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Studies on *in vitro* Antioxidant Activity of Marine Edible Seaweed From East Coastal Region, Peninsular Malaysia Using Different Extraction Methods

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Abstract: In this present study the antioxidant activity of two edible seaweeds *Eucheuma cottonii* and *Padina* sp. were determined. The two extraction methods such as conventional and soxhlet were used to extract secondary metabolites by using methanol as a solvent medium. Total phenolic content of crude seaweed extracts were analysed by standard method. The different antioxidant assays 2,2-Diphenyl-1-Picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Power (FRAP) and β-carotene bleaching assays were studied. The DPPH and FRAP assays showed positive correlation with expressed higher total phenolic content of methanolic seaweed extracts. Also, β-carotene bleaching assay lower activity compare with BHT as reference control. IR spectra showed the phenolic related functional groups are present the seaweed extract. It mainly responsible for higher rate of antioxidant activity. The methanolic extracts of *Padina* sp. showed better radical scavenging and higher phenolic content than *Eucheuma cottonii*. Also, the soxhlet extraction showed higher yield and better radical scavenging activity compared to conventional method. Moreover, the studies confirmed both seaweeds are an effective candidate for the control the free radical scavenging activity.

Key words: Edible seaweeds, phenolic compound, extraction, DPPH, FT-IR

INTRODUCTION

Seaweeds are multicellular macroalgae using potential renewable resource in the field of medical and commercial environment. Seaweed explores numerous pharmacologically important bioactive compounds such as flavanoids, carotenoids, dietary fiber, protein, essential fatty acids, vitamins and minerals are studied in detail. Nowadays seaweeds are used as dietary food supplements in daily life and it regulates the human health (Ganesan *et al*., 2008).

Eukaryotic system have number of cellular defence systems which include enzymatic scavengers such as catalase, glutathione peroxide and Superoxide Dismutase (SOD) are evolved to prevent the oxidation of cells using oxidizable substrates (O'Sullivan *et al*., 2011). Reactive Oxygen Species (ROS) and free radicals can only be eliminated efficiently under normal conditions but pathological conditions it induces various chronic diseases. The balance between generation and elimination of ROS cannot be achieved under clammy condition (Je *et al*., 2009). Other than peroxidation of lipids, the ROS will also, result in extensive oxidative damage which induce of serious human diseases including atherosclerosis, rheumatoid arthritis, muscular dystrophy, some neurological disorders, aging and cancer (Heo *et al*., 2003; Devi *et al*., 2011). However, uses of the synthetic antioxidants such as butylated hydroxyanisol butylated hydroxytoluene as food preservatives have been suspected to be a possible cause for liver damage and carcinogenesis (Ahn *et al*., 2004; Kumar *et al*., 2008; Vijayabaskar and Shiyamala, 2012). Consequently, nowadays most of the literatures are more focusing on finding alternative antioxidants from natural origin.

According to that novel findings of marine seaweed is a valuable antioxidant source, it consists of high level of antioxidants compounds (Yan *et al*., 1998; Duan *et al*., 2006; Kuda *et al*., 2005). Based on that seaweeds and their extracts are beneficial to health and some even have been reported to retain biological activity of potential medicinal value. Hence, the present study investigated on the antioxidant activity of edible seaweeds and studies on their percentage of phenolic content in the seaweed sample.

MATERIALS AND METHODS

Chemicals used: The 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Butylated Hydroxytoluene (BHT), Folin Ciocalteu's phenol reagent, 2,4,6-Tri (2-pyridyl)-1,3,5-

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Triazine (TPTZ) were purchased from Sigma, Malaysia. All other solvents and chemicals were of analytical grade used throughout the experiment.

Collection and processing of seaweeds: The sample *Eucheuma cottonii* and *Padina* sp. was collected from Pulau Besar, Mersing, Malaysia. The seaweeds were washed thoroughly with tap water to remove undesired particles and epiphytes. Then, the samples were dried in oven at 50°C for 2 days. The dried seaweeds were cut into smaller pieces and grind into powder and used for the extraction studies.

Extraction of seaweeds: Conventional method: A 10 g of seaweed powder were weighted and 100 mL of methanol was added into the clean conical flask and kept in shaker, 120 rpm at room temperature for 72 h. Extracted sample were filtered in a whatman filter paper was used for filteration. The solvent were removed by using rotary evaporator under vacuum at 40°C. The method was followed by Souza *et al.* (2011) with some modification. Soxhlet method: 20 g of seaweed powder was weighted put in a thimble and placed into soxhlet apparatus and extracted using 200 mL of methanol at 60°C for continually repeating 12 cycles. The solvent was evaporated by using a rotary evaporator under vacuum at 40°C. Both the extracts obtained were left air dried in a fume hood to make a dried sample. The dried extracts were stored at -20°C for further use.

Measurement of extraction yield: The yield of the extracts obtained from different extraction methods. The dry weight of the seaweed samples was calculated the total weight (Heo *et al*., 2006).

Determination of total phenolic content: The total phenolic contents of methanolic seaweed extract were performed by Taga *et al.* (1984). Sample (10 mg mL^{-1}) of 100 μL was added to 2 mL of 2% Na_2CO_3. The mixture was then left to stand for 2 min for incubation at room temperature.

Further the reaction mixture adding with 100 μL of 50% Folin-Ciocalteau's phenol reagent and incubated the reaction mixture at room temperature in the dark for 30 min. After 30 min by read the absorbance of all the sample at 720 nm. A gallic acid calibration curve was constructed to determine the phenolic contents in term of Gallic Acid Equivalents per gram (GAE/g) of extract:

$$C = c \times \left(\frac{V}{m}\right)$$

Where:

C = Total phenolic content of sample extract in mg GAE/g
c = Concentration of gallic acid established from calibration curve in mg/g
V = Volume of the extract (mL)
m = Weight of sample extract (g)

DPPH free radical scavenging assay: The 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) radical scavenging ability of the seaweed extracts was monitored by Nurul *et al.* (2011) method. Initially, the 0.16 mM DPPH solution was prepared freshly in methanol. A volume of 100 μL of DPPH solution was added to 100 μL of samples with different concentrations in 96 well plates and incubated at 37°C for 30 min. The methanol used as negative control and while positive control was BHT with the addition of DPPH solution, respectively. After incubation, the absorbance of the samples were measured using the Tecan Infinite M200 PRO microplate reader at 515 nm. The scavenging activity of the samples was determined by following equation:

$$\text{DPPH scavenging activity (\%)} = \left[1 - \frac{A_{sample} - A_{blank}}{A_{control}}\right] \times 100$$

Where:

A_{sample} = The absorbance of test samples
A_{blank} = The absorbance of samples
$A_{control}$ = The absorbance of control (BHT)

FRAP (Feeric Reducing Antioxidant Powder) assay: FRAP assay was performed to measure the scavenging activity of the seaweed extracts according to modified method of Benzie and Strain (1999). The FRAP reagent solution was freshly prepared with 300 mM acetate buffer (pH 3.6), 10 mM TPTZ 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in the ratio of 10:1:1. The reagent solution was warmed to 37°C before use. Briefly, 200 μL of the reagent solution was dispensed into the 96 wells plate followed by adding 20 μL crude extracts to initiate the reaction. The reaction mixtures were left for 10 min and the absorbance was read at 593 nm using a Tecan Infinite M200 PRO microplate reader. Ascorbic acid with concentration of 2 mg mL^{-1} was used as positive control:

$$\text{FRAP scavenging (\%)} = \left[\frac{Abs_{sample} - Abs_{control}}{Abs_{sample}}\right] \times 100$$

β-carotene bleaching assay: The total antioxidant activity of seaweed extracts and standards (BHT) was measured according to the method of Velioglu *et al.* (1998). About

1 mL of β-carotene solution (0.2 mg mL^{-1} chloroform) was dispersed into a round-bottom flask containing 0.02 mL of linoleic acid and 0.2 mL of 100% 20. The mixture solution chloroform was evaporated at 40°C for 10 min using rotary evaporator. Once the evaporation was completed the 100 mL of distilled water was immediately added to the mixture. The mixture mix well and it was formed like foam. The BHT and sample solutions prepared in 2 mg mL^{-1} were added with 5 mL aliquots of the emulsion. The tubes were mixed gently and placed in a water bath at 45°C for 2 h. A blank, consisting of an emulsion without β-carotene and methanol in 5 mL of the above emulsion as control were also prepared. The changes of absorbance was measured at 470 nm. The assay was calculated by using the following equation:

$$\beta\text{-carotene bleaching assay} = 1-\frac{A0-At}{A°0-A°t}\times 100$$

Where:

A0 and A°0 = The absorbance values measured at initial time of the incubation for samples and control, respectively

At and A°t = The absorbance values measured in the samples or standards and control at t = 120 min

Fourier Transform Infra-Red spectroscopy (FT-IR): The dried crude extract of seaweed samples was mixed with potassium bromide to prepare as a pellet. The disc prepared was analyzed in Perkin Elmer, Spectrum 100 FT- IR-Spectrometer between 4000-400 cm^{-1}.

Statistical analysis: All the assays were carried out in triplicate and the values are expressed in the mean±standard error. The SPSS 20.0 was used for the experiment one-way Analysis of Variance (ANOVA) to compare the mean values of the intensity. Then, the analysis was proceed through Turkey's multiple range test statistical significance at $p \leq 0.05$.

RESULTS AND DISCUSSION

Table 1 shows the extraction yield of the methanolic crude extracts of *E. cottonii* ranged from 2.76-5.03% while the *Padina* sp. crude extracts total yield from 1.21-2.87% in conventional and soxhlet extraction, respectively. For both methods, the methanolic extracts of *Eucheuma cottonii* showed higher yield as compared with the *Padina* sp. From the result, it showed an increased of extraction yield by using different extraction method with same solvent. The yield increased at around 50% when using Soxhlet Extraction Method compared to Conventional Extraction Method. Table 2 shows the total phenolic contents of *Padina* sp. in conventional and soxhlet extraction were 14.58±0.12 and 15.28±0.11 mg GAE/g, respectively while *E. cottonii* were 8.71±0.09 and 9.04±0.05 mg GAE/g, respectively.

Table 1: The extraction yields of the methanolic extracts of *Eucheuma cottonii* and *Padina* sp. from conventional and Soxhlet Extraction Method

	Yield (%) w/w	
Seaweeds	Conventional	Soxhlet
Eucheuma cottonii	2.76	5.03
Padina sp.	1.21	2.87

Table 2: Total phenolic content of seaweed extracts in different extraction methods

	Total phenolic content (mg GAE/g)	
Seaweed	Conventional	Soxhlet
E. cottonii	8.71±0.09[a]	9.04±0.05[b]
Padina sp.	14.58±0.12[a]	15.28±0.11[b]

Table 3: Free radical scavenging activities of conventional and soxhlet extraction of *E. cottonii* and *Padina* sp.

		Antioxidant assays		
Seaweed extract	Extraction methods	DPPH	FRAP	β-carotene
E.cottonii	Conventional	32.74±0.16[a]	40.54±0.62[a]	27.86±0.08[a]
	Soxhlet	31.44±0.08[b]	46.22±0.13[b]	34.72±0.28[b]
Padina sp.	Conventional	29.64±0.05[a]	47.06±0.22[a]	21.45±0.24[a]
	Soxhlet	32.45±0.01[b]	56.03±0.37[b]	28.67±0.02[b]
Standard		61.29±0.02	86.10±0.01	66.84±0.37

[a]Comparision of conventional extract with [b]soxhlet extract

Table 4: Studies on FT-IR functional groups of *E. cottonii* and *Padina* sp. extracts

IR frequency (cm^{-1})	Bond	Functional groups	*E. cottonii*	*Padina* sp.
3500-3200	O-H stretch	Alcohols, phenols	+	+
3100-3000	C-H stretch	Aromatics	-	-
3300-2500	O-H stretch	Carboxylic acids	+	+
1760-1665	C=O stretch	Unknown	-	-
1500-1400	C-C stretch	Aromatics	+	+
1320-10000	C-O stretch	Alcohols, carboxylic acids, esters, ethers	+	+
1000-650	C-H bend	Alkenes	+	+
900-675	C-H	Aromatics	+	+

Table 3 illustrates the DPPH activity of *E. cottanii* and *Padina* sp. of conventional and soxhlet extractions were 32.74±0.01 and 39.41±0.00 mg mL^{-1}, respectively. These readings were higher than BHT (61.29±0.02 mg mL^{-1}), lower compared to *E. cottonii* of conventional (29.74±0.16 mg mL^{-1}) and soxhlet extraction (32.04±0.08 mg mL^{-1}). Thus, this study showed that the antioxidant activity of *Padina* sp. is higher than *E. cottonii* lower than the positive control of BHT. Table 4 shows ferric reducing power of *E. cottonii* and *Padina* sp. extracts with the increased concentration of the extract. At concentration 2 mg mL^{-1}, the antioxidant content of *Padina* sp. extract from conventional and

soxhlet extraction 47.06±0.73 and 56.03±0.22%, respectively. Compared with *E. cottanii* was showed effective values in both extraction such as 40.54±0.62 and 46.22±0.13, respectively. The ascorbic acid expressed 86.10±0.01% higher than the *E. cottonii* and *Padiana* sp. The beta carotene scavenging activity of *Padiana* sp. shows 27.86±0.08 and 34.72±0.28%, respectively. Similarly *E. cottonii* expressed moderate scavenging properties, the value is 21.45±0.24 and 28.69±0.12 for conventional and soxhlet methanolic seaweed extracts. The inhibition of the beta carotene bleaching activity of seaweed extracts and BHT at various time intervals were measured. The total antioxidant activity between seaweed extracts of *E. cottonii*, *Padina* sp. and BHT did not showed significant difference ($p>0.05$). At the same time different extraction methods did not affect the antioxidant activity of the seaweeds as well.

FTIR analysis shows the functional group of *E. cottonii* and *Padina* sp. shown in Table 4. The *E. cottonii* and *Padina* sp. strong peaks in the region of 3500-3200 cm^{-1} is (O-H stretch) indicates phenolic active compounds, 1500-1400 cm^{-1} (C-C stretch) of aromatics clusters, 1000-650 cm^{-1} (C-H bend-alkenes) and 900-675 cm^{-1} (C-H stretch-aromatic). Furthermore, the FT-IR spectrums *E. cottonii* and *Padina* sp. also, showed moderate peaks in 1680-1640 cm^{-1} region it may be (-C = C stretch) alkanes.

Naturally seaweeds contains novel antioxidant compounds which is control the free radical formation. Some active therapeutic metabolites are identified in different marine seaweed such as *Sargassum* sp., *Padina* sp. and *Eisenia bicyclis* were reported. These edible seaweeds contain phlorotannins, fucoxanthin, polyphenols and phylopheophylin. The bioactive metabolites might be involved in the metabolic regulation to regulate the normal mechanism (Kim *et al*., 2005; Anagnostopoulou *et al*., 2006).

Kalia *et al*. (2008) stated that Soxhlet Extraction Method that contributed to maximum yield among other extraction methods. Jimenez-Escrig *et al*. (2001) brown seaweed showed significantly higher phenolic content than red seaweeds. The total phenolic contents of seaweed extracts is different concentration were observed in different methods. where TPC of soxhlet extract is higher than conventional extract.

According to Nakamura *et al*. (1996), phaeophyta consist of a kind of polyphenol called phlorotannins. This polyphenol had been reported as potential antioxidant, anticancer, antibacterial and antifungal compounds. The extraction methods do not have significant influence *Padina* sp. compare with *E. cottonii*. This happened might be due to Soxhlet Method extracted out the non-effective antioxidant that reacts with Folin-Ciocalteu reagent (Tomsone *et al*., 2012). Meenakshi *et al*. (2011) reported the brown seaweed *S. wightii* possessed higher antioxidant content. The reducing power property indicated the *Padina* sp. and *E. cottonii* extracts shows the consist of antioxidant compounds in the seaweed which are electron donors and will be able to reduce the oxidized intermediates of the lipid per oxidation process (Yen and Chen, 1995). Likewise Devi *et al*. (2008) stated that the presence of phenolic compounds in a seaweeds extract has the probably in affecting their antioxidant activity. Phenols in a plant significant constituents due to the present of hydroxyl groups which have the capability of scavenging of free radicals. Type of solvent as essential factor for the isolation of antioxidant compounds. Also, polar solvents are extracted considerable crude metabolites including polyphenols and tannins. These natural compounds have been exhibiting considerable antioxidant potential (Hatano *et al*., 1989). The infrared peaks of aromatic and hydroxyl groups revealed that *E. cotton* and *Padina* sp. probably consists of phenolic compounds (Meenakshi *et al*., 2009).

Therefore, further analyses using different antioxidant assays are essential in identifying the active compounds that are responsible for the activity. This finding is beneficial for future study and determining the major antioxidant compounds in the seaweed extract.

CONCLUSION

The *E. cottonii* and *Padina* sp. can be utilized as a source of natural antioxidant compounds as their methanolic crude extracts possessed higher antioxidant activity. The results indicate that the antioxidant activity of the seaweeds is related to the presence of phenolic compounds. The Soxhlet Extraction Method seems to be able to maximize the yield of extracts and more effective in extracting the phenolic compounds compared to Conventional Extraction Method. FTIR showed there are present of potential phenolic compounds in the methanolic seaweed extract. These seaweed have potent bioactive compounds that important in food as well as medical applications.

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