CHAPTER-5: IN VITRO ANTIOXIDANT ACTIVITIES

5.1. INTRODUCTION

A function of marine biodiversity is a unique source of novel natural products, also known as secondary metabolites. The ocean enclose very stable chemical and physical environment where nutrients are readily accessible and constantly being recycled, thus contributing to the development of the high biodiversity inherent in this environment. Marine organisms (invertebrates, algae and micro-organisms) utilize their secondary metabolites as a form of chemical defence against predators or in a chemically mediated response to inter-species competition for nutrients or space on a coral reef. Not surprisingly, many chemists in the 1960’s turned to marine organisms, than a previously unexplored source of chemical diversity for possible new drug leads. Free radicals are known to be generated through biological and environmental interactions (Ames et al., 1993) and they have been implicated in more than one hundred disease conditions in humans, including cardiovascular, brain and ocular dysfunctions, arthritis, ischemia and reperfusion carcinogenesis, and AIDS (Halliwell et al.,1992). Antioxidants are chemical substances which can scavenge free radicals and are implicated in the prevention of heart diseases, cancer, ageing etc. Gastropods have not been utilised and screened for their antioxidant potential.

Over the last five decades, the collaborative efforts among chemists and pharmacologists have yielded numerous marine natural compounds for the pharmaceutical industry, particularly novel anticancer drugs. Andersen and Williams (2000) the complexity and uniqueness of many marine natural products isolated so far, reflects possible novel biosynthetic pathways or evolutionary adaptations of ubiquitous pathways in marine organisms, resulting from the enormous evolutionary
time frame in which these pathways have been able to evolve in the unique environment provided by the sea.

Antioxidants may have a positive effect on human health since they can protect human body against deterioration by free radicals and reactive oxygen species (ROS), including singlet oxygen, hydrogen peroxide, superoxide anion, and hydroxyl radicals. ROS and free radicals attack macromolecules such as DNA, proteins and lipids, leading to many health disorders including inflammatory, aging, diabetes, neurodegenerative, cardiovascular and cancer diseases (Butterfield et al., 2006 and Ngo et al., 2011).

The antioxidant activity of bioactive peptides derived from marine has been determined by different in vitro methods, such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), carbon-centered, hydroxyl and superoxide anion radical scavenging activities which have been detected by Electron Spin Resonance (ESR) spectroscopy method as well as intracellular free radical scavenging assays. The beneficial effects of antioxidant marine bioactive peptides are well known in scavenging ROS and free radicals or in preventing oxidative damage by interrupting the radical chain reaction of oxidation (Ravindra Tanaji Pawar et al., 2013).

The anti-oxidative activity has been confirmed contributing different kinds of cancer and inflammatory preventions among its multiple functional roles. The production of oxidants is a typical event associated with aerobic metabolism. When oxygen is supplied in excess, Reactive Oxygen Species (ROS) or free radicals such as superoxide anions, hydroxyl radicals and hydrogen peroxide are generated (Kris-Etherton et al., 2004). Accumulation of the free radicals in body organs can cause oxidative damage to biomolecules and membranes of cell, eventually leading to
many chronic diseases and degenerative diseases (Wang et al., 2004). In the last 50 years, antioxidant activities of extracts from medicinal or food additives have been extensively investigated.

Many pharmacological studies reported that extracts of some marine organisms possess anti-inflammatory, anti-allergic, anti-tumor, anti-bacterial, anti-mutagenic and anti-viral activities to a greater or lesser extent (Schinella et al., 2002). Free radicals liberated from phagocyte cells are important in inflammatory processes, because they are implicated in the activation of nuclear factor κB, which induces the transcription of inflammatory cytokines and cyclo oxygenase 2 (Winrow et al., 1993).

To retard peroxidation processes in food, many synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tertiary butylhydroquinone (TBHQ) and Propyl Gallate (PG) have been used. However, the use of these synthetic antioxidants must be strictly controlled due to their potential health hazards. Hence, search for natural antioxidants as safe alternatives to synthetic products is an important in the food industry. Recently, the use of natural antioxidants available in food and other biological substances has attracted significant interest due to their presumed safety, nutritional and therapeutic values (Park et al., 2001; Pena and Xiong, 2001 and Ajila et al., 2007).

Antioxidant activities of bioactive peptides are mainly due to the presence of hydrophobic amino acids, some aromatic amino acids and histidine. Gelatin peptides are rich in hydrophobic amino acids, and the abundance of these amino acids favours a higher emulsifying ability. Hence, marine gelatin peptides possess higher antioxidant effects than peptides derived from other proteins because of the high
percentage of glycine and proline (Mendis et al., 2005). Compounds isolated from molluscs were also used in the treatment of rheumatoid arthritis and osteoarthritis (Chellaram and Edward, 2009).

Literature survey revealed the urgent need to explore marine molluscs and not much work has been carried out in *K. ornata*. Hence, the sea slug molluscs are chosen for the present study with well defined executable objectives. So the antioxidant property of the sea slug may have great potential use in pharmaceuticals, nutraceuticals and it act as substitute for synthetic antioxidants.

During normal metabolic processes or due to the exogenous factors and agents, reactive oxygen species (ROS) in the forms of superoxide anion radical (O$_2^-$), hydroxyl radical (OH), hydrogen peroxide (H$_2$O$_2$) may be generated. Formation of ROS can cause oxidative damage to human cells, leading to various diseases such as diabetes, cardiovascular diseases, inflammatory conditions, cancer and ageing (Joyce, 1987; Velioglu et al., 1998).

5.2. MATERIALS AND METHODS

5.2.1. *In vitro* antioxidant Assays

5.2.1.1. DPPH Radical Scavenging Activity

The scavenging effects of samples for DPPH radical were determined according to reference Blois, (1958) with a slight modification. The sea slug *K. ornata* crude and fraction were taken in different concentration (20, 40, 60, 80 and 100 μg/ml) and mixed with DMSO. The 1.5 ml of 0.2 mM DPPH in ethanol and mixed with different concentration of test samples. The mixture was mixed vigorously and allowed to stand at room temperature in the dark for 30 min. the absorbance of the resulting solution was measured at 517 nm. The change in absorbance with respect
to the control (containing DPPH only without sample, expressed as 100% free radicals) is calculated as percentage scavenging. Ascorbic acid (20, 40, 60, 80 and 100 μg/ml) was used as standard antioxidant. Scavenging activity was expressed as the percentage inhibition.

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\text{Percentage of inhibition} = \left( \frac{O.D \text{ of control} - O.D \text{ of test}}{O.D \text{ of control}} \right) \times 100
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5.2.1.2. Hydroxyl Radical Scavenging Activity

Hydroxyl (OH) radical assay was performed according to the modified method of Halliwell and Gutteridge, (1989). The different concentration of sea slug crude and fraction were mixed with reagents consisted of 0.1 ml of 10 mM FeSO₄, 0.1 ml of 10 mM EDTA, 0.5 ml of 10 mM α-deoxyribose, 0.9 ml of sodium phosphate buffer (pH 7.4) and 0.2 ml. H₂O₂ (0.2 ml, 10 mM). The reaction mixture was incubated at 37°C for 1 hrs. 1.0 ml of 2.8% trichloroacetic acid (TCA) and 1.0 ml of 1.0% thiobarbituric acid (TBA, in 50 mM aqueous NaOH) were added and boiled for 15 min. After cooling, the absorbance of the mixture was measured at 532 nm. DMSO with samples used as a blank, ascorbic acid. (20, 40, 60, 80, 100 μg/ml) was used as standard antioxidant. Hydroxyl (OH) radicals scavenging activity was evaluated as the inhibition rate of α-deoxyribose oxidation by hydroxyl radical.

5.2.1.3. Nitric Oxide Scavenging Activity

Nitric oxide scavenging activity was determined according to the method reported by Green et al., 1982 and Marcocci et al., 1994. The Nitric oxide scavenging activity was measured by using ‘Griess’ reagent 2 ml of 10 mM sodium nitro prusside in 0.5 ml phosphate buffer saline pH 7.4 was mied with 0.5 ml of extract at various concentration of sea slug crude and fraction and mixture incubated at 25°C for 150 min. From the incubated mixture 0.5 ml was taken out and added into 1.0 ml
sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. Finally 1.0 ml naphthylethylene diamine dihydrocholoride (0.1% w/v) was mixed and incubated at room temperature for 30 min before measuring the absorbance at 540 nm was measured with a spectrophotometer. The nitric oxide radicals scavenging activity was calculated. Ascorbic acid (10, 20, 25, 50 and 100 μg/ml) was used as standard antioxidant.

5.2.1.4. Reducing power assay

The reducing powers of the sea slug crude and active fractions were determined according to reference of Oyaizu, (1986). The sea slug crude and active fractions were taken in different concentration (20, 40, 60, 80 and 100 μg/ml). Phosphate buffer (2.5 Ml, 0.2M, pH 6.6) and potassium ferricyanide (K₃ Fe (CN)₆) (2.5Ml, 1%)were added to the test samples. The mixed samples were incubated at50°C for 20 min. a portion (2.5 ml) trichloro acetic acid (10%) was added to the mixed test samples, followed by centrifugation at 1000rpm for 10 min. the upper layer (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride and the absorbance was measured at 700 nm. Ascorbic acid ((10, 20, 25, 50 and 100 μg/ml) was used as standard antioxidant. Higher absorbance of the reaction mixture indicated greater reducing power.

5.2.1.5. Hydrogen peroxide (H₂O₂) scavenging activity

H₂O₂ scavenging activity was determined according to the method of Muller, (1985). The different concentration of sea slug crude and fractions 30 μl were mixed with reagents consist 100 μl of 0.1 M phosphate buffer (pH 5.0). then, 20 μl of 2 M H₂O₂ was added to the mixture, followed by incubation at 37°C for 5 min. after incubation, 30 μl of 1.25 mM ABTS and peroxide (1 unit/ml) each were added to the mixture, followed by further incubation at 37°C for 10 min. after incubation the
absorbance of mixture was measured at 405 nm. DMSO with sample was used as a blank. A tocopherol (20, 40, 60, 80, 100 µg/ml) was used as standard antioxidant.

5.3. RESULTS

5.3.1. DPPH Radical Scavenging Activity

DPPH is a free radical compound and has been widely used to test the free radical scavenging abilities on various concentrations. The results of the DPPH radical scavenging activity analyses are presented in (Fig. 27.). In this assay crude and fraction of sea slug *K. ornata* showed powerful scavenging activity. Crude extract showed 51.4±0.52 (20 µg/mL) to 65.27±0.42 (100 µg/mL) whereas fraction showed 53.52±0.41 (20 µg/mL) to 74.76±0.33 (100 µg/mL) scavenging activity in various concentrations.

5.3.2. Hydroxyl Radical Scavenging Activity

In hydroxyl radical scavenging assay crude and fractions of sea slug *K. ornata* showed high hydroxyl radical scavenging activities (Fig. 28.). The scavenging activities were oriented with increasing sea slug concentration. In this the Crude extract of sea slug *K. ornata* exhibited 56.9±0.67 (20 µg/mL) to 73.65±0.45 (100 µg/mL) whereas fraction yielded 51.27±0.36 (20 µg/mL) to 77.06±0.73 (100 µg/mL) of hydroxyl radical scavenging activity in various concentration.

5.3.3. Nitric Oxide Scavenging Activity

In this assay crude and fractions of sea slug *K. ornata* showed promising results. The scavenging activities were directly proportional to the increasing sea slug concentration. *K. oranaaua* crude extract showed 47.06±0.07 (20 µg/mL) to 62.43±0.75 (100 µg/mL) and fractions witnessed 51.14±0.55 (20 µg/mL) to 74.16±0.74 (100 µg/mL) (Fig. 29.) nitric oxide radical scavenging activity in various concentration.
Fig. 27. DPPH radical scavenging activity of crude and fraction of sea slug *K. ornata*

Fig. 28. Hydroxyl radical activity of crude and fraction of sea slug *K. ornata*

Fig. 29. Nitric oxide scavenging activity of crude and fraction of sea slug *K. ornata*
5.3.4. Reducing power assay

The concentration dependency of antioxidant activity was investigated as a function of reducing power (Fig. 30.), as this gives a general view of reductones present in the sample. The values of reducing power were found to be directly proportional to the concentration of the extracts. In this assay crude exhibited 0.47±0.10 (20 μg/mL) to 1.59±0.26 (100 μg/mL) whereas fraction showed 0.52±0.01 (20 μg/mL) to 1.73±0.07 (100 μg/mL) reducing power.

5.3.5. Hydrogen peroxide (H₂O₂) scavenging activity

Measurement of H₂O₂ scavenging activity is known to be one of the most useful methods for determining the ability of an antioxidant to decrease the levels of pro-oxidants such as H₂O₂. In this assay the sea slug crude and fractions showed potent scavenging activities (Fig. 31.). The *K. omaia* crude showed 58.63±0.78 (20 μg/mL) to 80.06±0.21 (100 μg/mL) while the fractions evidenced 62.09±1.09 (20 μg/mL) to 85.98±0.40 (100 μg/mL).
Fig. 30. Reducing power activity of crude and fractions of sea slug *K. ornata*

Fig. 31. Hydrogen peroxide scavenging activity of crude and fractions of sea slug *K. ornata*
5.4. Discussion

Animals have been blessed with complicated but efficient setup of antioxidants in form of proteinaceous substances. The animal antioxidants are chiefly confined to specific vital organs liver and kidney (Shahidi et al., 1994). A free radical is a chemical moiety which posse’s single unpaired electron in its outermost electron shell (Halliwell and Gutteridge, 1989). These unpaired electrons are usually highly reactive, so are likely to take part in various chemical reactions like intracellular killing of pathogens (Abbas et al., 1996) a free radical may be an atom or a molecule with one or more unpaired electrons. The free radicals are capable of independent existence and can cause oxidative tissue damage (Singh et al., 1997).

Free radicals are produced in vivo from various biochemical reactions and also from the respiratory chain as a result of occasional leakage (Fridovich, 1978). Increased production of reactive oxygen species or a decreased efficiency of an antioxidant system appears to be a major contributing factor to a number of degenerative processes such as cancer, coronary heart diseases, cataract, arthritis, ageing, and AIDS (Prakash, 1998).

The antioxidant activity is suggested due to the specific scavenging of oxygen containing compounds, or metal-chelating ability, scavenging of radicals formed during peroxidation, (Jun et al., 2004). The methanolic extract of K. ornata shows maximum scavenging activity in crude extract ranges from 65.27±0.42 at the dose of 100 µg/mL. In fraction the maximum scavenging activity value was recorded in 74.76±0.33 at the dose of 100 µg/mL. The minimum activity of 60.83±0.48 at 20 µg/mL concentrations and the highest activity of 81.56±0.36 at 100 µg/mL
concentrations were recorded in standard (Vitamin C). In similar results were obtained and reported by Sadeeshkumar et al. (2012) that the methanolic extracts of Babyonila zeylanica showed maximum total antioxidant activity (%) of 78.6±0.40 at 10 mg/ml and minimum activity of 65.3±0.10 at 0.1 mg/ml was recorded where as 77.2±0.35 and 82.5±0.50 at 0.1 mg/ml was recorded in standards (BHA and Ascorbic acid) respectively. The activity was found increased with concentration increasing concentration of the B. zeylanica.

Sivaperumal et al. (2014) explained the free radical scavenging activity of protein from green mussel Perna viridis extract was assessed by the DPPH assay. The result shows that crude tissue extract from green mussel had the significant DPPH scavenging activity (76.9%) at 100 μg/ml. The reducing capacity of P. viridis crude protein compared to standard Ascorbic acid. The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity (Meir et al., 1995).

The effect of Methanolic extracts of K. ornata on oxidative damage induced by Fe3+/H2O2 deoxyribose. An inhibition (%) of 73.65±0.45 (100 μg/mL) was observed as the highest concentration in crude extract of K. ornata and 56.9±0.67 (20 μg/mL) as the minimum concentration was observed. Whereas in fraction the minimum activity was 51.27±0.36 at 20 μg/mL and highest activity 77.06±0.73 (100 μg/mL) inhibition was observed respectively.

The reducing properties are generally associated with the presence of reductones (Pin-Der and Duh, 1998). The antioxidant action of reductones was based on the breaking of the free-radical chain by donating a hydrogen atom. Earlier reports of Sasikumar et al. (2009) explained the reducing power of the samples might be due
to the di and mono hydroxyl substitutions in the aromatic ring which possess potent hydrogen donating abilities.

From our study, the maximum activity was absorbed in the extract of *K. ornata* (1.73±0.07) at 100 µg/mL whereas the minimum activity was observed (0.047±0.01) in 20 µg/mL. The results are similar to our present study reported by Pachiyappan *et al.* (2014) that the maximum activity was absorbed in the methanolic extract of *Meritrix casta* (0.158) and *P. viridis*(0.127) whereas the minimum activity was observed for *Hemifuses conchlidium* (0.024) and *Sorghum bicolor* (0.02) respectively.

Hydroxyl radical is one of the most reactive free radicals and can react with all bio-macromolecules in living cells (Huang *et al.*, 2005 and Sun *et al.*, 2009). However, no enzymes found in organisms are known to degrade hydroxyl radical (Akashi *et al.*, 2004). Therefore, screening of hydroxyl radical scavenging effect from natural resources is extremely important for the increased function of the antioxidant defense system in the living cells. In hydroxyl radical assay crude and fractions of sea slug *K. ornata* showed high hydroxyl radical scavenging activities. The scavenging activities were oriented with increasing sea slug concentration. In this the crude extract of sea slug *K. ornata* showed 56.9±0.67 (20 µg/mL) to 73.65±0.45 (100 µg/mL) whereas fraction showed 51.27±0.36 (20 µg/mL) to 77.06±0.73 (100 µg/mL) hydroxyl radical scavenging activity in various concentration. Similar kind of result reported by Jiang *et al.* (2011) the crude showed strong scavenging activity in certain doses.
The highest $\text{H}_2\text{O}_2$ scavenging activity of the crude methanolic extract from *K. ornata* shows $80.06\pm0.21$ at 100 $\mu$g/mL and the minimum was found at $58.63\pm0.78$ at 20 $\mu$g/mL. The fraction extract of *K. ornata* shows maximum of $85.98\pm0.40$ at 100 $\mu$g/mL and the minimum was found at $62.09\pm1.09$ at 20 $\mu$g/mL. When comparing to the crude extract the fraction extracted compound shows high scavenging activity.

Likewise, the nitric oxide scavenging activity was also observed and compared with the crude and fraction extract obtained from sea slug of *K. ornata*. The maximum scavenging activity was $62.43\pm0.75$ at 100 $\mu$g/mL and the minimum was found at $47.06\pm0.07$ at 20 $\mu$g/mL. In fraction extract, the maximum scavenging activity was $74.16\pm0.74$ at 100 $\mu$g/mL and the minimum was found at $51.14\pm0.55$ at 20 $\mu$g/mL. In conclusion, the concentration is directly proportional to the scavenging activity.

Nitric oxide (NO) interacts with oxygen to produce stable products, nitrite, and nitrate. Scavengers of nitric oxide compete with oxygen, leading to a reduced production of nitrite. The concentration of nitrite in aqueous or alcohol solutions have been assayed spectrophotometrically (Marcocci *et al.*, 1994).

The different relative radical scavenging capacity of individual mussel extract against different testing radicals may be explained by the different mechanisms involved in the radical antioxidant reactions. Other factors, such as stereo selectivity of the radicals or the solubility of the mussel extracts in different testing systems, may also affect the capacity of the mussel extract to react and quench different radicals (Gorinstein *et al.*, 2003). In our study the crude and fraction of sea slug *K.ornata* showed maximum anti oxidant activity in DPPH, hydroxyl radical, reducing power, hydrogen peroxide and relatively moderate activity is found in nitric oxide assay.