Antioxidant Potential of *Ecklonia cava* on Reactive Oxygen Species Scavenging, Metal Chelating, Reducing Power and Lipid Peroxidation Inhibition

Mahinda Senevirathne[1], Soo-Hyun Kim,[1*] Nalin Siriwardhana,[2] Jin-Hwan Ha[1], Ki-Wan Lee[2] and You-Jin Jeon[2,*]

[1]*Department of Food Bioengineering, Cheju National University, Jeju 690–756, South Korea*
[2]*Faculty of Applied Marine Science, Cheju National University, Jeju 690–756, South Korea*

The antioxidative potential of different fractions (respective organic and aqueous fractions of *n*-hexane, chloroform and ethyl acetate) of 70% methanol extract of *Ecklonia cava* (a brown seaweed) was evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide anion, hydrogen peroxide, hydroxyl radical, nitric oxide, ferrous ion chelating, reducing power and lipid peroxidation inhibition (conjugated diene hydroperoxide and thiobarbituric acid-reactive substances production) assays. The 70% methanol extract showed significant ($p<0.05$) activities in all antioxidant assays and contained a high level of total phenolic content. It was observed that the level of hydrophilic phenolic content was higher than that of hydrophobics. Among those organic solvent fractions, ethyl acetate fraction exhibited significant activities due to the highest level of total phenolic content and their IC_{50} values were 0.013 mg/mL, 0.009 mg/mL and 0.33 mg/mL in DPPH, hydrogen peroxide and nitric oxide radical inhibition, respectively. These activities were superior to those of a commercial synthetic and natural antioxidants tested. The aqueous chloroform and ethyl acetate fractions also exhibited significant ($p<0.05$) activities in reactive oxygen species (ROS) scavenging and metal chelating, attributed to the high amount of hydrophilic phenolics. Moreover, *E. cava* extracts showed strong reducing power and a notable capacity to suppress lipid peroxidation.

Key Words: *Ecklonia cava*, brown seaweed, antioxidant, lipid peroxidation, reactive oxygen species

INTRODUCTION

Reactive oxygen species (ROS), which consist of free radicals such as superoxide anion ($O_2^{\cdot -}$) and hydroxyl (HO·) radicals and non-free radical species such as H_2O_2 and singled oxygen ($^1O_2$), are different forms of activated oxygen (Halliwell and Gutteridge, 1999; Yildirim et al., 2000; Gulcin et al., 2002b). ROS are produced by all aerobic organisms and can easily react with most biological molecules including proteins, lipids, lipoproteins and DNA. Thus, ample generation of ROS proceed to a variety of pathophysiological disorders such as arthritis, diabetes, inflammation, cancer and genotoxicity (Kourounakis et al., 1999; Gulcin et al., 2002a). Therefore, living organisms possess a number of protective mechanisms against the oxidative stress and toxic effects of ROS. Antioxidants regulate various oxidative reactions naturally occurring in tissues and are evaluated as a potential anti-aging agents. Hence, antioxidants can terminate or retard the oxidation process by scavenging free radicals, chelating free catalytic metals and also by acting as electron donors.

Antioxidants have been widely used as food additives to provide protection from oxidative degradation of foods and oils. Hence, antioxidants are used to protect food quality mainly by the prevention of oxidative deterioration of constituents of lipids. The most extensively used synthetic antioxidants are propylgallate (PG), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ) (Sherwin, 1990). However BHT and BHA have been suspected of being responsible for liver damage and carcinogenesis (Grice, 1986; Wichi, 1988; Hettiarachchy et al., 1996). Natural antioxidants are able to protect from ROS as well as other free radicals and retard the progress of many chronic diseases and lipid oxidative rancidity in foods (Pryor, 1991; Kinsella et al., 1993; Lai et al., 2001; Gulcin et al., 2003).

Polyphenols are widely distributed in plants and phenolic antioxidants have been found to act as free radical scavengers as well as metal chelators (Shahidi and Wanasundara, 1992; Sanchez-Moreno et al., 1999).

**To whom correspondence should be sent (e-mail: kshyun@cheju.ac.kr and youjinj@cheju.ac.kr).*
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It has also been reported that some types of polyphenols such as catechin, epicatechin, epigallocatechin, catechin gallate, epicatechin gallate and epigallocatechin gallate are present in the seaweeds like *Halimada* algae (Yoshie et al., 2002).

E. cava, a brown seaweed, is distributed in the temperate costal areas of the Korean peninsula and forms dense populations in clear waters (Kang et al., 2001). However it is a representative unutilised marine bioresource because of its bitter taste. Enzymatic hydrolysates of *E. cava* have showed higher radical scavenging activities towards DPPH free radical and significantly lower peroxide value in fish oil (Heo et al., 2003).

In the present study, characterisation and distribution of the antioxidants present in aqueous and organic fractions of *E. cava* was examined in different ROS scavenging, ferrous ion chelating, reducing power and lipid peroxidation assays in order to evaluate its natural antioxidant properties.

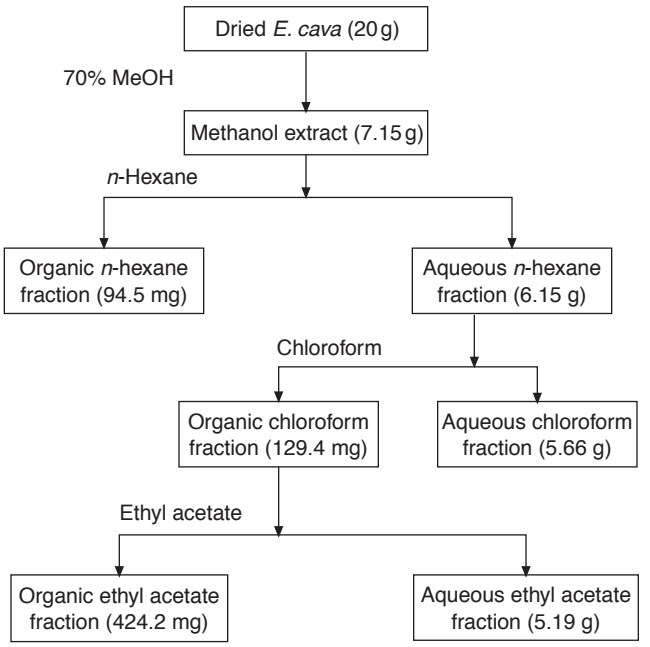

Figure 1. Scheme of solvent fractionation of *E. cava.*

MATERIALS AND METHODS

Materials

Butylated hydroxytoluene (BHT), α-tocopherol, dimethyl sulphoxide (DMSO), 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitro blue tetrazolium salt (NBT), xanthine, xanthine oxidase (XOD), fish oil, thiobarbituric acid (TBA), trichloroacetic acid (TCA), Folin-Ciocalteu reagent, sodium nitroprusside and sulphanilic acid were purchased from Sigma Co. (St. Louis, USA) and N-(1-Naphthyl) ethylenediamine dihydrochloride was purchased from Hayashi Pure Chemical Industries Ltd (Osaka, Japan). Ethylenediaminetetraacetic acid (EDTA), peroxidase, 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), $FeSO_4 \cdot 7H_2O$ and deoxyribose were purchased from Fluka Co. (Buchs, Switzerland). All other chemicals used were of analytical grade supplied by Fluka or Sigma Co.

Methods

Extraction and Solvent Fractionation

E. cava was collected from the coastal area of Jeju Island off South Korea in May 2004. Epiphytes, salt and sand were removed using tap water, then samples were rinsed with deionised water before freeze-drying. Then, freeze-dried *E. cava* was pulverised into a fine powder. A 20 g sample of the dried *E. cava* powder was mixed in 70% methanol (1,000 mL) and kept in the shaking incubator at 25 °C for 3 days and filtered in vacuum using Whatman No. 1 (Whatman Ltd, UK) filter paper. Later, solvent fractionation of 70% methanol extract was separately done with *n*-hexane, chloroform and ethyl acetate (Figure 1). After solvent fractionation, both aqueous and organic fractions were evaluated for antioxidant activities.

DPPH Radical Scavenging Assay

DPPH scavenging potential of different *E. cava* fractions was measured based on scavenging ability of stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals by *E. cava* antioxidants. The method modified by Brand-Williams (1995) was employed to investigate the free radical scavenging activity. Freshly prepared 2 mL DPPH (3×10^{-5} M in DMSO) solution was thoroughly mixed with 2 mL of different *E. cava* fractions. The reaction mixture was incubated for 1 h at room temperature. Absorbance of the resultant mixture was recorded at 517 nm using UV-VIS spectrophotometer (Opron 3000 Hanson Tech. Co. Ltd, Korea).

Superoxide Anion ($O_2^{\cdot -}$) Scavenging Assay

The superoxide scavenging ability of different *E. cava* fractions was assessed by the method of Nagai et al. (2001). The reaction mixture contained 0.48 mL of 0.05 M sodium carbonate buffer (pH 10.5), 0.02 mL of 3 mM xanthine, 0.02 mL of 3 mM EDTA, 0.02 mL of 0.15% bovine serum albumin, 0.02 mL of 0.75 mM NBT and 0.02 mL of *E. cava* fractions. After incubation for 20 min at 25 °C, 6 mU XOD was added to the mixture to initiate the reaction, which was carried out for 20 min at 25 °C. Reaction was terminated by adding 0.02 mL of 6 mM CuCl. The absorbance of the mixture was recorded at 560 nm.

Hydrogen Peroxide (H_2O_2) Scavenging Assay

The hydrogen peroxide scavenging ability of different *E. cava* fractions was investigated based on the scavenging of the hydrogen peroxide in ABTS-peroxidase system described by Muller (1995). A measurement of 80 μL of each *E. cava* fraction and 20 μL of 10 mM hydrogen peroxide was mixed with 100 μL of phosphate buffer (pH 5.0, 0.1 M) in a 96-microwell plate and the samples were incubated for 5 min at 37 °C. Subsequently, 30 μL of freshly prepared 1.25 mM ABTS and 30 μL of peroxidase were added and incubated for another 10 min at 37 °C. Absorbance of the resulting mixture was recorded using ELISA reader (Sunrise; Tecan Co. Ltd, Austria) at 405 nm.

Hydroxyl Radical (HO·) Scavenging Assay

The ability of different *E. cava* fractions to scavenge the hydroxyl radical generated by the Fenton reaction was measured according to the modified method of Chung et al. (1997). The Fenton reaction mixture containing 200 μL of 10 mM $FeSO_4{\cdot}7H_2O$, 200 μL of 10 mM EDTA and 200 μL of 10 mM 2-deoxyribose was mixed with 1.2 mL of 0.1 M phosphate buffer (pH 7.4) containing 200 μL of *E. cava* fractions. Thereafter, 200 μL of 10 mM H_2O_2 was added to the mixture before incubation for 4 h at 37 °C. Later, 1 mL of 2.8% TCA and 1 mL of 1% TBA were added and placed in a boiling water bath for 10 min. Then, the resultant mixture was allowed to cool up to room temperature and centrifuged at $395 \times g$ for 5 min. Absorbance was recorded at 532 nm in a UV-VIS spectrophotometer.

Nitric Oxide Radical (NO·) Scavenging Assay

Sodium nitroprusside in aqueous solution at physiological pH, spontaneously produce nitric oxide, which reacts with oxygen to produce nitrite ions, which can be determined by the use of the Griess Illosvoy reaction (Garrat, 1964). Griess Illosvoy reagent was slightly modified using naphthylethylenediamine dihydrochloride (0.1% w/v) instead of 1-naphthylamine (5%). Scavengers of nitric oxide compete with oxygen and reduce the production nitric oxide (Marcocci et al., 1994). The reaction mixture (3 mL) containing 2 mL of 10 mM sodium nitroprusside, 0.5 mL of phosphate buffer saline (pH 7.4, 0.01 M) and 0.5 mL of extract was incubated for 150 min at 25 °C. Thereafter, 0.5 mL of the reaction mixture containing nitrite was pipetted and mixed with 1 mL of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotisation. Then, 1 mL of naphthylethylenediamine dihydrochloride (0.1%) was added, and allowed to stand for 30 min in diffused light. The absorbance of the pink coloured chromophore was measured at 540 nm against the corresponding blank solutions in a 96 well plate using ELISA reader.

Ferrous Ion Chelating Ability

The published method by Decker and Welch (1990) was used to investigate the ferrous ion chelating ability of different *E. cava* fractions. A 5 mL amount of each *E. cava* fraction was mixed with 0.1 mL of 2 mM $FeCl_2$ and 0.2 mL of 5 mM ferrozine solutions. The absorbance at 562 nm was determined after reaction for 10 min. A complex of Fe^{2+}/ferrozine showed strong absorbance at 562 nm.

Measurement of Reducing Power

Reducing power was investigated using the method developed by Oyaizu (1986). A 2.5 mL fraction of *E. cava* was mixed with 2.5 mL of phosphate buffer (200 mM, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was placed in a water bath for 20 min at 50 °C. The resulting solution was cooled rapidly, mixed with 2.5 mL of 10% trichloroacetic acid and centrifuged at 3,000 rpm for 10 min. A 5.0 mL fraction from the supernatant was mixed with 5 mL of distilled water and 1 mL of 1% ferric chloride. Absorbance of the resultant mixture was measured at 700 nm after 10 min. The higher the absorbance value the stronger the reducing power.

Oxidation of Fish Oil

Fish oil was exposed to accelerated oxidation similar to the method used by Abdalla and Roozen (1999). Fish oil samples (20 g) containing 0.1%, 0.05% and 0.01% of organic fractions of *E.* cava were incubated at 60 °C in darkness for 11 days. Initial 6 h incubation was done without closing the cap of the bottles in order to remove the methanol which was added to dissolve the organic fractions of *E. cava*.

Thiobarbituric Acid-reactive Substances Assay (TBARS)

This assay was based on the method described by Madsen et al. (1998) and the basic principle of this method is the reaction of one molecule of malonaldehyde with two molecules of TBA to form a red coloured malonaldehyde–TBA complex. A 1 g sample of the fish oil was dissolved in 3.5 mL of cyclohexane and 4.5 mL of TCA–TBA mixture (7.5% TCA and 0.34% TBA) subsequently. The resultant mixture was vortexed for 5 min and centrifuged at $2{,}780 \times g$ for 15 min. The TCA–TBA phase was removed and heated in a boiling water bath for 10 min. Absorbance was recorded at 532 nm and the antioxidant capacity was expressed as equivalent μmol of malonaldehyde per kg

oil. TBARS concentration was calculated using a standard curve based on tetraethoxypropane.

Conjugated Diene Hydroperoxides (CDH) Assay

CDH content was detected every 2 days as described by Roozen et al. (1994). Of each fish oil sample, 50 mg (stored under accelerated oxidation conditions) was mixed with 5 mL of cyclohexane and vortex. CDH absorbance was recorded at 234 nm.

Total Phenolic Assay

Total phenolic compounds were determined according to the protocol described by Chandler and Dodds (1993). Of each *E. cava* fraction 1 mL 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 resultant mixture was allowed to react for 5 min and 1 mL of 5% Na_2CO_3 was added. It was mixed thoroughly and placed in dark for 1 h and absorbance was recorded at 725 nm in the UV-VIS spectrophotometer. A gallic acid standard curve was obtained for the calculation of phenolic content.

Calculation of 50% Inhibition Concentration (IC_{50})

The concentration of the extract (mg/mL) that was required to scavenge 50% of radicals was calculated by using the percent scavenging activities of five different extract concentrations. Percent scavenging activity was calculated as $[1 - (A_i - A_j)/Ac] \times 100$.

Where: A_i is the absorbance measured with different *E. cava* fractions in the particular assay with a ROS source; A_j is the absorbance measured with different *E. cava* fractions in the particular assay but without a ROS source; A_c is the absorbance of control with particular solvent (without *E. cava* fractions).

Statistical Analysis

All experiments were conducted in triplicate ($n = 3$) and an ANOVA test (using SPSS 11.5 statistical software) was used to compare the mean values of each treatment. Significant differences between the means of parameters were determined by using the Duncan test ($p < 0.05$).

RESULTS AND DISCUSSION

DPPH Radical Scavenging Activity

The free radical scavenging activity was investigated in DPPH assay. The highest DPPH radical scavenging effect was detected in organic ethyl acetate fraction (IC_{50} 0.013 ± 0.002 mg/mL, Table 1) followed by aqueous *n*-hexane and aqueous chloroform fractions (IC_{50} 0.0160 ± 0.002 mg/mL and 0.018 ± 0.002 mg/mL respectively). Those activities were significantly higher ($p < 0.05$) than that of BHT (IC_{50} 0.360 ± 02 mg/mL) but less than that of α-tocopherol (IC_{50} 0.010 ± 0.003 mg/mL). When considering the organic fractions of *E. cava*, the DPPH radical scavenging capacities increased towards the ethyl acetate fraction with increasing the polarity of the solvent. Also, DPPH radical scavenging activities were increased with an increased content of total phenolics in organic fractions. Further, all aqueous fractions showed higher DPPH scavenging activities and positively correlated with total phenolic content.

According to the studies carried out by Yuan et al (2005), IC_{50} value of dulse, *P. palmata*, extract was 12.5 mg/mL and Tepe et al. (2005) have recorded IC_{50} 140, 125, 110 μg/mL for their experiment conducted with various extracts of *Salvia tomentosa* Miller (Lamiaceae). Siriwardhana et al. (2003) have also reported higher DPPH scavenging activities for a water and methanol extract of *Hizikia fusiformis* (a brown alga), while ethanol, chloroform and ethyl acetate extracts also indicated strong inhibition activities over 50%. All those activities were reported for crude extracts, but lower than that of the authors values obtained for the crude extract. According to Suja et al. (2005), the IC_{50} value of purified extract was 5.49×10^3 μg/mL which was lower than that of the all aqueous fractions and methanol extract as well as the organic ethyl acetate fraction of *E. cava*. Therefore the authors sample showed significant activity in DPPH scavenging when compared with other crude extracts as well as purified samples.

Superoxide Anion ($O_2\cdot^-$) Scavenging Activity

The highest superoxide anion scavenging activity was reported for the 70% methanol fraction (IC_{50} 0.051 ± 0.003 mg/mL), which was significantly higher ($p < 0.05$) than that of α-tocopherol (IC_{50} 1.3 ± 0.03 mg/mL). Next highest values (IC_{50} 0.367 ± 0.01 mg/mL and 0.477 ± 0.04 mg/mL) were shown by aqueous ethyl acetate and aqueous *n*-hexane fractions, respectively. Those activities were slightly lower when compared with the activity of BHT but significantly higher ($p < 0.05$) than that of α-tocopherol. Also it is noteworthy to mention that higher scavenging activities were observed in aqueous fractions than that of organic fractions. Hence, it is quite relevant to increase the amount of total phenolics in aqueous fractions. Further, $O_2\cdot^-$ scavenging properties of *E. cava* may be attributed to both neutralisation of $O_2\cdot^-$ radicals via hydrogen donation and inhibition of xanthine oxidase by various phenolics present in *E. cava* fractions. Rajapakshe et al. (2005) have reported IC_{50} 220 μg/mL value for fermented mussel sauce which is

Table 1. Antioxidative effect of different fractions of *E. cava.*

Fraction	Total Phenol Content (± mg/100 g d.b.)	IC_{50} (mg/mL, mean ± SD n = 3)[1]					
		DPPH	$O_2 \cdot^-$	H_2O_2	HO·	NO·	Metal Chelating
70% Methanol	8,299 ± 12	0.019a ± 0.002	0.051a ± 0.003	0.065ab ± 0.002	0.023a ± 0.003	0.337a ± 0.02	0.436a ± 0.03
Organic *n*-hexane	8.22 ± 14	0.850d ± 0.03	2.420f ± 0.2	1.450d ± 0.1	0.025a ± 0.005	1.210c ± 0.1	0.839b ± 0.01
Aqueous *n*-hexane	7,262 ± 8	0.016a ± 0.002	0.477bc ± 0.04	0.104bc ± 0.02	0.054c ± 0.006	0.720ab ± 0.06	0.660ab ± 0.03
Organic chloroform	68.5 ± 6	0.111b ± 0.04	0.514bc ± 0.04	0.167c ± 0.04	0.071d ± 0.002	0.765b ± 0.04	1.501cde ± 0.3
Aqueous chloroform	6,628 ± 35	0.018a ± 0.002	1.468e ± 0.1	0.073ab ± 0.003	0.023a ± 0.003	0.330a ± 0.03	2.800f ± 0.2
Organic ethyl acetate	509 ± 13	0.013a ± 0.002	0.609c ± 0.06	0.009a ± 0.004	0.045b ± 0.001	0.330a ± 0.05	1.290cd ± 0.1
Aqueous ethyl acetate.	5,049± 11	0.038a ± 0.001	0.367b ± 0.01	0.065ab ± 0.006	0.068d ± 0.002	0.386a ± 0.04	1.200c ± 0.4
BHT		0.360c ± 0.02	0.165a ± 0.02	0.073ab ± 0.004	0.023a ± 0.004	1.590d ± 0.2	1.600de ± 0.02
α-tocopherol		0.010a ± 0.003	1.300d ± 0.03	0.127bc ± 0.03	0.046b ± 0.002	2.100e ± 0.6	1.720e ± 0.2

[1]Values within a column followed by different letters are significant different ($p < 0.05$).

lower than the values we obtained for 70% methanol crude extract (IC_{50} 0.051 ± 0.003 mg/mL). Further, these values are significant when compared with values of Rajapakshe et al. (2005) up to second purified stage (IC_{50} 66 and 52 μg/mL in first and second steps of purification respectively). Moreover present data are more significant when compared with other values (IC_{50} 0.5 μg/mL) obtained for crude extracts such as aqueous extract of potato peel (Singh and Rajini, 2004). Hence, *E. cava* fractions have strong activities in superoxide anion scavenging compared to other works done with crude extracts as well as purified extracts and is thus a useful functional food.

Hydrogen Peroxide (H_2O_2) Scavenging Activity

Hydrogen peroxide converts into the singlet oxygen ($^1O_2$) and hydroxyl radicals, which then become very powerful oxidising agents. Not only $^1O_2$ and HO· but also H_2O_2 can cross membranes and may oxidise a number of compounds.

Organic ethyl acetate fraction of *E. cava* showed strong H_2O_2 scavenging activities (IC_{50} 0.009 ± 0.004 mg/mL) which was significantly ($p < 0.05$) higher than those of commercial antioxidants (IC_{50} 0.073 ± 0.004 mg/mL for BHT and 0.127 ± 0.03 mg/mL for α-tocopherol). Aqueous ethyl acetate, chloroform fractions and 70% methanol extract also showed higher H_2O_2 scavenging activities (IC_{50} 0.065 ± 0.003 mg/mL, 0.073 ± 0.003 mg/mL and 0.065 ± 0.002 mg/mL, respectively). Among those activities, aqueous ethyl acetate and 70% methanol fractions showed comparatively higher activities than those of both BHT and α-tocopherol while the aqueous chloroform fraction showed equal activity with BHT and higher activity than that of α-tocopherol. Almost all aqueous fractions showed good activities in H_2O_2 scavenging indicating the potential of hydrophilic total phenolics.

Hydroxyl Radical (HO·) Scavenging Activity

Hydroxyl radical is the most reactive among ROS and it bears the shortest half-life compared with other ROS. Hydroxyl radical scavenging of aqueous chloroform fraction and 70% methanol extract exhibited equal higher activities (IC_{50} 0.023 ± 0.003 mg/mL) which were similar to that of BHT, but significantly ($p < 0.05$) higher than that of α-tocopherol (IC_{50} 0.046 ± 0.002 mg/mL). Organic *n*-hexane fraction also showed strong activity (IC_{50} 0.025 ± 0.003 mg/mL) which was almost similar to the activity of BHT but significantly ($p < 0.05$) higher than that of α-tocopherol. According to these results, hydrophilic phenolics are dominant in *E. cava* and to which are attributed the radical scavenging properties. Significant activities were exhibited by the crude extract of *E. cava* compared to the activities of crude extracts of Nagai et al. (2003) and Singh and Rajini (2004) that reported good hydroxyl radical activities for water extract of propolis, and water extract of potato peel respectively, but all those activities were lower than the activities exhibited by the authors crude extract. When compared the fractions of the authors crude extract with purified extracts of Xing et al. (2005) and Wettasinghe and Shahidi (2000), almost all fractions showed higher activities than those reported by Xing et al. (IC_{50} 1.25 mg/mL) and some fractions (70% methanol, organic *n*-hexane and aqueous chloroform) showed similar or higher activities than values given by Wettasinghe and Shahidi (2000).

Nitric Oxide (NO·) Scavenging Activity

Nitric oxide and superoxide anion cause ischemic renal injury separately and these radicals work together to bring about further damage. The toxicity and damage caused by NO· and $O_2^{\cdot -}$ is multiplied as they react to produce reactive peroxynitrite ($ONOO^-$), which leads to serious toxic reactions with biomolecules, like protein, lipids and nucleic acids (Moncada et al., 1991; Radi et al., 1991a, 1991b; Yermilov et al., 1995). Suppression of NO· released may be partially attributed to direct NO· scavenging, as all fractions of *E. cava* decreased the amount of nitrite generated from the decomposition of sodium nitroprusside *in vitro*.

Organic ethyl acetate and aqueous chloroform fractions showed strong activities on NO· scavenging (IC_{50} 0.33 ± 0.05 mg/mL and 0.33 ± 0.03 mg/mL respectively). Those activities were significantly ($p < 0.05$) higher than that of BHT and α-tocopherol (IC_{50} 1.59 ± 0.2 mg/mL and 2.1 ± 0.6 mg/mL respectively). All aqueous fractions also showed significantly higher activities than commercial antioxidants tested. Aqueous fractions of *n*-hexane and chloroform showed higher activities than their organic counterparts but organic ethyl acetate fraction showed higher activities than its aqueous counterpart. These results showed that hydrophilic antioxidants are abundantly present and showed different chemical properties.

Ferrous Ion Chelating Ability

Ferrozine can quantitatively form complexes with Fe^{2+} but in the presence of ion chelating agents, the complex formation is disrupted, resulting in a decrease in the red colour of the complex. Among organic and aqueous fractions of *E. cava*, 70% methanol fraction showed the highest ferrous iron chelating ability (IC_{50}.436 ± 0.03 mg/mL). These abilities were significantly higher ($p < 0.05$) than that of BHT and α-tocopherol (IC_{50} 1.6 ± 0.02 mg/mL and 1.72 ± 0.2 mg/mL respectively). Second highest abilities showed in aqueous *n*-hexane fraction (IC_{50} 0.660 ± 0.02 mg/mL), which was also significantly ($p < 0.05$) higher than the values of commercial antioxidants. Further aqueous

ethyl acetate fraction also showed higher activities than that of BHT and α-tocopherol. As reference antioxidants, BHT and α-tocopherol showed relatively lower activity when compared to the abilities obtained from the organic and aqueous fraction of *E. cava*. The iron(II) chelating properties of the antioxidant extract may be attributed to their endogenous chelating agents, mainly phenolics. Certain phenolic compounds have properly oriented functional groups, which can chelate metal ions (Thompson and Williams, 1976). Thompson and Williams (1976) reported that the stability of the metal-antioxidant complex is higher in six-membered than five-membered ring complexes. Metal chelating activity of methanol extract and aqueous *n*-hexane fraction of *E. cava* was equal or slightly higher than that of the chelating activity showed by 100 ppm concentrations of borage crude extracts and its purified fractions but slightly lower than that of the values showed by evening primrose at same concentration (Wettasinghe and Shahidi, 2000).

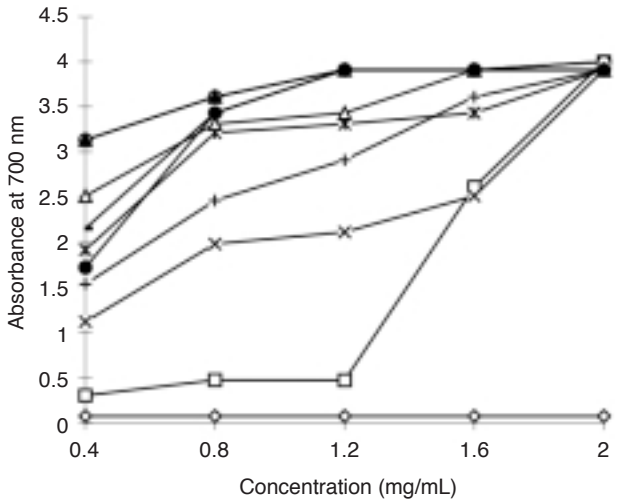

Figure 2. Reducing power of different fractions of *E. cava*: (❄) 70% methanol, (□) organic n-hexane, (—) aqueous n-hexane, (×) organic chloroform, (△) aqueous chloroform, (○) organic ethyl acetate, (+) aqueous ethyl acetate, (▲) BHT, (●) tocopherol, (◇) control.

Reducing Power

The reducing ability of a compound generally depends on the presence of reductones (Pin-Der-Duh, 1998), which have been exhibited antioxidative potential by breaking the free radical chain, donating a hydrogen atom (Gordon, 1990). The presence of reductants (i.e. antioxidants) in the fractions *E. cava* extract causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, the Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Figure 2 shows the reducing capacities of different fractions of *E. cava.* The organic ethyl acetate fraction of *E. cava* showed the highest reducing ability of all other fractions tested. That activity was higher than that of even BHT and α-tocopherol. Aqueous chloroform, *n*-hexane, ethyl acetate fractions and 70% methanol extract showed higher activities indicating that more hydrophilic phenolics are present in those fractions which affect those interesting values in reducing capacities. Also the reducing ability of each fraction was dose dependent and significantly higher than the control. Guo et al. (2001) have reported that the aqueous and methanol extracts of stem and leaf of broccoli showed higher reducing power at the concentration of 4 mg/mL but lower reducing abilities than that of the fractions of *E. cava* (2 mg/mL level showed highest reducing abilities). Kuda et al. (2005) have reported that crude fucoidan and crude alginate showed the reducing abilities (absorbance less than 1.0) at the concentration 10 mg/mL which were lower than that of the authors reducing abilities. At the concentration of 0.8 mg/mL chitosan, the highest absorbance value reached by a purified sample was 0.26 (Xing et al., 2005) while the authors' fractions were above 1.0 absorbance except for organic *n*-hexane fraction, indicating that crude extract of *E. cava* showed excellent activities in reducing power.

Conjugated Diene Hydroperoxides (CDH) Formation Inhibition

Lipids oxidation leads to conjugated diene hydroperoxides formation as a result of hydrogen capture from the unsaturated fatty acids; and also undergo further radical formation. Among three organic fractions tested, ethyl acetate fraction showed the highest inhibition on CDH formation (Table 2). Results showed that the CDH formation inhibitory effect of the organic fractions of *E. cava* was dose dependent and 0.1% concentration of organic fractions showed significantly higher ($p < 0.05$) inhibitory activities than that of BHT and α-tocopherol. The 0.05% level of ethyl acetate fraction also showed higher activities than that of α-tocopherol as well as higher activities than BHT up to day 7 of incubation. The inhibition of CDH by *E. cava* is important in the early stages of lipid peroxidation reactions, as it prevents further chain reactions. Siriwardhana et al. (2004) have also reported that the rate of CDH formation decreased significantly in the fish oil and linoleic acid treated with commercial antioxidants and *H. fusiformis* methanolic extracts at 0.1% level and those results were almost similar to the results obtained for *E. cava*. Comparing conjugated diene hydroperoxide values of *E. cava* with the values obtained for catnip, hyssop, L. balm, oregano, sage and thyme (in sunflower oil

Table 2. Effect of different *E. cava* organic fractions on the formation of conjugated diene hydroperoxides (CDH) in fish oil during storage in the dark under accelerated oxidation conditions (60 ± 1 °C).

	Conjugated Diene Hydroperoxides (mean ± SD, n = 3)[1]					
	Storage Time (days)					
Sample	1	3	5	7	9	11
Fish oil	0.068g ± 0.008	0.119f ± 0.027	0.162b ± 0.069	0.219c ± 0.046	0.348f ± 0.087	0.544f ± 0.092
+*n*-hexane 0.1%	0.047cd ± 0.006	0.069bc ± 0.005	0.129b ± 0.018	0.162bc ± 0.051	0.218abcd ± 0.064	0.311bc ± 0.058
+*n*-hexane 0.05%	0.058efg ± 0.002	0.091de ± 0.006	0.144b ± 0.015	0.205c ± 0.065	0.268cdef ± 0.061	0.398cd ± 0.054
+*n*-hexane 0.01%	0.064fg ± 0.005	0.109ef ± 0.002	0.154b ± 0.019	0.211c ± 0.078	0.329ef ± 0.057	0.519ef ± 0.075
+Chloroform 0.1%	0.036ab ± 0.006	0.061b ± 0.009	0.096ab ± 0.019	0.114ab ± 0.018	0.187abc ± 0.018	0.254ab ± 0.049
+Chloroform 0.05%	0.049cde ± 0.003	0.075bcd ± 0.006	0.119ab ± 0.029	0.138abc ± 0.015	0.224abcd ± 0.067	0.321bc ± 0.051
+Chloroform 0.01%	0.054def ± 0.004	0.085cd ± 0.007	0.142b ± 0.061	0.198c ± 0.028	0.298def ± 0.062	0.516ef ± 0.078
+Ethyl acetate 0.1%	0.026a ± 0.003	0.036a ± 0.009	0.054a ± 0.015	0.074a ± 0.016	0.132a ± 0.037	0.213ab ± 0.031
+Ethyl acetate 0.05%	0.035ab ± 0.008	0.063b ± 0.008	0.098ab ± 0.019	0.108ab ± 0.020	0.174abc ± 0.041	0.295ab ± 0.036
+Ethyl acetate 0.01%	0.041bc ± 0.007	0.071bc ± 0.009	0.126b ± 0.064	0.162bc ± 0.074	0.234bcde ± 0.054	0.402cd ± 0.072
+BHT 0.01%	0.058efg ± 0.005	0.080bcd ± 0.007	0.111ab ± 0.007	0.114ab ± 0.011	0.154ab ± 0.008	0.187a ± 0.012
+α-Tocopherol 0.01%	0.059efg ± 0.007	0.094de ± 0.007	0.129b ± 0.009	0.184bc ± 0.012	0.259cdef ± 0.016	0.432de ± 0.062

[1]Conjugated diene hydroperoxide values expressed as absorbance at 234 nm of 50 mg fish oil. Values within a column followed by different letters are significant different ($p < 0.05$).

Table 3. Effect of different *E. cava* organic fractions on the formation of thiobarbituric acid reactive substances (TBARS) in fish oil during storage in the dark under accelerated oxidation conditions (60 ± 1 °C).

	Tiobarbituric Acid Reactive Substances[1] (μmol/kg, mean ± SD, n = 3)					
	Storage Time (days)					
Sample	1	3	5	7	9	11
Fish oil	9.6i ± 0.34	15.2i ± 0.56	19.4i ± 0.32	24.26g ± 0.99	28.61i ± 1.07	32.5g ± 1.12
+*n*-hexane 0.1%	5.3e ± 0.22	8.2c ± 0.48	11.1e ± 0.28	13.1c ± 0.22	15.7e ± 0.55	15.1bc ± 0.68
+*n*-hexane 0.05%	5.9f ± 0.26	9.2d ± 0.32	13.7f ± 0.11	15.9e ± 0.28	17.5f ± 0.39	18.4d ± 0.28
+*n*-hexane 0.01%	6.7g ± 0.17	12.8h ± 0.33	15.7h ± 0.27	18.1f ± 0.22	20.6h ± 0.41	21.6e ± 0.84
+Chloroform 0.1%	4.1c ± 0.03	7.5a ± 0.21	9.7c ± 0.13	10.2b ± 0.28	12.1bc ± 0.34	16.1c ± 0.56
+Chloroform 0.05%	4.9d ± 0.15	9.1d ± 0.11	10.6d ± 0.14	13.4c ± 0.21	14.3d ± 0.33	18.9d ± 0.54
+Chloroform 0.01%	6.8g ± 0.37	11.4g ± 0.37	14.2g ± 0.11	16.3e ± 0.11	19.4g ± 0.37	19.4d ± 0.62
+ Ethyl acetate 0.1%	2.1a ± 0.11	7.1a ± 0.31	7.6a ± 0.17	9.4a ± 0.08	10.5a ± 0.28	11.4a ± 0.27
+ Ethyl acetate 0.05%	3.4b ± 0.09	7.6ab ± 0.19	9.7c ± 0.11	10.1b ± 0.19	11.5b ± 0.22	14.3b ± 0.36
+ Ethyl acetate 0.01%	4.9d ± 0.16	9.8e ± 0.33	10.6d ± 0.28	14.1d ± 0.13	16.2e ± 0.39	18.4d ± 0.51
+BHT 0.01%	3.9c ± 0.14	8.1bc ± 0.17	8.6b ± 0.18	10.7b ± 0.16	12.8c ± 0.33	14.2b ± 0.41
+α-tocopherol 0.01%	8.3h ± 0.27	10.5f ± 0.37	14.2g ± 0.29	17.6f ± 0.27	19.7g ± 0.37	24.6f ± 0.39

[1]TBARS values expressed as malonaldehyde equivalents of fish oil. Values within a column followed by different letters are significant different ($p < 0.05$).

system), all fractions showed higher inhibition activities at the lowest concentration of 0.01% compared to 1,200 ppm level (Abdalla and Roozen, 1999). It is important to inhibit the CDH formation at the early stages of lipid peroxidation, in order to prevent the subsequent formation of reactive lipid radicals which can undergo further chain reaction from which oils can be protected.

Thiobarbituric Acid-reactive Substances Formation Inhibition (TBARS)

During lipid peroxidation, low molecular-weight end products, probably malonaldehyde, are formed by oxidation of polyunsaturated fatty acids that can be reacted with two molecules of thiobarbituric acid to give a pinkish red chromogen. The TBARS formation inhibitory effect of the organic fractions of *E. cava*, BHT and α-tocopherol were significantly higher than that of control (fish oil without antioxidants) and the inhibition effect was dose dependent (Table 3). Almost all 0.1% levels of organic fractions showed significantly ($p<0.05$) higher inhibition activities than that of α-tocopherol. The inhibition of 0.1% and 0.05% levels of ethyl acetate fraction were higher than that of α-tocopherol and almost compatible with the values of BHT. Comparing TBARS values of *E. cava* with the values obtained for borage and primrose triacylglycerols, all fractions showed higher inhibition activities at the lowest concentration of 0.01% compare to 200 ppm level (Khan and Shahidi, 2001).

Total Phenolic Compounds

A number of studies has focused on the biological activities of phenolic compounds, which are potential antioxidants and free radical scavengers (Rice-Evans et al., 1995; Marja et al., 1999; Sugihara et al., 1999). Total phenolic content of different *E. cava* fractions were solvent dependent. Aqueous fractions of *E. cava* showed higher amounts of phenolics while their counterparts showed lower phenolic content. The content of total phenolics in aqueous fractions decreased in the order of 70% methanol > *n*-hexane > chloroform > ethyl acetate fraction while their respective organic fractions decreased in the order ethyl acetate > chloroform > *n*-hexane fraction (Table 1). As different *E. cava* fractions exhibited different reactive oxygen species scavenging activities, there may be different kinds of total phenolic compounds (hydrophilic and hydrophobic) in different *E. cava* fractions.

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