Turk. J. Fish.& Aquat. Sci. 19(3), 209-219
http://doi.org/10.4194/1303-2712-v19_3_04

RESEARCH PAPER

Turkish Journal of FISHERIES and AQUATIC SCIENCES

Antioxidant Activity of the Extracts from Freshwater Macroalgae (*Cladophora glomerata*) Grown in Northern Thailand and Its Preventive Effect against Lipid Oxidation of Refrigerated Eastern Little Tuna Slice

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Article History
Received 18 August 2017
Accepted 28 March 2018
First Online 05 April 2018

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Keywords
Freshwater macroalgae
Cladophora glomerata
Phenolic compounds
Eastern little tuna
Euthynnus affinis

Abstract

Characteristic and antioxidant activity of the extracts from freshwater macroalgae (*C. glomerata*) as influenced by extraction media, i.e., water or ethanol (20-100%, v/v) were studied. The highest yield (28.0%) was obtained when water was used. Ethanolic extracts had significantly lower yields (2.98-15.5%) ($P<0.05$). However, total phenolic and chlorophyll contents were lower in water extract. Among all extracts, the 60% ethanolic extract (E60) had the highest antioxidant activities, including DPPH and ABTS radical scavenging activities, ferric reducing antioxidant power and metal chelating activity. Based on LC/MS of E60, quercetin, isoquercetin and hydroquinin were the major phenolic compounds. When E60 at different levels (100, 200 and 400 mg/kg) were added to eastern little tuna (*Euthynnus affinis*) slices, the reduction in lipid oxidation was dose-dependent ($P<0.05$) as shown by smaller increases in peroxide and thiobarbituric acid-reactive substances (TBARS) values, compared to the control. Therefore, *C. glomerata* could be a source of alternative natural antioxidant in lipid based muscle food.

Introduction

Fish contain high amounts of polyunsaturated fatty acids, which undergo oxidation leading to the development of undesirable odors and flavors, and toxic products, thereby limiting the shelf-life of fresh fish. Several synthetic antioxidants have been widely used due to their high efficacy in controlling lipid oxidation (Maqsood, Benjakul, Abushelaibi, & Alam, 2014). However, the use of synthetic antioxidant may have a negative effect on consumers' health and have become restricted in some countries (Maqsood *et al*., 2014). As a consequence, natural antioxidants may provide safe alternatives. Several studies have shown that plant extracts, especially phenolic compound and essential oil, could be used as antioxidants (Maqsood *et al*., 2014). Cai, Cao, Li, Wu, Zu and Li (2015) reported that the turbot fillets sprayed with essential oils from clove, cumin and spearmint had lower microbial counts and also showed a better chemical quality than control fillet during 20 days of refrigerated storage.

Algae, apart from having high nutritive value, also contain various bioactive compounds including pigments (carotenoids, chlorophylls and tocopherols), sulphated polysaccharides (fucoidan), amino acids, and mono- and polyphenols (Farvin & Jacobsen 2013). These compounds have a role in protecting the algal cells against stressful conditions, e.g. ultraviolet radiation, temperature changes and fluctuation in nutrient. Algal phenolic compounds were reported to have antioxidant, anticancer, antibacterial, antiviral and anti-inflamatory activities (Jónsdóttir, Geirsdóttir, Hamaguchi, Jamnik, Kristinsson, & Undeland, 2016; Laungsuwon & Chulalaksananukul, 2013; Peerapornpisal, Amornlertpison, Rujjanawate, Ruangrita, & Kanjanapothib, 2006). Phenolic compounds, the secondary metabolites of plants, can act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators (Tsao & Deng, 2004).

Cladophora glomerata is freshwater green macroalgae, which grows naturally in the North of Thailand, especially in the Nan river. It is known locally

as 'Kai'. It is usually sold fresh or dried for direct consumption. Recently, it has become more economically important as it is being used in such products as crisps, baked goods and noodle as well as a medical ingredient (Peerapornpisal *et al*., 2006). Peerapornpisal *et al.* (2006) also reported that the ethanolic extracts from *C. glomerata* and *Nostochopsis lobatus* have been used as therapeutic agents. Laungsuwon and Chulalaksananukul (2013) found that the ethyl acetate extract of *C. glomerata* was a source of antioxidant and anticancer activity against the KB cell line. Pornpimol, Wannabutr and Rattanaporn (2015) found that crude extract from dried Kai prepared using alkaline extraction (0.3 N NaOH) in combination with heating at 60 °C had the highest total phenolic substances (1070 ± 20 mg gallic acid equivalence (GAE/100 g)), while the extract obtained using 2% HCl had the maximum antioxidant activity against DPPH radicals (88%). However, little information regarding the antioxidant activity and potential uses in food systems has been found for extracts from Kai. This study aimed to elucidate the effect of extraction media on the characteristic and antioxidant activity of the extracts. The effect of algae extracts on lipid oxidation of refrigerated eastern little tuna slices during storage was also studied.

Materials and Methods

Chemicals

2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 1,1,3,3-tetramethoxypropane, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid sodium salt (ferrozine) and 2,4,6-tripyridyl-triazine (TPTZ) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Thiobarbituric acid (TBA), potassium persulphate, cumene hydroperoxide and Folin Ciocalteu's phenol reagent were obtained from Fluka (Buchs, Switzerland). All chemicals were of analytical grade.

Analytical-grade standards of 10 phenolic compounds (quercetin, gallic acid, kaempferol, catechin, isoquercetin, tannic acid, rutin, hydroquinin, eriodictyol and apiginin (≥98% purity) were obtained from Sigma-Aldrich.

Materials

Northern freshwater macroalgae (*C. glomerata*) were gathered from the Nan river, Tha Wang Pha, Nan Province, Thailand (latitude 19°05'12.12''N and longitude 100°47'14.91''E) between November 2016 and March 2017, when the algae were at peak biomass (0-2 m in depth). *C. glomerata* is a filamentous green alga that naturally grown under eutrophic condition in the river. Harvested algae were placed in polyethylene bag and transported at 0-5 °C to the Faculty of Agro-Industry, Chiang Mai University, Chiang Mai, Thailand within 6 h. Upon arrival, algae were washed with tap water to remove impurities. The clean samples were dried at 60 °C using a cabinet rotary dryer (Mextech, Seoul, South Korea) to obtain a final moisture content <10% and cut with scissor into small pieces (1 cm in length). The algal samples were ground into a fine powder using a blender (Panasonic, Model MX-898N, Berkshire, UK) and sieved through a stainless steel sieve (60 mesh). The powders were placed in a polyethylene bag, sealed and kept in a refrigerator (4 °C) until use within 24 h.

Four kilograms of eastern little tuna (*Euthynnus affinis*) with an average size of 20-25 cm and weight of 0.3-0.4 kg/fish were purchased from a local market in Hat Yai, Thailand during June and July, 2017. After 24 to 36 h since capture, fish were stored in ice and transported to the Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Thailand within 30 min. Upon arrival, fish were washed, filleted, de-skinned and cut with a knife into slices with a weight of 50 g (thickness ~1.5-2 cm). The slices were placed in polyethylene bags and stored in ice until use (not longer than 2 h).

Preparation of Algae Extract

Algae powder was extracted using the method of Santoso, Yoshie-Stark, and Suzuki (2004) with a slight modification. Briefly, 10 g of algae powder was mixed with 250 mL of distilled water or ethanol at different concentrations (20, 40, 60, 80 or 100%, v/v). The mixtures were homogenized at 10,000 × g for 2 min using an IKA Labortechnik homogenizer (Selangor, Malaysia). Thereafter, the mixtures were stirred continuously for 12 h at room temperature (25-28 °C) using a stirrer (IKA Labortechnik stirrer). Mixtures were centrifuged at 8,000×g for 20 min at room temperature using a RC-5B plus centrifuge (Beckman, JE-AVANTI, Fullerton, CA, USA). The supernatant was collected and filtered through Whatman No.1 filter paper (Whatman International, Ltd., Maidstone, England). The solvent was then evaporated at 40 °C under vacuum using a rotary evaporator (Tokyo Rikakikai, Co. Ltd., Tokyo, Japan). All extracts were lyophilized using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). Dried extracts were powdered using a mortar and pestle. The powders prepared using 20, 40, 60, 80 and 100% (v/v) ethanol were referred to as E20, E40, E60, E80 and E100, respectively. The powders were kept in polyethylene bag and stored at 4 °C for not longer than 24 h for analyses.

Analyses

Determination of Yield

The yields of algae extract were calculated using

the following equation:

$$Yield\ (\%) = \frac{weight\ of\ dried\ algae\ extract}{weight\ of\ starting\ dried\ algae} \times 100$$

Determination of Total Phenolic Content

Total phenolic content of algae extracts was determined using Folin–Ciocalteu reagent using the method of Slinkard and Singleton (1977). The concentrations of total phenolic compounds in the samples were calculated from the standard curve of gallic acid (≥ 99% purity) in the range of 0 to 200 µg/mL and expressed as g gallic acid equivalents (GAE)/kg solids.

Determination of Total Chlorophyll Content (TCC)

TCC was determined spectrophotometrically using AOAC (2000) method No. 942.04. All extracts were adjusted to obtain 5 mg powder/mL using the corresponding solvent. Absorbance was measured at 660 and 642 nm using a UV-16001 spectrophotometer (Shimadzu, Kyoto, Japan). For the blank, the corresponding solvents were used instead of extracts. Total chlorophyll content (TCC) was calculated after blank subtraction using the following equation:

$$TCC\ (mg/100\ g\ extract) = \frac{7.12A_{660} + 16.8A_{642}}{C} \times 100$$

where C is the concentration of algae extracts

Determination of Color

The color of all algae extracts was measured using a colorimeter (ColourFlex, Hunter Lab, Reston, VA, USA) and reported in the CIE system. L^*, a^*, b^* and ΔE^* representing lightness, redness/greenness, yellowness/blueness, total difference of color, respectively, were reported. ΔE^* was calculated as follows:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where ΔL^*, Δa^* and Δb^* are the differentials between the color parameters of the samples and the color parameters of the white standard (L^* = 94.48, a^* = -0.56, b^*= 3.25).

Antioxidant Activities

DPPH Radical Scavenging Activity

DPPH radical scavenging activity was determined using the method of Binsan, Benjakul, Visessanguan, Roytrakul, Tanaka, and Kishimura (2008). Sample solution (1.5 mL) was added to 1.5 mL of 0.1 mM DPPH in 95% ethanol. The mixture was incubated at room temperature (27-30 °C) for 30 min in the dark. The resulting solution was measured at 517 nm. The control was prepared in the same manner except that distilled water was used instead of the sample. The DPPH radical scavenging activity was calculated from a Trolox standard curve (0-60 µM) and expressed as µmol Trolox equivalents (TE)/g solids.

ABTS Radical Scavenging Activity

ABTS radical scavenging activity was determined using the method of Binsan *et al*. (2008). The stock solutions included 14.8 mM ABTS solution and 5.2 mM potassium persulphate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 mL of ABTS solution with 50 mL of methanol to obtain an absorbance of 1.1 ± 0.02 units at 734 nm. A standard curve of Trolox ranging from 0 to 600 µM was prepared. The activity was expressed as µmol Trolox equivalents (TE)/g solids.

Ferric Reducing Antioxidant Power (FRAP)

FRAP was assayed using the method of Benzie and Strain (1996). FRAP reagent (a freshly prepared mixture of 10 mM TPTZ solution in 40 mM HCl, 20 mM $FeCl_3.6H_2O$ solution and 300 mM acetate buffer, pH 3.6 (1:1:10 v/v/v) (2.85 mL) was incubated at 37 °C for 30 min prior to mixing with 150 µL of sample. The reaction mixture was allowed to stand in the dark for 30 min at room temperature. Absorbance at 593 nm was measured. A standard curve was prepared using Trolox ranging from 0 to 600 µM. The activity was expressed as µmol Trolox equivalents (TE)/g solids.

Metal Chelating Activity

Chelating activity toward Fe^{2+} was measured using the method of Thiansilakul, Benjakul, and Shahidi (2007). Sample (200 µL) was mixed with 800 µL of distilled water. Thereafter, 0.1 mL of 2 mM $FeCl_2$ and 0.2 mL of 5 mM ferrozine were added. The mixture was allowed to react for 20 min at room temperature. The absorbance was then measured at 562 nm. The standard curve of EDTA was prepared in distilled water (without pH adjusted) to obtain final concentrations of 0 – 30 µM. The blank was prepared in the same manner except that distilled water was used instead of the sample. The ferrous chelating activity was expressed as EDTA equivalents (µmol EE/g solids).

Determination of Phenolic Compositions by LC-MS

Phenolic compositions of selected extract with the

highest antioxidant activity were determined using 1100 Series Capillary LC system (Agilent Technologies, Palo Alto, CA, USA) coupled to an ion trap mass analyzer (G1956B, Agilent Technologies). The separation was done using a LichroCART reverse phase-18 column (150 × 4.6 mm, 5 µm) (Purosher STAR, Merck, Darmstadt, Germany). The column temperature was 40 °C. The sample injection volume was 20 µL. The flow rate was 1 mL/min. Eluent A was acetonitrile, while eluent B consisted of 10 mM ammonium formate buffer with formic acid (pH 4). The elution program was run as follows: isocratic at 100% B from 0 to 5 min, linear gradient of 10− 20% A from 5 to 10 min, isocratic at 20% A from 10 to 20 min and a linear gradient of 20-40% A from 20 to 60 min. The UV diode array was monitored at 270, 330, 350 and 370 nm. The column effluent was further monitored in the positive ion mode of the mass spectrometer. The conditions were as follows: nebulizer, 60 psi; N_2 gas (≥ 99.9% purity), 13.0 L/min; gas temperature, 320 °C; ion trap, scan from 100-700 m/z. MS was used to capture and fragment the most abundant ion as a full scan mass spectra. Ions at m/z 188 and 209 were monitored for gallic acid, while 185, 329, 503 and 649 were monitored for catechin, isoquercetin, tannic acid and rutin, respectively. The ions at m/z 289, 327 and 341 were used to monitor for hydroquinin, eriodictyol and quercetin, respectively. Ion at m/z 271 and 287 were monitored for apiginin, while 309 and 325 were also monitored for kaemferol.

For quantification, the calibration curve was prepared for each corresponding standards. A linear correlation was obtained between the peak area and concentration, with $R^2 \geq 0.976$. The linear equations obtained were used to convert peak areas to concentrations of the target compounds in the extracts and expressed as mg/kg dry extract.

Antioxidant Activity of the E60 Extract with Eastern Little Tuna Slices

Preparation of the Eastern Little Tuna Slices With E60

Eastern little tuna slices (50 g) were placed in a petri dish. E60 (5, 10 and 20 mg) was dissolved separately in 1 mL of distilled water and uniformly coated on the prepared slices to obtain the final concentrations of 100, 200 and 400 mg/kg, respectively. The extract was manually manipulated until homogeneity was obtained (~5 min) using high personal hygiene. For the control sample, distilled water (1 mL) was added. Butylated hydroxyanisole (BHA) at a final concentration of 100 mg/kg was used as the positive control. All samples were transferred to polyethylene bags, packed in air and kept at 4 °C for 10 days. At the designated times (0, 2, 4, 6, 8 and 10 days), the slices were removed and ground using the blender in a walk-in cold room (4 °C) for analyses.

Determination of PV

PV was determined using the method of Richards and Hultin (2000). Ground sample (4.5 to 5.5 g) was homogenized at a speed of 13,500 rpm for 2 min in 11 ml of chloroform/methanol (2:1, v/v). The homogenate was then filtered using a Whatman No.1 filter paper. To 7 mL of filtrate, 2 ml of 0.5% NaCl were added. The mixture was vortexed at a moderate speed for 30 s, followed by centrifugation at 3,000 ×g for 3 min at 4 °C using a refrigerated centrifuge to separate the sample into two phases. To the lower phase (3 mL), 2 mL of cold chloroform/methanol (2:1) mixture, 25 µL of 30% (w/v) ammonium thiocyanate and 25 µL of 20 mM iron (II) chloride were added. The reaction mixture was allowed to stand for 20 min at room temperature prior to reading the absorbance at 500 nm. The blank was prepared in the same manner, except distilled water was used instead of ferrous chloride. A standard curve was prepared using cumene hydroperoxide (≥98% purity) at 0.5-2.0 ppm. Peroxide value was expressed as mg of hydroperoxide/kg of dry sample.

Determination of TBARS

TBARS were determined using the method of Buege and Aust (1978). A ground sample (0.5 g) in a walk-in cold room (4 °C) was homogenized with 2.5 mL of a solution containing 0.375% (w/v) thiobarbituric acid, 15% (w/v) trichloroacetic acid, and 0.25 M HCl. The mixture was heated in a boiling water bath (95 to 100 °C) for 10 min to develop a pink color, cooled with running tap water, and centrifuged at 3,600 ×g at 25 °C for 20 min. The absorbance of the supernatant was measured at 532 nm. A standard curve was prepared using 1,1,3,3 tetramethoxypropane (99% purity) from 0 to 10 ppm. TBARS were calculated and expressed as mg malonaldehyde/kg dry sample.

Statistical Analysis

Experiments were run in triplicate using three lots of algae and tuna. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out using Duncan's multiple range tests (Steel & Torrie, 1980). Statistical analysis was done using the Statistical Package for Social Science (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA). The significance level was set at $P<0.05$.

Result and Discussion

Effect of Extraction Media On Characteristic And Antioxidant Activity of the Extract from Freshwater Macroalgae

Yield and Total Phenolic Content

Yield and total phenolic content of algae extracts

prepared using water or ethanol at different concentrations are shown in Figure 1. Higher yield (28.0%) was obtained when water was used for extraction, compared with any of the ethanolic extracts (P<0.05). The result indicated that the compounds with high polarity might be dominant in the algae and could be potentially extracted using a polar solvent. Boonchum *et al.* (2011) reported that a higher yield of marine algae extracts was obtained when water was used compared to ethanol. Water soluble component contained in red seaweed *(Palmria palmata*) were soluble polysaccharide, protein and peptides (Galland-Irmouli *et al.*, 1999). Moreover, Honold, Jacobsen, Jónsdóttir, Kristinsson, and Hermund (2016) noted that water was able to extract not only phenolic compounds but also metals and chlorophyll derivatives. Mungmai, Jiranusornkul, Peerapornpisal, Sirithunyalug, and Leelapornpisid (2014) also found that water extract from *Rhizoclonium hieroglyphicum* (C. Agardh) Kützing from North Borneo had higher yield (21.6%) than that of 95% ethanolic extracts (12.6%). Among all the ethanolic extracts, E80 showed the highest extraction yield (15.5%). Variation in the yields of different extracts was probably attributed to the polarities of different compounds in the algae. Additionally, the yield of algae extracts also depends on pH, extraction time and temperature as well as on the chemical compositions of the algae (López, Rico, Rivero, & Suárez de Tangil, 2011).

Total phenolic contents (TPC) of algae extracts are shown in Figure 1B. The highest TPC was found in E40 or E60. The result showed that phenolic compounds in the algae were more soluble in polar organic solvents (40-60%) than water. Generally, phenolic compounds are bound to the cell walls of plants (Galland-Irmouli *et al.*, 1999). The use of organic solvent was able to inhibit polysacharide/protein-polyphenol complex formation during extraction by breaking hydrogen bonds between phenolic groups, and carboxyl groups of protein or hydroxyl groups of polysaccharides (Kallithraka, Garcia-Viguera, Bridle, & Bakker, 1995). As a consequence, more phenolic compounds were extracted. Nevertheless, the efficiency of extraction was lowered when ethanol concentration was >60% (P<0.05). Wang, Jonsdottir, and Ólafsdóttir (2009) found that 70% acetone was appropriated for extraction of phenolic compounds from 8 species of edible Icelandic seaweeds. Acetone and ethanol with *Fucus vesiculosus* extracts had the highest amount of phenolic compounds and carotenoids (Honold *et al*., 2016). Various phenolic compounds, including quercetin, myricetin (flavonoles), genistin, daidzein (iso-flavones), hesperidin (flavanones), and lutein (flavones) were found in methanolic extract of red, brown, and green seaweeds (Yoshie *et al*., 2000;

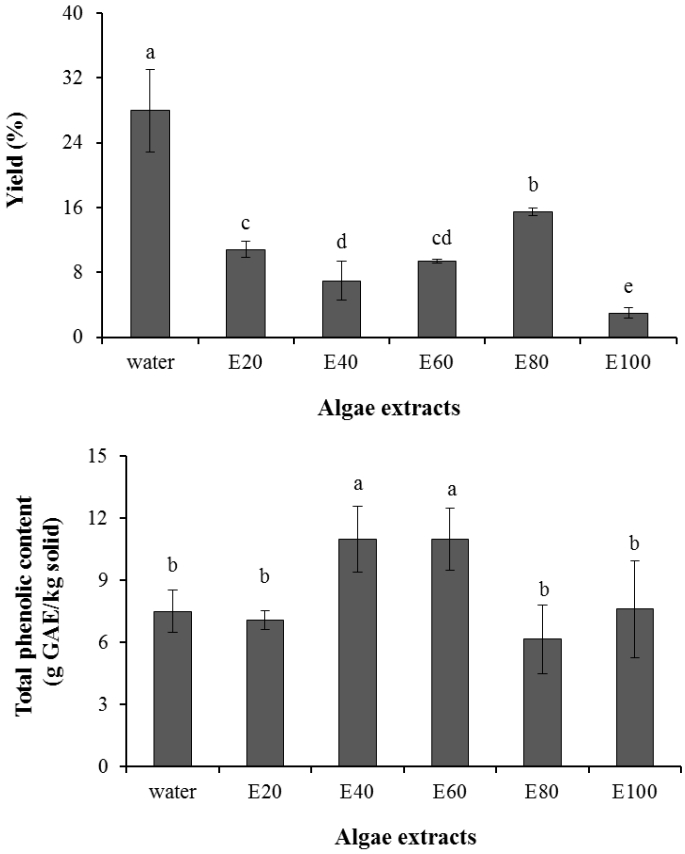

Figure 1. Yield (A) and total phenolic content (B) of different extracts from Northern freshwater macroalgae prepared using water or ethanol at different concentrations (20, 40, 60, 80 and 100%, v/v). Bars represent standard deviation (n=3). Different letters on the bars indicate significant differences (P < 0.05).

Yangthong *et al.*, 2009; Santoso *et al*., 2004). The results suggested that extracting media with different polarity mainly affected the extraction of phenolic compounds from these seaweeds. Although water was an effective media for extraction, there was no correlation between yield and TPC. Higher yields of water extract were possibly due to the water soluble polysaccharides, proteins or organic acids being simultaneously co-extracted with the phenolic compounds. Nevertheless, organic solvents with the right polarity could increase the extraction of phenolic compounds from freshwater macroalgae.

Total Chlorophyll Content (TCC) and Color

TCC and color of different algae extracts are shown in Table 1. Lower TCC was found in water extracts than those of ethanolic extracts. Since chlorophyll contains several non-polar moieties, it can be extracted using non-polar solvents. However, E20-40 showed lower values than water extracts. This was presumably due to the different cellular forms of chlorophyll (Wong, 1989). Chlorophyll and its derivatives with polar constituent were more soluble in water than 20-40% ethanol. Furthermore, TCC increased as the concentration of ethanol increased ($P<0.05$). E100 had the lowest L* and the highest a* and ΔE* values as evidenced by the darkest green color. Burrows (1991) reported that the bright green color associated with Chlorophyta extract was attributed to the major pigments, including chlorophyll a and b, β-carotene and xanthophylls. Ethanol was reported to be an effective solvent for chlorophyll extraction (Sartory & Grobbelaar 1984). Benjakul, Kittiphattanabawon, Sumpavapol, and Maqsood (2014) reported that phenolic compounds and mimosine from lead seed could be effectively released when the water was used as the extraction medium, while lower amounts of chlorophyll were extracted. In general, a green colored extract might be an obstacle for further application. Moreover, Lanfer-Marquez, Barros, and Sinnecker (2005) noted that chlorophyll could act as a photooxidizer. Chlorophylls as well as oxidized products in the extract might severe as pro-oxidant, in which singlet oxygen derived from photooxidation could be formed (Andersen, Skibsted, & Risbo, 2010). Wanasundara and Shahidi (1998) reported that the presence of chlorophyll in tea extracts was responsible for a pro-oxidant effect with marine oils.

***In Vitro* Antioxidant Activities**

Antioxidant activities are shown in Figure 2.

DPPH and Scavenging Activity

DPPH radical scavenging activity is shown in Figure 2A. DPPH radical scavenging activity increased when the concentration of ethanol increased up to 60% (E60) ($P<0.05$) and decreased at higher than 60%. DPPH radical scavenging activity of extracts reflects their hydrogen donating ability (Maqsood, Kittiphattanabawon, Benjakul, Sumpavapol, & Abushelaibi, 2015) which could reflect different compounds. The interaction of phenolic compounds with DPPH depended on the number of available hydroxyl groups and structural features such as O-H bond dissociation energy, resonance delocalizaion of the antioxidant and steric hindrance from bulky groups (Klompong & Benjakul, 2015). López *et al*. (2011) also reported that the extracting medium significantly affected TPC and antioxidant activities of several extracts from algae (*Stypocaulon scoparium*). Polarity of the extraction media determined their ability to extract different groups of antioxidant compounds (Zhou & Yu, 2004). Ethanol is low polarity solvent while water is strongly polar. E60 could extract the most algal phenolics with DPPH radicals scavenging activity. It was observed that the extracts with the most TPC scavenged more free radicals, which slowed oxidation.

ABTS Radical Scavenging Activity

ABTS radical scavenging activity is shown in Figure 2B and again E60 showed the highest ABTS radicals scavenging activity ($P<0.05$). However, the activity decreased sharply with E80 and E100. ABTS radical activity has been used to determine both hydrophilic and hydrophobic antioxidant capacities, while DPPH radical activity has been used to determine antioxidant properties in hydrophobic systems (Neelamegam & Valantina, 2015). Phlorotannin and fucoxannin-enriched ethyl acetate fraction of *F. vesiculosus* could contribute

Table 1. TCC and color of different extracts from Northern freshwater macroalgae as affected by extraction media

Extracts	TCC (mg/100 g extract)	Color			
		L*	a*	b*	ΔE*
Water	4.6±2.3^{b}	29.0±0.1^{a}	-0.42±0.05^{b}	2.02±0.05^{d}	65.5±0.2^{c}
E20	1.21±0.0^{c}	29.2±0.1^{a}	-0.48±0.03^{b}	1.83±0.03^{d}	65.3±0.1^{c}
E40	1.14±0.07^{c}	29.1±0.1^{a}	-0.62±0.02^{b}	1.97±0.03^{d}	65.4±0.2^{c}
E60	6.2±1^{b}	25.6±0.1^{c}	-1.45±0.00^{c}	5.32±0.08^{a}	68.9±0.1^{b}
E80	5.9±1^{b}	26.4±0.1^{b}	-1.41±0.04^{c}	4.56±0.07^{a}	68.2±0.5^{b}
E100	21±1^{a}	22.1±0.1^{d}	0.44±0.04^{a}	2.9±0.2^{c}	72±1^{a}

* Values are mean ± standard deviation (n=3).

** Different lowercase superscripts within a column denoted significant differences ($P<0.05$).

to high radical scavenging capacity (Honold, Jacobsen, Jónsdóttir, Kristinsson, & Hermund, 2016). The result indicated that an extraction medium that had the appropriate polarity could extract both hydrophilic and hydrophobic compounds.

Ferric Reducing Antioxidant Power

All extracts could reduce TPTZ–Fe (III) complex to TPTZ–Fe (II) complex as shown in Figure 2C. FRAP again was maximum for E60 ($P < 0.05$). Medina *et al*. (2007) reported that the reducing capacity measured the ease of donating electrons to Fe (III) (Klompong *et al*., 2008). Maqsood *et al*. (2015) also reported that 60% ethanol extracts of date seed had the highest FRAP activity.

Metal Chelating Activity

Metal chelating activity is shown in Figure 2D. Ethanolic extracts, especially E60-E-100, showed the highest activity ($P < 0.05$) with E60 again the highest ($P < 0.05$). No differences were observed when water and ethanol at low concentrations (20-40%) were used ($P \geq 0.05$). On the other hand, Wang *et al.* (2009) as well as Klompong and Benjakul (2015) found that water extracts from Icelandic brown algae and Bambara ground nut seed coats, respectively, had higher metal chelating activity than those extracted with other solvents. Santoso *et al*. (2004) reported that metal chelating ability has been related to the phenolic structure and quantity as well as the position of hydroxyl groups. Chelating activity is measured as the inhibition of ferrozine-Fe^{2+}complex formation (Sofidiya & Familoni, 2012). Ferrous ion (Fe^{2+}) is a pro-oxidant and can interact with hydrogen peroxide in a Fenton reaction to produce reactive oxygen species and hydroxyl (OH•) radicals, leading to the initiation and/or acceleration of lipid oxidation (Sakihama *et al.*, 2002).

Therefore, based on the above, E60 was selected for further study.

Identification and Quantification Of Phenolic Compounds in E60

Phenolic compounds in E60 were identified and quantified using LC/DAD/MSD (Table 2). The elution profile of phenolics is shown in Figure 3. E60 contained quercetin (2880 mg/kg), isoquercetin (1,940 mg/kg) and hydroquinin (1410 mg/kg) as major compounds. Gallic acid, catechin, tannic acid, rutin and kaempferol were also found. Simple phenolics (gallic, syringic, protocatechuic, and chlorogenic acids) and the flavonoids (+) catechin and (−) epicatechin were observed in methanolic *Euglena cantabrica* extracts (Jerez-Martel *et al*., 2017). Gallic acid, catechin and epicatechin were found as the predominant polyphenols of aqueous extracts from brown algae (*Stypocaulon scoparium*) (López *et al*., 2011). López *et al*. (2011) noted that solvent polarity did not change the total amounts of

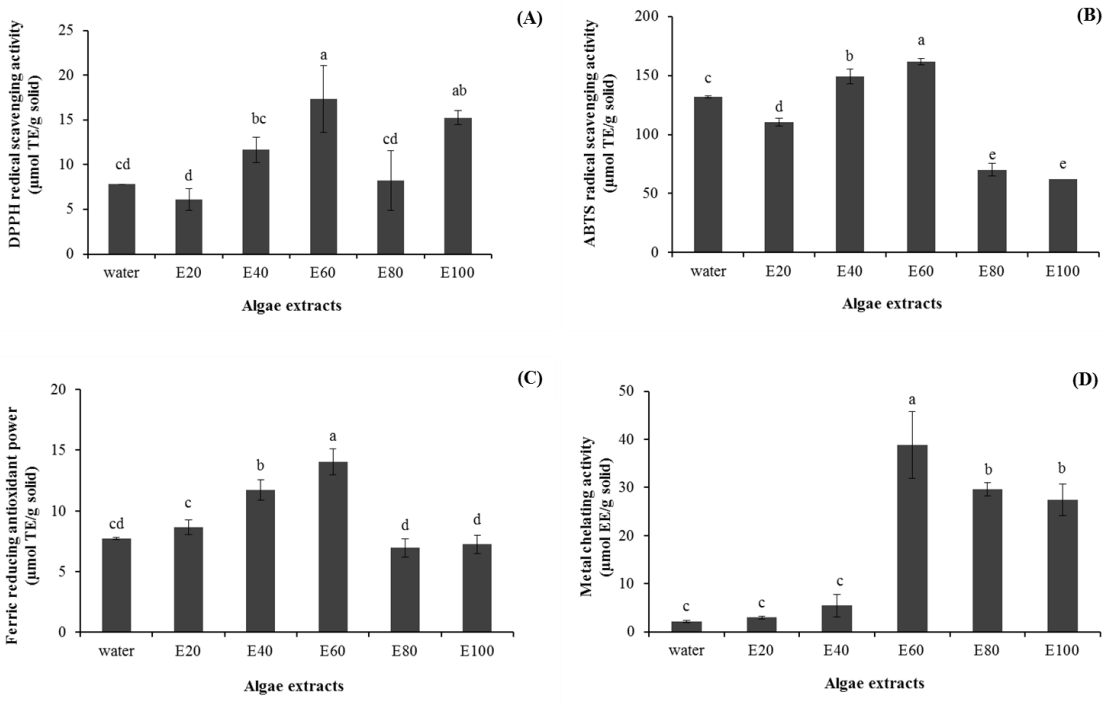

Figure 2. Antioxidant activities of different extracts from Northern freshwater macroalgae prepared using various extraction medium as determined using DPPH radical scavenging activity (A), ABTS radical scavenging activity (B), ferric reducing antioxidant power (FRAP) (C) and metal chelating activity (D). Bars represent the standard deviation (n=3). Different letters on the bars indicate significant differences ($P<0.05$).

a phenolics group, but it affected the phenolic profile. Quercetin is a plant flavonoid polyphenol. It has been reported to have antioxidant activity (Huber, Rupasinghe, & Shahidi 2009). Heijnen *et al*. (2002) reported that the catechol group in the B ring and the OH group at position 3 of the AC rings were the optimal configuration of quercetin for free radical scavenging. Huber *et al.* (2009) reported that C-3 glycosylation increased the antioxidant efficiency of quercetin in a bulk oil model.

Antioxidant Effect of E60 With Eastern Little Tuna Slices

Changes in PV

Changes in PV of tuna slices treated with E60 at different levels in comparison with BHA (100 mg/kg) during storage at 4ºC are shown in Figure 4A. PV of all sample tended to increase as storage time increased ($P < 0.05$). In the absence of an additive (control), a marked increase in PV was observed within the first 6 days of storage ($P < 0.05$), followed by a decrease up to day 10. The increase in PV of the samples indicated the increased formation of hydroperoxides, a primary lipid oxidation product. Generally, lipid oxidation of depot and membrane lipids occurs in tuna muscle during extended storage (Thiansilakul, Benjakul, Grunwald, & Richards, 2012). Moreover, heme proteins, especially in the dark muscle, accelerated lipid oxidation in fish muscle, particularly when non-heme iron was released (Thiansilakul *et al*., 2012). The subsequent decrease in PV indicated that the hydroperoxides decomposed to other secondary oxidation compounds. The PV pattern of samples containing E60 at 100 mg/kg was similar. However, the E60 slowed the increase in PV compared to the control. The slowing of PV occurred in a concentration dependent manner ($P < 0.05$). However, BHA slowed the increase in PV more effectively than E60 throughout storage. E60 presumably functioned as a radical scavenger or metal chelator (Figure 3). The

Table 2. Types and contents of phenolic compounds in E60

Peak No.	Retention time (min)	Phenolic compounds	Phenolic contents ($\times 10^2$ mg/kg)
1	7.33	Gallic acid	6.63
2	12.5	Catechin	8.64
3	12.9	Tannic acid	6.02
4	16.0	Rutin	1.99
5	16.7	Isoquercetin	19.4
6	23.9	Hydroquinin	14.1
8	33.1	Quercetin	28.8
10	43.2	Kaempferol	0.776

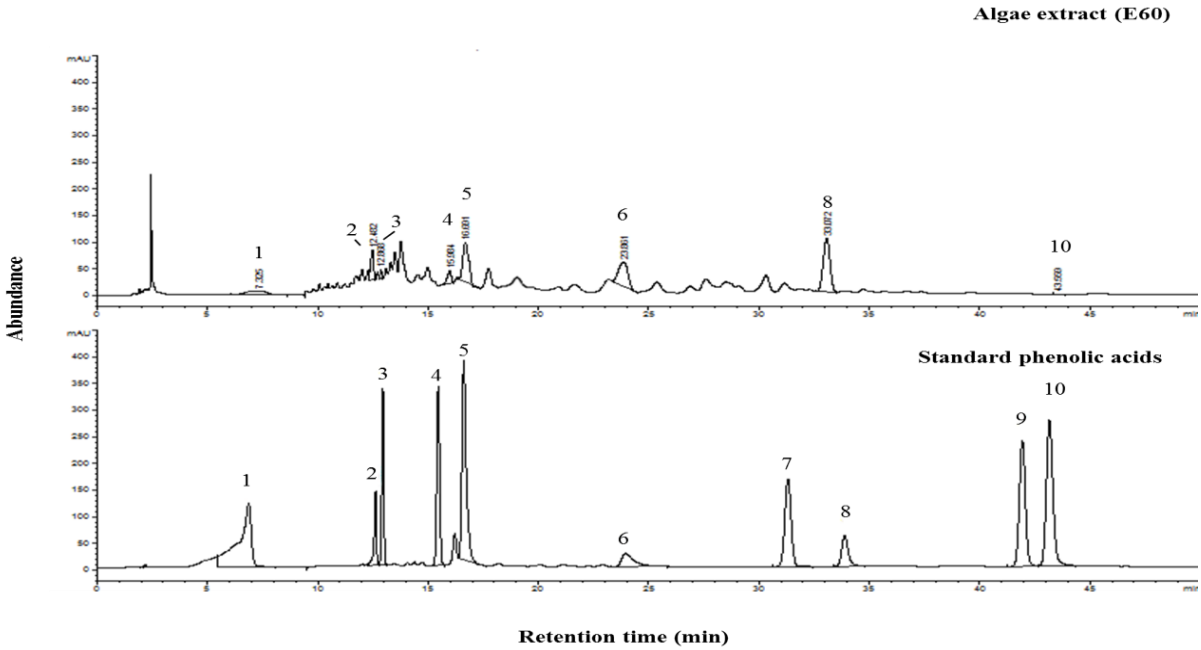

Figure 3. LC-MSD chromatogram of the polyphenolic compounds in E60. (1): Gallic acid; (2): Catechin; (3): Tannic acid; (4): Rutin; (5): Isocuercetin; (6): Hydroquinin; (7): Eriodictyol; (8): Quercetin (9): Apigenin; (10) Kaempferol.

mixtures of polar and non-polar phenolics in E60 could be dissolved and localized in both the polar and non-polar aspects of the fish muscle. It was noted that E60 at 400 mg/kg came close to the activity of 100 mg/kg BHA. Maqsood and Benjakul (2011) reported that free radical antioxidants interfere with the initiation and propagation step of lipid oxidation by quenching lipid radical. Thiansilakul, Benjakul, and Richards (2013) reported that tuna slices with tannic acid had markedly lower PV during extended storage when stored under MAP. Jónsdóttir *et al*. (2016) reported that brown seaweed extract was high in total phenolic compounds and strongly inhibited hemoglobin mediated lipid oxidation in a fish muscle model system. In addition, Halldorsdottir *et al.* (2014) reported that the use of natural antioxidants extracted from Icelandic brown seaweed (*F. vesiculosus*) could protect cod bone mince protein hydrolysates from lipid deterioration.

Changes in TBARS

The changes during storage at 4 °C are shown in Figure 4B. TBARS formation showed similar trends to those of PV (Figure 4A). TBARS values have been used to determine the secondary products of lipid oxidation, especially aldehydes and decomposition products of hydroperoxides (Thiansilakul *et al*., 2012). During the first 2 days of storage, TBARS in all samples increased slightly. Thereafter, sharp increases in TBARS were observed in the control, followed by a decrease at day 8 of storage ($P < 0.05$). For the samples containing E60, TBARS formation was significantly increased after day 4-6 ($P < 0.05$). It was noted that efficacy of E60 in prevention of TBARS formation was dose dependent. Nevertheless, the rate of increase in TBARS formation was lower than with BHA. The decreases in TBARS at the end of storage, as observed in control and those containing E60 at 100 mg/kg, might be caused by a loss of low molecular weight volatile compounds and the reaction of aldehydes with other compounds, especially proteins (Thiansilakul *et al*., 2013). This was in agreement with Benjakul, Kittiphattanabawon, Shahidi, and Maqsood (2013) who reported that TBARS formation of a control mackerel mince (without additive) peaked at day 8, followed by a decrease at the end of iced storage (day 10). The addition of lead seed extracts could reduce TBARS formation of the mackerel mince (Benjakul *et al*. 2013). Steamed chicken mince with the seed coat of Bambara ground nut extracts also slowed the increase in TBARS throughout 15 days at 4 °C (Klompong & Benjakul, 2015). Wang *et al.* (2009) noted that phlorotannin assisted the ethyl acetate fraction in

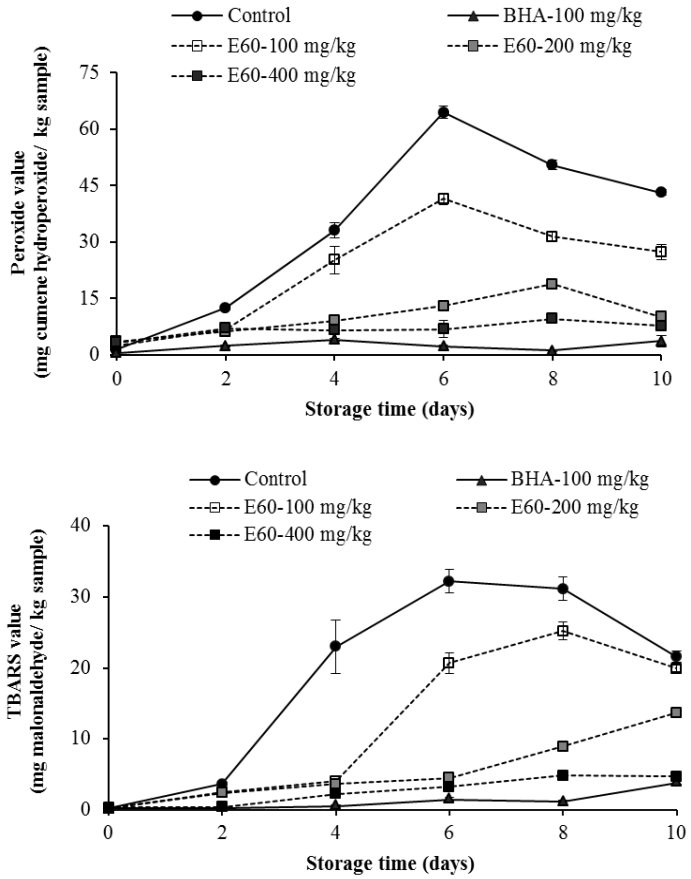

Figure 4. Changes in peroxide (A) and TBARS (B) values of eastern little tuna slices treated with E60 at different concentrations (100-400 mg/kg) during 10 days of refrigerated storage. BHA at a concentration of 100 mg/kg was used as the positive control. The bars represent the standard deviation (n=3).

significantly inhibiting the development of rancid odors in cod muscle and its protein isolate. Ortiz, Vivanco, and Aubourg (2014) also reported that the addition of different seaweed extracts from cochayuyo, sea lettuce, ulte, and red luche in the covering liquid led to the decrease of secondary peroxidation in canned salmon during 170 days of canned storage at 40 °C compared to the control sample. The addition of E60, especially at 400 mg/kg, could be a promising way to decrease the deterioration associated with lipid oxidation in post-mortem fish.

Conclusions

Freshwater macroalgae could be an alternative source of natural antioxidants. Ethanol (60%, v/v) gave the highest antioxidant activity. E60 containing quercetin, isoquercetin and hydroquinin as its major components could retard lipid oxidation in tuna fish slices in a dose dependent manner. E60 at 400 mg/kg was able to prevent lipid oxidation during 10 days of refrigerated storage, but its efficacy was slightly lower than that of BHA. E60 might therefore serve as an alternative natural antioxidant to prevent lipid oxidation of fish during post-mortem storage or transportation. However, the presence of chlorophyll might have a negative effect on sensory properties. Thus, the effect of dechlorophyllization must be studied.

Acknowledgements

This work was supported by the Thailand Research Fund to Dr. Suthasinee Yarnpakdee (MRG5980086). Further funding for this research was provided under the Functional Food Research Center for Well-being, Chiang Mai University, Thailand. Faculty of Agro-industry, Chiang Mai University and Prince of Songkla University, Hat Yai, Thailand are gratefully acknowledged for instrument supports and use of their facilities.

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