The Antioxidant Potentials of the New Zealand Surf Clams

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The Antioxidant Potentials of the New Zealand Surf Clams

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Date approved

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Signed 

Name ODELEYE TINUOLUWA

Date 9th March, 2015.

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Abstract

The antioxidant activities of the aqueous and ethanolic extracts of three New Zealand surf clam species (Tua Tua (*Paphies donacina*), Diamond Shell (*Spisula aequilatera*) and Storm shell (*Mactra murchisoni*)) were evaluated and presented as relative activities by comparing with a standard synthetic antioxidant (ascorbic acid). The ethanolic extracts were further fractioned into four parts (petroleum ether (pe), ethyl acetate (ea), n-butanol (nb) and the final aqueous residue (w)).

Two types of antioxidant assays were employed to test for antioxidant activity. The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, to test the scavenging ability of the clam fractions, and the Cupric Ion Reducing Antioxidant Capacity (CUPRAC) assay to evaluate their copper ion reducing ability.

Results showed that the *in vitro* antioxidant activities of all fractions of the three NZ surf clams tested exhibited dose dependency, and increased with increasing concentration of the extract. The pe and ea fractions of all clam species showed higher cupric ion reducing capacity than ascorbic acid and the other fractions tested. All fractions had very strong radical scavenging ability, with the aqueous fraction of the Diamond shell having the least DPPH radical scavenging ability of 57.08% at 50µg/mL.

This study forms the first comprehensive report on the nutraceutical properties of the New Zealand surf clams as natural sources of antioxidants.

CHAPTER ONE

1. INTRODUCTION

1.1 BIOACTIVES DERIVED FROM SEAFOOD

The wide diversity of marine organisms contained in the world's oceans offers a rich source of natural products (Wijesekara, Pangestuti, & Kim, 2011, Aneiros, & Garateix, 2004) and, as such, is regarded as the largest remaining reservoir of natural molecules to be evaluated for drug activity (Gerwick, 1987). Marine organisms are rich in functional materials including polyunsaturated fatty acids (PUFA), polysaccharides, essential minerals and vitamins, antioxidants, enzymes and bioactive peptides (Kim et al., 2008, Kim & Wijesekara, 2010; Ngo, Wijesekara, Vo, Van Ta, & Kim, 2010) and provide an ample scope for the extraction of drugs and chemicals for therapeutic purposes (Chakraborty& Ghosh, 2010) and medical research (Ruggieri, 1975).

Seafood contributes at least 15% of average animal protein consumption to 2.9 billion people and as much as 50% for some small islands and West African states. As well as being a great source of Omega-3 fatty acids essential for brain development, seafood also provides important micronutrients for the poor. As a source of livelihood, capture fisheries and aquaculture employed 43.5 million people in 2006 and 520 million people relied on income from seafood production. Seafood is also the most highly traded food commodity internationally (Smith et al., 2010).

Due to the increasing demand by consumers for high-quality healthy food, there is a focus on trying to reduce the impact of some risk factors of current agricultural production methods on human health. To date nutritional theories have focused on the health implications of the fatty acid profile of the diet; in particular the relationship between saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), long-chain polyunsaturated fatty acids (PUFA) of both the n-3 and n-6 categories. An important feature of polyunsaturated fatty acids, in particular n-3 fatty acids, is their role in the prevention and modulation of certain diseases that are common in industrialized countries. Reports have shown that lipids of marine fish or seafood species are

characterised by low levels of linoleic acid (18:2 n-6) and linolenic acid (18:3 n-3), as well as high levels of long chain n-3 polyunsaturated fatty acids. Eicosapentaenoic acid (20:5 n-3) and docosahexaenoic acid (22:6 n-3) are the predominant n-3 fatty acids (Valfré, Caprino, & Turchini, 2003).

While some marine animals especially bivalves (oysters, clams, scallops, etc.) contain carotenoids which show structural diversity (Maoka, 2009) and are antioxidants (Table 1), there is an increasing focus on another source of potential antioxidant activity, namely the marine-derived peptides.

Marine-derived bioactive peptides have been shown to possess many physiological functions including antihypertensive action or angiotensin-I-converting enzyme (ACE) inhibition (Je et al., 2005). Several studies have indicated that peptides derived from marine fish proteins have antioxidant properties (Jun, Park, Jung, & Kim, 2004) and a number of such peptides have shown higher antioxidative potential than α-tocopherol (Ngo, Wijesekara, Vo, Ta, & Kim, 2011).

The peptide (Glu-Ser-Thr-Val-Pro-Glu-Arg-Thr-His-Pro-Ala-Cys-Pro-Asp-Phe-Asn) for example, which was isolated from the peptic hydrolysate of hoki (*Johnius belengerii*) frame protein, has inhibited lipid peroxidation higher than that of α-tocopherol and efficiently quenched different sources of free radicals (Kim et al., 2007). An Antioxidant Reptide from Bullfrog Skin Protein (APBSP) also inhibited lipid peroxidation higher than that of α-tocopherol (Qian, Jung, & Kim, 2008). Fish and shellfish protein hydrolysates have shown a greater lipid peroxidation inhibition and are considered as useful candidates in search of effective non-toxic substances with differing antioxidant mechanisms (Mendis, Rajapakse, Byun, & Kim, 2005).

Bioactive peptides can be obtained from an organism's protein by enzymatic hydrolysis (Je et al., 2005; Lee, Hong, Jeon, Kim, & Byun, 2009) and have been reported effective and safe in recent years (Nazeer, Prabha, Kumar, & Ganesh, 2013).

The antioxidant activity of marine derived bioactive peptides has been determined by various *in vitro* methods such as DPPH (2,2-diphenyl-1-picrylhydrazyl), peroxide, hydroxyl and superoxide anion radical scavenging activities which have been detected by electron spin resonance (ESR)

spectroscopy method as well as intra-cellular free radical scavenging assays such as DNA oxidation, reactive oxygen species scavenging, membrane protein oxidation and membrane lipid oxidation (Ngo, Wijesekara, Vo, Ta, & Kim, 2011).

Table 1: Reported marine seafood that possess antioxidant peptides and their mode of action.

Marine animal	Mode of Peptide Action	References
*Meterix casta (*Chemnitz	i) Free radical scavenging ii) Lipid peroxidation inhibition	Nazeer, Prabha, Kumar, & Ganesh, 2013
Giant squid (*Dosidicus gigas)*	i) Free radical scavenging	Rajapakse, Mendis, Byun, & Kim, 2005
Fermented marine blue mussel	i) Superoxide radical scavenging ii) Hydroxyl radical scavenging iii) Carbon-centered radical scavenging iv) Lipid peroxidation inhibition v) DPPH radical scavenging	Rajapakse, Mendis, Jung, Je, & Kim, 2005
Jumbo squid (*Dosidicus gigas*)	i) Hydroxyl radical scavenging ii) Carbon centered radical scavenging iii) Metal ion chelation	Mendis, Rajapakse, Byun, & Kim, 2005
Oyster (*Crassostrea gigas*)	ii) Hydroxyl radical scavenging iii) Superoxide radical scavenging	Qian, Jung, Byun, & Kim, 2008
Side-gill Sea slug (*Pleurobranchus forskalii*)		Wesson & Hamann, 1996
Jellyfish (*Rhopilema)*	i) Superoxide anion scavenging ii) Hydroxyl radical scavenging iii) Copper- chelating	Zhuang, Sun, Zhao, Wang, Hou, & Li, 2009

1.2 SURF CLAMS

Extensive research has shown the usefulness of the surf clam, both nutritionally and medically. Clams are important recreational and commercial resource in many countries (Ludien, Brey, & Arntz, 2003; McLachlan et al., 1996). The flesh of *Mactra veneriformis* is consumed in China as a delicious food and it is also used in traditional Chinese medicine (Luan, Wang, Wu, Jin, & Ji, 2011).

Beentjes and Baird (2004) identified surf clams as one of the most important aquatic organisms. Surf clams are also very important in the surf zone food webs (McLachlan et al., 1996; Menn, 2002) as they feed on phytoplankton and detritus, and are consumed by birds, fish and crabs (Laudien, Brey, & Arntz, 2003). As well as playing an important ecological role, surf clams can be exploited for bait and harvested for human consumption (Ludien, Brey, & Arntz, 2003). A large quantity of surf clam is traded internationally (Manalo & Campos, 2010; Agasen et al. 1998).

1.2.1 Biology of Surf Clam

Following Linnaeus' system of organism classification, Fay, Neves, & Pardue, (1983) classified surf clams as shown in Figure 1 below.

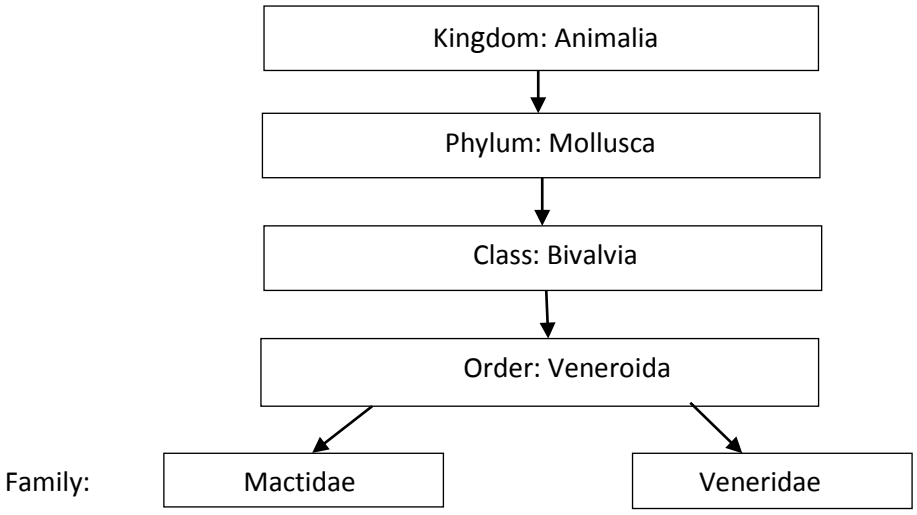


Figure 1: Classification of surf clam (Redrawn from Fay, Neves, & Pardue, 1983).

In 1980, Barnes reported the phylum *Mollusca* as one of the largest phyla in the animal kingdom with more than 100,000 extant species living in marine, fresh water and terrestrial habitats. Just two decades later, it was reported to have an estimated diversity of up to 200,000 species (Pechenik, 2000). *Mollusca* are subdivided into seven classes: *Gastropoda, Monoplacophora, Polyplacophora, Aplacophora, Bivalvia, Scaphopoda and Cephalopoda*. Of these, classes *Gastropoda* (over 75,000 extant species) and *Bivalvia* (over 20,000 living species) constitute 95% of the representatives of molluscs (Kantha, 1989; Chapman, 2009). The three prominent classes of molluscs are gastropods, bivalves and cephalopods (Kantha, 1989). They are prominent majorly because they are the commonly seen of the seven known classes (Bunje, 2003). Other animals in this phylum are snails, squids, oysters, mussels, scallops etc. Surf clams fall into the class Bivalvia because of their paired shells ("Massachusetts Marine Fisheries Shellfish Purification Plant", 2013).

A number of environmental variables such as water temperature (Ansell, 1968; Mann, 1979; Robert & Debra, 1992; Orban et al., 2004; Karakoltsidis, Zotos, & Constantinides, 1995), water depth (Jones, 1980; Ambrose et al., 1980) and population density (Cargnelli et al., 1999) can influence the growth of surf clams (Douglas et al., 1983). It is reported that sea water temperature variations also influence the immune functions in molluscs such as *Mactra veneriformis* (Yu et al., 2009; Renault et al., 2006). In *Chamelea gallina,* for example*,* the phagocytic activity of haemocytes was significantly decreased at 30°C (Marin et al., 2007). The lysosomal and cell membranes of haemocytes from the mussel, *Mytilus edulis*, appeared destabilised at 0 °C with respect to those of haemocytes from mussels acclimated at 10 °C. Significant reductions in lysosomal stability were also recorded in haemocytes from the oyster, *Ostrea edulis*, maintained at 15 °C (Camus, Grøsvikb, Børseth, Jones, & Depledge, 2000; Hauton, Hawkins, & Hutchinson, 2001).

Other factors that may also influence the growth of surf clams are geographical location, food availability (Robert & Debra, 1992; Karakoltsidis, Zotos, & Constantinides, 1995; Beninger & Lucas, 1984), salinity (Orban et al., 2002;Gardner and Thompson, 2001; Islay, 2004; Orban et al., 2006), turbidity or quantity of sediments present in the water (Bricelj,

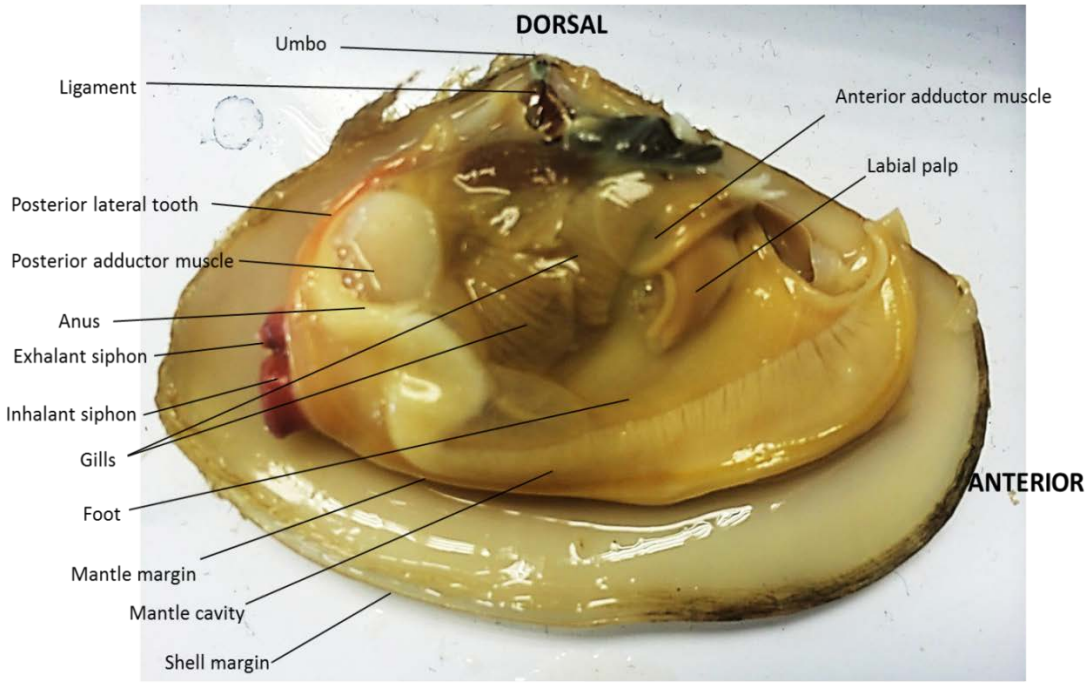
Malouf, & Quillfeldt, 1984; Aldridge, Payne, & Miller, 1987) and dissolved oxygen in ocean bottom waters (Fay, Neves, & Pardue, 1983).

Sometimes, man's activities such as dredging and navigational operations in water bodies, where clams can be found, also cause some of these deleterious impacts on aquatic organisms (Aldridge et al., 1987). Surf clams can live for up to 35 years. On average, those living in open water live longer than those living inshore (Fishwatch U.S. Seafood Facts, 2014).

Surf clams have two shells which are mirror images of each other, joined at one end by a flexible hinge (Klappenbach, 2014) and can close tightly together for protection with their whole body inside (University of Michigan, 2002; Klappenbach, 2014). It has been reported by the National Oceanic and Atmospheric Administration (2013) that Bivalves make their own shells whereby the mantle secretes calcium carbonate so that as the inner invertebrate grows, the outer shell provides a roomier home. The shells do not close fully and gape slightly (Fishwatch U.S. Seafood Facts, 2014).

As explained by Gosling (2003), clams occupy the broadest range of habitats among the four bivalve groups, and are found from open coasts to sheltered, saline and estuarine locations. Like fish, bivalve molluscs breathe through their gills. As filter feeders, bivalves gather food also through their gills (NOAA, 2013). Surf clams have a funnel-like siphon into which water and food can flow, and use this siphon in reproduction and locomotion (Cargnelli, Griesbach, Packer, & Weissberger, 1999)**.**

All marine bivalves, except scallops, possess gonads that produce the gametes necessary for their reproduction (Uddin et al., 2012). As male and female surf clams are identical externally, the sexes are differentiated through the histological examination of gonads (Gaspar, Ferreira, & Monteiro, 1999; Joaquim et al., 2008; Aljadani, 2013). Surf clams spawn from late spring through early fall, shedding their eggs and sperm directly into the water column. Larvae spend about three weeks in the water column as plankton before settling to the bottom to live. Sometimes reproduction in surf clams is possible by the age of one but most of them spawn by the end of their second year (Fishwatch U.S. Seafood Facts, 2014).



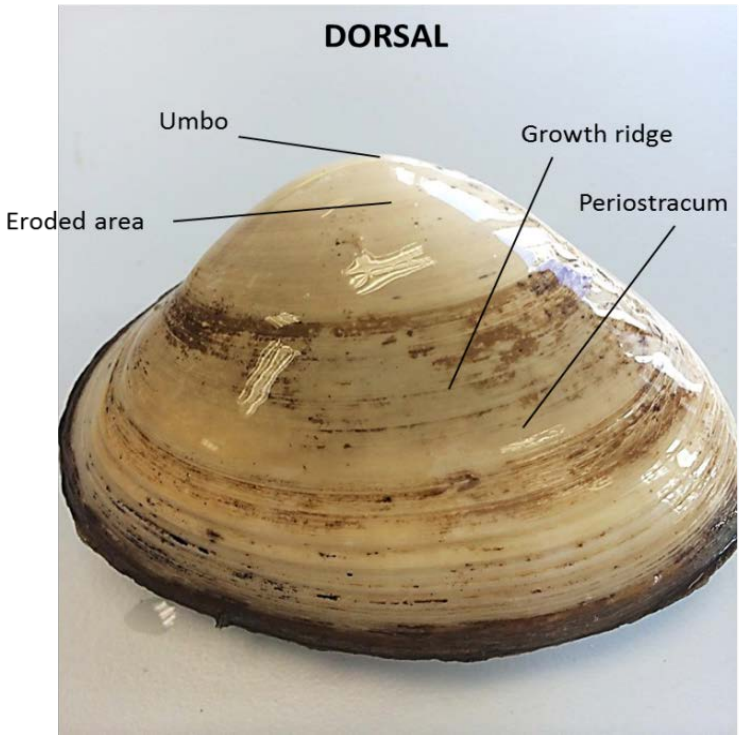


Figure 2: Surf Clam structure

1.2.2 Surf Clams in New Zealand

Surf clams are found in the surf zone of exposed sandy beaches throughout New Zealand (Cranfield, & Michael, 2001). According to the Ministry for Primary Industries (2012), three families of sub-tidal surf clams occur in New Zealand: Veneridae, Mactridae, and Mesodesmatidae. There are seven main species of surf clams in New Zealand; *Paphies donacina* (PDO), *Spisula aequilatera* (SAE), *Mactra discors* (MDI), *Mactra murchisoni* (MMI), *Dosinia anus* (DAN), *Dosinia subrosea* (DSU) and *Bassina yatei* (BYA), four out of which are given more attention; PDO, SAE, MMI and DAN (Ministry for Primary Industries, 2012). The reason for this could probably be as a result of their different depth ranges. The optimal depth range for PDO, SAE, MMI and DAN in the North Island is 2-8m, while other species like DSU and BYA range from 6-10m (Aljadani, 2013). Thus, the total catch across PDO, SAE, MMI and DAN will probably be higher than the catch in other species.

Cloudy Bay Clams (2014) has described these four species; *Paphies donacina* (PDO), *Spisula aequilatera* (SAE), *Mactra discors* (MDI), *Mactra murchisoni* (MMI), and *Dosinia anus* (DAN). Tua Tua (*Paphies donacina*) which is harvested from the low tide zone (2 to 4 metres) has a smooth clean shell and a full meat at nearly 33% meat to shell. The smooth low profile shell is cream to light moss in colour, weighing approximately 12-30 pieces per kg. Moon Shell (*Dosinia anus*) is found in the deeper parts of the surf zone (at 5-10 metres) with its beautiful mustard coloured circular shell. It has a depth of flavour and firm texture like no other clam and has a 20-25% meat to shell ratio, approximately 12-25 pieces per kg. Diamond Shell (*Spisula aequilatera*) is harvested from the 3 to 5 metre surf zone, naturally full of plump meat, and yielding 28- 35% meat to shell. The Diamond Shell clam has a beige coloured shell with a rich, deep coloured meat and white tongue, approximately 12-50 pieces per kg. The Storm shell clam (*Mactra murchisoni*) is harvested in the deeper regions of the surf zone (4 to 8 metres) and has a distinctive angular shell which is white with pale straw coloured bands. The smoother deep cupped shell holds a clam which is almost two separate parts- a long pearl white tongue and a deep ochre coloured body. With a total meat to shell ratio of more than 30%, the Storm Clam is unique in the world of shellfish, approximately 5-15 pieces per kg.

New Zealand surf clams grow rapidly in their first 3 years but considerably slower thereafter. Individuals grow quickly in summer but hardly at all in winter. South Island clams grow faster and to a larger size as compared to the same species from the North Island. It is also reported that at a growth size of 7cm, the deeper water species, such as the moon shell (*Dosinia anus*), and the diamond shell (*Spisula aequilatera*), a species most abundant in shallower waters, had a life expectancy of up to 25 years and about 5 years respectively (NIWA, 2008).

1.3 OXIDATIVE STRESS

Individuals are exposed to oxidants, both endogenous and exogenous, since the moment of conception (Fraga et al., 1991). Reactive oxygen and nitrogen species are generated *in vivo* and cause damage to DNA, lipids, proteins and other bio-molecules (Halliwell, 1996). DNA damage can occur, for example, when hydroxyl radical (a highly ROS) reacts with DNA bases by adding to double bonds of DNA bases and by abstracting an H atom from the methyl group of thymine and each of the C-H bonds of 2´ -deoxyribose (Cooke, Evans, Dizdaroglu, & Lunec, 2003). Antioxidants are, therefore, needed to prevent the formation and to oppose the actions of oxidants.

Antioxidants can be consumed in the diet and are synthesised *in vivo* in humans. Synthetic antioxidants have been invented, tested for acute toxicity and proposed as an addition to naturally occurring antioxidants. They are chemically pure and produce consistent antioxidant activity and are commercially available (Pokorný, 2007). Their advantages include, in some cases, lower production cost and higher antioxidant capabilities than naturally produced antioxidants. Synthetic antioxidants have also found to be less polar than their natural equivalents and are therefore more soluble in lipids (Kulawik, Özogul, Glew, & Özogul, 2013). Unfortunately, recent reports have revealed that these synthetic antioxidants may be associated with toxic and carcinogenic effects (Zhang et al., 2010).

As a part of society's demand for a better lifestyle and increased longevity, consumers have developed an increasing interest towards consuming 'nutraceuticals' and functional foods rich in natural bioactive compounds (Fung, 2012). In an attempt to address this interest, there has been a dramatic increase in the number of investigations aimed at identifying dietary compounds from natural sources which may be effective in preventing diseases caused by oxidative damage (Tierney, Croft, & Hayes, 2010; Yangthong, Hutadilok-Towatana, & Phromkunthong, 2009; Dudonné, Vitrac, Coutiére, Woillez, & Mérillon, 2009).

Antioxidants sourced from our diets generally contain radical chain reaction inhibitors, metal chelators, oxidative enzyme inhibitors and antioxidant enzyme cofactors that can prevent damage caused by oxidative stress (Huang, Ou, & Prior, 2005). However, it is important to be able to measure the antioxidant potency of food material containing antioxidants in order to assess the potential of the food to produce antioxidant effects and to assist in the diagnosis and treatment of diseases (Özyürek, Güçlü, & Apak, 2011).

Marine organisms are known to be a rich source of many different kinds of bioactive substances. The richness is believed to be due to the fact that they are living in a very exigent, competitive and aggressive surroundings, very different in many aspects from the terrestrial environment, a situation that demands the production of quite specific and potent active molecules (Kim & Wijesekara, 2010). One such marine organism of great importance to man as a dietary component and which also has antioxidant potential is the surf clam.

1.4 OXIDATION OF BIOLOGICAL MOLECULES

Oxygen is the most prevalent element in the earth's crust (Halliwell & Gutteridge, 1999) and is vital for most organisms for respiration. As well as being essential for most life, paradoxically, oxidative respiration also leads to the generation of molecules that cause sometimes life threatening damage to key biological sites (Serafini, 2006). The basis of energy production in animals, oxidation, is at the same time a major cause of irreversible deterioration and ultimate death (Scott, 1993).

Except for a small number of anaerobic bacteria, all living organisms use oxygen for energy production through the coupling of oxidation to energy transfer via the phosphorylation of Adenosine diphosphate (ADP) (Winston & Di Giulio, 1991) and it is thus essential for life as we know it (Magder, 2006). In the absence of oxygen, the electron transport chain is inhibited and glucose metabolism is shunted down the glycolytic pathways. The resultant depression of cellular metabolism is incompatible with life in higher organisms (Maltepe & Saugstad, 2009).

The reduction of molecular oxygen to H_2O via mitochondrial respiration complexes provides Adenosine triphosphate (ATP) (Bhattacharyya, Chattopadhyay, Mitra, & Crowe, 2014).
A consequence of this reaction is the formation of toxic ROS that can damage various classes of biological molecules and contribute to cell death (Bhattacharyya, Chattopadhyay, Mitra, & Crowe, 2014).

Oxygen is a diradical as it contains two electrons that are not spin-paired, with each electron residing in an orbital of its own. This unconventional distribution of electrons in the oxygen molecule makes it impossible for oxygen to accept a spin-matched pair of electrons until one of its unpaired electrons undergoes a spontaneous spin reversal to make pairing possible. Occasionally, oxygen does manage to steal away electrons from other molecules by non-enzymatic auto-oxidations. Since it cannot accommodate a spin-matched pair, it must settle for stealing electrons one at a time. This breaking-up of electron pairs results in the formation of free radicals (McCord, 2000). The univalent reduction of oxygen is superoxide radical, the divalent and trivalent reductions are hydrogen peroxide and hydroxyl radical (Cadenas & Davies, 2000) respectively as shown in Figure 3. One can agree with Aruoma (1994) and Halliwell (2007) that oxygen is a toxic gas.

Oxygen is the ultimate electron acceptor in the electron flow system that produces energy in the form of ATP (Pérez & Aguilar, 2013). Oxidation, the transfer of electrons from one atom to another, occurs when oxygen comes in contact with other substances, thereby causing them to lose one or more electrons (Stevens, 2011). Oxidation occurs in over one-quarter of

the known chemical reactions catalysed by enzymes in living cells (Khan, Tania, Zhang, & Chen, 2010).

Oxidants occur naturally as part of the normal body process; however harmful oxidants or free radicals, which are forms of oxygen, can cause damage to body cells. Biological systems are continuously exposed to oxidants, either generated endogenously by metabolic reactions (e.g. from mitochondrial electron transport during respiration or during activation of phagocytes) or exogenously like the air pollutants or cigarette smoke (Rahman & Adcock, 2006).

1.4.1 Free Radicals

Atoms contain a nucleus and electrons move around the nucleus, usually in pairs (Halliwell, 1994). A free radical is any atom or molecule that has a single unpaired electron in an outer shell (Erbas & Sekerci, 2011). The unpaired electrons alter the chemical reactivity of an atom or molecule, usually making it more reactive than the corresponding non-radical (Halliwell, 1994). Free radicals could be positively charged, negatively charged or electrically neutral (Battino, Bullon, Wilson, & Newman, 1999), and are generated during normal cellular function and are part of the natural physiological processes of all living beings (Dasgupta & Klein, 2014). Free radicals can be formed *in situ* in our tissues and cells by;

(i) Impact of radiation, which may be ionizing, ultra-violet, visible or thermal.

(ii) Redox reactions catalysed by transition metals such as iron or copper which can undergo unit changes in valence state (e.g. $Fe^{3+} + e^{-} \longrightarrow Fe^{2+}$).

(iii) enzymic-catalysis that often involves flavoproteins or hemoproteins (Slater, 1988; Freeman & Crapo, 1982).

During normal conditions, free radicals are generated at a low rate and subsequently taken care of by the well-developed scavenger and antioxidant system. However, a greatly increased rate of free radicals may exceed the capacity of the cellular defence system (Sjödin, Westing, & Apple, 1990). Free radicals, if not neutralised, can work against the immune system and cause the development of degenerative diseases (Veg-it.com, 2001) such as cancer, cardiovascular disease (CVD) and other disorders (Gutteridge, 1995) such

as inflammatory diseases (Butterfield et al., 2002) and even affect the aging process (Harmon, 1956). Excess free radicals can also cause destructive effects on foods (Wang, Zhao, Zhao, & Jiang, 2007).

1.4.2 Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) are various forms of activated oxygen (Kumar et al., 2005) which are unstable molecules with unpaired electrons. They are capable of initiating the process of oxidation (Kirkham & Rahman, 2006). ROS include free radicals (such as hydroxyl ($OH^{\bullet}$), superoxide ($O_2^{\bullet -}$), nitric oxide ($NO^{\bullet}$) and peroxyl ($ROO^{\bullet}$) (Aruoma, 1994), hydroperoxyl ($HO_2^{\bullet}$), alkoxyl ($RO^{\bullet}$), carbon dioxide radical ($CO_2^{\bullet -}$), (Halliwell, 2006) and non-free-radical species (such as hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen ($^1O_2$) and hypochlorous acid (HOCl) (Aruoma, 1994)).

Different ROS can be generated in different ways. Some sources of ROS are produced in the human body (endogenous) while others are not (exogenous) (Serafini, 2006). As explained by Temple and Machner in 2001, ROS can be generated in the body through leakage of electrons from the biological membranes, reactions with polyunsaturated fat, reduction of tissue oxygen by transitional metals such as copper and iron and from the activated phagocytes such as neutrophils, macrophages and eosinophils.

When phagocytes are exposed to bacteria or other appropriate stimuli, they undergo a radical metabolic change in which their oxygen uptake increases abruptly and they begin to produce large quantities of superoxide ($O_2^{\bullet -}$) (Babior & Curnutte, 1987) as part of their killing mechanism (Halliwell, 1996). This oxidative burst is mediated by the Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase system and this results in a marked increase in oxygen consumption and the production of superoxide ($O_2^{\bullet -}$). NADPH is composed of several subunits that assemble at the plasma membrane and fuse with intracellular phagocytic vesicles or the outer membrane. This allows the concentrated release of oxidants formed subsequently.

NADPH oxidase: $2O_2 + \mathrm{NADPH} \longrightarrow \underset{\text{Superoxide}}{2O_2^{\bullet -}} + \mathrm{NADPH}^+ + \mathrm{H}^+$

Superoxide is converted to hydrogen peroxide (H_2O_2) either spontaneously or more rapidly when catalyzed by superoxide dismutase, an enzyme that occurs in two isoforms, one of which is inducible by inflammatory cytokines such as tumour necrosis factor-α (TNF-α) (Hitchon & El-Gabalawy, 2004).

Spontaneous conversion: $2O_2^{\bullet -} + 2H^+ \longrightarrow [2HO_2^{\bullet}] \longrightarrow O_2 + H_2O_2$

Hydroperoxyl Radical

Superoxide dismutase: $2O_2^{\bullet -} + 2H^+ \longrightarrow O_2 + H_2O_2$

ROS is also known as Reactive Oxygen Intermediates (ROI) (Bhattacharyya, Chattopadhyay, Mitra, & Crowe, 2014), and they are formed as a natural by-product of the normal metabolism (Valko et al., 2007) of oxygen (Temple, 2000; Frei, 1994; Matés, 2000). In other words, the incomplete reduction of molecular oxygen produces ROS (Baud & Ardaillou, 1986). Thus, energy transfer or sequential univalent reduction of ground state triplet oxygen leads to the generation of ROS (Powers & Hamilton, 1999), as shown below.

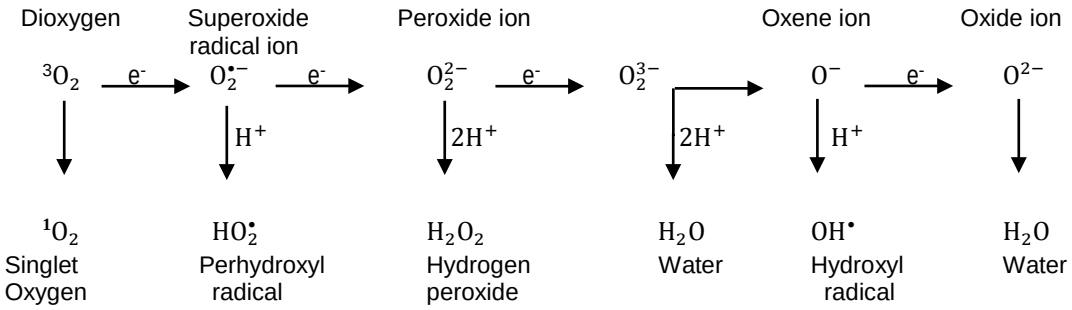


Figure 3: The electron reduction products of oxygen

Although molecular O_2 contains an even number of electrons, it has two unpaired electrons in its molecular orbitals, and is said to be in a triplet ground state (Cadenas, 1989). Ground state triplet molecular oxygen is a bio-radical with its two outermost valence electrons occupying separate orbitals with parallel spins. To oxidize a nonradical atom or molecule, triplet oxygen would need to react with a partner that provides a pair of electrons with parallel spins that fit into its free electron orbitals. However, pairs of electrons typically have opposite

spins and thus fortunately impose a restriction on the reaction of triplet molecular oxygen with most organic molecules (Powers & Hamilton, 1999).

Hydrogen peroxide, H_2O_2, for example, can be produced in this way by a 2-electron reduction of molecular oxygen as shown in (i) below.

$$O_2 + 2e^- + 2H^+ \longrightarrow H_2O_2 \qquad \text{(i)}$$

It is also produced by dismutation of the superoxide anion radical (non-enzymatic) (Özyürek, Bektaşoğlu, Güçlü, Güngör, & Apak, 2010) as shown in (ii) below;

$$O_2^{\bullet -} + e^- + 2H^+ \longrightarrow H_2O_2 \qquad \text{(ii)}$$

As mentioned earlier, tissue oxygen can be reduced by transition metals to produce ROS. Activation of oxygen to ROS is energy-dependent and requires an electron donation. In biological systems, transition metal ions (Fe^{2+}, Cu^{+}) can act as electron donors. $OH^{\bullet}$ radicals are generated from the less-damaging ROS, superoxide radical anion and hydrogen peroxide in a Fenton or Haber−Weiss reaction catalysed by ferrous or cuprous ions (Hevroni, Sayer, Blum, & Fischer, 2014) as shown in the reactions below:

$$Fe^{3+} + O_2^{\bullet -} \longrightarrow Fe^{2+} + O_2 \qquad \text{(iii)}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\bullet} \qquad \text{(iv)} \quad \text{(Kehrer, 2000)}$$

The ability of these metal ions to occupy multiple valence states and to undertake facile redox cycling and, thereby activating molecular oxygen has been utilised by a variety of enzymes. However, unregulated redox-active metals will inappropriately react with oxygen to generate ROS. Hence, the same properties that cells harness for beneficial means become destructive when the regulatory processes breakdown (Barnham, Masters, & Bush, 2004).

Additionally, the neutrophil-associated enzyme myeloperoxidase can oxidize halides such as chloride (Cl^-) [(v) below)] and also converts hydrogen peroxide (H_2O_2) into hypochlorous acid (HOCl), which then can interact with amino acids to form chloramines.

Myeloperoxidase: $Cl^- + H_2O_2 \longrightarrow OCl^- + H_2O \qquad \text{(v)}$

Similar reactions can occur with other halides such as bromide and iodide. Further reaction of hydrogen peroxide with hypochlorous acid produces singlet oxygen, another highly reactive and damaging radical. Reactions of hypochlorous acid with amino acids lead to aldehyde production (shown in (vi) below). Superoxide can also react with nitric oxide (NO) {synthesized from the deimination of L-arginine by nitric oxide synthase (NOS)} and produce the highly reactive peroxynitrite radical ($ONOO^-$) (equation (vii) (Hitchon & El-Gabalawy, 2004).

$$\underset{\text{Amino acids}}{R-CHNH_2-COOH} + HOCl \longrightarrow \underset{\text{Chloroamines}}{R-CHNHCl-COOH} + H_2O \longrightarrow \underset{\text{Aldehydes}}{R-CHO} + CO_2 + NH_4^+ + Cl^- \quad \text{(vi)}$$

$$NO^\bullet + O_2^{\bullet -} \longrightarrow ONOO^- \quad \text{(vii)}$$

Other endogenous sources of ROS *in vivo* are oxidative bursts in enzyme systems and the metabolic pathways (Yan, 2014) such as xanthine oxidase (Serafini, 2006), the mitochondria, peroxisomes, lipoxygenases, (Manea, 2010), cytochrome P450 oxidase (Finkel & Holbrook, 2000), glucose oxidase, monoamine oxidase (Cadenas & Davies, 2000; Yan, 2014) and cyclo-oxygenase (Bhattacharyya, Chattopadhyay, Mitra, & Crowe, 2014).

Exposure to electromagnetic radiation (both natural and man-made) can lead to the production of ROS in the body. A typical example is hydroxyl radical ($OH^\bullet$). Hydroxyl radical can be generated when low wavelength electromagnetic radiation (e.g. gamma rays) splits water in the body (Halliwell, 1994). Ultraviolet light is insufficiently energetic to split H_2O but it can cleave the O-O covalent bond in H_2O_2 to give $2OH^\bullet$. The viciously reactive $OH^\bullet$, once generated, attacks whatever is next to it, that is, it reacts at its site of formation (Halliwell, 1996). UVA and UVB radiation can be absorbed by many cellular components and cause oxidative damage by photo-dynamically generating reactive oxygen intermediates such as hydroperoxyl radical $HO_2^\bullet$, singlet oxygen $^1O_2$, hydroxyl radical $HO^\bullet$, etc. (Dulap & Yamamoto, 1995). Ultrasound and microwave radiation can also generate ROS (Sies, 1993).

Physical exercise is yet another source of ROS. The production of ROS is an outcome of the obligatory increased oxidative metabolism associated with exercise (Ha & Zemel, 2003). Physical activity increases the generation of free radicals in several ways which can result in

damage to cells (Clarkson & Thompson, 2000). As oxidative phosphorylation increases in response to exercise, there will be a concomitant increase in free radicals. During exercise, two of the potentially harmful free radical generating sources are semi-quinone in the mitochondria, and xanthine oxidase in the capillary endothelial cells. During high intensity exercise, the flow of oxygen through the skeletal muscle cells is greatly increased at the same time as the rate of ATP utilisation exceeds the rate of ATP generation. The metabolic stress in the cells causes several biochemical changes to occur, resulting in a markedly enhanced rate of production of oxygen free radicals from semi-quinone and xanthine oxidase (Sjödin, Westing, & Apple, 1990). Besides this, catecholamines that are released during exercise can lead to free radical production (Urso & Clarkson, 2003).

Furthermore, pollutants, foods and nutrition, chemotherapy, drugs and xenobiotics can also lead to the generation of ROS. A number of drugs (such as quinone, and hydroquinone) and chemicals activate oxygen to oxygen radicals. Oxygen is enzymatically reduced by a one-electron reduction step. The intermediates formed then transfer the extra electron to the molecular oxygen (Kappus, 1987). A common mechanism with quinones and related species is redox cycling, in which the compound is reduced by a flavoenzyme such as cytochrome P450 reductase to a radical that then reacts with oxygen to generate superoxide. Examples are the herbicide paraquat (as in equation (viii) below), the anticancer drug doxorubicin and the diabetogenic compound, alloxan (Winterbourn, 2008).

$$\text{Paraquat} \xrightarrow{reductase} \text{paraquat radical} + O_2 \leftrightarrows \text{paraquat} + O_2^{\bullet -} \qquad \text{(viii)}$$

1.4.3 The Two Sides of ROS

Beneficial actions of ROS occur at low concentrations and they involve physiological roles in cellular responses against infectious agents (Alexieva, Markova, Nikolova, Aragane, & Higashino, 2010). ROS have important roles in cell signalling (Nemoto, Takeda, Yu, Ferrans, & Finkel, 2000) and homeostasis (Harrison, Griendling, Landmesser, Hornig, & Drexler, 2003; Stocker & Keaney, 2004; Cooper, Patel, Brookes, & Darley-Usmar, 2002; Finkel & Holbrock, 2000).

Hydrogen peroxide, for instance, is considered to be the most important signalling messenger considering the specificity of its production, reaction and removal (Forman, Maiorino, & Ursini, 2010). The generation of ROS by phagocytic cells also constitutes an essential host defence mechanism necessary to combat infection. Likewise, cytosolic ROS produced in response to stimulation by growth factors are involved in regulating the proliferative response (Finkel, 1998). Studies have also shown that ROS actively participate in a diverse array of biological processes including protein phosphorylation, transcription factor activation (Rajendran et al., 2014), normal cell growth, induction and maintenance of the transformed state, programmed cell death, immune function (Serafini, 2006), regulation of vascular tone, monitoring of oxygen tension in the control of ventilation and erythropoietin production (Alexieva et al., 2010), cellular senescence (Finkel, 2003), adaptation to stress and the regulation of lifespan (Van Raamsdonk & Hekimi, 2010; D'Autreaux & Toledano, 2007).

During times of environmental stress {e.g., exposure to UV, heat, cigarette smoke, air pollution (Temple, 2000)} and physical exercise (Serafini, 2006; Leeuwenburgh & Heinecke, 2001), however, the ROS levels can increase dramatically which is a situation known as 'hyperoxia' (Maltepe & Saugstad, 2009). This results in significant damage to cell structures (Devasagayam et al., 2004). This is known as oxidative stress which leads to oxidative damage (Sies, 1991; Joyner-Matos, Downs & Julian, 2006).

The production of ROS can also be increased due to certain conditions such as hypercholesterolemia, diabetes, hypertension (Landmesser et al., 2003; Kushiro et al., 2005), smoking and Chronic Obstructive Pulmonary Disease (COPD) (Chapple, 1997; Rytilä et al., 2006; Boots, Haenen, & Bast, 2003), aging, nitrate intolerance (Harrison, et al., 2003), Hepatitis C (Korenaga et al., 1995), hyperthermia, chemotherapeutic agents (Finkel & Holbrook, 2000) and obesity (Furukawa et al., 2004).

ROS can cause direct tissue injury (Kirkham & Rahman, 2006) and can exert deleterious effects by oxidizing biologically essential molecules such as lipids (Livingstone, 2001), nucleic acids (Parthasarathy, Steinberg, & Witztum, 1992), proteins (Stadtman, 1992), carbohydrates, and DNA (Niki, 2012; Devasagayam et al., 2004). Lipid peroxidation causes aging in organisms and cancer promotion as well as food deterioration (Sakata, 1997). All

these happen either as a result of overproduction of ROS or inadequate antioxidant defence/ decrease in antioxidant defence system against ROS. (Farber 1994; Akpinar, Toker, Ozdemir, Bostanci, & Aydin, 2013).

Low levels of certain free radicals and ROS can stimulate the growth of fibroblasts and epithelial cells in culture whereas higher levels may result in tissue injury (Battino, Bullon, Wilson, & Newman, 1999). Thus, ROS act as a double edged sword by exerting both harmful and beneficial effects (Niki, 2012).

Reports have stated that ROS, formed *in vivo* and imported from outside, induce oxidative damage of cellular membranes, tissues, and enzymes (Niki, 2012). This may eventually lead to disorders and diseases such as atherosclerosis, neurological diseases (Temple, 2000), cancer (Matanjun, Mohamed, Mustapha, Muhammad, & Ming, 2008) and even Alzheimer's disease (Chauhan and Chauhan, 2006). Retinal damage (Nath, Gupta, Prasad, Pandav, & Thakur, 1999), schizophrenia (Reddy & Yao, 1999), skin-aging, nephritis, reperfusion injury, asthma, diabetes mellitus (Harman, 1998; Nazeer, Saranya & Naqash, 2012) are all also associated with ROS. It is reported that ROS are involved in apoptotic processes by activating/deactivating many enzymes (Calió et al., 2014). In addition to established concerns regarding ROS and chronic disease risk, some investigators believe that ROS accumulation delays muscle recovery and may also impair performance (Ha, & Zemel, 2003).

Unfortunately, the higher the metabolic rate of an organism, the greater the production ROS and hence the shorter is the life span (Finkel & Holbrook, 2000).

This only proves one thing that the metabolic rate of a species ultimately determines its life expectancy (Sugamura & Keaney, 2011).

1.4.4 Oxidative Stress and Diseases in Man

The oxygen consumption inherent in cell growth leads to the generation of a series of free radicals of oxygen which are the most abundant and characteristic species in the phenomenon known as ''oxidative stress'' (Yen, Duh, & Tsai, 2002). Oxidative stress is a serious imbalance between the generation of reactive oxygen species and the *in vivo* antioxidant protection in favour of the former, causing excessive oxidative damage (Halliwell, 2010; Redón et al., 2003; MacNee, 2000; Palmer & Kitchin, 2010). The balance between

ROS production and antioxidant defences determines the degree of oxidative stress (Finkel & Holbrook, 2000).

Alternatively, oxidative stress can also occur when there is a decrease in the antioxidant capacity of a cell. Non-enzymatic antioxidant levels (vitamin E, vitamin C, glutathione, etc.) and enzymatic antioxidant levels (superoxide dismutase, glutathione peroxidase, and catalase) in the cell can be decreased through modification in gene expression, decrease in their uptake in the diet or the levels can be overloaded in ROS production which creates a net increase in the amount of oxygen free radicals present in the cell (Barber & Harris, 1994). The formation of oxidative stress may result in damage to critical cellular macromolecules including DNA, membrane lipids, and proteins which can cause cell death (Klaunig et al., 1998; Mahajan & Tandon, 2004).

Oxidative stress, which involves chronically elevated ROS levels (Paravicini & Touyz, 2006), plays critical roles in the pathogenesis of various diseases (Brownlee, 2001; Furukawa et al., 2004) such as cardiovascular disease (CVD) (Andallu, Shankaran, Ullagaddi, & Iyer, 2014), Huntington's (Chandra, Samali, & Orrenius, 2000), diabetes (Furukawa et al., 2004), septic shock (Salvemini & Cuzzocrea, 2002), rheumatoid arthritis (Baynes & Thorpe, 1999), AIDS and atherosclerosis (Ohara, Peterson, & Harrison, 1993). A case in which it fits and is particularly well understood is the role of oxidative stress in CVD. Here, the oxidation of low density lipoprotein (LDL) seems to trigger the process of ''atherogenesis'' which leads to atherosclerosis and then ultimately to CVD (Pérez & Aguilar, 2013).

In the diseases that have a high impact on the health sector, Diabetes Mellitus is one of the most known. Diabetes is a risk factor for atherosclerosis. Like atherosclerosis, diabetes is progressive and is associated with enhanced oxidative stress (Madamanchi, Vendrov, & Runge, 2005). In the past few decades, type 2 diabetes mellitus (T2DM) has rapidly increased in the world. It has been estimated that the number of diabetic patients will more than double within 15 years. Moreover, although T2DM was previously considered a slow-onset disease of middle-aged and older subjects, an emerging issue is the recent increase in diagnosis of T2DM and pre-diabetic conditions in children (Ceriello & Motz, 2004). The World Health Organization (WHO) estimates that there are just over 180 million diabetics worldwide and the likelihood to double this number in 2030 is quite high. Countries like China, India,

United States of America and Mexico are at the top of this pathology. In Mexico, this condition is a major cause of mortality and morbidity with approximately 10 million individuals suffering from diabetes, out of which 22.7% did not know they are sick while 55% do not have good control of their condition (Pérez & Aguilar, 2013).

Cancer is the second leading cause of death after myocardial infarction. Despite the great advances in modern medical science in the last century, most cancers are not yet curable. This is partly due to the complexity of the pathogenesis of cancer and the difficulties in developing efficient treatments. Among the numerous factors, oxidative stress plays an important role in cancer initiation, promotion and progression by inducing the DNA damage and by interfering with the intracellular signal transduction pathways. Since antioxidant enzymes play crucial roles in protecting cells from oxidative stress, dysregulation or defects in the activity of antioxidant enzymes [such as superoxide dismutase (SOD), catalase (CAT) and glutathione-peroxidase (GPX)] are associated with cancer (Khan, Tania, Zhang, & Chen, 2010).

The decreased activities of antioxidant enzymes or the down-regulation of their expression were found to be associated with several types of cancers, including prostate cancer, bladder cancer, breast cancer, hepatic cancer, multiple myeloma (Khan et al., 2013).

More than 50 million Americans experience systolic hypertension. Hypertension is a risk factor for many other vascular diseases including atherosclerosis and stroke (Madamanchi, Vendrov, & Runge, 2005). Oxidative stress is thought to play a critical role in the pathogenesis of hypertension. The precise mechanisms remain to be elucidated. However, there is general consensus that ROS play a role by mediating oxidative damage to target organs, decreasing nitric oxide bioavailability and giving rise to endothelial dysfunction (Ward et al., 2004). Observations have strongly suggested that oxidative stress is a modulator of hypertension and a risk factor for atherosclerosis (Madamanchi, Vendrov, & Runge, 2005).

The importance of oxidative stress in chronic heart failure can be gauged by the fact that antioxidants prevent the progression of several pathological processes, such as cardiac hypertrophy, cardiac myocyte apoptosis, ischemia-reperfusion and myocardial stunning-which lead to heart failure in animal models. Emerging evidence demonstrates that oxidative stress, in general, and NAD(P)H oxidase–derived ROS, in particular, are important in human

cardiac failure. In the failing myocardium of patients with ischemic or dilated cardiomyopathy, NAD(P)H oxidase–derived ROS were up regulated. Plasma TNF- levels and platelet-derived NAD(P)H oxidase activity were also elevated in patients with heart failure. In addition, NAD(P)H oxidase activation and increased translocation of regulatory p47phox from the cytosol to the sarcolemmal membrane were recently observed in failing human myocardium. These combined results suggest that oxidative stress has a role in the pathophysiologic cardiac dysfunction in heart failure (Madamanchi, Vendrov, & Runge, 2005).

Rheumatoid arthritis is a chronic systemic disease that affects the joints, connective tissues, muscle, tendons and fibrous tissue. It tends to strike during the most productive years of adulthood, between the ages of 20 and 40, and is a chronic disabling condition often causing pain and deformity (WHO, 2014). Rheumatoid arthritis has been ranked as the 42nd highest contributor to global disability, according to analysis of data from the Global Burden of Disease 2010 study and it was ranked just below malaria and one place above iodine deficiency (Arthritis Research UK, 2014). Based on a 2010 - 2012 data from the National Health Interview Survey (NHIS), an estimated 52.5 million (22.7%) of adults have self-reported and doctor-diagnosed arthritis and 22.7 million (9.8% of all adults) have arthritis and arthritis-attributable activity limitation. Unfortunately, based on another NHIS data (2003), a projected 67 million (25%) adults aged 18 years or older will have doctor-diagnosed arthritis by the year 2030 and an estimated 37% (25 million adults) of those with arthritis will report arthritis-attributable activity limitations by the year 2030 (Centers for Disease Control and Prevention (CDC), 2014). Arthritis is the single greatest cause of disability in New Zealand. More than half a million people will be affected by arthritis during their lifetime (Ministry of Health, 2014).

Chronic obstructive pulmonary disease (COPD) is a major worldwide health problem that has an increasing prevalence and mortality (Repine et al., 1997).

According to WHO (2014), 65 million people have moderate to severe COPD. In 2002, COPD was the fifth leading cause of death. Total deaths from COPD are projected to increase by more than 30% in the next 10 years unless urgent action is taken to reduce the underlying risk factors, especially the tobacco usage. Estimates show that COPD becomes, in 2030, the third leading cause of death worldwide.

COPD has a substantial impact on the health of New Zealanders. Although often undiagnosed, it affects an estimated 15 percent of the adult population over the age of 45 years (at least 200,000 New Zealanders). In New Zealand, COPD is the 4th leading cause of death after cancer, heart disease and stroke. It is ranked 2nd in men and 5th in women with regards to its health impact (Asthma Foundation, 2012).

Exacerbations of obstructive airway diseases (whether asthma or COPD) are among the most common emergency admissions to hospitals and they place a large burden on health resources. There has been considerable interest in the hypothesis that an oxidant/antioxidant imbalance may be important in the pathogenesis of COPD (Rahman, Morrison, Donaldson, & Macnee, 1996).

A current hypothesis in the pathogenesis of COPD is that the increased oxidant burden, both directly as a result of smoking and/or indirectly by the release of increased amounts of reactive oxygen species from airspace leucocytes may not be adequately counterbalanced by the lung antioxidant systems and thence resulting in oxidative stress. An excess of oxidants may then lead to enhanced pro-inflammatory gene expression and protein release, inactivation of anti-proteases and oxidative tissue injury leading to COPD (Drost et al., 1996).

Oxidative stress also accounts for the dysfunction or death of hepatocytes and other liver cells and subsequently contributes to the pathogenesis of acute and chronic liver diseases (Medina & Moreno-Otero, 2005). The deleterious effects of increased free radicals causing potential biological damage (oxidative stress) are termed as “oxidative damage” (Chapple, 1997; Valko et al., 2007). Whiteman and Halliwell (2004) defined oxidative damage as the bio-molecular damage caused by attack of reactive species upon the constituents of living organisms.

Ultimately, under the normal conditions, increased radical concentrations would lead to damage of most biomolecules and, among them, of the antioxidant enzymes. Increased rate of radical production frequently elicits as response, an increase in the levels of antioxidant enzymes. However, in the initial rates of radicals input and/or under high rates of radicals input, the enzyme inactivation prevails and the enzymatic activities are reduced leading to autocatalysis of the oxidative damage process (Escobar, Rubio, & Lissi, 1996).

The basic tenet of oxidative stress hypothesis is that senescence-related loss of function is due to the progressive and irreversible accrual of molecular oxidative damage (Sohal, & Weindruch, 1996).

1.5 ANTIOXIDANTS

An anti-oxidant is any substance that when present at low concentrations, compared to those of an oxidise-able substrate, significantly delays or prevents oxidation of that substrate (Halliwell, 1997; Battino, Bullon, Wilson, & Newman, 1999). It does so by inhibiting the initiation or propagation of oxidizing chain reactions (Velioglu, Mazza, Gao & Oomah, 1998). Antioxidants are man-made or natural substances that may prevent or delay some types of cell damage (MedLine Plus, 2014) caused by free radicals. In its broadest sense, an antioxidant denotes an agent which is capable of inhibiting various types of oxidation reactions (Kahl & Hildebrandt, 1986). Antioxidants interfere with the oxidation process by reacting with free radicals, chelating catalytic metals, acting as oxygen scavengers (Shahidi, Janitha & Wanasundara, 1992; Buyukokuroglu et al., 2001), breaking chain reactions and by repairing the molecules damaged by free radicals (Temple and Machner, 2001), thereby preventing oxidative damage.

1.5.1 Benefits of Antioxidants

A number of diseases are associated with oxidative stress, being the basis of an antioxidant therapy (Sies, 1991). Experimental evidence has shown that plasma total antioxidant capacity (TAC) of patients affected by different chronic diseases such as diabetes, AIDS, ulcerative colitis, Crohn's disease, meningitis, CVD, colorectal, lung and breast cancer is lower compared to healthy controls, suggesting the deep impairment of the antioxidant network during the development of these pathologies (Serafini, 2006). The body has several defence systems to synthesize and accumulate molecules that would avidly react with and annihilate active oxygen species before they could inflict oxidative damage to vital components (McCord, 2000). To counter oxidative stress, cells constitutively express

enzymes that detoxify the reactive oxygen species and repair the damage caused by them (Storz & Imlay, 1999).

Aerobic organisms survive the presence of oxygen only because they contain antioxidant defences (Halliwell, 2007). These comprise of the endogenous enzymes including catalase, glutathione reductase [for the elimination of hydrogen peroxide and organic peroxides] and superoxide dismutase [for the elimination of superoxide radical]. Endogenous factors (including glutathione, urate and coenzyme Q), nutritional factors (principally the antioxidant nutrients, especially β-carotene and other carotenoids), vitamin C [ascorbic acid], vitamin E [α-tocopherol], bioflavonoids and selenium (Temple, 2000; Tierney, Croft, & Hayes, 2010) and metabolic antioxidants like ceruloplasmin, albumin, bilirubin, ferritin, transferrin, uric acid and lactoferrin are also a part of these defences (Mahajan & Tandon, 2004).

Catalase is a porphyrin-containing enzyme which catalyses two electron-dismutation of hydrogen peroxide into oxygen and water (Chaudièare & Ferrari-Iliou, 1999). It is located in the cytoplasm of red blood cells but compartmentalised in the peroxisomes of other cells.

$$2H_2O_2 + O_2 \xrightarrow{\text{Catalase}} O_2 + 2H_2O$$

(Parker, 1995).

Glutathione peroxidase (GP_x), which contains active selenium, is involved not only in hydrogen peroxide removal but also in converting lipid hydroperoxides (LOOH) to their corresponding alcohols (LOH) and in oxidising GSH to glutathione disulphide (GSSG) (Serafini, 2006).

$$ROOH + GSH \xrightarrow{\text{Glutathione peroxidase}} GSSG + ROH$$

Reduced Thiol
Glutathione

(Parker, 1995).

Since iron and copper catalyse radical generation, they need to be sequestered in order to prevent toxicity. Transferrin and lactoferrin-bound iron, ceruloplasmin-bound copper and albumin can scavenge radicals and can bind copper ions (Serafini, 2006).

β -Carotene, a water-soluble provitamin A, is a free-radical scavenger that controls the propagation of reactive species and influences lipoxygenase activity. Vitamin C (ascorbic acid), one of the first lines of defence from oxidative stress, can prevent lipid peroxidation by

trapping water-soluble peroxyl radicals before their diffusion into lipid membranes. It reacts with superoxide, peroxy and hydroxyl radicals and is important in recycling other antioxidants such as vitamin E. Vitamin E has lipid-soluble properties that allow it to act as a chain-breaking reagent in lipid peroxidation (Hitchon & El-Gabalawy, 2004).

Reports have shown that cells are protected by secondary and tertiary layers of damage removal and repair systems (e.g. proteinases, lipases, DNA repair enzymes). These layers help to make life in an oxygen- rich environment possible (Forman, Davies, & Ursini, 2014).

The rate of an antioxidant-oxidant reaction, which would be a second-order reaction, is determined by the equation:

$$\textbf{Reaction rate} = \boldsymbol{k}[\mathbf{A}][\mathbf{B}]$$

where k is a second rate constant, $[\mathrm{A}]$ is the concentration of the antioxidant and $[\mathrm{B}]$ is the concentration of the reactive species.

Hydroxyl radical, for example, is produced by radiolysis of water or decomposition of hydrogen peroxide. This extremely reactive radical reacts with practically all molecules present in a cell with a rate constant approaching the rate of diffusion limitation.

$$\mathrm{HO^{\bullet} + AH \longrightarrow A^{\bullet} + H_2O} \quad > 10^9 \mathrm{M^{-1}s^{-1}}$$

The only efficient protection mechanism is to prevent its formation instead of trying to scavenge it after it is formed. Such prevention *in vivo* is by reduction of $\mathrm{H_2O_2}$ to water and this antioxidant reaction is enzyme catalysed. Catalase, an endogenous antioxidant enzyme, can dismutate $\mathrm{H_2O_2}$ to $\mathrm{H_2O}$ and $\mathrm{O_2}$ (Forman, Davies, & Ursini, 2014).

To sum it up, the major classes of endogenous protective mechanisms (i.e., enzymatic and non-enzymatic antioxidants) work as a unit to reduce the harmful effects of ROS in cells (Powers, & Hamilton, 1999).

These defences are inadequate, however, in cases where the rates of intracellular $\mathrm{O_2^{\bullet -}}$ and$\mathrm{H_2O_2}$ formation are accelerated (Storz & Imlay, 1999) or if the balance between ROS and

antioxidants is disturbed (Chapple, 1997) or if the balance between the generation and elimination of ROS is broken (Qian, Jung, & Kim, 2008).

Abiotic stresses such as dehydration, low and high temperatures and excess irradiation can disturb this balance such that increased ROS initiates some signalling responses that include enzyme activation, gene expression, programmed cell death (PCD) and cellular damage (Neill, Desikan, & Hancock, 2002). It is also worth knowing that these enzymatic antioxidants are not 100% effective in eliminating the formation of all free radicals. The very reactive hydroxyl free radical, $HO^{\bullet}$, for example, is not eliminated by these mechanisms (Rose & Bode, 1993). Therefore, identification and development of safer, cheaper and effective antioxidant from natural sources is essential (Rehman, 2003).

Antioxidants have, therefore, become a topic of increasing interest recently. A literature search revealed that the number of publications on antioxidants and oxidative stress has nearly quadrupled in the past decade (Huang, Ou, & Prior, 2005).

Antioxidants are micronutrients that have gained importance in recent years due to their ability to neutralise free radicals (Shukla, Mehta, Mehta, & Bajpai, 2012). Antioxidants have become very important in almost all industries (Huang, Ou, & Prior, 2005; Shen, Ji, & Zhang, 2007) such as the pharmaceutical industry, in the production of cosmeceuticals (Kim, Ravichandran, Khan, & Kim, 2008), nutraceuticals (Ye, Jia, Tang, &Chen, 2014) and cosmetics and in the food and nutrition industry (McCarthy, Kerry, Kerry, Lynch, & Buckley, 2001).

1.5.2 Synthetic and Natural Antioxidants

There are two basic categories of antioxidants, namely, synthetic and natural (Velioglu, Mazza, Gao, & Oomah, 1998). In general, synthetic antioxidants are compounds with phenolic structures of various degrees of alkyl substitution, whereas, natural antioxidants can be phenolic compounds (tocopherols, flavonoids, and phenolic acids), nitrogen compounds (alkaloids, chlorophyll derivatives, amino acids, and amines), carotenoids as well as ascorbic acid (Hall and Cuppett, 1997).

The interest in natural antioxidants has increased considerably (Löliger, 1991), this might be due to their reduced or non-cytotoxicity or their low side effects compared with chemically synthesised drugs (Fung, 2012). Reports have shown that there has been a growing interest in replacing commercial antioxidants with natural ingredients due to the possible adverse effects of synthetic antioxidants such as butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT) which now have restricted uses in foods as they are suspected to be carcinogenic (Madhavi et al., 1995).

As a result of their safety and toxicity problems, synthetic antioxidants such as BHA, BHT, propyl gallate (PG), and *tert*-butylhydroquinone (TBHQ) have been reported to cause the following symptoms; enlarged liver, increased liver microsomal enzyme activity and conversion of some ingested materials into toxic and carcinogenic substances, especially if they are present in excessive amounts (Amarowicz, Naczk, & Shahidi, 2000; Jayathilakan, Sharma, Radhakrishna, & Bawa, 2007; Ito et al., 1986). BHA has been implicated in stomach cancer and urinary bladder cancer. BHT has been linked to urinary bladder cancer and thyroid cancer. In a study conducted in rats, moreover, BHT was shown to cause haemorrhage and death (Kulawik, Özogul, Glew, & Özogul, 2013). In fact, most chemically synthesised drugs are highly cytotoxic, not only to tumour cells but also to normal cells. Thus these agents cannot be used for cancer prevention (Ravindran, Prasad, & Aggarwal, 2009).

Also, the lack of biocompatibility of many of the synthetic metal chelators triggered the search for naturally occurring metal chelators (Hevroni, Sayer, Blum, & Fischer, 2014). Some Cu/Fe-chelators, for example phytic acid, proved to be promising antioxidants. However, most others which include tea catechins, hydroxytyrosol, and chlorophyll derivatives are found showing low antioxidant activity. Some of them even exhibited pro-oxidant properties (Yoshioka, Semba, Saito, & Hayakawa, 2001; Hevroni, Sayer, Blum, & Fischer, 2014).

Such problems are not seen when natural antioxidants are used (Aparadh, Naik, & Karadge, 2012). Naturally-occurring antioxidants can be formulated to give nutraceuticals that can help to prevent oxidative damage from occurring in the body (Wong, Leong, & Koh, 2006). Because of this, there is a motivation to search for safe and natural antioxidants from various bio-resources (Byun,Lee, Park, Jeon, & Kim, 2009) which are considered to be safe, environment-friendly, and play a very important role in the physician's therapeutic armamentarium (Andallu, Shankaran, Ullagaddi, & Iyer, 2014).

When ROS are generated in a living system, a wide variety of antioxidants comes into play. The relative importance of these as protective agents depends on which ROS is generated, how it is generated, where it is generated, and what target of damage is measured (Halliwell & Gutteridge, 1995). The enormous benefits of antioxidants to man cannot be over-emphasized. One of these is its ability to shield the lungs from free proteolytic and oxidative damage (Rahman & MacNee, 2000; Hector, Griese, & Hart, 2014).

1.6 POTENTIAL THERAPEUTIC EFFECTS OF SURF CLAM EXTRACTS

It has been reported that the extracts of certain molluscs like abalone, oyster and clams have shown both antibacterial and antiviral activities (Li et al., 1972; Lin-rui, 2012). Research on surf clams have been carried out in different parts of the world, as it is known to exhibit a variety of biological activities. It is common practice in China that the molluscs of *Mactra veneriformis* are not only utilised as a delicious food but also as a traditional Chinese medicine with the functions as antihyperglycemic, anticancer, anticoagulant, protecting vascular system and so on (Liu et al., 2012; Ji et al., 2013). Certain substances present in the common clam *(Mercenaria mercenaria)* possess significant antibacterial and antiviral activity *in vitro* and *in vivo* (Li et al., 1965). An extract from the edible clam *Mercenaria mercenaria* prevented the development of transplanted sarcoma 180 and Krebs-2-ascites tumors in Swiss mice (Schmeer & Huala, 1965). It has also been reported to show detoxification, cyst elimination, protecting the body cells from mutation and decrepitude (Leng, Liu, & Chen, 2005), as well as decreasing blood sugar and blood fat levels (Levy, 1985).

Clam antioxidant properties have been supported by several studies in different species including *Cyclina sinensis* (Jiang, Wang, Liu, Gan, & Zeng, 2011), *Venerupis (Ruditapes) philippinarum* (Lassudrie et al., 2014; Zhang, Pan, & Tao, 2014), *Corbicula fluminea* (Ren, Luo, Ma, Wang, & Ma, 2013; dos Santos & Martinez, 2014), *Mactra veneriformis* (Wang, Wu, Chang, & Zhang, 2011), *Ruditapes decussatus* (Geret, Serafim, & Bebianno, 2003; Sellami

et al., 2014), *Meretrix casts (Chemnitz)* (Nazeer, Prabha, Sampath, Kumar & Ganesh, 2013) and *Merecenaria merecenaria* (Rubin, Tanguy, Perrigault, Espinosa, & Allam, 2014).

Some health benefits of clam extracts are briefly described below.

1.6.1 Anticancer Activity

Clinical interest in the pharmacology of medicinals from marine organisms has heightened due to anticancer effects indicated for *Mercenaria* marine clam components (Schmeer, 1979). Evaluations into the apoptotic effects of the hard clam extract have shown it to possess inhibitory effects on human cancer cell proliferation (Pan, Huang, Chan, Ho, & Pan, 2007). The liver extract of this clam is a general inhibitor of cancer growth, as it has been reported to have a therapeutic effect upon Leukaemia 1210 in mice and a prophylactic effect against adenovirus 12-induced-tumor formation in hamsters (Ruggieri, 1975). The hot water extract of *Corbicula fluminea* and the aqueous, ethanol, and ethyl acetate extracts of *Meretrix lusoria* have been reported to have significant antitumor activities and significant inhibitory effects on growth of human gastric cancer cells (SGC7901) and human ovarian carcinoma cells (SKOV3 and A2780) respectively (Liao, Chen, & Xingqian, 2013; Kong, Chiang, Fang, Shinohara, & Pan, 1997; Pan, Huang, Chang, Ho, & Pan, 2006; Pan, Huang, Chan, Ho, & Pan, 2008). Furthermore, extracts derived from *Cyclina sinensis* have been found to induce apoptosis in human gastric cancer cells (Jiang, Wang, Liu, Gan, & Zeng, 2011).

1.6.2 Anticoagulant Activity

A substance with potent blood anticoagulant properties was first isolated by McLean in 1916 from bovine liver and heart; it was called heparin (McLean, 1959).

Subsequently, similar substances have been isolated from a variety of mammalian tissues including lung and liver of whale and tumours of animal and human. Potent anti-coagulants with comparable structure have also been isolated from such disparate sources as fish scales and other organs of fish and from the common surf and ocean clam (Lasker, 1984).

Heparin-like polysaccharides with high anticoagulant activity have been isolated from 2 species of clams, *Mactra spissula* and *Artica islandica.* These purified polysaccharides, termed as mactin-A and mactin-B, are related to but not identical with mammalian heparin. They possess a greater *in vivo* activity and a favourable therapeutic index when compared with heparin preparations of equal in *vitro* activity (Frommhagen, Fahrenbach, Brockman Jr., & Stokstad, 1953). They also possess structural features and anticoagulant activity identical to the mammalian polysaccharides (Hovingh & Linker, 1993). The presence of this heparin-like substance in the common clam, *Spissula solidissima* was demonstrated by Thomas in 1951 (De Meio, Lin, & Narasimhulu, 1967). According to Thomas, the 1954 discovery of the *Spissula* anticoagulant stemmed from previous studies by Heilbrunn and his students which were concerning the biological significance of heparin and related substances (in 1949). There is an increasing amount of evidence that such substances may be of rather general importance (Thomas, 1954). After this, further research went into the anticoagulant properties of clams. One of such was by Burson et al., (1956) and they showed that the alkali and ammonium sulphate extract of *Cyprina (Arctica) islandica* had anti-coagulating effects.

1.6.3 Antiviral Activity

Studies have been carried out on the effects of a number of substances from marine animals as potential antiviral agents. The results have indicated that certain substances present in the abalone *(Holiotis rufescens),* oyster *(Crassosfrea virginica)*, common clam *(Mercenaria rnercenaria)*, queen conch *(Strombus gigas),* squid *(Loligo pealii)*, sea snail (*Tegulu gallina)*, and *Mya arenaria* (Dang, Benkendorff, & Speck, 2011) possess significant antiviral activity *in vitro* and *in vivo* (Li et al., 1965).

Recent investigations conducted by Chatterji et al. in 2002 showed that extracts prepared from economically important Indian marine bivalves including the estuarine clam (*Meretrixcasta*), black clam (*Villorita cyprinoides*) and mud clam (*Polymesoda erosa*) were found to possess high antiviral activity when tested with influenza virus strains type-A (A/Missisipi 1/85/H3N2) and type-B (B/Harbin 7/94).

Li and Traxler (1972) reported that the aqueous extract of *Mye arenaria* had antiviral activity *in vivo*. Moreover, a new type of D-galactan sulfate was isolated from *Meretrix petechialis*. Its

structure was characterised and its antiviral activity was examined (Amornrut et al., 1999). It has been reported that these polysulfates exert their anti-HIV activity by either interfering with CD4 binding to gp 120 thus inhibiting the syncytica formation (Uzair, Mahmood, & Tabassum, 2011) or by binding to the V3 loop, stopping infectivity (Hayashi, Hayashi, & Kojima, 1996). Most of the sulphated polysaccharides, that have been found to inhibit HIV replication, appear to inhibit syncytia formation (Amornrut et al., 1999).

1.6.4 Antibacterial Activity

More than 100 species of marine organisms have been shown to exhibit antimicrobial activity (Ramasamy & Murugan, 2005). Two clam species; *Meretrix casta* and *Tridacna maxima* were screened for antibacterial activity using whole body tissues extracts obtained from different solvents. The ethanol and methanol extracts were able to inhibit all the pathogens, exhibiting broad spectral antibiotic activity. Ethanol extracts of *Meretrix casta* showed highest activity against *Escherichia coli*, *Staphylococcus aureus* while aqueous extracts showed highest activity against *Escherichia coli, Salmonella typhi* and *Staphylococcus aureus*. And the other extracts showed lowest activity against *Klebsiella pneumoniae* and *Lactobacillus vulgaris*. Similarly, the ethanol extract of *Tridacna maxima* exhibited highest activity against *Klebsiella oxytoca*, *Proteus mirabilis* and *Staphylococcus aureus* (Mariappan & Balasubramanian, 2012).

1.6.5 Antioxidant Activity

The antioxidant activity of surf clams has been evaluated over the years. The role of *P. malabarica* extracts in inhibition of free radical mediated oxidation of lipids as well as its reducing potential and radical scavenging properties have been demonstrated, showing that the seafood clam, *P. malabarica,* is a natural source of antioxidants (Pawar, Nagvenkar, & Jagtap, 2013).

Chang, Li, Sun, Yang, & Sun (2012) reported that polysaccharides from the Chinese surf clam (*Mactra chinensis*) have scavenging activity against superoxide anion and hydroxyl free radicals in a concentration-dependent way within a certain range of concentrations. Also, the

aqueous and alcoholic extracts from *Mactra veneriformis* showed antioxidant potentials using various *in vitro* assays (Luan, Wang, Wu, Jin, & Ji, 2011). The protein hydrolysates, prepared from the body, foot and viscera of the marine clam, *Meretrix casta* by enzymatic hydrolysis, have been reported to have potent antioxidant properties (Nazeer, Prabha, Kumar, & Ganesh, 2013).

Clam therapeutic properties are not limited to the above only. Surf clam extracts have also shown to have hepatoprotective (Hsu, Hsu, & Yen, 2010; Chen, Lin, Hiao, & Pan, 2011; Lin, 2013), antihypertensive (Tsai, Lin, Chen, & Pan, 2006), antineoplastic (Schmeer, 1964; Li, Prescott, Eddy, Chu & Martino, 1968), antimicrobial (Defer, Bourgougnon, & Fleury, 2009; Ramasamy & Balasubramanian, 2012), hypocholesterolemic (Lin, Tsai, Hungi, & Pan, 2010), and antiulcer *(*Ajithkumar, 2012) effects.

Clam extracts, besides the aforementioned, have been associated with ACE inhibition (Tsai, Chen, & Pan, 2008), leukaemia therapy (Li, Prescott, Liu, & Martino, 1968), Low-Density Lipoprotein (LDL) oxidation inhibition (Chen, Lin, Hiao, & Pan, 2008), chronic hepatitis treatment (Peng et al., 2008), enhanced IgM secretion (Kong, Chiang, Fang, Shinohara, & Pan, 1997), anti-HIV activity (Amornrut et al., 1999), skin wound healing (Badiu, Luque, Dumitrescu, Craciun, & Dinca, 2010), decreasing Haemorrhagic Shock–Induced Liver (HSL) injury (Lee, Subeq, Lee, Hsu, & Peng, 2011), anti-hyperglycaemia (Wang, Wu, Chang, & Zhang, 2011) and reducing cholesterol level and hepatic lipids (Chijimatsu, Tatsuguchi, Oda, & Mochizuki, 2009).

Surf clams are known not only for their abundance in nutrition. Consumption of these foods by humans may also significantly influence their health status (Peng, 2008).

1.7 ANTIOXIDANTS DERIVED FROM SURF CLAM (DIETARY ANTIOXIDANTS)

Many antioxidants have been found in shellfish which are of marine origin such as carotenoids, taurine and *n*-3 polyunsaturated fatty acids (Luan et al., 2011). Surf clams contain antioxidant substances that may either be hydrophilic or lipophilic. Antioxidants

released during digestion of seafood, including peptides with antioxidative capacity (AOC), have recently attracted considerable attention.

Early reports concluded that the AOC of seafood is mainly due to the aqueous fraction, referred to as press juice (PJ) (Jensen, Abrahamsen, Maehre, & Elvevoll, 2009).

This section will cover some active antioxidant compounds in clams.

1.7.1 Carotenoids

Carotenoids are lipophilic colorants present in numerous fruits and vegetables (Mangels, Holden, Beecher, Forman, & Lanza, 1993). There have been several reports on carotenoids in marine shellfish such as *Mytilus edulis, Pectene maximus, Patinopectene yessoensi*s, *Mytilus coruscus, Mactra chinensis, Crassostrea gigas* and *Fushinus perplexus*. Carotenoids have also been extracted from clams; *Corbicula japonica*, *Corbicula sandai, Corbicula* sp. (Chinese freshwater *corbicula* clam) (Maoka, Fujiwara, Hashimoto, & Akimoto, 2005), *Rudiapes philippinarum* and *Meretrix petechialis* (Maoka, Akimoto, Murakoshi, Sugiyama, & Nishino, 2010). Carotenoids interact synergistically with other antioxidants (Stahl & Sies, 2003). Carotenoids are reported as effective radical scavengers, responsible for scavenging radicals including singular molecular oxygen and peroxyl radicals, and forming a part of the antioxidant defence system in the human organism (Yan, Chuda, Suzuki, & Nagata, 1999; Stahl & Sies, 2003).

Fucoxanthin, a marine carotenoid possessing allenic, conjugated carbonyl, epoxide, and acetyl groups in its molecule, and fucoxanthinol esters have been isolated from the Chinese surf clam, *Mactra chinensis* (Maoka, Fujiwara, Hashimoto, & Akimoto, 2007). Carotenoids, including fucoxanthin, are also well known as antioxidants due to their singlet oxygen-quenching activity. The physical quenching reaction involves the transfer of excitation energy from $^3O_2$ to the carotenoid, resulting in the formation of the carotenoid triplet, as shown in reaction (i). Then in a subsequent reaction (ii), the excitation energy is harmlessly dissipated through rotational and vibrational interactions between the carotenoid triplet and the solvent, regenerating the original carotenoid molecule (Krinsky, 1989).

$^1O_2$ + CAROTENOID $\rightarrow$ $^3O_2$ + ^{3}CAROTENOID (i)

^{3}CAROTENOID $\rightarrow$ CAROTENOID + HEAT (ii)

Besides scavenging reactive oxygen species, carotenoids also influence cellular signalling. Carotenoids may cause triggering of redox sensitive regulatory pathways, stimulation of gap junctional communications and production of an impact on the regulation of cell growth and induction of detoxifying enzymes, such as cytochrome P450-dependent monooxygenases (Stahl, Ale-Agha, & Polidori, 2002). Carotenoids have attracted attention because a number of epidemiological studies have revealed that an increased consumption of a diet rich in carotenoids is correlated with a diminished risk for several degenerative disorders, and in the prevention of various types of cancers, cardiovascular or ophthalmological diseases (Mayne, 1996).

1.7.2 Free Amino Acids

Free amino acids are substrates for energy metabolism, protein synthesis and osmoregulation (Hummel et al., 1996). Extracts of fish meat contains free amino acids (FAAs) and peptides which are not incorporated in proteins. The FAAs have been implicated to be responsible for the characteristic taste of seafood, playing an important role in free radical scavenging activity (Wun, Chen, & Shiau, 2003). Also, a good correlation between the content of FAAs and antioxidant activity has been proved (Wu, Chen & Shiau, 2003). FFAs (e.g. alanine, β-alanine, arginine, aspartic acid, glutamine, Glycine, isoleucine, lysine, etc.) have been isolated and investigated in the soft tissues of clams, including *Macoma balthica* (Sokolowski, Wolowicz, & Hummel, 2003) and *Mercenaria mercenaria* (Jeffries, 1972).The contents of FAAs investigated in *Mactra veneriformis* have been positively correlated with its metal chelating activities (Luan et al., 2011).

Taurine (2-aminoethanesulfonic acid) is a free acid amino that is found in high concentrations in most types of animal tissues (Militante & Lombardini, 2004). Several different roles have been ascribed to taurine. These include conjugation of bile acids, calcium flux modulator, a neuronal activity modulator, a cellular osmo-regulator (Rose, 1996). The benefits of taurine supplementation have been reviewed with respect to diabetes,

cardiomyopathy, and hypertension (Militante & Lombardini, 2004). Claims have also been made for membrane stabilisation via antioxidant properties, a role essentially carried out by hypotaurine (the metabolic precursor of taurine (Rose, 1996) and cysteamine (Aruoma, Halliwell, Hoey, & Butler, 1988).

Taurine is formed by the oxidation of hypotaurine, which is a scavenger of hypochlorous acid (HOCl) and hydroxyl radical ($OH^{\bullet}$) (Green, Fellman, Eicher, & Pratt, 1991).

Taurine maintains the viability of human lymphocyte-derived cultured lymphoblastoid cells after long periods of aerobic incubation in the presence of the oxidants like ferrous sulfate and ascorbate (Redmond, Stapleton, Neary, & Bouchier-Hayes, 1998).The observation of a protective role for taurine against oxidant induced damage in the lymphocyte would suggest that it acts as an antioxidant in these cells (Pasantes-Morales, Wright, Gaull, 1985). Also, in a cell culture system where taurine concentrations can be controlled, taurine enhances the proliferation of human lymphoblastoid cells in a concentration-dependent manner. This action of taurine could result from its role of counteracting the antiproliferative effects of oxidative and peroxidative products, which may otherwise cause extensive damage and subsequent cellular death (Wright, Tallan, & Lin, 1986). Furthermore, previous studies have reported that taurine can act as a direct antioxidant by scavenging reactive oxygen species (ROS) and/or as an indirect antioxidant by preventing changes in membrane permeability due to oxidant injury (Gürer, Özgünes, Saygin, & Ercal, 2001). Other studies have revealed that the administration of taurine partially protected cells from oxidative damage and showed morphological protection for surviving cells (Rodríguez-Martínez, Rugerio-Vargas, Rodríguez, Borgonio-Pérez, & Rivas-Arancibia, 2004).

1.7.3 Polyunsaturated Fatty Acids

Free fatty acids can function as an energy source, as a constituent of the cell membrane and as a physiologically active substance. In nature the three types of fatty acids are; saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids (PUFAs) (Nishikiori, Iizuka, Ichiba, Sadamoto, & Fukushima, 2014). Mammals do not possess enzymes able to synthesise essential nutrients with double bonds at the n-3 and n-6 positions of the carbon

chain of a fatty acid, therefore, humans must obtain the essential fatty acids linoleic acid (C18:2n-6) and alpha linolenic acid (ALA, C18:3n-3) from dietary sources (Demott, & Müller-Navarra, 1997; Kromhout, Yasuda, Geleijnse, & Shimokawa, 2012). In the last 40 years, a growing body of evidence has accumulated on the beneficial effects of n-3 poly-unsaturated fatty acids (n-3 PUFAs) on cardiovascular diseases (CVD) in reducing triglyceride levels, controlling inflammation processes, improving endothelial function, regulating platelet aggregation and decreasing the incidence of arrhythmias. n-3 PUFAs are among the drugs recommended for managing dyslipidemia and preventing CVD. In addition, higher circulating n-3 PUFA levels have very recently been reported to be associated with lower total mortality, especially deaths due to coronary heart disease (CHD) in older adults (Bilato, 2013). Consumption of *n*-3 fatty acids like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) can mitigate the progression of diseases in which oxidative stress represents a common underlying biochemical process (Gao et al., 2007). It has been concluded that these unsaturated fatty acids extracted from surf clams contribute to its antioxidant activity (Luan et al., 2011).

1.7.4 Nucleoside

Nucleoside analogues display a wide range of biological activities as antitumor, antiviral and chemotherapeutic agents. In the last decades, nucleosides with a six-membered carbohydrate moiety have been evaluated for their potential antiviral and antibiotic properties and as building blocks in nucleic acid synthesis. One series of uncommon six-membered nucleoside analogues, the unsaturated ketonucleosides, is well established for its antineoplastic activity and immunosuppressive effects. It appears that these nucleosides not only exhibit growth inhibitory activity against a variety of tumor cells *in vitro* and L1210 leukemia *in vivo* but they may also constitute important synthetic intermediates in the nucleoside field owing to their chemical reactivity in various media (Spanou, Manta, Komiotis, Dervishi, & Kouretas, 2007). It was also proved that the introduction of a fluorine atom in the sugar moiety of the unsaturated nucleosides increases the activity, raises the lipophilicity and makes the penetration of the drug through the cell membrane easier (Lipnick & Fissekis, 1980). A study reports the antioxidant activity of a series of modified nucleoside analogues of N4-benzoyl cytosine and N6-benzoyl adenine for the first time. It is noteworthy

that most of the tested compounds showed significant activity to protect DNA from the strand breaking activity of ROO• radicals. What is also important is that the molecules with a α,β-unsaturated keto system were the most potent against activity of the ROO• radicals which can be explained by a radical stabilisation resonance effect (Spanou, Manta, Komiotis, Dervishi, & Kouretas, 2007).

1.7.5 Other Antioxidant Compounds

Various proteins and amino acids present in seafood have a capacity to scavenge free radicals by virtue of their ability to chelate metals, thereby, serving as synergists with other antioxidants (Jensen, Abrahamsen, Maehre, & Elvevoll, 2009). Other antioxidants present in seafood include flavonoids or polyphenols. Polyphenols act as metal chelators and synergists which enhance the activity of other antioxidants such as α-tocopherol. Many seafood species also contain significant amounts of chlorophyll a, purpurin, and bromophenols, which have antioxidant activity (Kulawik, Özogul, Glew, & Özogul, 2013).

1.8 MECHANISM OF ACTION OF ANTIOXIDANTS

Different responses of radical chain reactions are anticipated with regard to the type of antioxidants involved (Fung, 2012). For convenience, antioxidants have been traditionally divided into two classes; primary or chain-breaking antioxidants and secondary or preventative antioxidants, depending on their mode of operation (Vulic, Vitarelli, & Zenner, 2002).

1.8.1 Primary Antioxidants

Primary antioxidants are radical scavengers or hydrogen donors or chain reaction breakers. The major molecules of primary antioxidants include hindered phenols and secondary aryl amines (Wang, 2000).

Chain-breaking mechanisms:

$$L^{\bullet} + AH \longrightarrow LH + A^{\bullet}$$

$$LO^{\bullet} + AH \longrightarrow LOH + A^{\bullet}$$

$$LOO^{\bullet} + AH \longrightarrow LOOH + A^{\bullet}$$

(Where $L^{\bullet}$ is the lipid radical, $LO^{\bullet}$ peroxyl radical, $LOO^{\bullet}$ alkoxyl radical, and AH; antioxidant).

As illustrated above, radical initiation (by reacting with a lipid radical) or propagation (by reacting with peroxyl or alkoxyl radicals) steps are inhibited (Apak et al., 2013).

Four different reactions have been proposed for the initial step of the chain-stopping action. And each is supported by some experimental evidence. Each of the reactions provides for interruption of the free radical chain mechanism by interaction of a propagating radical with the antioxidant. The four mechanisms that have been proposed are:

(a) Hydrogen donation to $RO_2^{\bullet}$ by the antioxidant,

(b) Electron donation to $RO_2^{\bullet}$ by the antioxidant,

(c) Addition of $RO_2^{\bullet}$ to the aromatic ring of the antioxidant and

(d) Formation of a π-complex between $RO_2^{\bullet}$ and the aromatic ring of the antioxidant.

The first reaction [(a)] listed above in which the antioxidant donates a labile hydrogen to a peroxy radical to form a hydroperoxide has generally been considered to be the most probable reaction in the case of phenols and amines (other than tertiary) (Shelton, 1959). This is illustrated below;

$RH \longrightarrow R^{\bullet}$	Initiation	(i)
$R^{\bullet} + O_2 \longrightarrow RO_2^{\bullet}$	Addition of O_2	(ii)
$RO_2^{\bullet} + RH \longrightarrow ROOH + R^{\bullet}$	H- Atom exchange	(iii)

Once a free radical $R^{\bullet}$ has been generated, then reactions (ii) and (iii) form a chain reaction. As the chain cycles through (ii) and (iii), many lipid molecules (R-H) are converted into lipid hydroperoxide (ROOH) and thus resulting in oxidation and rancidity of fats. Reaction (ii) is

very fast, ca. 10^9 M^{-1} s^{-1}, whereas (iii) is much slower, typically 10^1 M^{-1} s^{-1} (Wright, Johnson, & DiLabio, 2001; Lucarini et al., 1999).

The second reaction [(b)] listed above by which an antioxidant can deactivate a free radical is electron transfer, in which the radical cation is first formed and then followed by rapid and reversible deprotonation in solution, according to the equations below;

$$RO_2^{\bullet} + ArOH \longrightarrow RO_2^{-} + ArOH^{+}$$ Electron transfer (iv)

$$ArOH^{+} + H_2O \rightleftharpoons ArO^{\bullet} + H_3O^{+}$$ Deprotonation equilibrium (v)

$$RO_2^{-} + H_3O^{+} \rightleftharpoons ROOH + H_2O$$ Hydroperoxide formation (vi)

However, if the radical cation, $ArOH^{+}$ has sufficient lifetime it can attack suitable substrates (Wright, Johnson, & DiLabio, 2001).

Polyphenols (strong chain- breaking antioxidants) are the most abundant antioxidants in the human diet. Current evidence strongly supports a contribution of polyphenols in the prevention of cardiovascular diseases, cancers and osteoporosis and suggests a role in the prevention of neurodegenerative diseases and diabetes mellitus (Scalbert, Johnson, & Saltmarsh, 2005). It employs the first mechanism [(a) above], inhibiting lipid peroxidation by rapidly donating a hydrogen atom to the peroxyl radical ($ROO^{\bullet}$) to form alkyl hydroperoxide (ROOH).

$$ROO^{\bullet} + PPH \longrightarrow ROOH + PP^{\bullet}$$

The polyphenol phenoxyl radical ($PP^{\bullet}$) produced is then stabilised by further donation of a hydrogen atom and formation of quinines or by reacting with another radical, including another phenoxyl radical, thereby, interrupting the initiation of a new chain reaction (Prior, Wu, & Schaich, 2005; Fung, 2012).

It has thus been reported that non-tertiary aromatic amines and phenols are highly effective radical-trapping antioxidants. This retards the rates of oxidative degradation of organic materials of commercial and biological importance (MacFaul, Ingold, & Lusztyk, 1996).

1.8.2 Secondary Antioxidants

Secondary antioxidants, on the other hand, retard the rate of oxidation e.g., transition-metal ion chelators may inhibit Fenton-type reactions that produce hydroxyl radicals;

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-}$$ (Apak et al., 2013).

Deactivation of metal catalysts of oxidation is very important since it is well known that many metal ions, such as iron and copper, when present will accelerate the oxidation process and bring about a rapid deterioration of the material. Organic compounds capable of forming coordination complexes in which the metal is held in an inactive form will thus retard the oxidation process. These compounds usually have more than one coordination centre, so that cyclic structures are formed. This effectively encloses the metal ion in a cage. For this reason they are frequently referred to as chelating agents and as sequestering agents (Shelton, 1959).

Secondary antioxidants are also peroxide decomposers (Wang, 2000). Peroxide decomposers include those materials which stoichiometrically reduce hydroperoxides to the corresponding alcohol;

$$ROOH \xrightarrow{2H} ROH + H_2O$$ (Burton & Ingold, 1984).

Where 2H = preventive antioxidant

$$ROH \xleftarrow[\text{antioxidant}]{\text{Preventive}} ROOH \quad \text{Chain} \cdots\cdots // \cdots\cdots \xrightarrow[\text{processes}]{\text{Initiative}} ROO^{\bullet};\ RO^{\bullet};\ HO^{\bullet}$$ (Burton & Ingold, 1983).

Or catalytically decompose it to non- radical products;

$$R_3COOH \xrightarrow{(H^+)} R_2C = O + ROH$$

In living organisms a variety of these enzymes (such as catalase, glutathione, and peroxidase) act as preventive antioxidants by destroying hydroperoxides without generating free radicals (Burton & Ingold, 1984).

This type of antioxidant thus inhibits oxidation by reducing the rate of peroxide initiation of the free radical oxidation mechanism (Shelton, 1959).

1.9 ANTIOXIDANT CAPACITY ASSAYS

Antioxidant capacity is related with compounds which are capable of protecting a biological system against the potentially harmful effect of processes or reactions involving reactive oxygen species (ROS). These protective effects of antioxidants have resulted in the requirement of simple, convenient, and reliable antioxidant capacity determination methods.

Many methods which differ from each other in terms of reaction mechanisms, oxidant and target/probe species, reaction conditions and expression of results have been developed and tested in the literature (Karadag, Ozcelik, & Saner, 2009). The most commonly used antioxidant capacity assays are divided into two main categories: the hydrogen atom transfer (HAT) reaction-based assays and the electron transfer (ET) reaction-based assays (Tierney, Croft, & Hayes, 2010).

1.9.1 HAT Based Assays

HAT based assays measure the ability of an antioxidant to quench a free radical by hydrogen donation to form stable compounds (Prior et al. 2005). The majority of HAT-based assays involve a reaction scheme in which antioxidant and substrate compete for thermally generated peroxyl radicals through the decomposition of azo compounds. These assays include inhibition of induced low-density lipoprotein autoxidation, oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP) and crocin bleaching assays (Huang, Ou, &. Prior, 2005). Most of these assays are kinetic-based, meaning that they are more concerned with the rate rather than thermodynamic conversion efficiency of the radical reaction with the antioxidant (Apak, Güçlü, Özyürek, Çelik, 2008).

$$AH + X^{\bullet} \longrightarrow XH + A^{\bullet}$$

HAT reactions are solvent and pH independent and are usually quite rapid, typically completed in seconds to minutes. However, the presence of reducing agents, including metals, is a complication in HAT assays and this can lead to erroneously high apparent reactivity (Prior, Wu, & Schaich, 2005).

1.9.2 ET Based Assays

In ET-based antioxidant assay methods, the ability of a potential antioxidant to transfer an electron to reduce any compound (including metals, carbonyls and radicals) is detected (Tierney, Croft, & Hayes, 2010). ET-based methods involve two components in the reaction mixture, antioxidants and oxidant (probe) (Karadag, Ozcelik, & Saner, 2009). Spectrophotometric ET-based assays measure the capacity of an antioxidant in the reduction of an oxidant, which changes colour when reduced (Apak, Güçlü, Özyürek, Çelik, 2008). The degree of colour change (either an increase or decrease of absorbance of the probe at a given wavelength) is correlated to the concentration of antioxidants in the sample (Apak et al., 2013).

$$\mathrm{M(III)} + \mathrm{AH} \longrightarrow \mathrm{AH^{\bullet}} + \mathrm{M(II)}$$

ET-based assays include ABTS/TEAC, DPPH [though ABTS/TEAC and DPPH are considered as mixed HAT/ET-based assays by some researchers (Özyürek, Güçlü, & Apak, 2011)], Folin–Ciocalteu reagent (FCR), FRAP, ferricyanide and CUPRAC using different chromogenic redox reagents with different standard potentials (Apak et al., 2013).

ET and HAT mechanisms cannot be differentiated with distinct boundaries (Huang, Ou, Prior, 2005; Özyürek, Güçlü, & Apak, 2011), as both almost always occur together in all samples (Karadag, Ozcelik, & Saner, 2009).

1.9.3 DPPH Radical Scavenging Assay

This method was developed by Blois in 1958 with the viewpoint to determine the antioxidant activity in a similar manner by using a stable free radical α, α-diphenyl-β-picrylhydrazyl (DPPH; $C_{18}H_{12}N_5O_6$, M=394.33) (Kedare & Singh, 2011).

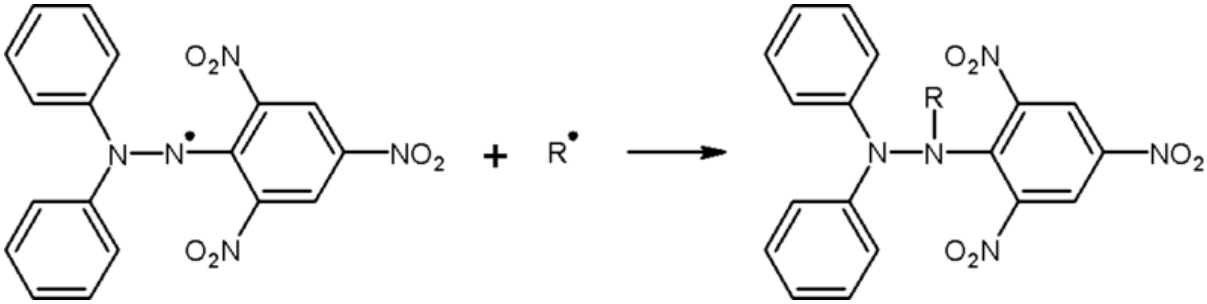
DPPH (2,2-diphenyl-1-picrylhydrazyl or 1,1-diphenyl-2-picrylhydrazyl) radical is one of the few stable organic nitrogen centred free radical, which is effectively scavenged by antioxidants (Fung, 2012). DPPH is considered as a stable radical because of the paramagnetism conferred by its odd electron (delocalisation of the spare electron over the molecule as a whole) (Jansuk, 2007). The odd electron in the DPPH free radical gives a

strong absorption maximum at 517 nm and is purple in colour. The colour turns from purple to yellow as the molar absorptivity of the DPPH radical at 517nm reduces from 9660 to 1640 when the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H. The resulting decolourization is stoichiometric with respect to number of electrons captured (Prakash, Rigelhof, & Mille, 2001).

Representing the DPPH radical by $R^{\bullet}$ and the donor molecule by AH, the primary reaction is;

$R^{\bullet} + AH = RH + A^{\bullet}$

(Where RH is the reduced form and $A^{\bullet}$ is free radical produced in this first step).



DPPH- Free radical form | $R^{\bullet}$ - Donor molecule | DPPH- Reduced

Figure 4: DPPH Inhibition.
Retrieved from http://commons.wikimedia.org/wiki/File:DPPHInhibition.png

1.9.4 Cupric Ion Reducing Antioxidant Capacity (CUPRAC) Assay

The CUPRAC method of antioxidant capacity measurement, which is introduced by the analytical chemistry laboratory of Istanbul University to world literature, is based on the absorbance measurement of the CUPRAC chromophore, Cu(I)-neocuproine (Nc) chelate which were formed as a result of the redox reaction of antioxidants with the CUPRAC reagent, bis(neocuproine)copper(II) cation [Cu(II)-Nc] where absorbance is recorded at the maximal light absorption wavelength of 450 nm (Apak et al., 2013). CUPRAC method is hinged on the reduction of light-blue colored cupric neocuproine complex (Cu(II)–Nc) by antioxidants to the yellow-orange coloured cuprous chelate (Cu(I)–Nc) (Özyürek et al., 2014; Çelik, Özyürek, Güçlü, Çapanoğlu, & Apak, 2014) in the following manner;

$nCu(Nc)_2^{2+} + n\text{-}electron\ reductant \rightarrow nCu(Nc)_2^{+} + n\text{-}electron\ oxidized\ product + nH^{+}$

(Fung, 2012).

The use of iron in this assay rather than copper ions is referred to as the ferric reducing antioxidant power (FRAP) assay. However, copper ions have a lower redox potential than iron and hence giving the CUPRAC assay less interference from sugars and other common interfering substances. The copper reaction also responds faster than iron which consequently gives the CUPRAC assay a shorter completion time than the FRAP assay (Prior, Wu, & Schaich, 2005; Mak, 2012). Also, the CUPRAC reagent has been shown to be much less dependent on phenolic lipophilicity, steric effects, solvent (mobile phase) composition, pH, dissolved oxygen, and daylight (Çelik, Özyürek, Güçlü, & Apak, 2010).

1.9.5 Pros and Cons of DPPH and CUPRAC Assays

The DPPH radical scavenging assay is a technically simple procedure (Huang, Ou, & Prior, 2005) that is rapid, inexpensive (Mensor at al., 2001) and widely used (Sagar, Kedare, & Singh, 2011). Also, antioxidant efficiency is measured at ambient temperature, which eliminates the risk of the thermal degradation of molecules tested (Bondet, Brand-Williams, & Berset, 1997). The drawbacks of this technique, however, include the loss of DPPH colour through mechanisms like radical reaction, reduction and steric accessibility. Antioxidants that react quickly with peroxyl radicals involved in lipid peroxidation may not react to DPPH due to steric inaccessibility (Prior, Wu, & Schaich, 2005; Fung, 2012). One very important drawback is that the reactional mechanism between the antioxidant and $DPPH^{\bullet}$ depends on the structural conformation of the antioxidant. Some compounds react very quickly with the DPPH radical, reducing a number of $DPPH^{\bullet}$ molecules equal to their number of available hydroxyl groups. However, for the majority of the compounds tested, the reactions are slower and the mechanisms seem to be more complex (Bondet, Brand-Williams, & Berset, 1997). Also, various research groups have used widely different protocols which differed in the concentration of DPPH (22.5 - 250µM), incubation time (5 minutes to 1 hour), reaction solvent (methanol, buffered methanol, ethanol) and pH of the reaction mixture. As a result of these differences in reaction conditions, the $1C_{50}$ values for even the standard antioxidants

like ascorbic acid and BHT have been reported to vary a lot (Sharma & Bhat, 2009). These complications can lead to imprecise estimations of antioxidant capacity (AOC) (Prior, Wu, & Schaich, 2005).

The CUPRAC method, on the other hand, is advantageous over other ET- Based methods as it measures the antioxidant capacity at nearly physiological pH (i.e. pH 7) so it better simulates the physiological action of these antioxidants. The chromogenic oxidising reagent of the CUPRAC assay, bis (2,9-dimethyl-1,10-phenanthroline) copper (II) (abbreviated as Cu(II)-Nc), is simple, stable and easily available at low cost (Özyürek et al., 2014).The CUPRAC reagent is fast enough to oxidise thiol-type antioxidants, whereas the FRAP method does not measure certain thiol-type antioxidants like glutathione. The reagent is more stable and accessible than other chromogenic reagents (e.g., ABTS, DPPH). The redox reaction which gives rise to a coloured chelate of Cu(I)-Nc is relatively insensitive to a number of parameters adversely affecting certain reagents such as DPPH, i.e., air, sunlight, humidity and pH to a certain extent (Apak, Güçlü, Özyürek, & Çelik, 2008). Also, the univalent- charged CUPRAC chromophore ($Cu(Nc)_2^+$) is soluble in both aqueous and organic solvents, enabling the simultaneous determination of hydrophilic and lipophilic antioxidants (Özyürek, Güçlü, & Apak, 2011).

1.10 OBJECTIVES

The aims of this study were as follows:

(1) to explore the pharmacological activity and the potential of three New Zealand surf clam's species as a natural resource of bioactive compounds;
(2) to investigate the antioxidant activities of extracts, fractions and sub-fractions of New Zealand surf clam's species;
(3) to compare and identify which fraction of surf clam has the highest antioxidant activity;
(4) to examine the differences in the DPPH scavenging activity and the reducing activity (using CUPRAC assay) between fractions of three New Zealand surf clam species.

CHAPTER TWO

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals and reagents

Methanol, n-Butanol, Petroleum spirit and Ethyl acetate were purchased from Global Science (Auckland, New Zealand) while ethanol was purchased from Thermofisher (Auckland, New Zealand) and were all of HPLC grade. 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) and Neocuproine, ≥98% were purchased from Sigma- Aldrich (St. Louis, MO, USA). Stock cultures of DPPH were prepared in methanol. All other reagents, solvents and chemicals used were obtained from the Auckland University of Technology (AUT) Applied Sciences laboratory and were of analytical grade.

2.1.2 Surf clam Processing

All clams used were cleaned and blanched. Clams were obtained from Cloudy Bay Clams and they were ready to eat. As such, no further processing was required. But according to Cloudy Bay Clams (2014), the surf clams are harvested using their environmentally friendly “winnowing clam rake” and sorted by species on board by the harvester. The shellfish are then held submersed in sea water in bulk bins on the deck of the vessel with frequent exchanges of aerated water for allowing them to continue natural respiration. At the conclusion of harvesting, the water is drained and the fish remain in the bulk bins in this “dry” state until they arrive at the wet store facility. The clams are placed in a single layer on perforated trays and stacked in specially designed tanks in a manner that allows uniform flow of water across them. They remain in this oxygen enhanced seawater that is drawn in from the open sea through a vast infiltration gallery, used once and then returned to sea. Both flesh and water are tested regularly for any contamination from either bacterial and bio toxin

causes. This process allows them to purge themselves of any sand or grit they may have in them. The fish remain in this environment with regular cleaning and care until dispatched live into both the local and international market or to the local processor. All water used in this process is USFDA approved and regularly monitored to maintain the highest possible quality. Prior to export, the water is cooled to below 10 degrees Celsius as required by NZFSA. The clams are packed into Polystyrene boxes (to regulate temperature) and freighted dry to the end user (AUT University).

2.2 METHODS

2.2.1 Sample Preparation

Drying

Frozen clams were prepared. The flesh was taken out by shucking the shells with a knife. The clam juice was drained and the frozen flesh was thawed at room tepmperature. The flesh was laid onto plastic trays as shown in Figure 5 (below) and then it was dried in an hot air oven at 60° to constant weight for 4 days. Flesh was turned two to three times daily to prevent it from sticking.

Dried sample was then pulverised at low speed in a laboratory blender (model: 800watts Sunbeam Multiblender-Pro blender). Milled clam flesh was transferred into glasswares, weighed and recorded. Sample was stored in the dark at room temperature until when it was needed.

Figure 5a: Flesh thawing prior to drying

Figure 5b: Dried flesh

Preparation of extracts and fractions of storm clam

All clam extraction and measurements were carried out in dim light to reduce any possibility of oxidation. Extraction method from Luan et al., 2011 was employed.

Aqueous extraction

Dried, pulverised clamsample was extracted in distilled water and stirred constantly using a magnetic bar at room temperature for an hour. At the end of one hour the solvent was filtered out using a Whatman No.1 (Diameter: 9cm, Pore size: 11µm) filter paper (Thermofisher, New Zealand) to remove all solids. This procedure was repeated at least 7 times and all supernatant was combined. Solvent was concentrated and stored at -20°C until use. The extract was named ‘cd’.

Solvent extraction

Dried, pulverised flesh was extracted several times (one hour per operation) with ethanol absolute and stirred using a magnetic bar at room temperature for an hour. This process was repeated until the solvent was colourless. All solvents from each extraction process were combined and this was filtered using a Whatman No.1 filter paper (Thermofisher, New Zealand) to remove the solids. The clear solution was collected and evaporated under reduced pressure using a Rota evaporator (Buchi Rotavapor R-215) until complete dryness. Semisolid extract was stored at -20°C.

Figure 5c: Extraction process using the Rota evaporator.

Fractionation

The crude extracts of the ethanol extraction were further fractionated by liquid-liquid extraction. The ethanol extract was fractioned into four sub-fractions according to the polarity of the extractive medium and using petroleum ether, ethyl acetate, n-butanol, and water. The extracts were dissolved in 100ml distilled water and fractionated with petroleum ether. Extracts ('pe') were collected and concentrated under reduced pressure. Further step-by-step fractionation was done using ethyl acetate and lastly with n-butanol, which resulted in the fractions of 'ea' and 'nb' respectively. Each fractionation process was repeated until the solvent was colourless. The final aqueous fraction was named 'w'. All four fractions (pe, ea, nb, and w) were evaporated to dryness and stored in the fridge at -20ºC until they were ready to be used for the antioxidant analyses (DPPH and CUPRAC).

2.2.2 DPPH Radical Scavenging Activity

Preparation of stock solution

The working solutions of test extracts were prepared by dissolving the required quantity of test extracts in distilled water and then diluting in methanol absolute to give the concentrations of 0.5µg/mL, 1µg/mL, 10µg/mL, and 20µg/mL.

Preparation of standard solution

Ascorbic acid was used as the reference standard. The standard solution was prepared by a series of dilution in methanol absolute.

DPPH Analysis

The free radical scavenging capacity of the aqueous and ethanol extracts was determined using DPPH (Duan, Zhang, & Wang, 2006). 0.16mM DPPH solution (6.31mg/100mL) was prepared in absolute methanol. 2mL of freshly prepared DPPH solution was mixed with 2mL of the sample in a cuvette so that the final volume was 4mL. The reaction tubes in triplicates were shaken properly and after 30 min of incubation at room temperature, the absorbance was read at 517nm using a spectrophotometer (ULTROSPEC 7000). Control sample was prepared containing the same volume without any extracts. Absolute methanol was used to

zero the spectrophotometer as the blank. Percentage scavenging of the DPPH free radical was measured using the following equation:

$$\text{Scavenging (\%)} = \left[1 - \left(\frac{Abs_{sample} - Abs_{sampleblank}}{Abs_{control}}\right)\right] X\ 100$$

Where Abs_{sample} = the absorbance of the sample with treatment

$Abs_{sampleblank}$ = the absorbance of sample with 2.0ml of methanol

$Abs_{control}$ = the absorbance of 2.0ml of methanol with the treatment.

2.2.3 CUPRAC Assay

Preparation of stock solution

The working solutions of test extracts were prepared by dissolving the required quantity of test extracts in distilled water and then diluting in ethanol absolute to give the concentrations of 0.1µg/mL, 0.5µg/mL, 1.5µg/mL, 2.5µg/mL, 3.5µg/mL, 5µg/mL, 7.5µg/mL, 10µg/mL, 15µg/mL, and 20µg/mL.

Preparation of other solutions

Ascorbic acid was used as the reference standard. The standard solutions (0.1µg/mL, 0.5µg/mL, 1.5µg/mL, 2.5µg/mL, 3.5µg/mL, 5µg/mL, 7.5µg/mL, 10µg/mL, 15µg/mL, and 20µg/mL) were prepared by a series of dilution in ethanol absolute.

The copper (II) chloride ($CuCl_2$) solution (10mM) and ammonium acetate (NH_2Ac) buffer solution (1M, pH7) were prepared in pure distilled water and Neocuproine solution (7.5mM) was prepared in absolute ethanol (Çelik, Özyürek, Güçlü, & Apak, 2010).

CUPRAC Analysis

Cupric reducing antioxidant power (CUPRAC) was determined as described by Apak, Güçlü, Özyürek, & Karademir (2004). 1mL each of Cu (II), Nc, and NH_2Ac buffer solutions were

added to a cuvette. Antioxidant standard solutions (1.0mL) and distilled water (0.1mL) were added to the initial mixture so as to make the final volume of 4.1mL. The cuvettes were manually shaken and after 30 min standing at room temperature, the absorbance at 450nm (A450) was recorded against a reagent blank. The reading on the spectrophotometer (ULTROSPEC 7000) was zeroed using distilled water as the blank. The absorbance of the samples was recorded. The standard calibration curves of each antioxidant compound were constructed in this manner as Absorbance vs. Concentration. The CUPRAC molar absorptivity of each antioxidant was found from the slope of the calibration line concerned. The scheme for normal measurement of antioxidants is summarized as:

Cu (II) (1mL) + Nc (1mL) + NH_2Ac buffer (1mL) + sample (1mL) + H_2O (0.1mL);

Total volume = 4.1mL (measured at 450nm against a reagent blank after 30 min of reagent addition).

2.2.4 Statistical Analysis

Analysis of data was done with GraphPad Prism (GraphPad Software, San Diego, CA). Analyses of variance were performed using one-way ANOVA, with post hoc Barlett's test in GraphPad Prism, where significant differences occurred. Assays for both the DPPH and CUPRAC analyses were repeated three times. Where shown, results were expressed as mean ± standard deviation. A nominal two sided $p < 0.05$ was used to assess significance. Correlation analysis was done using Microsoft Excel (2013).

CHAPTER THREE

3. RESULTS

3.1 ANTIOXIDANT ACTIVITY

Crude extract and four fractions of three New Zealand surf clam species were tested for the antioxidant activity using the DPPH and CUPRAC assays. The experimental results for both assays are presented in the figures below, where crude extracts and all four fractions were established to possess antioxidant activity. The antioxidant properties of all fractions, determined using DPPH and CUPRAC assays, were compared with that of ascorbic acid.

3.2 DPPH Assay

Proton-radical scavenging action is an important mechanism of an antioxidant, which is measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay.

DPPH is a stable free radical that shows maximum absorbance at 517 nm in ethanol. When DPPH encounters a proton-donating substance such as an antioxidant, the radical would be scavenged and the absorbance is reduced. Based on this principle, the antioxidant activity of the substance can be expressed as its ability in scavenging the DPPH (Wu, Chen, & Shiau, 2003).

The radical scavenging activity was observed in all tested samples in a concentration-dependent manner (Figures 6-10 below). The present results reveal that the clam extracts probably contained peptides, which were electron donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction.

The percentages of inhibition of all clam extracts and fractions at concentrations ranging from 0.5 - 20μg/mL were however lower than ascorbic acid. The ea fraction in all samples showed very high scavenging ability. At a concentration of 20μg/mL, the percentage inhibitions for Diamond shell, Tua tua and Storm shell were 73.29%, 76.14% and 73.69% respectively. It can be observed that the pe, cd, and w fractions had very similar scavenging

abilities, as they closely followed the ea fraction. As shown in Table 2, the DPPH radical scavenging activity was significantly higher ($p < 0.05$) in Diamond shell ($p < 0.0001$) and Storm shell ($p < 0.0001$), followed by Tua tua ($p = 0.017$). It was observed that DPPH had radical scavenging in the range of 0.5µg/mL - 20µg/mL.

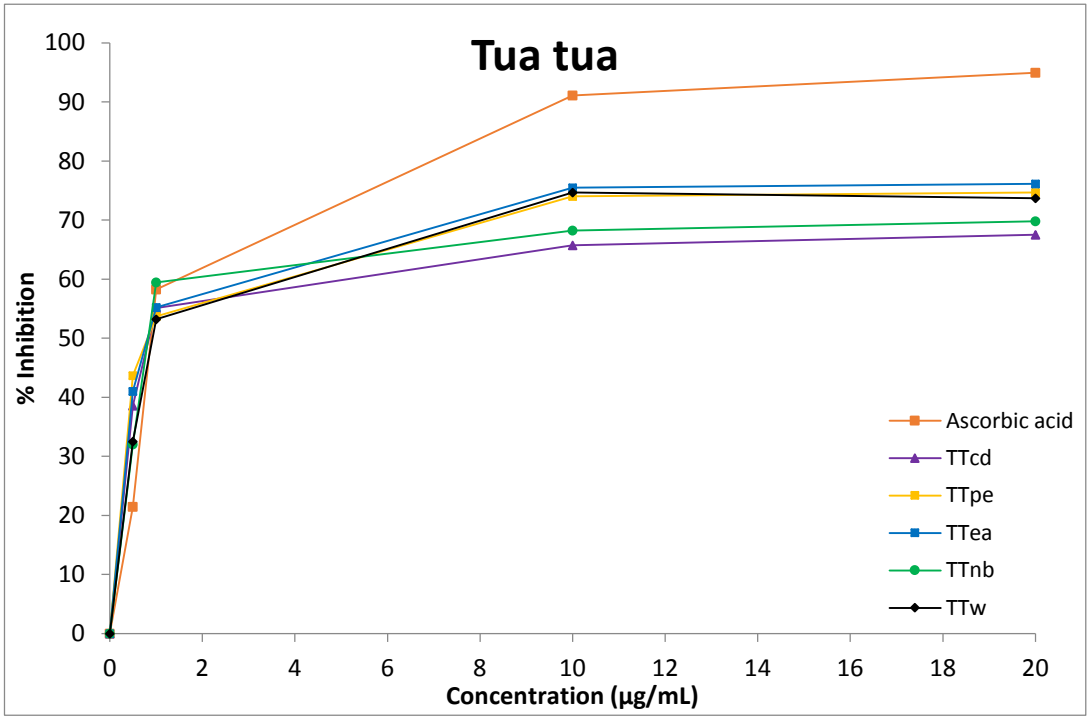


Figure 6: DPPH radical scavenging activity of Tua tua (crude extract- cd, petroleum ether-pe, ethyl acetate- ea, n-butanol-nb, and aqueous-w fractions), and Ascorbic acid. Values are means from three independent tests.

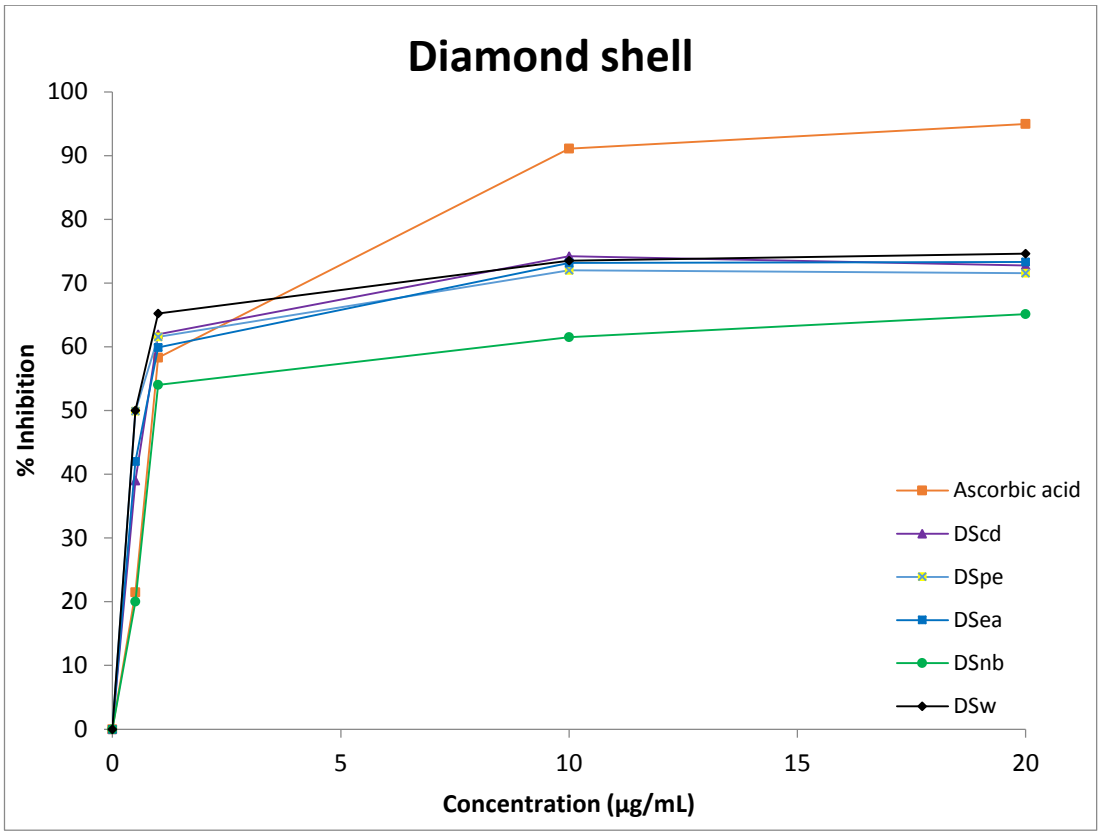


Figure 7: DPPH radical scavenging activity of Diamond shell (crude extract-cd, petroleum ether-pe, ethyl acetate- ea, n-butanol-nb, and aqueous-w fractions), and Ascorbic acid. Values are means from three independent tests.

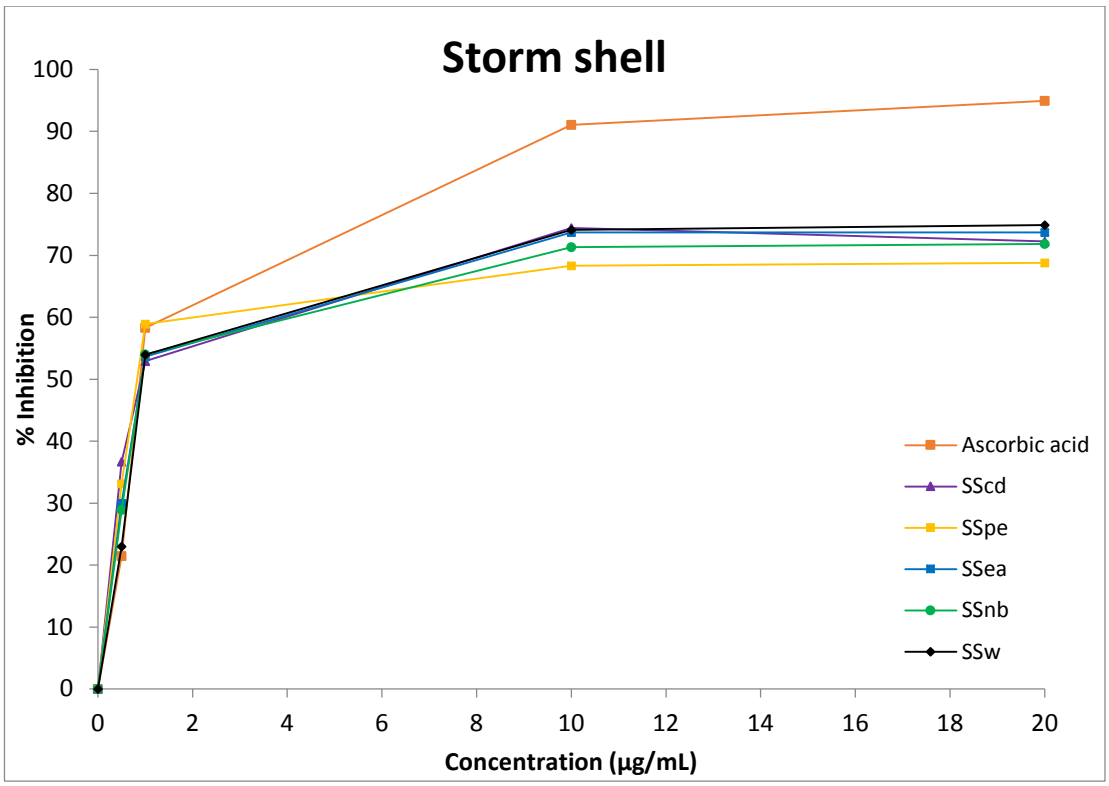


Figure 8: DPPH radical scavenging activity of Storm shell (crude extract-cd, petroleum ether-pe, ethyl acetate- ea, n-butanol-nb, and aqueous-w fractions), and Ascorbic acid. Values are means from three independent tests.

Table 2: DPPH radical scavenging activities by the New Zealand surf clam extracts.

Fraction	Diamond shell (%)	Tua tua (%)	Storm shell (%)
cd	73.01 ± 1.53	64.02 ± 8.27	72.83 ± 4.89
pe	71.78 ± 1.34	77.25 ± 6.46	68.52 ± 1.83
ea	73.81 ± 2.40	75.83 ± 2.15	73.59 ± 0.76
nb	73.24 ± 1.58	68.93 ± 2.09	72.18 ± 2.41
w	74.73 ± 1.41	68.90 ± 9.52	74.48 ± 2.26

All the values are mean ± standard error of three samples.

3.3 CUPRAC Assay

Figures 9, 10 and 11 show the reducing power of surf clam samples on copper ions using the CUPRAC assay. The CUPRAC assay investigated the reducing ability of the sample, with higher absorbance indicating higher reducing ability of the sample. All fractions tested exhibited the ability of reducing copper ions from Cu (II) to Cu (I) in a concentration-dependent manner. All three clam species, Diamond shell, Tua tua and Storm shell showed very high copper ion reducing activity (p <0.0001). The absorbance of the cd, pe, and ea fractions in all three samples were significantly high, compared to the nb and w fractions. At all concentrations, the pe and ea fractions were even higher than ascorbic acid. In all three clam species, the n-butanol (nb) and aqueous (w) fractions had the least reducing activity, as the decrease in absorbance in the CUPRAC assay is linked to a decrease in reducing activity (Apak et al., 2007). The results show that there is a linear correlation (Table 3) between concentrations of surf clam extracts and copper ion reducing ability within the applied concentrations.

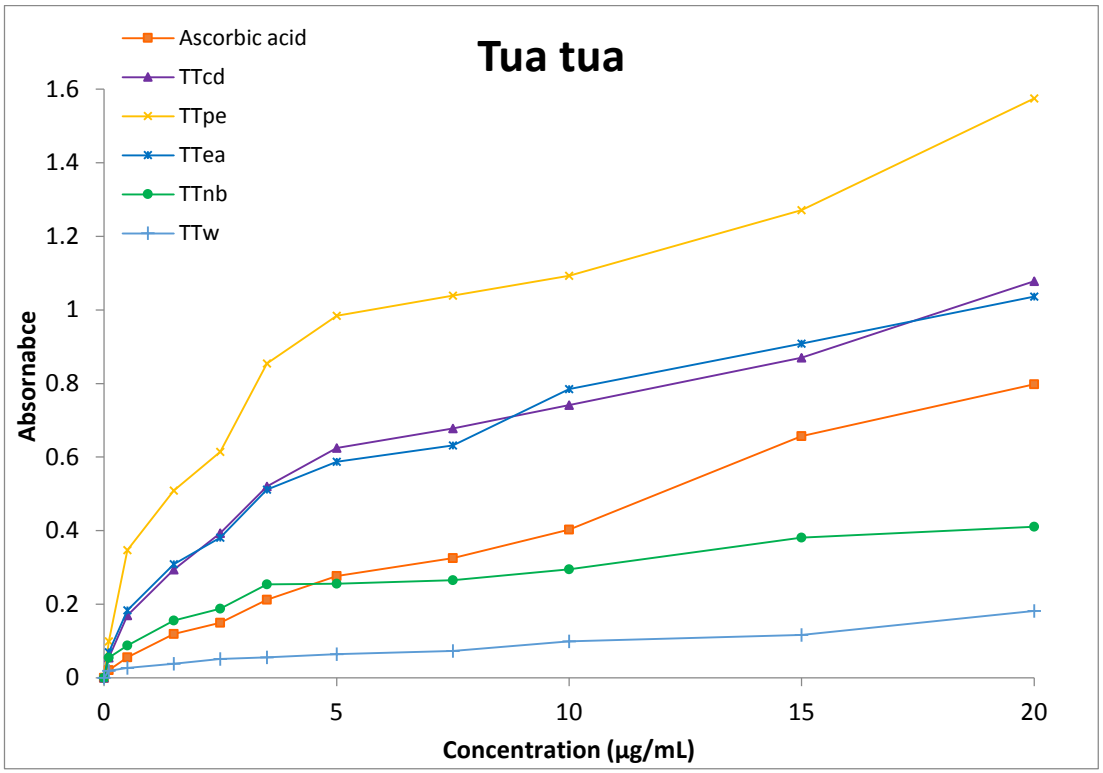


Figure 9: Total antioxidant capacity of Tua tua (crude extract-cd, petroleum ether-pe, ethyl acetate-ea, n-butanol-nb, and aqueous-w fractions), and Ascorbic acid with the CUPRAC reagent. Values are means from three independent tests.

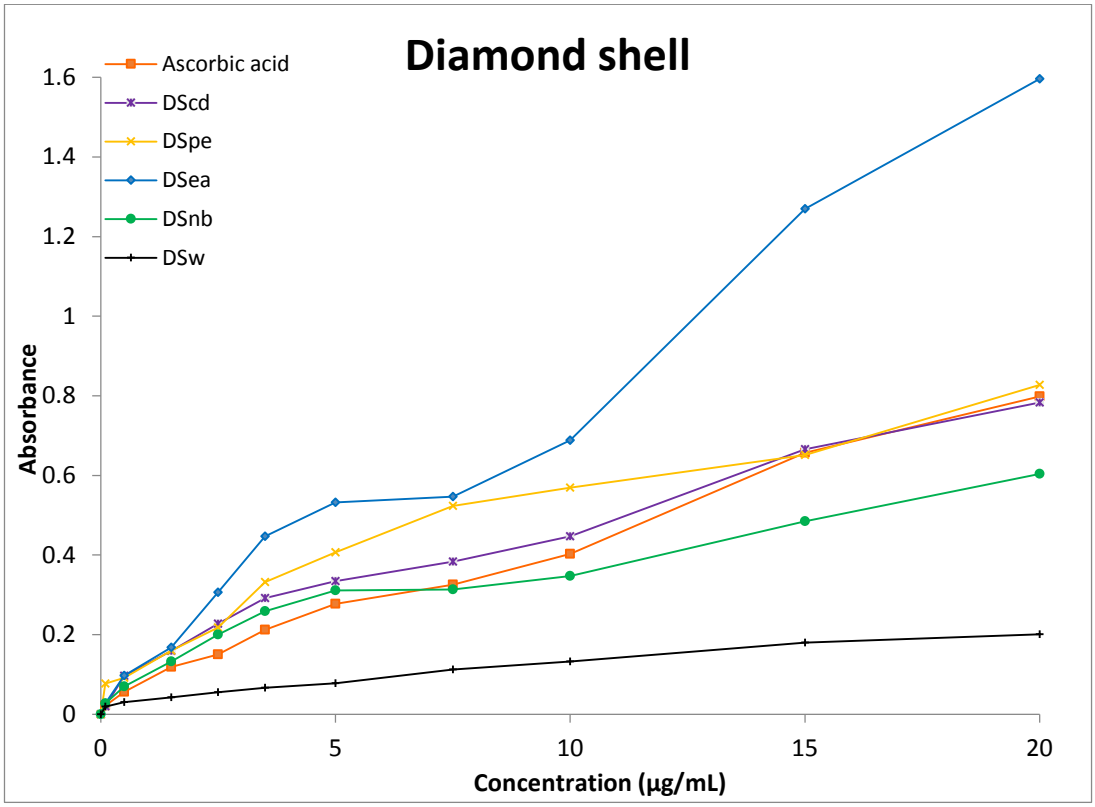


Figure 10: Total antioxidant capacity of Diamond shell (crude extract-cd, petroleum ether-pe, ethyl acetate-ea, n-butanol-nb, and aqueous-w fractions), and Ascorbic acid with the CUPRAC reagent. Values are means from three independent tests.

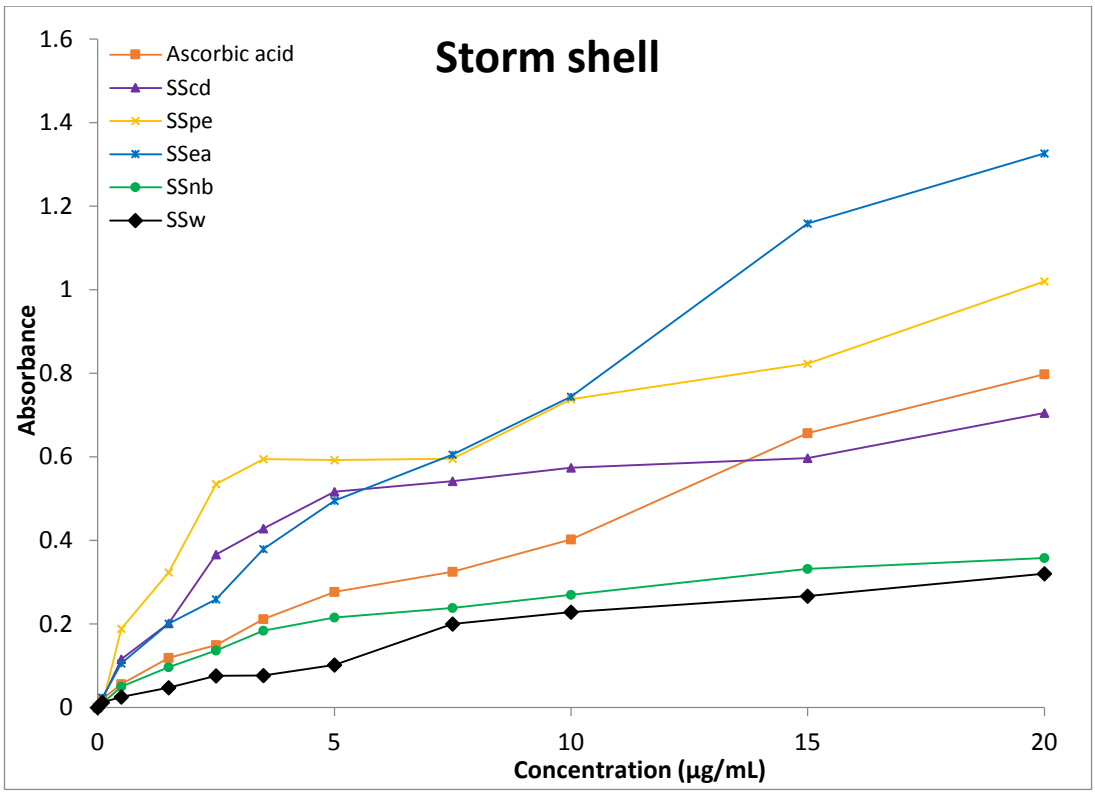


Figure 11: Total antioxidant capacity of Storm shell (crude extract- cd, petroleum ether-pe, ethyl acetate-ea, n-butanol-nb, and aqueous-w fractions), and Ascorbic acid with the CUPRAC reagent. Values are means from three independent tests.

3.4 Statistical Significance

The CUPRAC assay R^2 values (Table 3 below) shows that the regression lines approximate the real data points, and proves that the concentration of a fraction is directly proportional to its antioxidant activity, as the higher the R^2 value, the less variable it is from the ideal relationship of concentration and absorbance.

Table 3: CUPRAC assay R^2 values of surf clam fractions

	Diamond shell ($p<0.0001$)	**Tua tua** ($p<0.0001$)	**Storm shell** ($p<0.0001$)
Crude extract	0.9793	0.9466	0.8701
Petroleum ether fraction	0.9781	0.9369	0.9252
Ethyl acetate fraction	0.9876	0.9535	0.928
n-Butanol fraction	0.9806	0.9267	0.7536
Aqueous fraction	0.9244	0.8346	0.8202

The crude extracts and solvent fractions of all samples were further analysed at a higher concentration of 50µg/mL and then compared amongst each other. The results represented in Figures 12a- 16b below show that the antioxidant activity of fractions tested increased with an increase in concentration.

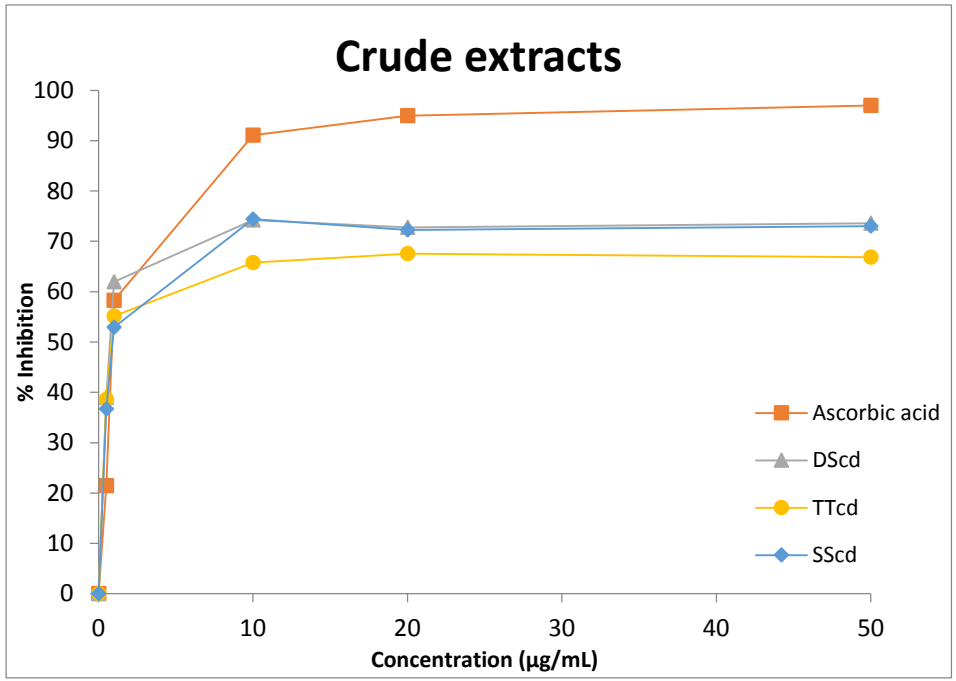


Figure 12a: DPPH radical scavenging activity of all crude extracts

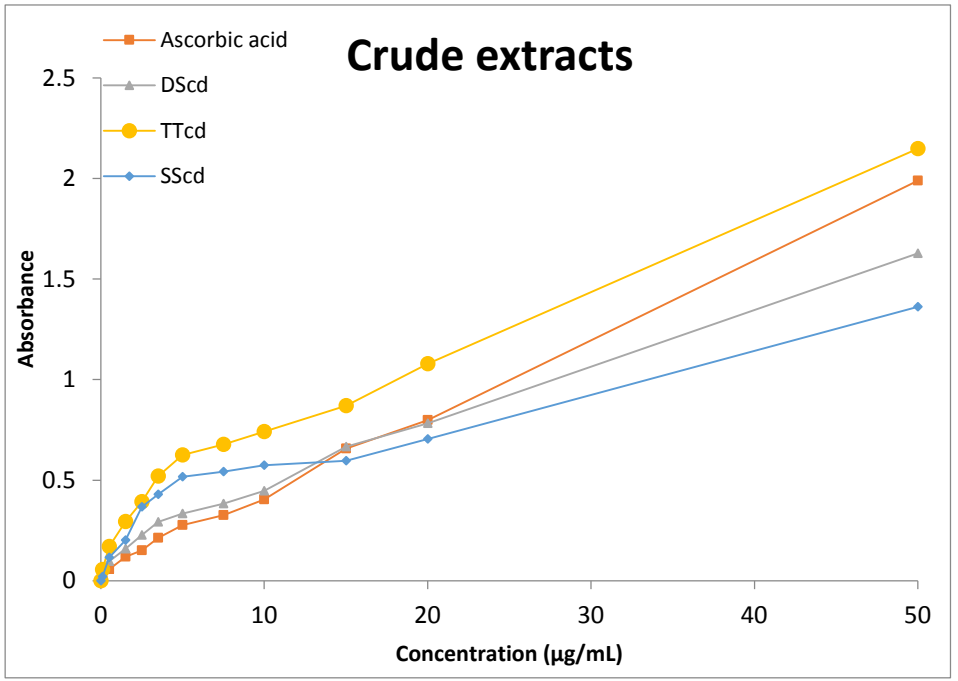


Figure 12b: Copper ion reducing activity of all crude extracts

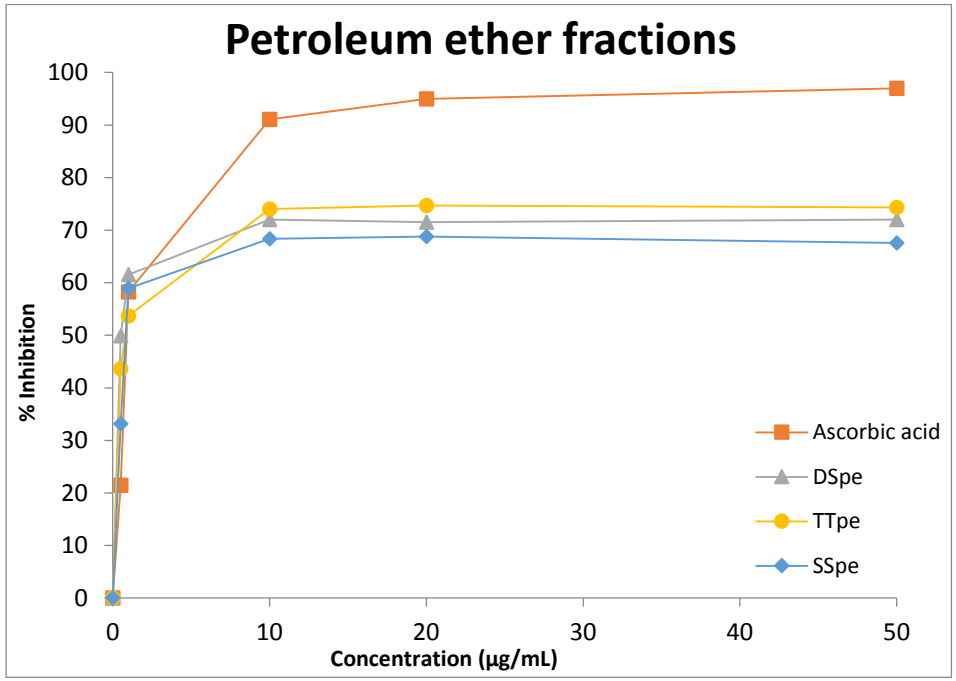


Figure 13a: DPPH radical scavenging activity of all pe fractions

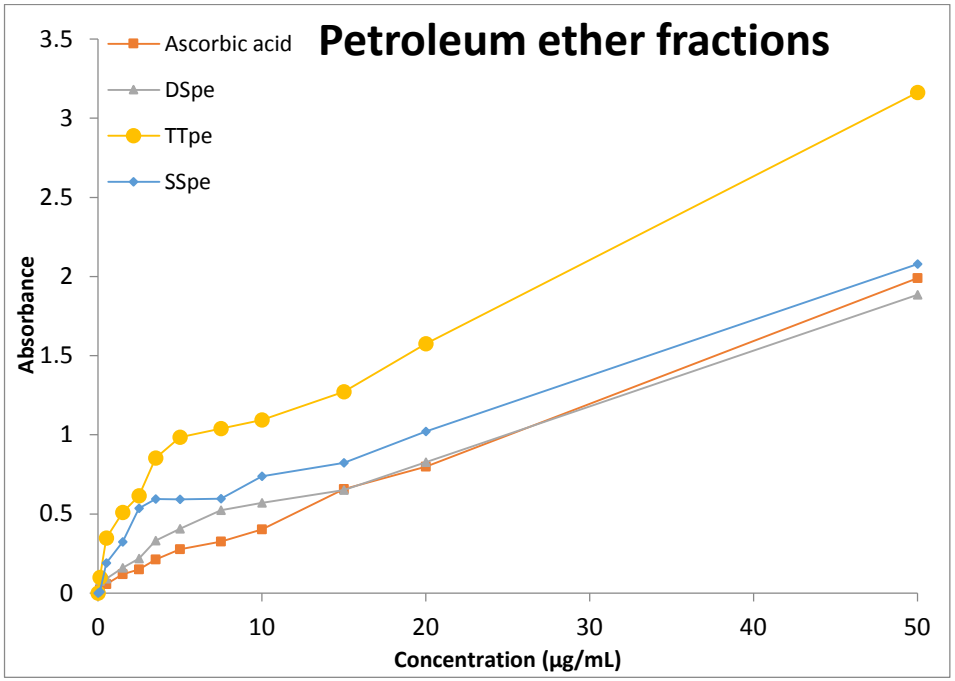


Figure 13b: Copper ion reducing activity of all pe fractions

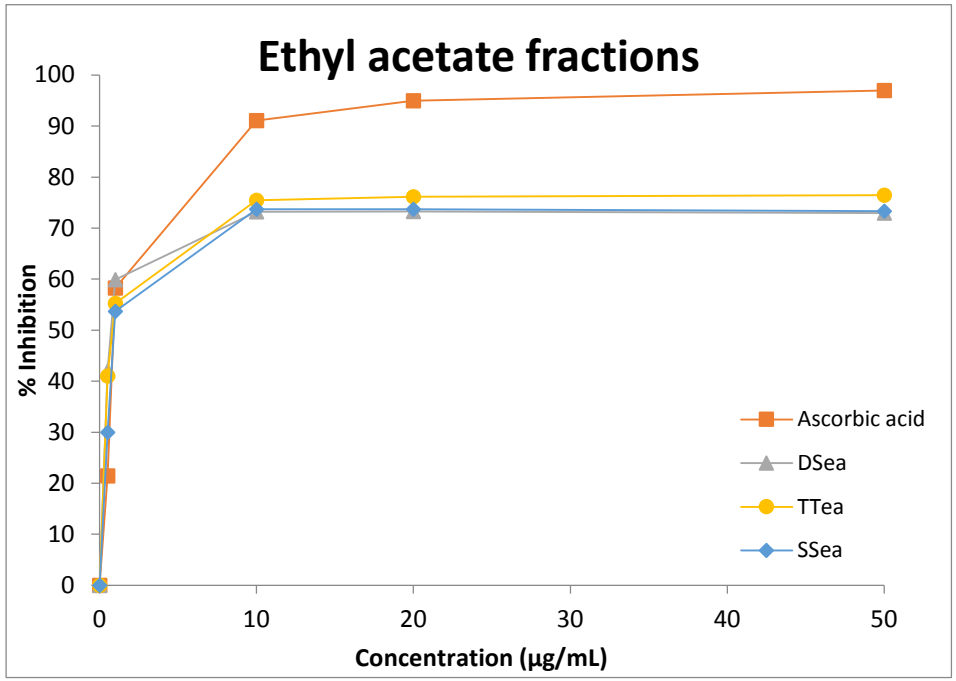


Figure 14a: DPPH radical scavenging activity of all ea fractions

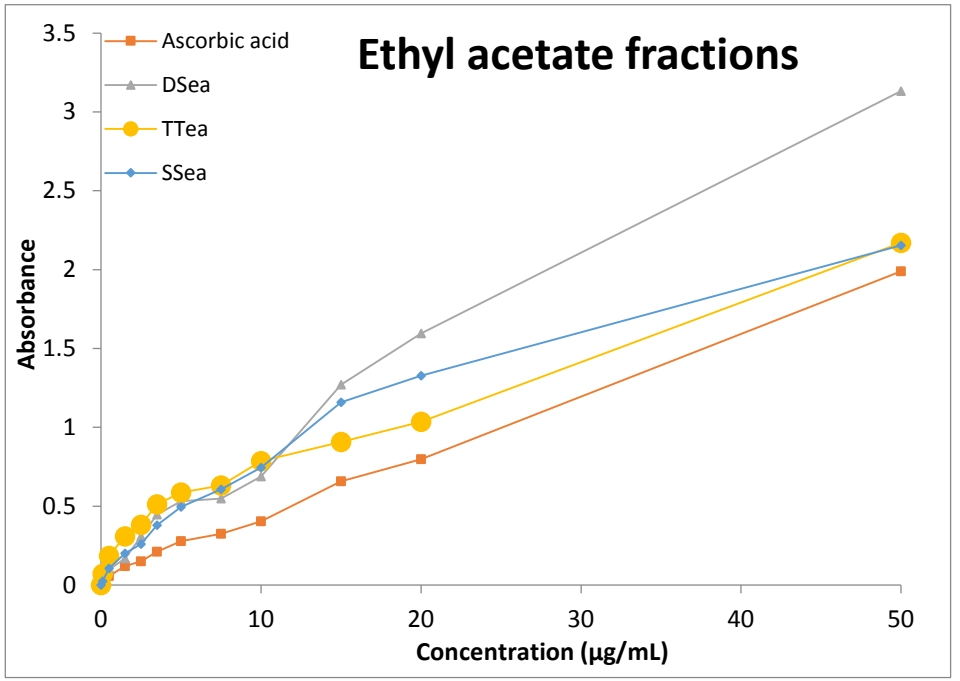


Figure 14b: Copper ion reducing activity of all ea fractions

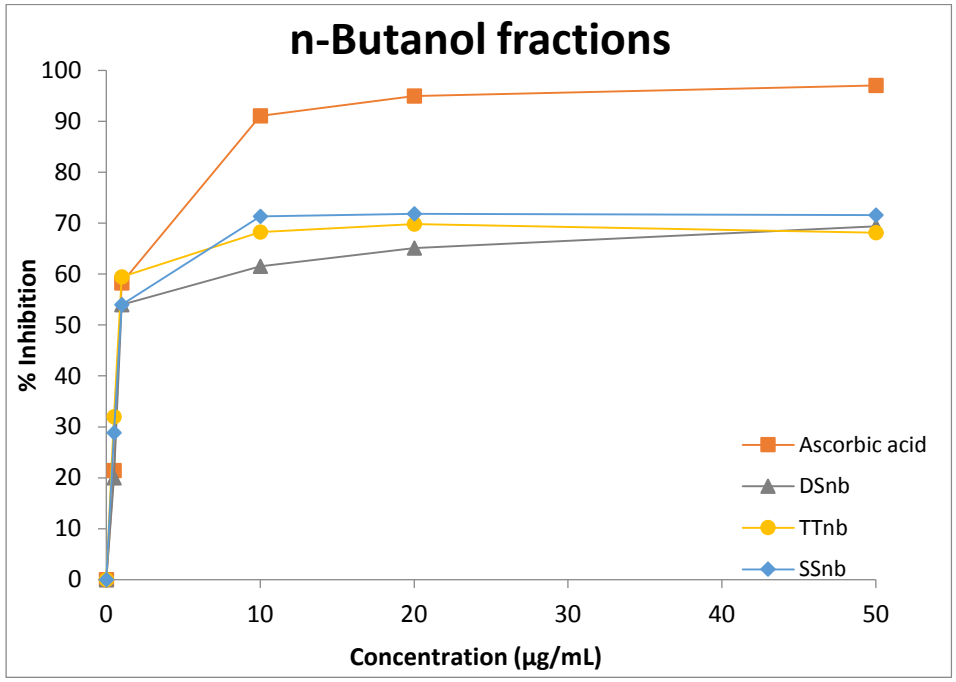


Figure 15a: DPPH radical scavenging activity of all nb fractions

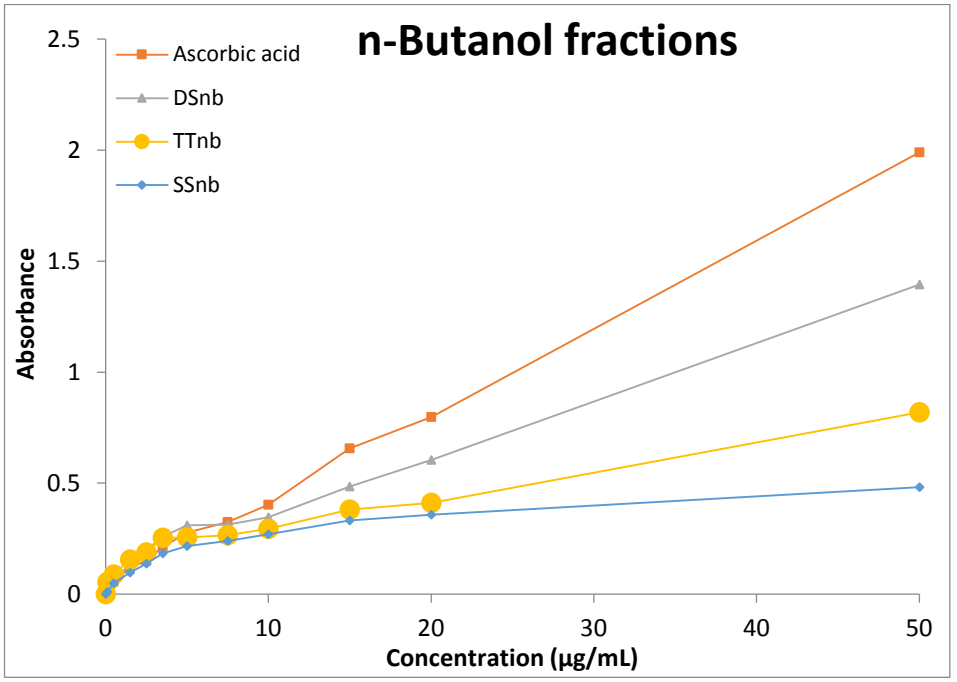


Figure 15b: Copper ion reducing activity of all nb fractions

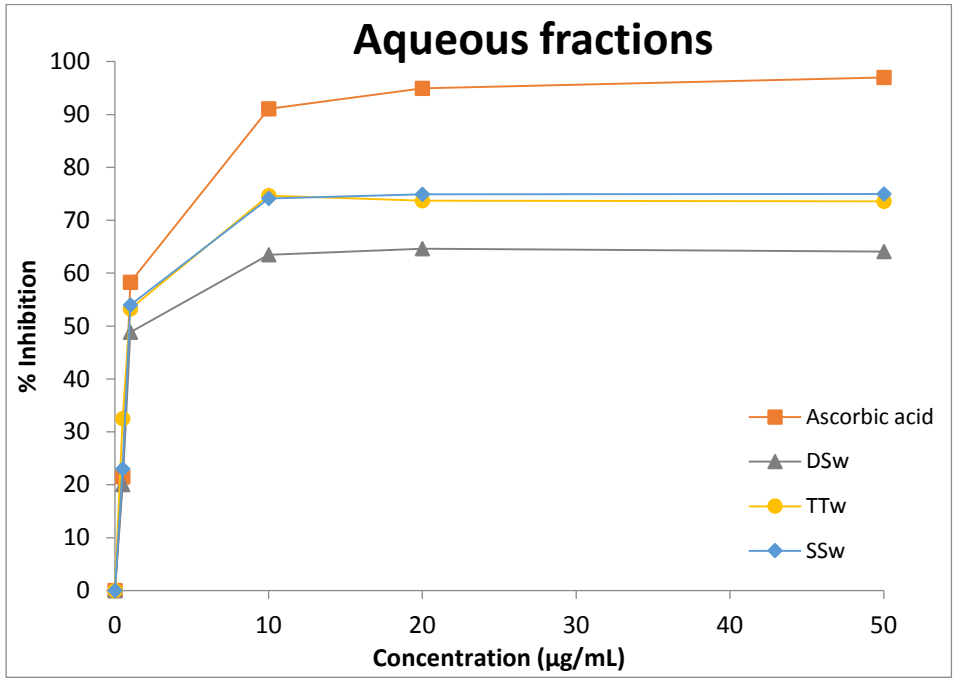


Figure 16a: DPPH radical scavenging activity of all w fractions

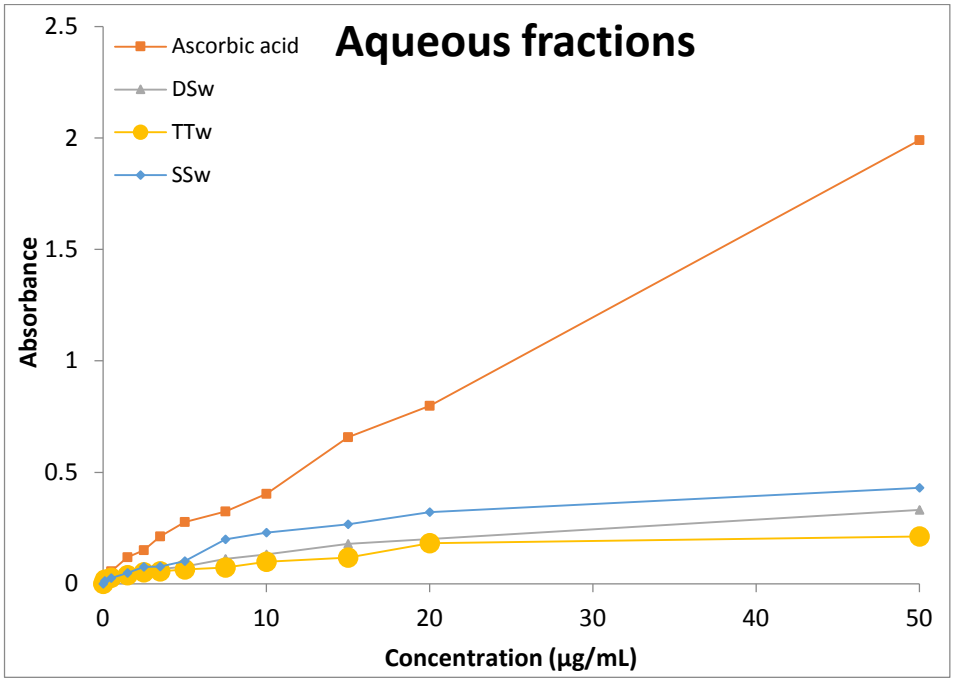


Figure 16b: Copper ion reducing activity of all w fractions

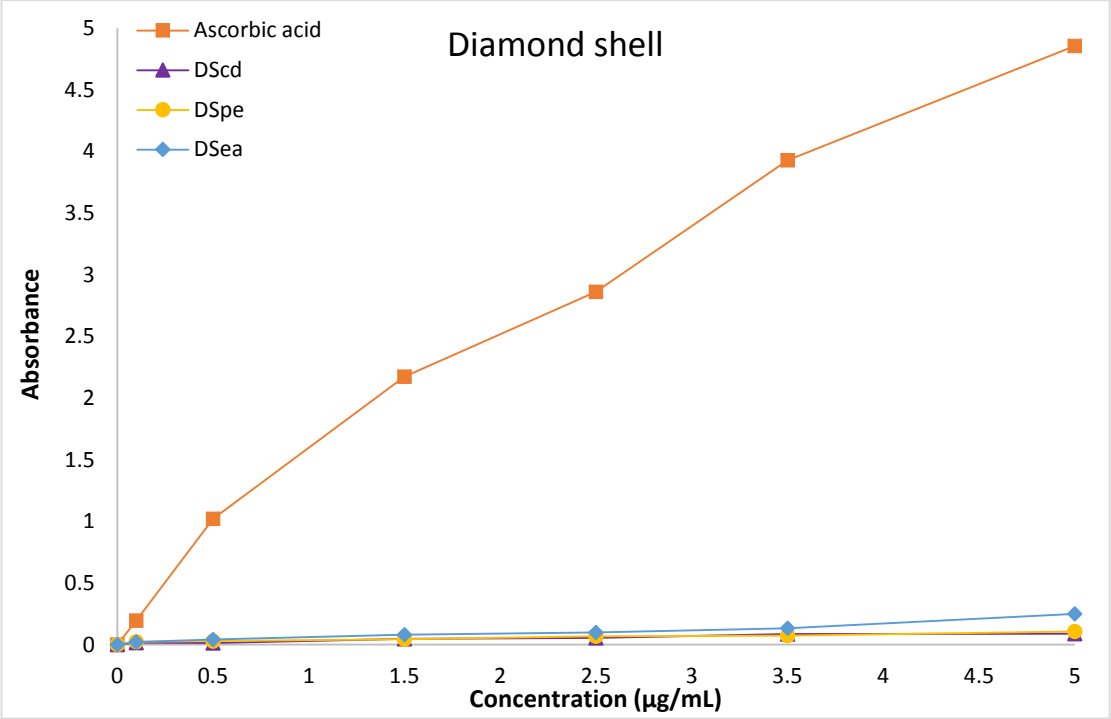


Figure 17: Diamond shell CUPRAC assay: Sample stock solution 10mg/mL; Standard stock solution 10mg/mL.

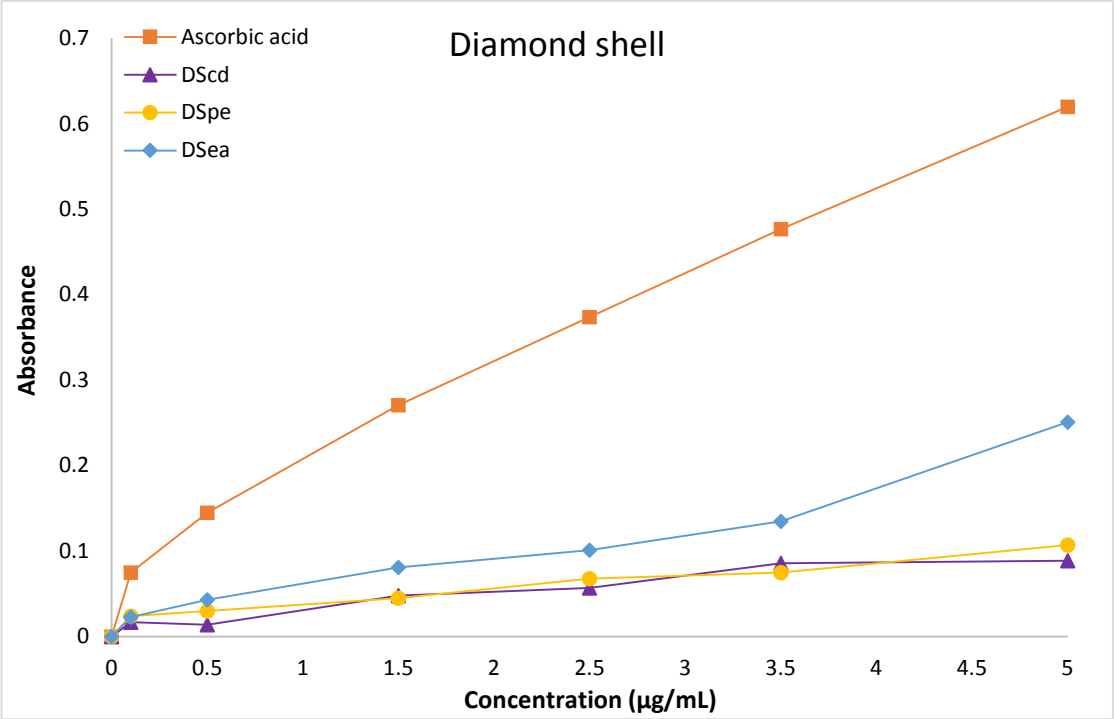


Figure 18: Diamond shell CUPRAC assay: Sample stock solution 10mg/mL; Standard stock solution 1mg/mL.

CHAPTER FOUR

4. DISCUSSION

Free radical and active oxygen species oxidise biological molecules such as lipids, proteins, DNA, and sugars to induce their modification (Wang, Sun, Cao, & Wang, 2010) which could lead to oxidative stress. Shellfish of marine origin have been found to be good sources of antioxidants (Luan et al., 2011). Three New Zealand surf clam species were evaluated for their antioxidant potentials. The extraction of all clam species yielded five clam fractions; cd (crude extract), pe (petroleum ether fraction), ea (ethyl acetate fraction), nb (n-butanol fraction) and w (aqueous fraction). Due to the presence of different components in the crude extracts of biological tissue samples, it was relatively difficult to measure each antioxidant component separately. Therefore, two assays (DPPH and CUPRAC) were employed to evaluate the total antioxidant activity of the three NZ surf clam extracts in this study. The CUPRAC assay measures the ability of reducing antioxidants to reduce copper ions from Cu(II) to Cu(I) utilising bis(neocuproine) (Fung, 2012). Hence, CUPRAC measured the reducing activity of the surf clam extract. In this study, the experimental results showed that all fractions exhibited the ability to reduce copper ions from Cu (II) to Cu (I).

It is well accepted that the antioxidants can scavenge the DPPH radical through hydrogen-donating behaviour (Chen & Ho, 1995). DPPH has been used as a free radical to determinate reducing substances (Cotelle et al., 1996; Luan et al., 2011). All fractions tested exhibited a dose dependent behaviour on DPPH radical scavenging activity within the test range of 0.5- 50µg/mL. This might be due to the fact that the surf clam extracts possibly contained substances which were electron donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction. This data clearly demonstrates that the NZ surf clam extracts have significant antioxidant properties.

4.1 Major Active Compounds in the NZ Surf Clam Extracts

The correlations between the major active compounds in the NZ surf clam extracts with their antioxidant activities have been briefly reported below.

4.1.1 Crude Extracts (cd)

The inhibition of ascorbic acid towards DPPH radicals was higher than that obtained by the crude extracts. (Figure 12a). Conversely, with the CUPRAC assay (Figure 12b), it can be seen that at concentrations between 0.1µg/mL and 10µg/mL, all three crude extracts from the clam species showed higher inhibitions than ascorbic acid, indicating that they possess stronger ability to reduce copper ions.

The component contents evaluation in the surf clam, *Mactra veneriformis*, revealed that the crude extract possessed free amino acids (140.49 ± 5.93µmolg^{-1}) and oligosaccharides (360.52 ± 11.64 µmolg^{-1}) (Luan et al., 2011).

Previous reports have shown that superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) (endogenous antioxidant enzymes) activities increased significantly in plasma from rats fed with Feruloyl oligosaccharides- FOs (the ferulic acid ester of oligosaccharides from wheat bran). The increase in activities of SOD, CAT and GSH-Px in plasma from rats fed coincides with the decrease of the levels of oxidative stress biomarkers (Wang, Sun, Cao, & Wang, 2010). Also, Ou et al. (2007) reported that feeding FOs increased the level of antioxidant capacity and significantly increased SOD and GSH-Px activity in the serum of diabetic rats compared to feeding the sodium ferulate and vitamin C. Furthermore, FOs can enhance the ability of human lymphocytes to resist H_2O_2 induced oxidative DNA damage and inhibit the non-enzymatic glycation of protein in a glucose/BSA system under *in vitro* conditions. Therefore the favourable influence of phenolic compounds on antioxidant enzyme activity merits attention. And this would be very helpful in understanding the mechanism of prevention of chronic diseases by intake of diets rich in phenolic compounds (Wang, Sun, Cao, & Wang, 2010).

The amino acid composition of the *Meretrix casta* peptides showed the presence of both essential and non-essential amino acids including Proline, Glycine, Alanine, Phenylalanine, Histidine, Glutamic

acid, etc. The HPLC analysis for amino acid composition and clam peptides revealed that the quantity of essential amino acids (milligrams of amino acid/gram of protein) in all samples was higher than the recommended values for human adults. These antioxidant active peptides contained glutamic acid, histidine, threonine, arginine in good percentage than remaining amino acids. The presence of amino acids like histidine and methionine might be the reason for good antioxidant activity (Nazeer, Prabha, Kumar, & Ganesh, 2013). Some amino acids such as Tau, Tyr, Met, Cys, and Arg have shown obvious antioxidant activities and constituted 14.59% of the total amino acid contents of *Mactra veneriformis.* Tau could scavenge reactive oxygen species and prevent changes in membrane permeability following oxidant injury (Luan et al., 2011).

Several amino acids such as Tyr, Met, His, Lys, and Trp are generally accepted as antioxidants and the presence of all these amino acids makes the clam peptide a potent antioxidant (Chen, Muramoto, Yamauchi, & Nokihara, 1996; Nazeer, Prabha, Kumar, & Ganesh, 2013).

4.1.2 Petroleum Ether Fraction (pe)

The results showed that the petroleum ether fraction was the strongest DPPH radical scavenger with a 77.25% inhibition (Table 2) and a very strong copper ion reducer, hence, there must be some compounds possessing very strong antioxidant properties occurring in the fraction. The pe results obtained in this study corresponds with *Psoralea corylifolia* L, (Jiangning, Xinchu, Hou, Qinghua, & Kaishun, 2005). Upon analysis, it was observed that the petroleum ether fraction contained a phenolic hydroxyl group and that there is a double bond directly connecting the phenol in the para-position of the phenolic hydroxyl group. This double bond extends the conjugation system of the phenolic oxygen free radical of the extract after donating a hydrogen atom to active free radicals. Hence, this group stabilises the phenolic oxygen free radical a lot and greatly strengthens the antioxidant activities of the compound. In other words, more resonance structures can be formed to stabilize the free radical because of the alkenic group (Jiangning, Xinchu, Hou, Qinghua, & Kaishun, 2005).

According to Luan et al. (2011), the highest content of unsaturated fatty acid was detected in the petroleum ether fraction. The n-3 series unsaturated fatty acid could act as indirect antioxidant in vascular endothelial cells, hence diminishing inflammation, and in turn the risk of atherosclerosis and cardiovascular disease.

The 4, 7, 10, 13, 16, 19-docosahexaenoic acid (DHA) and 5, 8, 11, 14, 17-eicosapentaenoic acid (EPA) lower serum lipid concentration, reduce incidence of CVD and prevent platelet aggregation and they have been used in clinical practice (Luan et al., 2011). It has also been established that these polyunsaturated fatty acids decrease the extent of cell proliferation in cultures of smooth muscle or medial cells (Gavino, Miller, Ikharebha, Milo, & Cornwell, 1981). *In vivo* studies (Gao et al., 2007; Frenoux, Prost, Belleville, &. Prost, 2001) have shown that the consumption of *n*-3 unsaturated fatty acid, especially EPA, lowered blood pressure, improved the total antioxidant activity status and decreased oxidative stress.

Furthermore, cellular evidence for the antioxidant actions of polyunsaturated acids has been provided. Fatty acid micelles scavenged superoxide in an unsaturation dependent manner, up to EPA which was the most effective $O_2^{\bullet -}$ scavenger. In particular, EPA and DHA decreased ROS production by 16.9% and 15.8%, respectively, as compared to stearic acid-supplemented controls. LC-PUFAs, including EPA, are also effective inhibitors of superoxide production by HAEC at more physiological concentrations. The inhibition of NAD(P)H oxidase by DHA and (presumably) other PUFAs might greatly explain the observed effects on ROS production. One other potential mechanism of action of LC-PUFAs would be that they act as a “sink” to trap free radicals, hence, becoming oxidised themselves (Richard, Kefi, Barbe, Bausero, & Visioli, 2008). These and more may be the reasons why the pe fractions of all samples showed very high antioxidant activities, especially with the CUPRAC assay.

4.1.3 Ethyl Acetate Fraction (ea)

At all concentrations evaluated (0.1µg/mL - 50µg/mL), the copper ion reducing abilities of the ea fraction of all three surf clam species were higher than ascorbic acid (Figure 14b). The highest correlation between a clam extract and the CUPRAC assay was found in the ea fraction (R^2 = 0.9876, p <0.0001). Compared with other fractions, the ea fraction (of Tua tua) also had the second highest DPPH radical scavenging ability (Table 2) with a 75.83% inhibition. This result was identical with those of *Hypericum hyssopifolium* (Cakir et al., 2003) where the ea fraction contained the highest amount of total phenolic compounds, which could be accepted as indication of antioxidant potential (Yıldırım, Mavi, & Kara, 2001), and *Polysiphonia urceolata* (Duan, Zhang, Li,& Wang, 2006) in which

the ethyl acetate-soluble fraction showed the most potent activity and indicated that compounds with strongest radical scavenging activity in *P. urceolata* are of medium polarity. Furthermore, the distribution of phenolic compounds in *P. urceolata* demonstrated that the ea-soluble fraction contained the highest amount and a high correlation was found between the total phenolic content and DPPH radical scavenging in *P. Urceolata* (Duan, Zhang, Li, & Wang, 2006).

The phenolic compounds of the ea fraction showed very high antioxidant activity. This could be responsible for its antioxidant activity as the free –OH groups in phenolic compounds have been linked with antioxidant activity (Weng & Wang, 2000; Cakir et al., 2003). It has been reported that if there is an electron donating group, especially a hydroxyl group located on phenolic compounds, their antioxidant activities are increased greatly (Weng, 1993).

The gas chromatography-mass spectrometric analysis of the ea fraction of the freshwater clam showed that it was composed mainly of fatty acids and steroids (Hua et al., 2012). Steroids have been found in plant and animal extracts (Akinmoladun, Ibukun, Afor, Obuotor, & Farombi, 2007) and may be responsible for their medicinal value. Steroids have been reported to be an active compound in *Solanum aculeastrum* where they are showing high antioxidant properties (Koduru, Jimoh, Grierson, & Afolayan, 2007).

Fatty acids, on the other hand, have the ability to augment free radical generation (Das, 1991).

4.1.4 n- Butanol Fraction (nb)

The nb fraction revealed the highest nucleoside contents. And the identification of hypoxanthine, xanthine, inosine and guanosine was determined by high-performance liquid chromatography (HPLC) analysis (Luan et al., 2011). Xanthine is known to have a quenching effect on the production of hydroxyl radicals, as well as on oxidative DNA breakage by hydroxyl radicals. Xanthine bind to copper ions, leading to the reduction of Cu(II) to Cu(I). This leads to a prooxidant action capable of inducing DNA breakage. However, such an activity requires a relatively high concentration of the compounds and copper ions (Azam, Hadi, Khan, & Hadi, 2003). Nucleoside analogues with the α,β-unsaturated keto system, compounds 9, 10 and 11 were potent against the activity of $ROO^{\bullet}$ (Spanou, Manta, Komiotis, Dervishi, & Kouretas, 2007). In their study, Gudkov et al. (2006) stated that purine nucleotides (especially guanosine and inosine) are natural antioxidants and protect DNA *in vitro* from

damage by ROS generated by ionizing radiation and heat. These nucleotides decrease heat-induced generation in aqueous solutions of such ROS as hydrogen peroxide and hydroxyl radical. It was found that guanosine and inosine, when injected intraperitoneally, function as radio-protectors that increase the survival rate of mice exposed to lethal doses of γ-radiation and that they are most effective when injected shortly after irradiation. A considerable radioprotective effect was also observed in mice that consumed nucleosides after irradiation with drinking water. Today, inosine (under the commercial name riboxin) is used in clinical practice for treating for the ischemic heart disease, arrhythmias, myocardial infarction and other pathologies. The conversion of hypoxanthine to uric acid is particularly principal as uric acid is regarded to be an important antioxidant in human adult plasma. It can directly react with free radicals and may interfere with Fenton's chemistry by chelating catalytic metal ions (Žitňanová et al., 2004). However, consistent with previous observations, the nb fraction showed the least antioxidant activity (especially with the CUPRAC assay) (Figure 15b). This might be due to the fact that little correlation was found between the nucleoside contents and the antioxidant activity of the extract, due to their stearic inaccessibility or the fact that nucleosides have higher antioxidant activity *in vivo* than *in vitro* (Luan et al., 2011; Spanou, Manta, Komiotis, Dervishi, & Kouretas, 2007; Gudkov, Shtarkman, Smirnova, Chernikov, & Bruskov, 2006).

4.1.5 Aqueous Fraction (w)

The w fraction of all three clam species showed antioxidant activity. Although this fraction was the least copper ion reducing fraction (CUPRAC assay), it showed relatively high DPPH radical scavenging activity. A spectrum of oligosaccharides was analysed from the aqueous fraction of *Mactra veneriformis*. This mainly consisted of monosaccharide, disaccharide and trisaccharide which positively correlated with its metal ion chelating activities (Luan et al., 2011). In a dose-response relation, agar oligosaccharides exhibited lipid peroxidation inhibition. They also showed antioxidative activities in scavenging superoxide anion and hydroxyl free radical (Wang, Jiang, Mou, & Guan, 2004). *In vitro* antioxidant studies showed that agaro-oligosaccharides expressed different antioxidant abilities. And it is quite significant that the *in vivo* animal experiment for agaro-oligosaccharides is quite consistent with the *in vitro* assays. Besides successful protection of liver damage by efficiently inhibiting MDA formation and decreasing AST and ALT, agaro-oligosaccharides enhance the activities

of antioxidant enzyme system of the host including SOD, GSH-Px etc (Chen, Yan, Zhu, & Lin, 2006). The w fractions of two species (Diamond shell and Storm shell) showed the highest DPPH radical scavenging ability, with 74.73% and 74.48% respectively (Table 2). Interestingly, this agrees with Yuan et al. (2005) and Chen & Yan, (2005) that oligosaccharides showed excellent scavenging activity on DPPH radicals, which may be attributable to its strong hydrogen-donating ability compared to other samples.

4.2 Comparison of antioxidant activity among species

All species tested showed very high antioxidant activity. Tua tua had the highest DPPH radical scavenging activity of 76.14% at 20µg/mL, and the fraction with the least scavenging activity was TTcd with a 67.53% inhibition at 20µg/mL (Figure 6). Storm shell was the next on the DPPH radical scavenging scale, with SSw as its fraction with the highest inhibition, and SSpe as its fraction with the lowest inhibition. SSw had a 74.88% inhibition at 20µg/mL, while SSpe had an inhibition of 68.79% at 20µg/mL (Figure 8). The Diamond shell was the surf clam species with the least DPPH radical scavenging activity. DSw had the highest inhibition, while DSnb had the least with 74.6% and 65.12% inhibitions respectively (Figure 7). Comparing among species, the fractions with the highest inhibition rate would be in this order; TTea > SSw > DSw, and fractions with the lowest inhibition rate as follows; SSpe > TTcd > DSnb. It is interesting to observe that among all three species tested in this study, Diamond shell was the species with the least DDPH radical scavenging ability.

The Diamond shell (which was the species with the least DPPH radical scavenging ability) had the highest copper reducing activity (Figure 14b), with DSea having an absorbance of 1.596 at 20µg/mL. DSw was the fraction that showed the least copper reducing ability, with an absorbance of 0.201 at 20µg/mL (Figure 10). The Diamond shell was closely followed by Tua tua, with TTpe (highest Tua tua copper reducing fraction) having a 1.575 absorbance and TTw (lowest Tua tua copper reducing fraction) a 0.182 absorbance at 20µg/mL respectively (Figure 9). Storm shell was the species with the least copper reducing ability. At 20µg/mL, its highest absorbance was 1.327 (SSea), and its lowest (SSw) 0.321. The order of copper ion reducing activities among surf clam species is DSea > TTpe >

SSea. As a matter of interest, the ea fraction seemed to have very high copper reducing abilities than other fractions tested. The DSea and SSea were the highest copper reducers in the Diamond shell and Storm shell respectively. In Tua tua however, the ea fraction was the second highest copper reducer (after the pe fraction), with an absorbance of 1.036 at 20µg/mL.

4.3 Comparison of antioxidant activity in the NZ surf clam and other marine species and plant products

Compared with other bivalves, the free radical scavenging ability of the NZ surf clam can be said to have very strong antioxidant activity. In contrast with concentrations used in this study, the free radical scavenging ability of most bivalves in many researches was analysed at rather high concentration ranges, e.g. Oyster (*Crassostrea talienwhannensis*) - 0.63 to 20 mg/mL (Dong et al., 2010), Oyster (*Saccostrea cucullata*) - 0.25 to 1mg/mL (Umayaparvathi et al., 2014), Black mussel (*Mytilus galloprovincialis*) - 2mg/mL (Monchevaa et al., 2004), and *Mytilus edulis* - 10mg/mL (Wang et al., 2013). The percentage of free radical scavenging activity of the smooth hound (*Mustelus mustelus*) hydrolysate (76.7% at 3mg/mL) (Bougatef et al. 2009) was significantly lower than the TTea fraction which was 76.45% at 50µg/mL (Figure 14a). Hence, one can say that the NZ surf clam is a potential source for antioxidant activity.

In comparison with seaweed (1000µg), *Sargassum marginatum* (11.00%), *Padina tetrastomatica* (14.78%), *Turbinaria conoides* (17.23%) (Chandini, Ganesan, & Bhaskar, 2008) had relatively lower percentage inhibitions than DSw which had an inhibition of 64.09% at 50µg/mL, and was the fraction with the lowest free radical inhibition (Figure 16a). At 2mg/mL, *Undaria pinnatifida* (neutrase extract) had a percentage inhibition of 57.08% (Je et al., 2009), while *Gelidiella acerosa* had an inhibition of 72.5% at 100µg/mL (Devi, Suganthy, Kesika, Pandian, 2008). These results were attained by most clam fractions at concentrations less than 50µg/mL. TTpe, TTea, TTw, SScd, SSea, SSnb, SSw and all DS fractions had over 70% inhibition at a concentration as low as 20µg/mL, whereas the F1, F2 and F3 fractions of the New Zealand *Undaria pinnatifida* (Harvey) Suringar required a concentration of 4000µg/mL to reach similar percentage (Mak, 2012).

At concentrations of 10, 20 and 30µg/mL, the kiwifruit had DPPH radical scavenging percentages of 4.9, 14.8 and 16.7% respectively (Bursal & Gülçin, 2011), which was nowhere close to any of the fractions tested in this study at 0.5µg/mL. An earlier study on Apples reported that the maximal percentage inhibition of 69% was reached at a concentration of 1mg/mL (Chaudhary et al., 2006), which was attained by most fractions, including all ea and pe (with the exception of SSpe which had an inhibition of 68.79%) fractions at 20 µg/mL (Figures 13a & 14a). In another study (Leontowicz et al., 2003), the apple pulp extract at a concentration of 5 mg/mL was reported to quench only 54.4% of DPPH radicals, and Pear pulp extract at concentrations of 2 and 5 mg/mL quenched only 6.5 and 10.8% of DPPH radicals, respectively. It is evident that the NZ clam extracts showed very strong proton donating ability and could serve as free radical inhibitors or scavengers, and could possibly act as primary antioxidants.

Unfortunately, the CUPRAC assay values could only be compared with a couple of studies due to more common application of other assays in other studies. One of such studies (Fung, Hamid, & Lu, 2013) reported that in July (the month with the highest copper reducing activity), processed New Zealand *U. pinnatifida* had an absorbance of 0.93, while fresh *U. pinnatifida* had an absorbance of 1.41. These absorbances were obtained at 1mg/g, which was several times higher than concentrations used in this study. Boğa, Hacıbekiroğlu, & Kolak, (2011) also reported the copper ion reducing ability of some edible plants. At 100µg/mL, the dichloromethane extracts of Celery, Spinach, Red cabbage and Cinnamon had absorbance values of 0.369, 0.171, 0.729, and 1.073 respectively. These values were very low compared to those obtained in this study, as they were exceeded by all cd, pe and ea fractions at 50µg/mL. Thus the NZ surf clam, compared with edible plants and marine algae, possess higher copper reducing abilities

4.4 Correlations

The results revealed that most fractions tested had very high copper reducing activity. Comparing between clam species, the Diamond shell had the highest correlation, as all DS fractions had higher R^2 values than the same fraction in other clam species. The DSea fraction had the highest correlation of all surf clam fractions, with an R^2 value of 0.9876.

Interestingly, the aqueous fractions of both the Diamond shell and Tua tua species were the fractions with the least correlation, with R^2 values of 0.9244 and 0.8346 respectively. This is indicated by the antioxidant activity of the fractions, as they (DSw and TTw) were the fraction samples with the least antioxidant activity in the two species (Figures 9 and 10).

Among all fractions tested, the least linear correlation based on concentration versus absorbance was found in the SSnb fraction with an R^2 value of 0.7536, followed by the SSw and TTw fractions with R^2 values of 0.8202 and 0.8346 respectively. It should be known, however, that the R^2 value does not always indicate the adequacy of a model (Frost, 2013). This is true with the SSnb fraction which had the least R^2 value of 0.7536, yet had higher absorbance at concentrations between 0.1 and 20µg/mL than SSw (Figure 11), which had an R^2 value of 0.8202. The same is also true for the pe and ea fractions of the Tua tua species. TTpe showed higher copper reducing activity than TTea (Figure 9), but had lower linear correlation (R^2 value = 0.9369) than TTea (R^2 value = 0.9535).

In this study, a nominal two sided $p < 0.05$ was used to assess significance. A decrease in the p value of an experiment indicates an increase in its significance. The results show that all fractions tested with the CUPRAC assay were statistically significant, with $p < 0.0001$ in all three clam species. With the DPPH assay, results showed that surf clam species also had significantly high radical scavenging ability- Diamond shell ($p < 0.0001$) Storm shell ($p < 0.0001$), and Tua tua ($p = 0.017$). Thus the observed copper reducing and DPPH radical scavenging abilities actually reflect characteristics of the New Zealand surf clam rather than just sampling errors.

4.5 Differences in DPPH scavenging activity and CUPRAC reducing activity

Comparing the DPPH radical scavenging capacity of the New Zealand surf clam to their copper ion reducing ability, it can be seen that all samples possessed higher copper ions reducing abilities than radical scavenging, with most fractions showing higher reducing values than ascorbic acid. This might be due to the difference in stock solution concentrations used, not necessarily because the extracts are better copper ion reducers than DPPH radical scavengers (See 5.1 - Caveats). Another reason for

the differences in the radical scavenging ability and copper reducing ability of the surf clam could be due to the selectivity of antioxidants that react differently in each assay. The CUPRAC assay, moreover, can simultaneously measure hydrophilic and lipophilic antioxidants, whereas the DPPH radical scavenging assay is solvent dependent (Prior et al., 2005; Fung, 2012).

The differences in antioxidant activities of each fraction (or active compounds in each fraction), moreover, may be due to their solubility effect. Phenolic compounds of the ethyl acetate fraction have been reported to be soluble in oil phase, hence the very high antioxidant activity (Figures 14a & b). Lower antioxidant activities of other fractions (which naturally choose to move water phase in an oil-in-water emulsion) may be due to this solubility effect (Cakir et al., 2003). Compared to all other fractions tested, a very lower copper ion reducing rate was observed in the w fraction. This could be due to the fact that enzymatic extraction is aqueous based and may not be as effective in extracting the active principles like in the case of solvent extraction (Ganesan, Kumar, & Bhaskar, 2008).

CHAPTER FIVE

5. CONCLUSION

5.1 Caveats

Sample stock solutions for the DPPH assay were prepared at a very low concentration of 1mg/10mL to investigate the strength of the surf clam antioxidant capacity.

Low working sample concentrations were also used (0.1µg/mL- 20µg/mL) as opposed to those of previous studies (Ganesan, Kumar, & Bhaskar, 2008; Cakir et al., 2003; Srivastava, Harish, & Shivanandappa, 2006; Mak, 2012). Interestingly, the results have shown that the three New Zealand surf clams tested have very high DPPH radical scavenging activity at such low concentrations. This (low working sample concentration) may be one of the reasons why none of the fractions tested had higher antioxidant abilities than ascorbic acid in the DPPH test. A higher concentration of the stock solution (and perhaps higher working sample solution concentrations) would probably have shown higher scavenging activity. Furthermore, reports (Zhou et al., 2012) have shown that an increase in incubation time is directly proportional to an increase in antioxidant activity. In this study, incubation time was 30 minutes. An increase in incubation time might have yielded higher percentage inhibition values.

DPPH assay is based on kinetic derived process, that is, it may take one minute (e.g. ascorbic acid) to more than 180 min (e.g. curcumin) and therefore the assay should be performed in kinetic mode and estimation of antioxidant capacity of unknown antioxidant compounds must be counter-measured by using other antioxidant assays e.g. ABTS, FRAP, ORAC, etc (Mishra, Ojha, & Chaudhury, 2012).

The different relative radical scavenging capacity of individual clam fractions against different DPPH radicals may be explained by the different mechanisms involved in the radical–antioxidant reactions. Other factors, such as stereoselectivity of the radicals or the solubility of the clam extracts in different testing systems, may also affect its capacity to react and quench different radicals. The mechanisms involved in the beneficial actions of antioxidants in biological systems included directly quenching of free radicals to terminate the radical chain reaction, chelating transition metals to suppress the

initiation of radical formation, acting as reducing agents, or stimulating the antioxidative defence enzyme activities (Monchevaa et al., 2004).

On the other hand, with the CUPRAC assay, an initial standard solution of 10mg/mL against a 10mg/mL sample stock solution was used. But the results showed very little to no effect vis-à-vis copper ion reduction (Figure 17). The standard stock solution was reduced to 1mg/mL. Again, results showed a minuscule reducing effect (Figure 18). To obtain results within the standard curve range, the concentration of sample stock solution was increased. A sample stock solution of 50mg/mL was finally used and results showed very high copper ion reducing effect.

Processed food have been found to contain fewer antioxidant vitamins than fresh and uncooked foods, as preparation exposes food to heat and oxygen (Henry & Heppell, 2002). The New Zealand surf clam, like most foods, might have had some vitamins destroyed, or some enzymes deactivated due to heat processing. There might have been a significant increase in the antioxidant activities of the New Zealand surf clam if they were not oven dried.

In this study, whole soft dried parts of surf clams were used. This implies that different tissues with different antioxidant capacities were mixed together, without distinguishing what is happening in a particular tissue, for instance, in the digestive gland, gills (Monchevaa et al., 2004), foot, mantle, mouth or viscera.

It should be known that many natural antioxidants are less potent than synthetic antioxidants but they can be used at higher concentrations than the synthetic ones, due to the very restrictive toxicological parameters of the latter (Kim, Je, & Kim, 2007). Thus, consuming foods possessing natural antioxidants like the surf clam could deliver desirable nutritional and functional properties.

5.2 Future Research Directions

This *in vitro* study has shown that the New Zealand surf clam have antioxidant effects, with some fractions more potent than the ascorbic acid standard. The significantly potent antioxidant effects was

found in DPPH free radical scavenging, and in reducing copper ion. This indicates that the clams may possess some novel compounds with antioxidant activities. It would be of great research interest to further elucidate the exact molecular structures responsible for its antioxidant activities.

The New Zealand surf clam extracts showed higher antioxidant activities than many seaweed, bivalves and marine products. This could be due to the fact that the NZ surf clam contained peptides with higher antioxidant activities than other most samples. A post-column HPLC and the application of nuclear magnetic resonance (NMR) spectroscopy could be used to analyse a wide range these endogenous metabolites from surf clam tissues, detect and obtain more structural information on any polysaccharides present that may be responsible for the clams' high antioxidant ability. In order to identify new compounds, moreover, gel permeation chromatography- based separation could be employed.

Surf clam extracts have been reported to have therapeutic effect on Leukaemia 1210 in mice, and proven to inhibit the growth of other human cancer cells (Pan, Huang, Chan, Ho, & Pan, 2008; Ruggieri, 1975). It would be useful to test the anticancer/ antitumor activity of the New Zealand surf clam on human cancer cell lines, especially breast, lung, melanoma and colorectal cancer cell lines, as these have been reported to have significantly increased in New Zealand (Ministry of Health, 2008).

It has been reported that for a number of compounds tested for DPPH free radical scavenging ability, the reactions are slow with very complex mechanisms (Bondet, Brand-Williams, & Berset, 1997). Further research to help understand the mechanism of action of the surf clam vis-à-vis its DPPH scavenging activity could also be carried out.

Among all the oxidative radicals, hydroxyl radicals are reported to be the most reactive free radicals formed in biological systems. They can almost react with all the substances in the living cells and induce severe damage to the cells (Luan et al., 2011). Hydroxyl radical scavenging is therefore said to be the most effective method to estimate the behaviour of antioxidants. It would be highly advantageous if the hydroxyl radical scavenging activity of the surf clam is also tested.

5.3 Overall Conclusion

In conclusion, the surf clams from Cloudy Bay Clams, New Zealand, were found to have great antioxidant potential for pharmaceutical developments. The Diamond shell, Tua tua and Storm shell showed high DPPH radical scavenging and copper reducing properties.

The broad range of antioxidant activity of the extracts indicates the potential of the clam as a source of natural antioxidants or nutraceuticals with potential application to reduce oxidative stress with consequent health benefits.

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