Effects of phenolic antioxidants extraction from four selected seaweeds obtained from Sabah

Carmen Wai Foong Fu, Chun Wai Ho, Wilson Thau Lym Yong, Faridah Abas, Chin Ping Tan

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. Single-factor experiments were proven as an effective tool to determine and quantify the relationship between a single factor and a single response variable.

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1 **Effects of Phenolic Antioxidants Extraction from Four Selected Seaweeds Obtained from**
2 **Sabah**
3
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6
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20
21 **Abstract:** Algal have attracted attention from biomedical scientists as they are a valuable natural
22 source of secondary metabolites that exhibit antioxidant activities. In this study, single-factor
23 experiments were conducted to investigate the best extraction conditions (ethanol concentration,
24 solid-to-solvent ratio, extraction temperature and extraction time) in extracting antioxidant
25 compounds and capacities from four species of seaweeds (*Sargassum polycystum, Eucheuma*
26 *denticulatum* , *Kappaphycus alvarezzi variance Buaya* and *Kappaphycus alvarezzi variance*
27 *Giant*) from Sabah. Total phenolic content (TPC) and total flavonoid content (TFC) assays were
28 used to determine the phenolic and flavonoid concentrations, respectively, while 2,2-azinobis-3-
29 ethylbenzothiazoline-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picylhydrazyl (DPPH) radical
30 scavenging capacity assays were used to evaluate the antioxidant capacities of all seaweed
31 extracts. Results showed that extraction parameters had significant effect ($p < 0.05$) on the
32 antioxidant compounds and antioxidant capacities of seaweed. *Sargassum polycystum* portrayed
33 the most antioxidant compounds (37.41 ± 0.01 mg GAE/g DW and 4.54 ± 0.02 mg CE/g DW)
34 and capacities (2.00 ± 0.01 µmol TEAC/g DW and 0.84 ± 0.01 µmol TEAC/g DW) amongst
35 four species of seaweed. Single-factor experiments were proven as an effective tool to determine
36 and quantify the relationship between a single factor and a single response variable.
37
38 **Keywords:** seaweeds, antioxidants, single-factor experiments, total phenolic content assay,
39 total flavonoid content assay, 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid radical

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40 scavenging capacity assay and 2,2-diphenyl-1-picylhydrazyl radical scavenging capacity
41 assays.
42
43 **Introduction**
44
45 For centuries, seaweed has been used in the preparation of salads, soups and also as low-calorie
46 foods in Asia (Jiménez-Escrig & Sánchez-Muniz, 2000). Japanese are the main consumers of
47 seaweed with an average consumption of 1.6 kg (dry weight) per year per capita (Dhargalkar &
48 Pereira, 2005). Most Europeans and Americans use processed seaweed as additives in their food
49 preparation (Boukhari & Sophie, 1998). However, in India, seaweeds are exploited mainly for
50 the industrial production of phycocolloids such as agar-agar, alginate and carrageenan; and not as
51 cookery item or for recovering beneficial biomolecules. In 1978, seaweed cultivation was
52 introduced in Sabah and had increasingly become an economically important natural resource for
53 Malaysia, particularly for Sabah. The interest for seaweed escalates tremendously in recent years
54 due to the demand caused by abalone farmers (Vasquez, 1999) the development of new products
55 such as organic fertilisers and use for human food (Alejandro *et al.*, 2008).
56
57 In recent years, seaweed products have received special attention as a source of natural
58 antioxidants (Lim *et al.*, 2002) and some of them possess biological activity of potential
59 medicinal value (Satoru *et al.*, 2003). Natural antioxidants are perceived to be safe by consumers
60 because they are naturally found in plant materials and have been used for centuries (Frankel,
61 1996). Natural antioxidants have shown to play a significant role in preventing a number of
62 chronic diseases such as heart disease, cancer, Alzheimer's and Parkinson's diseases (Weinreb *et*
63 *al.*, 2004).
64
65 Several researchers have reported the antioxidant properties of both brown and red seaweeds
66 from across the globe (Heo *et al.* 2005). Some active antioxidant compounds from marine algae
67 were identified as phylopheophylin in *Eisenia bicyclis* (Cahyana, Shuto & Kinoshita, 1992),
68 phlorotannins in *Sargassum kjellamanianum* (Yan *et al.*, 1996) and fucoxanthinin in *Hijikia*
69 *fusiformis* (Yan *et al.*, 1999). Furthermore, there are evidences available to show the potential
70 protective effects of seaweed against oxidative stress in target tissues and lipid oxidation in foods
71 (Rajamani *et al.*, 2011).
72
73 Therefore, the main objective of this study was to evaluate the effect of extraction conditions
74 (ethanol concentration, solid-to-solvent ratio, extraction temperature and extraction time) in
75 extracting antioxidant compounds as well as antioxidant capacities of the four selected seaweeds
76 (*Sargassum polycystum, Eucheuma denticulatum , Kappaphycus alvarezzi variance Buaya* and
77 *Kappaphycus alvarezzi variance Giant*) and determine the best extraction conditions for the
78 seaweeds.
79

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Materials and Method

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,

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119 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
120 rpm for 2 h.
121
122 Factor 3: Extraction Temperature
123 An amount of ethanol and deionised water (best ethanol concentration obtained from section
124 Factor 1) were added to each sample according to the best solid-to-solvent ratio obtained from
125 section Factor 2. They were then placed in a water bath shaker at 5 different temperatures (25,
126 35, 45, 55 and 65 °C) at 150 rpm for 2 h.
127
128 Factor 4: Extraction Time
129 An amount of ethanol and deionised water (best ethanol concentration obtained from section
130 Factor 1) were added to each sample according to the best solid-to-solvent ratio obtained from
131 section Factor 2. They were then placed in a water bath shaker at the best temperature of each
132 sample obtained from section Factor 3 at 150 rpm for a range of time set in 5 levels (1, 2, 3, 4
133 and 5 h).
134
135 *Total phenolic content (TPC) assay*
136 Total phenolic content (TPC) was determined using Folin-Ciocalteu (F-C) assay (Lim, Lim &
137 Tee, 2007) 500 µL of crude extracts obtained from extraction were added into Eppendorf falcon
138 tubes (2 mL) followed by 500 µL of Folin-Ciocalteu's reagent (diluted 10 times with water).
139 After 4 min, 400 µL of 7.5 % (w/v) sodium carbonate were added. The blank was prepared by
140 replacing 500 µL of sample with 500 µL of deionised water. Subsequently, the falcon tubes were
141 vortexed for 10 s with vortex mixer (VTS-3000L, LMS, Japan). They were incubated in the dark
142 environment at room temperature for 2 h. Absorbance was measured against the blank reagent at
143 765 nm using UV light spectrophotometer (Model XTD 5, Secomam, France). Each extract was
144 analyzed in triplicate and TPC were expressed as gallic acid equivalent (GAE) in mg per 100 g
145 dry weight (DW).
146
147 *Total flavonoid content (TFC) assay*
148 The determination of flavonoids was based on the procedures described in the study (Ozsoy *et*
149 *al.*, 2008) with slight modifications. 50 µL of crude extract added to 250 µL of deionised water,
150 followed by the addition of 15 µL of 5 % sodium nitrite in Eppendorf falcon tubes (2 mL). After
151 6 min, 30 µL of 10 % aluminium chloride hexahydrate was added into the mixture and was
152 allowed to stand for further 5 min. Then, 100 µL of 1 M sodium hydroxide and 55 µL of
153 deionised water were added. The blank was prepared by replacing the 50 µL sample with 50 µL
154 of deionised water. The falcon tubes were mixed thoroughly by using a vortex mixer (VTS-
155 3000L, LMS, Japan) for 10 s. Then, absorbance readings were immediately taken at 510 nm
156 using the UV light spectrophotometer (Model XTD 5, Secomam, France). Each extract was
157 analyzed in triplicate and TFC were expressed as catechin equivalent (CE) in mg per 100 g dry
158 weight (DW).

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159

160 *2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging capacity assay*

161 Antioxidant capacity was determined by measuring the scavenging activity of the radical 2,2-
162 azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) based on the method (Surveswaran,
163 2007) with slight modifications. 10 mL of 7 mM ABTS solution and 10 mL of 2.45 mM
164 potassium persulfate ($K_2S_2O_8$) solution were transferred into a 100 mL light protected amber
165 bottle. The solution were mixed by vortex mixer (VTS-3000L, LMS, Japan) for 10 s and allowed
166 to stand in a dark environment at room temperature for 16 h to give a dark blue solution. This
167 solution was diluted with 95 % ethanol until the absorbance was equilibrated to 0.7 (±0.02) at
168 734 nm. 975 µL ABTS solution with equilibrated absorbance of 0.7 ± 0.02 was added to 25 µL
169 of the undiluted extract in an Eppendorf falcon tube (2 mL). Negative control was prepared by
170 replacing 25 µL of undiluted crude extract with 25 µL of 95% ethanol whereas blank was
171 prepared by using 95 % ethanol solely. The reaction was allowed to occur at room temperature
172 for 6 min and the absorbance at 734 nm was immediately recorded against blank using the UV
173 light spectrophotometer (Model XTD 5, Secomam, France). Both the crude extracts and negative
174 control were carried out in triplicate. Trolox solution was used to calibrate the standard curve.
175 The mean ± SD results of triplicate analyses were expressed as µmol trolox equivalent per 100 g
176 dried sample (µmol TEAC/100 g dried sample).

177

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

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

179

180 *2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay*

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

193

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

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

195

196 *Statistical analysis*

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197 The experimental results were analyzed with Minitab statistical software (Version 16, Minitab
198 Inc., USA). Every measurement of each assay was performed in triplicate, and every sample was
199 duplicated. All values were expressed as the means ± standard errors (SE) of six measurements
200 (n=6) and the calculations were performed using Microsoft Office Excel 2007 (version 12.0,
201 Microsoft Corp., USA). One-way analysis of variance (ANOVA) with Tukey's test was used to
202 determine the significant differences ($p < 0.05$) between the means.
203
204
205 **Results**
206
207 From Figure 1, it could be seen that the amount of phenolic compounds increased as the ethanol
208 concentration increased until a peak was reached, and then it decreased slightly. However, the
209 highest antioxidant content from each species was obtained with different ethanol concentrations.
210 SP, KAB and KAG achieved a maximum TPC value of 23.58 mg GAE/100 g DW, 23.65 mg
211 GAE/100 g DW and 18.48 mg GAE/100 g DW at a 50% ethanol concentration, respectively; ED
212 achieved a maximum of 10.08 mg GAE/100 g DW at a 75% ethanol concentration. The trend for
213 the TFC value is about the same as for TPC; it increased as the ethanol concentration increased,
214 and then decreased after a peak was reached. It is obvious that flavonoids in KAG were
215 significantly higher than in the other species (3.1 mg CE/g DW). Antioxidant capacities of all
216 seaweeds species were significantly affected by the ethanol concentration as shown in Figure 1.
217 The trend exhibited by both assays agrees well with the TPC and TFC results.
218

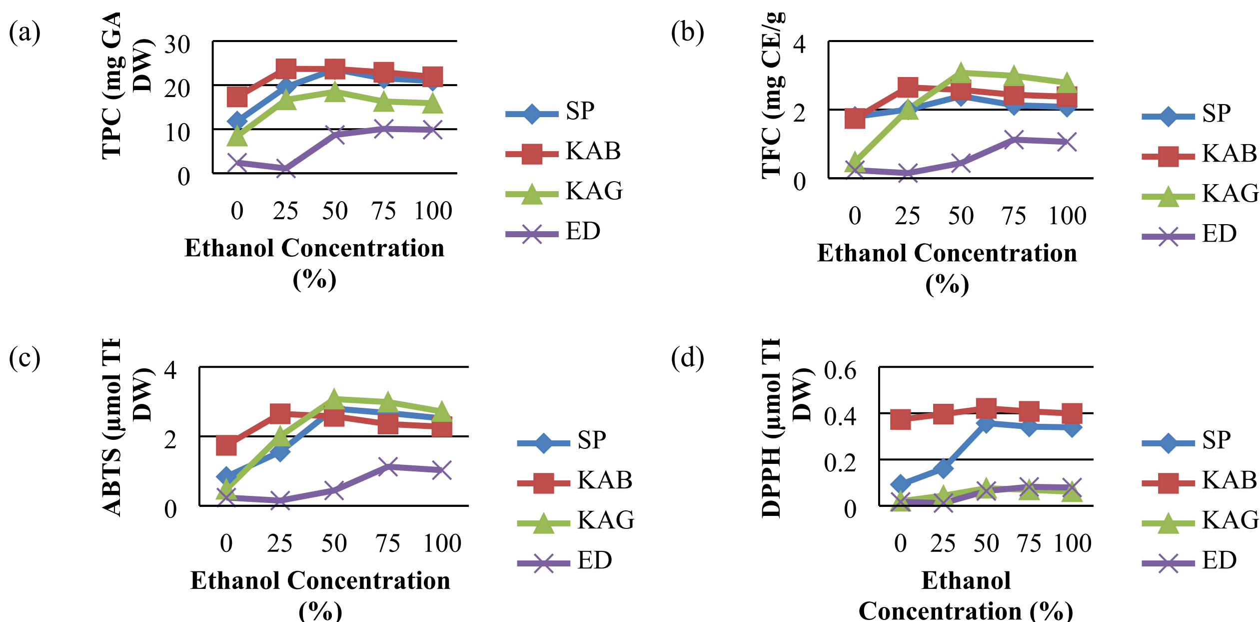

Figure 1 **Effects of ethanol concentration towards (a) TPC, (b) TFC, (c) ABTS and (d) DPPH of *Sargassum polycystum* (SP), *Kappaphycus alvarezzi variance Buaya* (KAB), *Kappaphycus alvarezzi variance Giant* (KAG) *and Eucheuma denticulatum* (ED).**

219

220 Figure 2 showed a significant effect ($p<0.05$) of the solid-to-solvent ratio on TPC, TFC, ABTS
221 and DPPH for the four seaweeds. In a preliminary test, a ratio of 1:5 was used, but no results
222 were obtained. The samples absorbed the solvent and expanded during the extraction, forming a
223 thick and viscous semisolid mass. This could be attributed to insufficient solvent to penetrate the
224 sample and therefore, no extraction occurred. Hence, it is concluded that solid-to-solvent ratio of
225 1:5 is too low to extract phenolics in the samples, so this ratio was not included in this
226 experiment. At a solid-to-solvent ratio of 1:10, the TPC and TFC reached a maximum for all four
227 seaweeds. Both TPC and TFC for the four seaweeds decreased at ratios greater than 1:10.
228 According Figure 2, the radical scavenging capacities of ABTS and DPPH were significantly
229 affected ($p < 0.05$) by the solid-to-solvent ratio. At the lower ratio of 1:10, both ABTS and
230 DPPH showed significantly high radical scavenging capacities for all four seaweeds. This trend
231 agreed with the results from the antioxidant compound assay performed earlier.
232

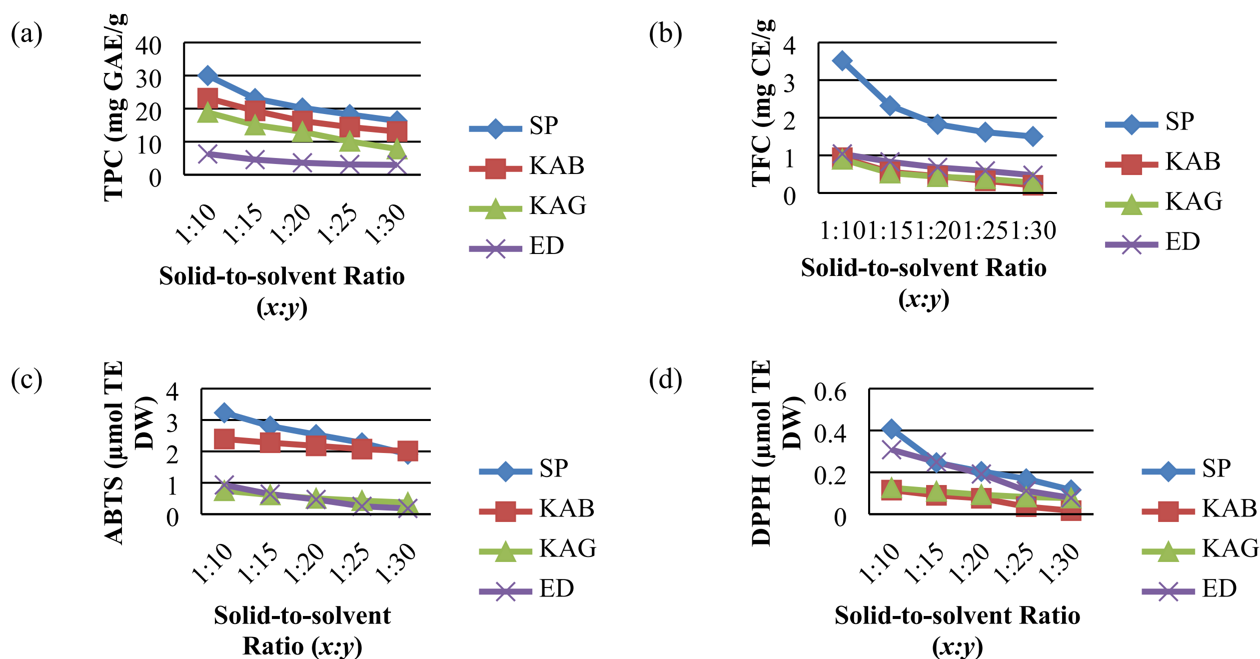

Figure 2 **Effects of solid-to-solvent ratio towards (a) TPC, (b) TFC, (c) ABTS and (d) DPPH of *Sargassum polycystum* (SP), *Kappaphycus alvarezzi variance Buaya* (KAB), *Kappaphycus alvarezzi variance Giant* (KAG) *and Eucheuma denticulatum* (ED).**

233

234 Figure 3 showed an increasing trend for TPC and TFC, and reached a peak at 65 °C for all
235 seaweeds. However, a preliminary test, a temperature of 75 °C was used to extract phenolics. It
236 caused a significant decline in both the amount of antioxidant compounds and the antioxidant
237 capacity. Therefore, 75 °C was not included in the range of extraction temperature used in this
238 study. ABTS was not significantly affected by temperature (as shown in Figure 3); while DPPH
239 presented increasing trend and peaked at 65 °C.
240

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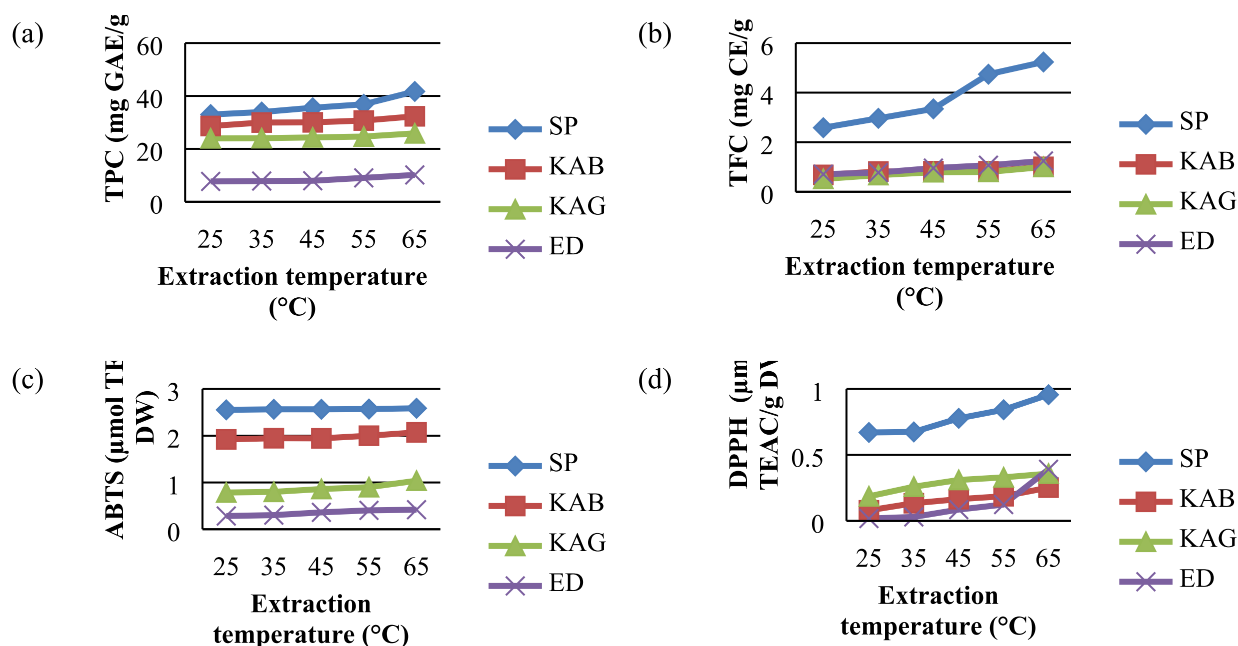
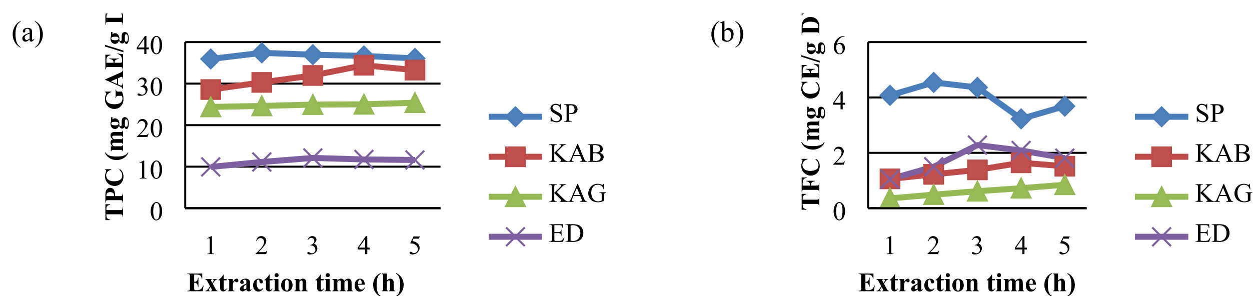


Figure 3 **Effects of extraction temperature towards (a) TPC, (b) TFC, (c) ABTS and (d) DPPH of *Sargassum polycystum* (SP), *Kappaphycus alvarezzi variance Buaya* (KAB), *Kappaphycus alvarezzi variance Giant* (KAG) *and Eucheuma denticulatum* (ED).**

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 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.



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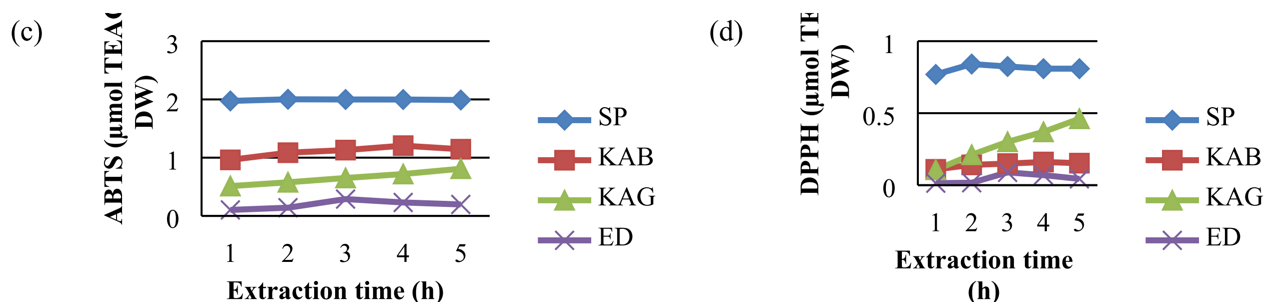

Figure 4 **Effects of extraction time towards (a) TPC, (b) TFC, (c) ABTS and (d) DPPH of *Sargassum polycystum* (SP), *Kappaphycus alvarezzi variance Buaya* (KAB), *Kappaphycus alvarezzi variance Giant* (KAG) and *Eucheuma denticulatum* (ED).**

255

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

256

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

257
258 **Discussion**
259
260 *Effects of ethanol concentrations*
261
262 The nature of the solvent used determines the types of phenols extracted from the plant material
263 (Liyana-Pathirana & Shahidi, 2005). A dual solvent system is more desirable than a mono-
264 solvent system (Wang *et al*., 2008) because it creates a moderately polar medium which
265 enhances the extraction of more water soluble polyphenols. Studies show that an ethanol and
266 water mixture extracts flavonoids (Spigno, Tramelli & DeFaveri, 2007), catechin, rutin and
267 quercetin (Angela & Meireles, 2008). The ethanol concentration affects extraction significantly,
268 whereby low ethanol concentration would favour impurities extraction (Chirinos *et al.*, 2007)
269 while high ethanol concentration tends to extract lipid components (Wang *et al*., 2008). Hence,
270 different samples should have their best ethanol concentration to extract maximum phenolics.
271 Results showed in this experiment can be explained by the different type and structure of phenols
272 contained in each species (Zhang, Li & Wu, 2008). It was believed that the highly active
273 phenolic compounds present in SP, KAB and KAG were balanced between polar and non-polar
274 because both ABTS and DPPH reached a maximum at 50% ethanol concentration. On the other
275 hand, ED reached a maximum at 75% ethanol concentration, which indicated that it contains

276 moderately polar active phenolic compounds. SP, KAB and KAG presented 50 % as the best
277 ethanol concentration; while ED showed 75 % as the best ethanol concentration.
278
279 *Effects of solid-to-solvent ratios*
280
281 Evaluating effects of solid-to-solvent ratios is imperative in an industry viewpoint – to ensure
282 efficient and economic phenolics extraction. In the preliminary test, ratio of 1:5 was tested.
283 However, no results were obtained. This is due to insufficient solvent to penetrate the samples,
284 leading to occurrence of viscous semisolid mass on the filter paper. As portrayed in the results, a
285 ratio of 1:10 was the best for all of the samples. Nonetheles, when the ratio was increased, the
286 amount of extracted phenolics in the extract remained the same but was diluted with the extra
287 solvent added. The decreases in ABTS and DPPH can be explained by the decreased values of
288 TPC and TFC obtained in the earlier experiment. Dilution by excessive solvent affects the
289 antioxidant capacity significantly. In addition, the lesser total phenolic compounds present in the
290 extract, the lower the antioxidant capacity it possessed. It was reported that the antioxidant
291 activity of a plant extract often originates from phenolic compounds (Amarowicz, Naczk &
292 Shahidi, 2000). A solid-to-solvent ratio of 1:10 was chosen as the best condition to extract the
293 highest amount of antioxidant compounds and capacity from SP, KAB, KAG and ED.
294
295 *Effects of extraction temperature*
296
297 65 °C was the best extraction temperature for all four species of seaweeds. In a preliminary test,
298 75 °C were tested, but a sharp decrease occurred. It was believed that phenolics were degraded at
299 that temperature. Increasing temperature promotes analyte solubility. This is mainly because
300 incubation in hot water weakens the cellular constituents of the seaweeds, releasing more bound
301 phenols into the solvent (Spigno, Tramelli & DeFaveri, 2007). Furthermore, a higher extraction
302 temperature reduces solvent viscosity and surface tension, thus, accelerating the extraction
303 process and increasing the diffusion coefficient. Additionally, studies showed that the rate of
304 recovery of thermally stable antioxidants at an elevated temperature (up to 65 °C) was greater
305 than the rate of decomposition of less soluble phenolics (Liyana-Pathirana & Shahidi, 2005).
306 Despite an increasing in the amount of antioxidant compounds extracted at a higher temperature,
307 Figure 3 shows that ABTS does not significantly change during extraction at high temperature.
308 This is likely because the bioavailability of phenolics or bioactive compounds was negatively
309 affected by the relatively high temperature. Nevertheless, the antioxidant capacity of the sample
310 could experience thermal destruction (Spigno, Tramelli & DeFaveri, 2007), in turn reducing its
311 antioxidant activities, therefore resulting in almost no change in ABTS. Nevertheless, DPPH was
312 significantly increased for all four seaweeds. DPPH is known to react well with low molecular
313 weight compounds (Paixão, 2007). Furthermore, DPPH radicals reacted with phenolic
314 compounds even at high temperatures. It is concluded that the four seaweeds contain a high
315 proportion of heat-resistant low molecular weight active phenolic compounds.

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316
317 *Effects of extraction time*
318
319 Extraction time is determined purely by the molecular size, quantity and chemical structure of
320 the phenolic compounds in the sample (Chirinos *et al.*, 2007). Different species of seaweeds
321 contain a different composition of bioactive compounds as well as of phenolic compounds. For
322 instance, some phenols require a longer extraction time because the phenols are bound with fiber
323 (Benjama & Masniyom, 2011). Phenols that are tightly bound to cell-wall polymers may need a
324 longer extraction time compared than free phenolic compounds. Therefore, a different optimum
325 extraction time resulted for each of the four seaweeds. The time required for the solvent to
326 interact with the solid material is critical for solute recovery. According to Fick's second law of
327 diffusion, final equilibrium is attained between the solution concentration in the solid matrix and
328 the solvent after a particular time (Pinelo, Arnous & Meyer, 2006). Results of antioxidant
329 compounds and antioxidant capacities were compatible; this is likely because the phenolic
330 compounds extracted are active. The decrease of ABTS and DPPH after the peak is believed to
331 results from the prolonged extraction time. This leads to the decomposition of active compounds
332 (Liyana-Pathirana & Shahidi, 2005) due to long exposure to the environment (i.e., temperature,
333 light and oxygen) (Lafka, Sinanoglou & Lazos, 2007), increasing the chance that the phenolic
334 compounds become oxidized, which decreases the antioxidant capacity. Furthermore,
335 undesirable reactions such as enzymatic oxidation and polymerization might be favoured by the
336 extended extraction time (Biesaga & Pyrzynska, 2013). The best extraction times were set as
337 follows: for SP (2 h), KAB (4 h), KAG (5 h) and ED (3 h).
338
339 **Conclusions**
340
341 The best extraction conditions (ethanol concentration, solid-to-solvent ratio, extraction
342 temperature and time) for four selected seaweeds were successfully identified by single-factor
343 experiments. However, *Sargassum polycystum* possessed the most antioxidant compounds and
344 capacities amongst the four species. The results obtained from this study are important in the
345 development of industrial extraction processes of phenols from seaweed. Purification and
346 identification of the phenolic components in seaweed can be done to identify phenolic
347 compounds that are responsible for the antioxidant characteristics.
348
349
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355 **Additional Information and Declaration**

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361 *Author Contributions*
362 Carmen Wai Foong Fu designed and performed the experiment and wrote the manuscript;
363 Wilson Yong collected and prepared the samples; Chun Wai Ho and Faridah Abas supervised the
364 sample analysis and characterization. Chin Ping Tan revised the manuscript. All authors read and
365 approved the manuscript.
366
367 *Conflicts of Interest*
368 The authors declare no conflict of interest.
369
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