
OS	oxidative stress
P	probability
PG	prostaglandin
PGE	prostaglandin E
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
pH	potential of hydrogen
PLA2	phospholipase A2
PT Ea	*Padina tetrastromatica* ethyl acetate
PT Et	*Padina tetrastromatica* ethanol
R&D	research and development
RO	alkoxy
ROO	peroxyl
ROS	Reactive Oxygen Species
rpm	revolutions per minute
Rv	reversed
S.E.M	standard error mean
SD	Sprague – Dawley
sec	second
SOD	superoxide dismutase
sp.	Species
Stds	standards

TEAC	Trolox Equivalent Antioxidant Capacity Assay
TEGx	Trolox equivalent galvinoxyl quenching activity
Tween 80	Polyoxyethylenesorbitan Monooleate
TXA_2	thromboxane A_2
UGIC	upper gastrointestinal complications
UMACC	University of Malaya Algae Culture Centre
UV	ultra violet
V_l	Left paw volume
V_{l0}	Left paw initial volume
V_r	Right paw volume
V_{r_0}	Right paw initial volume
w/v	weight/volume
µg/ml	microgram per milliliter
µl	microliter

1.0 INTRODUCTION

Seaweeds and their extracts have been studied for several decades and were reported to possess biological activity of potential medicinal values (Konig *et al*., 1994; Moore, 1978; Satoru *et al*., 2003; Tutour *et al*., 1998). Biological activities found in seaweeds were antibiotic, antiviral, antioxidant, anticoagulant, anti-inflammatory, and antitumor (Manish *et al*., 1993; Osselaer *et al*., 1996; Pereira, 1994; Salvador *et al*., 2007; Xue *et al*., 1998; Zang *et al*., 1995). These studies were conducted due to the world wide usage of natural compounds in food and medical industries. They are more desirable than synthetic counterparts as they are presumed to be safe due to the usage of natural sources (Arnao *et al*., 1999). Many studies have reported that seaweeds contain high levels of minerals, vitamins, essential amino acids, indigestible carbohydrates and dietary fiber (Ismail *et al*., 2002; Jimenez-Escrig *et al*., 1999). They have been used for a long time in traditional medicine to treat high blood pressure (Novaczek *et al*., 2001), rheumatism and also as animal feed (Prud'home van Reine *et al*., 2001).

One of the major interests of studies on seaweeds and microalgae biological activities was on their antioxidant properties. For many years, marine algae have received special attention as a source of natural antioxidants (Matsukawa *et al*., 1997). Antioxidants were known to scavenge ROS such as superoxide, hydroxyl radicals, peroxyl radicals, hydrogen peroxide, singlet oxygen, nitric oxide and peroxy nitrate (Halliwell, 1991). They inhibit lipid peroxidation and other free radical mediated processes that contributed to several diseases (Czinner *et al*., 2001). Studies have showed that diets rich in vegetables, fruits and grain products help to reduce the risk of cardiovascular disease and certain cancers (Beecher, 1999). Similarly, Potterat (1997) has reported that dietary antioxidants from seaweeds were believed to help prevent free radical mediated diseases. Besides

protecting human body from diseases, antioxidants have been used in food industries as food additives because of their unique properties of enhancing the shelf life of food products without any damage to the nutritional values (Madhavi *et al*., 1996).

Reactive oxygen species (ROS) are free radicals formed during biochemical reactions. They are intermediate products of oxygen formed during this process (Wu *et al*., 2003). ROS are toxic as they can disrupt cell membrane and DNA which leads to cell death (De Groot, 1994; Nakawaza *et al*., 1996; Toykuni, 1999). This induces the oxidative stress in the body which leads to various pathological conditions including cardiovascular dysfunction, neurodegenerative disease, gastroduodenal pathogenesis, metabolic dysfunction of almost all vital organs, cancer and premature aging (Thomas *et al*., 1997). To protect against these free radicals, antioxidants are manufactured within the body system (Kaczmarski *et al*., 1999). They are called endogenous antioxidants which consist of enzymatic and non enzymatic antioxidants (Singh *et al*., 2004; Fernandez-Checa *et al*., 1997). These endogenous antioxidants defenses by body are not sufficient to fight diseases caused by free radicals. Therefore, diet-derived antioxidant was suggested to help protect human body against free radicals (Halliwell, 1996., Vendemiale *et al*., 1999). A study by Okawa *et al*. (2001) supported this statement as it reported that the antioxidant substances in diets were the principle agents responsible for the protective effects in eliminating free radicals. These antioxidants act as free radical scavengers, hydrogen-donating compounds, singlet oxygen quenchers and metal ion chelators.

Many studies have revealed that a major part of antioxidant activity may be from compounds such as flavonoids, isoflavones, flavones, anthocyanin, catechin and other phenolics (Kahkonen *et al*., 1999). Antioxidant substances such as α-tocopherol,

carotenoids, polyphenols, phycobiliproteins and vitamin C were found in algae (Plaza *et al*., 2008) and it was reported by Chandini *et al*. (2008) that these compounds were useful as natural antioxidants in food and medical applications. Seaweeds are exposed to extreme environments which lead to formation of free radicals but they seldom suffer from serious oxidative damage. This suggests that they have protective antioxidative mechanisms and compounds (Matsukawa *et al*., 1997). A study by Matanjum *et al*. (2008) showed significant findings of antioxidant properties in brown, red and green seaweeds from north Borneo.

Studies were also carried out on microalgae to determine its biological properties. Miranda *et al*. (2001) and Shibata *et al*. (2003) have reported that microalgae contain compounds that exhibit high antioxidant and anti-inflammatory potentials. Since microalgae can be cultivated under controlled environment, it may also be served as a reliable source of natural antioxidant (Chen, 1996). Antioxidants were also detected in plants such as tea, fruits, medicinal plants, vegetables and flower (Kalt *et al*., 1999; Lin *et al*., 1998; Wetwitayaklung *et al*., 2008; Yang *et al*., 2007;). They possess phenolic compounds, nitrogen containing compounds, carotenoids, ascorbic acids, glutathione and uric acid which are powerful antioxidants (Fujimoto *et al*., 1985; Larson, 1988; Paya, *et al*., 1992 and Potterat, 1997).

Several in vitro methods have been developed to measure antioxidant activity of biological samples. The most commonly used methods were ascorbic acid equivalence antioxidant capacity (AEAC) or trolox equivalence antioxidant capacity (TEAC), DPPH, and Total Phenolic by Folin-Ciocalteu reagent (FCR) assays. Other methods that were used include Galvinoxyl, total radical trapping antioxidant parameter (TRAP),

ferric ion reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) assays (Huang *et al*., 2005; Perez *et al*., 2000). These assays differ from each other in terms of substrates, reaction conditions and quantitation methods. Due to these differences, many authors have suggested the need to carry out more than one assay as there is no single assay that could accurately quantify all antioxidant in a sample (Frankel *et al*., 2000; Prior *et al*., 2005). Yang *et al*. (2007) have reported in a study that, selection of solvent for samples' extraction plays a vital role in the detection of antioxidant properties as there is no one solvent that could extract all the biological compounds from a sample. Other factors that were studied and reported to interfere with the antioxidant properties were storage, temperature, drying and grinding methods (Cronin *et al*., 1995; Laurrauri *et al*., 1997; Silva *et al*., 1998).

Many researchers have carried out studies to determine the presence of other biological activities in seaweeds. Several studies have reported that seaweed extracts given orally to animal effectively reduce inflammation, pain and ulcer lesion (Paya *et al*., 1993; Raghavendran *et al*., 2004). Nevertheless, in this study, we hope to contribute in the finding of useful biological activities of seaweeds.

Objectives of study

The aims of this study were to investigate the biological activities of seaweeds and other extracts used in this study. The objectives were to:

(a) evaluate antioxidant activities of extracts using AEAC, DPPH, Galvinoxyl and Total Phenolic assays.

(b) study the effect of solvent polarity on antioxidant activities when samples were extracted using methanol and boiled water.

(c) study the effect of temperature on antioxidant activities when two different grinding methods were used to prepare samples before extraction.

(d) investigate antioxidant activities between samples.

(e) quantitate toxicity level (LD_{50}) of selected seaweeds extracts using animal model.

(f) study the anti-inflammatory, antinociceptive and anti-ulcerogenic activities of selected seaweed extracts using animal model.

2.0 LITERATURE REVIEW

2.1 Natural Source of Biological Activities

2.1.1 Seaweeds as a Source of Antioxidant and Other Biological Activities

Seaweeds have become a major food ingredient in products especially in Japan, Korea and China. Boukhari *et al*. (1998) reported that most Europeans and Americans use processed seaweeds as additives in their food preparation. In Asia, seaweeds have been used for centuries in the preparation of salads, soups and also as low-calorie foods (Jiménez-Escrig *et al*., 2000). Seaweeds belong to a group of marine plants known as algae. The estimated range of seaweeds is probably around 45,000 species (Bequette *et al*., 1997). Seaweeds can be divided into three categories based on their colour; red (Rhodophyta) (4,500 species), green (Chlorophyta) (900 species) and brown (Phaeophyta) (1,000 species). Seaweeds have been used as food, fertilizer and for medicinal purposes for a long time and now, antioxidant are intensively focused due to the growing demand from pharmaceutical industries. Like other plants, seaweeds contain various kinds of inorganic and organic substances which probably benefit human health. It has been reported that seaweeds contain high levels of minerals, vitamins, essential amino acids, indigestible carbohydrates, and dietary fiber (Jiménez-Escrig *et al*., 1999; Ismail *et al*., 2002). Seaweeds and their extracts have been studied for over several decades as a novel source and have been reported to possess biological activity of potential medicinal value (Moore, 1978; Konig *et al.*, 1994; Tutour *et al.*, 1998; Satoru *et al.*, 2003).

Padina tetrastromatica is a brown algae which is used as seasoning in dried flake form and as table salt replacement for high blood pressure patients (Novaczek *et al*., 2001). *Kappaphycus alvarezzi*, formerly termed as *Eucheuma cottonii* (Rönnbäck *et al.*, 2002) is a

red algae and the main usage of this algae is its main product of commercial importance, carrageenan. It is a popular species for aquaculture and being farmed at places with strong wave action such as on the reef edge. *Caulerpa racemosa* is an example of green algae that is usually served raw as salad or eaten cooked. In addition, it is used as animal feed and in folk medicine to reduce blood pressure and to treat rheumatism (Prud'homme van Reine *et al*., 2001). It has been reported by Matanjun *et al.* (2008) that there were significant findings of antioxidant properties in brown, red and green seaweeds.

Many researches have been carried out mainly on anti-tumor activity, anti-cholesterolemic activity and antioxidant activity using seaweeds. Almost all seaweeds and photosynthesizing plants are exposed to light and high oxygen concentrations which lead to formation of free radicals but they seldom suffer from serious photodynamic damage. This implies that they have protective antioxidative mechanisms and compounds (Matsukawa *et al.*, 1997). Algae can be considered as an important source of antioxidant compounds that could be suitable for protecting human bodies against the reactive oxygen species formed by our metabolism or induced by external factors (pollution, stress or UV radiation). In algae there are antioxidant substances of very different nature, among which are vitamin E (or α-tocopherol) and carotenoids that can be highlighted within the fat-soluble fraction, whereas the most powerful water-soluble antioxidants found in algae are polyphenols, phycobiliproteins and vitamins (vitamin C) (Plaza *et al*., 2008). Carotenoids have been reported to have multiple antioxidant activities, including the ability to scavenge singlet oxygen and peroxyl radicals (Krinsky, 1989; Terao *et al.*, 1992). Bioactive compounds found in seaweeds have many potential and it was stated by Chandini *et al.* (2008) that, the compounds found in seaweeds await a major breakthrough for a variety of food or medical applications as they have the potential for application of such compounds

as natural antioxidants in different food or pharmaceutical products. Many studies have also been carried out on microalgae, such as *Spirulina* and *Chlorella*, to study their antioxidant activities. Miranda *et al.* (2001) has reported that *Spirulina* contains phenolic acids, tocopherols and β-carotene which are known to exhibit antioxidant properties. Recently, it was reported by Dartsch (2008) that, *Spirulina* has high antioxidant and anti inflammatory potential. Antioxidant activity of *Chlorella* has been reported by Shibata *et al.* (2003) where in their study, *Chlorella* showed strong antioxidant effect compared to various vegetables that were tested.

2.1.2 Other Source of Antioxidant

Besides studies on seaweeds and microalgae antioxidant activity, Kalt *et al.* (1999) have published work on strong antioxidant compounds found in fruits. Teas, for example green teas and black teas, have also been extensively studied for antioxidant properties since they can contain up to 30% of the dry weight as phenolic compound (Lin *et al.*, 1998). Some researchers have also done research on antioxidant properties of flowers. Wetwitayaklung *et al*. (2008) had studied total polyphenolic content and antioxidant activities of 24 Thai edible flowers.

2.2 Introduction of Antioxidants

2.2.1 Free Radicals

Free radicals have been associated with a variety of chronic diseases in human and antioxidants are believed to protect human body against it (Thomas *et al*., 1997). Free radical is any atom or molecule (e.g. oxygen or nitrogen) with at least one unpaired electron in the outermost shell and is capable of independent existence (Karlsson, 1997). Electrons are more stable when paired together in orbitals: the two electrons in a pair have different directions of spin. Hence, radicals are generally less stable than non-radicals although their reactivity varies. The free radicals are capable of reacting indiscriminately with any molecules that they came in contact. Once radicals are formed, they can either react with another radical or with another non-radicals molecule by various interactions. If two radicals meet, they can combine their unpaired electrons, thus forming a covalent bond. However, most molecules found *in vivo* are nonradicals. In this case, a radical might donate its unpaired electron to the other molecule or might take one electron from it, transforming its radical character. At the same time, a new radical is formed. The feature that is becoming clear is that, a radical generates another radical, leading to the chain reaction (Nonhebel *et al.*, 1980; Mimić-Oka *et al.*, 1999).

One chemical element frequently involved in free radical formation is oxygen. Molecular oxygen is essential for cell function because it plays a pivotal role in a series of biochemical reactions occurring in the respiratory chain, which is responsible for most of the production of adenosine triphosphate (ATP), hence providing the energy required for a multitude of cellular reactions and functions. Molecular oxygen can accept a total of four electrons, one at a time and the corresponding number of protons to generate two molecules of water. During this process, different oxygen radicals are successively formed

as intermediate products including superoxide ($O_2^{\bullet -}$), which normally exists in cells as hydrogen peroxide (H_2O_2); and the hydroxyl radical ($^{\bullet}OH$). Superoxide, peroxide and hydroxyl radicals are considered the primary reactive oxygen species (ROS), which have sparked major research on the role of free radicals in biology and medicine (Wu *et al.*, 2003). However, because they are unstable and rapidly react with additional electrons and protons, most of these ROS are converted to water before they can damage cells. It has been estimated that only about 2 to 3 percent of the oxygen (O_2) consumed by the respiratory chain is converted to ROS (Chance *et al.*, 1979). The toxic effects of oxygen in biological systems such as breakdown of lipids, inactivation of enzymes, introduction of changes in the DNA and the destruction of cell membrane and cells are attributable to the reduction of O_2 to ROS (De Groot, 1994; Nakawaza *et al.*, 1996; Toykuni, 1999). It is also known that free radical can be produced by inflammation, xenobiotic metabolism and hyperoxia (Halliwell *et al*., 1999).

It is well known that ROS generates spontaneously in the living cell during several metabolic pathways. These comprise of biological electron transport systems such as photosynthetic, mitochondria, microsomal, including various enzymes and biomolecules such as neutrophil, xanthine oxidase, cyclooxygenase, lipooxigenase and autooxidation of catechol amines (Halliwell *et al*., 1984). Another source of ROS, which can be formed in the liver is by cytochrome P450 mixed-function oxidase (Lieber, 1997). Other sources of ROS in the body are two types of immune cells called macrophages and neutrophils, which help body defends against microorganism (Rosen *et al.*, 1995). It has been well established that over production of ROS occurs at site of chronic inflammation (Halliwell, 1995). These ROS production is called endogenous oxidants.

Besides ROS generated by the body, external cause such as exposure to environmental free radical, including ROS in the form of radiation, ultra violet (UV) light, smog, tobacco smoke and certain compounds referred to as redox cycling agents, which includes some pesticides can induce ROS formation (Wu *et al.*, 2003). These ROS production is called exogenous oxidants.

2.2.2 Reactive Oxygen Species

The molecule oxygen has bi-radical nature where it readily accepts unpaired electrons to give rise to a series or partially reduced species ROS. This includes superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl ($^{\bullet}HO$), peroxyl (ROO), alkoxy (RO) and nitric oxide ($NO^{\bullet}$), until it is itself completely reduced to form water (Singh *et al.*, 2004). Besides ROS, there are many free radical species that exists, for example carbonyl, ozone, thyil and nitroxyl radicals. There are a many types of ROS, but few are mostly studied. Some of the free radicals that are mostly studied will be explained briefly in this chapter.

i). Superoxide radical ($O_2^{\bullet-}$)

Superoxide is generated by a small percentage of electrons leakage from the main stream of the mitochondrial respiratory chain. The quantitative importance of oxygen-derived free radicals can be realized by the fact that about 250 grams of oxygen are consumed everyday by human organism. Of this, about 2 to 5 % would be converted to superoxide (Rice-Evans, 1994). Most of the superoxide radicals are formed in the mitochondrial and microsomal electron transport chain. The majority of superoxides radical generated by mitochondrial electron transport chain are enzymatically dismutated

by superoxide dismutase (SOD) (Mimić-Oka *et al.*, 1999) to H_2O_2 (hydrogen peroxide) (Singh *et al.*, 2004).

ii). Hydroxyl ($^{\bullet}OH$)

The hydroxyl radical is the most reactive among the other ROS. In the presence of free iron or copper ion, a sequence of two reaction steps can occur to hydroxyl production. First, hydroxyl peroxide can produce hydroxyl radical by removing an electron from the participating metal ion. In the second step it involved the superoxide radical, where the original metal ions are generated so that they can again be available for the reaction with hydrogen peroxide. Because of iron's critical contribution to hydroxyl radical formation, anything that increases the levels of free iron in the cells can promote ROS generation and oxidative stress (Wu *et al.*, 2003).

iii). Hydrogen peroxides (H_2O_2)

In human cells, superoxide is quickly transformed into hydrogen peroxide. Reaction catalyzed by enzymes such as monoamine oxidase and L-amino acid oxidase can produce hydrogen peroxides directly (Singh *et al.*, 2004). Hydrogen peroxide is a potent oxidant and in sufficient concentration will kill any cells. The further reduction of hydrogen peroxide stabilizes the interoxygen bond, resulting in a cleavage to produce OH^- and OH^+ (Mimić-Oka *et al.*, 1999). Hydrogen peroxide is produced *in vivo* and it is unique because it can be converted to the highly damaging hydroxyl radical or be catalyzed and excreted to water (Alessio *et al.*, 1997). If hydrogen peroxide is not converted into water, singlet oxygen ($^1O_2$) is formed (Karlsson, 1997).

iv). Singlet oxygen ($^1O_2$)

Singlet oxygen radical is form when the spin of one of the electrons of the two outer orbitals is inverted, removing the quantum mechanical spin restrictions of molecular oxygen (Mimić-Oka *et al.*, 1999). Singlet oxygen violated Hund's rule of electron filling in that it has eight outer electrons existing in pairs leaving one orbital of the same energy level empty. When oxygen is energetically excited, one of the electrons can jump to an empty orbital, creating unpaired electrons. Singlet oxygen is not a free radical but it can be formed during radical reactions and also cause further reactions (Karlsson, 1997).

v). Nitric Oxide ($NO^{\bullet}$)

Nitric oxide can react with superoxide radical to form highly toxic peroxynitrite (ONOO) where this can mediate cell death (Jacobson, 1996). When peroxynitrite reacts with human body fluids, nitrotyrosines are generated, which have been detected in human brain and may be increased in neurodegenerative disease, especially because glial cells and macrophages generates nitric oxide (Halliwell *et al.*, 1984). Nitric oxide has recently emerged as an important mediator of cellular and molecular events which impacts the pathophysiology of myocardial ischemia. An increase in intracellular Ca^{2+} could activate the enzymes nitric oxide (NO) synthase (Bredt *et al.*, 1994), which catalyzes the synthesis of NO from L-arginine and molecular oxygen. Nitric oxide may cause cytotoxicity through formation of iron-NO complexes with several enzymes including mitochondrial electron transport chain, oxidation of protein sulphydryls and DNA nitration (Bredt *et al.*, 1994; Xie *et al.*, 1996). Peroxynitrite decomposes to hydroxyl free radical, which is a potent activator of lipid peroxidation (Mimić-Oka *et al.*, 1999).

2.3 Free Radicals and Health

2.3.1 Lipid Peroxidation

One of the most destructive effects of oxygen free radicals is the initiation of lipid peroxidation, which can result in runaway reactions (Schoneich *et al.*, 1992). Lipids that contain phosphate groups (i.e., phospholipids) are essential components of the membranes that surround the cells as well as other cellular structures such as the nucleus and mitochondria. Damage to the phospholipids will compromise the viability of the cells and the complete degradation of lipid is a hallmark of oxidative damage. Polyunsaturated fatty acids present in the membranes are sensitive to the attack by hydroxyl radicals and other oxidants. A single hydroxyl radical can result in the peroxidation of many polyunsaturated fatty acid molecules because the reactions involved in this process are part of a cyclic chain reaction. Lipid peroxidation can result in formation of reactive products that they themselves can react with damage proteins and DNA (Wu *et al.*, 2003).

2.3.2 The Positive and Negative Effect of Free Radicals

Although free radicals causes several diseases like heart disease, cataract, cognitive dysfunction, cancer, chronic inflammatory disease of gastrointestinal tract and many more, free radicals are also important in several processes in human body. Both positive and negative effects of free radicals are explained briefly in this chapter.

i). Positive Effects

Free radicals are involved in several normal biological processes *in vivo*. They are part of cascade event in antimicrobial action of phagocytic cells via NADPH-oxidase, in the arsenal of defense cells such as neutrophils, monocytes, macrophages and eosinophils. This process is central to the human antimicrobial defence system where they will damage the membranes, DNA and other cellular component of the invading organism (Del Maestro *et al.*, 1980). Free radicals also act as regulatory molecules in biochemical processes, for example, lymphocytes and fibroblast constantly generate small amounts of superoxide radicals as growth regulators (Jacobson, 1996). Nitric oxide from endothelial cells is involved in the regulation of vascular tone, including relaxation of smooth muscle cells. Macrophage-derived nitric oxide has been implemented in killing tumor cells and bacteria. Other nonphagocytic cells such as endothelial and arterial smooth muscle cells can be stimulated to release superoxide (Bredt *et al.*, 1994). Free radicals are also involved in mechanism action of certain enzymes such as ribonucleoside diphosphate reductase, cytochrome P-450 and prostaglandin synthase (Jacobson, 1996).

ii). Negative Effects

a. Heart Disease

Heart disease such as atherosclerosis has been widely accepted that it has a connection with oxidative stress (Mimić-Oka *et al.*, 1999). Recent studies suggest that oxidative modification of low-density lipoproteins (LDL) is a critical factor that may be oxidatively modified by all major cell types of the arterial wall via their extracellular release of ROS (Morel *et al.*, 1984; Parthasaratry *et al.*, 1992). The three most important cell types in the arterial wall are endothelial cells, smooth muscle cell and macrophage, where they can release free radical, which effect lipid peroxidation. A continuation of lipid

oxidation will cause blood vessel damage to the reaction process and can lead to generation of foam cells and plaque, which are the symptoms of atherosclerosis. Further oxidized LDL is cytotoxic and can directly damage endothelial cells (De Whallel *et al.*, 1990). Atherosclerotic lesions from human aorta contain lipid peroxides and the peroxides content correlates with the extent of atheroma (Glavind *et al.*, 1952). Detectable levels of oxidatively modified LDL are also found in human plasma and elevated plasma peroxides levels have been found in diabetics, smokers and patients with coronary disease (Sato *et al.*, 1979; Blackman *et al.*, 1984).

5 Aging

The free radical theory of aging proposed that ROS cause oxidative damage over the life span of a living organism. It is the cumulative and potentially increasing amount of accumulated damage that accounts for the dysfunctions and pathologies seen in normal aging (Irshad *et al.*, 2002). Oxidative damage has also been reported in several other age-related neurodegenerative diseases, including Huntington disease, progressive supranuclear, amyotrophic lateral sclerosis and prion disorders, all of which have abnormal protein aggregation as a major component of their pathology. Intrinsic oxidative stresses and presumably cell damage increase with age due to either diminished defenses or the increase in mitochondrial dysfunction. These age-related effects, compounded with genetic and environmental factors and high-energy dependence but relatively low level of antioxidants in the brain, may provide a unifying mechanism for the high incidence of neurodegenerative disorders in the aged population (Klein *et al.*, 2003).

c. Cancer

Free radicals or oxidative injury now appears to be the fundamental mechanism underlying a number of human neurologic and other disorders for example cancer (Atawodi, 2005). In carcinogenesis, ROS are responsible for initiating the multistage carcinogenesis process starting with DNA damage and accumulation of genetic events in one or few cell lines which leads to progressively dysplastic cellular appearance, deregulated cell growth and finally carcinoma (Tsao *et al.*, 2004)

2.4 Antioxidants

2.4.1 Antioxidant in Biological System

Antioxidant means “against oxidation”. Antioxidants are effective because they are willing to give up their own electrons to free radicals where, when a free radical gain electron from antioxidant, it no longer attack the cells and the oxidation chain reaction is broken (Dekkers *et al.*, 1996). By right, after donating an electron, antioxidant becomes free radical but antioxidants in this state is not harmful because they have the ability to accommodate the change without being reactive. Antioxidants are manufactured within the body and also by intake of food such as fruits, vegetables, seeds, nuts, meats and oil (Kaczmarski *et al.*, 1999). Biological system has defensive system against free radicals.

Antioxidant protection can be viewed as consisting of four levels of defensive activities which are:

- The first level of defense is largely enzymatic that involves enzymes, where it is concerned with the control of formation and proliferation of primary radical species derived from molecular oxygen.

- The second level involves vitamins C and E and probably carotenoids where it is concerned with the prevention of the proliferation of secondary radicals in chain reaction such as lipid peroxidation, which is initiated and driven by primary radicals.

- The third level is the enzymatic prevention formation of secondary radicals from chain-terminated derivatives and enabling the removal of such molecules from an environment in which metal-catalyzed reactions might cause further oxidative damage.

- The final level is the adaptation in antioxidant mechanism where free radicals work as a signal in inducing the synthesis and the transport of the appropriate antioxidants to the site of action.

(Diplock, 1994)

There are two types of antioxidant mechanisms that evolved to protect cells against ROS. They are enzymatic and non-enzymatic antioxidants (Yu, 1994).

i). Enzymatic Antioxidants

Enzymes involved directly in the elimination of ROS are superoxide dismutases (SODs), catalase and glutathione peroxidase (Singh *et al.*, 2004). SODs catalyze the rapid removal of superoxide radicals. Catalase and glutathione peroxidase system both help to remove hydrogen peroxidase. Catalase is an iron-containing enzyme found primarily in small membrane-enclosed cell components called peroxisomes where it serves to detoxify hydrogen peroxide and various other molecules (Wu *et al.*, 2003). Glutathione is the most significant component that directly quenches ROS such as lipid peroxides and plays a major role in xenobiotic metabolism (Singh *et al.*, 2004). The glutathione peroxidase system consists of several components including the enzyme glutathione peroxidase and glutathione reductase and the cofactors glutathione (GSH) and reduced nicotinamide adenosine dinucleotide phosphate (NADPH). These molecules effectively remove hydrogen peroxide (Wu *et al.*, 2003). These antioxidants are called endogenous antioxidants because it is produced by the body system.

ii). Non-enzymatic Antioxidants

There are two types of non-enzymatic antioxidants. One, is present in the body which are GSH and NADPH and the second is exogenous antioxidant which are non-enzymatic antioxidant that enters the body through food intake such as Vitamin C and E. GSH consist of three amino acid, which serves as cofactor for an enzyme called glutathione transferase which helps remove drugs and chemicals as well as other reactive molecules from the cells. Besides that, GSH can interact directly with hydroxyl radical to

detoxify them as well as performing other critical activities in the cell (Fernandez-Checa *et al.*, 1997). NADPH is involved in the glutathione peroxidase system. Therefore, NADPH or enzymes that generate this compound are sometimes considered antioxidants (Wu *et al.*, 2003). Vitamin C (ascorbic acid) is a water-soluble antioxidant in extracellular fluids where it is capable of neutralizing ROS in the aqueous phase before lipid peroxidation is initiated. Vitamin E is a lipid-soluble antioxidant and is the most effective chain-breaking antioxidant within the cell membrane where it protects membrane fatty acids from lipid peroxidation (Halliwell, 1994; Jacob, 1995).

2.5 Antioxidant Assay

Major antioxidant assays can be roughly divided into two categories that are hydrogen atom transfer reaction based assay and single electron transfer reaction based assay. Antioxidant activity measured by individual assay reflects only the chemical reactivity under the specific condition applied in that assay.

2.5.1 Ascorbic Acid Equivalent Capacity Assay (AEAC)

AEAC assay is quite similar to Trolox Equivalent Antioxidant Capacity Assay (TEAC), which was first reported by Miller and Rice-Evans (1993) and later improved by Re *et al.* (1999). The only difference between the two assays is, ascorbic acid is used as a standard for AEAC assay whereas Trolox is used as standard for TEAC assay. In the improved method, $ABTS^{\cdot-}$, the oxidant is generated by persulfate oxidation of 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{2-}$), where the blue green

colour formed will be reduced in the presence of antioxidants. Due to its operational simplicity, this assay has been used in many researches for antioxidant studies. Apparently, the reaction rate differences between antioxidants and oxidants are not reflected in the AEAC/TEAC value because this is an end-point assay (Huang *et al*., 2005).

2.5.2 Diphenyl-1-picryl-hydrate (DPPH) Free Radical Photometric Assay

DPPH is one of a few stable and commercially available organic nitrogen radicals. DPPH is soluble in ethanol (Brand-Williams *et al.*, 1995) and the antioxidant activity is measured by the decreased in the absorbance at 515nm. Upon reduction in the presence of antioxidant molecules, the solution colour fades from its violet colour solution. The DPPH assay was believed to involved hydrogen atom transfer reaction but recent paper suggested otherwise (Huang *et al*., 2005). On the basis of the kinetic analysis of the reaction between phenols and DPPH, it was suggested that the reaction behaved like electron transfer (Foti *et al.*, 2004). This assay provides information on the ability of a compound to donate a hydrogen atom, the number of electrons a molecule can donate and the mechanism of antioxidant actions.

2.5.3 Galvinoxyl Free Radical Quenching Assay

For most radicals, to directly monitor the decay of free radical (Romay *et al.*, 1996), it requires a pulsed production of the radicals and fast techniques for the detection of the radical decay. This can be done by employing stable radicals of relatively long lifetimes, such as galvinoxyl (2,6-Di-tert-butyl-alpha-(3,5-di-ter-butyl-4-oxo-2,5-cyclohexadien-1-ylidene)-p-tolyloxy). Galvinoxyl is introduced to determine the hydrogen donating efficacies of phenols. Since Galvinoxyl is already stable, it accepting an electron or hydrogen will cause Galvinoxyl to reduce irreversibly. This result in decolorization that is stoichiometric to the number of electrons taken up. This type of methodology can give information regarding the quantity and reactivity of the added compounds **(**Perez *et al.*, 2000). Galvinoxyl shows a strong absorption at 428nm in methanol. The main drawback is that the kinetics of the process is usually complex (Campos *et al*., 1997; Alliaga *et al*., 1998). This species usually owe their stability to a combination of odd electron delocalization and steric hindrance to dimerization.

2.5.4 Total Phenols Assay by Folin-Ciocalteu Reagent (FCR)

FCR was initially intended for the analysis of proteins taking advantage of the reagent's activity towards protein tyrosine (containing a phenol group) residue (Folin *et al*., 1927). Many years later, Singleton and co-workers extended this assay to the analysis of total phenol in wine and since then the assay has found many applications (Singleton *et al.,* 1999). FCR assay is commonly known as total phenol (phenolic) assay. FCR actually measures a sample's reducing capacity but this is not reflected in the name "total phenolic

assay". FC reagent is non-specific to phenolic compounds (e.g. vitamin C). Phenolic compounds react with FCR only under basic conditions (adjusted by sodium carbonate solution). Dissociation of a phenolic proton leads to a phenolate anion, which is capable of reducing FCR where a blue compound is formed between the phenolate and FCR. Disregarding the chemistry principles, the total phenol assay by FCR has the following advantages:

- FCR is commercially available and the procedure is rather standardized
- The long-wavelength absorption minimizes interferences from the sample matrix, which is often colored.
- It is commonly accepted assay and routinely practiced in dietary antioxidant research laboratories around the world.
- A large body of comparable data has been produced.

(Huang *et al*., 2005)

2.6 Animal Studies

As discussed in section 2.3.2, free radicals have positive and negative effects on human body. Many studies have been carried out using animal model to investigate the biological activities in seaweed extracts. Paya *et al*. (1993) and Raghavendran *et al*. (2004) have reported seaweed extracts given orally to animal effectively reduce inflammation, pain and ulcer lesion.

2.6.1 Introduction

i). Inflammation

As explained earlier, free radicals can be produced by environmental causes such as light or ionizing radiation. However, there are three physiological processes that can result in extraordinarily high levels of radical species in the body. These include the mixed-function oxidase systems of endoplasmic reticulum, the NADPH oxidase system of inflammatory cells and the presence of high levels of autoxidation-mediating charge-transfer agents. Production of these activated species can exceed the capacity of local protective mechanisms and produce tissue injury (Proctor, 1989). Unbalance between ROS and its inhibitors or antioxidants can stimulate anti-inflammatory pathways, or damage lipids, proteins or DNA (Rice-Evans, 1990). Physiologically, the production of ROS is found in all aerobic organisms and arises from the secondary production of superoxide, hydrogen peroxide and the reaction of superoxide with nitric oxide (peroxynitrite) during metabolic and extra-metabolic processes of all cells. Such oxidative damage occurs as one of the earliest aberrations in the disease indicates a major role of free radical damage in

both etiology and pathogenesis (Casadesus *et al.*, 2007). In addition to direct oxidation damage of cellular macromolecules, free radicals formation can lead to damage indirectly by activating other harmful mechanism such as inflammation (Smith *et al*, 1997). In this regard, oxidative stress inflammations are reciprocally linked such that inflammatory processes lead to an increase in ROS production (Wang *et al.*, 2004) and vice versa (Qin *et al.*, 2004). Figure 2.1 summarized the role of ROS in inflammation (Proctor, 1989).

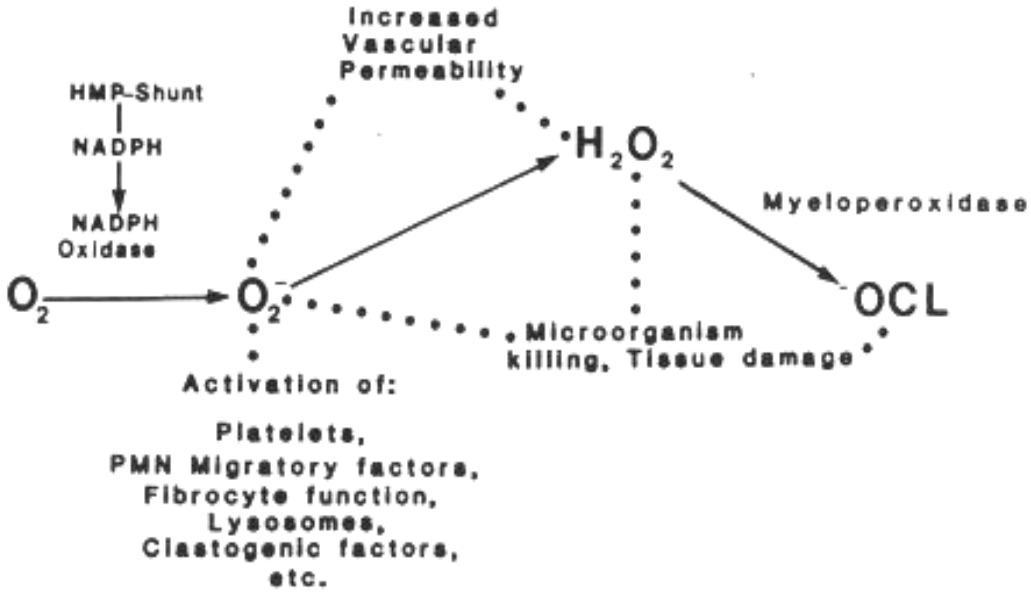

Figure 2.1: Role of reactive oxygen species in inflammation.

(Proctor, 1989)

Given this interdependence, it is perhaps not surprising that ROS and inflammation can both be attenuated by individually targeted treatments, i.e., antioxidant or non-steroidal anti-inflammatory drugs (NSAIDs) treatment (Hou *et al.*, 2003; Godbout *et al.*, 2004; Wang *et al.*, 2004). NSAID worked mainly through the inhibition of cyclooxygenase, which is a critical component of the inflammatory response (Sung *et al.*, 2004). Therefore, anti-inflammatory drugs such as NSAID have become the focus of several new treatment strategies (Gasparini *et al.*, 2004; Imbimbo, 2004). Non-steroidal anti-inflammatory drugs

will be discussed further in this chapter. The group of novel molecules that establish the link between inflammation and oxidative stress are prostaglandins and isoprostanes, respectively (Basu, 2003; Basu, 2004). Prostaglandin will also be discussed further in this chapter.

Inflammatory responses occur within tissue microenvironments. Such environments are complex and composed of many different cell types, often at different stages of activation and differentiation. Host responses to tissue injury involve a complex interplay of diverse cellular, humoral and connective tissue elements, which prevent tissue invasion and which, ultimately, re-establish normal tissue integrity (Buckley, 2003). During early stage of inflammation response, large numbers of leucocytes are recruited from the peripheral blood in response to injury of infection (Buckley *et al.*, 1997). Inflammation processes involve major cells of the immune system, including neutrophils, basophils, mast cells, T-cells, B-cells and others. However, examination of a range of inflammatory lesion demonstrates the presence of specific leucocytes in any given lesions. This is why the inflammatory process is regulated in such way as to ensure appropriate leucocytes are recruited, as described above. These events are controlled by a host of extracellular mediators and regulators including cytokines, growth factors, eicosanoids (prostaglandin, leucotrines and others), complement and peptides. These extracellular events are matched by equally complex intracellular signaling control pathways mechanism, with the ability of cells to assemble and disassemble an almost bewildering array of signaling pathways as they move from inactive to dedicated roles within the inflammatory response and site (Punchard *et al.*, 2004).

The classic description of inflammation accounts for the visual changes such as redness and swelling. The redness resulted from heat which is caused by the increased movement of blood through dilated vessels into the environmentally cooled extremities (due to the additional number of erythrocytes passing through the area). Swelling (oedema) is the result of increased passage of fluid from dilated and permeable blood vessels into the surrounding tissues, infiltration of cells into damage area and in prolonged inflammatory responses deposition of connective tissue. All these changes results in pain which is due to the direct effects of mediators, either from initial damage or that resulting from the inflammatory response itself and the stretching of sensory nerves due to oedema (Punchard *et al.*, 2004).

ii). Pain

Pain serves as a warning to protect an organism from injury and is one of the main reasons that a patient goes to a physician (Meyer *et al.*, 2005). During the inflammation produced by mild tissue damage, primary afferent fibers are activated and the pain produced is persistent (Omote *et al.*, 1998). Mild tissue damage, nerve damage and infection produce inflammation and associated pain. Besides the direct stimulation of peripheral afferents, noxious stimuli produce local pH changes, vascular permeability and local blood flow alterations, immune cells migration to the site of injury and release of cytokines (e.g. interleukins, tumor necrosis factor, interferons) and possibly opioids and the release from damaged cells of a ‘chemical soup’ of algogens, including cations (proton K+), kinins (bradykinin, kallidin), neurokinins (substance P, neurokinin A), prostanoids (prostaglandins, leukotrienes), purines (adenosine, ATP), amines (histamine, serotonin) and free radicals (e.g. nitric oxide) (Besson *et al.*, 1987; Treede *et al.*, 1992; Levine *et al.*,

1993; Dray, 1995). These events can result in long-term changes in the sensitivity of nociceptive afferents (i.e. peripheral sensitization) and the spinal neurons to which they project (i.e. central sensitization) (Coderre *et al.*, 1993; Dickenson, 1995; Woolf, 1995). Nociceptors rapidly transmit information about the degree and site of damage to the central nervous system (CNS) (Koppert, 2007).

Opioids are among the most powerful analgesics in clinical use for the treatment of nociception pain. Nevertheless, opioid treatment of neuropathis pain is often discouraged due to concern relating to the development of analgesic tolerance, the risk of addition and other debilitating adverse effects (Carver *et al*., 2001). Side effects of opioid treatment include nausea, vomiting, sweating, fatigue, difficult micturation, constipation, phychomimetic disturbance, dependence and immunosuppressive effects, particularly those which affect cell-mediated immunity (Beilin *et al.*, 1992). Opioids analgesics or opiates, have been used for thousands of years to treat pain. This class of agents produces potent analgesics effects by activating mu, delta or kappa opioid receptors. These receptors normally regulate nociceptions, mood and responses to stress by interacting with endogenous endorphins. However, these agents are not effective in the treatment of all types of chronic pain (Ashburn *et al.*, 2001; McQuay, 2001; Ballantyne *et al.*, 2003; Prous Science Drug R&D Backgrounds, 2005). Numerous nonopioid analgesics with various mechanisms of actions have been discovered and have been marketed or are under development. These agents include NSAIDs, cyclooxygenase type 2 (COX-2) inhibitors, nitric oxide synthase (NOS) inhibitors, superoxide dismutase mimetics, GABA receptors agonists, phospholipase A2 (PLA2) inhibitors, ion channel modulators, cannabinoids, nociception receptors antagonists, antidepressants and histamine antagonists, among others (Prous Science Drug R&D Backgrounds, 2005). Tolerance (analgesic) development is not

based on intensified pain sensation, but can be observed even without overt pain experience. However, not only decreasing analgesic (or analgesia, which refers to the relieve of pain) effects are observed clinically following administration of opioids (during long-term application), but pain may also increase above preexisting level of hyperalgesia (characterized by a lowered pain threshold and an increase pain sensitivity to mechanical and heat stimuli that are normally painful) may occur (Koppert, 2007).

NSAIDs are used clinically to decrease peripheral inflammation, as well as for depressing inflammation-induced pain. These effects occur by inhibition of COX, which catalyzes arachidonic acid to prostaglandin. While an obvious site of action of NSAIDs is in the periphery, at the site of the inflammation, recent work has suggested that a site of the analgesic action of COX inhibitors may also be central (Jurna *et al.*, 1990) especially at the level of the spinal cord (Jurna *et al.*, 1992). The peripheral mechanism of action has been suggested to be of an anti-inflammatory nature, which in turn attenuates sensitization of peripheral nociceptors, attenuates afferent nociceptive activity and thereby attenuates C-fiber mediated central sensitization (Dahl *et al.*, 1993). Peripheral sensitization can be observed particularly during inflammation and other pathological tissue changes. They can sensitize nociceptors locally by lowering their activation thresholds or *de novo* sensitize primarily insensitive, so-called ‘silent’ nociceptors (Bessou *et al.*, 1969; Meyer *et al.*, 1981; Reeh *et al.*, 1987). Central sensitization is characterized by the increased spontaneous activity and expansion of receptive fields of dorsal horn neurons (Raja *et al.*, 1984; LaMotte *et al.*, 1991; Ali *et al.*, 1996). One crucial event of this process is the activation of spinal N-methyl-d-aspartate (NMDA-) receptors by glutamate (Woolf *et al.*, 1991; Schaible *et al.*, 1991; Dickson, 1995). Central sensitization process can, thus, not

only initiate but also maintain pain conditions that long out-last the triggering event (Koppert, 2007).

iii). Ulcerogenesis

The distinction between erosions and ulcers depends on pathological and endoscopic definitions, with erosion defined as lesions confined to the mucosa (without endoscopically appreciable depth) and ulcers defined as lesions that penetrate to the level of submucosa (involving endoscopically evident depth) that would give rise to major bleeding, perforation or obstruction (Peura *et al.*, 2005).

Reactive oxygen species generated by the metabolism of arachidonic acid, platelets, macrophages and smooth muscle cells may contribute to gastric damage. Therefore, by scavenging free radicals, the reactive oxygen metabolites might be useful by protecting the gastric mucosa from oxidative damage or by accelerating healing of ulcers (Hahm *et al.*, 1997). Gastric mucus is an important protective factor for the gastric mucosa and consists of a viscous, elastic, adherent and transparent gel formed by 95% water and 5% glycoproteins that covers the entire gastrointestinal mucosa. Moreover, mucus is capable of acting as an antioxidant, and thus can reduce mucosal damage mediated by oxygen free radicals. The protective properties of the mucus barrier depend not only on the gel structure but also on the amount or thickness of the layer covering the mucosal surface (Penissi *et al.*, 1999). The decrease in gastric mucus renders the mucosa susceptible to injuries induced by acid, aspirin or cold restraint stress (Cross *et al.*, 1984). If some oxygen radicals are generated in surface epithelium containing mucus, intracellular mucus could scavenge them and prevent additional damage (Seno *et al.*, 1995).

Prostaglandins have been shown to increase the amounts of luminal mucus and the resistance of the gastrointestinal tract to injury in several experimental models. They are believe to exert their cytoprotective actions through the stimulation of mucus and bicarbonate secretion, the maintenance of mucosal blood flow and by enhancing the resistance of epithelial cells to injury induced by cytotoxins (Repetto *et al.*, 2002). Inhibition of prostaglandin synthesis is another reason that evokes formation of mucosal disturbance besides free radicals (Vasiliauskas *et al.*, 2004). Cyclooxygenase (COX) is the enzyme responsible for conversion of arachidonic acid to prostaglandins. COX exists in 2 isoforms (Vane, 1971). COX-1 is a ubiquitous constitutive isozyme producing prostaglandins responsible for homeostatic functions such as maintenance for gastrointestinal (GI) mucosal integrity. COX-2 is largely a cytokine-induced isozyme producing prostaglandins that mediate pain and inflammation. Problem arises when NSAIDs inhibit both COX-1 and COX-2 to varying degree (Crofford *et al.*, 2000). The therapeutic effects of conventional NSAIDs are derived from inhibition of COX-2, while the adverse effects of these agents, particularly in the upper GI tract, arise from inhibition of COX-1 activity (Gierse *et al*. 1995; Gierse *et al.*, 1999). COX will be discussed further in this chapter.

Most anti-inflammatory drugs have been associated with gastrointestinal side effects. Gastrointestinal mucosa damage can range from endoscopic lesions with no clinical manifestations to serious upper gastrointestinal complications (UGIC) that, in some instance, may be fatal (Rodriguez *et al.*, 2001). As explained earlier, an example of an anti-inflammatory drug is NSAID. The association between NSAIDs and gastrointestinal erosion and ulcers is well established. The relative risk for experiencing

serious adverse GI events is approximately three times greater for NSAIDs users than for non-users (Gabriel *et al.*, 1991). Several risk factors are known substantially an individual's risk for NSAID-induced GI events (Laine, 2001). These include a history of ulcer, presence of *Helicobacter pylori* infection, use of more than one NSAID (including aspirin), use of high-dose NSAIDs, concurrent anticoagulant or corticosteroid use, a serious underlying disease and age greater than 75 years (Peura *et al.*, 2005).

Helicobacter pylori (*H. pylori*) have been recognized as a causative agent for gastritis and gastroduodenal ulcer. Furthermore, it has been identified as an important risk factor for gastric cancer (Yamaguchi *et al.*, 2001; Janulaityte –Gunther *et al.*, 2003). *H. pylori* infection has been associated with generation of reactive oxygen species (ROS), which leads to oxidative stress (OS) in gastric mucosa (Naito *et al.*, 2002). *H. pylori* induce infiltration and activation of neutrophils, which produce inflammatory mediators that include ROS (Ernst, 1999). According to National Institute of Health Consensus Development Panel on *Helicobacter pylori* in Peptic Ulcer Disease, 1994, in patients who are not using NSAIDs, *H. pylori* infection is present in more than 95% of those with duodenal ulcer and 80% of those with gastric ulcer.

Besides all mentioned above, the excessive secretion of hydrochloric acid (HCl) acid in the stomach is considered an important factor in the formation of ulcer (Walpole *et al*., 1940; Hay *et al.*, 1942). HCl acid is known to produce ulceration and digestion of the stomach tissue (Dragstedt, 1917) as well as to reduce the neutralizing capability of the stomach mucus secretions (Florey *et al.*, 1935; Rider *et al.*, 1965). High levels of acid secretion increase the risk of an ulcer developing, where the basic mechanism of ulcer formation involves a disruption of mucosal resistance. A peptic ulcer may develop

whenever the balance between acid, pepsin and mucosal resistance shifts towards acid and pepsin. Stated simply, this shift may occur because of excess levels of acid and pepsin, reduced mucosal resistance. Traditional teaching has emphasized the important of acid as the cause of this imbalance. For example, a person with high levels of acid secretion, such as in the Zollinger-Ellision syndrome, have a high incidence of duodenal ulceration (Peterson, 1990).

2.6.2 Arachidonic Acid and Prostaglandin

Arachidonic acid (AA), a 20-carbon polyunsaturated fatty acid, is normally found esterified to cell membrane glycerophospolipids. In response to several stimuli, AA can be released from these cellular pools by phospholipases and can serve as a precursor to several biological active compounds. The three major enzymatic pathways for AA oxidation are the cycloxygenase (COX) pathway, the lipoxygenase (LOX) pathway and the cytochrome P450 monoxygenase pathway (Fitzpatrick *et al.*, 1989; McGiff, 1991; Capdevila *et al.*, 1992). AA is a slippery molecule that owes its mobility to its four *cis* double bonds. These are the source of its flexibility, keeping the pure fatty acid liquid, even at subzero temperature and helping to give mammalian cell membrane their correct fluidity at physiological temperature. This can happen nonenzymatically, contributing to oxidative stress or through the actions of the three of oxygenase as listed previously (COX, LOX, cytochrome P450). While the products of these enzymes and of the nonenzymatic transformations have well-substantiated bioactivities, unchanged arachidonic acid itself has biological activity (Brash, 2001). Arachidonic acid is converted to prostaglandin G_2 (PGG_2) within the COX site, which is reduced to prostaglandin H_2 (PGH_2) by the

peroxidase site, before conversion to the biologically important derivatives prostaglandin E_2 (PGE_2), prostacyclin, thromboxane A_2 (TXA_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) through the cell (Smith and Marnett, 1991).

Prostaglandin is a major mediator of the inflammatory response and lowers nociceptors thresholds, thereby potentiating the effects of agents that cause pain (Vane, 1971). Prostaglandins are formed in numerous types of cells within an organism. PGE_2 can be produced by PGE synthase from many different cell types including neurons, endothelial cells and neutrophils. Their effects are complex and depend on the type of target cells. Prostaglandin is important in the regulation of thrombocyte aggregation, inflammatory processes, pain and fever induction, the regulation of vessel perfusion and many other processes. PGE_2 released in inflamed tissue sensitize the terminal of afferent nerve fibers, thereby enhancing nociceptive processing within the spinal cord and brain to evoke hyperalgesia (Svensson *et al.*, 2002). In the joints, prostaglandin induces and perpetuates inflammation by causing vasodilation, allowing an influx of more inflammatory cells and mediators. In the upper gastrointestinal (GI) tract, they protect the mucosal lining by reducing acid secretion and by increasing the production of mucus and bicarbonate. In the kidney, they are necessary to maintain renal function when renal perfusion is reduced and they are necessary for normal platelet (Huang, 2000).

According to the mechanism of action put forward by Vane (1971), the unwanted side effects of NSAIDs can also be explained which arise from a blockade of the physiological effects of prostacylin, PGE_2 and thromboxane A_2 (Steinmeyer, 2000).

2.6.3 COX and NSAIDs

A prostaglandin deficiency caused by NSAIDs is due to cyclooxygenase (COX) inhibition. There are two isoforms of COX which are COX-1 and COX-2. COX-1 is found constitutively expressed in various tissues, including the stomach (Feng *et al.*, 1993), while COX-2 does not appear to be expressed, or at least at very low levels in most tissues and is rapidly up regulated in response to growth factors and cytokines (Kennedy *et al.*, 1993). This tissue specificity leads to the contention that COX-1 is critical for housekeeping action in the gastric mucosa (Chan *et al.*, 1995), whereas COX-2 is responsible for inflammation (Langenbach, 1995). Indeed, studies using selective COX-2 inhibitors showed that the ulcerogenic property of NSAIDs is brought about by inhibition of COX-1 but not COX-2 (Futaki *et al.*, 1993). However, Wallace *et al.* (2000), recently showed that inhibition of both COX-1 and COX-2 is required for NSAID-induced gastric injury, suggesting a "housekeeping" role of COX-2 as well as COX-1 in the stomach (Tanaka *et al.*, 2001).

Cyclooxygenase (COX), also known as prostaglandin H synthase, is the key enzyme in the synthesis of prostaglandins (PGs). Elucidation of the two COX isoforms gave rise to the concept that the constitutive enzymes COX-1 is responsible for the production of the PGs with homeostatic functions in tissue such as the stomach, kidney and platelets, whereas COX-2, the inducible enzymes is responsible for the production of proinflammatory PGs (Langenbach *et al.*, 1995; Seibert *et al.*, 1997; and Vane, 1998). There is extensive evidence based on animal as well as human studies supporting the role of COX-2 in the development of inflammation (Tomlinson *et al.*, 1994; Seibert *et al.*, 1997). Animal models of inflammation have demonstrated that COX-2 mRNA and protein

as well as PGs increase in a time-dependent manner parallels in the inflammatory process (Anderson *et al.,* 1996). Inflammatory cytokines and endotoxins can induce a 10- to 80-fold increase in the level of COX-2 expression in monocytes, macrophages, chondrocytes, fibroblast and endothelial cells (Anderson *et al.,* 1996; Buttar, 2000). The contribution of COX-2 to inflammation is further supported by demonstration that the expression of COX-2 and production of PGs can be inhibited by anti-inflammatory cytokines and glucocorticoids (Ristimaki *et al.,* 1996; Fernandez-Morata *et al.,* 2004). But, it is now believed that COX-1 is responsible for the initial prostanoid response to inflammatory stimuli, whereas COX-2 becomes the major contributor to prostanoid synthesis as inflammation progresses (Langenbach *et al.,* 1995; Noguchi *et al.,* 1996; Gilroy *et al*., 1998; Tilley *et al*, 2001). COX-1 mRNA has a half-life of about 12 to 15 hours, whereas COX-2 has a shorter half-life of less than 3.5 hours (Lukiw and Bazan, 1997), suggesting a close temporal link between tissue injury, COX-2 expression, and elevated PGE_2 in comparison to constitutively expressed COX-1 (Khan *et al.,* 2007).

2.6.4 Nonsteroidal anti-inflammatory drugs (NSAIDs)

The nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely used medications in the USA because of their demonstrated efficacy in reducing pain and inflammation (Abramson *et al*., 2005). More than one-third of the elderly take NSAIDs daily and 70% reported taking NSAIDs at least once a week (Talley *et al.,* 1995).

Traditional NSAIDs can be grouped into three classes based on their modes of inhibition of COX:

Class I : Simple, competitive, reversible inhibitors that compete with arachidonic acid for binding to the COX active site.

Ibuprofen, Piroxican, Sulindac sulfide, Flufenamate, Mefenamic acid and Naproxen are included in this class.

Class II : Competitive, time dependent, reversible inhibitors that bind to the COX active site in a first phase to form reversible enzyme inhibitor complexes, that if retained for a sufficient time, cause a non covalent conformational change in the protein, associated with tighter binding.

Indomethacin, Flurbiprofen, Meclofenamic acid and Diclofenac are included in this class.

Class III: Competitive, time dependent, irreversible inhibitors that form an enzyme inhibitor complex after a covalent conformational change in the protein.

Aspirin also known as Dispirin is included in this class.

The acetylation of COX-1 by aspirin is an irreversible process that inhibits COX activity but not the peroxidase activity.

(Vane *et al.*, 1998; Smith *et al.*, 1995; and Meade *et al.*, 1993)

Although there are minor differences in the mechanism of NSAIDs, they are all inhibitors of COX enzymes, thus, they prevent conversion of arachidonic acid into prostaglandin. Although nonspecific inhibition of prostaglandin synthesis is beneficial in terms of reducing inflammation and pain, it may cause upper GI, renal and platelet dysfunction (Huang, 2000). In the upper GI tract, prostaglandin E protects the mucosal lining by decreasing acid secretion and by increasing bicarbonate and mucus production. By inhibiting prostaglandin production, NSAIDs have been shown to cause endoscopically demonstrable upper GI ulcer as well as other serious upper GI complications, including perforation, gastric outlet obstruction and clinically significant upper GI bleeding (Silverstein *et al.*, 1995). Therefore, many researches have been done and are going on to search an alternative to NSAIDs.

3.0 MATERIALS AND METHODS

3.1 Samples Used In This Study

In this study, 16 samples were used which comprised of nine seaweeds, two microalgae, two teas, two dried fruit skins and one dried flower. All the seaweeds used were collected from Cape Rachado and Pantai Dickson, Port Dickson except for *Kappaphycus alvarezzi, Eucheuma denticulatum* and *Gracilaria changii* that were bought from Sabah by the Algae Lab, University Malaya. As for the microalgae, both *Spirulina platensis* and *Chlorella vulgaris* were grown in the Algae Lab, University Malaya using the stock culture from UMACC (University Malaya Algae Culture Centre). The two different teas were bought, where 1 was taken from the Lipton Tea sachet while the other tea was bought from Vietnam. Dried fruit skins used were from pomelo and mangosteen (obtained from supervisor). As for dried flower sample, *Bougainville*a spp. (Paper Flower) was used. Various scientists have studied some of the samples stated above. All samples studied were briefly discussed in this chapter and summarized in Table 3.1.

i.) ***Kappaphycus alvarezzi*** **(a)** Doty ex P. Silva

Figure 3.1: *Kappaphycus alvarezzi*

Common name: Eucheuma

Family: Areschougiaceae

Colour: Light brown

Collected: Bought from LKIM, Sabah and arrived at lab on April 2005

Location: Pulau Sibangkat, Sabah

This sample is turgid and brittle. It has spiny branches and its thalli may reach several kilograms in weight. It branches but is not truly apposite. It tends to grow scattered among soft and hard corals in tropical reef areas. *K. alvarezii* was reported to possess antioxidant activity and it was suggested that these extracts could be considered for curing disease arising from oxidative damage (Kumar *et al*., 2008).

ii). ***Gracilaria changii*** Abbott, Zhang, and B. Xia

Figure 3.2: *Gracilaria changii*

Family: Gracilatiaceae

Colour: Dark green

Collected: Bought from LKIM, Sabah and arrived at lab on April 2005

Location: Pulau Sibangkat, Sabah.

This seaweed have thalli and irregular branches. It is slightly swollen at distal end of stipe, tapering towards the tip. The transverse section of frond shows thick walled, large and rounded medullary cells. Zandi *et al*. (2007) found that crude water of *G. salicornia* exhibit anti-viral activity against Herpes Simplex Virus type 2.

iii). *Kappaphycus alvarezzi* (b)

Common name: Eucheuma

Family: Areschougiaceae

Colour: Light brown

Collected: Collected on 25 May 2005 and was bought from LKIM, Sabah on 1 June 2005. Sample arrived at lab on 6 Jun 2005.

Location: Pulau Sibangkat, Sabah

This sample is turgid and brittle. It has spiny branches and its thalli may reach several kilograms in weight. It branches but is not truly apposite (Figure 3.1). It tends to grow scattered among soft and hard corals in tropical reef areas.

iv). ***Eucheuma denticulatum*** Burman Collins and Hervey

Figure 3.3: *Eucheuma denticulatum*

Common name: Eucheuma

Family: Areschougiaceae

Colour: Dark brown

Collected: Collected on 25 May 2005 and was bought from LKIM, Sabah on 1 June 2005. Sample arrived at lab on 6 Jun 2005.

Location: Pulau Sibangkat, Sabah

This sample is similar to *K. alvarezii* but with spiny branches. Thalli may reach several kilograms in weight. It is turgid, brittle and branching not truly opposite. Its colour ranges from light to dark brown. *E. denticulatum* is a red seaweed and is used mainly for its polysaccharide, carrageenan. Carrageenan had shown several potential pharmaceutical properties such as antitumor (Zhou *et al*., 2004), antihyperlipidemic (Panlasigui *et al*., 2004) and anticoagulant activities (Caceres *et al*., 2000).

v). ***Gracilaria salicornia*** C. Agardh Dawson

Figure 3.4: *Gracilaria salicornia*

Common name: Agar-Agar Caocaoyan

Family: Gracilariaceae

Colour: Dark brown

Collected: 9 June 2005

Location: Pantai Dickson, Port Dickson.

Sample was found near the shore and attached to small rocks as well as coral reefs. Its thalli consist of solid, brittle, cylindrical to compressed branches. A study by Sasidharan *et al*. (2009) reported antimicrobial activity of 5 different extracts of *G. changii*.

vi). ***Caulerpa racemosa*** Forsskal J. Agardh

Figure 3.5: *Caulerpa racemosa*

Common name: Sea grapes

Family: Caulerpaceac

Colour: Green

Collected: 9 June 2005

Location: Cape Rachado, Port Dickson. Sample attached to coral reefs.

It is commonly found on sandy or muddy bottom in shallow water. They have grape like appearance and can produce several vertical branches that extend above the substrate. A study carried out by Chew *et al*. (2008) reported antioxidant activity in 50% aqueous methanol extracts of *C. racemosa.*

vii). ***Padina tetrastromatica*** Hauck

Figure 3.6: *Padina tetrastromatica*

Family: Dictyotaceae

Colour: Green

Collected: 9 June 2005

Location: Cape Rachado, Port Dickson.

Samples were found attached to coral reefs. Its thalli are usually divided into several small lobes, regularly and distinctly. It is easily recognized due to dark double lines of sporangia, enclosing a line of colourless hairs in between. It has blades composed of two layers of cells. *P. tetrastromatica* was reported to possess anticoagulant (Prasada Rao *et al*., 1984) due to the presence of alginic acid.

viii). ***Turbinaria conoides*** J. Agardh Kützing

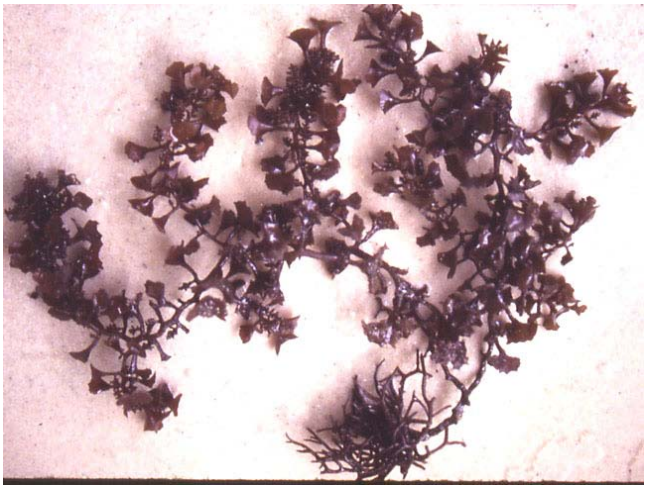

Figure 3.7: *Turbinaria conoides*

Common Name: Agar-Agar lesong

Family: Sargassaceae

Colour: Dark brown

Collected: 9 June 2005

Location: Cape Rachado, Port Dickson.

This sample is dark brown in colour. It is differentiated into holdfast, stipes, fronds, and fruiting bodies. It has a hard texture and attaches to coral, rocks and found in shallow tropical reef flats. It is also often found near *Sargassum* spp. A study carried out by Sheu *et al*. (1999) reported that oxygenated fucosterols isolated from *T. conoides*, showed cytotoxicity activity against various cancer cell lines.

ix). ***Sargassum binderi*** Sonder

Figure 3.8: *Sargassum binderi*

Common Name: Horsetail tangle

Family: Sargassaceae

Colour: Dark brown

Collected: 9 June 2005

Location: Cape Rachado, Port Dickson. Sample attached to coral reefs.

It is brown in colour. It is differentiated into holdfast, stipes, fronds, fruiting bodies and there are gas filled bladders. It is often found near coral reefs. They attach to coral or rocks. Ara (2001) found significant hypolipideamic, and cyctotoxicity activities of *S. binderi* ethanolic and methanolic extracts respectively.

x). *Chaetomorpha* spp. Kützing

Figure 3.9: *Chaetomorpha* spp.

Family: Cladophoraceae

Colour: Dark brown

Collected: 9 June 2005

Location: Pantai Dickson, Port Dickson. Sample attached to mangrove roots.

They have fine, unbranched filaments, consisting of large cells arranged in a single row end to end and green in colour. The individual filaments can be straight, curled or twisted while the whole plant can be either clumps of straight filaments, like a clump of grass, or a tangled mat. *Chaetomorpha* spp, was reported to possess bioactive compounds such as terpenoids, steroids and aminoacids which are antioxidant compounds (Sambamurty, 2005).

xi). Malaysian Tea (Lipton Brand)

Figure 3.10: Malaysia tea (Lipton brand)

Scientific Name: *Camellia sinensis*

Family: Theaceae

Colour: Light brown

Collected: Tea was packaged on April 2005

Location: Shopping mall

Lipton Yellow Label is a black tea blended from as many as 20 different teas. These teas were carefully selected, processed, packed and sold all over the world. Mehrabian (2007) have reported that black tea contains antioxidant, antimutagenic and anticarcinogenic activities.

xii). Vietnamese Tea

Figure 3.11: Vietnamese tea

Scientific Name: *Camellia sinensis*

Family: Theaceae

Colour: Dark brown

Collected: No collection date. Sample was given by supervisor.

Location: Vietnam

Vietnamese black tea is produced in many areas that have been known for tea-house "retreats" for hundreds of years, for example, located amidst immense tea forests of the Lamdong high lands. This is a cultural tourist area that produces a distinctive Vietnamese-flavored tea and has nearly 100-year old tea roots that are very hard to find and maintain.

xiii). Mangosteen Skin

Figure 3.12: Mangosteen

Scientific Name: *Garcinia mangostana*

Family: Clusiaceae

Colour: Dark brown

Collected: No collection date. Sample was given by supervisor.

Location: Malaysia

Mangosteen has a deep purple leathery skin which is often thrown away. Its interior has 5 to 7 segments of white flesh, some containing seed. Tadtong *et al*. (2009) recently reported mangosteen skin extract exhibited antityrosinase and antibacterial activities. Xanthones extracted from mangosteen skin was reported to possess anti-cancer effect (Akao *et al*., 2008).

xiv). Pomelo Skin

Figure 3.13: Pomelo

Scientific Name: *Citrus grandis*

Family: Rutacea

Colour: Light yellowish

Collected: No collection date. Sample was given by supervisor.

Location: Malaysia

The peel of the pomelo is usually used in Chinese cooking. The peel is often used in southern Chinese cuisine for flavouring. The peel is thick and sometimes used to make marmalade. Pomelo is an interesting source of pectin where it is used in various food products. It was reported by Wang *et al*. (2002) that the main source of pectin is from citrus fruit skin.

xv). *Bougainvillea* spp.

Figure 3.14: *Bougainvillea* spp.

Common name: Paper Flower

Family: Nyctaginaceae

Colour: Pink

Collected: No collection date. Sample was given by supervisor.

Location: Malaysia

The flower is small and pink. It is called “paper flower” because the bracts are thin and papery. Bougainvillea species flower have not been studied extensively. There are many studies carried out on its leaves which have shown anti-diabetic activity (Bates *et al*., 2000). A study carried out by Ali *et al*. (2005) showed that extract of pink colour flower inhibit the growth of plant.

xvi). *Spirulina platensis*

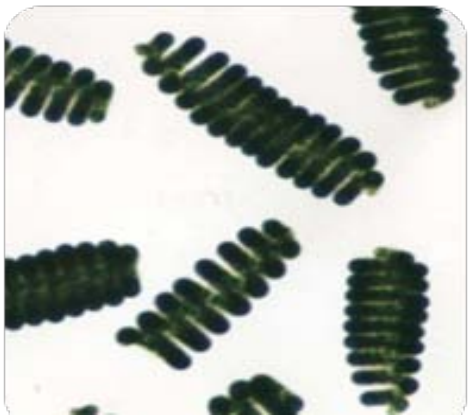

Figure 3.15: *Spirulina platensis*

Family: Cyanobacteria

Colour: Green

Collected: Sample was grown on 8 November 2005 using UMACC 161 and was harvested on the 18 November 2005.

Location: Algae lab, University Malaya

Spirulina is a photosynthetic, filamentous, spiral-shaped, multicellular and green-blue microalgae. It has been shown to be an excellent source of proteins, vitamins and minerals. Different fractions obtained during the phycocyanin purification process of *S. platensis* were reported to possess antioxidant activity (Estrada *et al*., 2001).

xvii). *Chlorella vulgaris*

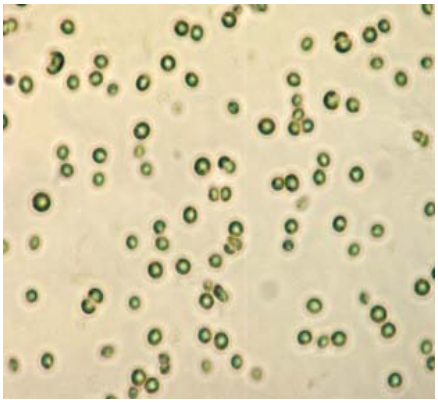

Figure 3.16: *Chlorella vulgaris*

Family: Oocystaceae

Colour: Green

Collected: Sample was grown on 8 November 2005 using UMACC 001 and was harvested on the 18 November 2005.

Location: Algae lab, University Malaya

Chlorella, a green microalgae is extremely high in enzymes, vitamins and minerals, including the full vitamin-B Complex. A study carried out by Rodriguez-Garcia *et al*. (2008) showed antioxidant activity in *C. vulgaris* ethanolic extract.

Samples	
Seaweed	*Kappaphycus alvarezzi* (a)
	Gracilaria changii
	Kappaphycus alvarezzi (b)
	Eucheuma denticulatum
	Gracilaria salicornia
	Caulerpa racemosa
	Padina tetrastromatica
	Turbinaria conoides
	Sargassum binderi
	Chaetomorpha spp.
Tea	Malaysian Tea (Lipton Brand)
	Vietnamese Tea
Fruit skin	Mangosteen Skin
	Pomelo Skin
Flower	*Bougainvillea* sp.
Microalgae	*Spirulina platensis*
	Chlorella vulgaris

Table 3.1: List of samples used in this study

3.2 Preparation of Samples

3.2.1 Collection of Seaweeds

Seaweeds were collected at Cape Rachado (02° 24' 58N, 101° 51' 24E), Pantai Dickson (02° 25' 02N, 101° 53' 41E), Negeri Sembilan, Malaysia. They were stored in iceboxes and transported from the collection site to Algae Laboratory on the same day. Seaweeds that were collected were *Chaetomorpha* spp, *Gracilaria salicornia, Turbinaria conoides, Caulerpa racemosa, Padina tetrastromatica* and *Sargassum binderi*. *Caulerpa racemosa* was washed and cleaned at the collection site. Then, it was blotted dry on tissue paper and kept in a plastic bag filled with silica gel to absorb all the water from it. This was also done to prevent *Caulerpa* from rotting and loosing its originality. *Kappaphycus alvarezzi, Gracilaria changii* and *Eucheuma denticulatum* were collected from Sabah. As for *Kappaphycus alvarezzi,* there were two different samples where *Kappaphycus alvarezzi* (a) was bought fresh from Sabah while *Kappaphycus alvarezzi* (b) were kept in the Algae lab for some period of time.

In the laboratory, the fresh seaweeds were stored at -20°C prior to washing. The seaweeds were cleaned as they were covered with dirt, epiphytes and fragments of seashells and coral. Tap water was used for washing, as the salt from the seaweeds provided seawater like environment to prevent hypertonic condition. The seaweeds were washed thoroughly and were fan dried for 3 days. *Sargassum binderi, Turbinaria conoides, Padina tetrastromatica* and *Caulerpa racemosa* were separated into 2 parts whereby 1 part was fan dried for 3 days and another part was blotted dry and ground using liquid nitrogen (cryogenic grinding). The seaweeds that were ground using liquid nitrogen were kept in plastic tube covered with aluminum foil to prevent light exposure. All the seaweeds were

then transported to Physiology department. The seaweeds that were ground using liquid nitrogen was kept at -79˚C. As for the seaweeds that were fan dried, they were dried again in an oven at the Physiology department in Medical faculty of University Malaya. Seaweeds were oven dried at 40˚C - 42˚C for three days. After 3 days, the seaweeds were ground using a grinder and the ground samples were kept in separate plastic bags at room temperature. The drying and grinding methodology of the seaweeds were summarized in Figure 3.17.

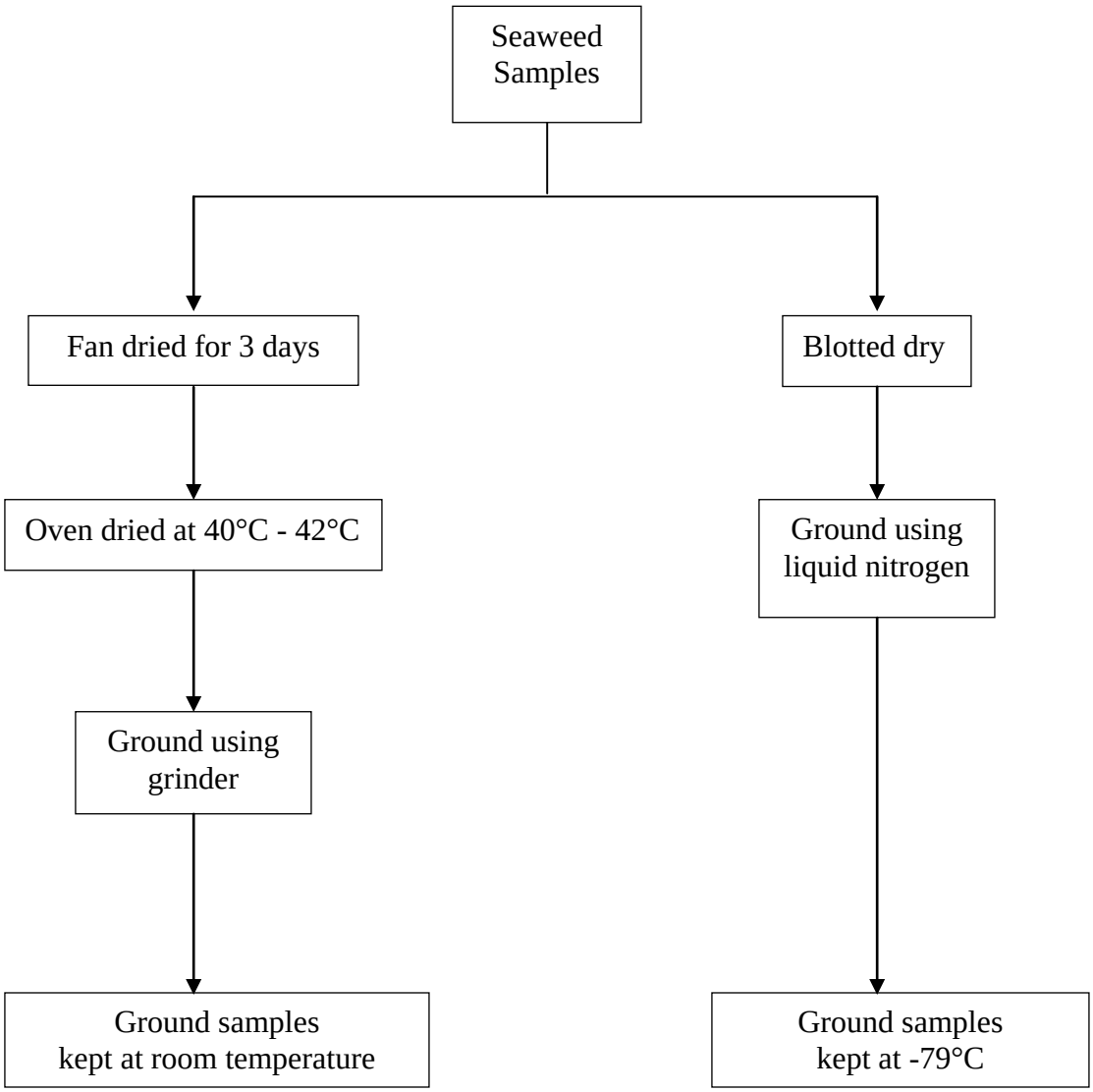

Figure 3.17: Flow chart of seaweeds drying and grinding methods

3.2.2 Microalgae harvesting

Two microalgae, *Spirulina platensis* (UMACC 161) and *Chlorella vulgaris* (UMACC 001) obtained from the University Malaya Algae Culture Centre (UMACC), were grown in Algae Lab, University Malaya. *Spirulina platensis* was cultured on Kosaric agar medium and *Chlorella vulgaris* was cultured on BBM agar medium for 2 weeks and these inoculums were transferred into their respective medium to grow.

Bold's Basal Medium (BBM) (Nichols and Bold, 1965) was used to grow *Chlorella vulgaris* and Kosaric Medium (modified after Zarrouk, 1966) was used to grow *Spirulina platensis*. The chemicals used to prepare both mediums were described in Appendix 7.1 and 7.2. One liter of each medium was prepared and the solutions were distributed into 10 conical flasks (100 ml per flask). All the flasks were covered and autoclaved for 20 minutes at 121˚C. After autoclaving, the flasks were kept in culture room to cool down. After the medium have cool down, *Spirulina platensis* (10 % inoculums) was transferred into each flask that contain Kosaric medium and *Chlorella vulgaris* (10 % inoculums) were transferred into each flask that contain BBM medium under a sterile condition in the laminar flow. The flasks were then labeled and kept in the culture room. The flasks were placed on shaker to promote the growth of *Spirulina* and *Chlorella*. After 10 days, the cultures were harvested.

Spirulina platensis was filtered using a strainer to remove the unnecessary portion (clumps) of the *Spirulina* medium. Cultures of *Spirulina platensis* were transferred into sterile centrifuge tubes and centrifuged at 30,000 rpm for 5 minutes. After all the culture mediums were centrifuged, the pellet of the microalgae was collected into a single

centrifuge tube. One ml of liquid nitrogen was added into the centrifuge tube and mixed using vortex to break the cell walls of the microalgae. Both microalgae were kept in freezer at –80˚C till future use. This process was repeated for *Chlorella vulgaris* except for the filtration process.

3.2.3 Collection of other samples

Besides seaweeds and microalgae, other samples that were used for this study were dried mangosteen skin, dried pomelo skin, Malaysian Tea (from Lipton brand tea bag), Vietnam Tea (bought from Vietnam) and *Bougainvillea* sp. These samples were obtained in dry condition. Therefore, the samples were not oven dried but was ground using a grinder upon received and kept in separate plastic bags at room temperature.

3.3 Extraction of Samples

3.3.1 Extraction of samples for antioxidant assays

Extraction was done using methanol and boiled water. Besides methanol and boiled water, acetone was also used to extract selected samples. Two different methods were used where forward extraction (Fw) was done on all samples whereas reverse extraction (Rv) was done on selected samples which are *Gracilaria salicornia, Caulerpa racemosa, Padina tetrastromatica, Turbinaria conoides, Sargassum binderi, Chaetomorpha* spp. All the samples were weighed using their dry weight except *Spirulina platensis* and *Chlorella*

vulgaris where they were weighed using their wet weight. Extraction method of samples was summarized in Figure 3.18.

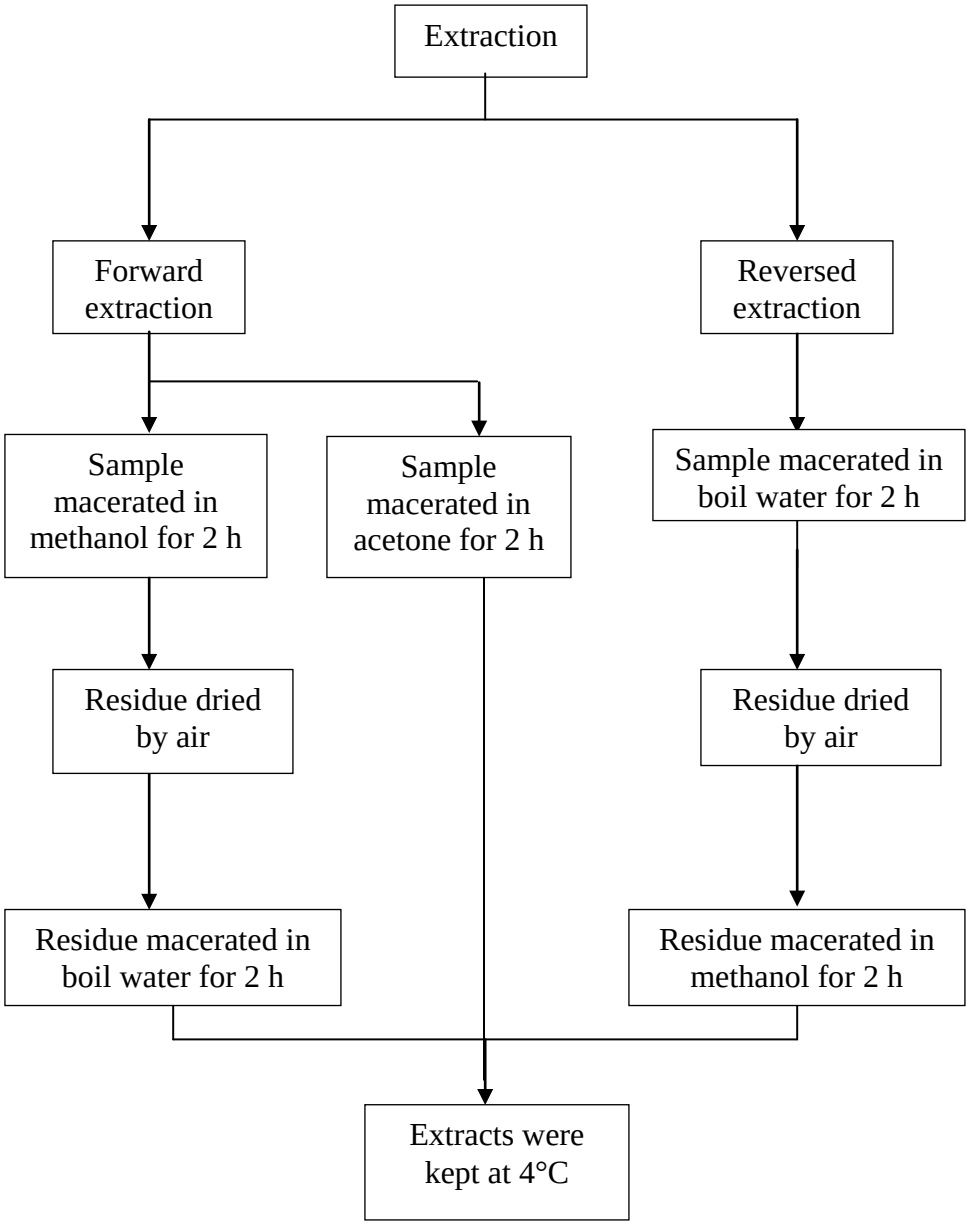

Figure 3.18: Flow chart of samples forward and reverse extraction.

i) Forward Extraction (Fw)

a). Methanol Extraction

0.8g (dry weight, except for microalgae where 0.8g wet weight was used) of samples were macerated with 8ml of methanol in test tubes and spun for two hours using Rugged Rotator at 80% speed. Test tubes were covered with aluminum foil and sealed with parafilm to minimized light exposure. After 2 hours, the test tubes were centrifuged at 5,000 rpm. The extracts (supernatant) were pipetted and kept in plastic tubes covered with aluminum foil. These tubes were then sealed and kept in freezer at 4˚C.

b). Boiled Water Extraction

The residues from methanol extraction were air dried to evaporate methanol from the residue. After the samples were completely dried, the residues were macerated in 8 ml of boiled water and spun for 2 hours using Rugged Rotator at 80% speed. The same extraction method used for methanol extraction was repeated for boiled water extraction. The extracts were kept in plastic tubes covered with aluminum foils, sealed and stored in freezer at 4˚C.

ii). Reverse Extraction (Rv)

Reverse extraction was carried out by samples macerated in boiled water first followed by methanol. The residues were oven dried after maceration with boiled water to evaporate all the water content before methanol extraction. Extracts were kept in plastic tubes, covered with aluminum foiled, sealed and stored in freezer at 4˚C. Flow chart of forward and reverse extractions was described in Figure 3.18.

iii). Acetone extraction

Acetone extraction was carried out on *Gracilaria salicornia, Caulerpa racemosa*, *Padina tetrastromatica*, *Turbinaria conoides*, *Sargassum binderi*, *Chaetomorpha spp*, Malaysian tea and Vietnamese tea. The same extraction method used for methanol extraction was used for acetone extraction (Figure 3.18).

3.3.2 Preparation of Seaweed Extracts for Animal Tests (Pharmacology Test)

i). Extraction of Sample

Seaweeds that were used for pharmacology test were *Padina tetrastromatica* and *Kappaphycus alvarezzi* (b) where these seaweeds were ground earlier for antioxidant tests. *P. tetrastromica* and *K. alvarezzi* were weighed, macerated with ethyl acetate and stirred for 3 days. The solvent extracts were filtered and the residues were air dried till ethyl acetate was evaporated off. After the residues were totally dried, the residues were macerated in ethanol and stirred for 3 days. The solvent extracts were filtered after 3 days.

The extracts were then concentrated using rotary evaporator until the solvents were completely absent from the extracts. Then concentrated extracts of *P. tetrastromatica* (both ethyl acetate and ethanol) was scrap, weighed, kept in a sterile bottle and stored in the freezer at 4˚C. As for *K. alvarezzi* ethanol extract, the concentrated extract was freeze dried as *K. alvarezzi* contains high level of water. After freeze drying, the extract was weighed and kept in freezer at 4˚C.

ii). Preparation of Dose Concentration

For *P. tetrastromatica*, both extracts of ethyl acetate and ethanol were used whereas for *K. alvarezzi*, only alcohol extract was used. *Padina* ethyl acetate extracts and ethanol extracts were weighed and dissolved with Tween 80 (Polyoxyethylenesorbitan Monooleate) because *Padina*'s extract could not be dissolve in distilled water. Tween 80 is a hydrophilic surfactant which acts as a compound that enhances solubility of compounds (Malingre et al., 2001). Each extract was stirred well and respective volume of distilled water was added to obtain the desired concentration. Extract solutions were kept in freezer

at 4°C. *K. alvarezzi* was dissolved in distilled water without Tween 80 as *Kappaphycus* can dissolve in water. Extract solution was kept in freezer at 4°C.

3.4 Antioxidant Assay

3.4.1 Ascorbic acid Equivalent Capacity (AEAC) assay

AEAC assay is a decolorizing assay using $ABTS^+$ free radicals that produce blue green color in solution. The color will be reduced in the presence of antioxidants when samples were added. This assay assessed antioxidant activity by comparing with ascorbic acid, which was tested in parallel.

i). Ascorbic Acid preparation

1.2mM ascorbic acid was prepared by dissolving 0.0021g ascorbic acid in 10 mL of deionized water. Concentrations ranging from 0 mM to 1.2 mM were prepared.

ii). $ABTS^+$ Stock Solution preparation

0.00769 g of $ABTS^+$ was dissolved in 20 ml of deionized water, followed by the addition of 0.0132 g of potassium persulfate. The solution was kept in a dark bottle for 12 to 16 hours at room temperature to give a dark blue solution. After that, 1 mL of the solution was aliquot into eppendorf tubes and kept in the freezer at –20°C till use.

iii). Phosphate Buffered Saline (PBS) Preparation

1 x PBS working solution was prepared by dissolving 0.1 g potassium dihydrogen phosphate (KH_2PO_4) and 4 g sodium chloride (NaCl) in 900ml of deionized water. The pH of the solution was adjusted to pH 7.4 and the solution was topped to 1 liter after the desired pH was achieved.

iv). AEAC assay procedure

AEAC working solution was prepared by diluting 1ml of the prepared stock solution ($ABTS^+$ solution) in 19 ml of PBS solution (5mM, pH 7.4). From the working solution, 200µl was dispensed into each of microtitration plate and a blank reading was taken at 630 nm using Tecan Reader, Sunrise model (03930005700). 20 µl of standard acid ascorbic (vitamin C), ranging from 0.0mM to 1.2mM were added into the working solution or samples of methanol or boiled water extracts. Each standard and samples were tested in duplicates where the standard was tested in parallel with the samples to serve as its individual standards. Each plate has its own standard. The absorbance was taken after 6 minutes at 630 nm. The change of the absorbance between the negative control and samples were calculated. The ascorbic acid antioxidant capacity for each sample and ascorbic acid was expressed as percentage of inhibition using the following formula:

$$\text{Inhibition \%} = \left[\frac{\text{Negative control} - \text{Sample or Standard after 6 min}}{\text{Negative control}} \right] \text{X } 100$$

A dose response linear regression was generated using ascorbic acid data and the percentage of sample inhibition was calculated. Antioxidant capacity of the samples was then determined by comparing to that of ascorbic acid and expressed as ascorbic acid equivalent capacity in mili molar (mM). Figure 3.19 summarized the AEAC assay.

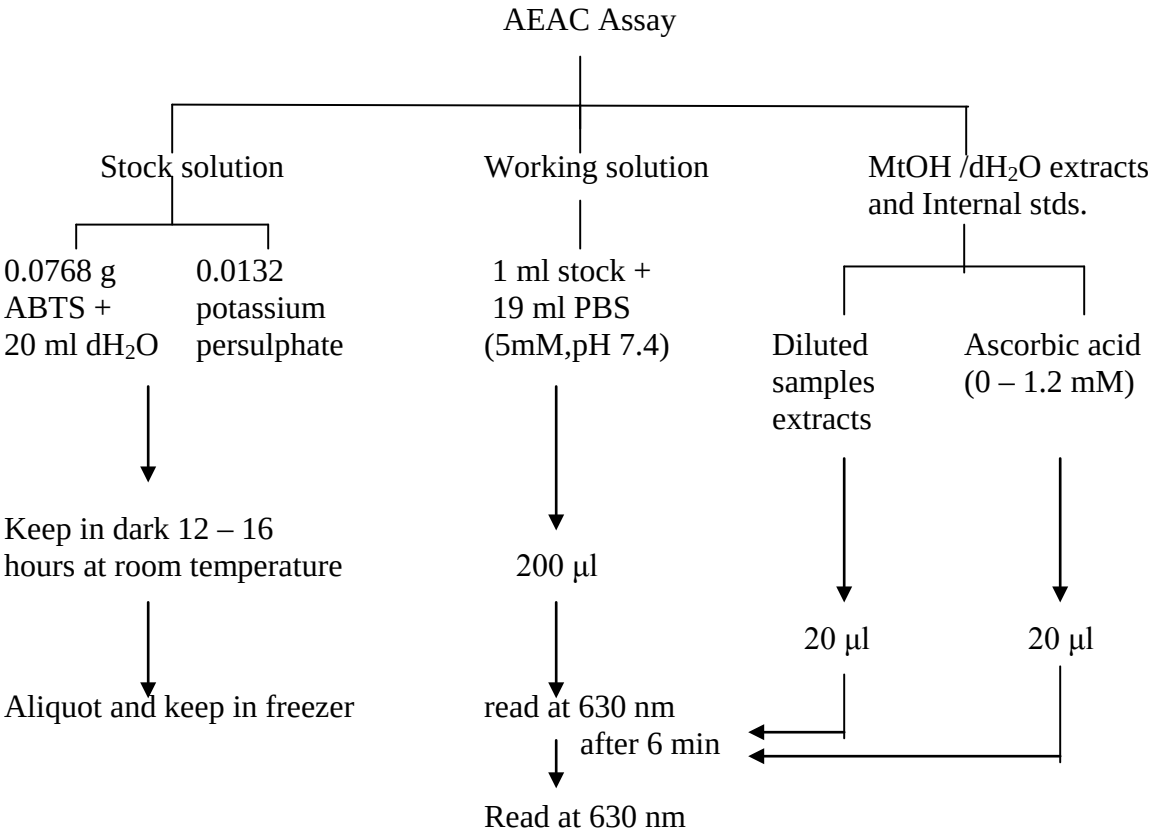

Figure 3.19: Flow chart of AEAC assay

3.4.2 Diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay

This assay evaluates the electron-donating ability of antioxidant by using Diphenyl-1-picryl-hydrazyl-hydrate. This compound is characterized as a stable free radical by its property of the delocalization of the electron pair over the molecule as a whole, thus the molecule do not dimerise as would be the case of most other free radicals (Molyneux, P., 2003). This assay was done using ascorbic acid as a standard to compare with both boiled water and methanol samples extraction.

i). Ascorbic acid preparation

1.2 mM ascorbic acid was prepared by dissolving 0.0021g ascorbic acid in 10 mL of deionized water. Concentrations ranging from 0 mM to 1.2 mM were prepared.

ii). DPPH solution preparation

0.008% w/v DPPH was prepared by dissolving 0.008g of DPPH in 100mL of 99.7% v/v min denatured ethyl alcohol (46.07g/mol). This solution was prepared in dark as DPPH solution is light sensitive. After preparation, solution was kept in freezer till use.

iii). DPPH assay procedure

This assay was divided into 3 major parts, which were ascorbic acid standard (DPPH solution and ascorbic acid), negative control [for boiled water (DPPH solution and boiled water), for methanol (DPPH solution and methanol)] and sample (DPPH solution and boiled water or methanol extracts). First, 20µl of samples or standards or negative control were dispensed into microtitration plate. Then, 180µl DPPH was added with the standards, samples and negative control. All samples, standards and negative control were

tested in duplicates. The standards and negative control were tested in parallel with the samples to serve as its individual standards and control where each plate has its own standard. The OD reading was taken at 515nm, using Tecan Reader, Sunrise model, 10 minutes after adding the DPPH solution into the wells. While adding the DPPH solution, the surrounding area must be dark as the DPPH solution is sensitive to light. The wells, which contain the samples and DPPH, will give the optical density (OD) reading of the antioxidant activity of the samples. Antioxidant capacity of each sample was then determined by comparing to that of ascorbic acid standard graph and expressed in percentage. Figure 3.20 shows the flow chart of DPPH assay. The final value was obtained by using the calculation below:

$$\% \text{ Inhibition} = \left(\frac{\text{Abs negative control} - \text{Abs standard or sample}}{\text{Abs negative control}} \right) \text{X } 100$$

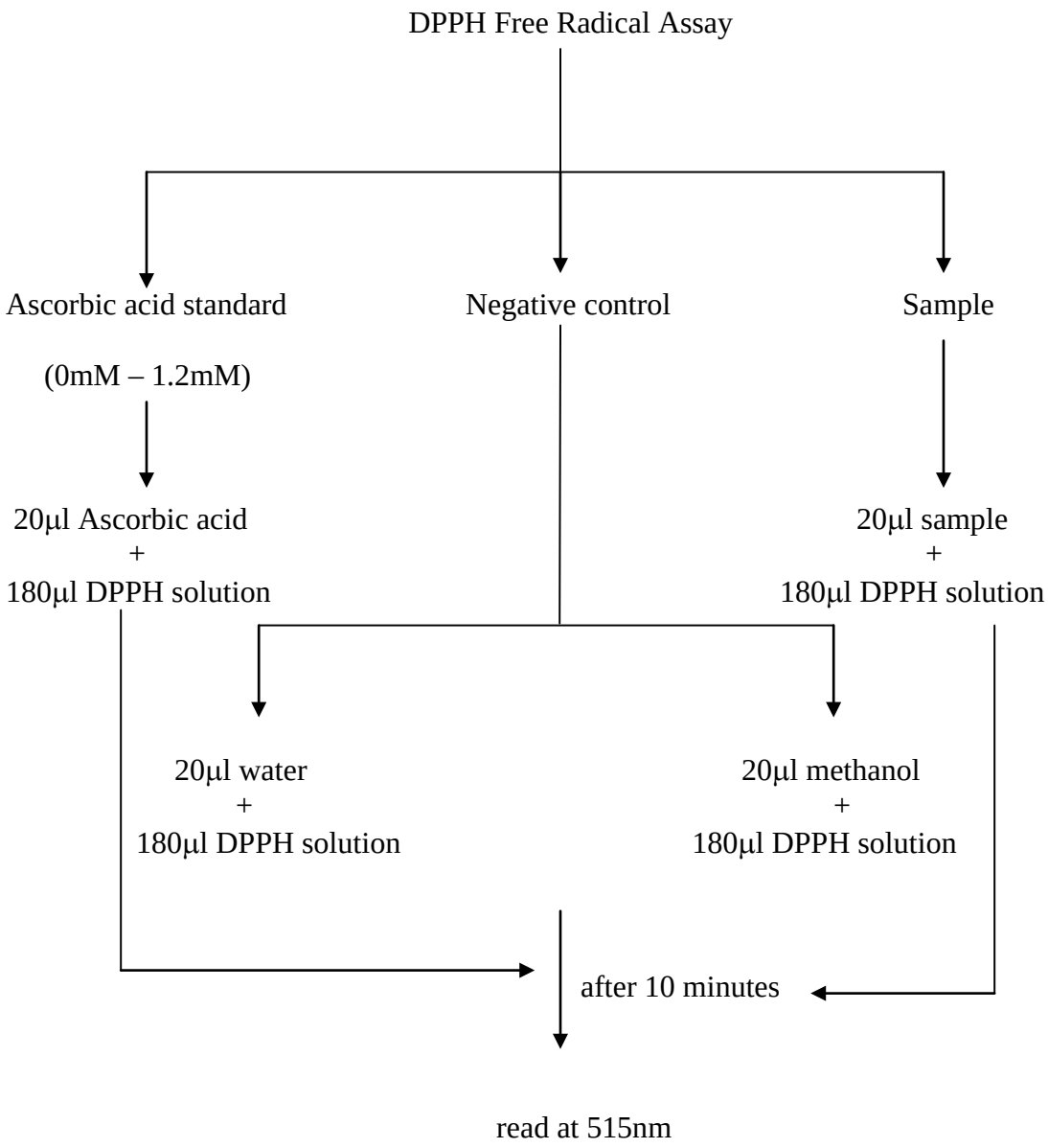

Figure 3.20: Flow chart of DPPH assay

3.4.3 Galvinoxyl Free Radical Quenching (GFRQ) Assay

Galvinoxyl can be reduced by hydrogen-donating free radical scavengers. The result of decolorization is stoichiometric with respect to the number of hydrogen taken up.

i). Galvinoxyl stock solution preparation

Galvinoxyl stock solution was prepared by dissolving 0.0042g of Galvinoxyl in 2ml of methanol.

ii). Working solution preparation

GFRQ working solution was prepared fresh on the day of the experiment and was kept in a dark bottle to prevent degradation of free radicals. The final concentration of the working solution was set at 10μM. The solution was prepared in 95% methanol.

iii). GFRQ assay procedure preparation

The standard that was used for this assay was trolox. A serial dilution of trolox ranging from 0μL to 250μL was used. Standard is required in order to produce a dose linear regression graph. This assay could only assess methanolic extracts. Samples were tested in duplicates. 200μL of freshly prepared galvinoxyl was dispensed into microtitration plate and the initial blank reading of the reagent was taken at 428 nm. The absorbance reading of the samples and standards dispensed into the wells containing the reagent was taken again after 20 minutes. Negative control for this assay was boiled water and methanol.

The percentage inhibition of Galvinoxyl was calculated using the formula shown below:

$$\text{Inhibition \%} = 100 - \left\{ \left[\frac{A_{428nm} \text{ at } 20^{th} \text{ minute}}{A_{428nm} \text{ at } 0 \text{ minute}} \right] \mathbf{X}\ 100 \right\}$$

A dose response linear regression graph was generated using Trolox data. The inhibition percentage of the end point reading of each extracts was related to the graph generated and expressed as µM (TEGx, Trolox equivalent galvinoxyl quenching activity). The scavenging activity and inhibition of each sample was compared with those in the same range of concentration. Figure 3.21 illustrated the flow chart of the assay.

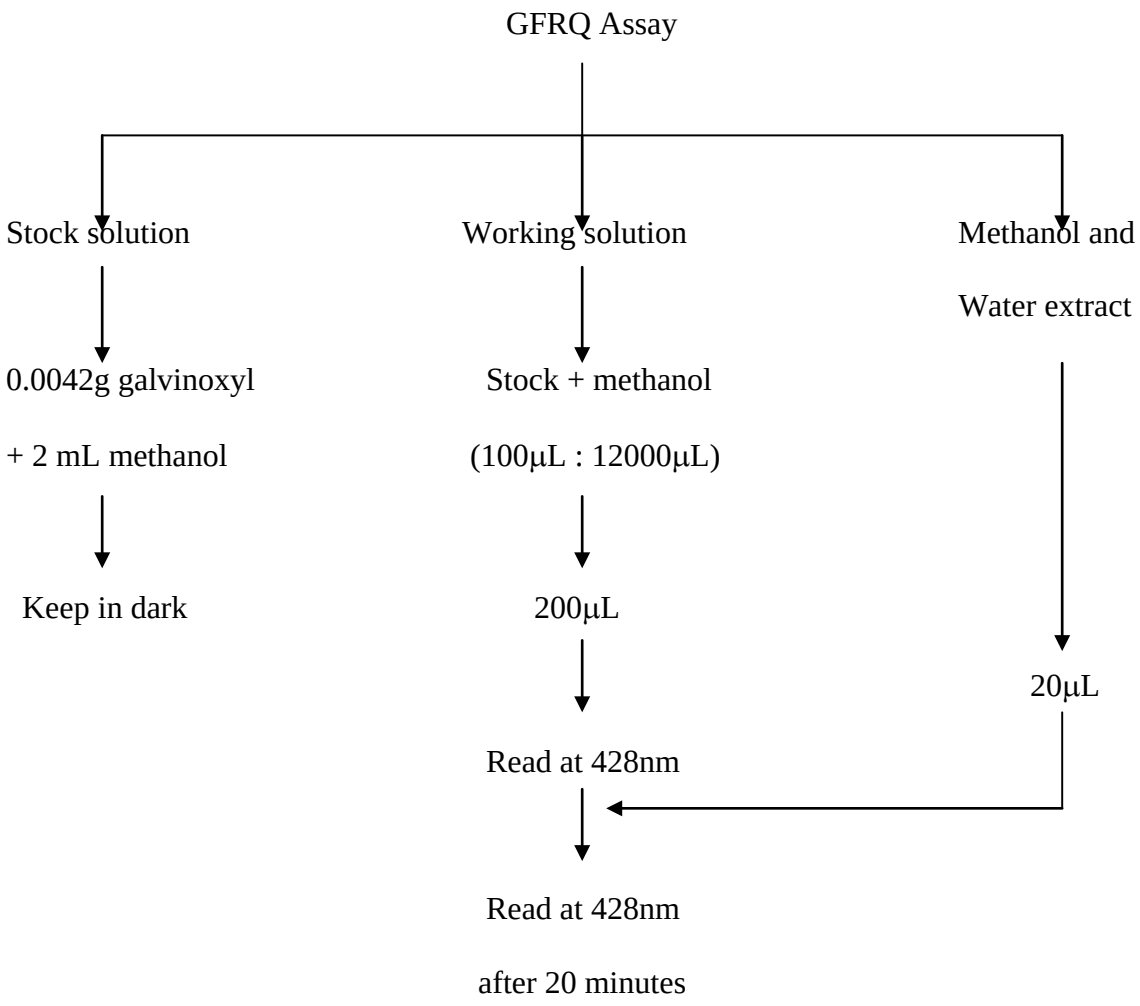

Figure 3.21: Flow chart of Galvinoxyl Free Radical Quenching assay

3.4.4 Total Phenolic Content

This Total phenolic assay was done using Folin – Ciocalteu reagent (FCR) and gallic acid as standard and 15 % Sodium Carbonate (Na_2CO_3) solution.

i). Folin – Ciocalteu Reagent (FCR)

FCR solution is light sensitive, therefore it must be kept in a dark bottle. FCR solution has an intense yellow colour. When there are any electrons transfer occur between the FCR and sample extracts, the solution will turn blue. If the solution was contaminated, a green colour solution would occur.

ii). Sodium Carbonate (Na_2CO_3) solution preparation

Sodium carbonate was prepared by adding the appropriate amount of deionized water into sodium carbonate powder to obtain a 15% solution concentration. This solution was prepared fresh each time a test was run.

iii) Gallic acid preparation

Gallic acid was used as a standard for this test. A stock of 80 mg/ml of gallic acid was prepared by adding 80mg of gallic acid into 1ml of methanol. Then, standards ranging from 0μg/ml to 400μg/ml were prepared from the stock. Methanol was used in gallic acid solution preparation because gallic acid powder only dissolves in methanol.

iv). Total Phenolic assay

First, 20µl of gallic acid serial dilution was dispensed into the microtitration plate. Then 20µl of samples' extracts were added into the wells. Then, 100µl of Folin-Ciocalteu reagent was added into the microtitration plate containing the extracts and gallic acid. While adding FCR reagent, the area must be in dark. After 5 minutes, 80µl of Sodium Carbonate (Na_2CO_3) (15% concentration) was added into the microtitration plate. Then, the microtitration plate was left for 1 hour 30 minutes before readings were taken using Tecan Reader, Sunrise model, at 700nm. Each standard and samples were tested in duplicates where the standard was tested in parallel with the samples to serve as its individual standards. Each plate has its own standard. The Total Phenolic content for each sample and gallic acid was expressed using the following formula:

$$\text{Inhibition} = \text{Absorbance} - \text{negative control}$$

From the equation, a standard graph of gallic acid was generated. Antioxidant capacity of each sample was then determined by comparing to that of gallic acid standard graph and expressed as gallic acid equivalent (GAE). Figure 3.22 summarized the Total Phenolic assay.

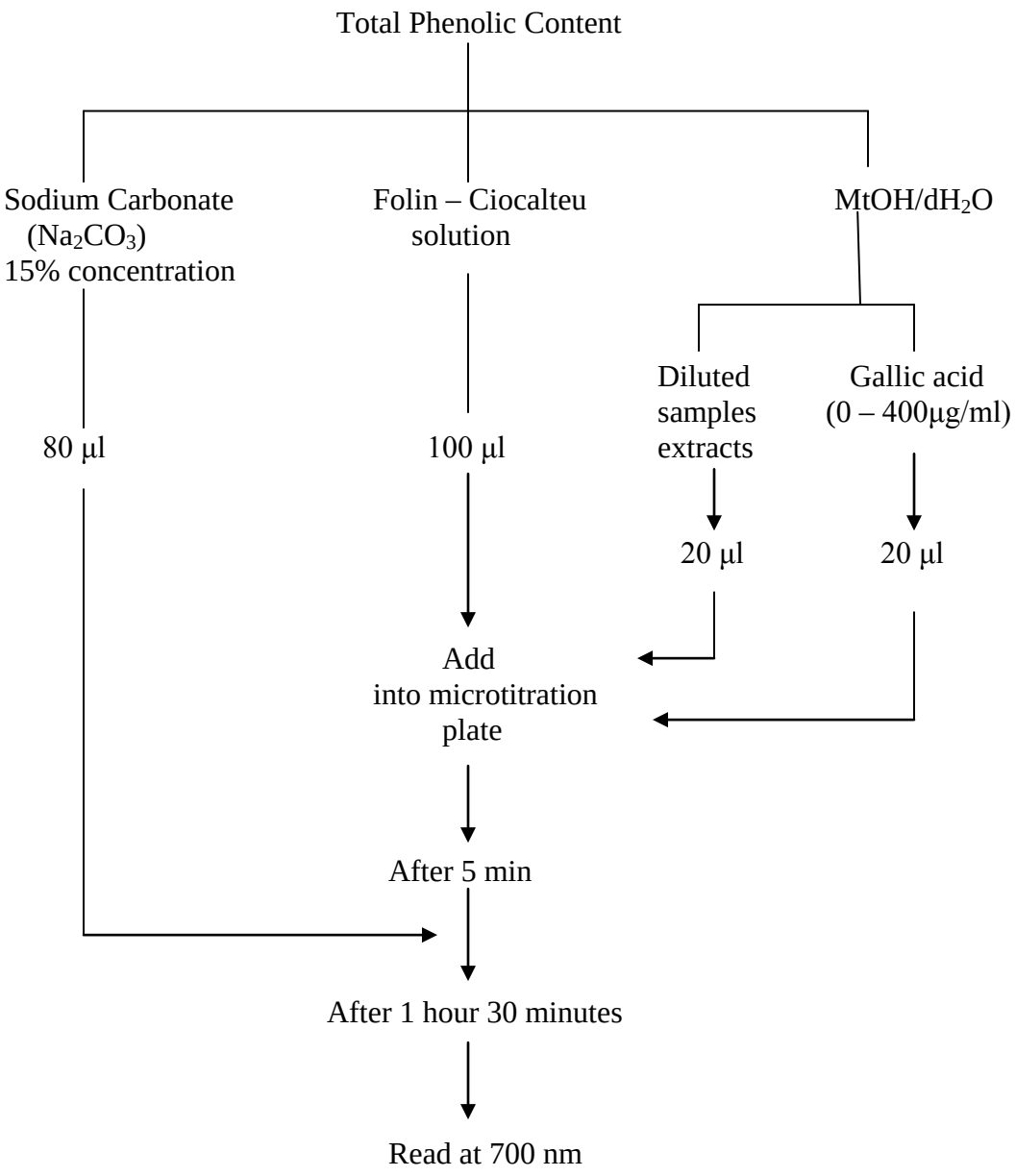

Figure 3.22: Flow chart of Total Phenolic assay

3.5 Animal Test

3.5.1 Determination of Lethal Dose (LD_{50})

100 mice (25 – 30 g each) were divided into 10 groups of 10 mice per group. These mice were weighed and marked. *Padina tetrastromatica* ethyl acetate extract, *P. tetrastromatica* ethanol extract and *Kappaphycus alvarezzi* alcohol were administrated intraperitoneal (i.p) into the mice where the volume varied from 1400 mg/kg to 2000 mg/kg. The time of administration was recorded and every 30 minutes the mice were examined carefully for any symptoms and abnormal behavior. The number of deaths in each group was also recorded. Percentage of deaths for each dose was determined. The probit units were obtained from the percentage of death using the statistical table (Finney, 1985). The probit units were plotted against log dose concentration of the volume of seaweed extracts used (Finney, 1985).

3.5.2 Anti-inflammatory activity (Carrageenan-induced rat paw oedema)

This test was performed in adult SD rats weighing 200-300 g. Rats were starved for 24 hours with free excess to water. Paw oedema in rats was induced by injection of 0.1 ml of 1% carrageenan into the plantar surface of the right hind paw (Winter et al, 1962). Then equal volume of saline was injected into the plantar surface of the left hind paw. Indomethacin (10 mg/kg), Dispirin (150 mg/kg) and 10% ethanol were given as control. Indomethacin was given intraperitoneally (i.p) where as dispirin, ethanol, *Kappaphycus alvarezzi* ethanol extracts (10, 30 and 100 mg/kg) and *Padina tetrastromatica* (10, 30 and 100 mg/kg) ethyl acetate and ethanol extracts were given orally 30 minutes prior to

carrageenan injection. The volumes of both hind paws of each rat were measured using Plethysmometer (Model 7140, Ugo Basile) (Figure 3.7) at every half-hour interval until the period of 7 hours after injection of carrageenan. Flow chart of anti-inflammatory study was shown in Figure 3.23. Hind paw swelling was calculated in a percentage by the formula below:

$$\% \text{ Swelling} = \left[\frac{Vr - Vr_0}{Vr_0} - \frac{V_l - V_{l0}}{V_{l0}} \right] X\ 100$$

Vr = Right paw volume

Vr_0 = Right paw initial volume

V_l = Left paw volume

V_{l0} = Left paw initial volume

Figure 3.23: Plethysmometer (Model 7140, Ugo Basile)

3.5.3 Plantar Test (Pain study)

Adult SD rats weighing 250-300 kg were used for this test. The rats were starved for 24 hours with free excess to water. 0.1 ml of 1% carrageenan was injected into the right hind paws of the rats followed by the same amount of saline into the left hind paw. Indomethacin (10 mg/kg), Dispirin (150 mg/kg) and 10% ethanol were given as control. Indomethacin was given intraperitoneally (i.p) where as dispirin, 10% ethanol, ethanol extracts of *Kappaphycus alvarezzi* (10, 30 and 100 mg/kg) and *Padina tetrastromatica* (10, 30 and 100 mg/kg) ethyl acetate and ethanol extracts were given orally 30 minutes prior to carrageenan injection. A thermocouple was placed under the heel of the hind paws (Figure 3.24) and the withdrawal latencies were recorded every 30 minutes for the duration of 7 hours 30 minutes. Figure 3.25 shows the flow chart of plantar test. Baseline value was recorded earlier before the hind paw was injected with carrageenan saline. Analgesic effect was detected when the right hind paw, injected with carrageenan prolong the withdrawal latencies compared to the baseline values (Hargreaves, K., et al., 1988).

Figure 3.24: A thermocouple was placed under the heel of the rat's hind paw

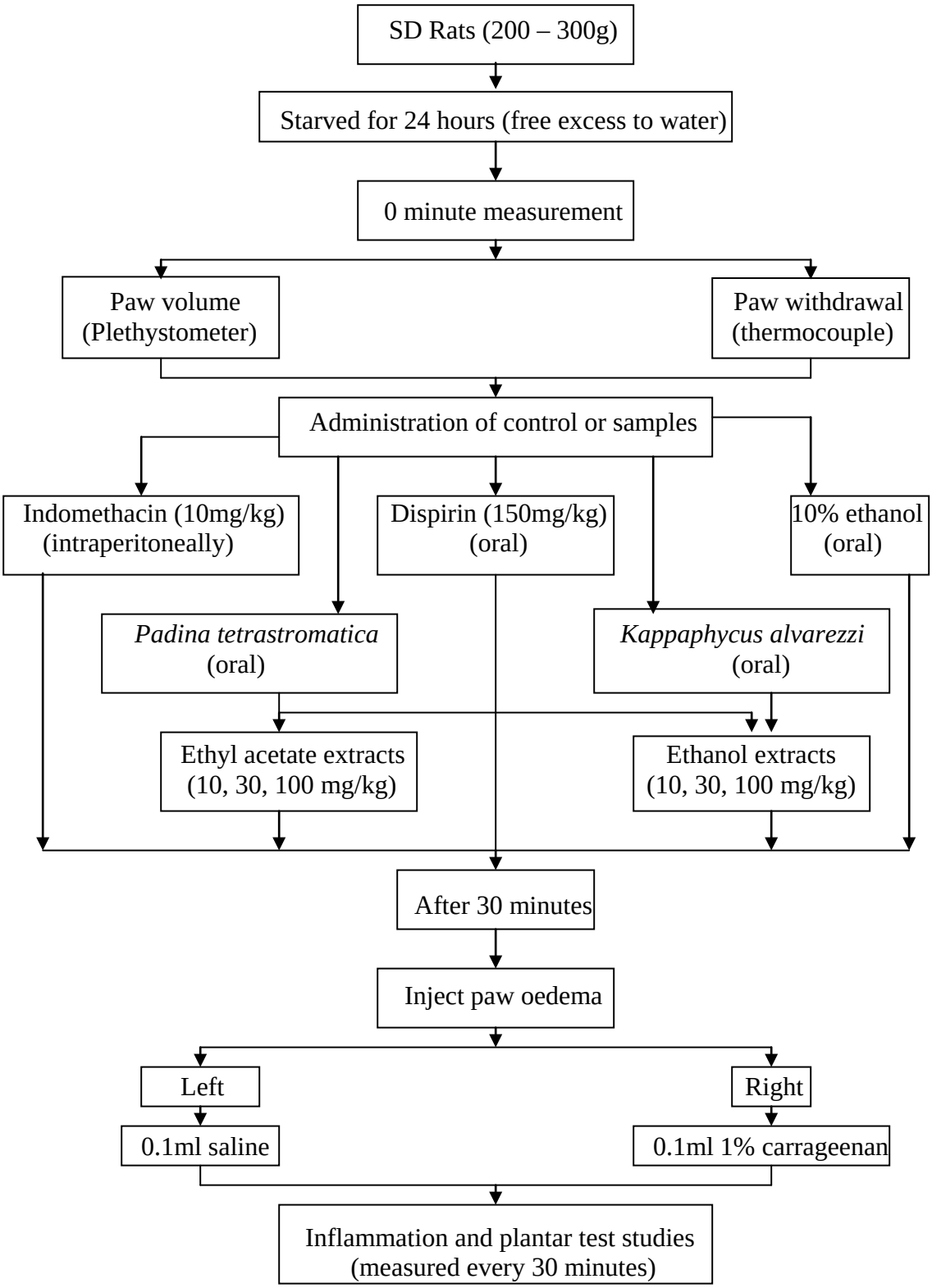

Figure 3.25: Anti-inflammation and plantar test studies procedures

3.5.4 Anti-Ulcerogenic Activity

This experiment was performed in adult SD rats, (n=6), weighing 150-250g. Rats were starved for 48 hours with free excess to water. In control group, the rats were given 10% Tween 80 solution and distilled water orally in 1 ml volume, while treated group were given *Padina tetrastromatica* and *Kappaphycus alvarezzi* ethanol extract 100mg/kg orally. After 30 minutes, the rats were given necrotic agents indomethacin (30mg/kg) and 0.6 M HCl (1ml) orally (Parmar, N.S., *et al*, 1987). After 8 hours, the rats were sacrificed by cervical dislocation and its stomach was dissected for examination of the total surface area of lesion. Photographs were taken (4.3 magnifications) and the lesion area of the stomach was traced with a transparent graph paper to count the total surface area of lesions in millimeter square (mm^2). Figure 3.26 shows the detailed method.

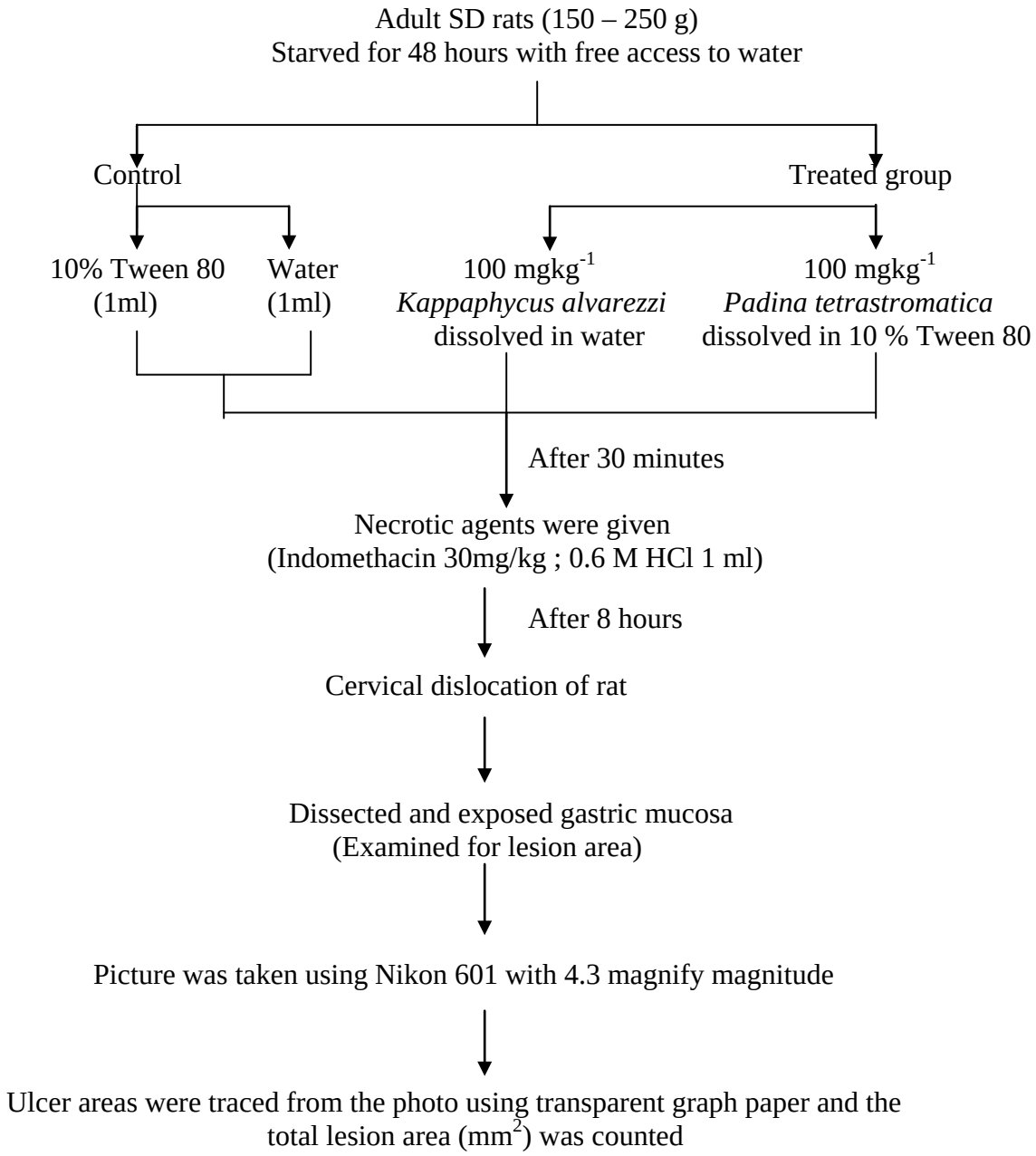

Figure 3.26: Flow chart of anti-ulcer procedure

4.0 RESULTS

4.1 Antioxidant Assays

A few different types of extraction methods were done which include; a) Forward extraction assay (extraction with methanol solvent followed by boiled water) (Fw) b) Reverse extraction assay (extraction with boiled water first followed by methanol solvent) (Rv) c) Grinding method using grinder (samples were oven dried and ground using grinder) using forward extraction d) Grinding method (samples were air dried and ground using liquid nitrogen) using forward extraction.

4.1.1 Ascorbic acid Equivalent Capacity (AEAC) assay

Ascorbic Acid Equivalent Antioxidant Capacity Assay (AEAC Assay) is a decolorization assay based on the scavenging of stable ABTS (2,2-azino-bis(3-ethylbenzo tiazoline-6-sulfonic-acid) radical cation. Therefore AEAC assay as explained by Rice-Evans and co-workers (1995) and Roberta *et al*. (1999) can be used to measure the antioxidant activity of a broad diversity of substances. In this study, the AEAC assay was carried out in screening 17 different types of samples consisting of seaweeds, micro algae, teas, fruit's skin and flower. Antioxidant activities were expressed in AEAC value (mM) for both methanol and boiled water extraction, by comparing to a dose linear regression of ascorbic acid. Ascorbic acid was used as control and was also tested at different temperature and time.

Table 4.1 showed that almost all the samples have lower AEAC value in methanol extract compared to boiled water extract. The highest AEAC value obtained for methanol extract was Vietnamese tea (2.14 mM). This was followed by Malaysian tea (2.08 mM), Mangosteen skin (1.51 mM), Pomelo skin (1.26 mM), *Chlorella vulgaris* (0.63 mM), *Bougainvillea* spp. (0.55 mM), *Padina tetrastromatica* (0.48 mM), *Spirulina platensis* (0.46 mM), *Turbinaria conoides* (0.31 mM), *Gracilaria salicornia* (0.26 mM), *Caulerpa racemosa* (0.19 mM), *Sargassum binderi* (0.17 mM), *Kappaphycus alvarezii* (b) (0.13 mM), *Kappaphycus alvarezii* (a) (0.05 mM), *Gracilaria changii* (0.04 mM) and *Eucheuma denticulatum* (0.03 mM). *Chaetomorpha* spp. (0.02 mM) gave the lowest AEAC value for methanol extract.

Table 4.1 showed that Vietnamese tea has the highest AEAC value for its boiled water extract which was 2.19 mM. This was followed by Malaysian tea (2.15mM) *S. binderi* (1.81mM), *P. tetrastromatica* (1.39 mM), Mangosteen skin (1.39 mM), Pomelo skin (1.34 mM), *Bougainvillea* spp. (1.27 mM), *T. conoides* (1.21 mM), *S. platensis* (0.78 mM), *Chaetomorpha* spp. (0.53 mM), *G. salicornia* (0.44 mM), *E. denticulatum* (0.27 mM), *K. alvarezii* (b) (0.14 mM) and *C. racemosa* (0.13 mM). The three lowest AEAC values for boiled water extract were *C. vulgaris* (0.11 mM), followed by *G. changii* (0.09 mM) and finally *K. alvarezii* (a) (0.04 mM).

Almost all boiled water extracts showed higher value when compared with methanol extract (Figure 4.1) except for four samples which showed the other way. This can be seen in *K. alvarezii* (a) where it has higher AEAC value in methanol extract (0.05 mM) compared to boiled water extract (0.04 mM), *C. racemosa* where its AEAC value in methanol extract was 0.19 mM and for boiled water extract was 0.13 mM, Mangosteen

skin where its methanol extract AEAC value was 1.51 mM compared to its boiled water extract 1.39 mM and finally *C. vulgaris* where its AEAC value for its methanol extract was 0.63 mM compared to its boiled water extract 0.11 mM.

Table 4.1: Antioxidant activities of forward methanol and boiled water extracts evaluated with ABTS Assay.

Samples	AEAC value (mM)			
	Forward extraction			
	Methanol	SD	Boiled water	SD
Kappaphycus alvarezii (a)	0.05	0.00	0.04	0.00
Gracilaria changii	0.04	0.01	0.09	0.01
Kappaphycus alvarezii (b)	0.13	0.00	0.14	0.01
Eucheuma denticulatum	0.03	0.02	0.27	0.01
Gracilaria salicornia	0.26	0.03	0.44	0.02
Caulerpa racemosa	0.19	0.02	0.13	0.01
Padina tetrastromatica	0.48	0.03	1.39	0.02
Turbinaria conoides	0.31	0.00	1.21	0.03
Sargassum binderi	0.17	0.00	1.81	0.03
Chaetomorpha spp.	0.02	0.01	0.53	0.07
Malaysian tea	2.08	0.02	2.15	0.00
Vietnamese tea	2.14	0.04	2.19	0.00
Mangosteen skin	1.51	0.05	1.39	0.05
Pomelo skin	1.26	0.02	1.34	0.00
Bougainvillea spp.	0.55	0.01	1.27	0.00
Spirulina platensis	0.46	0.02	0.78	0.02
Chlorella vulgaris	0.63	0.02	0.11	0.00

Forward extraction indicates methanol followed by boiled water extraction

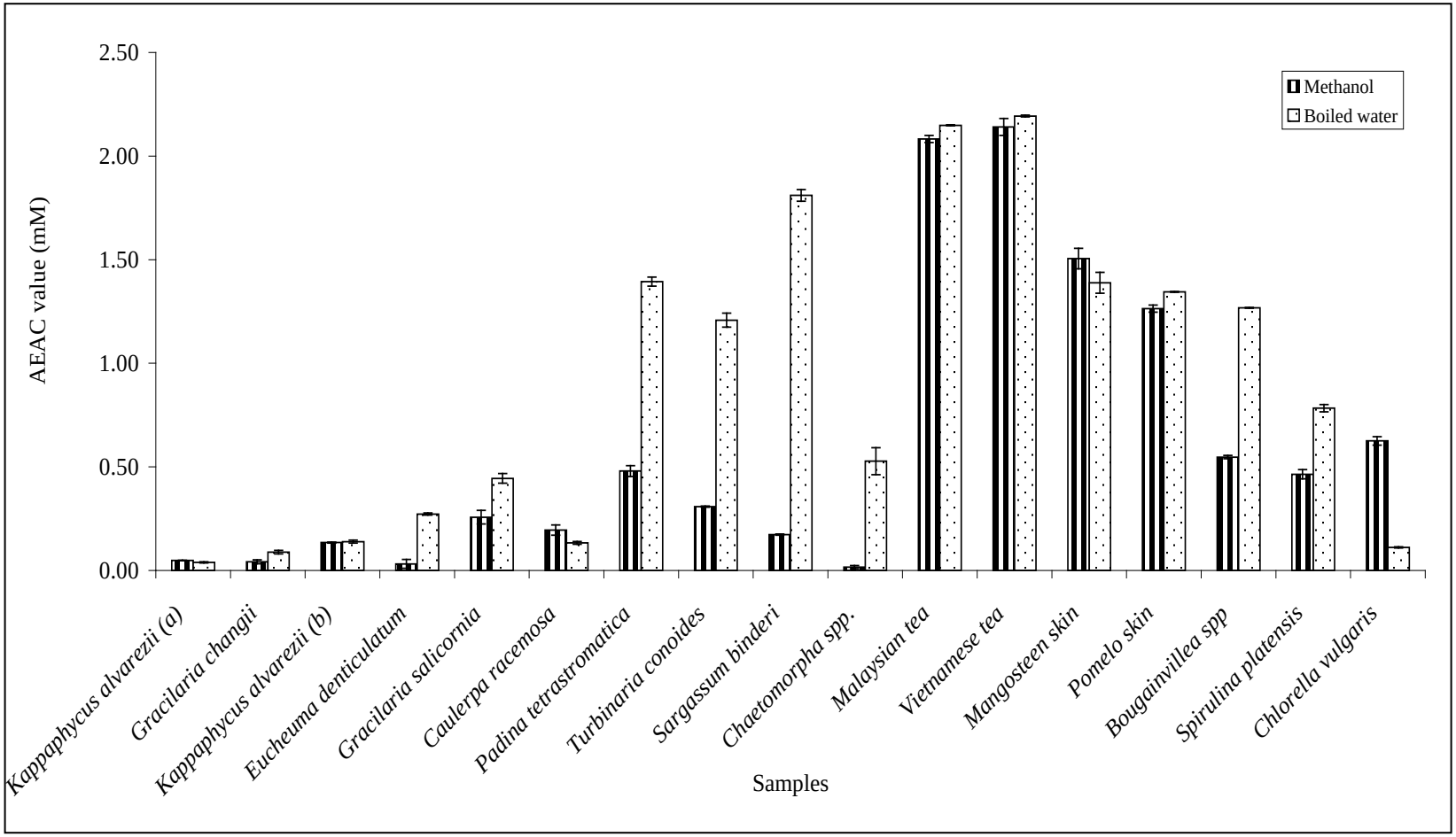

Figure 4.1: Antioxidant activities of forward methanol and boiled water extracts as evaluated by AEAC assay ($ABTS^{+}$ radicals)

Forward extraction indicates methanol extraction followed by boiled water extraction.

Forward and reverse extractions were carried out on selected samples. Table 4.2 (a) showed the antioxidant activity of forward and reverse methanol extracts. Figure 4.2 (a) showed that *P. tetrastromatica* has the highest AEAC value for its forward methanol (0.48 mM) extract and reverse (0.68 mM) extract whereby its reverse methanol extract showed higher AEAC value compared to forward extract. *C. racemosa* showed the same AEAC value for both forward and reverse methanol extracts which was 0.19 mM. All reverse methanol extracts showed higher AEAC value compared to forward extract except for *T. conoides.* The second highest AEAC value for reverse methanol extract was *G. salicornia* (0.39 mM), followed by *S. binderi* (0.38 mM), *Chaetomorpha spp.* (0.23 mM), *T. conoides* (0.20 mM) and finally *C. racemosa* (0.19 mM). The second highest AEAC value for forward methanol extract was *T. conoides* (0.31 mM) followed by *G. salicornia* (0.26 mM), *C. racemosa* (0.19 mM), *S. binderi* (0.17 mM) and finally *Chaetomorpha* spp. (0.02 mM). There was a huge difference between forward and reverse methanol extracts of *Chaetomorpha* spp. compared to the other seaweeds.

Table 4.2 (a): Antioxidant activities of forward and reverse methanol extracts evaluated with ABTS Assay

Samples	AEAC value (Mm)			
	Methanol			
	Forward	SD	Reverse	SD
Gracilaria salicornia	0.26	0.03	0.39	0.03
Caulerpa racemosa	0.19	0.02	0.19	0.00
Padina tetrastromatica	0.48	0.03	0.68	0.02
Turbinaria conoides	0.31	0.00	0.20	0.03
Sargassum binderi	0.17	0.00	0.38	0.01
Chaetomorpha spp.	0.02	0.01	0.23	0.01

Forward indicates methanol followed by boiled water extraction
Reverse indicates boiled water followed by methanol extraction

Table 4.2 (b) showed the comparison of antioxidant activity between forward and reverse boiled water extracts. In this study, 3 samples showed higher AEAC value for their forward extracts while another 3 samples showed higher AEAC value for their reverse extracts (Figure 4.2 (b)). Samples that showed higher AEAC value for their forward boiled water extracts compared to reverse extracts were *G. salicornia* where AEAC value for its forward extract was 0.44 mM and reverse extract was 0.38 mM, *P. tetrastromatica* where its forward extract AEAC value was 1.39 mM compared to its reverse extract AEAC value which was 0.98 mM and finally *Chaetomorpha* spp that showed 0.53 mM AEAC value for its forward extract and 0.21 mM for its reverse extract. Samples that showed higher AEAC value in their reverse boiled water extract compared to forward extract were *C. racemosa* that showed 0.21 mM AEAC value for reverse extract compared to 0.13 mM for its forward extract. The second sample was *T. conoides* that showed 1.40 mM AEAC value for its reverse extract and 1.21 mM for its forward extract. The third sample was *S. binderi* where the AEAC value for its reverse extract was 2.00 mM and its forward extract was 1.81 mM.

Table 4.2 (b): Antioxidant activities of forward and reverse boiled water extracts evaluated with ABTS Assay

Samples	AEAC value (mM)			
	Boiled water			
	Forward	SD	Reverse	SD
Gracilaria salicornia	0.44	0.02	0.38	0.00
Caulerpa racemosa	0.13	0.01	0.21	0.01
Padina tetrastromatica	1.39	0.02	0.98	0.01
Turbinaria conoides	1.21	0.03	1.40	0.00
Sargassum binderi	1.81	0.03	2.00	0.05
Chaetomorpha spp.	0.53	0.07	0.21	0.00

Forward indicates methanol followed by boiled water extraction
Reverse indicates boiled water followed by methanol extraction

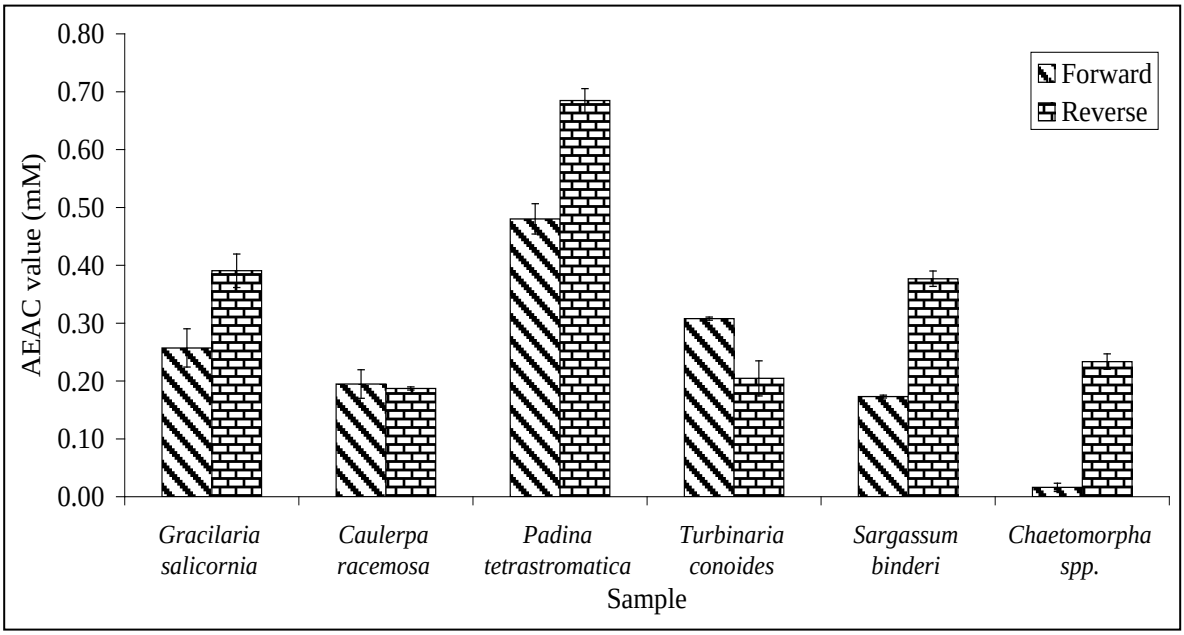

Figure 4.2 (a): Antioxidant activities of selected seaweeds forward and reverse methanol extracts as evaluated by AEAC assay ($ABTS^{+}$ radicals)

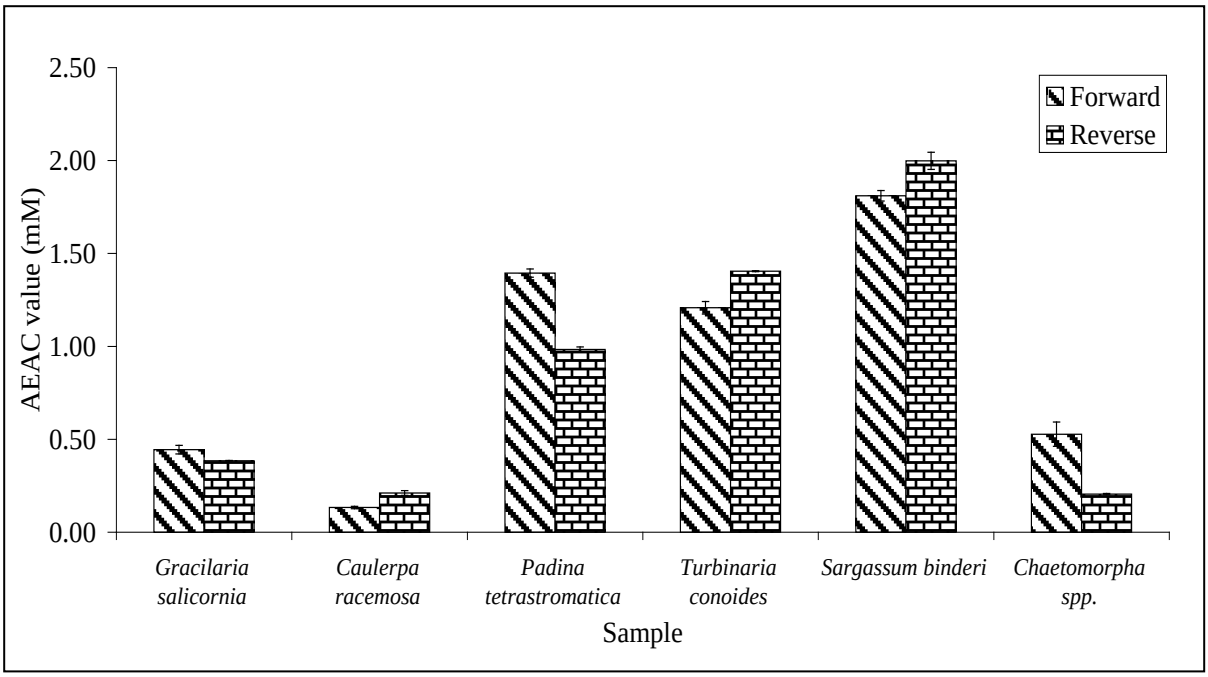

Figure 4.2 (b): Antioxidant activities of selected seaweeds forward and reverse boiled water extracts as evaluated by AEAC assay ($ABTS^{+}$ radicals)

Forward indicates methanol followed by boiled water extraction
Reverse indicates boiled water followed by methanol extraction

Selected samples were ground using two different methods and tested in AEAC assay. All the samples showed higher AEAC value for liquid nitrogen grinding method when compared to the grinder method for their methanol extracts (Figure 4.3 (a)). Table 4.3 (a) showed that *P. tetrastromatica* has the highest AEAC value for liquid nitrogen grinding method (1.16 mM) compared to grinder method (0.48 mM). This was followed by *S. binderi* where liquid nitrogen grinding showed AEAC value of 0.51 mM and the grinder method showed 0.17 mM. *C. racemosa* showed AEAC value of 0.41 mM for liquid nitrogen grinding whereas AEAC value for grinder method showed 0.19 mM. *T. conoides* showed the lowest AEAC value for liquid nitrogen grinding method which was 0.36 mM compared to grinder method 0.31 mM.

Table 4.3 (a): Antioxidant activities of selected seaweeds forward methanol extracts using two methods evaluated with ABTS Assay

Sample	AEAC value (mM)			
	Methanol			
	Grinder	SD	Liquid nitrogen	SD
Sargassum binderi	0.17	0.00	0.51	0.02
Turbinaria conoides	0.31	0.00	0.36	0.00
Padina tetrastromatica	0.48	0.03	1.16	0.03
Caulerpa racemosa	0.19	0.02	0.41	0.10

Grinder indicates extraction of samples dried in oven and ground using a grinder
Liquid nitrogen indicate extraction of samples that was blotted dry and ground using liquid nitrogen

Figure 4.3 (b) showed that boiled water extract of seaweeds ground with grinder has higher AEAC value compared to seaweeds ground with liquid nitrogen except for *C. racemosa*. For samples ground using grinder, Table 4.3 (b) showed that *S. binderi* has

the highest AEAC value, 1.81 mM, followed by *P. tetrastromatica* (1.39 mM), *T. conoides* (1.21 mM) and finally *C. racemosa* (0.13 mM). As for seaweeds ground with liquid nitrogen, *P. tetrastromatica* showed a higher AEAC value, 1.14 mM, followed by *T. conoides* (0.70 mM), *S. binderi* (0.41 mM) and finally *C. racemosa* (0.14 mM).

Table 4.3 (b): Antioxidant activities of selected seaweeds forward boiled water extracts using two methods evaluated with ABTS Assay

Sample	AEAC value (Mm)			
	Boiled water			
	Grinder	SD	Liquid nitrogen	SD
Sargassum binderi	1.81	0.03	0.41	0.01
Turbinaria conoides	1.21	0.03	0.70	0.01
Padina tetrastromatica	1.39	0.02	1.14	0.01
Caulerpa racemosa	0.13	0.01	0.14	0.01

Grinder indicates extraction of samples dried in oven and ground using a grinder
Liquid nitrogen indicate extraction of samples that was blotted dry and ground using liquid nitrogen

These results showed that methanol extracts have higher AEAC values compared to boiled water extracts for samples ground with liquid nitrogen

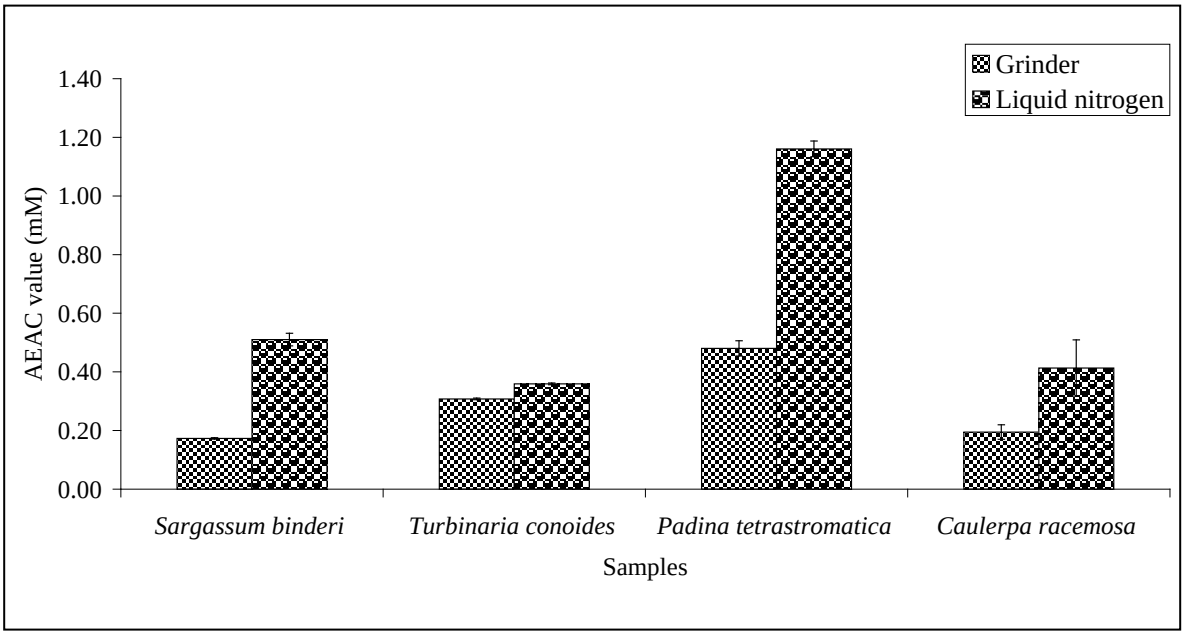

Figure 4.3 (a): Antioxidant activities of selected seaweeds forward methanol extracts using two methods as evaluated by AEAC assay ($ABTS^{+}$ radicals).

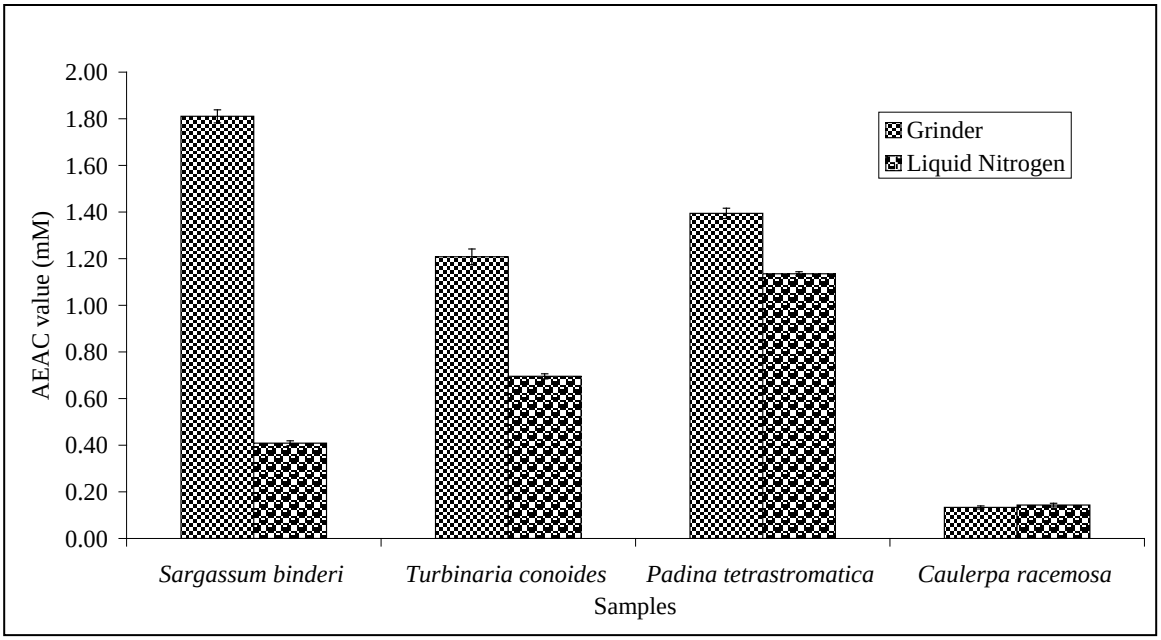

Figure 4.3 (b): Antioxidant activities of selected seaweeds forward boiled water extracts using two methods as evaluated by AEAC assay ($ABTS^{+}$ radicals)

Grinder indicates extraction of samples dried in oven and ground using a grinder
Liquid nitrogen indicates extraction of samples that was blotted dry and ground using liquid nitrogen

Figure 4.4 showed the AEAC values of ascorbic acid taken at different time interval. Table 4.4 showed that ascorbic acid has higher percentage of inhibition after 15 minutes and 5 minutes of its addition into $ABTS^{\cdot+}$ reagent. Similar results were obtained when ascorbic acid were incubated for 5 minutes and 15 minutes except for ascorbic acid at 2.4mM concentration, whereby higher inhibition value of 85.99% was obtained after 15 minutes of incubation as compared to 85.12% after 5 minutes of incubation.

Table 4.4: Percentage inhibition of $ABTS^{\cdot+}$ by ascorbic acid at 5, 10 and 15 minutes.

Ascorbic acid (mM)	Inhibition (%) of $ABTS^{\cdot+}$					
	5 minutes	SD	10 minutes	SD	15 minutes	SD
0	0.30	0.00	0.07	0.00	0.37	0.00
0.075	5.59	0.00	5.08	0.00	5.08	0.00
0.15	8.86	0.01	7.46	0.00	7.89	0.00
0.3	17.53	0.01	13.61	0.01	14.15	0.01
0.6	33.04	0.01	25.95	0.02	26.82	0.01
1.2	56.69	0.03	52.14	0.03	53.81	0.03
2.4	85.12	0.02	84.08	0.02	85.99	0.02

5 minutes = reading taken 5 minutes after incubation of ascorbic acid
10 minutes = reading taken 10 minutes after incubation of ascorbic acid
15 minutes = reading taken 15 minutes after incubation of ascorbic acid

This is the reason for allowing the ascorbic acid to equilibrate with the reagent for 5 minutes before the reading was taken as mentioned in ABTS method earlier.

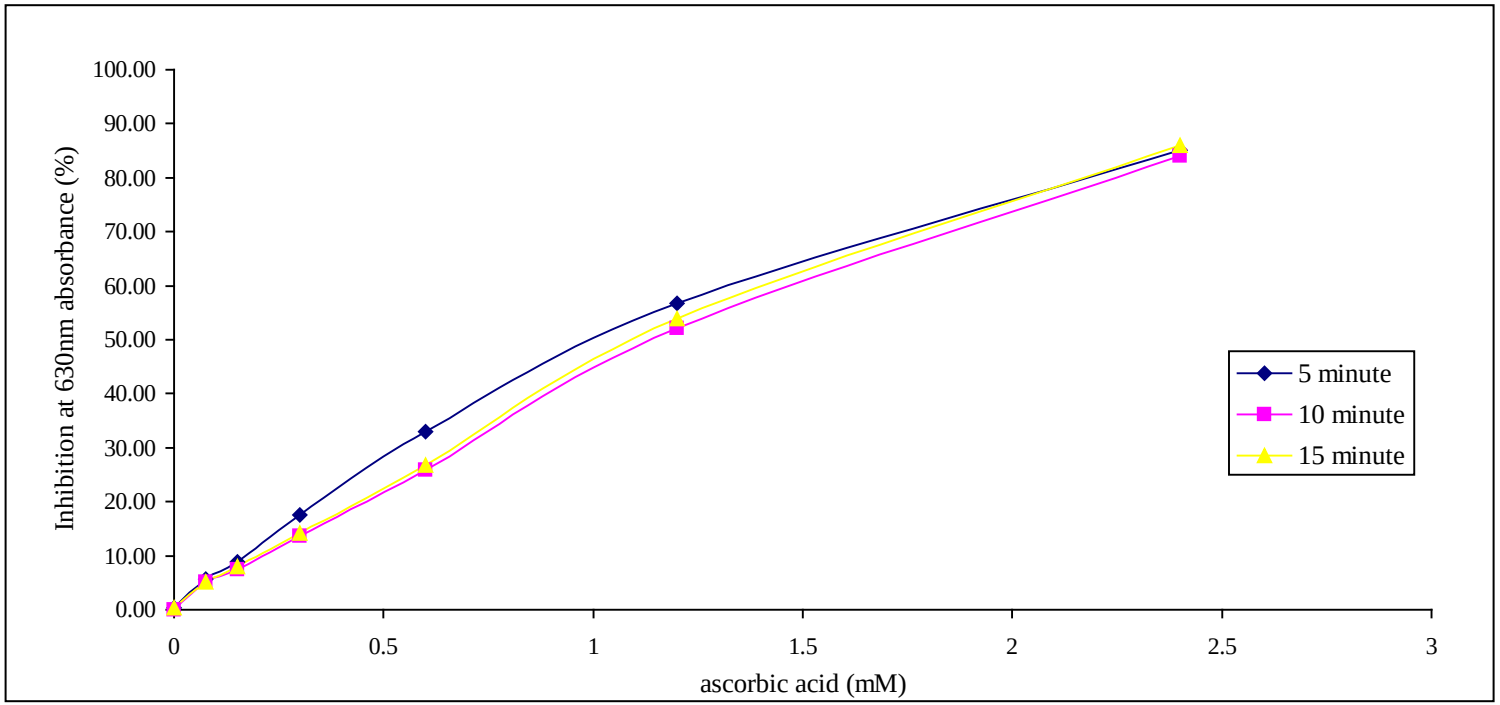

Figure 4.4: Absorbance reading of $ABTS^{\cdot+}$ inhibition by ascorbic acid at 630nm taken at 5, 10 and 15 minutes.

5 minutes = reading taken 5 minutes after incubation of ascorbic acid
10 minutes = reading taken 10 minutes after incubation of ascorbic acid
15 minutes = reading taken 15 minutes after incubation of ascorbic acid

Ascorbic acid was prepared fresh everyday for AEAC assay. This is because, ascorbic acid only showed the highest percentage of inhibition on the same day. On the second day, although the percentage of inhibition was still high, but it was lower than the 1st day (Table 4.5). From the graph (Figure 4.5), the 1st day of ascorbic acid (freshly prepared) gave better result of inhibition compared to the other 4 days. Therefore, referring to these results, it showed that ascorbic acid needs to be prepared fresh everyday.

Table 4.5: Percentage inhibition of $ABTS^{\cdot+}$ by ascorbic acid stored at 4°C for 5 different days.

Ascorbic acid (mM)	Inhibition (%) of $ABTS^{\cdot+}$									
	1st day	SD	2nd day	SD	3rd day	SD	4th day	SD	5th day	SD
0	0.30	0.00	0.24	0.00	0.24	0.00	0.88	0.01	1.11	0.00
0.075	5.59	0.00	4.86	0.00	3.72	0.00	2.85	0.00	3.19	0.00
0.15	8.86	0.01	8.43	0.00	6.62	0.00	6.34	0.00	7.05	0.00
0.3	17.53	0.01	16.04	0.00	16.13	0.00	14.54	0.00	14.81	0.00
0.6	33.04	0.01	30.28	0.01	29.50	0.00	26.33	0.01	27.40	0.00
1.2	56.69	0.03	53.01	0.01	51.24	0.00	50.56	0.00	50.44	0.00
2.4	85.12	0.02	81.89	0.03	84.63	0.01	76.48	0.03	76.76	0.04

1st = Reading taken on the 1st day of ascorbic acid preparation
2nd = Reading taken 2nd days after ascorbic acid preparation
3rd = Reading taken 3rd days after ascorbic acid preparation
4th = Reading taken 4th days after ascorbic acid preparation
5th = Reading taken 5th days after ascorbic acid preparation

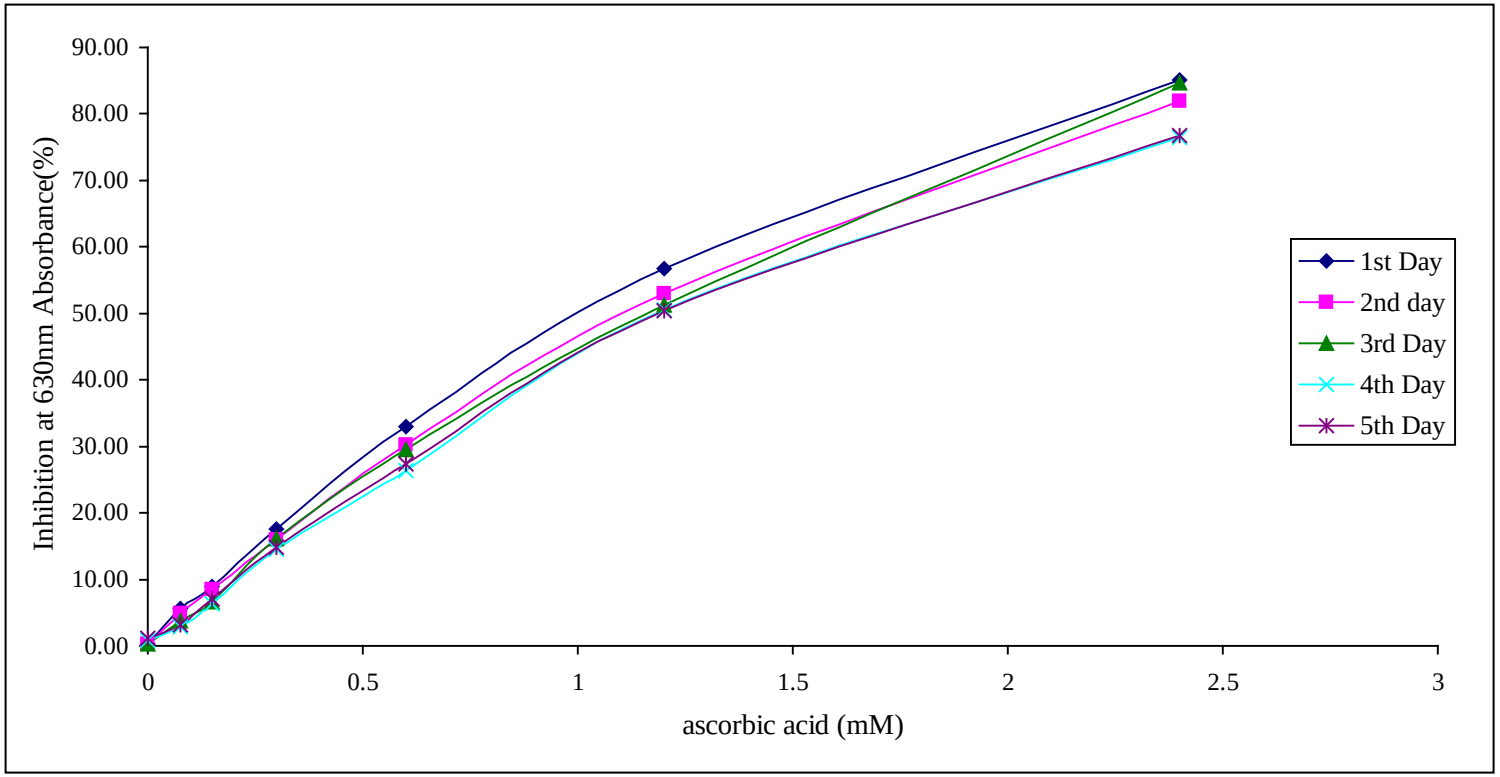

Figure 4.5: Absorbance reading of $ABTS^{\cdot+}$ inhibition by ascorbic acid refrigerated at $4^{o}C$ and used at successive days post-preparation

2^{nd} = after 24 hours at $4^{o}C$

1^{st} = Reading taken on the 1^{st} day of ascorbic acid preparation

2^{nd} = Reading taken 2^{nd} days after ascorbic acid preparation

3^{rd} = Reading taken 3^{rd} days after ascorbic acid preparation

4^{th} = Reading taken 4^{th} days after ascorbic acid preparation

5^{th} = Reading taken 5^{th} days after ascorbic acid preparation

A short test was carried to observe whether ascorbic acid will lose its antioxidant properties if it was stored at -20^{o}C for several days. According to the results obtained (Figure 4.6), it showed that ascorbic acid must be prepared fresh. Ascorbic acid gave an inhibition of 85.12% on the day it was prepared. After ascorbic acid was stored at -20^{o}C and thawed, the inhibition percentage was lower (81.35%) than the freshly prepared ascorbic acid (Table 4.6). Although the percentage difference between each day was not far, but based on the graph (Figure 4.6) it showed better correlation for freshly mixed ascorbic acid. This shows that fresh ascorbic acid is more sensitive and its properties is unaffected compared to stored ascorbic acid.

Table 4.6: Percentage inhibition of $ABTS^{\cdot+}$ by ascorbic acid stored at –20^{o}C for 5 different days.

Ascorbic acid (mM)	Inhibition (%) of $ABTS^{\cdot+}$									
	Before thaw	SD	1st day	SD	2nd day	SD	3rd day	SD	4th day	SD
0	0.30	0.00	1.02	0.00	0.90	0.00	0.85	0.00	1.88	0.00
0.075	5.59	0.00	3.43	0.00	3.45	0.00	4.58	0.00	4.10	0.00
0.15	8.86	0.01	5.61	0.00	6.72	0.00	7.49	0.00	7.22	0.00
0.3	17.53	0.01	11.28	0.00	11.17	0.00	13.01	0.00	12.12	0.00
0.6	33.04	0.01	22.56	0.01	23.64	0.01	19.86	0.00	25.22	0.00
1.2	56.69	0.03	41.35	0.00	45.97	0.01	46.39	0.00	47.68	0.00
2.4	85.12	0.02	81.35	0.01	80.91	0.03	67.84	0.02	72.06	0.02

Before freezing = Reading taken before ascorbic acid were placed in the freezer
1st = Reading taken after ascorbic acid freeze/thawed (1x) on 2nd day of preparation
2nd = Reading taken after ascorbic acid freeze/thawed (2x) on 3rd day of preparation
3rd = Reading taken after ascorbic acid freeze/thawed (3x) on 4th day of preparation
4th = Reading taken after ascorbic acid freeze/thawed (4x) on 5th day of preparation

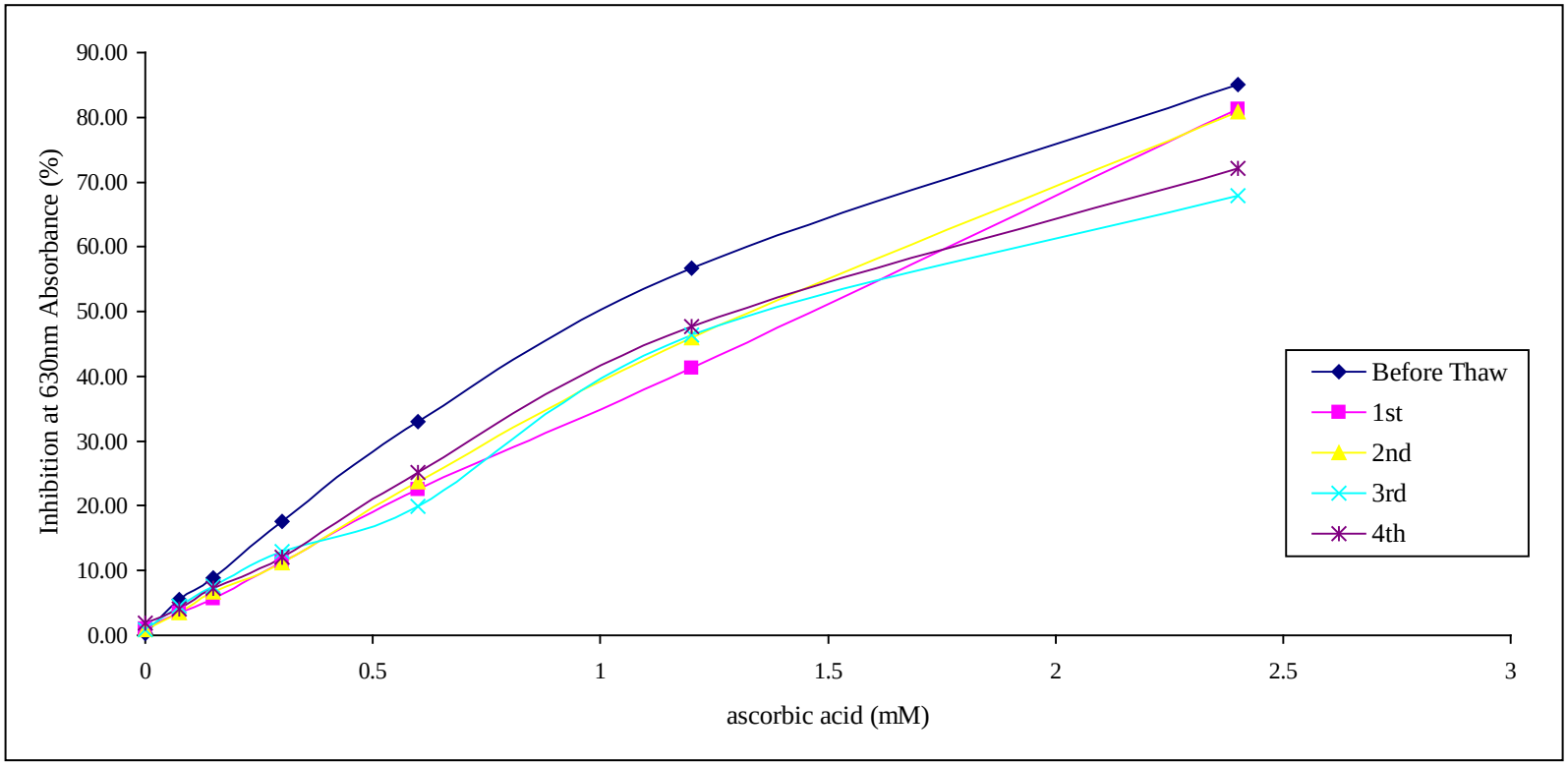

Figure 4.6: Absorbance reading of $ABTS^{\cdot+}$ inhibited by ascorbic acid refrigerated at –20°C and used at successive days post-preparation

1st = after 24 hours freeze at –20°C

Before freezing = Reading taken before ascorbic acid were placed in the freezer

1st = Reading taken after ascorbic acid freeze/thawed (1x) on 2nd day of preparation

2nd = Reading taken after ascorbic acid freeze/thawed (2x) on 3rd day of preparation

3rd = Reading taken after ascorbic acid freeze/thawed (3x) on 4th day of preparation

4th = Reading taken after ascorbic acid freeze/thawed (4x) on 5th day of preparation

4.1.2 Diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay.

This assay was used because DPPH react directly and rapidly with antioxidant compounds. Any substance that can donate a hydrogen atom (antioxidant) to the solution of DPPH can reduce the stable free radical and change the colour of solution from violet to pale yellow. DPPH is a purplish stable free radical in ethanol solvent and its odd electron gives a strong absorption at 515nm. When these free radical electron pair with electron from an antioxidant, it forms the reduced DPPH-H (diphenylpicryl hydrazine). The absorption fades and results in decolorization which is stoichiometric with respect to the number of electron captured.

Table 4.7 showed that most of the seaweeds did not show inhibition toward DPPH assay. *K. alvarezii* (a), *G. changii*, *K. alvarezii* (b), *E. denticulatum*, *G. salicornia*, *C. racemosa* and *Chaetomorpha* spp. did not show any inhibition for their boiled water extracts (Figure 4.7). *Bougainvillea* spp. did not show any inhibition for its boiled water extract as well. As for methanol extracts, *K. alvarezii* (a), *G. changii*, *C. racemosa*, *P. tetrastromatica*, *T. conoides*, *S. binderi*, *Chaetomorpha* spp, *S. platensis* and *C. vulgaris* did not show any inhibition. Samples that did not show inhibition for both its boiled water and methanol extracts were *K. alvarezii* (a), *G. changii*, *C. racemosa* and *Chaetomorpha* spp.

For boiled water extract, Malaysian tea has the highest percentage of inhibition (83.16 %), followed by Vietnamese tea (83.44 %). The third highest percentage of inhibition for boiled water extract was Mangosteen skin with 68.04% inhibition, followed

by Pomelo skin (55.79%), *S. binderi* (12.35%), *T. conoides* (7.36%), *S. platensis*, *C. vulgaris* (2.07%) and finally *P. tetrastromatica* (1.69%).

Malaysian tea has the highest percentage of inhibition for its methanol extract (89.68%) followed by Vietnamese tea (89.20%), Mangosteen skin (88.73%), Pomelo skin (26.81%), *Bougainvillea* spp. (14.96%), *G. salicornia* (5.42%), *K. alvarezii* (b) (1.70%) and finally *Eucheuma denticulatum* (0.41%).

Table 4.7: Antioxidant activities of forward methanol and boiled water extracts evaluated with Diphenyl-1-picryl-hydrazyl-hydrate (DPPH) Free Radical Assay

Samples	DPPH value (%)			
	Forward extraction			
	Methanol	SD	Boiled water	SD
Kappaphycus alvarezii (a)	0	0	0	0
Gracilaria changii	0	0	0	0
Kappaphycus alvarezii (b)	1.70	0.58	0	0
Eucheuma denticulatum	0.41	0.07	0	0
Gracilaria salicornia	5.42	0.91	0	0
Caulerpa racemosa	0	0	0	0
Padina tetrastromatica	0	0	1.69	0.33
Turbinaria conoides	0	0	7.36	0.98
Sargassum binderi	0	0	12.35	0.64
Chaetomorpha spp.	0	0	0	0
Malaysian tea	89.68	0.12	83.16	0.07
Vietnamese tea	89.20	0.06	83.44	0.33
Mangosteen skin	88.73	0.22	68.04	1.25
Pomelo skin	26.81	0.25	55.79	0.08
Bougainvillea spp.	14.96	1.44	0	0
Spirulina platensis	0	0	2.07	0.08
Chlorella vulgaris	0	0	2.07	0.08

Forward extraction indicates methanol followed by boiled water extraction

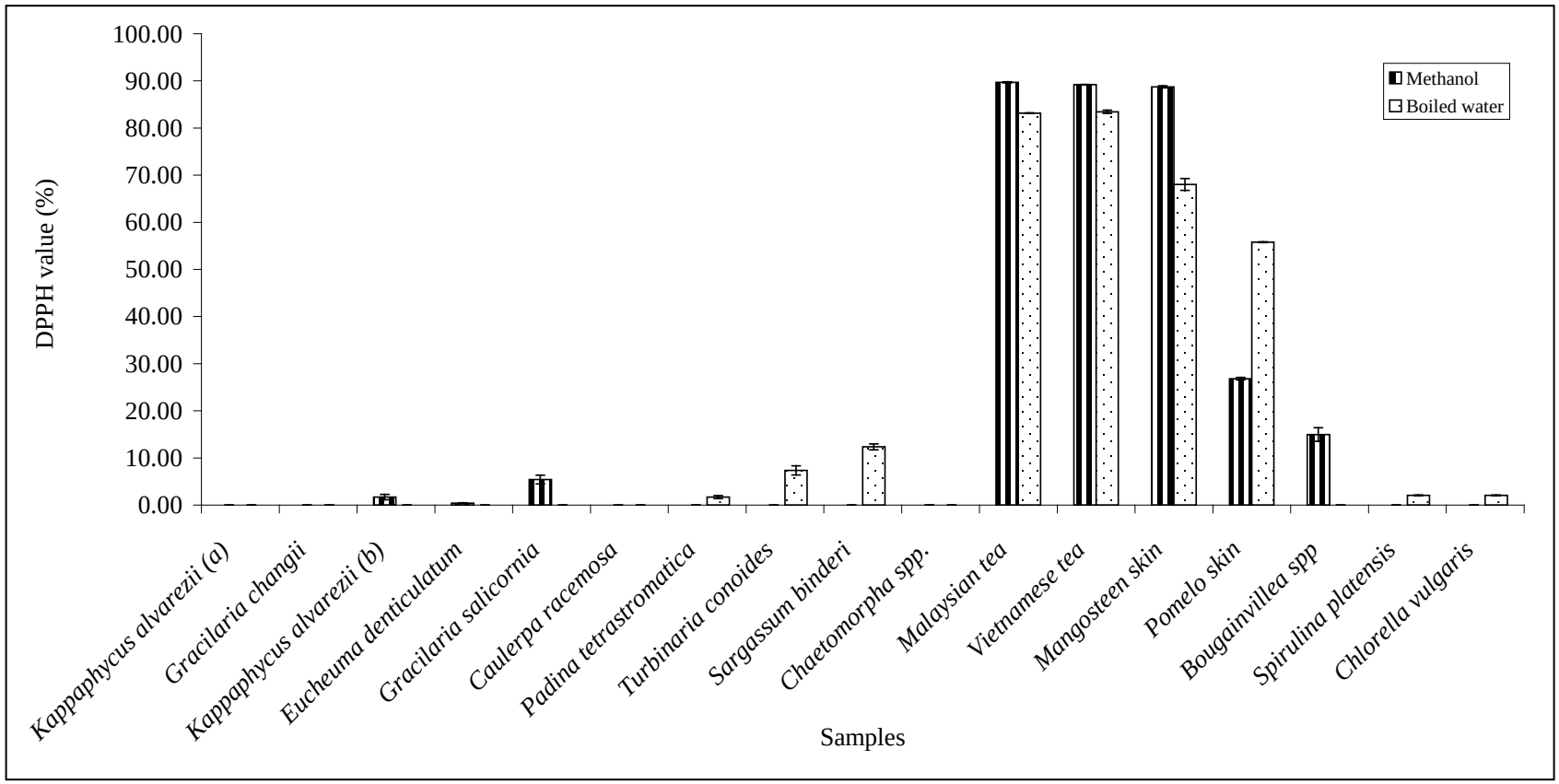

Figure 4.7: Antioxidant activities of forward methanol and boiled water extracts as evaluated by DPPH assay

Forward extraction indicates methanol followed by boiled water extraction

Figure 4.8 (a) showed the comparison between forward and reverse methanol extracts. Only *G. salicornia* showed inhibition whereby its forward methanol extract showed 5.42% of inhibition compared to its reverse extract where it only showed 2.18% of inhibition (Table 4.8 (a)). The other samples (*C. racemosa, P. tetrastromatica, T. conoides, S. binderi* and *Chaetomorpha* spp.) did not show any inhibition for both their forward and reverse methanol extracts.

Table 4.8 (a): Antioxidant activities of forward and reverse methanol extracts evaluated with DPPH assay

Samples	DPPH value (%)			
	Methanol			
	Forward	SD	Reverse	SD
Gracilaria salicornia	5.42	0.91	2.18	0
Caulerpa racemosa	0	0	0	0
Padina tetrastromatica	0	0	0	0
Turbinaria conoides	0	0	0	0
Sargassum binderi	0	0	0	0
Chaetomorpha spp.	0	0	0	0

Forward indicates methanol followed by boiled water extraction
Reverse indicates boiled water followed by methanol extraction

Figure 4.8 (b) showed the percentage of inhibition of forward and reverse boiled water extracts. *S. binderi* showed the highest inhibition (12.35%) for its forward boiled water extract compared to its reverse extract, 10.40% (Table 4.8 (b)). This was followed by *T. conoides*, which showed 7.36% of inhibition for its forward extract, compared to 6.74% of inhibition for its reverse extract. Finally, *P. tetrastromatica* showed 1.69% of inhibition for its forward boiled water extract whereas it did not show any inhibition for its reverse extract. *Chaetomorpha* spp., *C. racemosa* and *G. salicornia* did not show any inhibition for both their forward and reverse boiled water extracts.

Table 4.8 (b): Antioxidant activities of forward and reverse boiled water extracts evaluated with DPPH assay

Samples	DPPH value (%)			
	Boiled water			
	Forward	SD	Reverse	SD
Gracilaria salicornia	0	0	0	0
Caulerpa racemosa	0	0	0	0
Padina tetrastromatica	1.69	0.33	0	0
Turbinaria conoides	7.36	0.98	6.74	0.11
Sargassum binderi	12.35	0.64	10.40	0.14
Chaetomorpha spp.	0	0	0	0

Forward indicates methanol followed by boiled water extraction
Reverse indicates boiled water followed by methanol extraction

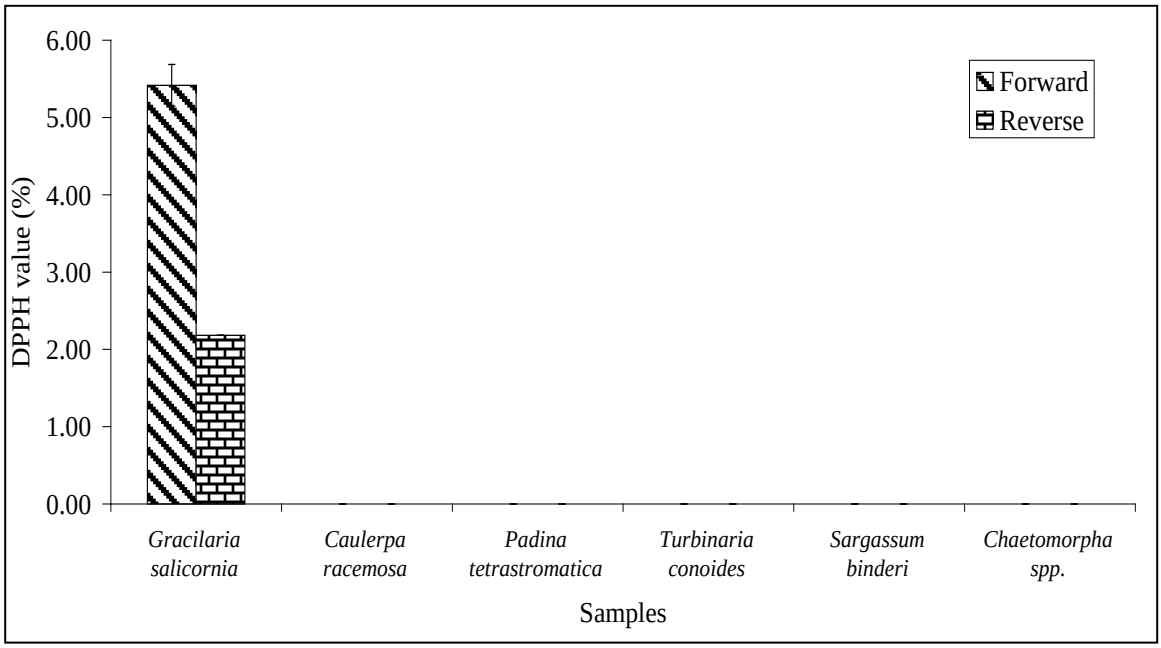

Figure 4.8 (a): Antioxidant activities of selected seaweeds forward and reverse methanol extracts as evaluated by DPPH

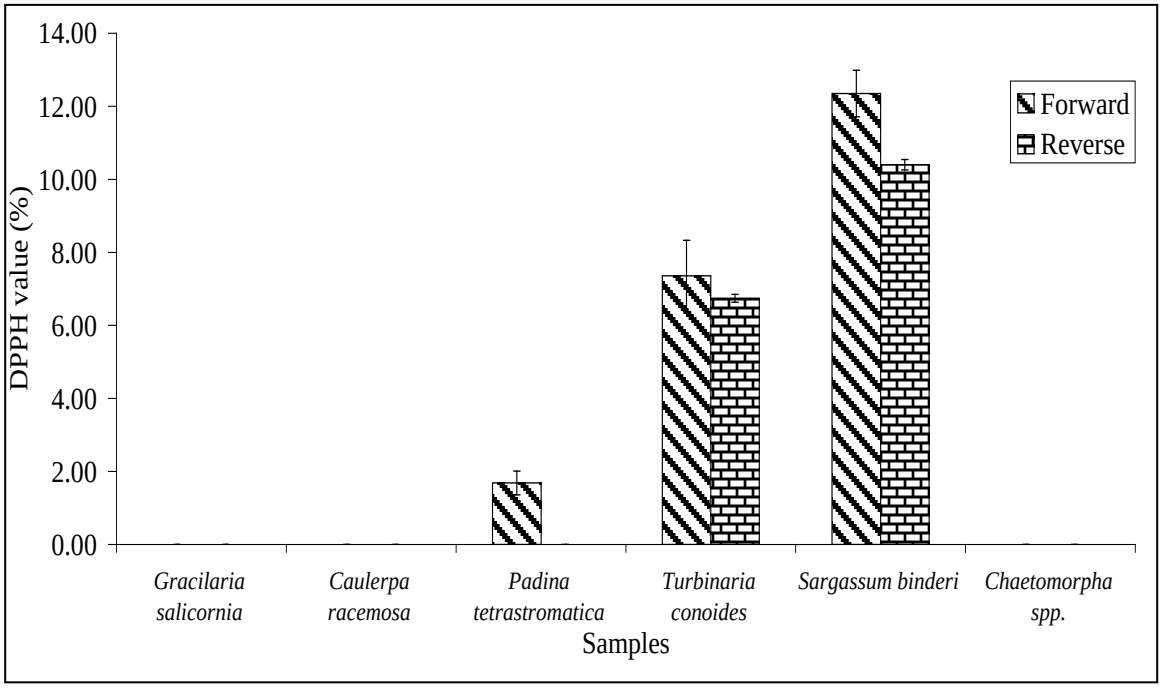

Figure 4.8 (b): Antioxidant activities of selected seaweeds forward and reverse boiled water extracts as evaluated by DPPH assay

Forward indicates methanol followed by boiled water extraction
Reverse indicates boiled water followed by methanol extraction

Table 4.9 (a) showed a comparison of methanol extract between samples ground with grinder and liquid nitrogen. All four samples (*S. binderi*, *T. conoides*, *P. tetrastromatica* and *C. racemosa*) did not show any inhibition for their methanol extract when the samples were ground using grinder but two samples showed inhibition when the samples were ground with liquid nitrogen (Figure 4.9 (a)). *T. conoides* showed 2.15% of inhibition whereas *P. tetrastromatica* showed the highest inhibition, 17.00%.

Table 4.9 (a): Antioxidant activities of selected seaweeds forward methanol extracts using two methods evaluated with DPPH Assay

Sample	DPPH value (%)			
	Methanol			
	Grinder	SD	Liquid nitrogen	SD
Sargassum binderi	0	0	0	0
Turbinaria conoides	0	0	2.15	1.10
Padina tetrastromatica	0	0	17.00	0.76
Caulerpa racemosa	0	0	0	0

Grinder indicates extraction of samples dried in oven and ground using a grinder
Liquid nitrogen indicate extraction of samples that was blotted dry and ground using liquid nitrogen

Figure 4.9 (b) showed the comparison of boiled water extracts between samples ground with grinder and samples ground with liquid nitrogen. From the table (Table 4.9 (b)), it showed that extract of samples ground with liquid nitrogen have lower percentage of inhibition compared to samples ground with grinder. *S. binderi* showed 12.35% of inhibition for grinder method compared to 0.77% for liquid nitrogen grinding method. Grinding method using grinder for *T. conoides* showed 7.36% inhibition whereas extract of sample ground using liquid nitrogen showed 1.89% of inhibition. *P. tetrastromatica* only showed inhibition for the extract of sample ground with grinder (1.69%). As for

C. racemosa, extract of sample ground with grinder did not show any inhibition whereas extract of sample ground with liquid nitrogen showed 1.36% of inhibition.

Table 4.9 (b): Antioxidant activities of selected seaweeds forward boiled water extracts using two methods evaluated with DPPH Assay

Sample	DPPH value (%)			
	Boiled water			
	Grinder	SD	Liquid nitrogen	SD
Sargassum binderi	12.35	0.64	0.77	0.75
Turbinaria conoides	7.36	0.98	1.89	1.34
Padina tetrastromatica	1.69	0.33	0	0
Caulerpa racemosa	0	0	1.36	0.25

Grinder indicates extraction of samples dried in oven and ground using a grinder
Liquid nitrogen indicate extraction of samples that was blotted dry and ground using liquid nitrogen

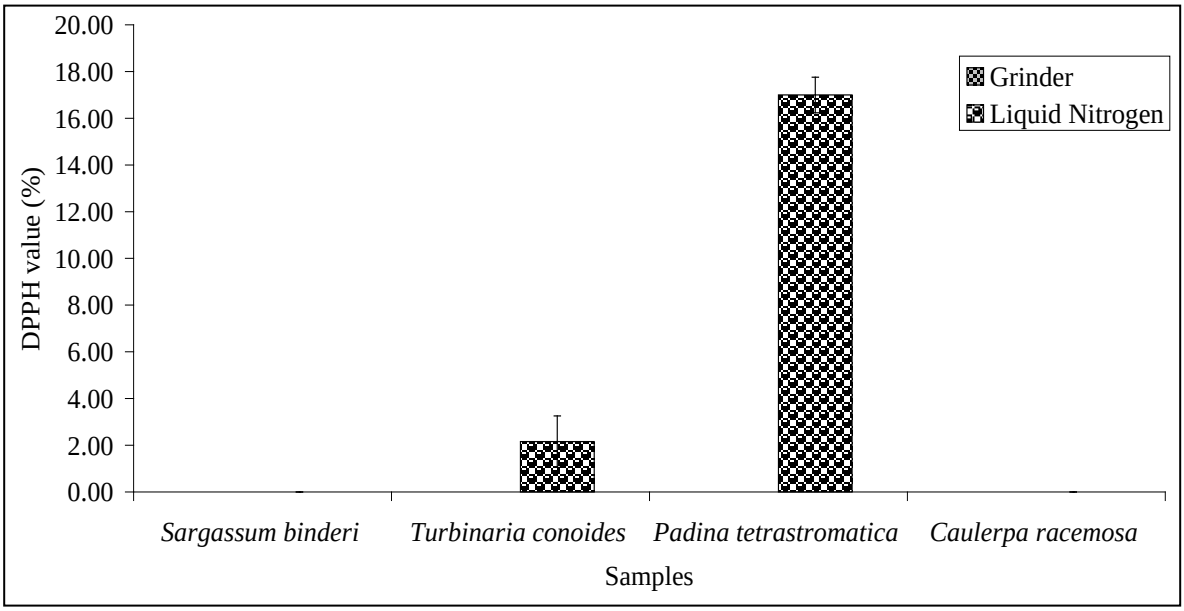

Figure 4.9 (a): Antioxidant activities of selected seaweeds forward methanol extracts using two methods as evaluated by DPPH assay

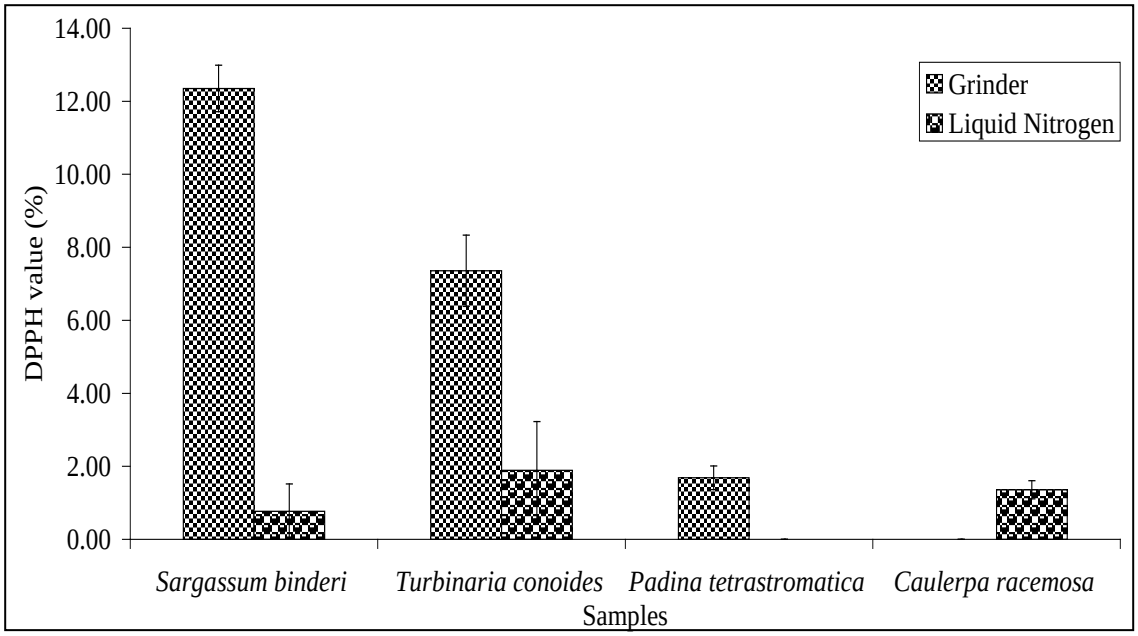

Figure 4.9 (b): Antioxidant activities of selected seaweeds forward boiled water extracts using two methods as evaluated by DPPH assay

Grinder indicates extraction of samples dried in oven and ground using a grinder
Liquid nitrogen indicates extraction of samples that was blotted dry and ground using liquid nitrogen

4.1.3 Galvinoxyl Free Radical Scavenging Assay for Sample Extract

Galvinoxyl is a stable phenoxyl radical used to determine the hydrogen donating efficacies of samples. Galvinoxyl radical accepts electron or hydrogen which will cause it to reduce where the decrease in absorbance is measured at 428nm (Honglian *et al*., 1998).

Table 4.10 showed the antioxidant activity of forward methanol extracts whereas table 4.11 showed the antioxidant activity of both forward and reverse extracts of samples for Galvinoxyl Free Radical Quenching Assay. Figure 4.10 showed the whole range of forward methanol extracts for this assay. From the table, it showed that for forward methanol extracts, Mangosteen skin has a higher TGEx value, 212.02 µM. This was followed by Malaysian tea (199.80 µM), Pomelo skin (194.03 µM) and Vietnamese tea (182.32 µM). *Bougainvillea* spp. gave a TGEx value of 86.92 µM, followed by *G. changii* 35.53 µM, *C. vulgaris* 31.96 µM, *K. alvarezii* (a) 23.01 µM, *K. alvarezii* (b) 22.66 µM, *S. platensis* 12.59 µM, *E. denticulatum* 10.28 µM, *Chaetomorpha* spp. 7.47 µM, *S. binderi* 6.15 µM and finally *G. salicornia* 6.01 µM. Three samples, *C. racemosa*, *P. tetrastromatica* and *T. conoides* did not show any inhibition.

Table 4.10: Antioxidant activities of forward methanol extracts evaluated with Galvinoxyl Free Radical Quenching Assay (TGEx).

Samples	TGEx value (μM)	
	Forward	
	Methanol	SD
Kappaphycus alvarezii (a)	23.01	0.44
Gracilaria changii	35.53	1.98
Kappaphycus alvarezii (b)	22.66	0.77
Eucheuma denticulatum	10.28	0.61
Gracilaria salicornia	6.01	0.97
Caulerpa racemosa	0.00	0.00
Padina tetrastromatica	0.00	0.00
Turbinaria conoides	0.00	0.00
Sargassum binderi	6.15	0.82
Chaetomorpha spp.	7.47	0.61
Malaysian tea	199.80	0.62
Vietnamese tea	182.32	5.81
Mangosteen skin	212.02	1.02
Pomelo skin	194.03	2.33
Bougainvillea spp.	86.92	3.28
Spirulina platensis	12.59	3.59
Chlorella vulgaris	31.96	9.88

Forward extraction indicates methanol followed by boiled water extraction.

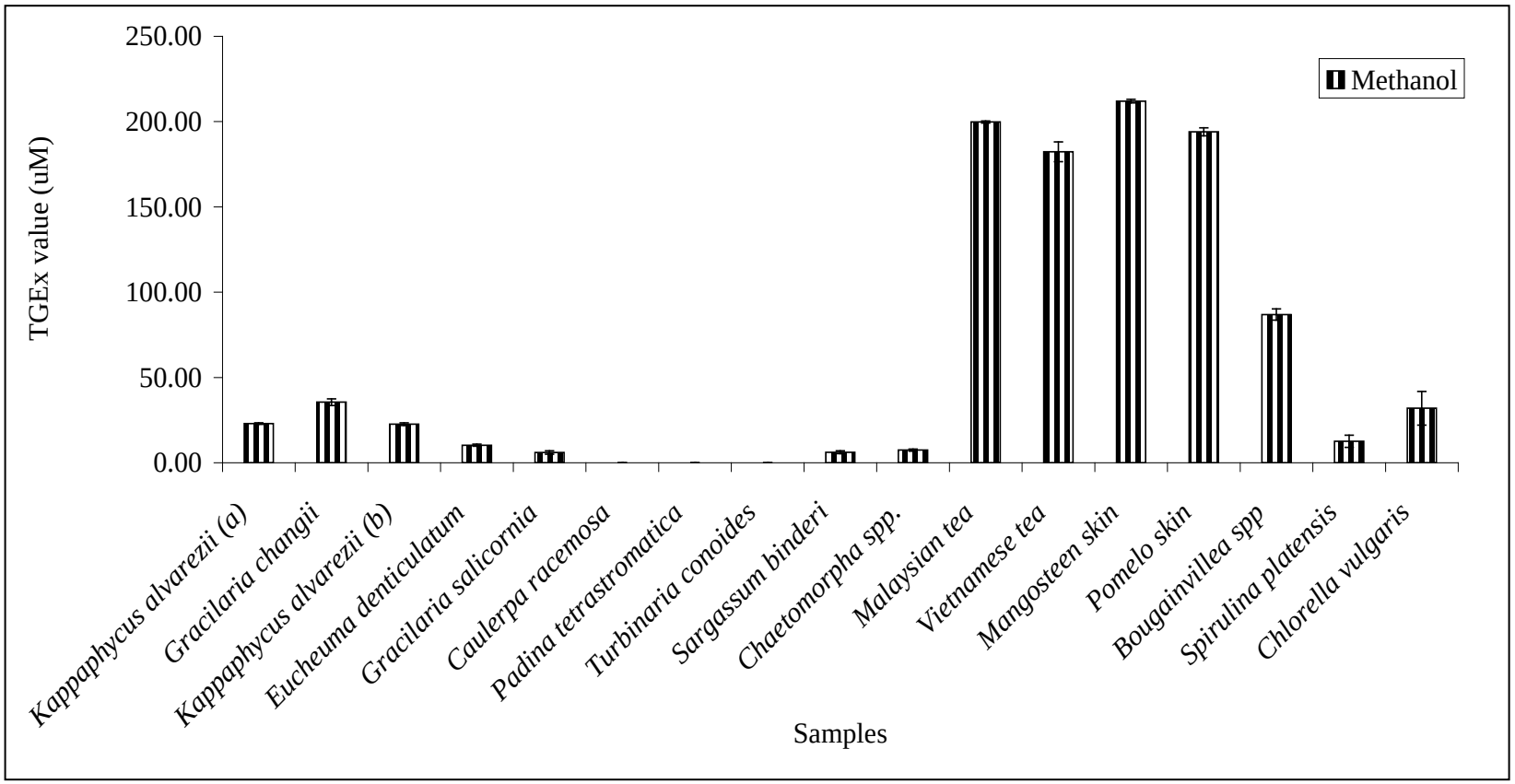

Figure 4.10: Antioxidant activities of forward methanol extracts as evaluated by Galvinoxyl assay.

Forward extraction indicates methanol followed by boiled water extraction.

As for reverse extraction, 3 samples did not show any inhibition and these samples were *P. tetrastromatica*, *S. binderi* and *Chaetomorpha* spp. According to Table 4.11 *S. binderi* and *Chaetomorpha* spp showed inhibition for forward methanol extracts whereas for their reverse extracts, both samples did not show any inhibition. The highest TGEx value for reverse extracts was *C. racemosa* 16.17 µM, followed by *G. salicornia* 2.94 µM and finally *T. conoides* 0.59 µM. For forward methanol extracts, *C. racemosa* and *T. conoides* did not show any inhibition whereas for reverse extracts, both samples showed inhibition whereby *C. racemosa* showed 16.17 µM and *G. salicornia* showed 2.94 µM. As for *G. salicornia,* it showed higher inhibition for forward extraction extract (6.01 µM) compared to its reverse extract (2.94 µM) (Figure 4.10).

Table 4.11: Antioxidant activities of forward and reverse methanol extracts evaluated with Galvinoxyl assay

Samples	TGEx value (µM)			
	Methanol			
	Forward	SD	Reverse	SD
Gracilaria salicornia	6.01	0.97	2.94	0.17
Caulerpa racemosa	0.00	0.00	16.17	0.18
Padina tetrastromatica	0.00	0.00	0.00	0.00
Turbinaria conoides	0.00	0.00	0.59	0.42
Sargassum binderi	6.15	0.82	0.00	0.00
Chaetomorpha spp.	7.47	0.61	0.00	0.00

Forward indicates methanol followed by boiled water extraction
Reverse indicates boiled water followed by methanol extraction

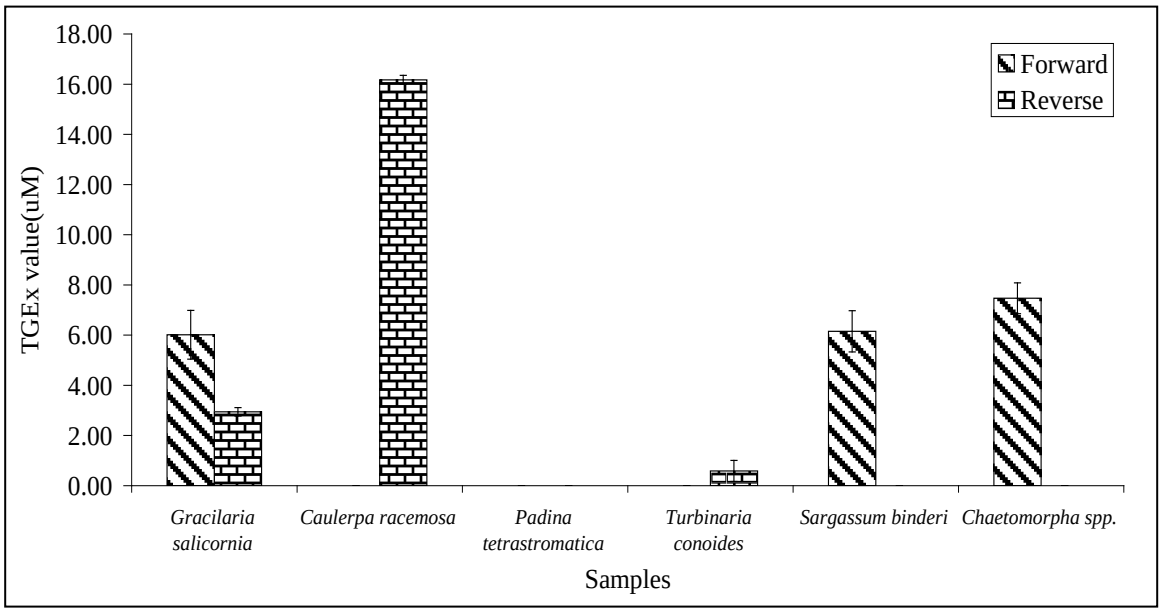

Figure 4.11: Antioxidant activities of selected seaweeds forward and reverse methanol extracts of seaweeds as evaluated by Galvinoxyl assay

Forward indicates methanol followed by boiled water extraction
Reverse indicates boiled water followed by methanol extraction

From the Table 4.12, it showed that extracts of liquid nitrogen grinding method have higher TGEx value compared to normal drying and grinding method using grinder. *S. binderi* showed the highest TGEx value of 82.40 µM, followed by *T. conoides* with 68.31 µM, *P. tetrastromatica* with 36.16 µM and finally *C. racemosa* with 5.31 µM. *T. conoides*, *P. tetrastromatica* and *C. racemosa* did not show any inhibition for extracts of samples ground by grinder whereas extracts of samples ground by liquid nitrogen showed high TGEx values (Figure 4.12). The only sample that showed inhibition value for extract of sample ground by grinder was *S. binderi* (6.15 µM). However, when compared to its liquid nitrogen method, its extract showed a very high inhibition value, 82.40 µM, where this sample showed the highest TGEx value compared to the other samples.

Table 4.12: Antioxidant activities of selected seaweeds forward methanol extracts using two methods evaluated with Galvinoxyl assay

Sample	TGEx value (µM)			
	Methanol			
	Grinder	SD	Liquid nitrogen	SD
Sargassum binderi	6.15	0.82	82.40	5.42
Turbinaria conoides	0.00	0.00	68.31	0.24
Padina tetrastromatica	0.00	0.00	36.16	3.80
Caulerpa racemosa	0.00	0.00	5.31	1.49

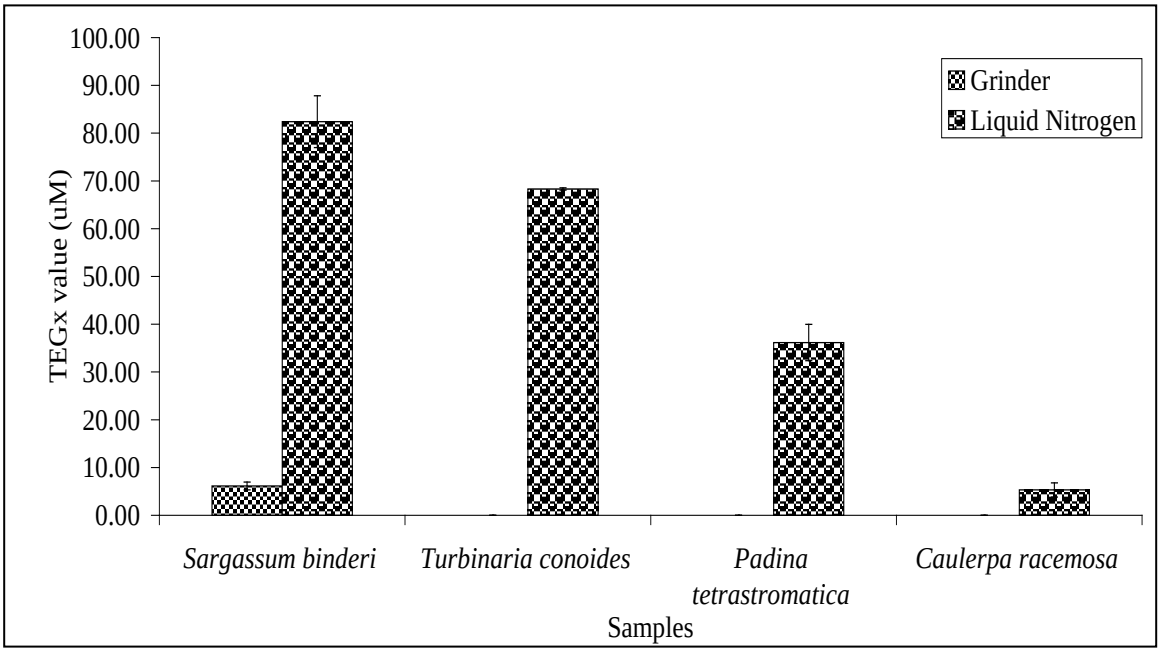

Figure 4.12: Antioxidant activities of selected seaweeds forward and reverse boiled water extracts using two methods as evaluated by Galvinoxyl (GFRQ) assay

Grinder indicates extraction of samples dried in oven and ground using a grinder
Liquid nitrogen indicates extraction of samples that was blotted dry and ground using liquid nitrogen

4.1.4 Total Phenols Assay by Folin – Ciocalteu Reagent

Total phenolic assay was carried out to determine the total phenolic content of the sample. Total Phenolic content was standardized against gallic acid and expressed as gallic acid equivalents (GAE). Dissociation of a phenolic proton from sample leads to a phenolate anion, which is capable of reducing FCR. This supports the notion that the reaction occurs through electron transfer mechanism forming a blue compound between phenolate and FCR.

Figure 4.13 showed the total phenolic assay results for all the samples that were tested with its forward methanol and boiled water extracts. For methanol extract, the highest GAE value was shown by Vietnamese tea where its GAE value was 448.41. Malaysian Tea showed the next higher GAE value of 405.32 followed by Mangosteen skin with 308.62 and Pomelo skin with 207.76. *Bougainvillea* spp. showed 45.00 GAE value followed by *C. vulgaris* with 33.65, *S. platensis* with 28.10, *P. tetrastromatica* with 19.24 and *C. racemosa* with 12.42. Samples that showed below 10 GAE values were *K. alvarezii* (b) with 9.62 followed by *G. salicornia* with 6.06, *G. changii* with 4.05, *Eucheuma denticulatum* with 2.48, *T. conoides* and *Chaetomorpha* spp. with 1.49 and finally the lowest GAE value was shown by *S. binderi* with 0.77.

From the table (4.13), it showed that Mangosteen skin has the highest GAE value for its boiled water extract, 684.81. This was followed by Pomelo skin with 489.42, Vietnamese tea with 453.97, Malaysian tea with 435.95 *Bougainvillea* spp. with 220.24 and *S. binderi* with 142.20. Samples that showed below 100 GAE were *P. tetrastromatica*

with 78.08 GAE, followed by *T. conoides* with 74.70, *S. platensis* with 49.68, *Chaetomorpha* spp. with 48.81, *K. alvarezii* (b) with 25.10, *E. denticulatum* with 11.81 and *G. salicornia* with 9.29. *G. changii* with 6.95, *C. vulgaris* with 5.16 and finally *K. alvarezii* (a) with 2.52 showed the lowest GAE value compared to the other samples.

Table 4.13: Antioxidant activities of forward methanol and boiled water extracts evaluated with Total Phenolic Assay (GAE Assay)

Samples	Total Phenolic content (GAE)			
	Forward extraction			
	Methanol	SD	Boiled water	SD
Kappaphycus alvarezii (a)	2.19	0.40	2.52	0.20
Gracilaria changii	4.05	0.20	6.95	0.40
Kappaphycus alvarezii (b)	9.62	0.40	25.10	0.61
Eucheuma denticulatum	2.48	0.00	11.81	0.00
Gracilaria salicornia	6.06	0.00	9.29	0.43
Caulerpa racemosa	12.42	0.43	5.05	0.00
Padina tetrastromatica	19.24	0.21	78.08	1.29
Turbinaria conoides	1.49	0.25	74.70	0.25
Sargassum binderi	0.77	0.25	142.20	0.76
Chaetomorpha spp.	1.49	0.25	48.81	1.01
Malaysian tea	405.32	9.09	435.95	0.34
Vietnamese tea	448.41	3.59	453.97	3.82
Mangosteen skin	308.62	3.43	684.81	6.26
Pummelo skin	207.76	1.90	489.42	0.82
Bougainvillea spp.	45.00	0.56	220.24	27.27
Spirulina platensis	28.10	0.22	49.68	1.57
Chlorella vulgaris	33.65	1.35	5.16	0.11

Forward extraction indicates methanol followed by boiled water extraction

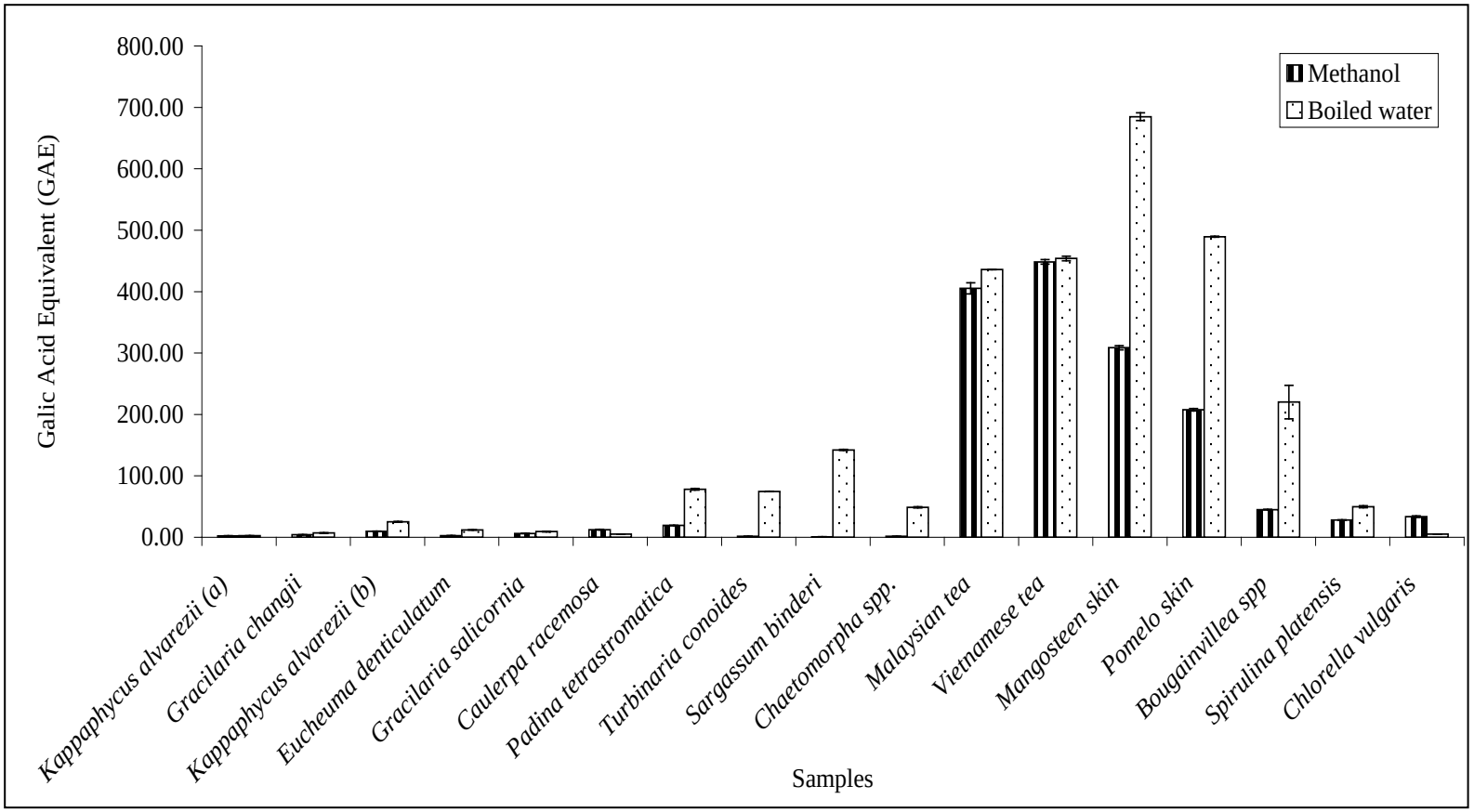

Figure 4.13: Antioxidant activities of forward methanol and boiled water extracts as evaluated by Total Phenolic assay

Forward extraction indicates methanol followed by boiled water extraction

Figure 4.14 (a) showed the GAE value for selected seaweeds that were tested using total phenolic assay for their forward and reverse methanol extracts. From table 4.14 (a) it showed that 4 out of 6 samples showed higher GAE value for their reversed methanol extracts compared to their forward methanol extracts. These samples were *P.tetrastromatica* (23.48), *Chaetomorpha spp*. (20.24), *S. binderi* (13.81) and *G. salicornia* (13.03). *T. conoides* did not show any GAE value for its reverse extract whereas for its forward extract it showed 1.49. *C. racemosa* showed higher GAE value for its forward extract (12.42) compared to reverse extract (10.91).

Table 4.14 (a): Antioxidant activities of forward and reverse methanol extracts using Total Phenolic assay

Samples	GEA value			
	Methanol			
	Forward	SD	Reverse	SD
Gracilaria salicornia	6.06	0.00	13.03	0.00
Caulerpa racemosa	12.42	0.43	10.91	1.29
Padina tetrastromatica	19.24	0.21	23.48	0.64
Turbinaria conoides	1.49	0.25	0.00	0.00
Sargassum binderi	0.77	0.25	13.81	0.51
Chaetomorpha spp.	1.49	0.25	20.24	3.03

Forward indicates methanol followed by boiled water extraction
Reverse indicates boiled water followed by methanol extraction

Figure 4.14 (b) showed the difference between forward and reverse boiled water extracts. Table 4.14 (b) showed the GAE value of all the samples tested for their Total Phenolic content. *P. tetrastromatica* showed a huge difference between its forward and reverse boiled water extracts. Its forward extract showed 78.08 GAE value whereas its reverse extract showed 39.90 GAE value. *Chaetomorpha* spp showed 48.81 GAE value for its forward extract and 6.31 GAE value for its reverse extract. *S. binderi* showed the highest GAE value for both its forward and reverse boiled water extracts where its forward

extract showed 142.20 GAE and its reverse extract showed 153.99 GAE which was higher than its forward extract. This showed that *S. binderi* reverse boiled water extract contained higher total phenolic content compared to its forward extract. For reverse boiled water extract, the second highest GAE value was *T. conoides*, 80.06, followed by *P. tetrastromatica* with 39.90, *G. salicornia* with 13.84, *Chaetomorpha* spp. with 6.31 and finally *C. racemosa* with 5.35.

Table 4.14 (b): Antioxidant activities of forward and reverse boiled water extracts using Total Phenolic assay

Samples	GAE value			
	Boiled water			
	Forward	SD	Reverse	SD
Gracilaria salicornia	9.29	0.43	13.84	0.00
Caulerpa racemosa	5.05	0.00	5.35	0.00
Padina tetrastromatica	78.08	1.29	39.90	1.71
Turbinaria conoides	74.70	0.25	80.06	0.76
Sargassum binderi	142.20	0.76	153.99	1.26
Chaetomorpha spp.	48.81	1.01	6.31	0.00

Forward indicates methanol followed by boiled water extraction
Reverse indicates boiled water followed by methanol extraction

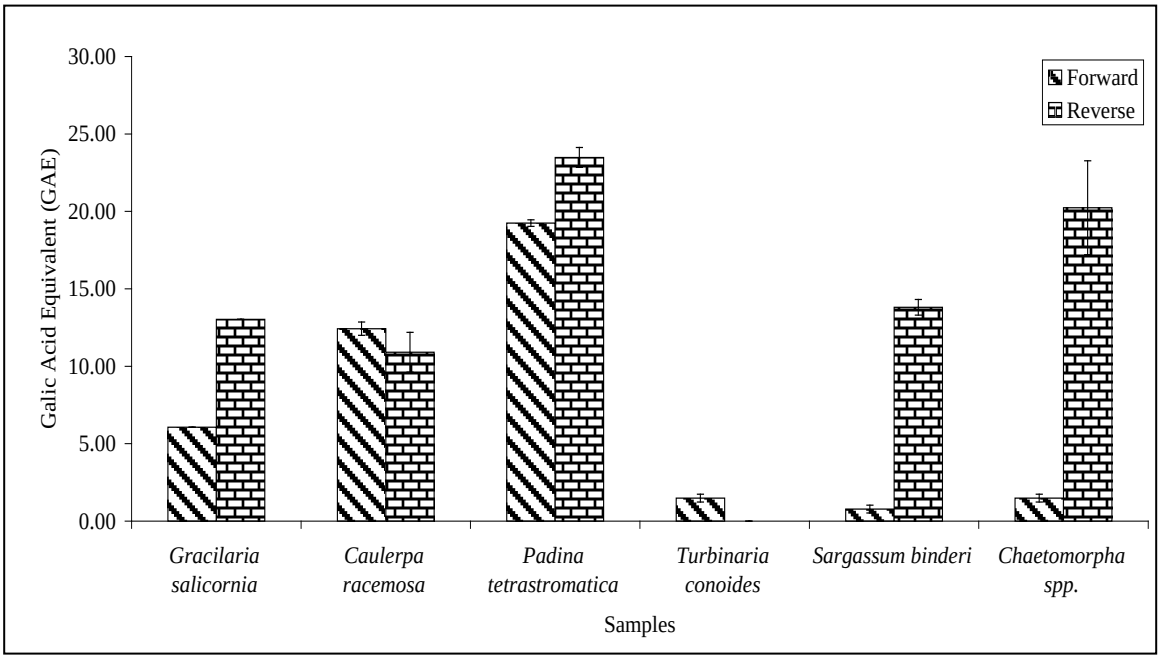

Figure 4.14 (a): Antioxidant activities of selected seaweeds forward and reverse methanol extracts as evaluated by Total Phenolic assay

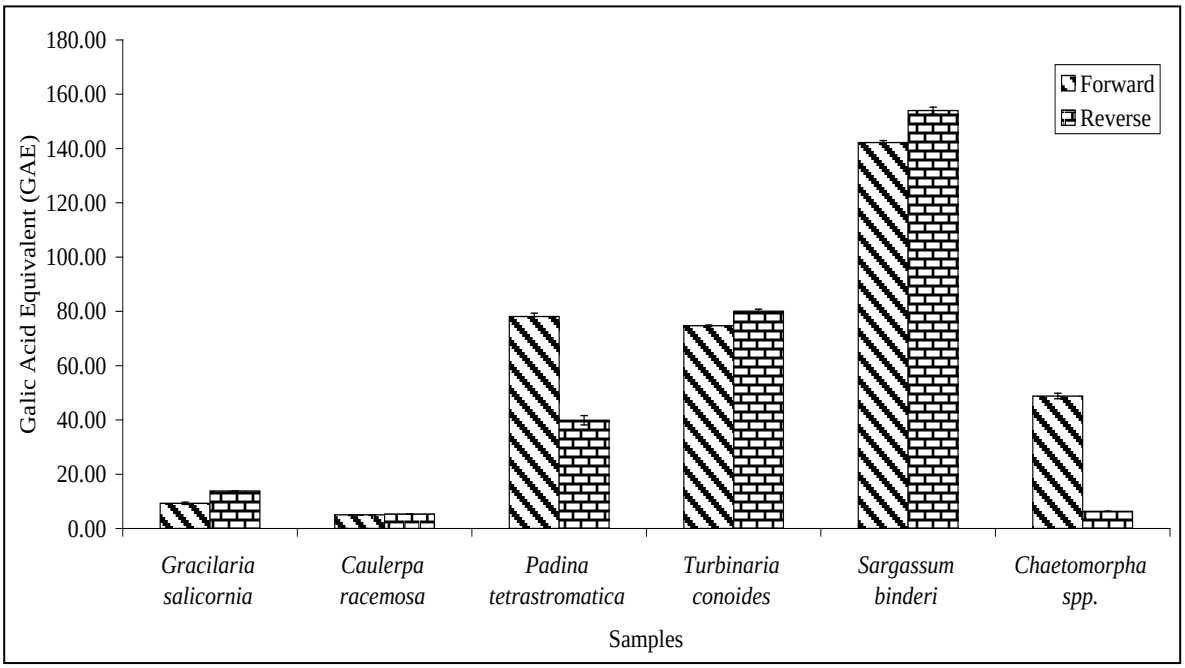

Figure 4.14 (b): Antioxidant activities of selected seaweeds forward and reversed boiled water extracts as evaluated by Total Phenolic assay

Forward indicates methanol followed by boiled water extraction
Reverse indicates boiled water followed by methanol extraction

Figure 4.15 (a) showed the GAE value for selected seaweeds of methanol extracts where the seaweeds were ground using two different grinding methods (grinding with grinder and grinding with liquid nitrogen). From the table below (4.15 (a)), the GAE values for samples ground with grinder was lower compared to the samples ground with liquid nitrogen. *T. conoides* showed the highest GAE value for liquid nitrogen grinding with 98.65 followed by *C. racemosa* with 41.11, *S. binderi* with 32.78 and finally *T. conoides* with 28.10. The highest GAE value for grinder method was shown by *Padina tetrastromatica* with 19.24, followed by *C. racemosa* with 12.42, *T. conoides* with 1.49 and finally *S. binderi* with 0.77.

Table 4.15 (a): Antioxidant activities of selected seaweeds forward methanol extracts using two methods evaluated with Total Phenolic Assay

Sample	Total Phenolic value (GAE)			
	Methanol			
	Grinder	SD	Liquid nitrogen	SD
Sargassum binderi	0.77	0.25	32.78	1.91
Turbinaria conoides	1.49	0.25	28.10	0.67
Padina tetrastromatica	19.24	0.21	98.65	1.91
Caulerpa racemosa	12.42	0.43	41.11	0.00

Grinder indicates extraction of samples dried in oven and ground using a grinder
Liquid nitrogen indicates extraction of samples that was blotted dry and ground using liquid nitrogen

Figure 4.15 (b) showed the total phenolic assay for forward boiled water extract compared between the two different methods of grinding. From the table (4.15 (b)), samples that were ground with liquid nitrogen showed lower GAE values compared to samples that were ground with grinder. The highest GAE value for grinder method was shown by *S. binderi* (142.20) whereas for its liquid nitrogen grinding, it showed 13.10 GAE value. The highest GAE value for liquid nitrogen grinding was shown by *P. tetrastromatica* (55.24), followed by *T. conoides* (28.73), *S. binderi* (13.10) and finally the lowest is *C. racemosa* (1.90).

Table 4.15 (b): Antioxidant activities of selected seaweeds forward boiled water extracts using two methods evaluated with Total Phenolic Assay

Sample	Total Phenolic value (GAE)			
	Boiled water			
	Grinder	SD	Liquid nitrogen	SD
Sargassum binderi	142.20	0.76	13.10	0.11
Turbinaria conoides	74.70	0.25	28.73	0.22
Padina tetrastromatica	78.08	1.29	55.24	0.00
Caulerpa racemosa	5.05	0.00	1.90	0.00

Grinder indicates extraction of samples dried in oven and ground using a grinder
Liquid nitrogen indicates extraction of samples that was blotted dry and ground using liquid nitrogen

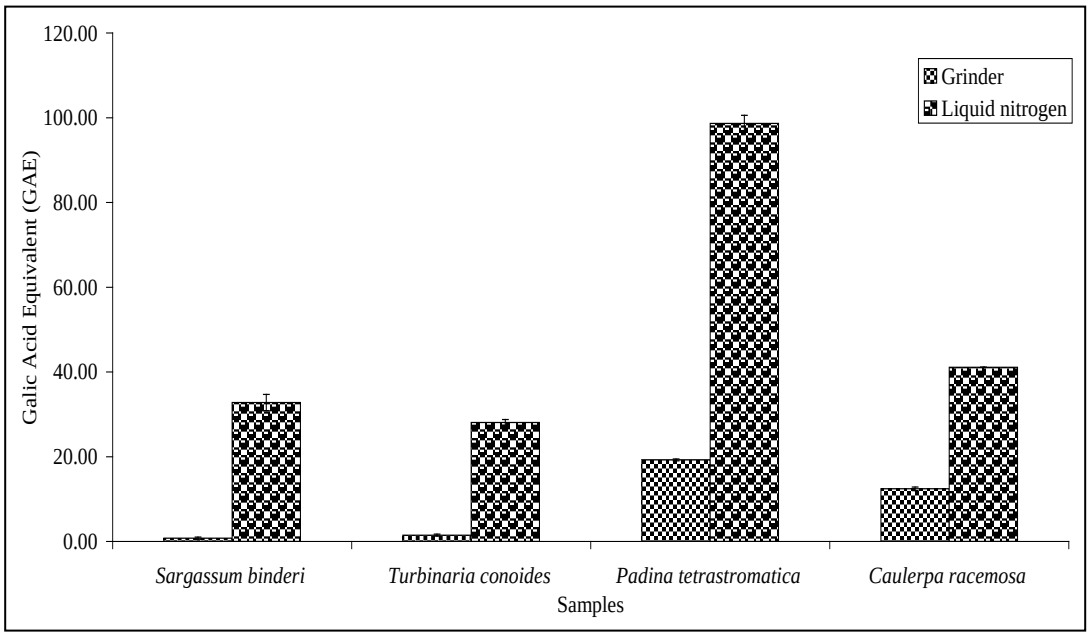

Figure 4.15 (a): Antioxidant activities of selected seaweeds forward methanol extracts using two methods as evaluated by Total Phenolic assay

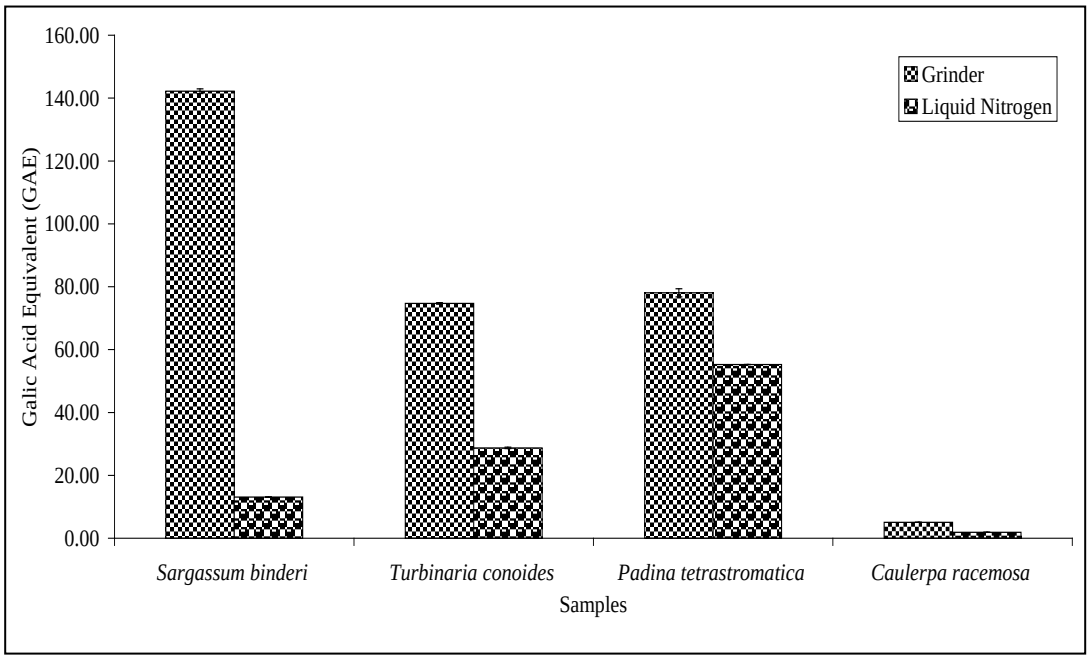

Figure 4.15 (b): Antioxidant activities of selected seaweeds forward boiled water extracts using two methods as evaluated by Total Phenolic assay

Grinder indicates extraction of samples dried in oven and ground using a grinder
Liquid nitrogen indicates extraction of samples that was blotted dry and ground using liquid nitrogen

Figure 4.16 showed the GAE value for acetone extraction of selected seaweeds and teas. From the table (4.16), it clearly showed that extracts of seaweeds from acetone extraction did not show any GAE value whereas Vietnamese tea showed the highest GAE value, 44.95 compared to Malaysian tea, 6.62.

Table 4.16: Acetone extracts of selected seaweeds and teas evaluated with Total Phenolic assay

Sample	Acetone	
	Value (GAE)	SD
Gracilaria salicornia	0.00	0.00
Caulerpa racemosa	0.00	0.00
Padina tetrastromatica	0.00	0.00
Turbinaria conoides	0.00	0.00
Sargassum binderi	0.00	0.00
Chaetomorpha spp.	0.00	0.00
Malaysia tea	6.62	0.21
Vietnam tea	44.95	1.29

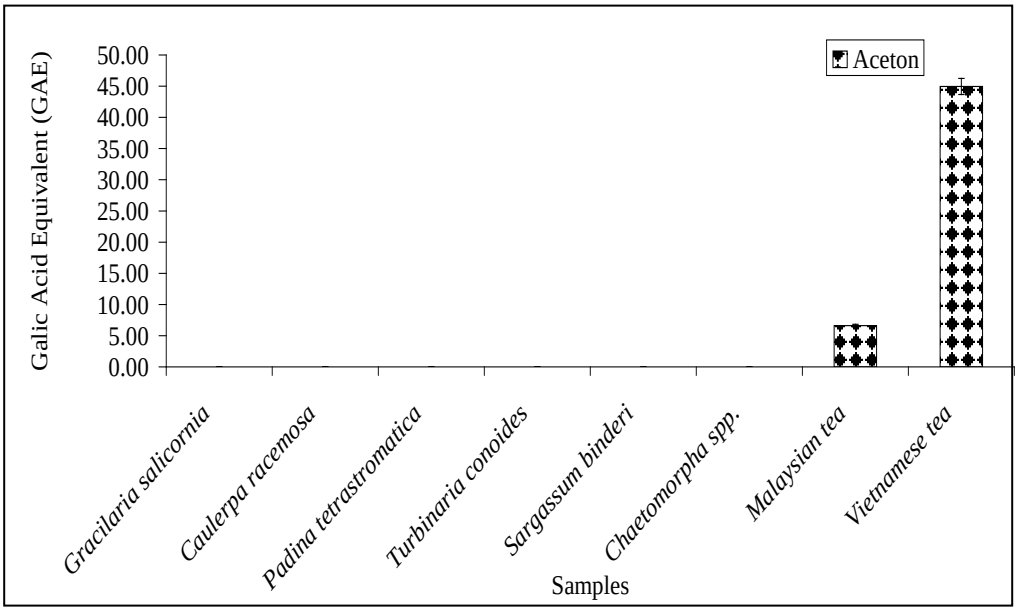

Figure 4.16: Antioxidant activities of acetone extracts as evaluated by Total Phenolic assay

4.2 Animal studies

4.2.1 Determination of LD_{50}

The LD_{50} for *Padina tetrastromatica* (ethyl acetate and ethanol extracts) and *Kappaphycus alvarezii* (b) (ethanol extract) were obtained from the log-dose response curve based on Probit Unit 5. Figure 4.17 (a), (b) and (c) shows the LD_{50} of *P. tetrastromatica* ethyl acetate extract, *P. tetrastromatica* ethanol extract and *K. alvarezii* (b) ethanol extract respectively. LD_{50} ethyl acetate extract of *P. tetrastromatica* was found to be 1845mg/kg. LD_{50} ethanol extracts of *P. tetrastromatica* was found to be 1596mg/kg and finally LD_{50} ethanol extract of *K. alvarezii* was found to be 1799mg/kg.

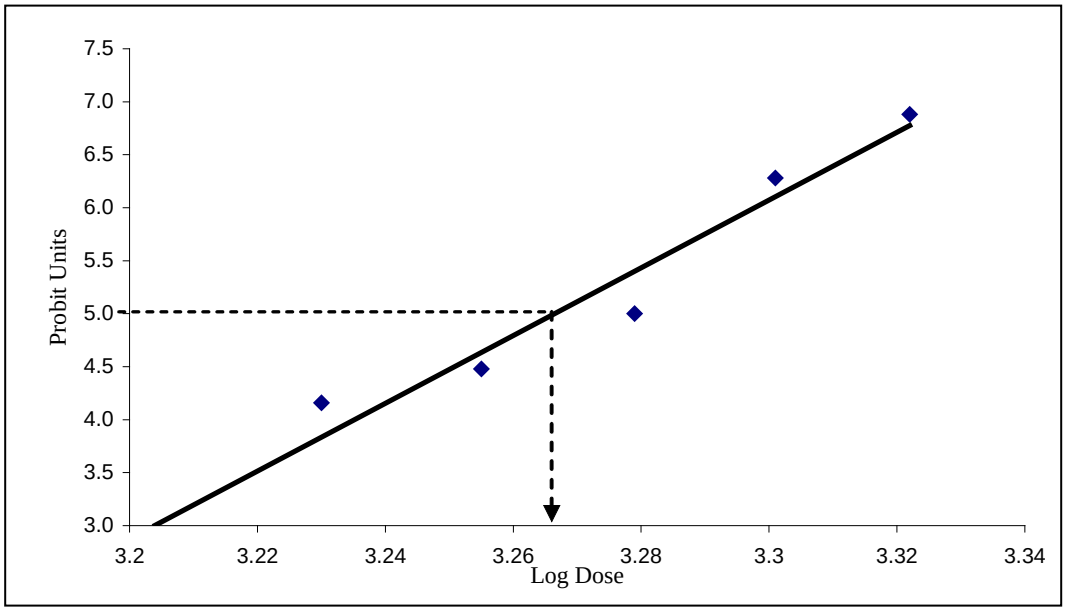

Figure 4.17 (a): Mortality of mice after being administered with *Padina tetrastromatica* ethyl acetate extracts at several doses.

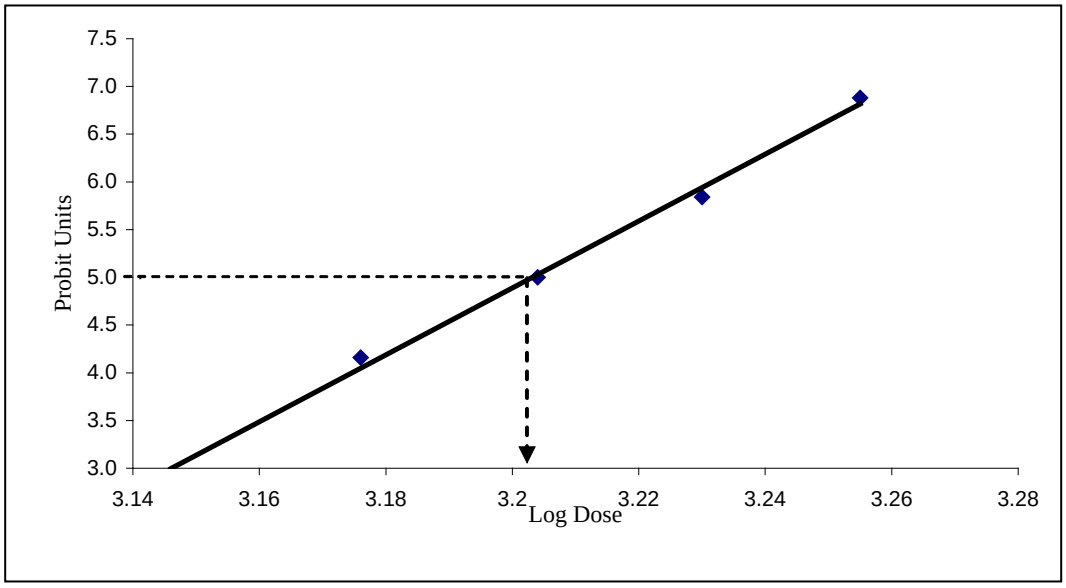

Figure 4.17 (b): Mortality of mice after being administered with *Padina tetrastromatica* ethanol extracts at several doses.

Doses were reported in mg/kg with the percentage of mortality transformed in probit unit

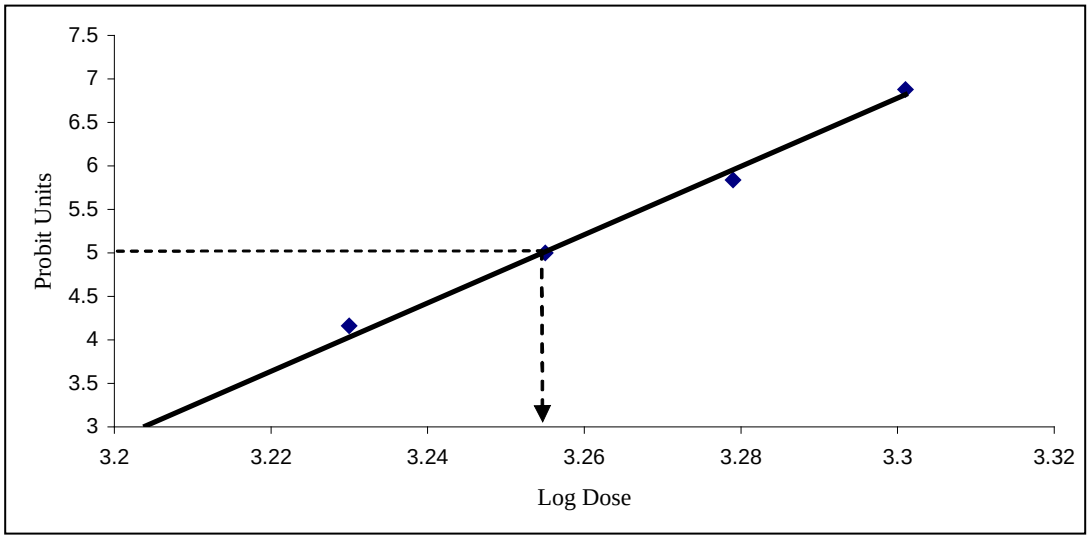

Figure 4.17 (c): Mortality of mice after being administered with *Kappaphycus alvarezii* ethanol extracts at several doses.

Doses were reported in mg/kg with the percentage of mortality transformed in probit unit

4.2.2 Effects of Carrageenan induced oedema on rat's right hind paw (10, 30 and 100 mg/kg of sample).

Paw oedema was induced by injecting 0.1mL of 1% carrageenan onto the plantar surface of the rat's right hind paw. The oedema effect by oral administration of 10, 30 and 100 mg/kg extracts of *P. tetrastromatica* (ethyl acetate and ethanol extracts) and *K. alvarezii* (ethanol extract) were shown in Figure 4.18 (a), (b) and (c), respectively.

Indomethacin (10 mg/kg) and dispirin (150 mg/kg) showed a significant decrease in percentage of swelling compared to control (10% ethanol). Indomethacin showed 82.40±6.5% and dispirin showed 88.33±5.73% in the inhibition of oedema. The optimum percentage of oedema in this test was calculated at 210 minutes as compared to the optimum oedema effect in control group as shown in Table 4.17 (a), (b) and (c), respectively.

Sample *Padina tetrastromatica* ethanol (PT Et) 10 mg/kg extract caused significant anti-inflammatory effect when compared to control (10% ethanol). *Padina tetrastromatica* ethyl acetate (PT Ea) at 10 mg/kg extract and *Kappaphycus alvarezii* ethanol (KA Et) at 10 mg/kg extract did not show any significant in percentage swelling induced by carrageenan as compared to control group. PT Et extract showed 54.48±10.28% in the inhibition of oedema as shown in table 4.17 (a). PT Et showed the onset of action began at 180 minutes of carrageenan injection and the duration lasted for an hour. Dispirin and indomethacin showed the onset of action started at 90 minutes but duration for dispirin lasted for 3 and a half hour whereas duration of action for indomethacin lasted for 5 and a half hour or probably longer (experiment was only conducted for 7 hours) as shown in Figure 4.18 (a).

Table 4.17 (a): Percentage inhibition of carrageenan – induced paw oedema in rats treated with 10 mg/kg *Padina tetrastromatica* ethyl acetate (PT Ea), *Padina tetrastromatica* ethanol (PT Et) and *Kappaphycus alvarezii* ethanol (KA Et) extracts.

Group	Paw withdrawal latencies (sec)	
	% of Oedema (mean ± S.E.M)	% Inhibition of Oedema (obtained from average value)
Control (10% Ethanol)	86.01 ± 5.08	0
Indomethacin 10 mg/kg	6.74 ± 3.58	82.04 ± 6.5 ***
Dispirin 150 mg/kg	8.82 ± 4.1	88.33 ± 5.73 ***
Padina tetrastromatica (PT Ea)	77.50 ± 11.11	9.5 ± 13.02
Padina tetrastromatica (PT Et)	45.05 ± 9.18	54.48 ± 10.28 **
Kappaphycus alvarezii (KA Et)	63.78 ± 4.72	23.38 ± 8.99

10 % ethanol and 150 mg/kg dispirin were given orally and 10mg/kg indomethacin was administered intraperitoneally at 210 minutes as compared to 100% swelling in control group. *P<0.05, **P<0.01, ***P<0.001 indicated significant difference using ANOVA followed by Tukey Test.

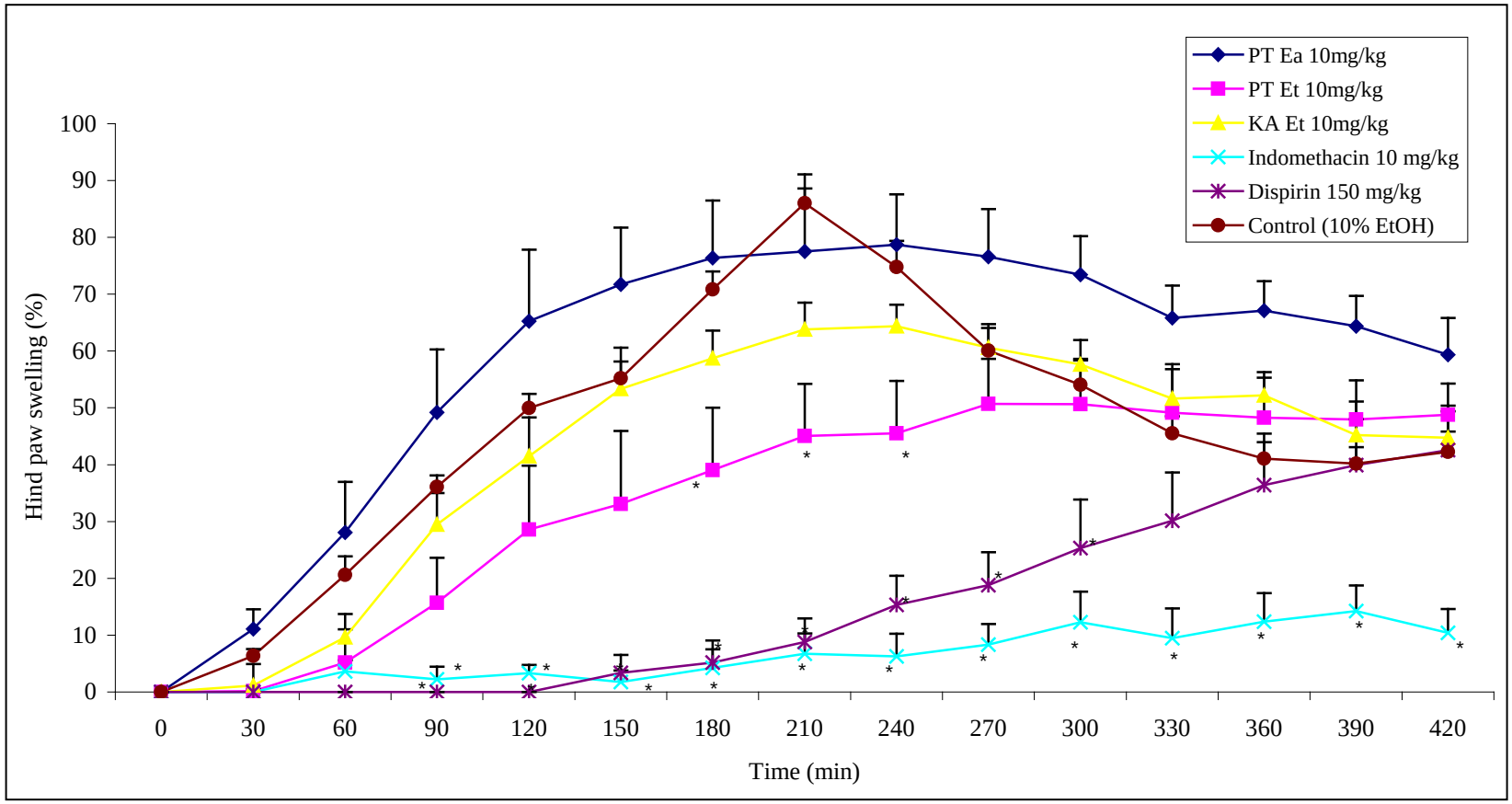

Figure 4.18 (a): Carrageenan induced paw oedema in rats treated with 10 mg/kg of *Padina tetrastromatica* ethyl acetate (PT Ea), *Padina tetrastromatica* ethanol (PT Et) and *Kappaphycus alvarezii* ethanol (KA Et) extracts.

10 % ethanol and 150 mg/kg dispirin were given orally and 10mg/kg indomethacin was administered intraperitoneally. Data presented as mean ± S.E.M (n=6). * $P<0.05$ compared with control group determined by ANOVA followed by Tukey Test.

At 30 mg/kg of PT Et, KA Et and PT Ea extracts, they showed a significant decrease in percentage of swelling were observed when compared to control. PT Et showed 84.90±3.8 % inhibition of oedema, KA Et showed 83.09±6.58 % and PT Ea showed 40.01±9.09 %, the lowest among the samples as shown in Table 4.17 (b). PT Et, KA Et, indomethacin and dispirin showed the onset of action started at 30 minutes. For PT Et, the duration of action lasted for 5 hours. KA Et and dispirin showed the duration of action lasted for 4 and a half hour and indomethacin action lasted for 5 and a half hour. As for PT Ea, the onset of action started at 180 minutes and the duration lasted for only 1 hour. The second onset for KA Et, PT Et and indomethacin started at 420 minutes and the duration cannot be detected because the test was carried out for 420 minutes as shown in Figure 4.18 (b).

Table 4.17 (b): Percentage inhibition of carrageenan – induced paw oedema in rats treated with 30 mg/kg *Padina tetrastromatica* ethyl acetate (PT Ea), *Padina tetrastromatica* ethanol (PT Et) and *Kappaphycus alvarezii* ethanol (KA Et) extracts.

Group	Paw withdrawal latencies (sec)	
	% of Oedema (mean ± S.E.M)	% Inhibition of Oedema (obtained from average value)
Control (10% Ethanol)	86.01 ± 5.08	0
Indomethacin 10 mg/kg	6.74 ± 3.58	82.04 ± 6.5 ***
Dispirin 150 mg/kg	8.82 ± 4.1	88.33 ± 5.73 ***
Padina tetrastromatica (PT Ea)	52.04 ± 8.40	40.01 ± 9.09 *
Padina tetrastromatica (PT Et)	13.35 ± 3.79	84.90 ± 3.8 ***
Kappaphycus alvarezii (KA Et)	13.81 ± 5.29	83.09 ± 6.58 ***

10 % ethanol and 150 mg/kg dispirin were given orally and 10mg/kg indomethacin was administered intraperitoneally at 210 minutes as compared to 100% swelling in control group. *P<0.05, **P<0.01 , ***P<0.001 indicated significant difference using ANOVA followed by Tukey Test.

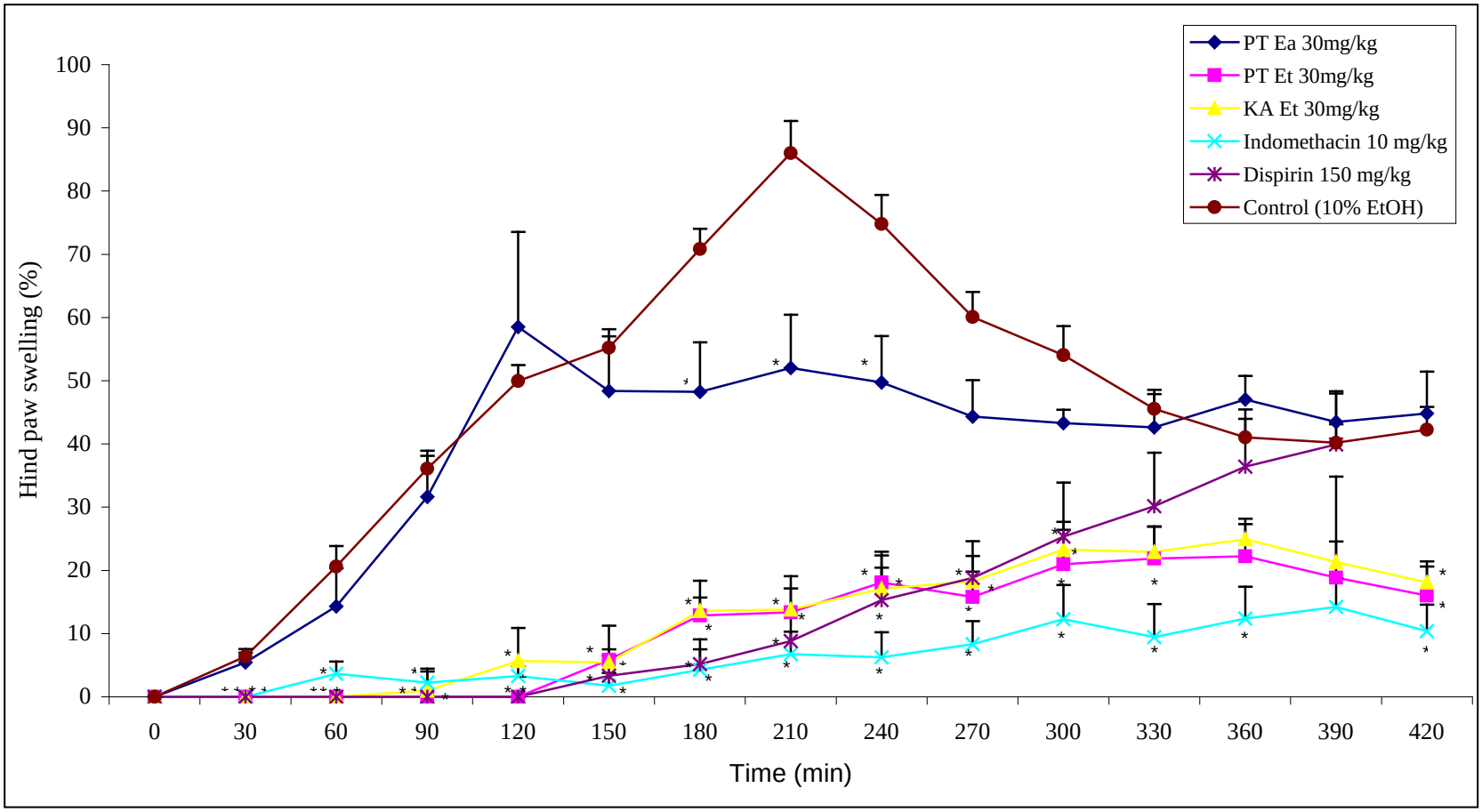

Figure 4.18 (b): Carrageenan induced paw oedema in rats treated with 30 mg/kg of *Padina tetrastromatica* ethyl acetate (PT Ea), *Padina tetrastromatica* ethanol (PT Et) and *Kappaphycus alvarezii* ethanol (KA Et) extracts

10 % ethanol and 150 mg/kg dispirin were given orally and 10mg/kg indomethacin was administered intraperitoneally. Data presented as mean ± S.E.M (n=6). * $P<0.05$ compared with control group determined by ANOVA followed by Tukey Test.

When 100 mg/kg of sample extracts were administered orally, only PT Et and KA Et showed a significant decrease in percentage of swelling induced by carrageenan as compared to control as shown in Table 4.17 (c). PT Ea showed no significant decrease in the percentage of swelling. PT Et showed 91.65±5.75 % whereas KA Et showed 70.55±6.14 % inhibition of oedema. The onset action of PT Et, KA Et, indomethacin and dispirin started at 60 minutes. The duration of action for all four lasted for 3 and a half hour. The second onset for PT Et started at 330 minutes and lasted for or less than half an hour and the third onset started at 390 minutes and lasted for or less than half an hour as shown in Figure 4.18 (c). The second onset for indomethacin started at 330 minutes and lasted for or less than 30 minutes. PT Et showed an increment in percentage of inhibition of oedema as the dose was increased, indicating a dose-dependency of anti-inflammatory effect of PT Et. At 100 mg/kg, anti-inflammatory effect of Pt Et was more effective than dispirin and indomethacin.

Table 4.17 (c): Percentage inhibition of carrageenan – induced paw oedema in rats treated with 100 mg/kg *Padina tetrastromatica* ethyl acetate (PT Ea), *Padina tetrastromatica* ethanol (PT Ea) and *Kappaphycus alvarezii* ethanol (KA Et) extracts.

Group	Paw withdrawal latencies (sec)	
	% of Oedema (mean ± S.E.M)	% Inhibition of Oedema (obtained from average value)
Control (10% Ethanol)	86.01 ± 5.08	0
Indomethacin 10 mg/kg	6.74 ± 3.58	82.04 ± 6.5 ***
Dispirin 150 mg/kg	8.82 ± 4.1	88.33 ± 5.73 ***
Padina tetrastromatica (PT Ea)	60.53 ± 11.97	28.00 ± 15.52
Padina tetrastromatica (PT Et)	6.13 ± 5.19	91.65 ± 5.75 ***
Kappaphycus alvarezii (KA Et)	23.95 ± 3.56	70.55 ± 6.14 ***

10 % ethanol and 150 mg/kg dispirin were given orally and 10mg/kg indomethacin was administered intraperitoneally at 210 minutes as compared to 100% swelling in control group. *P<0.05, **P<0.01, ***P<0.001 indicated significant difference using ANOVA followed by Tukey Test.

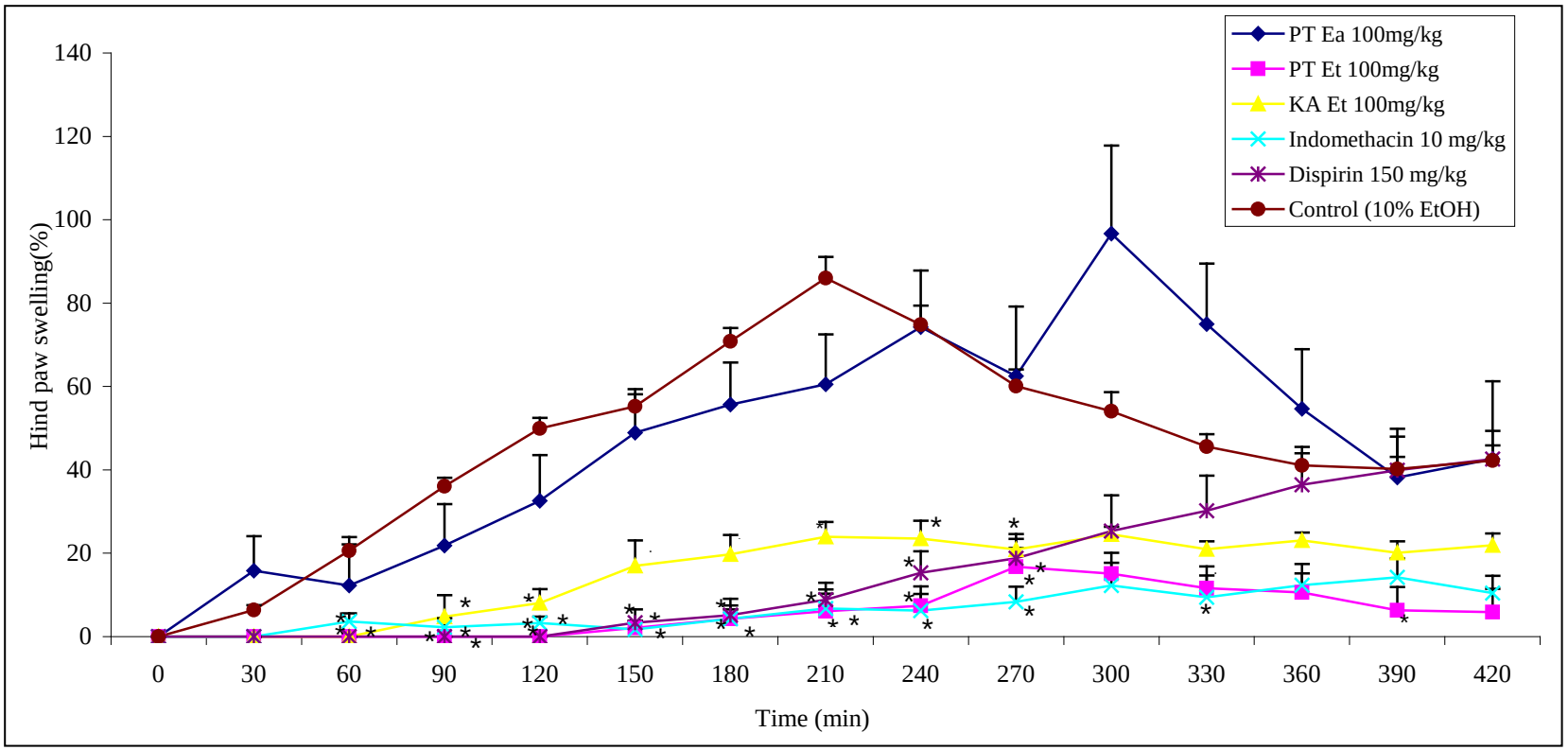

Figure 4.18 (c): Carrageenan induced paw oedema in rats treated with 100 mg/kg of *Padina tetrastromatica* ethyl acetate (PT Ea), *Padina tetrastromatica* ethanol (PT Et) and *Kappaphycus alvarezii* ethanol (KA Et) extracts.

10 % ethanol and 150 mg/kg dispirin were given orally and 10mg/kg indomethacin was administered intraperitoneally. Data presented as mean ± S.E.M (n=6). * $P<0.05$ compared with control group determined by ANOVA followed by Tukey Test.

4.2.3 Analgesic effects of seaweeds extracts

P. tetrastromatica ethyl acetate and ethanol extracts and *K. alvarezii* ethanol extract were studied for the peripheral antinociceptive effect on carrageenan-induced hyperalgesia of rat's right hind paw (Figure 4.19 (a), (b) and (c)) and central antinociceptive effect of the rat's left hind paw (Figure 4.20 (a), (b) and (c)) injected with saline using Hargreaves method (1988).

(i) Peripheral antinociceptive action on carrageenan-induced hyperalgesia on right hind paw (10, 30 and 100 mg/kg of sample).

Extracts of PT Ea, PT Et and KA Et at 10 mg/kg did not show any antinociceptive effect since the samples did not prolong rat's paw withdrawal latencies after being injected with carrageenan (Figure 4.19 (a)). Similar response was also observed with dispirin. As for indomethacin, a clinically used drug, it showed 2 onsets. The first onset started at 120 minutes and lasted for 1 and half hour. Another onset started at 450 minutes and the duration of action was not obtained because the test was carried out for 450 minutes only. Injection of 0.1 mL of 1% carrageenan onto rat's right hind paw produced hyperalgesic response in control group. In the control group which was given 10% ethanol, the paw withdrawal latency was at 10.65 ± 0.8 seconds at 0 minutes and it was shortened to 2.85 ± 0.45 seconds at 450 minutes. According to the results, samples given at 10 mg/kg orally were not significant in prolonging rat's paw withdrawal latency (PT Et; 6.97 ± 1.75 seconds, PT Ea; 4.25 ± 0.63 seconds and KA Et 4.10 ± 0.55 seconds) as compared to the control group (Table 4.18 (a)).

Table 4.18 (a): Effects of radiant heat on withdrawal latencies of rats' right hind paws injected with 1% carrageenan treated with 10 mg/kg of *Padina tetrastromatica* ethyl acetate (PT Ea), *Padina tetrastromatica* ethanol (PT Et) and *Kappaphycus alvarezii* ethanol (KA Et) extracts.

Group	Right paw withdrawal latencies (sec)	
	Baseline (0 hour)	At 7 hour 30 min
Control (10% Ethanol)	10.65 ± 0.8	2.85 ± 0.45
Indomethacin 10 mg/kg	14.0 ± 0.79	7.55 ± 1.61 *
Dispirin 150 mg/kg	12.28 ± 1.07	3.3 ± 0.51
Padina tetrastromatica (PT Ea)	6.03 ± 0.53	4.25 ± 0.63
Padina tetrastromatica (PT Et)	11.47 ± 1.26	6.97 ± 1.75
Kappaphycus alvarezii (KA Et)	11.28 ± 0.66	4.10 ± 0.55

10 % ethanol and 150 mg/kg dispirin were given orally and 10mg/kg indomethacin was administered intraperitoneally with left hind paw injected with 0.1ml saline. *$P<0.05$ indicate significant difference between control and treated group as determined with ANOVA followed by Tukey Test.

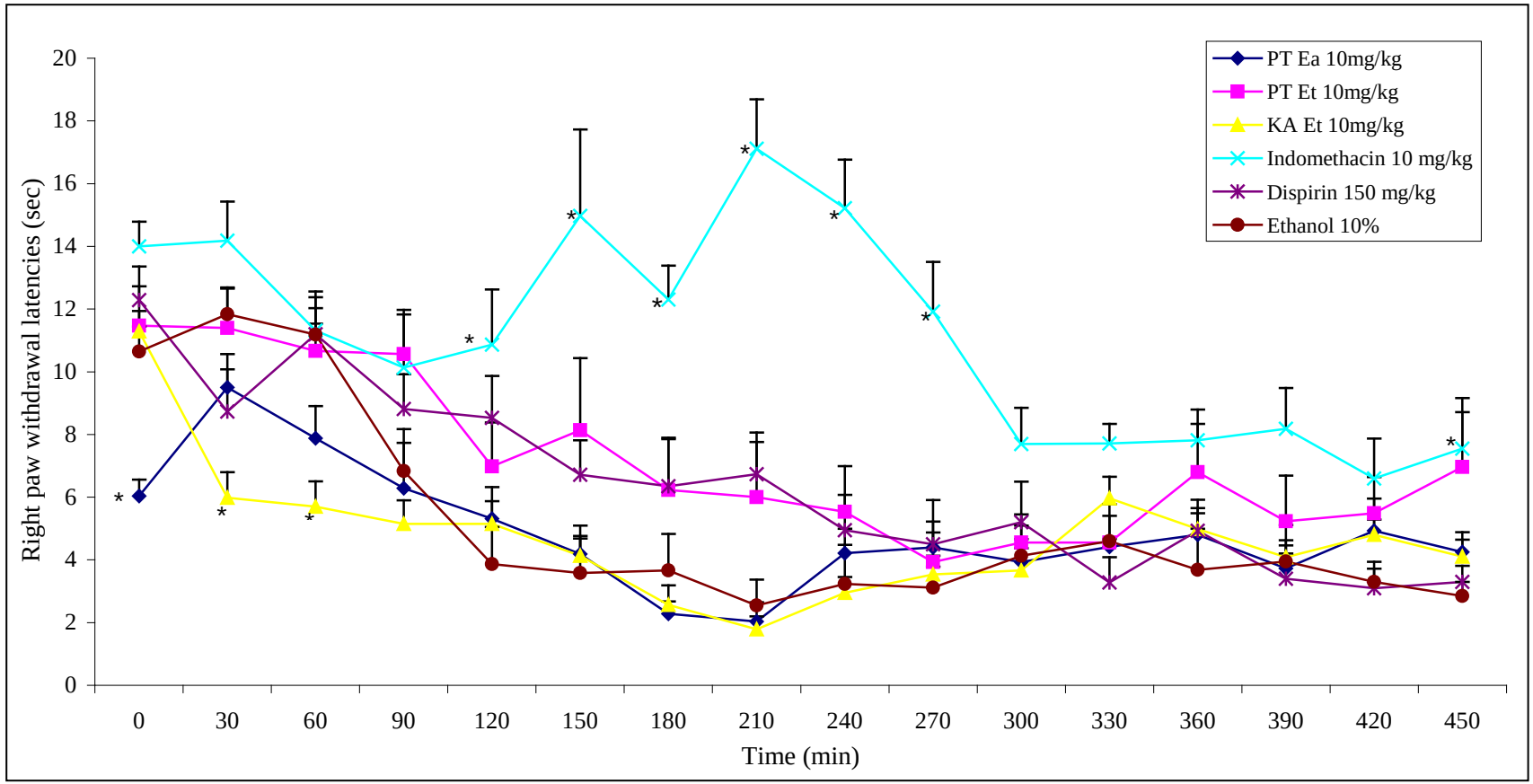

Figure 4.19 (a): Effects of radiant heat on withdrawal latencies of the rats' right hind paws injected with 1% carrageenan treated with 10 mg/kg of *Padina tetrastromatica* ethyl acetate (PT Ea), *Padina tetrastromatica* ethanol (Pt Et) and *Kappaphycus alvarezii* ethanol (KA Et) extracts.

10 % ethanol and 150 mg/kg dispirin were given orally and 10mg/kg indomethacin was administered intraperitoneally. Data presented as mean ± S.E.M (n=6). * $P<0.05$ compared with control group determined by ANOVA followed by Tukey Test.

PT Et administered orally at 30 mg/kg significantly reduced carrageenan induced hyperalgesia (7.75 ± 1.05 seconds) at 450 minutes (Table 4.18 (b)). The onset actions started at 210 minutes and lasted for less than half and hour, then started again at 270 minutes and lasted for less than half and hour. The onset started again at 330 minutes and lasted for more than half an hour and lastly started at 420 minutes and possibly lasted for more than half and hour (experiment was only performed for 450 minutes) (Figure 4.19 (b)). Samples PT Ea and KA Et given at 30 mg/kg orally were not significant in prolonging rat's paw withdrawal latency (PT Ea; 5.32 ± 1.03 seconds and KA Et 6.88 ± 0.65 seconds) as compared to the control group.

Table 4.18 (b): Effects of radiant heat on withdrawal latencies of rats' right hind paws injected with 1% carrageenan treated with 30 mg/kg of *Padina tetrastromatica* ethyl acetate (PT Ea), *Padina tetrastromatica* ethanol (PT Et)and *Kappaphycus alvarezii* ethanol (KA Et) extracts .

Group	Right paw withdrawal latencies (sec)	
	Baseline (0 hour)	At 7 hour 30 min
Control (10% Ethanol)	10.65 ± 0.8	2.85 ± 0.45
Indomethacin 10 mg/kg	14.0 ± 0.79	7.55 ± 1.61 *
Dispirin 150 mg/kg	12.28 ± 1.07	3.3 ± 0.51
Padina tetrastromatica (PT Ea)	10.10 ± 0.74	5.32 ± 1.03
Padina tetrastromatica (PT Et)	10.25 ± 0.98	7.75 ± 1.05 *
Kappaphycus alvarezii (KA Et)	10.18 ± 0.71	6.88 ± 0.65

10 % ethanol and 150 mg/kg dispirin were given orally and 10mg/kg indomethacin was administered intraperitoneally with left hind paw injected with 0.1ml saline. *$P<0.05$ indicate significant difference between control and treated group as determined with ANOVA followed by Tukey Test.

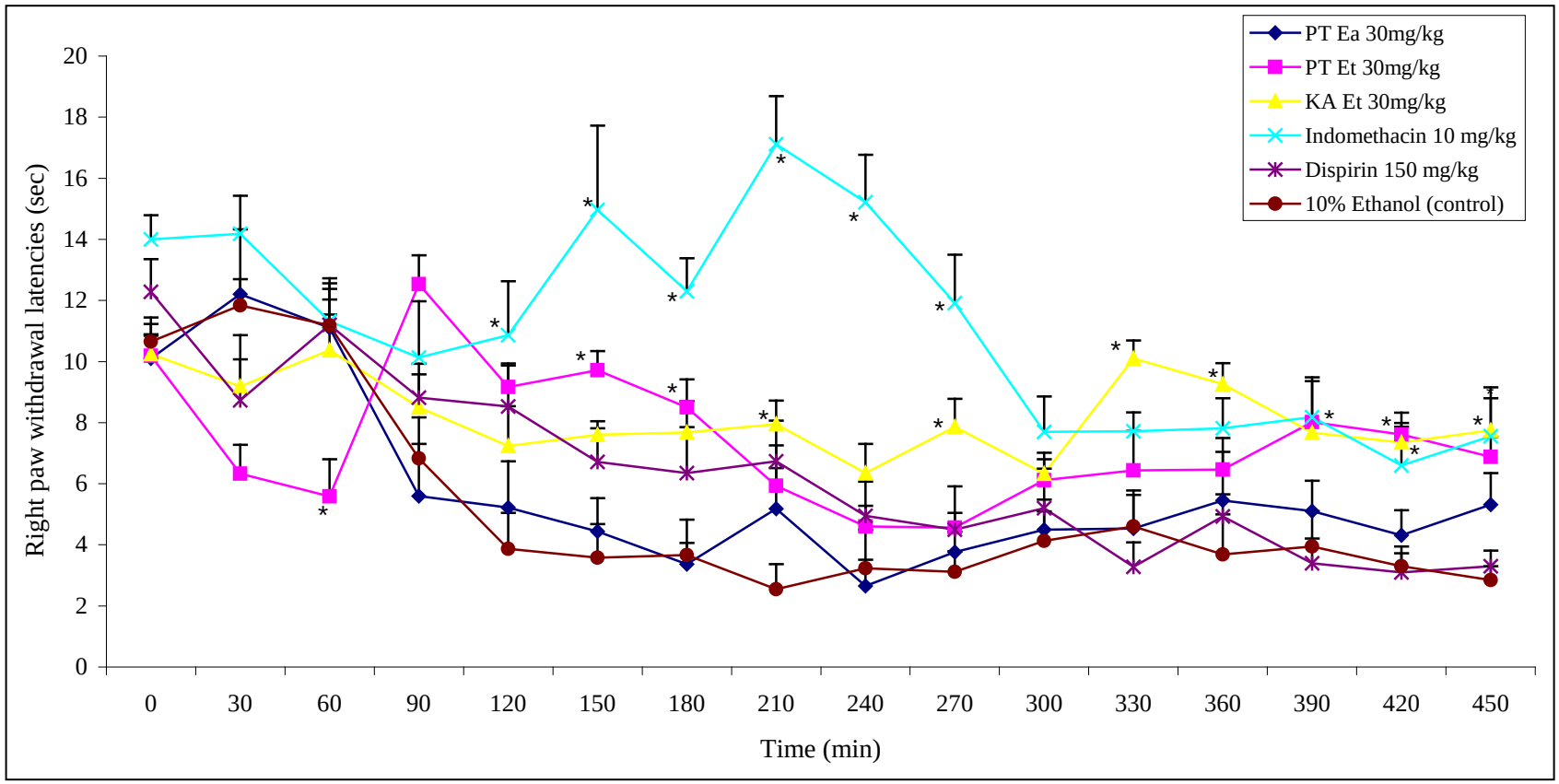

Figure 4.19 (b): Effects of radiant heat on withdrawal latencies of the rats' right hind paws injected with 1% carrageenan treated with 30 mg/kg of *Padina tetrastromatica* ethyl acetate (PT Ea),*Padina tetrastromatica* ethanol (Pt Et) and *Kappaphycus alvarezii* ethanol (KA Et) extracts.

10 % ethanol and 150 mg/kg dispirin were given orally and 10mg/kg indomethacin was administered intraperitoneally. Data presented as mean ± S.E.M (n=6). * $P<0.05$ compared with control group determined by ANOVA followed by Tukey Test.

PT Et and PT Ea administered orally at 100 mg/kg showed significant reduction in carrageenan induced hyperalgesia (Table 4.18 (c)). Both samples significantly prolong rat's paw withdrawal latency (PT Et; 10.03 ± 1.40 seconds and PT Ea; 10.70 ± 1.41 seconds) as compared to control group. There were 3 onsets of actions for PT Et where the first onset action of PT Et started at 180 minutes and lasted for one and half hour. The second onset started at 330 minutes and lasted for half and hour. The final onset for PT Et started at 420 minutes and lasted for half and hour or more (experiment was conducted for 450 minutes only). There were 4 onsets of actions for PT Ea as well. The first onset started the moment carrageenan was injected onto the rat's hind paw and lasted for less than half an hour. The second onset started at 150 minutes and lasted for one hour. The third onset started at 330 minutes and lasted for more or less than half an hour. The last onset started at 420 minutes and lasted for half an hour or more (Figure 4.19 (c).

Table 4.18 (c): Effects of radiant heat on withdrawal latencies of rats' right hind paws injected with 1% carrageenan treated with 100 mg/kg of *Padina tetrastromatica* ethyl acetate (PT Ea), *Padina tetrastromatica* ethanol (PT Et) and *Kappaphycus alvarezii* ethanol (KA Et) extracts.

Group	Right paw withdrawal latencies (sec)	
	Baseline (0 hour)	At 7 hour 30 min
Control (10% Ethanol)	10.65 ± 0.8	2.85 ± 0.45
Indomethacin 10 mg/kg	14.0 ± 0.79	7.55 ± 1.61 *
Dispirin 150 mg/kg	12.28 ± 1.07	3.3 ± 0.51
Padina tetrastromatica (PT Ea)	17.73 ± 1.69	10.70 ± 1.41 ***
Padina tetrastromatica (PT Et)	10.37 ± 0.53	10.03 ± 1.40 **
Kappaphycus alvarezii (KA Et)	10.85 ± 1.22	5.43 ± 0.74

10 % ethanol and 150 mg/kg dispirin were given orally and 10mg/kg indomethacin was administered intraperitoneally with left hind paw injected with 0.1ml saline. *P<0.05, **P<0.01, ***P<0.001 indicate significant difference between control and treated group as determined with ANOVA followed by Tukey Test.

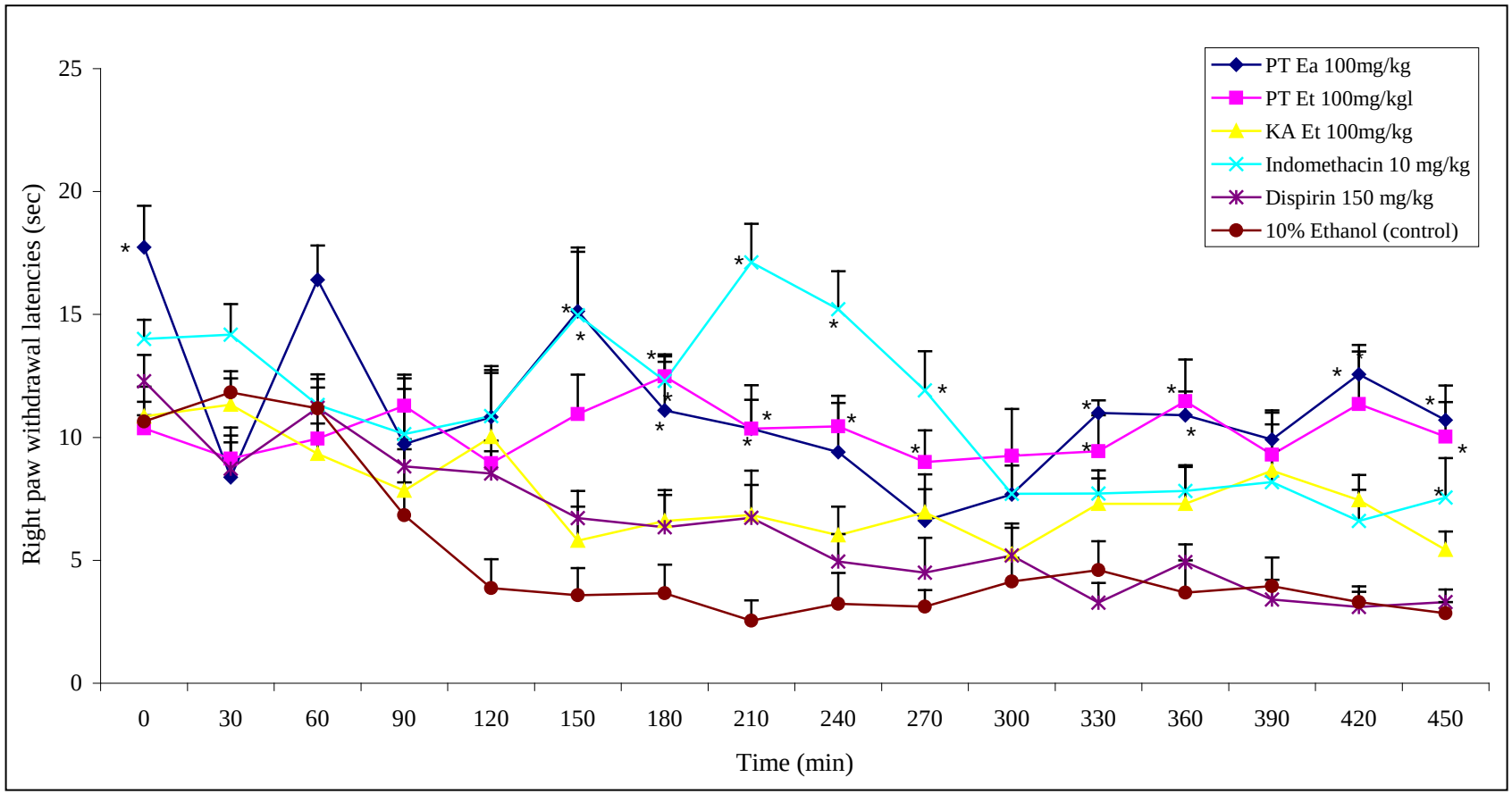

Figure 4.19 (c): Effects of radiant heat on withdrawal latencies of the rats' right hind paws injected with 1% carrageenan treated with 100 mg/kg of *Padina tetrastromatica* ethyl acetate (PT Ea),*Padina tetrastromatica* ethanol (Pt Et) and *Kappaphycus alvarezii* ethanol (KA Et) extracts.

10 % ethanol and 150 mg/kg dispirin were given orally and 10mg/kg indomethacin was administered intraperitoneally. Data presented as mean ± S.E.M (n=6). * $P<0.05$ compared with control group determined by ANOVA followed by Tukey Test

(ii) Central antinociceptive action on left hind paw injected with 0.1 ml saline (10, 30 and 100 mg/kg of sample).

The right hind paw of the rat was screened for carrageenan induced hyperalgesia whereas the left hind paw of the rat was injected with 0.1ml saline to study the possible involvement of central nervous system mechanism in producing antinociceptive effect.

10, 30 and 100 mg/kg of PT Et, PT Ea and KA Et were administered orally to rats that were injected with 0.1ml saline onto their left hind paw. From these samples, only PT Ea 100 mg/kg showed significant prolongation in paw withdrawal latencies at 450 minutes (10.65 ± 1.04 seconds) compared to control (Table 4.19 (c)). There were 3 onsets of action where the first onset started at 120 minutes and lasted for one and half hour. The second onset started at 270 minutes and lasted for half and hour. The last onset started at 390 minutes and lasted for half and hour or more (Figure 4.20 (a)). Indomethacin and dispirin did not show any significant of prolongation in paw withdrawal latencies (indomethacin; 7.30 ± 0.94 seconds and dispirin; 5.27 ± 0.36 seconds) compared to control. In the control group given 10% ethanol, the paw withdrawal latency was at 12.07 ± 0.67 seconds at 0 minutes and it was shortened to 7.20 ± 0.82 seconds at 450 minutes. Therefore, in this test, only PT Ea at 100 mg/kg caused a significant antinociceptive effect.

Table 4.19 (a): Effects of radiant heat on withdrawal latencies of rats' left hind paws injected with 0.1mL saline treated with 10 mg/kg of *Padina tetrastromatica* ethyl acetate (PT Ea), *Padina tetrastromatica* ethanol (PT Et) and *Kappaphycus alvarezii* ethanol (KA Et) extracts.

Group	Left paw withdrawal latencies (sec)	
	Baseline (0 hour)	At 7 hour 30 min
Control (10% Ethanol)	12.07 ± 0.67	7.20 ± 0.82
Indomethacin 10 mg/kg	11.88 ± 1.30	7.30 ± 0.94
Dispirin 150 mg/kg	12.30 ± 2.40	5.27 ± 0.36
Padina tetrastromatica (PT Ea)	6.71 ± 0.73	9.30 ± 0.39
Padina tetrastromatica (PT Et)	9.15 ± 0.87	8.70 ± 0.43
Kappaphycus alvarezii (KA Et)	11.20 ± 1.42	7.95 ± 0.34

10 % ethanol and 150 mg/kg dispirin were given orally and 10mg/kg indomethacin was administered intraperitoneally with right hind paw injected with 1% carrageenan. *P<0.05 indicate significant difference between control and treated group as determined with ANOVA followed by Tukey Test.

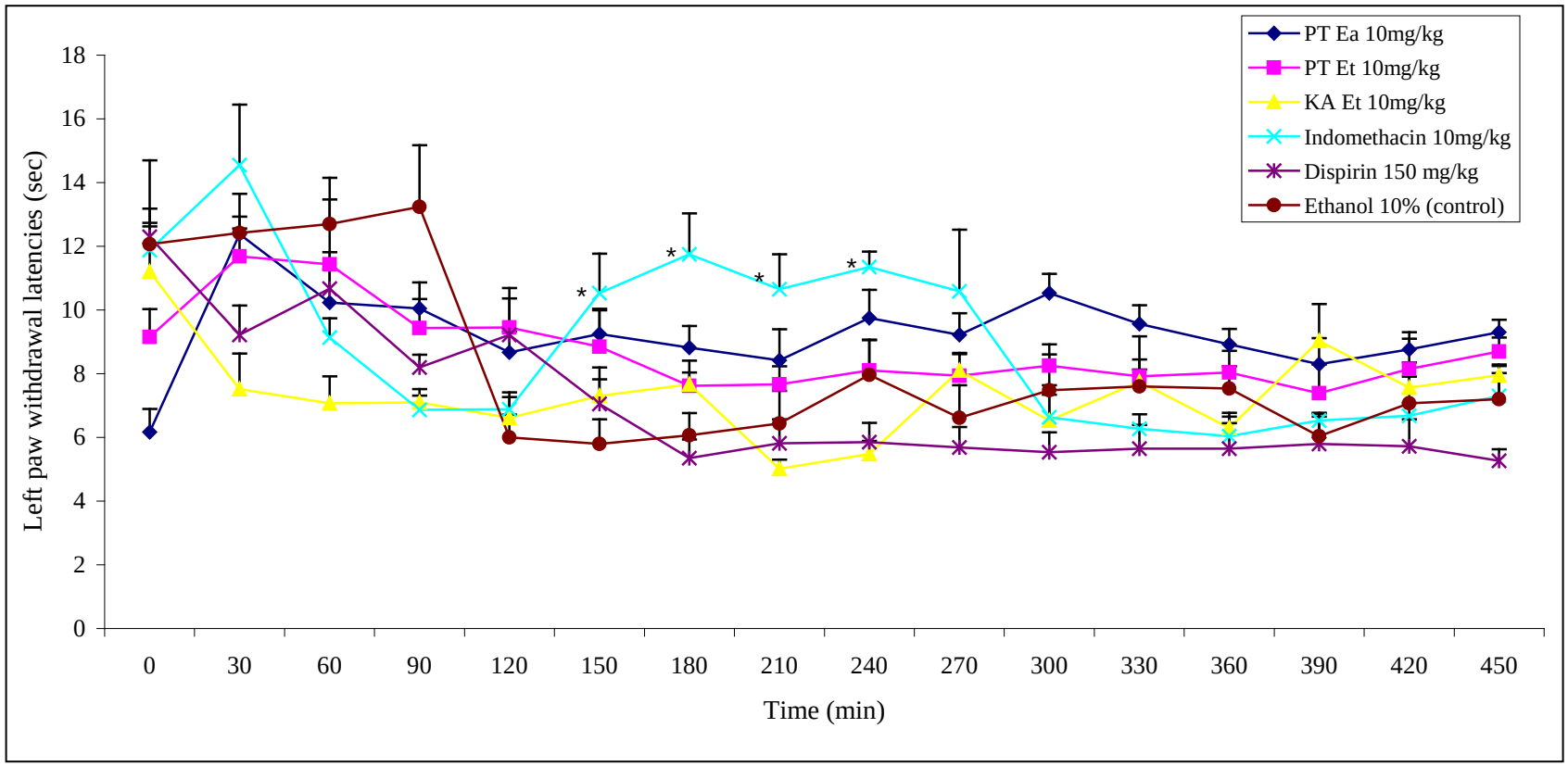

Figure 4.20 (a): Effects of radiant heat on withdrawal latencies of the rats' left hind paws injected with 0.1mL saline treated with 10 mg/kg *Padina tetrastromatica* ethyl acetate (PT Ea), *Padina tetrastromatica* ethanol (PT Et) and *Kappaphycus alvarezii* ethanol (KA Et) extracts.

10 % ethanol and 150 mg/kg dispirin were given orally and 10mg/kg indomethacin was administered intraperitoneally. Data presented as mean ± S.E.M (n=6). * P<0.05 compared with control group determined by ANOVA followed by Tukey Test.

Table 4.19 (b): Effects of radiant heat on withdrawal latencies of rats' left hind paws injected with 0.1mL saline treated with 30 mg/kg of *Padina tetrastromatica* ethyl acetate (PT Ea), *Padina tetrastromatica* ethanol (PT Et) and *Kappaphycus alvarezii* ethanol (KA Et) extracts.

Group	Left paw withdrawal latencies (sec)	
	Baseline (0 hour)	At 7 hour 30 min
Control (10% Ethanol)	12.07 ± 0.67	7.20 ± 0.82
Indomethacin 10 mg/kg	11.88 ± 1.30	7.30 ± 0.94
Dispirin 150 mg/kg	12.30 ± 2.40	5.27 ± 0.36
Padina tetrastromatica (PT Ea)	10.55 ± 0.69	8.83 ± 1.10
Padina tetrastromatica (PT Et)	7.37 ± 0.61	6.72 ± 0.95
Kappaphycus alvarezii (KA Et)	6.87 ± 0.35	6.37 ± 0.69

10 % ethanol and 150 mg/kg dispirin were given orally and 10mg/kg indomethacin was administered intraperitoneally with right hind paw injected with 1% carrageenan. *P<0.05 indicate significant difference between control and treated group as determined with ANOVA followed by Tukey Test.

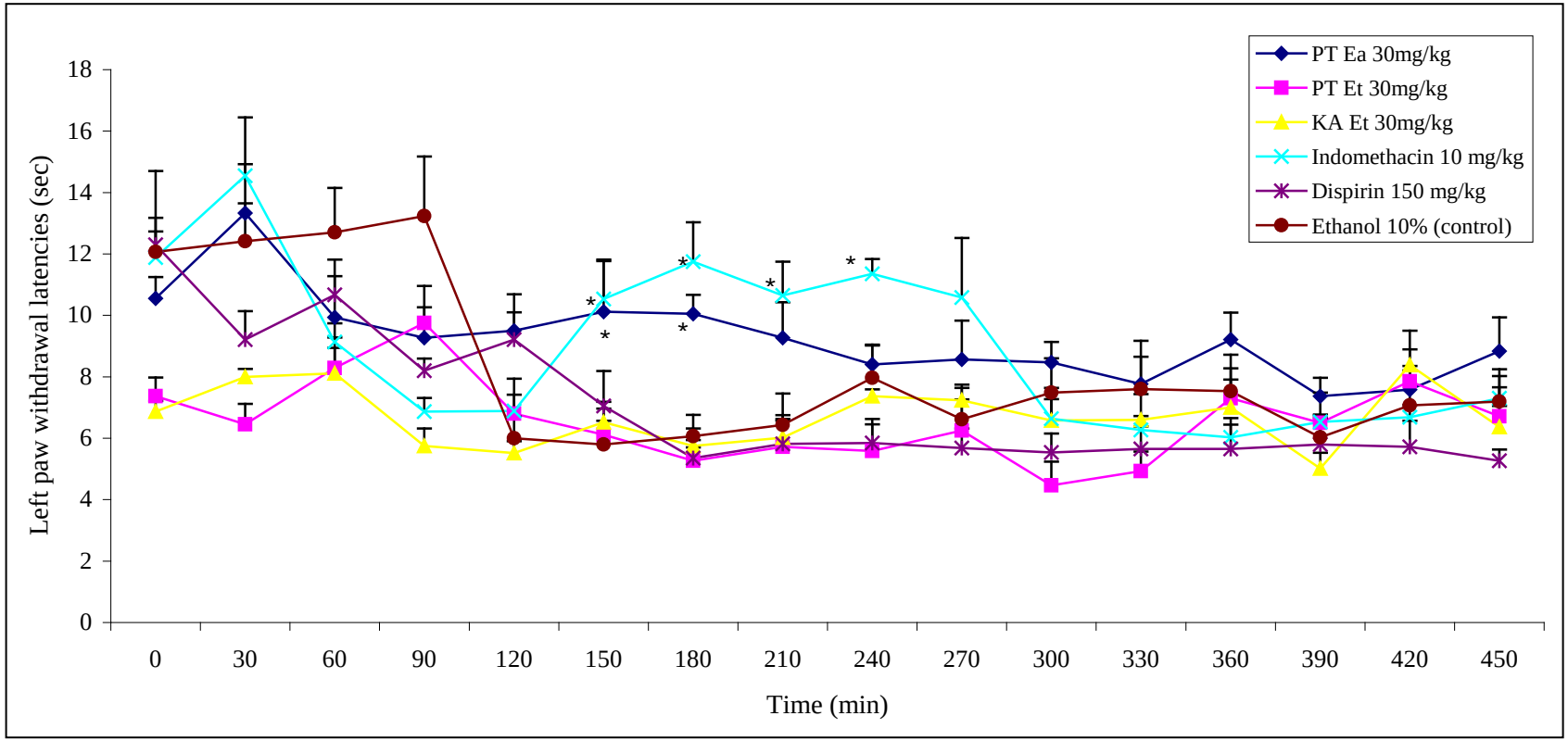

Figure 4.20 (b): Effects of radiant heat on withdrawal latencies of the rats' left hind paws injected with 0.1mL saline treated with 30 mg/kg *Padina tetrastromatica* ethyl acetate (PT Ea), *Padina tetrastromatica* ethanol (PT Et) and *Kappaphycus alvarezii* ethanol (KA Et) extracts.

10 % ethanol and 150 mg/kg dispirin were given orally and 10mg/kg indomethacin was administered intraperitoneally. Data presented as mean ± S.E.M (n=6). * $P<0.05$ compared with control group determined by ANOVA followed by Tukey Test

Table 4.19 (c): Effects of radiant heat on withdrawal latencies of rats' left hind paws injected with 0.1mL saline treated with 100 mg/kg of *Padina tetrastromatica* ethyl acetate (PT Ea), *Padina tetrastromatica* ethanol (PT Et) and *Kappaphycus alvarezii* ethanol (KA Et) extracts.

Group	Left paw withdrawal latencies (sec)	
	Baseline (0 hour)	At 7 hour 30 min
Control (10% Ethanol)	12.07 ± 0.67	7.20 ± 0.82
Indomethacin 10 mg/kg	11.88 ± 1.30	7.30 ± .94
Dispirin 150 mg/kg	12.30 ± 2.40	5.27 ± 0.36
Padina tetrastromatica (PT Ea)	16.58 ± 1.53	10.65 ± 1.04 *
Padina tetrastromatica (PT Et)	7.80 ± 0.35	9.65 ± 0.63
Kappaphycus alvarezii (KA Et)	10.07 ± 1.40	10.05 ± 0.83

10 % ethanol and 150 mg/kg dispirin were given orally and 10mg/kg indomethacin was administered intraperitoneally with right hind paw injected with 1% carrageenan. *P<0.05 indicate significant difference between control and treated group as determined with ANOVA followed by Tukey Test.

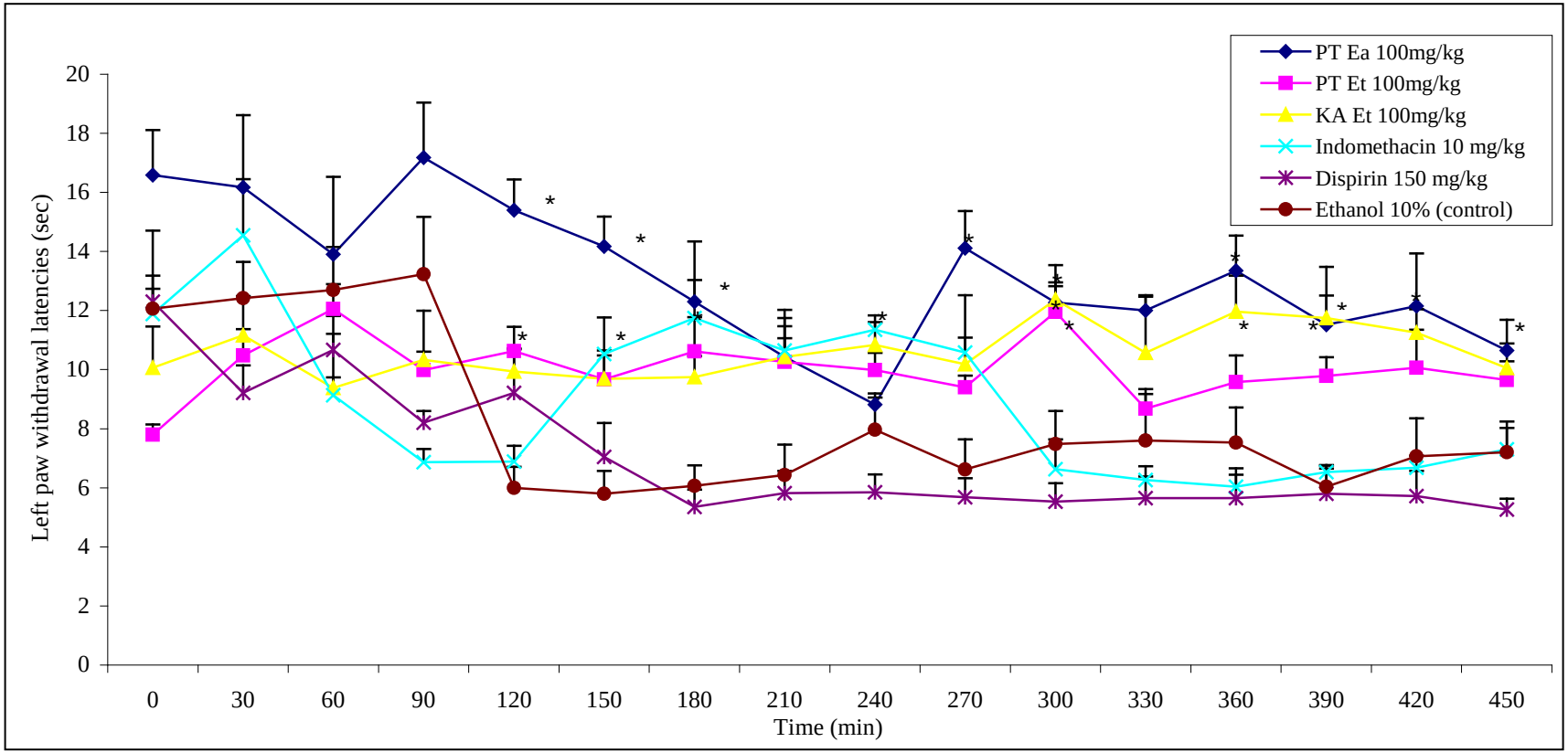

Figure 4.20 (c): Effects of radiant heat on withdrawal latencies of the rats' left hind paws injected with 0.1mL saline treated with 100 mg/kg *Padina tetrastromatica* ethyl acetate (PT Ea), *Padina tetrastromatica* ethanol (PT Et) and *Kappaphycus alvarezii* ethanol (KA Et) extracts.

10 % ethanol and 150 mg/kg dispirin were given orally and 10mg/kg indomethacin was administered intraperitoneally. Data presented as mean ± S.E.M (n=6). * P<0.05 compared with control group determined by ANOVA followed by Tukey Test

4.2.4 Anti-Ulcerogenic activity

P. tetrastromatica ethanol extract at 100 mg/kg significantly inhibited gastric mucosal lesion induced by indomethacin given with 1ml of Tween 80 from 142.67 ± 20.02 mm^2 (Figure 4.22 (a) and (b)) to 33.17 ± 7.96 mm^2 ($P<0.05$) (Figure 4.23 (a) and (b)) (Table 4.20). As for *K. alvarezii* ethanol extract at 100 mg/kg, it significantly reduced the total area of lesion caused by indomethacin given with 1ml of water (155.5 ± 25.49 mm^2) (Figure 4.24 (a) and (b)) to 69.67 ± 10.67 mm^2 ($P<0.001$) (Figure 4.25 (a) and (b)). This indicates that *K. alvarezii* is more significant in inhibiting gastric mucosal lesion induced by indomethacin.

The total area of lesion caused by HCl and Tween 80, 347.5 ± 24.07 mm^2 (Figure 4.26 (a) and (b)), was significantly reduced to 116.0 ± 12.06 mm^2 ($P<0.05$) by *P. tetrastromatica* ethanol extract (Figure 4.27 (a) and (b)). When water was given with HCl, the total area of lesion was 1236.67 ± 53.74 mm^2 (Figure 4.28 (a) and (b)) and it was reduced by *K. alvarezii* ethanol extract to 408.67 ± 49.96 mm^2 ($P<0.05$) (Table 4.20) (Figure 4.29 (a) and (b)). This indicates that both samples possessed cytoprotective effect which prevents the destruction of mucosa on the mucosa layer. Figure 4.21 shows the normal stomach of SD rat that was starved for 48 hours with free excess to water.

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Table 4.20: Effects of total area lesion induced by necrotic agents treated with 100 mg/kg *Padina tetrastromatica* ethanol (PT Et) and *Kappaphycus alvarezii* ethanol (KA Et) extracts.

Necrotic agents	Control (only ulcer agent) Total area lesion (mm^2)		Treated with 100 mg/kg sample Total area lesion (mm^2)	
Indomethacin 30mg/kg	Tween 80 (1ml)	Water (1ml)	*Padina tetrastromatica* (ethanol)	*Kappaphycus alvarezii* (ethanol)
	142.67 ± 20.02		33.17 ± 7.96*	
		155.5 ± 25.49		69.67 ± 10.67**
0.6M HCl	347.5 ± 24.07		116.0 ± 12.06*	
		1236.67 ± 53.74		408.67 ± 49.96*

*P<0.05, **P<0.01 indicate significant difference between control and treatment group using student t- test.

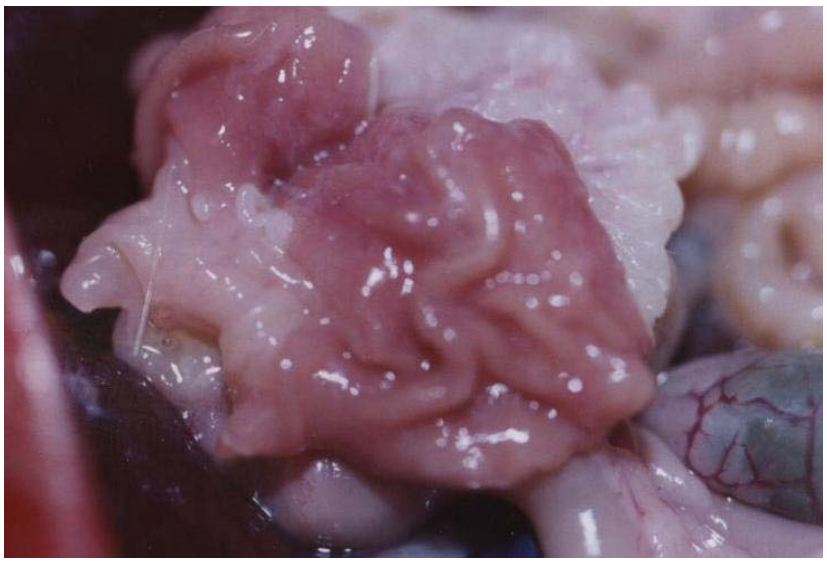
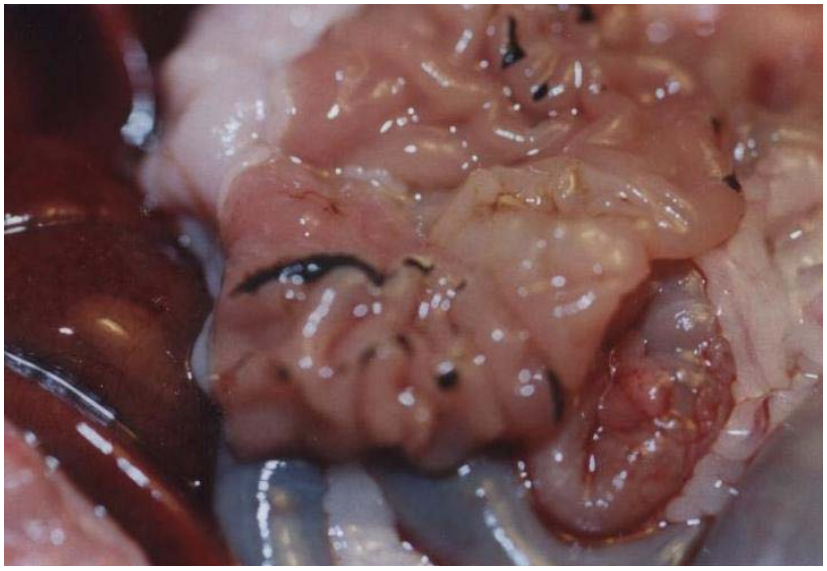
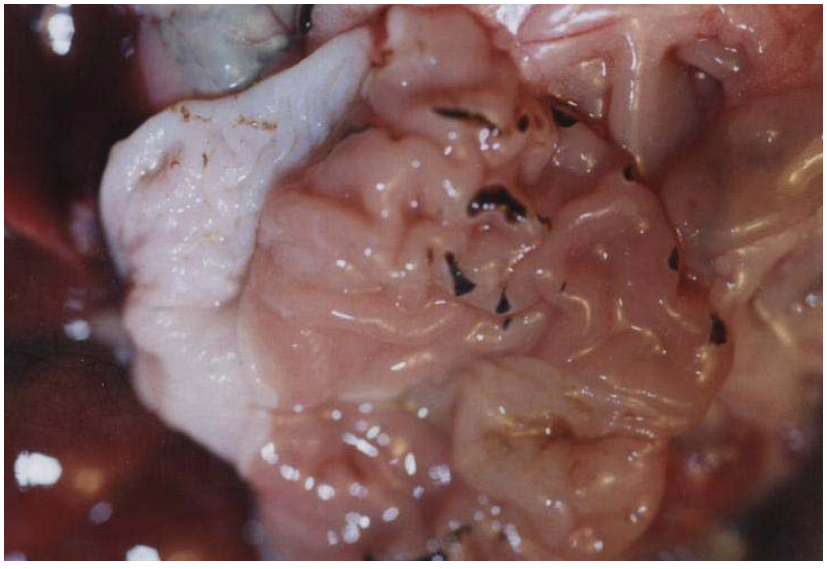


Figure 4.21: Normal stomach of SD rat starved for 48 hours with free excess to water.



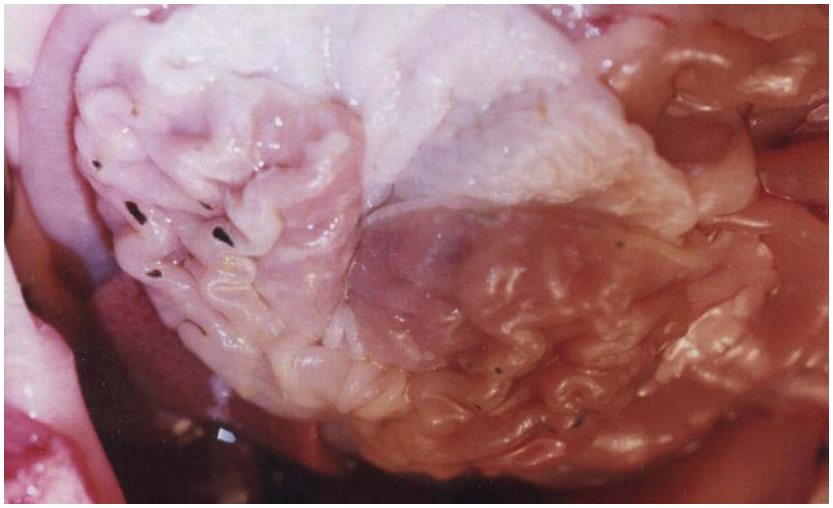
30 mg/kg
Indomethacin
+
10 %
Tween 80

Figure 4.22 (a): Ulcer induced by 30 mg/kg indomethacin after 10% of Tween 80 was given orally. Total lesion induced was 274 mm^2. (Left stomach view)



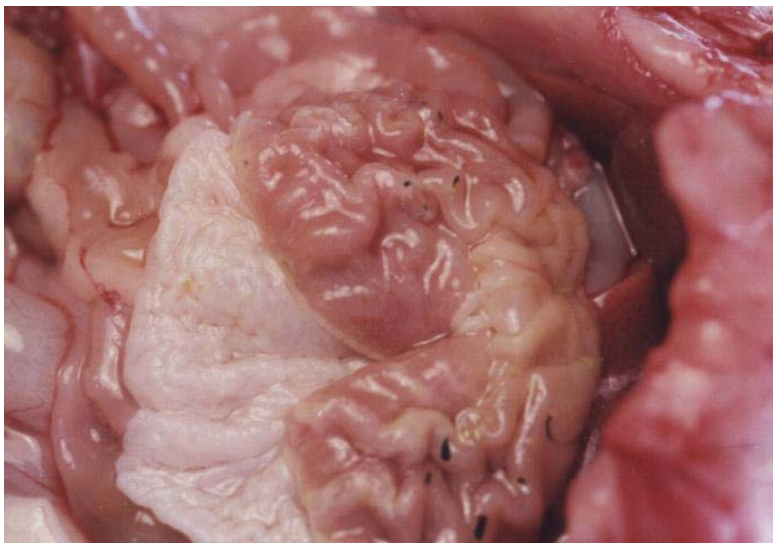
30 mg/kg
Indomethacin
+
10 %
Tween 80

Figure 4.22 (b): Ulcer induced by 30 mg/kg indomethacin after 10% of Tween 80 was given orally. Total lesion induced was 274 mm^2. (Right stomach view)



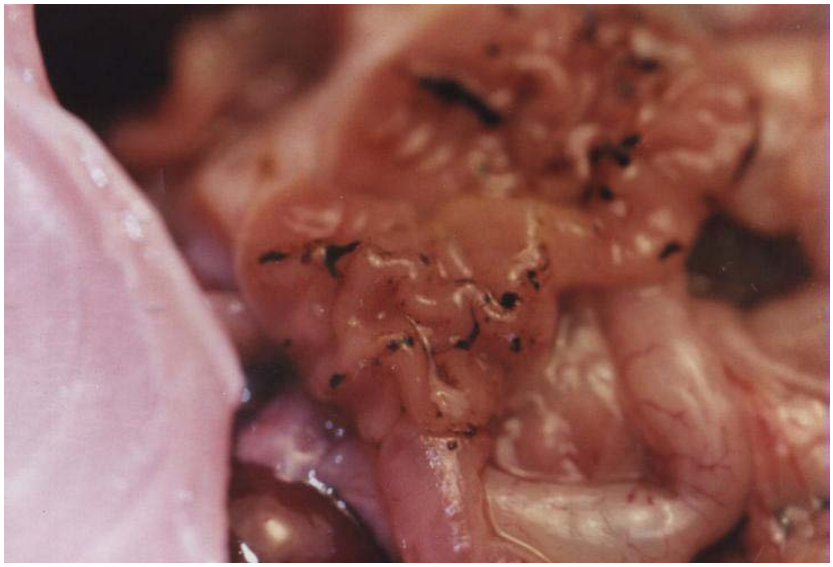
30 mg/kg
Indomethacin
+
100 mg/kg
Padina
tetrastromatica

Figure 4.23 (a): Ulcer induced by 30 mg/kg indomethacin after 100 mg/kg *Padina tetrastromatica* ethanol extract was given orally. Total lesion induced was 22 mm^2. (Left stomach view)



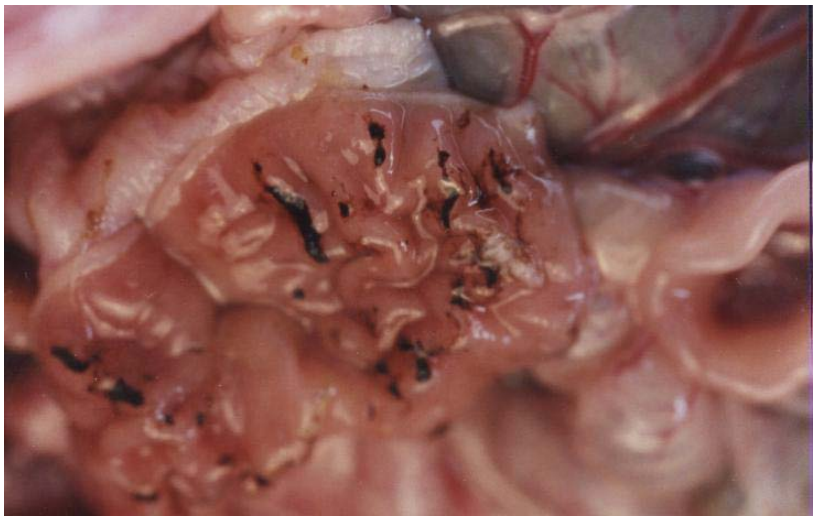
30 mg/kg
Indomethacin
+
100 mg/kg
Padina
tetrastromatica

Figure 4.23 (b): Ulcer induced by 30 mg/kg indomethacin after 100 mg/kg *Padina tetrastromatica* ethanol extract was given orally. Total lesion induced was 22 mm^2. (Right stomach view)



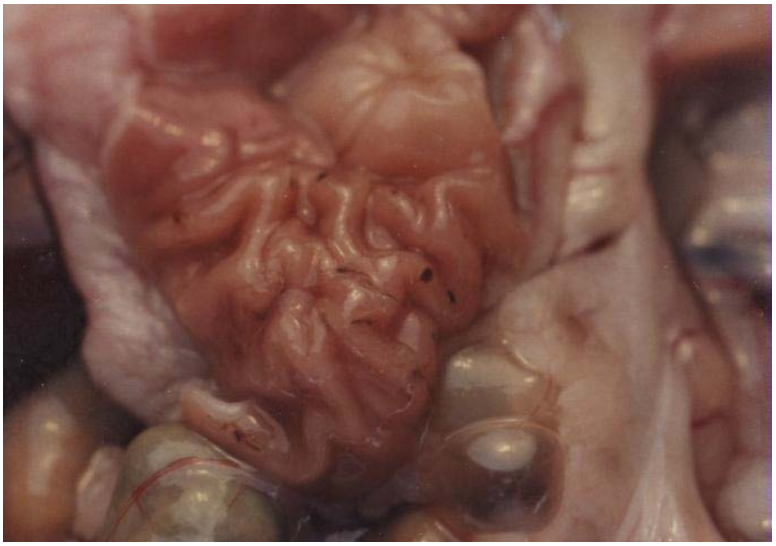
30 mg/kg
Indomethacin
+
1 mL dH_2O

Figure 4.24 (a): Ulcer induced by 30 mg/kg indomethacin after 1 mL dH_2O was given orally. Total lesion induced was 179 mm^2. (Left stomach view)



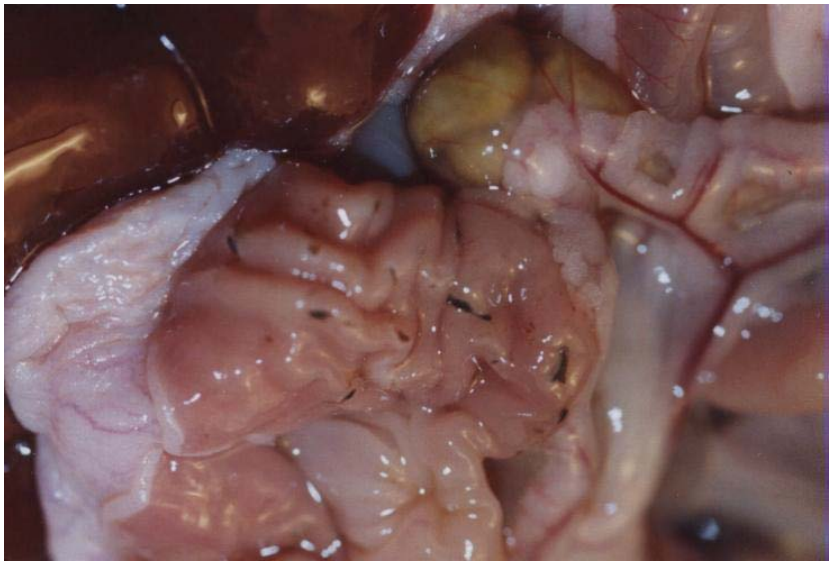
30 mg/kg
Indomethacin
+
1 mL dH_2O

Figure 4.24 (b): Ulcer induced by 30 mg/kg indomethacin after 1 mL dH_2O was given orally. Total lesion induced was 179 mm^2. (Right stomach view)



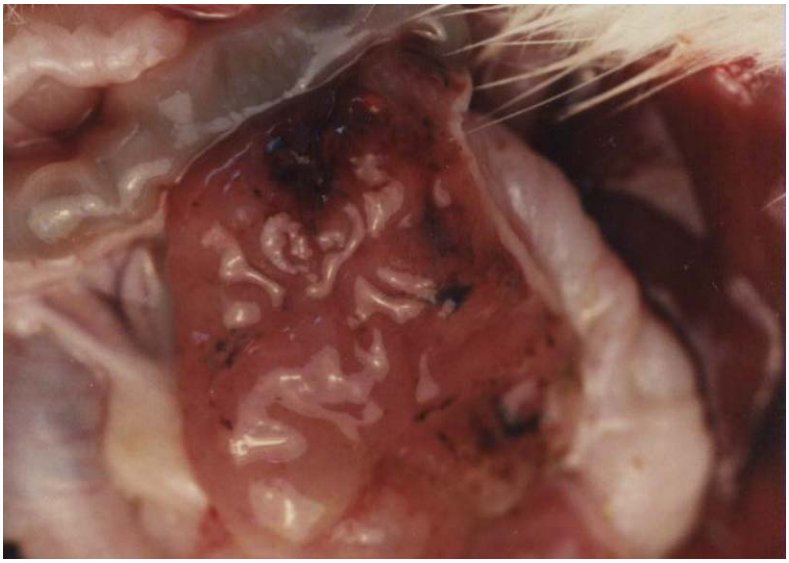
30 mg/kg
Indomethacin
+
100 mg/kg
Kappaphycus alvarezii

Figure 4.25 (a): Ulcer induced by 30 mg/kg indomethacin after 100 mg/kg *Kappaphycus alvarezii* ethanol extract was given orally. Total lesion induced was 44 mm^2. (Left stomach view)



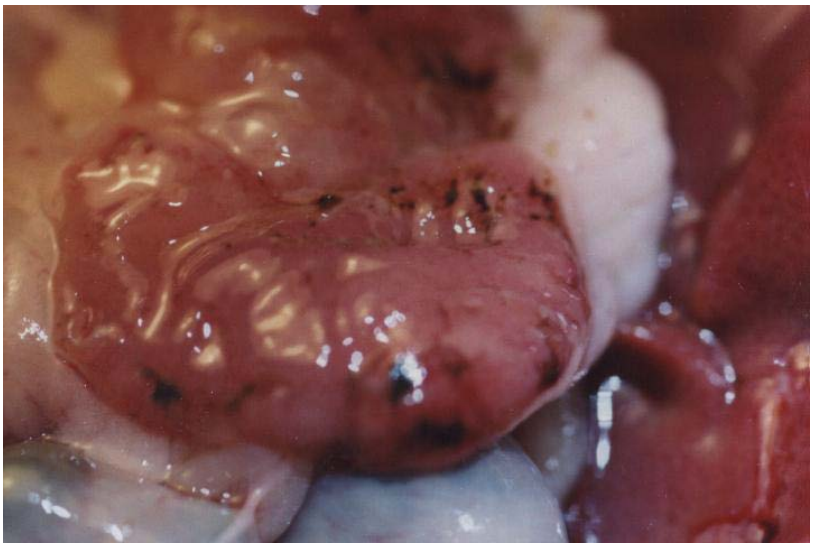
30 mg/kg
Indomethacin
+
100 mg/kg
Kappaphycus alvarezii

Figure 4.25 (b): Ulcer induced by 30 mg/kg indomethacin after 100 mg/kg *Kappaphycus alvarezii* ethanol extract was given orally. Total lesion induced was 44 mm^2. (Right stomach view)



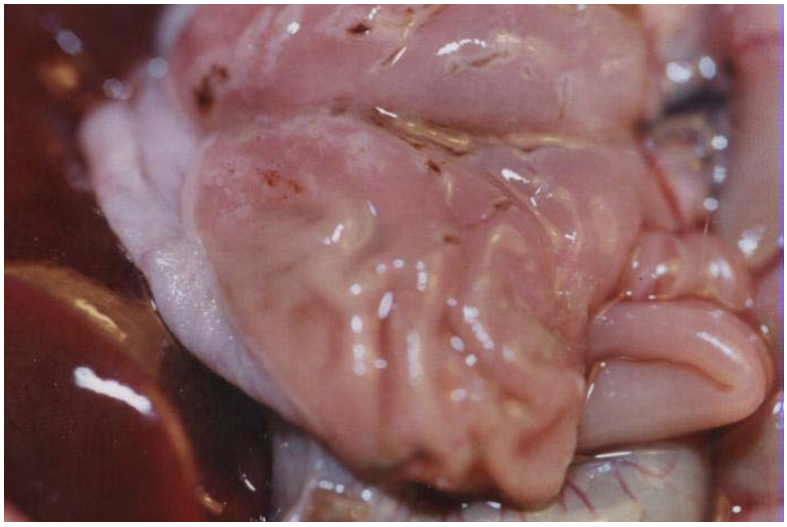
0.6 M HCl
+
10 %
Tween 80

Figure 4.26 (a): Ulcer induced by 0.6M HCl after 10% of Tween 80 was given orally. Total lesion induced was 869 mm^2. (Left stomach view)



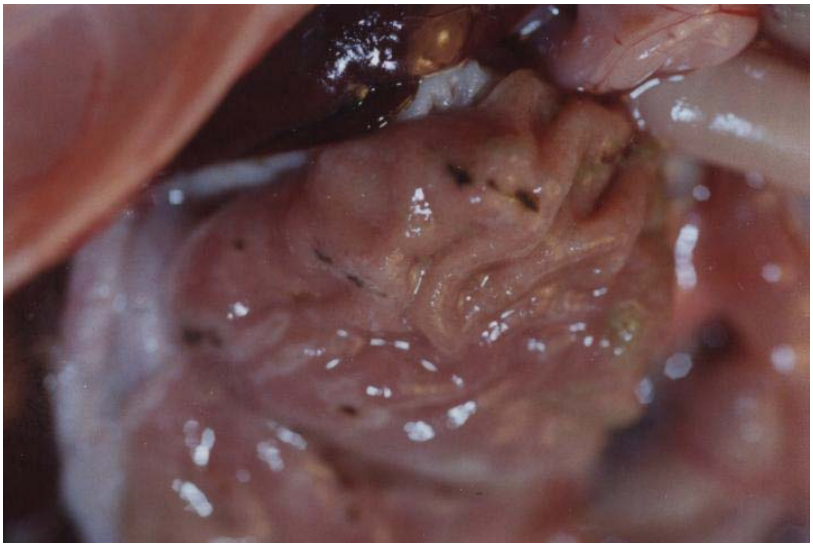
0.6 M HCl
+
10 %
Tween 80

Figure 4.26 (b): Ulcer induced by 0.6M HCl after 10% of Tween 80 was given orally. Total lesion induced was 869 mm^2. (Right stomach view)



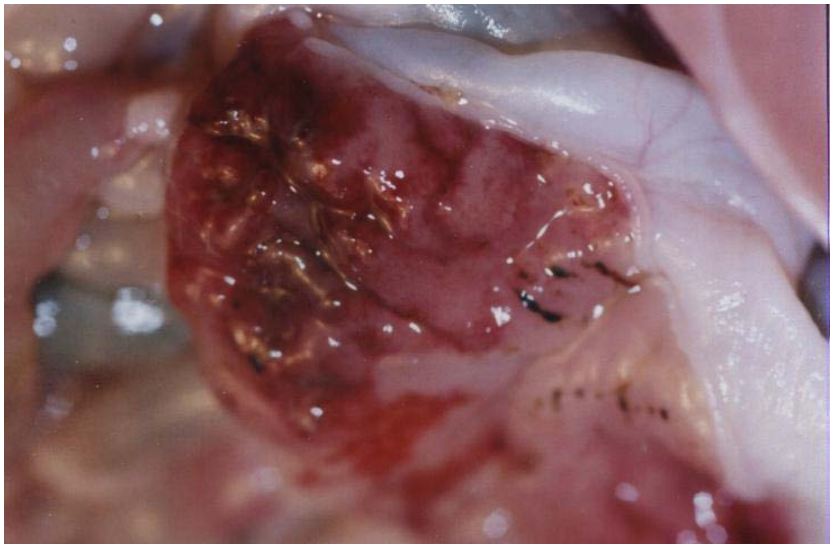
0.6 M HCl
+
100 mg/kg
Padina tetrastromatica

Figure 4.27 (a): Ulcer induced by 0.6M HCl after 100 mg/kg *Padina tetrastromatica* ethanol extract was given orally. Total lesion induced was 62 mm^2.
(Left stomach view)



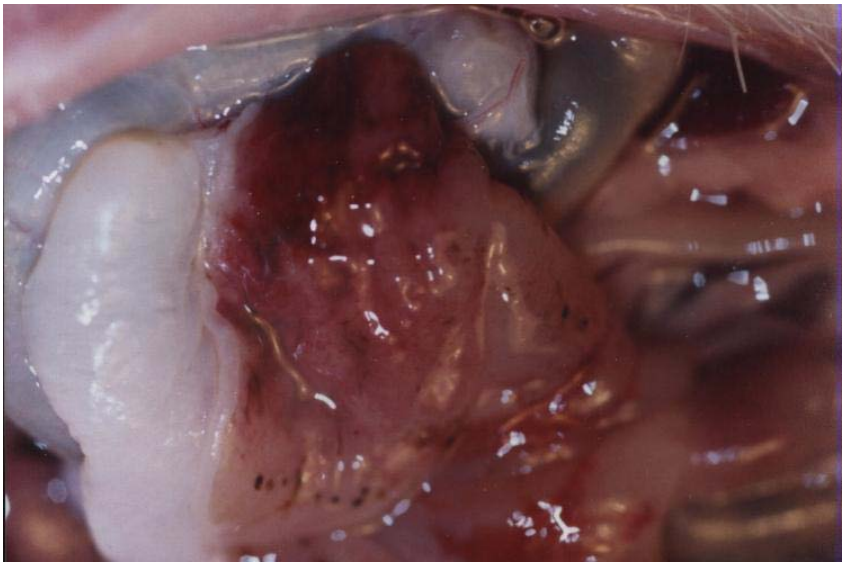
0.6 M HCl
+
100 mg/kg
Padina tetrastromatica

Figure 4.27 (b): Ulcer induced by 0.6M HCl after 100 mg/kg *Padina tetrastromatica* ethanol extract was given orally. Total lesion induced was 62 mm^2.
(Right stomach view)



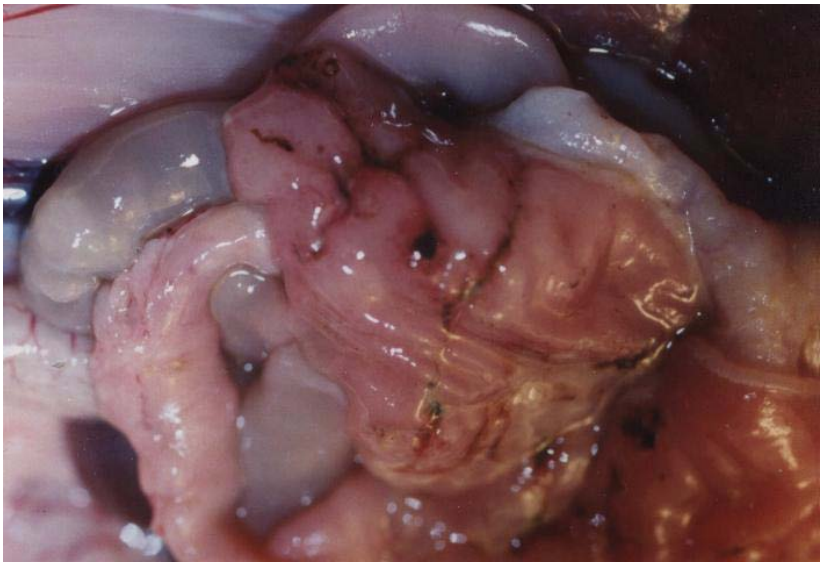
0.6 M HCl
+
1 mL dH_2O

Figure 4.28 (a): Ulcer induced by 0.6M HCl after 1 mL dH_2O was given orally. Total lesion induced was 2712 mm^2. (Left stomach view)



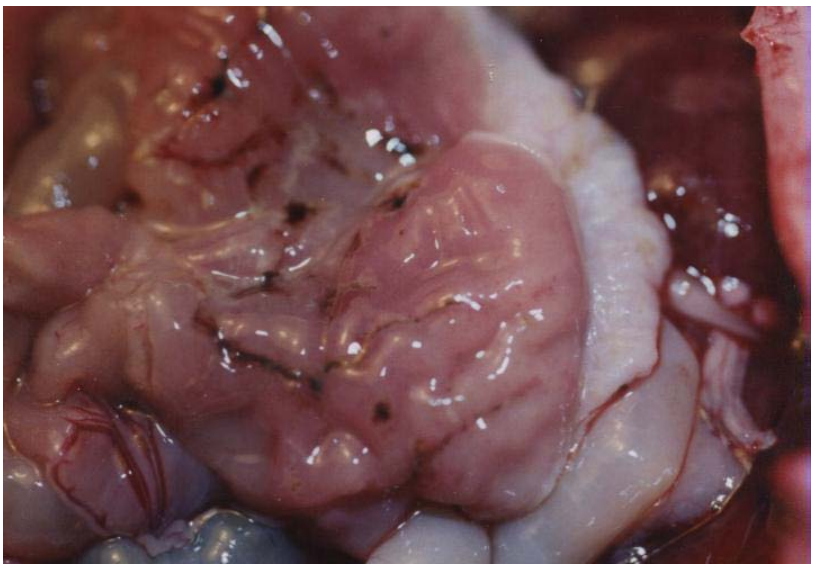
0.6 M HCl
+
1 mL dH_2O

Figure 4.28 (b): Ulcer induced by 0.6M HCl after 1 mL dH_2O was given orally. Total lesion induced was 2712 mm^2. (Right stomach view)



0.6 M HCl
+
100 mg/kg
Kappaphycus alvarezii

Figure 4.29 (a): Ulcer induced by 0.6M HCl after 100 mg/kg *Kappaphycus alvarezii* ethanol extract was given orally. Total lesion induced was 725 mm^2. (Left stomach view)



0.6 M HCl
+
100 mg/kg
Kappaphycus alvarezii

Figure 4.29 (b): Ulcer induced by 0.6M HCl after 100 mg/kg *Kappaphycus alvarezii* ethanol extract was given orally. Total lesion induced was 725 mm^2. (Right stomach view)

5.0 DISCUSSION

5.1 Antioxidant activities in sample extracts

5.1.1 Comparison between AEAC, DPPH and Galvinoxyl Assays.

There are many methods in the literature for assessing the antioxidant activity of compounds *in vitro*. Most methods assess the ability of compounds to donate H^+ (hydrogen ion) or electron to an oxidizing species introduced into the assay system. These assays differ from each other in terms of substrates, probes, reaction conditions and quantitation methods. Therefore, it is difficult to compare antioxidant results between these assays as mentioned by Frankel *et al*., (2000).

The two most widely used electron transfer antioxidant assays are AEAC and DPPH assays and these assays were used to evaluate antioxidant properties in this study. Both assays presented excellent stability in certain assay conditions but also showed several important differences in their responses to antioxidants and in their manipulation. Besides the mentioned assay, Galvinoxyl assay was also used to determine in vitro antioxidant activity in this study. Various limitations have been encountered for these assays (Arnao, 2000). Even though DPPH assay has many limitations, it is still the preferred assay compared to ABTS and Galvinoxyl assay because it is easy to perform, convenient and not tedious when it comes to screening for many samples. It was recommended by Katalinic *et al*. (2006), that DPPH assay is easy and accurate when measuring antioxidant activity of plant extracts.

Antioxidant activity methods using free radicals are fast, easy and simple. AEAC ($ABTS^{+\cdot}$) and DPPH assay is based on decolorization method. When an antioxidant compound interacts with ABTS free radical ($ABTS^{+\cdot}$) the oxidation of this free radical delayed the change of solution from dark blue color to colorless in accordance to the presence of antioxidant in the sample (Reis Lima *et al.*, 2005). In DPPH assay, antioxidant compounds reduce the free radical ($DPPH^{\cdot}$), changing the color of the solution from violet to pale yellow (Molyneux, 2003). The important difference between ABTS and DPPH assay is that, ABTS free radical can be solubilized in aqueous and organic media, which means antioxidant activity can be measured due to the hydrophilic and lipophilic nature of the compounds in samples (Cano *et al.*, 2000; Arnao *et al.*, 2001). In contrast, DPPH free radical can only be dissolved in organic media (especially in alcoholic media), not in aqueous media, which is an important limitation when interpreting the role of hydrophilic antioxidants (Lee *et al.*, 2003). This can be seen in the test result where ABTS assay (Table 4.1) showed inhibition for all its boiled water and methanol extracts used for antioxidant evaluation whereas in DPPH assay (Table 4.7), some boiled water and methanol extracts did not show any inhibition. This might be due to the insolubility of the extracts in the DPPH solution. Besides that, many antioxidants that can react quickly with peroxyl radicals may react slowly or even inert to DPPH assay (Huang *et al.*, 2005).

Another important difference between these two assays was their wavelengths. ABTS assay was read at wavelength 750 nm whereas DPPH assay was read at 515nm. Thus, at wavelength near visible range, like DPPH, antioxidant activity measured were underestimated due to sample interference. Interference can be greater in the presence of colored compounds (anthocianins, carotenoids, etc.) in sample or due to the apparition of secondary reaction products between chromogen and samples being analyzed. Besides that,

the higher the intensity of color in an extract, the smaller the absorbance decrease and the less antioxidant activity measured, even when working with minimal samples volumes. Therefore, these interferences were greater in DPPH compared to ABTS assay as ABTS wavelength is far away from the visible range (Arnao, 2001). Almost all the extracts used in this study possess color. This might be another reason why boiled water and methanol extracts did not show a good antioxidant properties when tested using DPPH assay.

Besides that, DPPH is concentration dependent as reported by Ismail *et al*. (2002). It was preferred that antioxidant efficiency is expressed as EC_{50}, determined as the concentration of substrates that causes 50% loss in absorbance in DPPH activity. This is due to different maximum activity by different antioxidants, therefore using only one concentration would not give accurate result whereby the antioxidant activity of the plants would be underestimated. Balasundram *et al.* (2005) suggested that the EC_{50} provides clearer and more direct indicator of the radical scavenging potency of a sample. In this study, EC_{50} of samples extracts were not studied. The concentration used in this study was 1g/10mL solvent which might be viscous for DPPH assay. Therefore, it increases the interference when the reading for this assay was taken.

There were not many studies done for Galvinoxyl assay. This assay was used to evaluate methanol extraction of sample extracts. This assay showed good antioxidant evaluation for all the samples except for three samples. These samples did not show any inhibition for its forward methanol extracts (Fw) *Padina tetrastromatica*, *Turbinaria conoides* and *Caulerpa racemosa* for this assay. These samples also did not show a good antioxidant activity for its methanol extracts in ABTS and DPPH assay. When liquid nitrogen was used to ground these samples, their methanol extracts showed antioxidant

activity in all three assays except for *C. racemosa* in DPPH assay which might be due to interferences. Therefore, more than one method was needed to evaluate antioxidant of extracts (Buenger *et al.*, 2006) due to interference that could affect antioxidant activity of samples extracts in an assay.

5.1.2 Correlation between Antioxidant Activities and Their Total Phenolic Content.

It is known that phenolic compounds are widely distributed in plants and seaweeds. Phenolic compounds have been associated with antioxidant action in biological system, acting as scavengers of singlet oxygen and free radical (Rice Evans *et al.*, 1995). In this study, the total phenolic content of samples was determined using Folin-Ciocalteu assay. The antioxidant properties were expressed as gallic acid equivalent (GAE).

Almost all the boiled water extracts in this study showed higher phenolic content compared to methanol extracts except for *C. racemosa* and *Chlorella vulgaris* (Table 4.11). This might be due to the higher phenolic content in the sample extract (Pourmorad *et al*., 2006) extracted with boiled water where generally, high radical scavenging activity shows high phenolic content. Boiled water can dissolve components ranging from low polarities to high polarities. Therefore, a very good correlation was observed in boiled water extracts compared to methanol extracts. Besides that, the higher antioxidant values obtained in boiled water extracts might be due to the non-phenolic compounds present in the extracts. Although methanol is known to extract compounds ranging from mid-polar to polar phenolic compounds, but from the test results, it clearly showed that boiled water extracts have higher phenolic content. Therefore, it can be concluded that the efficiency to

extract polar phenolic compounds might be higher in aqueous solvent as said by Yu *et al.* (2004). The limitation of this assay is that it cannot distinguish the types of phenolic compounds presents in the extracts because its reagent is not specific and it detects all the phenolic compounds found in the extracts, including extractable proteins (Naczka *et al.*, 2004). Another important disadvantage of using this assay is that it could be interfered by reducing substance such as ascorbic acid.

Besides that, solvent used for extraction is an important factor in extraction of phenolic compound from samples. When acetone was used as an extraction solvent in Folin-Ciocalteu assay, no phenolic content was observed for its seaweeds extract (Table 4.16), however, the same samples tested in this assay using their methanol and boiled water extracts showed phenolic content. Only Malaysian and Vietnamese tea showed antioxidant activity for their acetone extracts but they showed a very low phenolic content compared to their methanol and boiled water extracts. Acetone is known to be a less polar solvent compared to methanol and boiled water. Therefore, this solvent was only capable to dissolve low amount of non-phenolic compounds from the extracts, giving a lower phenolic content for this assay. Therefore, it can be concluded that solvent plays an important role in dissolving non-phenolic and phenolic compounds from the samples.

Extraction time also plays an important role. In this study, extraction was run for only two hours which might not be enough to dissolve most of the samples polar and non-polar compounds. The longer the solvent is in contact with the sample, the better the penetration of solvent into the samples cellular structure. To confirm this, further study of samples' extraction time must carried out.

5.1.3 Antioxidant Activities of Methanol and Boiled Water Extracts

Solvents such as water, ethanol, methanol, acetone or their mixture are commonly used to extract phytochemicals from plants and they are one of the important factors affecting both extraction yield and antioxidant activities of extracts. In this study, samples were extracted using methanol and boiled water as these two are the most widely employed solvents for hygienic and abundance reasons. The ranking of solvents polarity increase in the order of chloroform < acetone < ethanol < methanol < water (Zubrick, 1984). It is well known that a polar solvent is capable of dissolving polar compounds and a non-polar solvent is capable of dissolving non-polar compounds. Due to the complexities of both chemical characteristics of solvents and the diverse structures and the composition of the sample materials, the behaviors of the material solvents systems are different from each other and can hardly be predicted. Therefore, it is important to select the suitable solvent for extraction because as said by Yang *et al*. (2007), there is no single solvent that could extract all the antioxidant of different polarity and solubility in a sample. Methanol and alcohol 80% are polar solvents and it is thought that alcoholic solvents efficiently penetrate cell membranes, permitting the extraction of high amounts of endocellular components whereas a lower polarity solvent may wash out mostly extracellular materials (Ghisalberti, 1993). Water is seldom used alone to obtain a crude plant extract instead a mixture of aqueous-methanolic extract is generated. In this study, boiled water was used as most of the samples especially teas and seaweeds were boiled before used. Therefore, in order to detect the amount of antioxidant obtained from these samples, boiled water was used.

The effects of antioxidant activities are mainly attributed to their phenolic content. Phenolic or polyhenols constitute one of the most numerous and ubiquitously distributed group of plant secondary metabolites ranging from simple molecules (phenolic acids, phenylpropanoids, flavanoids) to highly polymerized compounds (lignins, melanin, tannins) and may also exist as complexes with carbohydrates, proteins and other plant components. Therefore, as said by Naczka *et al.* (2004), extractions of plant materials are always a mixture of different classes of phenolic.

Usually, extractions were done from less polar solvent to more polar solvent. In this study, the usual extraction method was followed where methanol extraction was carried out first followed by boiled water extraction (Fw). To compare the effect of an extraction done in the reverse polar, selected samples were extracted by boiled water first followed by methanol extraction (Rv). From all the results obtained from antioxidant test with reverse extraction for both water and methanol (Table 4.2 (a) and (b), 4.8 (a) and (b), 4.11 and 4.14 (a) and (b)), there was no clear evidence that reverse extraction showed better antioxidant results compared to forward extraction. The readings were not constant to make a conclusion. Therefore, it is best to extract samples using less polar solvent to more polar solvent.

From the tests results, it showed that most samples showed higher antioxidant values in boiled water extract compared to methanol. It has been reported by Siddhuraju *et al*. (2003) that methanol was more efficient as a solvent for antioxidant extraction compared to water. But in this study, boiled water was used instead of water and it has been said by Smith (2002) that boiled water can dissolve components ranging from low polarities to high polarities. This might be the reason why most boiled water extraction

showed higher antioxidant values although some samples still showed higher antioxidant values in methanol extracts. A very good correlation was seen between ABTS assay and Total Phenolic assay in boiled water extraction. In DPPH assay the antioxidant values were not constant between boiled water and methanol extracts (Table 4.6 (a) and (b)) where some samples showed high antioxidant values in boiled water extracts and no value for their methanol extracts while some showed higher value in methanol extracts and no value in boiled water extracts. Therefore, it cannot be concluded that in DPPH assay, methanol extracts was better than boiled water extracts or vise versa.

5.1.4 Comparison between Antioxidant Properties Using Different Drying and Grinding Method.

In this study, four different seaweeds were chosen, *Sargassum binderi*, *T. conoides*, *P. tetrastromatica* and *C. racemosa* for comparison. One part of the sample was dried using oven at 40°C and ground using grinder and another part was blotted dry and ground using liquid nitrogen (cryogenic grinding).

For samples ground with grinder, the boiled water extracts showed higher antioxidant values for the entire assay tested (ABTS, DPPH, Galvinoxyl and Total phenolic) compared to when the sample ground with liquid nitrogen. As for methanol extracts, samples ground with liquid nitrogen showed higher antioxidant properties for all the assays tested compared to extracts of samples ground using grinder. It is known that cryogenic grinding reduces the particle size of the samples and breaks the cell wall that contains secondary metabolites of the samples. Generally it was found that the smaller the

particle size the more efficient the extraction will be, thus giving higher yield of extraction (Silva *et al.*, 1998). In this study, samples were extracted using methanol first before boiled water extraction. It was shown in all the assays that methanol extraction of samples ground with liquid nitrogen showed higher antioxidant values compared to when the samples ground with grinder. Therefore, it can be concluded that during methanol extraction, the solvent dissolved most of the non-phenolic and phenolic compound of the samples ground with liquid nitrogen, hence, giving high antioxidant values for all the assays used. As for samples that were oven dried and ground with grinder, methanol did not effectively dissolve phenolic compounds. This can be the reason these samples showed lower antioxidant values for all the assays tested.

Samples that were oven dried and ground with grinder showed higher antioxidant values in the boiled water extracts compared to samples ground with liquid nitrogen. This shows that boiled water extracts of samples oven dried and ground with grinder dissolved more phenolic compounds because methanol extraction of these samples did not dissolve most of the phenolic compounds. As for samples ground with liquid nitrogen, methanol dissolved most of the phenolic compounds leaving low amount of phenolic compounds to be dissolve by boiled water during its extraction.

Besides that, there was no heat applied during liquid nitrogen grinding whereas temperature as high as 40°C was used for drying the samples that was ground with grinder. While grinding the dried samples, the samples were also heated by the grinder. Temperature plays an important role and heat is known to affect the compound stability due to chemical and enzymatic degradation and latter was suggested to be the main mechanism causing the reduction in polyhenols content (Laurrauri *et al.*, 1997). From the

study done, it can be concluded that liquid nitrogen grinding was more effective for methanol extraction and samples oven dried and grinded using grinder was more effective for boiled water extraction.

5.1.5 Comparison of Antioxidant Properties between Samples

Samples used in this study were seaweeds, teas, microalgae, fruit skins and flower. Due to their exposure to different environment stress, seaweeds, teas, microalgae, fruits and flower produce different level and types of antioxidants to protect themselves from oxidative stress that could damage their cells and finally leading to cell death (Collen *et al*., 1999). In this study, we compared the antioxidant activity between these sample extracts and our study supported this theory.

Higher plants are well equipped with enzymatic detoxification systems and antioxidants of different chemical groups diminishing oxidative stress by elimination or reduction of the ROS to less toxic and less reactive products (Larson, 1988). In addition to proteins with antioxidative properties, phenolic compounds such as flavanoids, coumarins and tocopherols, nitrogen containing compounds including alkaloids, chlorophyll derivates, amino acids and amines as well as other compounds such as carotenoids, ascorbic acid, glutathione and uric acid are powerful antioxidants in plants (Potterat, 1997). It is well known that tea possesses high antioxidant properties. Malaysian tea and Vietnamese tea are black tea (leaves that had been completely oxidized before desiccation). Vietnamese tea showed higher antioxidant properties in ABTS, Total Phenolic and DPPH assays compared to Malaysian tea but the values did not differ greatly

to conclude that Vietnamese tea is better than Malaysian tea. This is because it is known that tea extracts are powerful antioxidants due to the presence of flavanoids (Salah *et al.*, 1995). Polyphenols present in black tea possesses antioxidant properties. Catechins, theaflavins and gallic acid also contribute to the antioxidant characteristic of tea (Vinson *et al.*, 1998).

Although phenolic compounds can be the principle antioxidant compounds in many plant species (Cai *et al.*, 2004), they are less important as antioxidant in seaweeds. Like other plants, seaweeds contain various kinds of organic and inorganic substances and it had been reported that seaweeds contain high levels of minerals, vitamins, essential amino acids, indigestible carbohydrates and dietary fibers (Jiménez-Escrig *et al*., 1999). Some of the well-known antioxidants in seaweeds are ascorbic acid, ß-carotene and α-tocopherol. In general, seaweeds and microalgae have to cope with changing environmental conditions such as light, temperature, salinity and desiccation. Collen *et al*. (1999) have explained that an increase in environmental stress increases the activities of reactive oxygen scavenging enzymes. That is why different species showed different antioxidant properties in this study.

Seaweeds studied in this study were *P. tetrastromatica*, *T. conoides* and *S. binderi* (Brown seaweeds, Phylum of Phaeophyceae); *Kappaphycus alvarezii*, *Eucheuma denticulatum*, *Gracilaria changii* and *G. salicornia* (Red seaweeds, Phylum of Rhodophyta); *C. racemosa* and *Chaetomorpha* spp. (Green seaweeds, Phylum of Chlorophyta) and the microalgae studied were *Spirulina platensis* and *C. vulgaris*. *Spirulina* is classified under Family Cyanobacteria whereas *Chlorella* is classified under the Family Chlorophyta. Each Family poses different characteristic in its compounds. For

example, in Brown algae, it contains xanthophyll pigment called fucoxanthin that masks the other pigments such as Chlorophyll a and c, beta-carotene and other xanthophylls. As for Red algae, it contains high amount of phycoerythrin and phycocyanin pigments that mask Chlorophyll a, beta-carotene and a number of unique xanthophylls. Finally, Green algae possess Chlorophyll a and b in the same properties as the 'higher plants' (http://www.surialink.com, 2007). In the assays tested, Brown seaweeds showed higher antioxidant values in ABTS (Table 4.0), DPPH (Table 4.5) and Total Phenolic (Table 4.11) assays compared to the other seaweeds for its boiled water extracts. For methanol extract, Red seaweeds showed good antioxidant activities in all the assays tested especially in Galvinoxyl assay where they showed higher antioxidant values compared to the other seaweeds (Table 4.18). This shows that, many factors must be taken into account while measuring antioxidant properties of a sample as explained earlier. One of the important factors that was not explained earlier was storage of sample. This can be seen between *K. alvarezii* (a) and (b). *K. alvarezii* (a) which was an old stock showed lower antioxidant activities in the entire assay tested compared to *K. alvarezii* (b) which was a new stock. Therefore, it is best to use a fresh sample compared to a stored sample.

Spirulina spp. is a photosynthetic, filamentous, spiral-shaped, multicellular and blue-green microalgae which is edible. Its chemical composition includes proteins, carbohydrates, essential fatty acids, vitamins, minerals and pigments such as carotenes (especially ß-carotene which is essential as an antioxidant), chlorophyll a and phycocyanin (Switzer, 1980). *Spirulina* spp. has a soft cell wall made of complex sugars and protein and is different from most other algae in that it is easily digested (Richmond, 1992). *Chlorella* spp. is a microalgae that has high content of chlorophyll. The only set back of *Chlorella* spp. usage is its cell wall which is strong due to its indigestible cellulose cell wall (Guiry

and Guiry, 2007). The composition of *C. vulgaris* indicates that it has a high nutritional value to a wide range of essential nutrients, such as vitamins, minerals and proteins. Moreover, it contains other compounds such as n-3 and n-6 polynsaturated fatty acids, pro-vitamins and phenolic compounds (Miranda *et al.*, 2001). In the entire assay tested, boiled water extract of *S. platensis* showed higher antioxidant properties compared to *C. vulgaris* boiled water extract. For methanol extract, *C. vulgaris* showed higher antioxidant properties compared to *S. platensis* methanol extract except for DPPH assay where both samples did not show any inhibition (Table 4.5). This indicated that compounds from *C. vulgaris* dissolved better in methanol solvent compared to boiled water solvent.

Pomelo and mangosteen skins were tested for their antioxidant properties. Pomelo or scientifically known as *Citrus grandis*, is a giant citrus and parent of many citrus fruits such as grapefruits. It is well known that citrus juice contains bioactive compounds such as limonoids, flavanoids, pectin, coumarins, furanocoumarins and known antioxidants such as vitamin C and carotenoids (Girennavar *et al.*, 2006) but little is known in regard to its skin's bioactive compound. Peels are often the waste part of various fruits. These wastes have not generally received much attention with a view to being used or recycled rather than discharged. This might be due to their lack of commercial application (Soong *et al*., 2004). Mangosteen or known as *Garcinia mangostana* scientifically, contains high antioxidant properties. Xanthone derivatives are major secondary metabolites of mangosteen and the most abundant xanthone is α-mangostin (Chin *et al.*, 2008). Mangosteen skin showed higher antioxidant values in all the assays tested for both its boiled water and methanol extracts in comparison to Pomelo skin.

Bougainvillea is a flowering plant. *Bougainvillea spectabilis* bark has been tested by Purohit *et al*. (2006) for its anti-diabetic efficacy. In this study, we tested the flower of Bougainvillea for antioxidant properties and we found that this flower showed antioxidant properties when tested with AEAC (both boiled water and methanol extracts) (Table 4.1), DPPH (only methanol extract) (Table 4.7), Galvinoxyl (Table 4.10) assays and Total Phenolic (both boiled water and methanol extracts) (Table 4.13).

Ascorbic acid was used in our study as standard and it is known to have high radical scavenging ability due to its purity. Ascorbic acid (L-ascorbic) is used as antioxidant and reducing agent (Naidu, 2003) but it is unstable and sensitive to light, heat and air. Therefore in our study, ascorbic acid was prepared fresh. The stability of ascorbic acid was studied (Table 4.5 and 4.6) and we postulated that ascorbic acid scavenge free radical efficiently when it was prepared fresh. Besides that, the time taken for samples to incubate in the ABTS reagent was also studied and we postulated that the time (6 minutes) for incubation was the best as mentioned earlier (method 3.3.1). This was postulated based on our study (Table 4.4) that 5 minutes of incubation in ABTS was more sensitive compared to 10 and 15 minutes.

5.2 Animal Studies

For animal study, *Padina tetrastromatica* (ethyl acetate and ethanol extract) and *Kappaphycus alvarezii* (b) (ethanol extract) were used to study toxicity, inflammation, analgesic and ulcer effect on rats. *Padina*'s extract was dissolved using 10% Tween 80 while *Kappaphycus*'s extract was dissolved in distilled water.

5.2.1 Toxicity study

The LD_{50} of crude *P. tetrastromatica* ethyl acetate (PT Ea) extract was found to be 1845 mg/kg (Figure 4.17 (a)) and its ethanol (PT Et), LD_{50} was found to be 1596 mg/kg (Figure 4.17 (b)). As for *K. alvarezii* ethanol (KA Et) extract, its LD_{50} was found to be 1799 mg/kg (Figure 4.17 (c)). From the results, PT Ea showed a higher LD_{50} compared to the other two samples. It was observed that the mice were gasping, had stretching effects, hind limb placing and other normal reflexes during LD_{50} study before it died but the physiological effects were not recorded. Therefore, it is hard to conclude that the effects of the samples given were peripheral or central nervous system responses (Kim *et al.*, 1998).

5.2.2 Anti-Inflammatory Study

Carrageenan-induced inflammation is useful in detecting orally active anti-inflammatory agents (Di Rosa *et al.*, 1971; Ismail *et al.*, 1997). To study the anti-inflammatory effect of samples on rats, carrageenan-induced paw inflammation model was used and the samples used were PT Ea, PT Et and KA Et at 10 mg/kg, 30 mg/kg and 100 mg/kg extract. This model was chosen because it has long been used to evaluate inflammatory activity (Winter *et al.*, 1962). It was said by Otterness *et al.* (1985) that there was good correlation between the efficacies of anti-inflammatory in this model and human's activity. Besides that this model had been used to evaluate inflammatory COX enzymes (Vane, 1971; Ferreira, 1972). It is a well-established fact that NSAIDs exerts their anti-inflammatory activity by inhibition of prostaglandin biosynthesis (Vane, 1971). Furthermore, Mossa *et al*. (1995) have found that this model is useful for oral NSAIDs drugs by inhibiting the mediator of inflammation. From the explanation above, COX, inflammation, prostaglandin and NSAIDs drugs are all inter- related and this model is best to explain the anti-inflammatory effect of samples used in carrageenan induced oedema of rat's right hind paw.

Oedema formation due to carrageenan in the rat paw is a biphasic event (Vinegar *et al.*, 1969). The initial phase was attributed to the release of histamine, 5-hydroxytryptamin, bradykinin and cyclooxygenase products (Di Rosa and Willoughby, 1971) where as prostaglandin was detected in the late phase of inflammation (Salvemini *et al.*, 1996b). Carrageenan-induced paw oedema model is very useful for determining the acute phase of inflammation. Mossa *et al*. (1995) found that this model was also useful for detecting oral

anti-inflammatory agents in drugs which act via peripheral in inhibiting the mediators of acute inflammation.

In this study, the initial phase of oedema was observed at 30 minutes after administration and followed by a second phase that sustained for 180 minutes. The oedema was at its maximal at 210 minutes. The second phase for oedema in this study was not clear as the mode of action continued from its initial phase. For PT Et extract given orally at 10, 30 and 100 mg/kg showed significant decrease in the swelling of carrageenan-induced oedema on rat's right hind paw. PT Et extract at 10 mg/kg caused slow onset of action which started at 180 minutes and lasted for one and half hour (Figure 4.18 (a)). For PT Et at 30 mg/kg, the onset action started at 30 minutes, as soon as the oedema occurs and significantly reduced the oedema for 300 minutes (Figure 4.18 (b)). When a higher dose, 100 mg/kg PT Et was given orally, oedema were significantly inhibited at 60 minute and lasted for 210 minutes (Figure 4.18(c)). From the result, it was observed that PT Et extract significantly reduce oedema and it was concentration dependent where the percentage of oedema inhibited increases as the concentration of the extract increases. PT Ea extract only showed significant reduction in oedema when 30 mg/kg of extract was given orally and it showed the lowest percentage of oedema inhibition compared to 30 mg/kg of PT Et and KA Et given orally (Table 4.17 (b)). KA Et did not show any reduction in carrageenan induced oedema when this extract was given 10 mg/kg orally. At 30 mg/kg and 100 mg/kg, KA Et showed significant reduction of oedema. Extract KA Et given orally at 30 mg/kg (Table 4.17 (b)) showed higher percentage of oedema inhibition compared to when 100 mg/kg (Table 4.17 (c)) of its extract were given orally.

It is well known that in acute inflammatory process, vascular permeability increases and leukocyte migration occurs via peripheral. In this study, indomethacin and dispirin, an aspirin-like drug was used as positive control and it was found by Hargreaves *et al*. (1988) that oedema produced at the second phase was inhibited by indomethacin via peripheral mechanism. Besides that, dispirin is known to inhibit prostaglandin (Smith *et al*., 1971). Therefore, it can be concluded that PT Et (10, 30 and 100 mg/kg), KA Et (30 and 100 mg/kg) and PT Ea (30 mg/kg) possess peripheral anti-inflammatory effect.

Although the relative contributions of COX-1 and COX-2 to prostaglandin production are not known, it is likely that COX-2 is primarily responsible for the increased levels of prostaglandin but with continued production of prostaglandin by COX-1. There is extensive evidence based on animal studies supporting the role of COX-2 in the development of inflammation (Seibert *et al.*, 1997; Thomlison *et al.*, 1994; Wallace *et al.*, 1998). The effect of indomethacin, dispirin and aqueous crude extract of PT Et (10, 30 and 100 mg/kg), KA Et (30 and 100 mg/kg) and PT Ea (30 mg/kg) in inhibition of the second phased carrageenan could not be determined that it was inhibited via COX-1 or COX-2. To confirm the inhibition of COX-1 or COX-2, the hind paw liquid must be measured for its prostaglandin content (Seibert *et al.*, 1994). COX-2 is often referred to as the inducible isoform of COX, since levels of COX-2 increase in response to several forms of stimuli, including inflammation (Teather *et al.*, 2002). In contrast, COX-1 which is the constitutive form of COX, appear to be involved in housekeeping cellular function (Herschman, 1996). It is also known that second phased of oedema is due to release of prostaglandins, protease and lysosome (Vinegar *et al.*, 1969; Crunkhon *et al.*, 1971) and the second phase is sensitive to most clinically effective anti-inflammatory drugs (Vinegar *et al.*, 1969; Di Rosa *et al.*, 1971) in this study, indomethacin and dispirin. Therefore, from the results

obtained, it showed that all the samples used in this study significantly reduce oedema during the second phase and even after 210 minutes. Therefore, it can be postulated that the samples, PT Ea, PT Et and KA Et might inhibit prostaglandin production via COX-2.

Some researches demonstrated that inflammatory effect induced by carrageenan is associated with free radical. Free radicals, prostaglandins and nitric oxide will be released when administered with carrageenan for 1 to 6 hours (Dudhgaonkar *et al.*, 2006). Polymorphonuclear leukocytes which are the first cells to arrive at the inflammatory site of the body, release free oxygen radicals (O2-) and free hydroxyl radicals (HO-) (McCord *et al*., 1982). Del Maestro *et al*. (1980) explained that, at low concentrations of superoxide dismutase and catalase, they increase the susceptibility of extracellular components to oxygen radical injury and may stimulate chemotaxis for other inflammatory cells. Therefore, it can be postulated that the aqueous extract that inhibited oedema may act as antioxidant and as COX-2 inhibitor and this was supported by Salvemini *et al*. (1996a) and Boughton-Smith *et al*. (1999) where they reported the inhibition of second phase oedema may be explained by an inhibition of cyclooxygenase or exerting of anti-oxidative properties.

5.2.3 Antinociceptive Study (Plantar Test – Hargreaves method)

Antinociceptive effect of *P. tetrastromatica* ethyl acetate and ethanol and *K. alvarezii* ethanol extract were studied using paw withdrawal test which is known as thermal hyperalgesia. Hargreaves *et al*. (1988) designed this method.

Hargreaves and his colleagues (1988) introduced plantar radiant thermal test to study hyperalgesic mechanism in rat's hind paw. This model is suitable to determine anti-inflammatory effect and its mechanism of action either acting on COX-1 or COX-2 and via either peripheral or CNS response (Yaksh *et al.*, 2001). The paw withdrawal latency (PWL) decreases in hyperalgesic conditions (Hargreaves *et al.*, 1988). Hyperalgesia is defined as a decrease in pain threshold and an increased sensitivity to suprathreshold stimuli. Lewis (1942) and Hardy *et al*. (1950) had identified 2 types of hyperalgesia, primary and secondary hyperalgesia. Primary hyperalgesia is restricted to the site of injury and has been characterized by an increased sensitivity to both mechanical and thermal stimuli. Secondary hyperalgesia develops in the area that surrounds the injury site and it is thought to be sensitive to mechanical but not heat stimuli (Sluka *et al.*, 1994b); and Xu *et al.*, 2001). Several lines of evidence indicate that primary hyperalgesia is caused by increased responsiveness of primary afferent nociceptors, whereas secondary hyperalgesia is produced by enhanced responses of dorsal horn neurons to a given peripheral input (central sensitization) (Dahl *et al.*, 1992; Lamotte *et al.*, 1992; Millan, 1999; Sluka *et al.*, 1994a; Treede *et al*., 2000). Successive application of noxious radiant heat to the plantar surface of the hind paw may cause the animal to move, lift away the paw or lick its paw if it exhibits more intense pain and these behaviors were documented by Donahue *et al.*

(2001), Le Bars *et al*. (2001) and Simmons *et al*. (2002). All these behaviors were observed in this study as well.

In this study, all the extract of PT Et, PT Ea and KA Et given at 10 mg/kg did not significantly prolong rat's paw withdrawal latencies (PWL) on both right (Table 4.18 (a)) and left hind paw (Table 4.19 (a)). Extract of KA Et and PT Et given at 30 mg/kg significantly prolonged rat's PWL on right hind paw (Table 4.18 (b)). Only PT Ea extract given at 30 mg/kg significantly prolonged rat's PWL on the left hind paw (Figure 4.19 (b)). Samples PT Ea and PT Et given at 100 mg/kg, significantly prolonged rat's PWL on the right hind paw (Table 4.18 (c)) whereas PT Ea, PT Et and KA Et given at 100 mg/kg significantly prolonged rat's PWL on the left hind paw after carrageenan injection (Figure 4.19 (c)).

From the results, it clearly showed that PT Ea and PT Et significantly prolonged PWL in both rat's right and left hind paws when given orally at 100 mg/kg. These results indicated that the action of both these samples were via peripheral (right paw) and also via nervous system (left paw). Hargreaves *et al*. (1989) observed that when morphine was used to determine its effect using plantar test; it showed bilateral prolonged effect on both right and left hind paws. Sample KA Et given at 100 mg/kg significantly prolonged PWL in rat's left hind paw indicating that this sample at this concentration exerted the antinociceptive effect via CNS only. At lower concentration of 30 mg/kg, PT Ea only showed PWL at rat's left hind paw which shows that, PT Ea exerted its antinociceptive effect through CNS mechanism. PT Et and KA Et given at 30 mg/kg exerted its antinociceptive effect through peripheral mechanism as these two samples showed PWL at rat's right hind paw. Therefore, it can be concluded that at lower concentration, PT Ea

exerted the antinociceptive effect via peripheral mechanism while at higher concentration the effect of the sample exerted via peripheral and CNS mechanism. Sample PT Et exerted its antinociceptive via peripheral at lower dosage while at higher dosage, this sample exerted via peripheral and CNS mechanism. Its CNS mechanism showed slower onset and a short duration of action. At lower concentration, KA Et exerted its antinociceptive via its peripheral mechanism whereas at higher concentration, the effect was exerted via CNS mechanism. Its peripheral mechanism showed earlier onset (at 60 second) compared to its CNS mechanism (at 300 second).

Samples that exerted the antinociceptive effects via peripheral at lower concentration and via CNS at higher concentration might be due to the close relationship between stimulus intensity and response to acute stimuli in the injured, uninjured and inflamed side associated with hypersensitivity (Raja *et al.*, 1988). In response to tissue damage, peripheral nociceptors are stimulated, giving rise to acute or nociceptive pain. If pain input persists, as with postoperative pain, an inflammatory reaction may occur. Inflammatory pain seems to be elicited by a variety of C-fiber in the spinal dorsal horn (substance P, somatostatin, corticotropin-releasing factor) (Melzack *et al.*, 2001), excitatory amino acids (glutamate, aspartate) (Randic *et al.*, 1993) and other chemical mediators (cytokines, chemokines, bradykinin, prostaglandins) (Ahmadi *et al.*, 2002). These substances that promote tissue regeneration may directly act as irritants at nociceptors. With ongoing nociceptive input, there is modulation of the central nervous system through activation-dependent plasticity. As a result, allodynia (a reduction in pain threshold) and hyperalgesia (an increase in responsiveness to peripheral nociceptor signals) may occur and this process is called peripheral sensitization. Sensitization is reflected by increased spontaneous activity, reduced threshold, or increased responsiveness to afferent

inputs, prolonged discharge to repeated stimulation (Melzack *et al*., 2001) and expansion of the peripheral receptive fields of the dorsal horn neurons (McMahon *et al*., 1984; Woolf *et al*., 1990; Cameron *et al.*, 1991). Neuronal plasticity induces a memory effect, leading to a more rapid reaction of the central nervous system when re-experiencing the same or a similar stimulation. According to Coderre *et al*. (1992) and Kissin (1994), through a mechanism of maladaptive plasticity, further pain input will be amplified and longer lasting where this phenomenon is called central sensitization. Central sensitization contributes many forms of pain such as pain hypersensitivity, spontaneous pain, and chronic postoperative pain (Perkins *et al*., 2000; Macrae, 2001). This is in agreement with results obtained in our studies on the effect of PT Ea, PT Et and KA Et. In this study we also observed that at the later time after carrageenan injection, PT Et and PT Ea also prolonged PWL on the right hind paw. Therefore, it can be concluded that at 100 mg/kg of PT Et and PT Ea given orally to rats can exert the antinociceptive effect via both peripheral and central mechanism. Besides the explanation above in regard to central sensitization induction, Fisher *et al*. (2000) in his study found that NMDA receptor antagonists may have the potential for attenuation of central sensitization and prolonged pain states.

NSAIDs might act on the central nervous system as found by Malmberg *et al*. (1992) because these drugs can prolong paw withdrawal latency injected with saline or control vehicles. Indomethacin is a NSAID which is widely used for treatment of different rheumatic condition (Pemberton *et al*., 1979). Indomethacin given at 10mg/kg in this study significantly prolonged PWL on the right hind paw from and on the left hind paw after carrageenan injection. Indomethacin is a classic inhibitor of cyclo-oxygenase, which is known to mediate synthesis of prostaglandins such as prostaglandin E2 (PGE2) and prostaglandin I2. Indomethacin preferentially inhibits COX-1 (Smith *et al.*, 1994).

Indomethacin was found to prolong paw withdrawal latency on the right hind paw by Hargreaves *et al.* (1989) where its antinociceptive effect is via peripheral. Recent study by McCormack *et al*. (1991); Brune *et al.* (1991); Malmberg *et al*. (1992) and Bannwarth *et al.* (1993) showed that indomethacin act via peripheral and central mechanism. This was observed in our study as well when indomethacin was used. Dispirin (aspirin) given at 150 mg/kg did not significantly prolong PWL on the rat's right and left hind paw but it inhibited oedema induced by carrageenan in our study. Smith *et al*. (1971) found that aspirin ingested at 600 mg dose by selected individuals inhibited prostaglandin synthesis when their platelets were tested *ex vivo* for the level of prostaglandins. This inhibition of PG synthesis was also observed *in vivo* in animal preparation exhibiting PG release from the spleen (Ferreira *et al*., 1977). Dispirin significantly inhibited carrageenan induced oedema but was not effective to exert thermal induced hyperalgesia in our study. This might be due to the dose used in this study. A study done by Campbell *et al.* (2000), concluded that aspirin at 300 mg/kg inhibited hyperalgesia by approximately 50%. This showed that a higher dose is needed for dispirin to exert thermal induce hyperalgesia. This is the reason that dispirin could inhibit oedema induced by carrageenan but not to pain induced by heat.

Pain is perhaps the most significant manifestations of inflammation. Hyperalgesia typically accompanies inflammation and is produced by mediator substances in response to tissue injury and repair (Woolf *et al.*, 2000). Prostaglandins (PGs) are one of the key players in hyperalgesia where PGs are released by fibroblasts and numerous other tissue cells for example immune cells such as macrophages, mast cells and neutrophils (Levine *et al*., 1994). They are produced from arachidonic acid when cells membranes are disrupted. As explained earlier, PGs are formed via the action of COX which exists in two isoforms;

COX-1 and COX-2. COX-1 is distributed throughout the body, with a housekeeper role involve in cell homeostasis such as cytoprotection in GI tract, platelet function and renal perfusion, whereas COX-2 is inducible and rapidly up-regulated in response to inflammatory stimuli as a result of tissue damage (Simmons *et al.*, 2004). In pain pathways, cyclooxygenase (COX)-2 is known to have important roles in both central nervous system (CNS) and peripheral tissues. It has been reported that COX-2 is expressed in both the spinal cord (Beiche *et al.*, 1996; Ichitani *et al.*, 1997) and peripheral inflammatory tissue (Seibert *et al.*, 1994). It was also shown that central and peripheral COX-2 was relevant to inflammatory pain. Therefore, in our study, we postulated that samples of PT Et and PT Ea given at 100 mg/kg in rats possessed antinociceptive behavior via COX-2 inhibition and act via peripheral and central mechanism. This was also postulated based on a hypothesis by Ballou *et al.* (2000). In his study, he reported that COX-2 is involved in mediating both peripheral and central component of inflammatory pain or thermal hyperalgesia. Cyclooxygenase-2 (Beiche *et al.*, 1996; Samad *et al.*, 2001) and microsomal prostaglandin E synthase (Guay *et al.*, 2004) are induced in the spinal cord in response to peripheral inflammation. Spinal prostaglandin E2 (PGE2) is largely responsible for central sensitization following peripheral inflammation. Therefore, further study must be carried out to determine the effect of the samples tested in our study is via COX-2 or COX-1.

5.2.4 Anti-ulcerogenic Study

NSAIDs produce gastric damage as an adverse reaction in experimental animals and humans (Lanza, 1984; Whittle, 1981). Although there is other multiple elements are that involved in gastric lesion, a deficiency of endogenous prostaglandin (PGs) is the most important in the producing of ulcerogenic, with response to the usage of NSAIDs (Whittle, 1981; Vane *et al*., 1995). This connection was supported by the fact that NSAID-induced gastric lesions were effectively prevented by supplementation with exogenous PGs (Takuechi *et al.*, 1986; Okada *et al.*, 1989; Takeuchi *et al.*, 1991). NSAIDs inhibit only the cyclooxygenase reaction of the PGH synthase. Prostaglandins are important in the regulation of thrombocyte aggregation, inflammatory processes, pain and fever induction, the regulation of vessel perfusion, and many other processes (Steinmeyer, 2000).

Cyclooxygenase (COX) is an important enzyme family that comprises of enzymes responsible for the synthesis of prostaglandins from arachidonic acid that plays varied range of roles in various physiological and pathological conditions. COX exists in two isoforms, COX-1 and COX-2 (FitzGerald *et al*., 2001). COX-1 is expressed constitutively in the body. It produces prostanoids involved in physiological and housekeeping functions such as protection of gastrointestinal mucosa, regulation of renal haemodynamics and stimulation of platelet aggregation. COX-2 is inducible in most of the cells in response to tissue injury by pro-inflammatory or mitogenic agents and has a leading role in inflammatory process (Halter *et al.*, 2001).

Nonselective NSAIDs, those that inhibit both cyclooxygenase, COX-1 and COX-2 are known to be associated with an increased risk for serious upper gastrointestinal (GI)

complications, including perforation, symptomatic ulcers and bleeding, nephrotoxicity, including edema, hypertension, and acute renal insufficiency as well as congestive heart failure (Garcia Rodriguez *et al*., 2000; Hernandez-Diaz *et al*., 2001). These effects arise from the blockage of the physiological effects of prostacyclin, PGE2 and Thromboxane. Both PGE2 and prostacyclin are tissue hormones, cytoprotective towards the stomach where they stimulate the production of mucus and inhibit acid secretion. NSAIDs inhibit endogenous prostaglandin synthesis and in this way remove this cytoprotective effect; thus, the induction of ulcers is promoted (Steinmeyer, 2000). This led to the development of COX-2 selective inhibitors. COX-2-selective NSAIDs were developed with the intention of reducing the unwanted side effects of NSAIDs, particularly those relating to the GI system. It was reported that NSAIDs inhibits COX-2 but not the COX-1 enzyme at therapeutic plasma concentrations would have the beneficial anti-inflammatory and analgesic effects but not the gastrointestinal or renal toxicity as caused by nonselective NSAIDs (Vane *et al*., 2000). The use of COX-2-selective drugs such as rofecoxib and celecoxib (Deeks, 2002) also seems to be a safer option than using other NSAIDs. However, one limitation is that COX-2 might also have a role in healing ulcers of the stomach, and this was reported by Motilva *et al.* (2005) where these inhibitors caused a delay in ulcer healing in rats, highlighting the pivotal role of this isozyme in gastric ulcer healing.

Indomethacin is a non-selective NSAID and it was found that the mucosal PGE2 content were markedly reduced after administration of indomethacin, confirming a PG deficiency in the background for NSAID-induced gastric lesions (Wallace *et al.*, 2000). Gastric mucosal blood flow critically determines the ability of the stomach to withstand noxious injury. Reduction in mucosal blood flow exacerbates injury by ulcerogenic agents

(Ritchie, 1975; Whittle, 1977), while little or no damage occurs after necrotizing agents if blood flow is maintained at appropriate levels, for example with cytoprotective prostaglandins (Guth *et al.*, 1984). Indomethacin a potent inhibitor of endogenous prostaglandin synthesis (Whittle *et al.*, 1980), causes vascular endothelial damage (Rainsford *et al*., 1982) and reduces gastric mucosal blood flow (Whittle, 1977; Kauffman *et al.*, 1980) therefore induces stomach ulcer. Indomethacin was used as necrotic agents to induce ulcer in this study where indomethacin 30 mg/kg were given orally after oral administration of 100 mg/kg samples. From the test results, it showed that, both PT Et (*P. tetrastromatica* ethanol extract) and KA Et (*K. alvarezii* ethanol extract), given at 100 mg/kg orally significantly reduced the ulcer lesion induced by indomethacin (Table 4.20). PT Et reduced gastric mucosal lesion from 142.67 ± 20.02 mm^2 (induced by indomethacin) to 33.17 ± 7.96 mm^2 whereas KA Et significantly reduced gastric mucosal lesion from 155.5 ± 25.49 mm^2 to 69.67 ± 10.67 mm^2. This shows that these samples were able to act as one of the COX inhibitor as indomethacin is known to inhibit both COX-1 and COX-2. Further studies must be carried out to confirm the inhibition of COX-1 or COX-2 by the samples. From the test result for anti-inflammatory (Table 4.17 (c)), both samples significantly inhibited oedema; 91.65 ± 5.75% for PT Et and 70.55 ± 6.14% for KA Et at 100 mg/kg given orally. This shows that both samples selectively inhibited COX.

Another necrotic agent used in this study to induce ulcer was hydrochloric acid (HCl). Free hydrochloric acid has been generally found to be present in increased amount in patients with ulcers (Vanzant *et al.*, 1933). HCl is known to produce ulceration and digestion of the stomach tissue (Dragstedt, 1917) as well as to reduce the neutralizing capability of the stomach mucus secretions (Florey *et al.*, 1935; Rider *et al.*, 1965). Besides that, HCl induces wide haemorrhage bands in gastric corpus. The excessive secretion of

hydrochloric acid in the stomach is considered an important factor in the formation of peptic ulcer (Walpole *et al.*, 1940; Hay *et al.*, 1942). Gastric mucus is an important protective factor for the gastric mucosa and consists of a viscous, elastic, adherent and transparent gel formed by 95% water and 5% glycoproteins that cover the entire gastrointestinal mucosa. Mucus is capable of acting as an antioxidant, thus it can reduce mucosal damage mediated by oxygen free radicals (Penissi *et al*., 1999). A decrease in gastric mucus renders the mucosa susceptible to injuries induced by acid (Cross *et al.*, 1984). The exact mechanism of pathogenesis by HCl is still unclear (Matsumoto *et al.*, 1993), but it has been demonstrated that active oxygen species might be involved in the formation of gastric mucosal lesion (Itoh *et al*., 1985; Smith *et al.*, 1987; Yoshikawa *et al.*, 1990). In this study, *P. tetrastromatica* ethanol extract (PT Et) 100 mg/kg significantly reduced ulcer induced by HCl from 347.5 ± 24.07 mm^2 to 116.0 ± 12.06 mm^2 while *K. alvarezii* ethanol extract (KA Et) 100 mg/kg significantly reduced ulcer from 1236.67 ± 53.74 mm^2 to 408.67 ± 49.96 mm^2 (Table 4.20). In the HCl induced stomach lesion, the increase of COX-2 expression plays an important role in the healing and other events associated to tissue repair, supporting an important production of regulative PGs (Peskar *et al.*, 2001; Perini *et al.*, 2003). This can be postulated that these samples (PT Et and KA Et) at higher concentration can act as antioxidant as well as a mediator to increase prostaglandin level induced by COX-2 in stomach. Therefore, it can be concluded that these samples possessed anti-inflammatory, antinociceptive with gastro protective effects.

6.0 CONCLUSION

In this work, we studied the antioxidant activities of teas, fruits' skin, flower, seaweeds and microalgae. Animal study was also carried out which comprised of anti-inflammation, pain, anti-ulcer and lethal dose study.

All the samples were assayed with 4 different methods namely, AEAC, DPPH, Galvinoxyl and Total Phenolic assay to determine the antioxidant activity of our samples. We found that the antioxidant activity of each sample differs in each assay. Therefore, to obtain the antioxidant activity of the samples more than one assay was needed. The antioxidant activity obtained in our study for DPPH assay was inconsistent although DPPH is known to be a rapid and easy test. Some samples did not show any antioxidant activities but when the same samples were tested with other assays, they showed antioxidant activities. This might be due to the samples used were not studied for their EC_{50} and many other interferences that involved when DPPH assay was used to evaluate antioxidant activity. AEAC assay showed more consistent results compared to DPPH assay's results. We concluded that this might be caused by the ability of AEAC assay to assay both organic and aqueous extracts and its specific absorbance wavelength is away from the visible range, unlike DPPH assay. Galvinoxyl assay showed good antioxidant evaluation but this assay was only tested for methanol extracts. It was observed that all assays tested showed that boiled water extracts possessed higher antioxidant activities compared to methanol extracts. A good correlation was observed between AEAC and Total Phenolic assay for boiled water extracts compared to methanol extracts. This might be due to the ability of boiled water to extract low polarity to high polarity compounds whereas methanol was able to extract mid-polar to low polar compounds. Forward and reverse

extractions were carried out on selected seaweeds to study the effect of solvent polarity when extracted from high polar solvent to low polar solvent. From the observation, we concluded that there was difference between the two methods but the results did not differ much to determine that forward extraction was better than reverse extraction or vice versa. We also studied the different method of grinding to observe the effect of heat towards the sample's extract. Two tests were carried out whereby selected seaweeds were blotted dry and ground with liquid nitrogen as well as seaweeds that were oven dried and ground with grinder. We found that for methanol extracts, liquid nitrogen grinding method showed higher antioxidant activities compared to grinder method and for boiled water extracts grinder method showed higher antioxidant activities compared to liquid nitrogen grinding. We also found that the 5 different group of samples; teas, fruits' skins, flower, seaweeds and microalgae, gave different antioxidant activities. As a result, the antioxidant activity could not be compared between the samples but must be compared among the samples from the same group.

We carried out animal study using only two seaweeds which were *Padina tetrastromatica* (ethyl acetate (PT Ea) and ethanol (PT Et) extracts) and *Kappaphycus alvarezii* (ethanol extract) (KA Et). From the study of lethal dose, we observed that these samples possessed high LD_{50}. PT Ea showed a higher LD_{50} compared to the other two samples. For anti inflammation study, PT Et extract given orally at 10, 30 and 100 mg/kg showed significant decrease in the swelling of carrageenan-induced oedema on rat's right hind paw. Furthermore, it was also observed that PT Et extract significantly reduced oedema and it was concentration dependent where the percentage of oedema inhibited increased as the concentration of the extract increased. PT Ea extract only showed significant reduction in oedema when 30 mg/kg of extract was given orally and it showed

the lowest percentage of oedema inhibition compared to 30 mg/kg of PT Et and KA Et given orally. At 30 mg/kg and 100 mg/kg, KA Et showed significant reduction of oedema. Extract KA Et given orally at 30 mg/kg showed higher percentage of oedema inhibition compared to when 100 mg/kg of its extract was given orally. From the results obtained, it showed that all the samples used in this study significantly reduced oedema during the second phase and even after 210 minutes. We suggest that these samples, PT Ea, PT Et and KA Et, might inhibit prostaglandin production via COX-2. Antinociceptive effect (pain study) of *P. tetrastromatica* ethyl acetate and ethanol and *K. alvarezii* ethanol extract were studied using paw withdrawal test. We found that PT Et, PT Ea and KA Et given at 10 mg/kg did not significantly prolong rat's paw withdrawal latencies (PWL) on both right and left hind paws. Extract of KA Et and PT Et given at 30 mg/kg significantly prolonged rat's PWL on right hind paw. PT Ea was the only extract given at 30 mg/kg that significantly prolonged rat's PWL on the left hind paw. Extracts PT Ea and PT Et given at 100 mg/kg, significantly prolonged rat's PWL on the right hind paw whereas PT Ea, PT Et and KA Et significantly prolonged rat's PWL on the left hind paw. We suggest that samples of PT Et and PT Ea given at 100 mg/kg in rats might possess antinociceptive behavior via COX-2 inhibition and act via peripheral and central mechanism but further studies on this matter must be carried out to determine the samples behavior. In anti-ulcer study, we found that *P. tetrastromatica* (PT Et) at 100mg/kg reduced gastric mucosal lesion that was induced by indomethacin from 142.67 ± 20.02 mm^2 to 33.17 ± 7.96 mm^2 whereas *K. alvarezii* (KA Et) significantly reduce gastric mucosal lesion from 155.5 ± 25.49 mm^2 to 69.67 ± 10.67 mm^2. *P. tetrastromatica* ethanol extract (PT Et) at 100mg/kg significantly reduced ulcer induced by HCl from 347.5 ± 24.07 mm^2 to 116.0 ± 12.06 mm^2 while *K. alvarezii* ethanol extract (KA Et) at 100mg/kg significantly reduced ulcer from 1236.67 ± 53.74 mm^2 to 408.67 ± 49.96 mm^2. We postulated that these samples (PT Et and

KA Et) at higher concentration could act as antioxidant as well as a mediator to increase prostaglandin level induced by COX-2 in stomach.

Therefore, we concluded that these seaweed extracts studied in this study possessed antioxidant, anti-inflammatory, antinociceptive and anti-ulcerogenic activities.

7.0 APPENDICES

Appendix A: Preparation of Kosaric Medium (Modified after Zarrouk, 1966)

Chemicals used for Kosaric medium preparation to grow *Spirulina platensis* is as listed below:

1.	Chemical	gL^{-1}
2.	$NahCO_3$	9.0
3.	$K2HPO_4$	0.250
4.	$NaNO_3$	1.250
5.	K_2SO_4	0.5
6.	NaCl	0.5
7.	$MgSO_4.7H_2O$	0.1
8.	$CaCl_2$	0.02
9.	$FeSO_4.2\ H_2O$	0.005
10.	Micronutrient solution :	$0.5mlL^{-1}$
a.	- H_3BO_4	2.86
b.	- $MnCl_2.4\ H_2O$	1.81
c.	- $ZnSO_4.7\ H_2O$	0.22
d.	- $CuSO_4.5\ H_2O$	0.08
e.	MoO_3	0.01
f.	$CoCl_2.6\ H_2O$	0.01

Table 7.1: Chemicals used to prepare Kosaric medium to grow *Spirulina platensis*.

First, all the chemicals were weigh and mix together in a 2 liter conical flask and added with 0.5mlL^{-1} micronutrient solution which was prepared earlier in 1 liter stock. This mixture was then top up to 980 ml with distilled water. The pH for the medium was then adjusted to pH 8.5 and the solution were top up till 1 liter. The solution was then left to stir well for 1 hour. After that, the solution was distributed into 10 conical flaks (100ml each). Conical flasks were then covered and autoclaved 121˚C for 20 minutes.

Appendix B: Preparation of Bold's Basal Medium (BBM) (Nicholas & Bold, 1965)

Chemicals used for BBM medium preparation to grow *Chlorella vulgaris* is as listed below:

	Chemicals	per 400ml
1.	$NaNO_3$	10.0g
2.	$MgSO_4.7H_2O$	3.0g
3.	K_2HPO_4	4.0g
4.	KH_2PO_4	6.0g
5.	$CaCl_2$	1.0g
6.	NaCl	1.0g
7.	Trace element solutions:	per L
a.	ZnSO4	8.82g
b.	MoO_3	0.71g
c.	$Co(NO_3)_2.6H_2O$	0.49g
d.	MnCl2	1.44g
e.	$CuSO_4.5H_2O$	1.57g
		per 100ml
8.	H_3BO_4	1.14g
9.	EDTA-KOH solution	
a.	$EDTA.Na_2$	5.0g
b.	KOH	3.1g
		per L
10.	$FeSO_4.7H_2O$	4.98g
11.	Concentrated HCL	1.0ml

Table 7.2: Chemicals used to prepare BBM medium to grow *Chlorella vulgaris*

To prepare BBM medium, stock solutions of 1-6 were pipette 10.0ml each and stock solutions 7-10 were pipette 1.0ml each and all these solutions were mixed together in a 2-liter conical flask. The solution was top up till 980ml and the pH of the solution was adjusted to pH6.8 while stirring. Then, the solution was top up till 1 liter and stir for 1 hour. After 1 hour, the solution was distributed into 10 conical flasks (100ml each). Conical flasks were then covered and autoclaved 121˚C for 20 minutes.

Appendix C: Preparation of Agar Plate

From the Kosaric and BBM medium that was prepared, two conical flasks were taken from each group to prepare for alga plate. Before autoclaving, each flask was added with 2g (2% of volume) Bato™ Agar into the 100ml volume medium and was mixed well using stirrer. The flasks were then covered and autoclaved at 121˚C for 20 minutes. Laminar flow was used to prepare alga plate. Therefore, laminar flow was sterilized. The flasks were left to cool down in the laminar flow. After the flasks were cool down, the medium was pour into sterile plates, sealed and covered. The medium was left to harden in the culture room. *Spirulina platensis* and *Chlorella vulgaris* was obtained from the stock culture of University Malaya. These microalgae were cultured onto the algae plate under sterile condition and were sealed, marked and kept in the culture room.

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