Extraction of phenolic antioxidants from four selected seaweeds obtained from Sabah

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Abstract

Algal have attracted attention from biomedical scientists as they are a valuable natural source of secondary metabolites that exhibit antioxidant activities. In this study, single-factor experiments were conducted to investigate the best extraction conditions (ethanol concentration, solid-to-solvent ratio, extraction temperature and extraction time) in extracting antioxidant compounds and capacities from four species of seaweeds (*Sargassum polycystum, Eucheuma denticulatum , Kappaphycus alvarezzi* variance Buaya and *Kappaphycus alvarezzi* variance Giant) from Sabah. Total phenolic content (TPC) and total flavonoid content (TFC) assays were used to determine the phenolic and flavonoid concentrations, respectively, while 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picylhydrazyl (DPPH) radical scavenging capacity assays were used to evaluate the antioxidant capacities of all seaweed extracts. Results showed that extraction parameters had significant effect ($p < 0.05$) on the antioxidant compounds and antioxidant capacities of seaweed. *Sargassum polycystum* portrayed the most antioxidant compounds (37.41 ± 0.01 mg GAE/g DW and 4.54 ± 0.02 mg CE/g DW) and capacities (2.00 ± 0.01 µmol TEAC/g DW and 0.84 ± 0.01 µmol TEAC/g DW) amongst four species of seaweed.

Introduction

For centuries, seaweed has been used in the preparation of salads, soups and also as low-calorie foods in Asia (Jiménez-Escrig and Sánchez-Muniz, 2000). Japanese are the main consumers of seaweed with an average consumption of 1.6 kg (dry weight) per year per capita (Dhargalkar and Pereira, 2005). Most Europeans and Americans use processed seaweed as additives in their food preparation (Boukhari and Sophie, 1998). However, in India, seaweeds are exploited mainly for the industrial production of phycocolloids such as agar-agar, alginate and carrageenan; and not as cookery item or for recovering beneficial biomolecules. In 1978, seaweed cultivation was introduced in Sabah and had increasingly become an economically important natural resource for Malaysia, particularly for Sabah. The interest for seaweed escalates tremendously in recent years due to the demand caused by abalone farmers (Vasquez, 1999) the development of new products such as organic fertilisers and use for human food (Alejandro *et al.*, 2008).

In recent years, seaweed products have received special attention as a source of natural antioxidants (Lim *et al.*, 2002) and some of them possess biological activity of potential medicinal value (Satoru *et al.*, 2003). Natural antioxidants are perceived to be safe by consumers because they are naturally found in plant materials and have been used for centuries (Frankel, 1996). Natural antioxidants have shown to play a significant role in preventing a number of chronic diseases such as heart disease, cancer, Alzheimer's and Parkinson's diseases (Weinreb *et al.*, 2004).

Several researchers have reported the antioxidant properties of both brown and red seaweeds from across the globe (Heo *et al.*, 2005). Some active

antioxidant compounds from marine algae were identified as phylopheophylin in Eisenia bicyclis (Cahyana, Shuto and Kinoshita, 1992), phlorotannins in Sargassum kjellamanianum (Yan *et al.* 1996) and fucoxanthinin in Hijikia fusiformis (Yan *et al.*, 1999). Furthermore, there are evidences available to show the potential protective effects of seaweed against oxidative stress in target tissues and lipid oxidation in foods (Rajamani *et al.*, 2011).

Therefore, the main objective of this study was to evaluate the effect of extraction conditions (ethanol concentration, solid-to-solvent ratio, extraction temperature and extraction time) in extracting antioxidant compounds as well as antioxidant capacities of the four selected seaweeds (*Sargassum polycystum, Eucheuma denticulatum , Kappaphycus alvarezzi* variance Buaya and *Kappaphycus alvarezzi* variance Giant) and determine the best extraction conditions for the seaweeds.

Materials and Methods

Seaweed cultivation and collection

Sargassum polycystum (SP) and *Eucheuma denticulatum* (ED) were commercially farmed seaweed in Semporna, Sabah. They were harvested at week 6 (maturity stage). *Kappaphycus alvarezii* variance Giant (KAG) and *Kappaphycus alvarezii* variance Buaya (KAB) were tissue cultured seaweed, grown in Universiti Malaysia Sabah (Kota Kinabalu, Malaysia). 1.0 g of explants was cultured *in-vitro* for 10 - 12 weeks, producing 50.0 g of seedlings to acclimatize in the open sea. They were harvested at week 16 (maturity stage). Seaweeds were cleaned under running water and air-dried for 2 days. Then, they were placed in oven at 60 °C until they were completely dry. Dried seaweed were packed and delivered to Universiti Putra Malaysia (Serdang, Malaysia) for future analysis.

Sample preparation

500 g of dried seaweeds were ground in a laboratory grinder (Mikro-Feinmuhle-Culatti. MFC grinder, Janke and Kunkel GmbH and Co., Staufen,. Germany) with a particle size of 0.08 mm. Powdered samples were then vacuum-packed and stored in dark for further research.

Sample extraction

1 g of powdered sample of each species of seaweeds was accurately weighed into conical flasks (50 mL). The extraction processes were carried out by varying the experiment parameters for ethanol concentration, solid-to-solvent ratio, temperature and time. After the extractions, seaweed extracts were filtered by a glass funnel with Whatman No. 1 filter paper (Whatman International, England). The clear solution of crude extract was collected in a light-protected amber bottle (50 mL) for analysis without further treatment. All extractions were carried out in replicates.

Factor 1: Ethanol concentration

10 mL of ethanol and deionised water were mixed according to the ethanol concentration set in 5 levels (0, 25, 50, 75 and 100 %, v/v), added to 1 g of each sample. They were then placed in a water bath shaker at 40 °C at 150 rpm for 2 h.

Factor 2: Solid-to-solvent ratio

An amount of ethanol and deionised water (best ethanol concentration obtained from section Factor 1) was added to each sample according to the solid-to-solvent ratio set in 5 levels (1:10, 1:15, 1:20, 1:25 and 1:30, w/v). They were then placed in a water bath shaker at 40 °C at 150 rpm for 2 h.

Factor 3: Extraction temperature

An amount of ethanol and deionised water (best ethanol concentration obtained from section Factor 1) were added to each sample according to the best solid-to-solvent ratio obtained from section Factor 2. They were then placed in a water bath shaker at 5 different temperatures (25, 35, 45, 55 and 65°C) at 150 rpm for 2 h.

Factor 4: Extraction time

An amount of ethanol and deionised water (best ethanol concentration obtained from section Factor 1) were added to each sample according to the best solid-to-solvent ratio obtained from section Factor 2. They were then placed in a water bath shaker at the best temperature of each sample obtained from section Factor 3 at 150 rpm for a range of time set in 5 levels (1, 2, 3, 4 and 5 h).

Total phenolic content (TPC) assay

Total phenolic content (TPC) was determined using Folin-Ciocalteu (F-C) assay (Lim *et al.*, 2007) 500 μL of crude extracts obtained from extraction were added into Eppendorf falcon tubes (2 mL) followed by 500 μL of Folin-Ciocalteu's reagent (diluted 10 times with water). After 4 min, 400 μL of 7.5% (w/v) sodium carbonate were added. The blank was prepared by replacing 500 μL of sample with 500 μL of deionised water. Subsequently, the falcon tubes were vortexed for 10 s with vortex mixer (VTS-3000L, LMS, Japan). They were incubated

in the dark environment at room temperature for 2 h. Absorbance was measured against the blank reagent at 765 nm using UV light spectrophotometer (Model XTD 5, Secomam, France). Each extract was analyzed in triplicate and TPC were expressed as gallic acid equivalent (GAE) in mg per 100 g dry weight (DW).

Total flavonoid content (TFC) assay

The determination of flavonoids was based on the procedures described in the study (Ozsoy *et al*., 2008) with slight modifications. 50 µL of crude extract added to 250 µL of deionised water, followed by the addition of 15 µL of 5% sodium nitrite in Eppendorf falcon tubes (2 mL). After 6 min, 30 µL of 10% aluminium chloride hexahydrate was added into the mixture and was allowed to stand for further 5 min. Then, 100 µL of 1 M sodium hydroxide and 55 µL of deionised water were added. The blank was prepared by replacing the 50 µL sample with 50 µL of deionised water. The falcon tubes were mixed thoroughly by using a vortex mixer (VTS-3000L, LMS, Japan) for 10 s. Then, absorbance readings were immediately taken at 510 nm using the UV light spectrophotometer (Model XTD 5, Secomam, France). Each extract was analyzed in triplicate and TFC were expressed as catechin equivalent (CE) in mg per 100 g dry weight (DW).

2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging capacity assay

Antioxidant capacity was determined by measuring the scavenging activity of the radical 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) based on the method (Surveswaran, 2007) with slight modifications. 10 mL of 7 mM ABTS solution and 10 mL of 2.45 mM potassium persulfate ($K_2S_2O_8$) solution were transferred into a 100 mL light protected amber bottle. The solution were mixed by vortex mixer (VTS-3000L, LMS, Japan) for 10 s and allowed to stand in a dark environment at room temperature for 16 h to give a dark blue solution. This solution was diluted with 95% ethanol until the absorbance was equilibrated to 0.7 (± 0.02) at 734 nm. 975 µL ABTS solution with equilibrated absorbance of 0.7 ± 0.02 was added to 25 µL of the undiluted extract in an Eppendorf falcon tube (2 mL). Negative control was prepared by replacing 25 µL of undiluted crude extract with 25 µL of 95% ethanol whereas blank was prepared by using 95% ethanol solely. The reaction was allowed to occur at room temperature for 6 min and the absorbance at 734 nm was immediately recorded against blank using the UV light spectrophotometer (Model XTD

5, Secomam, France). Both the crude extracts and negative control were carried out in triplicate. Trolox solution was used to calibrate the standard curve. The mean ± SD results of triplicate analyses were expressed as µmol trolox equivalent per 100 g dried sample (µmol TEAC/100 g dried sample).

$$ABTS \text{ radical scavenging capacity } (\%) = [1 - (A_o / A_1)] \times 100 \% \quad (1)$$

Where A_o is A_{734} of the crude extract; A_1 is A_{734} of negative control in ethanolic ABTS solution.

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay

Antioxidant capacity was determined by measuring the scavenging activity of the radical, 2-diphenyl-1-picrylhydrazyl (DPPH) based on the method (Saha *et al*., 2004) with slight modifications. 25 µL of undiluted crude extract was added to 975 µL of ethanolic DPPH in the Eppendorf falcon tubes and vortexed for 1 min using the vortex mixer (VTS-3000L, LMS, Japan). They are allowed to stand in a dark environment at room temperature for 30 min. Absorbance was measured at 517 nm using UV light spectrophotometer (Model XTD 5, Secomam, France). Absolute ethanol was used as blank. Absorbance of negative control (25 µL of absolute ethanol and 975 µL of ethanolic DPPH) and absorbance of blank were also measured at 517 nm. Both sample and negative control were analyzed in triplicate. Trolox solution was used to calibrate the standard curve. The mean ± SD results of triplicate analyses were expressed as µmol trolox equivalent per 100 g dried sample (µmol TEAC/100 g dried sample). The capability to scavenge the DPPH radicals was calculated by using the equation below.

$$DPPH \text{ radical scavenging capacity } (\%) = [1 - (A_o / A_1)] \times 100 \% \quad (2)$$

Where A_c is A_{517} of the crude extract; A_1 is A_{517} of negative control in ethanolic DPPH solution.

Statistical analysis

The experimental results were analyzed with Microsoft Office Excel 2007 (version 12.0, Microsoft Corp., USA) and Minitab statistical software (Version 16, Minitab Inc., USA). Every measurement of each assay was performed in triplicate, and every sample was duplicated. All values were expressed as the means ± standard errors (SE) of six measurements (n=6) and the calculations were performed using Microsoft Office Excel 2007. One-way analysis

of variance (ANOVA) with Tukey's test was used to determine the significant differences ($p < 0.05$) between the means.

Results

From Figure 1, it could be seen that the amount of phenolic compounds increased as the ethanol concentration increased until a peak was reached, and then it decreased slightly. However, the highest antioxidant content from each species was obtained with different ethanol concentrations. SP, KAB and KAG achieved a maximum TPC value of 23.58 mg GAE/100 g DW, 23.65 mg GAE/100 g DW and 18.48 mg GAE/100 g DW at a 50% ethanol concentration, respectively; ED achieved a maximum of 10.08 mg GAE/100 g DW at a 75% ethanol concentration. The trend for the TFC value is about the same as for TPC; it increased as the ethanol concentration increased, and then decreased after a peak was reached. It is obvious that flavonoids in KAG were significantly higher than in the other species (3.1 mg CE/g DW). Antioxidant capacities of all seaweeds species were significantly affected by the ethanol concentration as shown in Figure 1. The trend exhibited by both assays agrees well with the TPC and TFC results.

Figure 2 showed a significant effect ($p < 0.05$) of the solid-to-solvent ratio on TPC, TFC, ABTS and DPPH for the four seaweeds. In a preliminary test, a ratio of 1:5 was used, but no results were obtained. The samples absorbed the solvent and expanded during the extraction, forming a thick and viscous semisolid mass. This could be attributed to insufficient solvent to penetrate the sample and therefore, no extraction occurred. Hence, it is concluded that solid-to-solvent ratio of 1:5 is too low to extract phenolics in the samples, so this ratio was not included in this experiment. At a solid-to-solvent ratio of 1:10, the TPC and TFC reached a maximum for all four seaweeds. Both TPC and TFC for the four seaweeds decreased at ratios greater than 1:10. According Figure 2, the radical scavenging capacities of ABTS and DPPH were significantly affected ($p < 0.05$) by the solid-to-solvent ratio. At the lower ratio of 1:10, both ABTS and DPPH showed significantly high radical scavenging capacities for all four seaweeds. This trend agreed with the results from the antioxidant compound assay performed earlier.

Figure 3 showed an increasing trend for TPC and TFC, and reached a peak at 65°C for all seaweeds. However, a preliminary test, a temperature of 75°C was used to extract phenolics. It caused a significant decline in both the amount of antioxidant compounds and the antioxidant capacity. Therefore, 75°C was not
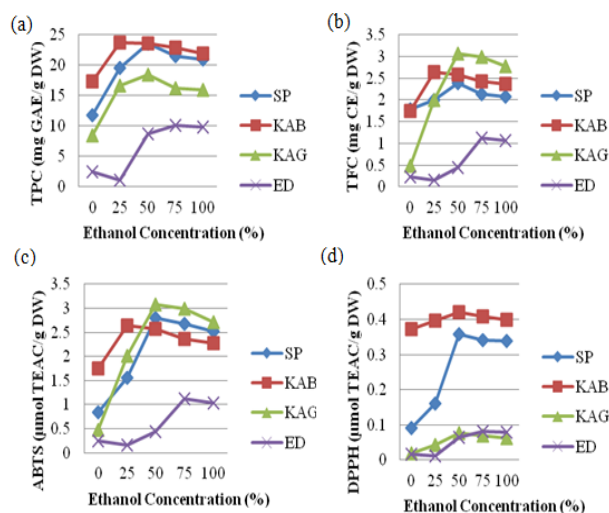

Figure 1. Effects of ethanol concentration towards (a) TPC, (b) TFC, (c) ABTS and (d) DPPH of *Sargassum polycystum* (SP), *Kappaphycus alvarezzi* variance Buaya (KAB), *Kappaphycus alvarezzi* variance Giant (KAG) and *Eucheuma denticulatum* (ED)
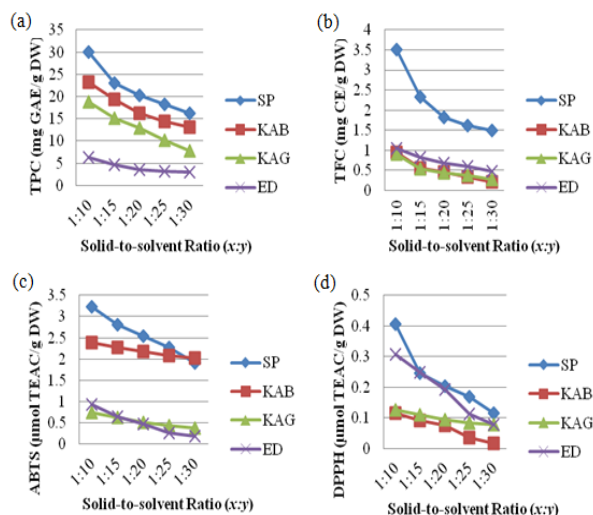

Figure 2. Effects of solid-to-solvent ratio towards (a) TPC, (b) TFC, (c) ABTS and (d) DPPH of *Sargassum polycystum* (SP), *Kappaphycus alvarezzi* variance Buaya (KAB), *Kappaphycus alvarezzi* variance Giant (KAG) and *Eucheuma denticulatum* (ED)

included in the range of extraction temperature used in this study. ABTS was not significantly affected by temperature (as shown in Figure 3); while DPPH presented increasing trend and peaked at 65°C.

From Figure 4, it is obvious that each of the seaweed had a different optimum extraction time for phenolic compounds. SP showed the highest TPC (37.41 mg GAE/g DW) at 2 hours; KAB had an optimum (34.43 mg GAE/g DW) time of 4 hours; KAG showed the highest TPC value (25.4 mg GAE/g DW) at 5 hours, and ED peaked (12.1 mg GAE/g DW of TPC) at 3 hours. In a preliminary test, we used a 6 hours extraction time for KAG. A significant decrease was observed, and so 6 hours of
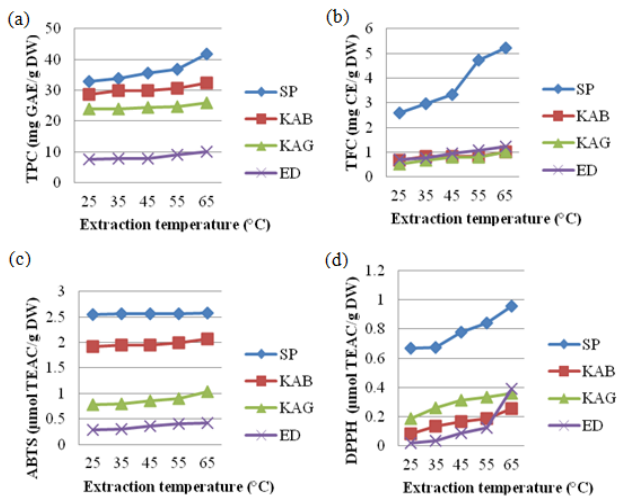
Figure 3. Effects of extraction temperature towards (a) TPC, (b) TFC, (c) ABTS and (d) DPPH of *Sargassum polycystum* (SP), *Kappaphycus alvarezzi* variance Buaya (KAB), *Kappaphycus alvarezzi* variance Giant (KAG) and *Eucheuma denticulatum* (ED)
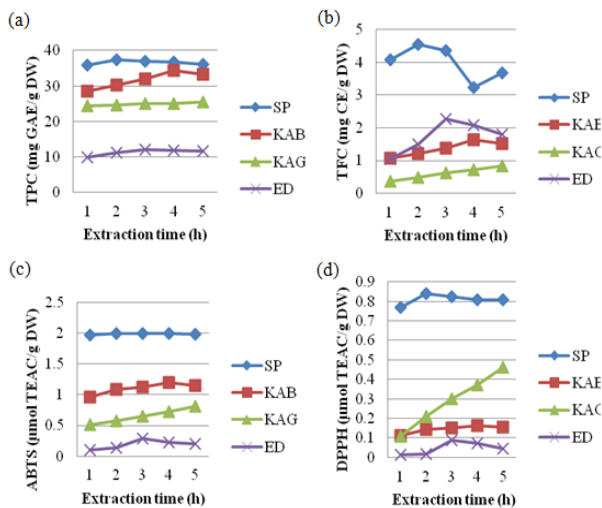

Figure 4. Effects of extraction time towards (a) TPC, (b) TFC, (c) ABTS and (d) DPPH of *Sargassum polycystum* (SP), *Kappaphycus alvarezzi* variance Buaya (KAB), *Kappaphycus alvarezzi* variance Giant (KAG) and *Eucheuma denticulatum* (ED)

extraction time was not included in this experiment. Figure 4 presented that the trend for the antioxidant capacities is almost the same as that for the amount of antioxidant compounds extracted.

Discussion

Effects of ethanol concentrations

The nature of the solvent used determines the types of phenols extracted from the plant material (Liyana-Pathirana and Shahidi, 2005). A dual solvent system is more desirable than a mono-solvent system (Wang *et al.*, 2008) because it creates a moderately polar medium which enhances

the extraction of more water soluble polyphenols. Studies show that an ethanol and water mixture extracts flavonoids (Spigno *et al.*, 2007), catechin, rutin and quercetin (Angela and Meireles, 2008). The ethanol concentration affects extraction significantly, whereby low ethanol concentration would favour impurities extraction (Chirinos *et al.*, 2007) while high ethanol concentration tends to extract lipid components (Wang *et al.*, 2008). Hence, different samples should have their best ethanol concentration to extract maximum phenolics. Results showed in this experiment can be explained by the different type and structure of phenols contained in each species (Zhang *et al.*, 2008). It was believed that the highly active phenolic compounds present in SP, KAB and KAG were balanced between polar and non-polar because both ABTS and DPPH reached a maximum at 50% ethanol concentration. On the other hand, ED reached a maximum at 75% ethanol concentration, which indicated that it contains moderately polar active phenolic compounds. SP, KAB and KAG presented 50% as the best ethanol concentration; while ED showed 75 % as the best ethanol concentration.

Effects of solid-to-solvent ratios

Evaluating effects of solid-to-solvent ratios is imperative in an industry viewpoint – to ensure efficient and economic phenolics extraction. In the preliminary test, ratio of 1:5 was tested. As portrayed in the results, a ratio of 1:10 was the best for all of the samples. Nonetheless, when the ratio was increased, the amount of extracted phenolics in the extract remained the same but was diluted with the extra solvent added. The decreases in ABTS and DPPH can be explained by the decreased values of TPC and TFC. Dilution by excessive solvent affects the antioxidant capacity significantly. In addition, the lesser total phenolic compounds present in the extract, the lower the antioxidant capacity it possessed. It was reported that the antioxidant activity of a plant extract often originates from phenolic compounds (Amarowicz *et al.*, 2000). A solid-to-solvent ratio of 1:10 was chosen as the best condition to extract the highest amount of antioxidant compounds and capacity from SP, KAB, KAG and ED.

Effects of extraction temperature

65°C was the best extraction temperature for all four species of seaweeds. In a preliminary test, 75°C were tested, but a sharp decrease occurred. It was believed that phenolics were degraded at that temperature. Increasing temperature promotes analyte solubility. This is mainly because incubation in hot water weakens the cellular constituents of the

Table 1. Best extraction condition (ethanol concentration, solid-to-solvent ratio, extraction temperature and time) for 4 selected seaweeds

Species	Ethanol concentration (%)	Solid-to-solvent ratio (x:y)	Extraction temperature (°C)	Extraction time (hours)
SP	50	1:10	65	2
KAB	50	1:10	65	4
KAG	50	1:10	65	5
ED	75	1:10	65	3

seaweeds, releasing more bound phenols into the solvent (Spigno et al., 2007). Furthermore, a higher extraction temperature reduces solvent viscosity and surface tension, thus, accelerating the extraction process and increasing the diffusion coefficient. Additionally, studies showed that the rate of recovery of thermally stable antioxidants at an elevated temperature (up to 65°C) was greater than the rate of decomposition of less soluble phenolics (Liyana-Pathirana and Shahidi, 2005). Despite an increasing in the amount of antioxidant compounds extracted at a higher temperature, Figure 3 shows that ABTS does not significantly change during extraction at high temperature. This is likely because the bioavailability of phenolics or bioactive compounds was negatively affected by the relatively high temperature. Nevertheless, the antioxidant capacity of the sample could experience thermal destruction (Spigno et al., 2007), in turn reducing its antioxidant activities, therefore resulting in almost no change in ABTS. Nevertheless, DPPH was significantly increased for all four seaweeds. DPPH is known to react well with low molecular weight compounds (Paixão, 2007). Furthermore, DPPH radicals reacted with phenolic compounds even at high temperatures. It is concluded that the four seaweeds contain a high proportion of heat-resistant low molecular weight active phenolic compounds.

Effects of extraction time

Extraction time is determined purely by the molecular size, quantity and chemical structure of the phenolic compounds in the sample (Chirinos et al., 2007). Different species of seaweeds contain a different composition of bioactive compounds as well as of phenolic compounds. For instance, some phenols require a longer extraction time because the phenols are bound with fiber (Benjama and Masniyom, 2011). Phenols that are tightly bound to cell-wall polymers may need a longer extraction time compared than free phenolic compounds. Therefore, a different optimum extraction time resulted for each of the four seaweeds. The time required for the solvent to interact with the solid material is critical for solute recovery. According to Fick's second law of diffusion, final equilibrium is attained between the solution concentration in the solid matrix and the solvent

after a particular time (Pinelo et al., 2006). Results of antioxidant compounds and antioxidant capacities were compatible; this is likely because the phenolic compounds extracted are active. Prolonged extraction time leads to the decomposition of active compounds (Liyana-Pathirana and Shahidi, 2005) due to long exposure to the environment (i.e., temperature, light and oxygen) (Lafka, Sinanoglou and Lazos, 2007), increasing the chance that the phenolic compounds become oxidized, which decreases the antioxidant capacity. Furthermore, undesirable reactions such as enzymatic oxidation and polymerization might be favoured by the extended extraction time (Biesaga and Pyrzynska, 2013). The best extraction times were set as follows: for SP (2 h), KAB (4 h), KAG (5 h) and ED (3 h).

Conclusions

The best extraction conditions (ethanol concentration, solid-to-solvent ratio, extraction temperature and time) for four selected seaweeds were successfully identified by single-factor experiments. However, Sargassum polycystum possessed the most antioxidant compounds and capacities amongst the four species. The results obtained from this study are important in the development of industrial extraction processes of phenols from seaweed. Purification and identification of the phenolic components in seaweed can be done to identify phenolic compounds that are responsible for the antioxidant characteristics.

Acknowledgment

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