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Effects of different extraction methods on contents, profiles, and antioxidant abilities of free and bound phenolics of *Sargassum polycystum* from the South China Sea

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Abstract: Total phenolic content (TPC), phenolic profiles, and antioxidant activity of free and bound extracts of *Sargassum polycystum*, obtained by different extraction solvents and hydrolysis methods, were investigated. Aqueous acetone afforded the highest free TPC and antioxidant ability, followed by aqueous ethanol and aqueous methanol. Twelve free phenolic compounds were identified by ultra-high-performance liquid chromatography-mass spectrometry (UHPLC-MS), including two hydroxycinnamic acids, seven flavonoids, one stilbene, and two phlorotannins. Three to nine different free phenolic compounds were extracted by these solvents with different compositions, including nine by 70% acetone and eight by 70% methanol, 70% ethanol, and 50% ethanol. The highest total content of free phenolic compounds determined by high-performance liquid chromatography-diode array detection was obtained from 70% ethanol. Alkaline hydrolysis afforded higher bound TPC (274.27 mg GAE/100 g DW) and antioxidant ability than acid hydrolysis. Five bound phenolic compounds were characterized by UHPLC-MS and five were released from alkaline hydrolysis, whereas two were released from acid hydrolysis. Total content of bound phenolic compounds released by alkaline hydrolysis was 14.68-fold higher than that by acid hydrolysis. The free and bound TPC, phenolic profiles, and antioxidant

activities depended on the extraction solvent used. These results indicate that *S. polycystum* is a potentially useful antioxidant source and contribute to the development of seaweed-based functional foods.

Practical Application: Phenolics are usually divided into free and bound forms based on their extractability and interaction with cell wall components. The nutritional effects of bound phenolics in algae have long been neglected. These topics contribute to the development of seaweed-based functional foods.

1 | INTRODUCTION

Marine seaweeds have been used as food and medicine for many centuries. Brown seaweeds have recently attracted particular attention for their phytochemicals with biological activities, including antioxidant, anti-inflammatory, antimelanogenesis, antibacterial, anticancer, antihyperglycemia, and hepatoprotective activities (Cotas et al., 2020). Phenolic compounds are found in brown seaweeds and have attracted interest due to their biological capacities and array of health-promoting effects (Santos et al., 2019).

Phenolics in plants are usually divided into free and bound forms based on their extractability and interaction with cell wall components. Free phenolics are generally thought to exist in plant cells vacuoles and are relatively easily absorbed in the small intestine, whereas bound phenolics are difficult to directly absorb due to their interaction with cellulose, pectin, and other polysaccharides (Acosta-Estrada et al., 2014). Therefore, the nutritional effects of bound phenolics have long been neglected. In marine brown seaweeds, research has particularly focused on free phenolics extracted with organic solvents or pressurized fluids, but bound phenolics have been neglected. Indeed, bound phenolics obtained via hydrolysis possess antioxidant and anti-inflammatory activities in vitro, and have been suggested to have different health benefits (Zhang et al., 2020). Most bound phenolics are released by microorganisms in the colon and are metabolized by microorganisms, and exert more durable physiological activities than free phenolics (Rio et al., 2013). Thus, further research on bound phenolics from marine brown seaweeds is warranted.

Phenolic compounds in seaweeds can be classified into many subclasses based on the structure, including phenolic acids, bromophenols, flavonoids, and phlorotannins (Cotas et al., 2020). Many of these phenolics are often bound with cellulose, pectin, and polysaccharides, mak-

ing them difficult to hydrolyze (Erpel et al., 2020; Koivikko et al., 2005). The diversity of these phenolic compounds has posed a challenge to their highly efficient extraction from seaweeds. For the extraction of free phenolics from marine brown seaweeds, a major issue is the choice of extraction solvent. Organic solvents (particularly methanol, ethanol, acetone, and their aqueous mixtures, and ethyl acetate) are most commonly used for extracting free phenolics from seaweeds (Koivikko et al., 2005). Although there is no consensus on the most efficient extraction solvent, higher extraction efficiency was obtained by organic aqueous mixtures, but not by a single solvent (Koivikko et al., 2005; Su et al., 2014). Higher extractability for free phenolic acids could be achieved using an ethanol-aqueous mixture or methanol-aqueous mixture, whereas the specific extraction of phlorotannins and flavan-3-ols could be realized with an acetone-aqueous mixture (Arivalagan et al., 2018; Erpel et al., 2020; Koivikko et al., 2005). For bound phenolics, alkaline hydrolysis and acid hydrolysis have been widely used. Most studies report that alkaline hydrolysis can more effectively release bound phenolics from plants (Su et al., 2014; Wang et al., 2020). Conversely, according to some reports, acid hydrolysis released bound phenolics more efficiently than alkaline hydrolysis (Bonoli et al., 2004). Although sporadic studies have focused on the bound phenolics of marine seaweeds, alkaline hydrolysis was demonstrated to be more efficient for releasing bound phlorotannins from brown seaweed *Fucus vesiculosus* than acid hydrolysis (Koivikko et al., 2005). Moreover, the composition of bound phenolics released from residues can differ among different hydrolysis methods (Su et al., 2014; Wang et al., 2020). Thus, the method for extracting phenolics should be selected based on the form and composition of phenolics (Alu'datt et al., 2019; Wang et al., 2020).

Sargassum polycystum, a tropical, edible, brown seaweed distributed primarily in Hainan, Guangdong, and

Guangxi provinces of China, Malaysia, and Japan has been reported to have health-promoting effects in traditional Chinese medicine and is locally used as food and medicine (Laboratory of Marine Biology of Institute of South China Sea Oceanography of Chinese Academy of Sciences, 1978; Matanjun et al., 2009). Recent studies have reported that *S. polycystum* contains a wide range of bioactive compounds that exert a broad spectrum of biological activities, including anti-inflammatory, antioxidant, anticancer, antihyperglycemia, and hepatoprotective activities (Chan et al., 2011; Johnson et al., 2019; Raghavendran et al., 2005). Recent research has mainly focused on sulfated polysaccharides and partly on the bioactivity of phenolic-enriched extracts, which have not been chemically characterized (Palanisamy et al., 2017; Raghavendran et al., 2005). Although several studies have reported that *S. polycystum* is rich in total phenolics in free form (extracted by organic solvents) with potent bioactivity (Chan et al., 2011; Johnson et al., 2019), the influences of extraction methods on the profiles of phenolics present in free and bound forms remain unclear. Therefore, the present study characterized and quantified phenolic compounds present in free and bound forms from *S. polycystum*, obtained by different extraction solvents and hydrolysis methods, using ultra-high-performance liquid chromatography-mass spectrometry (UHPLC-MS) and high-performance liquid chromatography-diode array detection (HPLC-DAD). Total phenolic content (TPC), total flavonoid content (TFC), and antioxidant ability of free and bound extracts obtained by these methods were also measured.

2 | MATERIALS AND METHODS

2.1 | Materials and reagents

Sargassum polycystum was collected in Fengjiawan Bay, Wenchang, Hainan Province, China (110°51′48″E, 19°31′16″N), in April 2019. The brown seaweed was carefully observed and identified by Prof. Xiubao Li from College of Marine Science, Hainan University. After rinsing in fresh water to remove visible surface contaminants, the seaweed was dried in an electric thermostat blast drying oven (DHG-9140A, Shanghai Yiheng Scientific Instrument, Shanghai, China) at 45°C for 4 h, ground through a 60-mesh sieve, and stored at −18°C.

Chlorogenic acid, cryptochlorogenic acid, baicalein, acacetin, diosmetin, hinokiflavone, hesperidin, rutin, quercitrin, trans-resveratrol, gallic acid, and apigenin for UHPLC-MS and HPLC-DAD were purchased from Qiyun Biotechnology (Guangzhou, China). 2,2′-Azobis (2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-

2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and Folin–Ciocalteu reagent were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Total antioxidant capacity assay kits employing the ferric-reducing antioxidant power (FRAP) method and 2,2′-azino-bis(3-ethylbenzthiazoline)−6-sulfonic acid (ABTS) radical scavenging activity method were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China).

2.2 | Extraction of free phenolic fraction of seaweeds

The free phenolic fractions were extracted in accordance with previous methods with some modifications (Su et al., 2014). Seaweed powder (2 g) was mixed with 60 ml of methanol, 70% methanol, 50% methanol, ethanol, 70% ethanol, 50% ethanol, acetone, 70% acetone, 50% acetone, or ethyl acetate, and homogenized using an XHF-D homogenizer (IKA-Labortechnik, Staufen, Germany) at 5000 rpm for 5 min at 4°C. Then, the mixture was centrifuged at 8000 rpm for 10 min at 4°C and the precipitate was used for repeated extraction. Finally, the supernatants from twice extractions were combined and concentrated to dryness on a rotating evaporator at 45°C, redissolved in 7 ml of methanol/water (85:15, v/v), and kept at −18°C.

2.3 | Extraction of bound phenolic fraction of seaweeds

2.3.1 | Alkaline hydrolysis method

The bound phenolic fractions of *S. polycystum* were extracted from the residue resulting from the free phenolic extraction using a slightly modified version of the alkaline hydrolysis method of Su et al. (2014). Briefly, the residue was supplemented with NaOH (60 ml, 2 mol/L), charged nitrogen gas for 5 min, and hydrolyzed for 1 h with continuous shaking in a water bath shaker at 37°C. After the mixture had been centrifuged (8000 rpm, 10 min at 4°C), the precipitate was used for repeated extraction. The supernatants from twice hydrolysis were combined, adjusted to pH 1−2, and then extracted with ethyl acetate six times. Next, the ethyl acetate fractions were mixed and concentrated to dryness on a rotating evaporator at 45°C, redissolved in 10 ml of methanol/water (85:15, v/v), and kept at −18°C.

2.3.2 | Acid hydrolysis method

The bound phenolic compounds of *S. polycystum* were extracted using the acid hydrolysis method reported by Su

et al. (2014) with some modifications. Briefly, the residue resulting from the free phenolic extraction using 70% ethanol was hydrolyzed with 60 ml of HCl (6 mol/L) for 1 h, with continuous shaking under nitrogen gas. After the mixture had been centrifuged (8000 rpm, 10 min at 4°C), the precipitate was used for repeated extraction. The supernatants from twice hydrolysis were combined, neutralized with sodium hydroxide (2 mol/L), and extracted with ethyl acetate six times. Next, the ethyl acetate fractions were combined and evaporated at 45°C till dry, redissolved in 10 ml of methanol/water (85:15, v/v), and kept at −18°C.

2.4 | Determination of TPC and TFC

TPC and TFC of free and bound phenolic extracts were measured using the Folin–Ciocalteu colorimetric method and the aluminum chloride colorimetric method, respectively, as described previously (Su et al., 2014). They were calculated by comparison with the calibration curves of gallic acid and (+)-catechin, respectively, and expressed as mg gallic acid or catechin equivalent per 100 g dry weight (DW) of seaweed (mg GAE/100 g DW or mg CE/ 100 g DW).

2.5 | Identification of phenolic composition

The free and bound phenolic compounds were detected via UHPLC-MS according to the method reported in our previous study with some modifications (Xiao et al., 2020). The detection was carried out using a UHPLC tandem with Xevo triple quadrupole mass spectrometer system (Micromass Waters, Milford, MA, USA) with an electrospray ionization source conducted in both negative and positive ionization modes. For the separation, an Acquity UHPLC BEH-C18 column (2.1 id × 100 mm, 1.7 μm), obtained from Waters (USA), was used, with elution A (0.25% formic acid–water) and elution B (0.25% formic acid–methanol) via the following procedure (0–1 min, 5% B; 8 min, 25% B; 11 min, 60% B; 13 min, 100% B; 16 min, 100% B; and 16.1−20 min, 5% B). The identification was achieved by multiple reaction monitoring, then after the UHPLC-MS analysis of all compounds present in the extracts and a comparison with the published mass spectrometry (MS) data (Kelebek et al., 2017), the basic structure of some peaks were deduced, and then further assignation was performed by comparison with the respective standards using UHPLC-MS. Eckol, diphlorethol/difucol, and coutaric acid were preliminarily identified by UHPLC-MS analysis and comparison with the published MS data without further assignation, due to the lack of their commercial standards.

MS data of unknown peaks that may be phenolic compounds were also shown, and the unknown peaks will be investigated in the further study. MS data were collected over a mass range of m/z 50−1000. The constant parameters used were as follows: capillary voltage, 2.0 kV; cone voltage, 30 V; drying gas (N_2) flow, 1000 L/h; and drying gas temperature, 500°C.

2.6 | Quantification of phenolic composition

After free and bound phenolic compounds had been tentatively identified, they were quantified by HPLC-DAD as our previously reported methods (Xiao et al., 2020). The separation was conducted on an Agilent Zebra-C18 column (250 × 4.6 mm, 5 μm) with elution A (0.4% acetic acid–water) and solution B (acetonitrile) in the following procedure: 0 min, 95% B; 40 min, 75% B; 45 min, 65% B; 50 min, 50% B; 55 min, 95% B; and 60 min, 95% B. 254, 280, 320, 350, and 520 nm were chosen as detection wavelength for HPLC-DAD and content measurements were carried out at 280 nm. Six-point calibration lines were established and used for the quantitative analysis. The content of seaweed was expressed as μg per g DW. Diphlorethol/difucol and eckol were quantified using standard curve of phloroglucinol, and coutaric acid was quantified using standard curve of gallic acid. And the others were quantified with the respective standard curve.

2.7 | Antioxidative activity

FRAP assay and ABTS scavenging activity assay were performed using commercial kits as per the manufacturer's protocols. The values of these two methods were calculated by comparison with the standard curves of $FeSO_4$ and Trolox, and expressed as mmol ferrous sulfate equivalent per g of dry seaweed powder (mM Fe(II)E/g DW) and mmol Trolox equivalent per g of dry seaweed powder (mM TE/g DW), respectively.

Oxygen radical absorbance capacity (ORAC) assay was performed following the method reported by Su et al. (2014). First, the extract and its respective extract solvent were appropriately diluted with 75 mmol/L (pH 7.4) phosphate buffer. Second, 20 μl diluted extract, Trolox (6.25−50 μmol/L), or the diluted extract solvent was added into triplicate wells in a black 96-well plate, and incubated with 200 μl of fluorescein (0.96 μmol/L) for 20 min at 37°C. After ABAP solution (119 mmol/L, 20 μl) had been added to each well, the fluorescence intensity was measured every 5 min for 35 cycles at excitation/emission wavelengths of 485/538 nm on an Infinite M200PRO plate reader (TECAN,

TABLE 1 Effects of different extraction solvents on the total phenolic content (TPC) and total flavonoid content (TFC) of free phenolic fraction from *S. polycystum*

Solvents	TPC (mg GAE/ 100 g DW)	TFC (mg CE/ 100 g DW)
Methanol	27.29 ± 3.04b	107.43 ± 0.84cd
70% Methanol	40.63 ± 3.25c	126.45 ± 6.46e
50% Methanol	43.99 ± 6.13c	50.04 ± 3.79a
Ethanol	19.46 ± 3.04a	103.00 ± 3.86bc
70% Ethanol	68.88 ± 5.55d	128.21 ± 2.29e
50% Ethanol	84.52 ± 8.69e	98.79 ± 5.10b
Acetone	25.26 ± 0.77b	113.46 ± 1.25d
70% Acetone	91.54 ± 2.03e	171.55 ± 5.43f
50% Acetone	90.55 ± 5.99e	174.25 ± 1.54f
Ethyl acetate	18.39 ± 1.52a	125.03 ± 8.73e

Each value was expressed as mean ± standard deviation ($n = 3$). Values with different letters within columns are significantly different ($p < 0.05$). Abbreviation: DW, dry weight.

Männedorf, Switzerland). The ORAC value was expressed as μmol Trolox equivalent per gram of dried seaweed (μM TE/g DW).

2.8 | Statistical analyses

All the data were expressed as mean ± standard deviation derived from triplicate extractions. Statistical analyses were performed with SPSS 16.0 using one-way ANOVA, followed by Duncan's post hoc test, and $p < 0.05$ was considered statistically significant.

3 | RESULTS AND DISCUSSION

3.1 | Effects of the different solvent extractions on free phenolics of *S. polycystum*

3.1.1 | TPC and TFC

The TPC and TFC of free phenolic fractions of *S. polycystum* are presented in Table 1. The highest TPC of the free phenolic fraction was obtained by extraction of 70% acetone, 50% acetone, and 50% ethanol, with insignificant differences among them ($p > 0.05$). The second highest TPC of the free phenolic fraction was obtained by the extraction of 70% ethanol, which was 75.25% of that of 70% acetone ($p < 0.05$). Notably, methanol and ethyl acetate afforded the lowest TPC. Moreover, 50% acetone and 70% acetone gave the highest TFC of free phenolic fraction, followed by 70% ethanol, 70% methanol, and ethyl acetate ($p < 0.05$). Furthermore, 50% methanol afforded the low-

est TFC. In consideration of both TPC and TFC of the free phenolic fraction of *S. polycystum*, 50% acetone and 70% acetone had the best efficiency, followed by 50% ethanol and 70% ethanol. At present, there is no consensus on the most efficient extraction solvent for free phenolics from *S. polycystum* due to lack of studies. Chan et al. (2011) found that phenolics are present in the ethanol extract of *S. polycystum*. Johnson et al. (2019) found that chloroform affords the highest TPC of *S. polycystum* among four extraction solvents: acetone, chloroform, petroleum ether, and methanol. Better extraction efficiency for free phenolics from brown seaweeds was obtained by organic aqueous mixtures, but not by a single solvent (Koivikko et al., 2005). Similarly, the present study found that organic aqueous mixtures afforded higher extraction efficiency for free phenolics than pure methanol, ethanol, and acetone. According to the principle of "the substances are more likely to dissolve in the solvent with the similar polarity to them," the extraction solvent with the polarity close to those of phenolic compounds gives a high extractability. The polarity of phenolic compound is dependent on its structure, including the basic structure, the number and position of phenolic hydroxyl groups, and binding forms and types of other groups with different polarity linked to the basic structure (Cheynier, 2012; Cotas et al., 2020). The polarity of phenolics can be increased if the phenolic acids and flavonoids are linked with polar groups, such as sugars (Rio et al., 2013).

Among solvents involved in this study, the relative polarity of water is the strongest, followed by methanol, ethanol, acetone, and ethyl acetate (Ma et al., 2019). Compared with pure organic solvents, the relative polarity of organic aqueous mixtures increased with the increasing ratio of water in them. Our results showed that the glycosides (e.g., rutin, hesperidin, and quercitrin) and special aglycones (e.g., hinokiflavone) with relatively high polarity were the main phenolic composition. As shown in the chromatograms of samples analyzed by HPLC-DAD (Figure S3), the number of peaks with retention time at the range of 0–10 min (compounds with relatively high polarity) increased significantly in 70% acetone, 70% methanol, 70% ethanol, compared with their respective pure organic solvent, demonstrating that more compounds with relatively high polarity were extracted with the increase in the polarity of solvent. Thus in this study, organic aqueous mixtures were more effective for extracting total phenolics than their pure organic solvents (methanol, ethanol, acetone), which coincided with the previous studies (Koivikko et al., 2005; Ma et al., 2019). Additionally, among all solvents used in this study, ethyl acetate, with the weakest polarity, afforded the lowest extractability for phenolic compounds from *S. polycystum*, which coincided with the previous studies (Sarikurkcua et al., 2020).

TABLE 2 Identification of free phenolic compositions in S. polycystum by ultra-high-performance liquid chromatography-mass spectrometry (UHPLC-MS)

Peak	λ_{max} (nm)	Tentative assignment	Model	Parents ions	Fragment ions	References	Polyphenols subclass
1	241,322	Chlorogenic acid[1]	–	353.2	191.0,179.2	Ivanescu et al., 2010	Hydroxycinnamic acid
2	243,315	Cryptochlorogenic acid[1]	–	353.2	191.0, 179.2	Shakya & Navarre, 2006	Hydroxycinnamic acid
3	276	Baicalein[1]	+	271.0	122.9	Li et al., 2011	Flavone
4	270,335	Acacetin[1]	+	285.0	153.0	Kim et al., 2015	Flavone
5	267,345	Diosmetin[1]	+	301.0	153.0, 111.0, 255.0, 257.0	Chen et al., 2019	Flavone
6	272,330	Hinokiflavone[1]	–	537.0	417.0, 284.0	Shan et al., 2018	Flavone
7	283,327	Hesperidin[1]	–	609.0	301.0	Mattonai et al., 2015	Flavanone
8	255,355	Rutin[1]	–	609.0	301.1, 270.9,178.7	Mattonai et al., 2015	Flavonol
9	257,356	Quercitrin[1]	–	447.0	301.0, 179.0, 151.0	Ivanescu et al., 2010;	Flavonol
10	280, 306	Trans-resveratrol [1]	+	229.0	135.0,107.1	Lambert et al., 2015	Stilbene
11	280	Diphlorethol/difucol	–	249.0	231.0, 207.0, 163.0, 113.0	Corona et al., 2017	Phlorotannin
12	280	Eckol	+	373.0	357.0, 319.0, 248.0, 231.0, 142.0	Hipólito & Gerardo, 2013	Phlorotannin
13	262,346	Unknown	–	791.41	765.41, 575.0, 531.3, 461.5, 313.12, 277.2		Flavonoid
14	274,310	Unknown	–	765.38	697.3, 644.7, 591.0, 413.2, 301.1		Flavonoid

Note: After the ultra-high-performance liquid chromatography-mass spectrometry (UHPLC-MS) analysis of all compounds present in the extracts and a comparison with the published mass spectrometry (MS) data, peaks 1–12 can be preliminarily deduced, then [1]peaks 1–10 were further recognized by comparison with related commercial standards using UHPLC-MS.

3.1.2 | Composition and content of free phenolic compounds

As shown in Table 2, in free phenolic fractions, two hydroxycinnamic acids (chlorogenic acid, cryptochlorogenic acid), four flavones (baicalein, acacetin, diosmetin, hinokiflavone), two flavonols (rutin, quercitrin), one flavanone (hesperidin), one stilbene (trans-resveratrol), and two phlorotannins (eckol, diphlorethol/difucol) were identified. Chlorogenic acid and cryptochlorogenic acid showed the same deprotonated ion (m/z 353.2) and the same daughter ions (m/z 191.0 [quinic acid−H]⁻, 179.2 [caffeic acid−H]⁻), with different retention times (Ivanescu et al., 2010; Shakya & Navarre, 2006). Baicalein was distinguished by its protonated ion at m/z 271.0, as well as its daughter ion at m/z 122.9, as reported by Li et al. (2011). Peaks 4 and 5, respectively, gave protonated ions at m/z 285.0 and 301.0, with the same product ion at m/z 153.0 due to Retro-Diels-Alder (RDA) cleavage of C-ring (Chen

et al., 2019; Kim et al., 2015); thus, they were deduced as acacetin and diosmetin, respectively. Peak 6 with [M−H]⁻ at m/z 537.0 and product ion at m/z 284.0 corresponded to hinokiflavone, which was in accordance with MS data from Shan et al. (2018). Hesperidin and rutin were distinguished by the same deprotonated ion (m/z 609.0), and the hesperitin fragment (m/z 301.0) and quercetin fragment (m/z 301.1) for the loss of glycoside, respectively. Quercitrin was distinguished by the deprotonated [M−H]⁻ ion at m/z 447.0, and the product ion m/z 301.0 (aglycone quercetin) for the loss of rhamnoside and m/z 179.0 and 151.0 as typical RDA fragmentation. Trans-resveratrol was characterized by the protonated [M+H]⁺ ion at m/z 229.0, and its product ions m/z 135.0 and m/z 107.1 due to loss of a phenol group and formation of a hydroxytropylium ion, respectively (Lambert et al., 2015). Peak 11 was deduced as diphlorethol or difucol by its deprotonated ion (m/z 249.0) as well as its daughter fragment m/z 231.0 for the loss of H_2O, in conformity with MS data reported

by Corona et al. (2016). Fucols and phlorethols are produced from phloroglucinol units with phenyl bonds and ether bonds, respectively. Their same molecular weight at the same degree of polymerization makes it difficult for them to be distinguished by MS. Thus, peak 11 was tentatively assigned as diphlorethol/difucol without their standards (Corona et al., 2016; Hipólito & Gerardo, 2013). Eckol was characterized by the protonated $[M+H]^+$ ion at m/z 373.0, and its product ions m/z 248.0 $[M+H−125]^+$ and 357.0 $[M+H−16]^+$, a characteristic pattern of eckol-type phlorotannin fragmentation, in conformity with MS data described by Hipólito and Gerardo (2013).

Phenolic compounds including flavonoids, phenolic acids, phlorotannins, and bromophenols are commonly present in free form in brown seaweeds (Cotas et al., 2020; Rajauria et al., 2016). Hydroxycinnamic acids such as chlorogenic acid were found in the free fraction of Irish brown seaweed *Himanthalia elongata* (Rajauria, 2018). Flavonoid derivatives such as flavones, flavonols, flavanones, flavanols, and flavanonols were also found in the free fractions of brown seaweeds. Quercetin-O-rutinoside, quercetin-O-glucoside, and hesperitin-O-rutinoside were identified as the free constituents of brown macroalgae by high-performance liquid chromatography-mass spectrometry (HPLC-MS) without further assignation for the linkage of between aglycone moity and glycosidic group (Olate-Gallego et al., 2019). In the present study, quercetin-3-O-rutinoside (rutin), quercetin-3-O-rhamnoside (quercitrin), and hesperitin-7-O-rutinoside (hesperidin), were identified by UHPLC-MS and further assignation with their commercial standards using UHPLC-MS. Phlorotannins are the major phenolic class found only in marine brown seaweeds, but not in green and red seaweeds (Corona et al., 2016; Cotas et al., 2020). Phlorotannin compounds such as diphlorethol/difucol, bieckol/dieckol, eckol, 7-phloroeckol, phlorofucofuroeckol, fucophloroethol, and phloroglucinol are found in brown seaweeds (Corona et al., 2016; Hipólito & Gerardo, 2013). Phlorotannins with different degrees of polymerization have been described in the free fractions of brown seaweeds such as *S. muticum* (Corona et al., 2016; Cotas et al., 2020; Olate-Gallego et al., 2019).

The number and content of phenolic compositions determined in the free phenolic fractions extracted by different solvents changed considerably (Table 3). Three to nine different free phenolic compounds were extracted by these solvents. All aforementioned phenolic compositions were determined in the free phenolic fraction extracted by 70% acetone, except for rutin, diosmetin, and hesperidin. Eight free phenolic compounds were extracted by 70% methanol, 70% ethanol, and 50% ethanol. However, only three free phenolic compounds (hinokiflavone, eckol,

and diphlorethol/difucol) were extracted by ethyl acetate. Moreover, the highest total content of free phenolic compounds was obtained from 70% ethanol, which was 1.10-fold higher than those of both 70% acetone and 50% methanol ($p < 0.05$). Here, 70% methanol gave the lowest total content of free phenolic compounds, followed by ethyl acetate and 70% ethanol ($p < 0.05$). Hinokiflavone and diphlorethol/difucol were extracted by all extraction solvents. The highest hinokiflavone level was found in the free phenolic fraction obtained by 50% methanol, followed by 70% methanol, 70% ethanol, and 70% acetone ($p < 0.05$). Additionally, acetone afforded the lowest hinokiflavone yield, which was 26.93% of that of 50% methanol ($p < 0.05$). Moreover, 70% ethanol, as well as ethanol, afforded the highest diphlorethol/difucol yield, followed by acetone and ethyl acetate ($p < 0.05$). The diphlorethol/difucol yield obtained from 70% ethanol was 1.69-, 2.61-, 5.18-, and 12.62-fold of that obtained from 50% ethanol, 70% acetone, 50% acetone, and 50% methanol, respectively ($p < 0.05$). Hinokiflavone and diphlorethol/difucol accounted for 90.7%−98.6% of the total content of free phenolic compounds in extracts obtained by methanol, 70% ethanol, ethyl acetate; 40%−50% of the total content of free phenolic compounds in extracts obtained by 50% methanol, 70% methanol, and 50% ethanol; and 26.60%−36.81% of the total content of free phenolic compounds in extracts obtained by 50% methanol and acetone-water solutions. Trans-resveratrol was only detected in 70% acetone and 50% acetone, which were 38.60% and 45.20% of their respective total contents of free phenolic compounds. Diosmetin was only detected in 70% ethanol and 50% ethanol, which were 36.01% and 48.01% of their respective total contents of free phenolic compounds. Hesperidin was only detected in 70% methanol and 50% methanol, which were 42.31% and 34.60% of their respective total contents of free phenolic compounds. Besides, baicalein was extracted by 50% methanol, 70% ethanol, 50% ethanol, acetone, 70% acetone, and 50% acetone with content ranging from 13.45 to 17.18 μg/g DW. Considering the number and total content of phenolic compounds detected in the free phenolic fraction from *S. polycystum*, 70% ethanol had the highest extraction efficiency, followed by 70% acetone.

The present study reported the effect of different solvents on the free phenolic profile of *S. polycystum* for the first time. Normally, the phenolic profiles depended on the extraction solvents used. The results from this study explained the significance of solvent polarity and solubility on extraction. Higher extractability of free phenolic acids could be achieved using an ethanol-aqueous mixture or methanol-aqueous mixture, whereas the specific extraction of phlorotannins and flavan-3-ols could be realized with an acetone-aqueous mixture (Arivalagan et al., 2018; Erpel et al., 2020; Koivikko et al., 2005). However,

TABLE 3 Contents of free phenolic compounds extracted by different extraction solvents determined by high-performance liquid chromatography-diode array detection (HPLC-DAD)

Phenolics	Content of free phenolic compounds (μg/g DW)									
	Methanol	70% Methanol	50% Methanol	Ethanol	70% Ethanol	50% Ethanol	Acetone	70% Acetone	50% Acetone	Ethyl acetate
Chlorogenic acid	nd	3.30 ± 0.29b	nd	2.66 ± 0.24a	nd	nd	2.37 ± 0.05a	3.15 ± 0.25b	nd	nd
Cryptochlorogenic acid	nd	2.73 ± 0.24a	3.31 ± 0.20b	nd	5.35 ± 0.39c	4.92 ± 0.35c	nd	6.51 ± 0.36d	5.3 ± 0.31c	nd
Total phenolic acids	nd	6.03 ± 0.26d	3.31 ± 0.20b	2.66 ± 0.24a	5.35 ± 0.39c	4.92 ± 0.35c	2.37 ± 0.05a	9.66 ± 0.35e	5.3 ± 0.31c	nd
Baicalein	nd	nd	13.63 ± 0.70a	nd	17.18 ± 0.42bc	13.48 ± 0.61a	17.12 ± 0.24c	16.46 ± 0.17b	13.45 ± 0.21a	nd
Acacetin	nd	2.86 ± 0.15c	3.43 ± 0.24d	nd	1.87 ± 0.14a	2.46 ± 0.32bc	27.31 ± 1.48e	1.73 ± 0.17a	2.42 ± 0.13b	nd
Diosmetin	nd	nd	nd	nd	45.05 ± 0.97a	48.05 ± 0.98b	nd	nd	nd	nd
Hinokiflavone	25.57 ± 1.00d	41.20 ± 1.24g	52.44 ± 2.05h	22.07 ± 1.12c	38.89 ± 1.19f	22.59 ± 1.09c	14.12 ± 0.48a	37.85 ± 2.60f	17.84 ± 1.03b	28.37 ± 0.95e
Hesperidin	nd	39.40 ± 3.57a	39.28 ± 4.90a	nd	nd	nd	nd	nd	nd	nd
Rutin	0.28 ± 0.03a	nd	nd	0.51 ± 0.09b	nd	nd	nd	nd	nd	nd
Quercitrin	0.10 ± 0.03a	0.30 ± 0.06b	0.56 ± 0.06c	nd	3.31 ± 0.28e	2.26 ± 0.29d	0.15 ± 0.02a	0.31 ± 0.03b	nd	nd
Total flavonoids	25.95 ± 0.96b	83.76 ± 2.76f	109.34 ± 3.67h	22.58 ± 0.98a	106.3 ± 1.01h	88.84 ± 0.89g	58.70 ± 1.34e	56.35 ± 2.35c	33.71 ± 0.87d	28.37 ± 0.95c
Trans-resveratrol	nd	nd	nd	nd	nd	nd	nd	44.11 ± 0.44b	33.96 ± 0.23a	nd
Diphlorethol/difucol[1]	5.01 ± 0.14e	3.01 ± 0.21c	0.87 ± 0.10a	11.53 ± 0.25h	10.98 ± 0.34h	6.48 ± 0.13f	8.01 ± 0.35g	4.21 ± 0.20d	2.12 ± 0.21b	8.52 ± 0.32g
Eckol[1]	0.50 ± 0.04c	0.34 ± 0.03b	nd	0.74 ± 0.02e	0.64 ± 0.04d	0.34 ± 0.03b	0.45 ± 0.04c	0.30 ± 0.03b	0.14 ± 0.02a	0.54 ± 0.06 cd
Total phlorotannins	5.51 ± 0.10e	3.35 ± 0.19c	0.87 ± 0.10a	12.27 ± 0.33h	11.62 ± 0.43h	6.82 ± 0.12f	8.46 ± 0.32g	4.51 ± 0.19d	2.26 ± 0.20b	9.06 ± 0.30g
Number of phenolics	5	8	7	5	8	8	7	9	7	3
Total phenolics	31.46 ± 1.05a	93.14 ± 4.27e	113.52 ± 3.78g	37.30 ± 0.87b	125.10 ± 0.98h	100.02 ± 0.97f	69.53 ± 1.34c	114.23 ± 2.5g	75.02 ± 0.99d	37.40 ± 0.85b

Note: After being identified, [1]diphlorethol/difucol and eckol were quantified by HPLC-DAD with the standard curve of phloroglucinol. The others were quantified by high-performance liquid chromatography-diode array detection (HPLC-DAD) with their own standard curves. The contents were expressed as means ± SD ($n = 3$). Values not sharing a common letter within the same row are significantly different ($p < 0.05$).

Abbreviations: DW, dry weight; nd, not detected.

TABLE 4 Antioxidant activity of free phenolic fraction from *S. polycystum* obtained by different extraction solvents

Solvents	Antioxidant activity		
	FRAP		
	(mM Fe(II)E/ g DW)	ABTS (mM TE/ g DW)	ORAC (μM TE/g DW)
Methanol	1.37 ± 0.13c	1.02 ± 0.21c	8.88 ± 0.41c
70% Methanol	2.30 ± 0.07d	1.79 ± 0.31d	8.93 ± 0.39c
50% Methanol	3.10 ± 0.02e	2.04 ± 0.15de	12.56 ± 0.29d
Ethanol	0.82 ± 0.11b	0.94 ± 0.14bc	6.87 ± 0.37b
70% Ethanol	5.15 ± 0.03f	2.09 ± 0.09e	13.81 ± 0.29e
50% Ethanol	6.18 ± 0.11 g	2.26 ± 0.12e	21.71 ± 0.27 g
Acetone	2.38 ± 0.09d	0.46 ± 0.09a	5.30 ± 0.05a
70% Acetone	6.79 ± 0.02h	2.55 ± 0.15f	20.15 ± 0.25f
50% Acetone	5.08 ± 0.18f	2.15 ± 0.11e	23.23 ± 0.33h
Ethyl acetate	0.29 ± 0.02a	0.67 ± 0.13ab	5.50 ± 0.22a

Note: Values not sharing a common letter within the same column are significantly different ($p < 0.05$).

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline)−6-sulfonic acid; FRAP, ferric-reducing antioxidant power; ORAC, oxygen radical absorbance capacity.

TABLE 5 Correlation coefficients (r) between total flavonoid content (TFC), total phenolic content (TPC), and antioxidant activity (ferric-reducing antioxidant power [FRAP], 2,2′-azino-bis(3-ethylbenzthiazoline)−6-sulfonic acid [ABTS], and oxygen radical absorbance capacity [ORAC]) of free phenolic fraction of *S. polycystum* obtained by different extraction solvents

	TFC	TPC	FRAP	ABTS	ORAC
TFC	1				
TPC	0.659[*]	1			
FRAP	0.454	0.956[**]	1		
ABTS	0.287	0.886[**]	0.863[**]	1	
ORAC	0.506	0.970[**]	0.896[**]	0.865[**]	1

*Correlation is significant at $p \leq 0.05$ level.

**Correlation is significant at $p \leq 0.01$ level.

in this study, considering the phenolic compound content detected in free phenolic fractions, 50% methanol and 70% ethanol achieved the highest total flavonoids, 70% acetone obtained the highest total phenolic acids, whereas ethanol and 70% ethanol obtained the highest total phlorotannins. This may have been due to the fact that different components have different polarities.

3.1.3 | Antioxidant activity of free phenolics

Multiple assays (FRAP, ABTS, and ORAC) were adopted to measure antioxidant activities of free phenolic fractions from *S. polycystum* (Table 4). Free phenolic fractions obtained from 10 different solvents exhibited remarkably different FRAP values in the range of 0.29−6.79 mM Fe(II)E/g DW ($p < 0.05$). Here, 70% acetone afforded the highest FRAP value, followed by 50% ethanol ($p < 0.05$). The FRAP value of the free phenolic fraction obtained from 70% acetone was 1.32-fold and 1.34-fold higher than those of 70% ethanol and 50% acetone, respectively ($p < 0.05$). Moreover, the ABTS value of 70% acetone was the highest, followed by 50% ethanol, 70% ethanol, 50% acetone, and 50% methanol ($p < 0.05$). The lowest FRAP antioxidant activity was detected in acetone. Furthermore, the free phenolic fraction obtained from 50% acetone had the highest ORAC value, followed sequentially by 50% ethanol, 70% acetone, and 70% ethanol ($p < 0.05$). Johnson et al. (2019) found that acetone extract had

the highest 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant ability and petroleum ether extract had the highest H_2O_2-scavenging activity among four phenolic-rich extracts of *S. polycystum* obtained from acetone, chloroform, petroleum ether, and methanol. Raghavendran et al. (2005) found protective effects of alcoholic extract from *S. polycystum* on endogenous antioxidant (reduced glutathione) and antioxidant enzymes of liver mitochondria in rats with acetaminophen-induced toxic hepatitis. These results indicated that the free phenolic fraction of *S. polycystum* exerted potent antioxidant ability and acetone-aqueous mixture afforded the highest antioxidant ability, followed by ethanol-aqueous mixture.

3.1.4 | Correlation of TPC, TFC, and antioxidant capacity of free phenolics

Pearson's correlation coefficients (r) among TPC, TFC, and antioxidant activity (FRAP, ABTS, ORAC) of free phenolic fractions of *S. polycystum* obtained from different solvents are shown in Table 5. A remarkably positive correlation between TPC and antioxidant activity was shown, in conformity with the positive correlation between TPC and antioxidant activity of seaweed extracts obtained from different solvents reported by previous studies (Airanthi et al., 2011; Su et al., 2014). Whereas, the present study showed an insignificant correlation between TFC and antioxidant activity (FRAP, ABTS, ORAC). Yin et al. (2015) found an insignificant correlation between ABTS and TFC, and a strong positive correlation between DPPH and TPC of *S. horneri* extract obtained by supercritical CO_2 with ethanol extraction.

TABLE 6 Effect of different hydrolysis methods on the total phenolic content (TPC) and total flavonoid content (TFC) and antioxidant activity of bound phenolic fraction of *S. polycystum*

	Alkaline hydrolysis	Acid hydrolysis
TPC (mg GAE/100 g DW)	274.27 ± 6.71b	63.73 ± 1.51a
TFC (mg CE/100 g DW)	310.71 ± 3.86b	144.98 ± 2.84a
FRAP (mM Fe(II)E/g DW)	7.58 ± 0.10b	1.71 ± 0.15a
ABTS (mM TE/g DW)	3.82 ± 0.19b	3.27 ± 0.14a
ORAC (μM TE/g DW)	94.39 ± 0.19b	8.79 ± 0.30a

Note: Values not sharing a common letter within the same row are significantly different ($p < 0.05$).

Abbreviations: ABTS, 2′-azino-bis(3-ethylbenzthiazoline)−6-sulfonic acid; FRAP, ferric-reducing antioxidant power; ORAC, oxygen radical absorbance capacity.

3.2 | Effects of different hydrolysis methods on bound phenolics of *S. polycystum*

3.2.1 | TPC and TFC

As shown in Table 6, TPC and TFC of bound phenolic fractions released by alkaline hydrolysis from the residue of *S. polycystum* were 4.30-fold and 2.14-fold higher than that released by acid hydrolysis, respectively ($p < 0.05$). Bound phenolics may interact with cellulose, pectin, and other polysaccharides via both reversible interactions via noncovalent forces (hydrogen and hydrophobic bonding) and van der Waals forces and irreversible interactions via covalent bonds (Acosta-Estrada et al., 2014). Alkaline hydrolysis and acid hydrolysis may disintegrate cell walls and facilitate the solubilization and diffusion of bound phenolic compounds. Moreover, alkaline hydrolysis was reported to more effectively release bound phenolics from fruits and cereals (Su et al., 2014; Wang et al., 2020) and release bound phlorotannins from brown seaweed *Fucus vesiculosus* than acid hydrolysis (Koivikko et al., 2005). However, Bonoli et al. (2004) reported that acid hydrolysis was more efficient for the release of bound phenolics of barley flour than alkaline hydrolysis.

3.2.2 | Composition and content of bound phenolic compounds

The results in Table 7 revealed that the compositions and contents of bound phenolics released from the residue of *S. polycystum* were clearly influenced by the hydrolysis method, in accordance with previous studies (Su et al., 2014; Wang et al., 2020). Five phenolic compounds were found in the bound phenolic fraction obtained from alkaline hydrolysis. Peak 1 at m/z 168.9 with its product ion m/z

125.0 due to loss of CO_2 was tentatively identified as gallic acid. Coutaric acid displayed the precursor ion $[M-H]^-$ at m/z 295.1 and the product ions at m/z 149.1 [tartaric acid−H]$^-$, 163.1 [coumaric acid−H]$^-$, and 119.1 [coumaric acid−H−CO_2]$^-$, which were in accordance with MS data from Monica et al. (2016). Peak 3 gave precursor ion [M + H]$^+$ at m/z 301.0, with the product ion at m/z 153.0 due to RDA cleavage of the C-ring (Chen et al., 2019), being deduced as diosmetin. Baicalein and apigenin were identified by their precursor ion $[M + H]^+$ at m/z 271.0, and product ions m/z 122.9 for baicalein and m/z 227.0 and 151.0 for apigenin, as reported by Li et al. (2011) and Ivanescu et al. (2010), respectively. However, only two phenolic compounds (gallic acid, coutaric acid) were discovered in the bound phenolic fraction obtained from acid hydrolysis. Moreover, contents of gallic acid and coutaric acid obtained from alkaline hydrolysis were 6.35-fold and 9.68-fold higher than those obtained from acid hydrolysis, respectively ($p < 0.05$). The total content of bound phenolic compounds released by alkaline hydrolysis determined by HPLC-DAD was 303.95 ± 3.49 μg/g DW, which was 14.68-fold higher than that released by acid hydrolysis ($p < 0.05$).

Bound phenolics from marine brown seaweeds have gained seldom attention, although only a few studies have identified the composition of bound phenolics released from soluble dietary fiber and insoluble dietary fiber from algae by LC-MS (Luo et al., 2020). This study investigated the method suitable for extracting bound phenolics from *S. polycystum* for the first time, indicated by the difference not only in TPC, TFC and antioxidant ability, but also in the profiles of bound phenolics between different hydrolysis methods. In a previous study, bound phlorotannins were released from the brown seaweed *Fucus vesiculosus*, but were not identified (Koivikko et al., 2005). Moreover, flavonoids, including cirsimaritin, kaempferol, morin, rutin, myricetin, hesperidin and quercitrin, were determined in bound phenolic fractions of several *Rhodophyta* species by HPLC-DAD (Santoso et al., 2002). Moreover, hydroxybenzoic acid derivatives including gallic acid have commonly been reported as free form constituents of brown macroalgal species (Rajauria et al., 2016). Apigenin was also identified in free extracts of brown seaweeds *D. antarctica*, *L. spicata*, and *M. integrifolia* by HPLC-MS (Olate-Gallego et al., 2019). Furthermore, baicalein, diosmetin, and coutaric acid were found in free extracts of *Radix scutellariae* (Li et al., 2011), citrus fruits, and Chinese herbal medicines (Chen et al., 2019) and rosé wines (Lambert et al., 2015), whereas these phenolics were not found in free or bound fraction of seaweeds in previous study. Thus, baicalein, diosmetin, and coutaric acid were found in seaweeds for the first time in this study.

TABLE 7 Identification and quantification of bound phenolic compounds in *S. polycystum*

Peak	λ_{max} (nm)	Tentative assignment	Model	Parents ions	Fragment ions	Reference	Polyphenols subclass	Content (μg/g DW)[3] Alkaline hydrolysis	Acid hydrolysis
1	271	Gallic acid[1]	–	168.9	125.0, 97.0	Mattonai et al., 2015	Hydroxybenzoic acid	9.59 ± 1.69b	1.51 ± 0.11a
2	230,321	Coutaric acid[2]	–	295.1	119.1, 149.1, 163.1	Monica et al., 2016	Hydroxycinnamic acid	176.11 ± 9.84b	18.20 ± 1.12a
3	276	Baicalein[1]	+	271.0	122.9	Li et al., 2011	Flavone	20.99 ± 1.28	nd
4	267,345	Diosmetin[1]	+	301.0	153.0, 111.0, 255.0, 257.0	Chen et al., 2019	Flavone	81.25 ± 3.52	nd
5	267,339	Apigenin[1]	+	271.0	227.0, 151.0	Ivanescu et al., 2010	Flavone	16.04 ± 0.44	nd
6	242, 315	Unknown	–	609.0	565.0, 493.0, 299.0, 283.0		Flavonoid		
7	252,326	Unknown	–	595.0	551.0, 421.0, 397.0, 325.0, 293.0		Flavonoid		
8	280	Unknown	–	679.0	623.0, 578.0, 430.0, 340.0		Phlorotannin/procyanidin		
9	280	Unknown	–	940.85	793.9, 686.8, 487.7,368.6, 249.4		Phlorotannin		

Note: After the UHPLC-MS analysis of all compounds exist in the extracts and a comparison with the published MS data, peaks 1–5 can be preliminarily deduced, then [1]peaks were further recognized by comparison with related commercial standards using UHPLC-MS. [2]Peak 2 was not further assigned by UHPLC-MS analysis of its commercial standard due to the lack of the commercial standard. [3]Coutaric acid was quantified with standard curve of gallic acid using HPLC-DAD. The other peaks were quantified by HPLC-DAD with their own standard curves. Values not sharing a common letter within the same row are significantly different ($p < 0.05$). Abbreviations: DW, dry weight; nd, not detected.

3.2.3 | Antioxidant activity of bound phenolics

Table 6 shows the antioxidant activity of bound phenolic compounds released from *S. polycystum* by alkaline hydrolysis and acid hydrolysis. FRAP, ABTS, and ORAC values revealed that the antioxidant activity of bound phenolic compounds released by alkaline hydrolysis was significantly higher than that of those released by acid hydrolysis ($p < 0.05$). FRAP, ABTS, and ORAC values from alkaline hydrolysis were 4.41-fold, 1.17-fold, and 10.74-fold of those from acid hydrolysis, respectively. This may have been due to the higher TPC and TFC of bound phenolic fractions obtained by alkaline hydrolysis than those by acid hydrolysis (Su et al., 2014; Wang et al., 2020). The correlation analysis revealed that antioxidant activity determined by the FRAP, ABTS, and ORAC assays positively correlated with TPC (r-values of 0.999 [$p \leq 0.01$], 0.904 [$p \leq 0.05$], and 0.999 [$p \leq 0.01$]), and with TFC (r-values of 0.999 [$p \leq 0.01$], 0.893 [$p \leq 0.05$], and 0.999 [$p \leq 0.01$]).

4 | CONCLUSION

This study revealed the TPC, TFC, phenolic compound profiles, and antioxidant activity of free and bound extracts of *S. polycystum* obtained by different extraction solvents and hydrolysis methods. The results showed that aqueous acetone afforded the highest free TPC and antioxidant ability, followed by aqueous ethanol and then aqueous methanol. Among 12 free phenolic compounds characterized by UHPLC-MS from *S. polycystum*, nine were extracted by 70% acetone and eight were extracted by 70% methanol, 70% ethanol, and 50% ethanol. The highest total content of free phenolic compounds was obtained from 70% ethanol. Given these findings, 70% ethanol may be the best extraction solvent for free phenolics. Alkaline hydrolysis was more efficient for the extraction of bound phenolics, as indicated by significantly higher bound TPC, antioxidant ability, and number and total content of phenolic compounds identified from alkaline hydrolysis than those from acid hydrolysis. *Sargassum polycystum* is a promising source of natural antioxidants.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

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