Radical Scavenging Potential of Hydrophilic Phlorotannins of *Hizikia fusiformis*

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Hizikia fusiformis is well known edible brown seaweed both in Korea and Japan. It has been intensively studied due to its pronounced health benefits. In this study, the radical scavenging (antioxidative) activities of its hydrophilic phlorotannin constituents were studied. An aqueous extract/original extract (OE) of *H. fusiformis* was initially prepared with heat, enzymes and pH control treatments. Then the original extract was further fractionated (with methylene chloride and methanol) and crude hydrophilic phlorotannin extract (CHPE) was prepared. The radical scavenging activities of both OE and CHPE were determined by using electron spin resonance (ESR) spectrophotometric assays such as 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl and alkyl radical assays. The CHPE reported significantly ($p < 0.05$) higher total phenolic (phlorotannins) content ($1.23 \ mg \cdot ml^{-1}$) than that of the OE ($0.21 \ mg \cdot ml^{-1}$). Both OE and CHPE have reported good radical scavenging activities and those activities were dose-dependent. The CHPE have demonstrated significantly higher radical scavenging activities than that of the OE. In comparison, the DPPH radical (6×10^{-5} M) scavenging activities of all the CHPE concentrations (0.25, 0.5 and $1 \ mg \cdot ml^{-1}$) tested were significantly higher (37.3, 78.2 and 91.6%, respectively) than that of the OE counterparts (11.4, 34.6 and 61.7%, respectively). Alkyl radical scavenging percentages of CHPE at 0.0625, 0.125, 0.25 and 0.5 $mg \cdot ml^{-1}$ were significantly higher (34.3, 69.2, 80.4 and 88.7%, respectively) than that of the OE (16.6, 41.4, 62.3 and 77.4%, respectively). The percentages of hydroxyl radical scavenging activities of CHPE at the concentrations of 0.25, 0.5 and $1 \ mg \cdot ml^{-1}$ were 32.5, 59.4 and 84.2 % respectively. Moreover, the hydroxyl radical scavenging activity of OE was quite lower than that of the CHPE. Therefore, these results suggest that the hydrophilic phlorotannins of *H. fusiformis* are potential radical scavengers thus, a great source of antioxidative nutraceuticles.

Key Words: Brown seaweed, *H. fusiformis*, hydrophilic phlorotannins, radical scavengers, ESR spectroscopy

INTRODUCTION

Oxidants like reactive oxygen species (ROS) and reactive nitrogen species (RNS) are able to damage a wide range of essential biomolecules including nucleic acids (DNA/RNA), proteins, lipids and carbohydrates (Halliwell and Gutteridge 1990). Antioxidants can scavenge biologically harmful oxidants such as superoxide, hydrogen peroxide, hydroxyl radicals, singlet oxygen, peroxyl radicals, alkyl radicals, nitric oxide and peroxy nitrate (Halliwell 1991). The imbalance between harmful oxidants and respective antioxidant defense mechanisms leads to oxidative modifications in biomolecules causing degenerative diseases including aging, cancer, arteriosclerosis, heart diseases and many other degenerative diseases (Kehrer 1993; Fridovich

1998; Kourounakis et al. 1998) Moreover, antioxidants effectively retard lipid peroxidation in food products and maintain the quality (Harris *et al.* 1992; Wagner et al. 1993). In particular, antioxidants can interfere with the oxidation process by scavenging free radicals, chelating free catalytic metals and also by acting as oxygen scavengers.

Synthetic antioxidants have been identified as mutagens and tumour promoters at high dosages (Kahl and Kappus 1993; Kahl 1994). Natural antioxidants impart promising safety even at higher dosages and those are not only antioxidants but also may impart other health benefits. In particular, natural antioxidants have a wide range of functional properties including inhibition of ROS and RNS generation, direct or indirect scavenging of free radicals and maintaining oxidative balance in the body. Therefore, the extensive search for natural antioxidants alternatives to synthetic ones is of great interest among present researches. Bioactive

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materials from natural sources have received a considerable attention of the pharmaceutical, nutraceutical and functional food industries. The proven beneficial effect on human health, well-known safety and growing demand for such materials are the key functional deems for involvement of enormous amount of researches in this area.

Polyphenols (e.g. phenolic acids, flavonoids, tannins) being widely distributed in plants are known to act as potential antioxidants. Also, polyphenols in human diet may exert a beneficial health effect via protecting against some diseases, including coronary heart disease and some cancers (Hotta *et al.* 2002). In general, phenolic compounds or polyphenols, have a similar basic structural chemistry including an "aromatic" or "phenolic" ring structure. Phytophenolic compounds have been associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals (Rice-Evans *et al.* 1995; Jorgensen *et al.* 1999). The protective effects of plant plolyphenols in biological systems are ascribed to their capacity to transfer electrons to ROS/ free radicals, chelate metal catalysts, activate antioxidant enzymes and inhibit oxidases. Polyphenols are basically of two class: condensed tannins and hydrolysable tannins derived from shikimic acid pathway. Polyphenols in marine brown algae are called as phlorotannins, which are formed by the polymerization of phloroglucinol (1,3,5 trihydroxybenzene) monomer units and synthesized in the acetate-malonate pathway (Ragan and Glombitza 1986). Phlorotannins are quite hydrophilic compounds with a wide range of molecular sizes (126Da ~ 650kDa).

Phlorotannins have been reported to have potential bioactivities specially as antioxidant, anticancer, antibacterial, antihyaluronidase antiglycosidases, antiplasmin compounds (Nakamura *et al.* 1996; Shibata *et al.* 2002; Shibata *et al.* 2002; Nagayama *et al.* 2002).

Hizikia fusiformis (class Phaeophyceae, order Fucales, family Sargassaceae), in Korean and Japanese sea has been consuming as a popular dish in both countries. Recent evidence suggests that *H. fusiformis* possesses a variety of biologically active compounds specially antioxidants, immuno-modulators and anticoagulants (Kim *et al.* 1998; Okai *et al.* 1998; Yan *et al.* 1999; Nagai *et al.* 2003). Our previous studies (Siriwardhana *et al.* 2003; Siriwardhana *et al.* 2005; Karawita *et al.* 2005) on *H. fusiformis* antioxidant activity investigation by means of ROS scavenging and lipid peroxidation inhibition showed potential lipid oxidation inhibitory effects. Most

of those antioxidants showed a marked tendency to accumulate in polar (hydrophilic) solvents than that of non-polar (hydrophobic) solvents. Moreover, the antioxidant or radical scavenging potential of crude hydrophilic phlorotannins of *H. fusiformis* water extract prepared by using commercial enzymes has not been previously studied. Accordingly, as a part of an overall investigation for effective utilization of bioactive materials existing in *H. fusiformis*, we focused on the water-soluble (hydrophilic) antioxidants and preparation of antioxidants- rich aqueous extracts. Moreover, we (Siriwardhana *et al.* 2003; Karawita *et al.* 2005) have reported the positive correlation between phlorotannins content and antioxidant activity of different hydrophilic and hydrophobic fractions of *H. fusiformis*.

The objective of the present study was to demonstrate the antioxidant potential of hydrophilic phlorotannis of *H. fusiformis* by using electron spin resonance (ESR) spectrophotometric assays. Further, to draw the attention of future antioxidative nutraceutical industries, thus to explore the utilization of hydrophilic phlorotannins of *H. fusiformis* as a potential bio-resource in the industry.

MATERIALS AND METHODS

Plant Material

Hizikia fusiformis was collected from the Jeju Island cost of Korea in March and June 2004. Epiphytes, salt and sand were removed using tap water and the samples were rinsed with deionised water before freeze-drying.

Chemicals

Ultraflo L (β-glucanases) and Alcalase 2.4 L FG (alkaline endopeptidase) enzymes were purchased from Novo Co. (Novozyme Nordisk, Bagsvaerd Denmark). 5,5-Dimethyl-1-pyrrolin N-oxide (DMPO), 2,2-azobis(2-amidinopropane) hydrochloride (AAPH), 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and low-melting agarose (LMA) were purchased from Sigma Co. (St Louis, USA). $FeSO_4 \cdot 7H_2O$ and H_2O_2 were purchased from Fluka Co (Buchs, Switzerland). All the other chemicals used were analytical grade supplied by Fluka or Sigma Co.

Enzymatic hydrolysis and preparation of crude extract of hydrophilic phlorotannins

Our previous protocol (Siriwardhana *et al.* 2004) used in the preparation of antioxidants by using enzymatic hydrolysis was further developed to obtain hydrophilic phlorotannis. Freeze dried *H. fusiformis* was ground into

a fine powder and 1 g was mixed with 100 ml of distilled water in a conical flask. Initially, the mixture was heated at 100°C for 45 min and allowed to cool up to ambient temperature (25°C). The pH and temperature of the resultant mixture was adjusted as 8.0 and 56°C, respectively. Alcalase (an alkaline endopeptidase/protease) and Ultraflo (a β-glucanase/ carbohydrase) enzymes were then added as 2% and 3%, respectively. After incubating for 1 day, the enzyme activity of hydrolysates was inactivated by heat (100°C for 10 min) and the pH of the mixture was increased until 12 (by using 1M NaOH) and placed in the shaking incubator for 12h. The resultant mixture was centrifuged and the supernatant was filtered in vacuum. The pH of the mixtures was adjusted to 7.0 by using 1M HCl. This extract hereafter referred to as original extract (OE). Finally, the OE was divided into two portions and one of the portions was fractionated with a 100 ml of organic solvent mixture (methylene chloride and methanol at 1:1 ratio) and the respective aqueous fraction (upper dark brown layer) was separated and its total phenol content was measured. Polyphenols in marine brown algae occur as a single structural class, the phlorotannins, which are formed by the polymerization of phloroglucinol (1,3,5 trihydroxybenzene) monomer units (Ragan and Glombitza 1986). The aqueous layer containing significantly higher amount of total phenolics (crude hydrophilic phlorotanin extract) was then placed in the vacuum drier for 2h (in order to evaporate methylene chloride and methanol). The crude hydrophilic phlorotannin extract hereafter referred as CHPE. Both OE and CHPE were then lyophilized and stored at -20°C until use for the experiments.

Total phenolic content

Content of the total phenolic compounds was determined according to the protocol similar to Chandler and Dodds (1993). One ml of the extract was mixed in a test tube containing 1 ml of 95% ethanol, 5 ml of distilled water and 0.5 ml of 50% Folin-Ciocalteu reagent. The mixture was allowed to react for 5 min and 1 ml of 5% Na_2CO_3 was added. Thoroughly mixed mixture was placed in a dark room for 1 h and absorbance was recorded at 725 nm in the UV-VIS spectrophotometer (Opron 3000 Hanson Tech. Co. Ltd., Korea). A gallic acid standard curve was used for the extrapolation of total phenolic content.
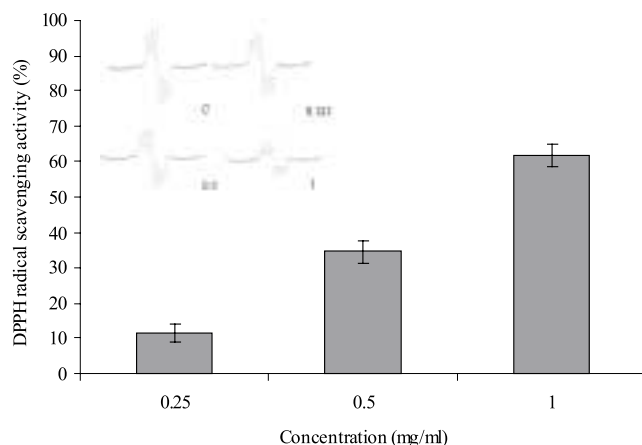
DPPH radical scavenging assay

This assay was based on the scavenging of stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals by the radical scavenging antioxidants in OE and CHPE. Method of Nanjo *et al.* (1996) was used to investigate the free radical scavenging activity in electron spin resonance (ESR) spectrophotometer. DPPH solution in dimethyl sulfoxide (DMSO) was prepared at the concentration of 6×10^{-5}M. A 60 μL fraction of extract (or water itself as control) was added to same volume of freshly prepared DPPH. Then the reactants were thoroughly mixed for 10 S. The reaction mixture was transferred into a 100 μL teflon capillary tube and fitted into the ESR spectrophotometer. The spin adduct was measured after 2 min. The measurement conditions of the JES-FA ESR (JEOL, Tokyo, Japan) were as follows; central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, mocrowave power 5 mW, gain 6.3×10^{-5}M and temperature 25°C.
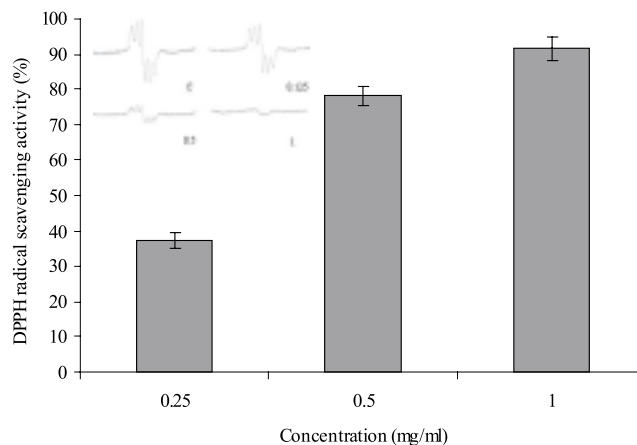
Alkyl radical scavenging assay

Alkyl radicals were generated by AAPH and their scavenging effects on extracts were investigated by the method of Hiramoto *et al.* (1993). The reaction mixture prepared containing 10 mM AAPH, 10 mM 4-POBN and indicated concentrations of extracts in phosphate buffer at pH 7.4, was incubated at 37°C for 30 min. The reaction mixture was transferred into a 100 μL teflon capillary tube and fitted in to the ESR spectrophotometer. The resultant spin adduct spectrum was recorded. The measurement conditions were as follows; central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 10 mW, gain 6.3×10^{-5}M and temperature 25°C.

Hydroxyl radical (HO·) scavenging assay

Ability of extracts to scavenge the HO· using ESR spectroscopy was investigated by the method of Rosen and Raukman (1980). Hydroxyl radicals were generated by the Fenton reaction and reacted rapidly with nitrone spin trap DMPO. The Fenton reaction mixture consisted: 200 μL of 10 mM $FeSO_4·7H_2O$, 200 μL of 10 mM H_2O_2 and 200 μL of 0.3 M DMPO in 0.1 M phosphate buffer (pH 7.4) mixed with 200 μL of extract. The resultant DMPO-HO adducts was investigated and the ESR spectrum was recorded after 2.5 min. The measurement conditions were as follows central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 5 mW, gain 6.3×10^{-5}M and

Fig. 1. DPPH radical scavenging activities of original extract (OE) extrapolated by ESR spectrum. The ESR spectrum (shown inside the graph) obtained at each OE concentration was marked as concentration in mg/ml and C represents the control (zero concentration).

Fig. 2. DPPH radical scavenging activities of crude hydrophilic phlorotannin extracts (CHPE) extrapolated by ESR spectrum. The ESR spectrum (shown inside the graph) obtained at each CHPE concentration was marked as concentration in mg/ml and C represents the control (zero concentration).

temperature 25°C.

Data analysis

All data were the means of three determinations and the data was analyzed using the SPSS package for Windows (Version 11.5). In the data analysis, variance analyses were performed using Students t test and the differences between means of treatment were determined. *P*-value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

### Crude extract of hydrophilic phlorotannin

Solvent fractionation (using methylene chloride and methanol) of original extract (OE) obtained after combined treatments (enxymatic hydrolysis, heat and pH control) could yield a hydrophilic aqueous fraction rich in total phenols (hydrophilic phlorotannins). Polyphenols in marine brown algae are called as phlorotannins, which are formed by the polymerization of phloroglucinol (1,3,5 trihydroxybenzene) monomer units (Ragan and Glombitza 1986). In the present study, the antioxidative potential of crude hydrophilic phlorotannin extract (CHPE) was compared with its original extract (OE). The total polyphenolic content of the CHPE (1.23 mg/ml) was significantly (p < 0.05) higher than that of the OE (0.21 mg/ml). Moreover, all the tested antioxidative activities of CHPE were also significantly (P<0.05) higher than that of the OE.

### Comparison of antioxidant scavenging activities using ESR assays

The unpaired/odd electrons (free radicals) are continuously formed in our body as a result of normal metabolism, immunological reactions or various unfavorable extracelluler conditions such as pollution, radiation, smoke and pesticides. ESR spectroscopy is a tool, which can detect unpaired electrons and their quantity. The unpaired electrons are absorbing microwave energy and the first derivative of the absorption of microwave energy is displayed as the ESR spectrum. When the unpaired electrons are paired (anti-oxidation/scavenging of free radicals) by antioxidants, the signal intensity of the ESR spectrum decreases accordingly. The more the ESR signal intensity decreases the higher is the antioxidative potential of testing compound.

DPPH radical scavenging effect

The organic compound DPPH is a radical, in which an unpaired/odd electron located on one of the nitrogen atoms of the molecule. Both OE and CHPE treated ESR spectrum of DPPH adduct and respective extrapolated scavenging activaties are shown in Fig. 1 and 2 respectively. The DPPH radical scavenging activities of both OE and CHPE were increased in a dose-dependent manner. In comparison, the DPPH radical (6×10^{-5}M) scavenging activities of all the CHPE concentrations (0.25, 0.5 and 1 mg/ml) tested were significantly (P<0.05) higher (37.3, 78.2 and 91.6 % respectively) than that of
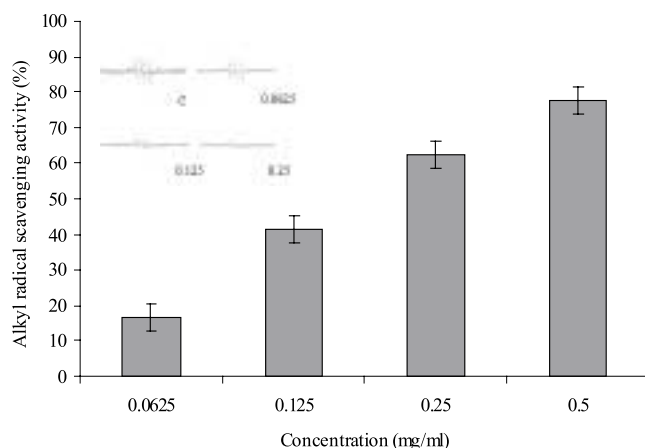
Fig. 3. Alkyl radical scavenging activities of original extract (OE) extrapolated by ESR spectrum. The ESR spectrum (shown inside the graph) obtained at each OE concentration was marked as concentration in mg/ml and C represents the control (zero concentration).
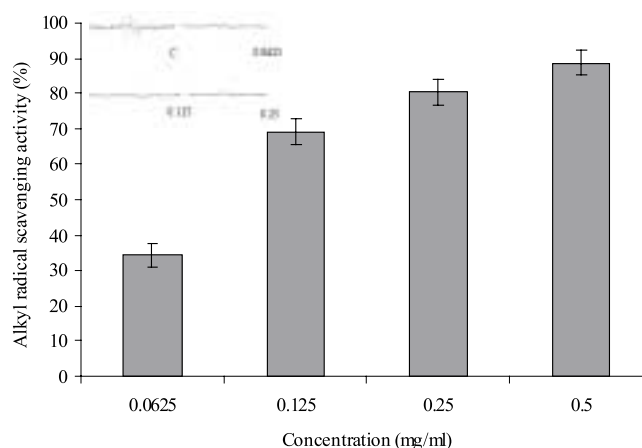
Fig. 4. Alkyl radical scavenging activities of crude hydrophilic phlorotannin extracts (CHPE) extrapolated by ESR spectrum. The ESR spectrum (shown inside the graph) obtained at each CHPE concentration was marked as concentration in mg/ml and C represents the control (zero concentration).

the OE counterparts (11.4, 34.6 and 61.7% respectively). These higher radical scavenging activities in CHPE can be attributed to its higher total phenolic (hydrophilic phlorotannins) content than that of the OE counterpart. We have reported higher positive correlations between total phenolics of *H. fusiformis* and DPPH radical scavenging (Siriwardhana *et al.* 2003). Also, the total phenolic content and antioxidative activities of hydrophilic solvent extracts (methanol and water extracts) were higher than that of the hydrophobic solvent extracts (diethyl ether, chloroform, ethyl acetate and acetone). With this study, it became clear that hydrophilic phlorotannin compounds attributed to the well-known antioxidative potential of *H. fusiformis*. Numerous studies conducted on brown algal phlorotannins have reported potential antioxidative activities. Kang *et al.* (2003) have isolated a potential free (DPPH) radical scavenging compound (eckstolonol 1) from *Ecklonia stolonifera* (Okamura). It has been suggested that compounds, which can scavenge free radicals, can inhibit mutations and numerous health complications (Buyukokuroglu *et al.* 2001; Nordberg and Arner 2001; Gulcin *et al.* 2003).
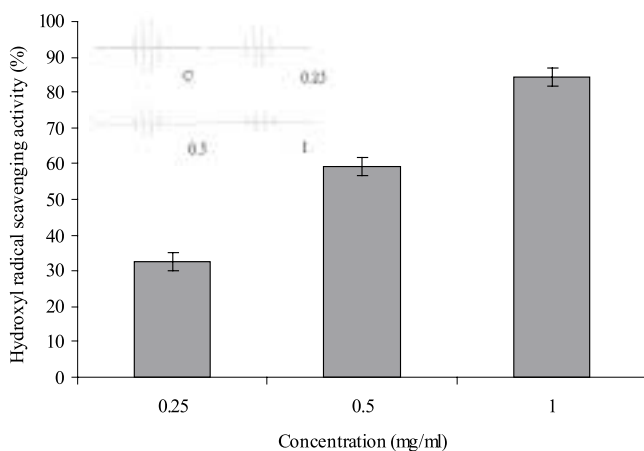
Alkyl radical scavenging effect.

Alkyl radicals are a series of sp^2 hybridised univalent groups of the general formula C_nH_{2n+1} derived from aliphatic hydrocarbons. In the presence of oxygen, alkyl radicals are rapidly transformed into the corresponding peroxyl derivatives (acylperoxyl and alkylperoxyl radicals). Alkyl radicals are directly produced by the

metal mediated free radical generation of unsaturated fatty acids ($Fe^{3+} + RH \rightarrow Fe^{2+} + R\cdot + \dot{H}^+$) Moreover, different kinds of carbon centered alkyl radicals are produced during lipid peroxidation and those alkyl radicals can further reacts with a variety of biomolecules (Chamulitrat and Mason 1990) Alkylation (the addition or substitution of one or more alkyl radicals into an organic compound) replaces certain hydrogen atoms in DNA, which leads to mutation, cancer or cell death. Alkyl radical scavenging percentages of CHPE (Fig. 3 and 4) at 0.0625, 0.125, 0.25 and 0.5 $mg \cdot ml^{-1}$ were significantly ($p < 0.05$) higher (34.3, 69.2, 80.4 and 88.7 % respectively) than that of the OE (16.6, 41.4, 62.3 and 77.4 % respectively). As similar to the DPPH and hydroxyl radical scavenging activities the alkyl radical scavenging activities of both OE and CHPE were also dose-dependent. Apparently, this results revealed that the alkyl radical scavenging activity of CHPE demonstrates a marked potential to act against harmful alkyl radicals even at extremely lower concentrations like 0.0625 $mg \cdot ml^{-1}$ (activity: 34.3%).

Hydroxyl radical (HO·) scavenging effect.

Hydroxyl radical is the most reactive among reactive oxygen species. Moreover, the cell-damaging action of HO· is the strongest among free radicals and its approximate half life is 10^{-9} S. HO· reacts with several biological materials oxidatively by hydrogen withdrawal, double bond addition, electron transfer, new radical formation, autoxidation initiation,

Fig. 5. Hydroxyl radical scavenging activities of crude hydrophilic phlorotannin extracts (CHPE) extrapolated by ESR spectrum. The ESR spectrum (shown inside the graph) obtained at each CHPE concentration was marked as concentration in mg/ml and C represents the control (zero concentration).

polymerization and fragmentation (Lui and Ng 2000). The antioxidative potential of OE and CHPE were investigated by ESR spectroscopy (Fig. 5). The percentage of DMPO-HO. adducts scavenging activities of CHPE at the concentrations of 0.25, 0.5 and 1 mg/ml was 32.5, 59.4 and 84.2 % respectively. The HO. scavenging activities of OE counterparts were significantly (p < 0.05) lower (results not shown) than that of the CHPE. Singh *et al.* (2004) have reported good hydroxyl radical activities in hydrophilic extracts (water extract) of propolis, and potato peel. In fact, HO. is an extremely reactive oxidant radical that will react with most biomolecules at diffusion controlled rates. Therefore, the strong hydroxyl radical scavenging activity of CHPE demonstrates the great potential of hydrophilic phlorotannins against a variety of complications of this extremely reactive radical.

Two principle mechanisms of action have been proposed for antioxidants. First is the chain-breaking mechanism, by which the primary antioxidant donates an electron to the odd electron/free radical present in the system (e.g., lipid radical). The second mechanism involves removal of ROS/RNS initiators (secondary antioxidants) by quenching chain-initiating catalysts. Primary antioxidants, such as polyphenols, tocopherol and ascorbic acid, can stop chain reaction by donating an electron to the free radical of the fatty acid, and thus stops the propagation steps. Compounds which can react with the initiating radicals, inhibit the initiating enzyme and reduce the oxygen level (without generating reactive

radical species), are secondary antioxidants. Antioxidant mechanisms of polyphenols are based on hydrogen donating ability and chelating metal ions. Karawita *et al.* (2005) have reported good metal chelating activities in aqueous (hydrophilic) fractions of *H. fusiformis*. Polyphenolic antioxidants are not only act as hydrogen donating or metal chelating antioxidants but also they act as synergistic compounds with other bioactive compounds (Komori *et al.* 1993). These results suggest that the hydrophilic phlorotannins of *H. fusiformis* is a great source of antioxidative nutraceuticles.

ACKNOWLEDGEMENT

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