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Proximate Composition, Phytochemical Analysis and Toxicity Assessment of Extracts of *Caulerpa lentillifera* using Autoclave- and Microwave-Assisted Extractions

(Komposisi Proksimat, Analisis Fitokimia dan Penilaian Ketoksikan Ekstrak *Caulerpa lentillifera* menggunakan Pengekstrakan Berbantu Autoklaf dan Mikrogelombang)

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ABSTRACT

Caulerpa lentillifera is a marine alga widely cultured and consumed in Asia and Oceania. Emerging green techniques are currently popular for phytochemical extraction. Therefore, this study aimed to investigate the proximate compositions, phytochemicals, and antioxidant properties of the *C. lentillifera* extracts using autoclave-assisted and microwave-assisted extraction methods (A-CLE and M-CLE, respectively). The toxicity of extracts was tested against human normal colon cells and freshwater crustacean *Daphnia magna*. We found that crude lipid, ash and moisture contents in A-CLE were lower than those in M-CLE, while crude protein and crude carbohydrate were higher in A-CLE. Total flavonoid content in A-CLE (1.474 ± 0.046 mg QE g^{-1} extract) was higher than that in M-CLE (1.179 ± 0.054 mg QE g^{-1} extract). There was no difference in total phenolic contents, triterpenoid contents and antioxidant activities between the extracts. The IC_{50} of A-CLE (12,260 ± 197 µg mL^{-1}) was higher than that of M-CLE (10,950 ± 169 µg mL^{-1}). In the acute toxicity test, the LC_{50} of A-CLE against *D. magna* (7.50 ± 0.28 g L^{-1}) was lower than LC_{50} of M-CLE (8.76 ± 0.26 g L^{-1}). This study suggest that autoclave-assisted and microwave-assisted extractions are effective green methods of extracting *C. lentillifera*. This study will be useful for further studies of *C. lentillifera* extracts to improve human and animal health.

Keywords: Antioxidant; autoclave-assisted extraction; *Caulerpa lentillifera*; microwave-assisted extraction; toxicity

ABSTRAK

Caulerpa lentillifera ialah alga marin yang dikultur secara meluas dan dimakan di Asia dan Oceania. Kemunculan teknik hijau kini popular untuk pengekstrakan fitokimia. Oleh itu, penyelidikan ini bertujuan untuk mengkaji komposisi proksimat, fitokimia dan sifat antioksidan bagi ekstrak *C. lentillifera* menggunakan kaedah pengekstrakan berbantu autoklaf dan mikrogelombang (A-CLE dan M-CLE). Ketoksikan ekstrak telah diuji terhadap sel kolon normal manusia dan krustasea air tawar *Daphnia magna*. Kami mendapati bahawa kandungan lipid mentah, abu dan lembapan dalam A-CLE adalah lebih rendah daripada M-CLE, manakala protein mentah dan karbohidrat mentah lebih tinggi dalam A-CLE. Jumlah kandungan flavonoid dalam A-CLE (1.474 ± 0.046 mg ekstrak QE g^{-1}) adalah lebih tinggi daripada M-CLE (1.179 ± 0.054 mg ekstrak QE g^{-1}). Tiada perbezaan dalam jumlah kandungan fenol, kandungan triterpenoid dan aktiviti antioksidan antara ekstrak. IC_{50} A-CLE (12,260 ± 197 µg mL^{-1}) adalah lebih tinggi daripada M-CLE (10,950 ± 169 µg mL^{-1}). Dalam ujian ketoksikan akut, LC_{50} A-CLE terhadap *D. magna* (7.50 ± 0.28 g L^{-1}) adalah lebih rendah daripada LC_{50} M-CLE (8.76 ± 0.26 g L^{-1}). Kajian ini mencadangkan bahawa pengekstrakan berbantu autoklaf dan mikrogelombang adalah kaedah hijau yang berkesan untuk mengekstrak *C. lentillifer*a. Penyelidikan ini adalah penting untuk kajian lanjut ekstrak C. lentillifera dalam meningkatkan kesihatan manusia dan haiwan.

Kata kunci: Antioksidan; *Caulerpa lentillifera*; ketoksikan; pengekstrakan berbantu autoklaf; pengekstrakan berbantu mikrogelombang

INTRODUCTION

Algae or seaweeds are used in various ways. Applications have been found for these organisms in aquaculture, livestock farming, pharmaceuticals, and water treatment. Algal extracts are used in cosmetics and food supplements (Wang et al. 2016; Wells et al. 2017). The nutritious components of algae include carbohydrates, proteins, lipids, minerals, vitamins, and bioactive substances that include antioxidants (Nagappan et al. 2021; Sun et al. 2022). Polyphenols are a class of antioxidants found in algae and plants that are beneficial to human health (Michalak & Chojnacka 2014; Pandey & Rizvi 2009). Previous studies found that polyphenolic compounds inhibited activities of α-amylase and α-glucosidase, leading to reduced carbohydrate absorption and reduced blood sugar in diabetic mice (Lee et al. 2014; Roy et al. 2011). In addition, marine algae produce secondary metabolites, such as triterpenoid compounds, that are anti-oxidative, anti-inflammatory, anti-cancer, anti-tumor, anti-bacterial and anti-microbial (Chung, Navaratnam & Chung 2011; Li, Himaya & Kim 2013; Nufus, Nurjanah & Abdullah 2017).

Algae play an especially important role in commercial aquaculture, where they are used as food for aquatic larvae. They have replaced more conventional types of food for crustaceans and ornamental fish to increase productivity and reduce mortality (Muller-Feuga 2000). Algal extracts from green algae can stimulate growth, anti-inflammatory, anti-oxidant and anti-pathogenic activities. Pacific white shrimp (*Litopenaeus vannamei*) fed with 0.75% of *Tetraselmis suecica* showed a 30% increase in growth, and the growth of tilapia (*Oreochromis niloticus*) fed a diet containing 15% component of *Chlorella* sp. was 69% higher than the control group (Nagappan et al. 2021). *Chlorella* extract contains the immunomodulator β-1,3-glucan, which acts as an antioxidant. *Chlorella* extract may also protect the liver and control blood lipids during malnutrition when the concentration of blood sugar is low and the concentration of hemoglobin is high (Koyande et al. 2019). Metabolites of the seaweed *Ulva rigida* directly affected the immune system of *L. vannamei* treated with algal extracts which CAT and PO activity levels increased, and MDA levels decreased (Akbary & Aminikhoei 2018). A recent study showed that polysaccharides from *Caulera racemosa* improved the immune response of *L. vannamei* by increasing phenoloxidase activity, total hemocyte count, phagocytosis and superoxide anion production. Polysaccharides from *C. racemose* also exhibited anti-oxidant activity in *L. vannamei* (Lee et al. 2020).

Caulerpa lentillifera, also known as sea grapes, is a green alga that is used as food for humans and aquatic animals (Mary et al. 2009). *C. lentillifera* contains essential amino acids, fatty acids, minerals, vitamins, pigments and other bioactive compounds (Ratana-arporn & Chirapart 2006; Syakilla et al. 2022). This seaweed has been reported to offer cardioprotective, anti-bacterial, anti-microbial, anti-cancer, anti-coagulant, anti-diabetic, anti-hyperglycemic, anti-inflammatory, antioxidant, and immunostimulatory benefits (Sun et al. 2019; Syakilla et al. 2022). An extract of *C. lentillifera* was recently reported to inhibit cell proliferation and induce the death of the brain cancer cell glioblastoma (Tanawoot et al. 2021). Previous studies also showed that *C. lentillifera* improved cardiovascular and metabolic health in rats fed high-cholesterol and high-fat diets (du Preez et al. 2020; Matanjun et al. 2010). The treatments reduced inflammation, plasma triglycerides, total cholesterol, non-esterified fatty acid and visceral adiposity, and increased HDL-cholesterol and the glucose metabolism rate. Moreover, *C. lentillifera* could modulate the gut microbiota of Wistar rats with diet-induced metabolic syndrome (du Preez et al. 2020). The addition of *C. lentillifera* to culture medium was reported to stimulate the growth of the zooplankton *Phronima pacifica* and increase the nutritional value of the species (Herawati et al. 2021).

Conventional methods of extracting the valuable natural compounds from marine seaweeds have been used for more than a century and are still widely practiced. These methods include refluxing, soxhlet, maceration, and boiling the sample with a solvent. The metabolites obtained from algal extracts depend on the extraction process used (Michalak & Chojnacka 2014). However, these methods usually take a long time to extract bioactive compounds. More environment friendly, green extraction methods are increasingly being studied and developed that could increase the yields of extracts by innovative combinations of energy, pressure, and biological agents. These emerging technologies include microwave-assisted, ultrasound-assisted, autoclave-assisted, and enzyme-assisted extractions (Getachew, Jacobsen & Holdt 2020). These emerging green technologies can reduce energy consumption and make use of alternative solvents such as water and agro-solvents (Chemat, Vian & Cravotto 2012). These emerging technologies are widely used for marine seaweed extraction (Garcia-Vaquero et al. 2021; Mohaddes-Kamranshahi et al. 2019). Autoclave-assisted extraction method is a hydrothermal extraction. The combination of high temperature and pressure is effective for breaking the algal cells to gain the high yields of targeted compounds (Garcia-Vaquero et al. 2021). Previously, brown alga *Sargassum plagiophyllum* were extracted in water using autoclave-assisted method. *S. plagiophyllum* extract had antioxidant activity and was not toxic for mice (Sengkhim et al. 2021), moreover, it could increase *Bifidobacterium* and prevented constipation in loperamide-induced mice (Khuituan et al. 2022). Furthermore, *Fucus vesiculosus* and *Pelvetia canaliculata* were extracted using autoclave-assisted extraction method. In both extracts, contents of

total phenolic compounds, phlorotannins, flavonoids and tannins were higher than maceration method (Garcia-Vaquero et al. 2021). Therefore, autoclave-assisted extraction is one of effective methods for extraction of bioactive compounds in algae. Another effective method is microwave-assisted extraction method which is a process using microwave energy to heat the solvents leading to cell rupture and releasing of organic compounds from the algal cells into the solvent (Getachew, Jacobsen & Holdt 2020). Previously, green alga *Caulerpa racemosa* was extracted using microwave-assisted method, and its extract consisted of phenolic compounds and had antioxidant activities (Li et al. 2012). The brown algae, such as *Sargassum vestitum*, *Cystoseira sedoides,* and *Ascophyllum nodosum*, were extracted by microwave-assisted extraction, and the extracts showed the antioxidant and anticancer activities (Abdelhamid et al. 2019; Dang et al. 2018; Garcia-Vaquero et al. 2020). Both autoclave- and microwave-assisted methods can reduce the energy consumption and extraction time. However, the efficiency comparisons of both extraction methods are not widely studied in green alga *Caulerpa lentillifera*.

Therefore, this study aimed to compared the bioactive compounds of *C. lentillifera* extracts obtained from two methods: autoclave-assisted extraction and microwave-assisted extraction. We focused on the aqueous extraction due to the extracts could be completely dissolved in water which would be safely used in aquaculture and safely discarded in environment. In this study, proximate compositions, total contents of phenolic, flavonoid and triterpenoid compounds were determined, and antioxidant activities were investigated. Toxicity tests were carried out against human normal colon cells and *Daphnia magna* which will be useful for further studies in human and aquatic organisms.

MATERIALS AND METHODS

Caulerpa Lentillifera CULTURE AND COLLECTION

C. lentillifera was bought from a farm in Trang, Thailand. *C. lentillifera* was dried at 60 °C until constant weight was reached. The dried *C. lentillifera* was ground to a fine powder and kept in air-tight plastic bags at -30 °C until used (Khuituan et al. 2022).

PREPARATION OF *Caulerpa lentillifera* EXTRACT (CLE)

To prepare the autoclave-assisted extract of *C. lentillifera* (A-CLE), 50 g of dried *C. lentillifera* were placed in 1000 mL of milliQ water and autoclaved at 121 °C and 15 psi for 20 min (Sengkhim et al. 2021). The product was filtered through cheesecloth, and the filtrate was centrifuged at 2200 × g for 10 min. The supernatant was collected and freeze-dried. To prepare the microwave-assisted extract of *C. lentillifera* (M-CLE), 50 g of dried *C. lentillifera* were placed in 1000 mL of milliQ water and extracted using microwave radiation at 140 W for 40 min (Getachew, Jacobsen & Holdt 2020). Subsequently, M-CLE was filtered, centrifuged and freeze-dried under the same conditions as A-CLE. Eight replications were performed for calculation of extraction yield which was calculated as extraction yield (%) = (weight of freeze-dried extract × 100)/weight of dried *C. lentilllifera.* Subsequently, 3 samples were used for analysis of proximate composition, and five samples were used for determination of total phenolic content, flavonoid content, triterpenoid content, and DPPH assay.

ANALYSIS OF PROXIMATE COMPOSITION

The moisture, crude lipid, crude protein, crude carbohydrate, and ash contents of dried *C. lentillifera*, A-CLE and M-CLE were analyzed following AOAC methods (AOAC 2000). A-CLE and M-CLE were dried at 100 °C in a hot-air oven (Memmert, SNB, Germany), and moisture content was evaluated. Crude lipids in A-CLE and M-CLE were extracted in 100% petroleum ether using a Soxhlet extractor (Foss, SOXTEC8000, Denmark). The extracted crude lipids were dried for 30 min at 105 °C, and lipid content was determined. To determine crude protein content, nitrogen content was measured using the Dumas combustion method (LECO, FP-528, USA). Crude protein content was then calculated by multiplying nitrogen content by a factor of 5.13 (Lourenço et al. 2002). Ash content was determined using a laboratory furnace (Carbolite, CWF1100, Germany) at 550 °C for 30 min. Crude carbohydrate was calculated from percentages of moisture, crude lipid, crude protein and ash contents (Sullivan & Carpenter 1993). Three replications were performed in this experiment.

TOTAL PHENOLIC CONTENT

Total phenolic contents were estimated using the Folin-Ciocalteu colorimetric assay following the method described by previous study (Chang, Lin & Lai 2012). Briefly, 100 µL of 100 mg mL^{-1} of A-CLE or M-CLE were mixed with 2 mL of 2% Na_2CO_3 and left at RT for 2 min. Then, 100 µL of 50% Folin-Ciocalteu reagent were added, mixed and the mixture was left at RT for 30 min. The absorbance of the reaction mixture was measured at 750 nm using a spectrophotometer. Total phenolic content was shown as mg gallic acid equivalent (GAE) g^{-1} extract. Five replications were performed.

FLAVONOID CONTENT

Flavonoid contents were estimated using the aluminium chloride colorimetric assay. Samples were prepared by mixing 100 µL of 100 mg mL^{-1} of A-CLE or M-CLE with 3.2 mL of milliQ, 100 µL of 1M potassium acetate and 100 µL of 10% $AlCl_3$. The samples were then left at RT for 30 min (Chandra et al. 2014). The absorbance of the reaction mixture was measured at 415 nm using a spectrophotometer. Flavonoid content was shown as mg quercetin equivalent (QE) g^{-1} extract. Five replications were performed.

TRITERPENOID CONTENT

To estimate triterpenoid contents, 100 µL of 100 mg mL^{-1} of A-CLE or M-CLE, 150 µL of 5% vanillin-glacial acetic acid, and 500 µL of 70% perchloric acid were mixed and heated at 60 °C for 45 min. The heated mixture was placed on ice and 2.25 mL of glacial acetic acid were added (Chang, Lin & Lai 2012). The absorbance of the reaction mixture was measured at 548 nm using a spectrophotometer. Triterpenoid content was shown as mg ursolic acid equivalent (UAE) g^{-1} extract. Five replications were performed.

DPPH RADICAL SCAVENGING ASSAY

Solutions of A-CLE and M-CLE in distilled water were prepared at 6 concentrations (0, 10, 20, 30, 40, and 50 mg mL^{-1}). Aliquots of 300 µL of each concentration were added directly to 300 µL DPPH (250 µM) and incubated for 30 min in darkness at RT. After incubation, absorbance was measured at 517 nm using a microplate reader (Synergy HT, BioTek). The absorbance of A-CLE or M-CLE was subtracted from the absorbance of the reacted mixture. The concentration of extract that inhibited 50% of DPPH free radicals was expressed as IC_{50} (Suo et al. 2022). Five replications were performed.

CYTOTOXICITY (MTT ASSAY)

Human normal colon cells were cultured in a humidified CO_2 incubator at 37 °C in a growth medium (GM) composed of Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal bovine serum. Wells of 96-well plates were seeded with 1×10^4 cells per well. The cells were allowed to grow for 24 h and then treated for 72 h with the growth medium containing ten concentrations of A-CLE and M-CLE (0, 1000, 2000, 4000, 6000, 8000, 10000, 12000, 14000, and 16000 µg mL^{-1}). Subsequently, the medium was replaced with 100 µL of MTT solution (0.5 mg mL^{-1}) and the plate was incubated at 37 °C. After 3 h of incubation, the solution was discarded and the crystals that had formed in the wells were dissolved in 100 µL DMSO. Absorbance was measured at 570-630 nm using a microplate reader (Synergy HT, BioTek). The cytotoxicity of the extract was expressed at 50% inhibition concentration (IC_{50}) (Suo et al. 2022). Four replications were performed.

ACUTE TOXICITY TEST IN *Daphnia magna*

To test the acute toxicity of A-CLE and M-CLE, sample solutions were prepared at eight different concentrations (0. 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, 10.5 and 11.5 g L^{-1}). Five *D. magna* neonates were placed in each well of a 6-well plate containing 10 mL of COMBO medium with test solution, and cultured for 48 h at 23 °C under a 16/8 h light and dark photoperiod. Surviving neonates were counted for LC_{50} calculations (OECD 2024). Five replications were performed. All *D. magna* were cultured following the institutional and national guidelines for the care and use of animals according to the approval of Animal Care and Use Committee, Prince of Songkla University (project license number: MHESI 68014/2348, Ref.111/2021).

STATISTICAL ANALYSES

All data were shown as means ± standard error. Differences between A-CLE and M-CLE groups were tested using the Student's t-test. Percentages of *D. magna* survival and cell viability were tested using one-way ANOVA followed by Tukey's HSD.

RESULTS AND DISCUSSION

ANALYSIS OF PROXIMATE COMPOSITION

Crude carbohydrate, protein, lipid, ash, and moisture of dried *C. lentillifera* were 30.900 ± 0.211, 4.923 ± 0.127, 0.753 ± 0.041, 56.663 ± 0.652 and 6.760 ± 0.420%, respectively. After extraction using autoclave-assisted method, extraction yield of A-CLE was 41.16 ± 3.92%, which was not different from extraction yield of M-CLE (40.71 ± 2.05%) using microwave-assisted method (p>0.05). These yields are higher than those produced by a conventional method using ethanol solvent (7-11%) (Nguyen, Ueng & Tsai 2011). In addition, crude lipid was significantly lower in A-CLE (0.030 ± 0.002%) than M-CLE (0.054 ± 0.001%) (p<0.05). Crude protein was significantly higher in A-CLE (1.373 ± 0.012%) than M-CLE (0.787 ± 0.009%) (p<0.05). Crude carbohydrate content was higher than crude lipid and protein contents in both extracts, and crude carbohydrate content was higher in A-CLE (23.410 ± 0.279%) than M-CLE (22.200 ± 0.313%) (p<0.05). Ash content was significantly lower in A-CLE (73.870 ± 0.229%) than M-CLE (75.400 ± 0.337%) (p<0.05), and the moisture content of A-CLE (1.319 ± 0.058%) was also significantly lower than that of M-CLE

(1.558 ± 0.024%) (p<0.05). The data of proximate composition analysis are presented in Table 1.

Our results showed that carbohydrate was higher than protein and lipid in all samples which was corresponded to the previous studies (Nguyen, Ueng & Tsai 2011; Ratana-arporn & Chirapart 2006; Sompong et al. 2020). Polysaccharide extracted from *C. lentillifera* showed high antioxidant activity, and they can prevent diabetes by inhibiting α-glucosidase (Chaiklahan et al. 2020; Fajriah, Fadhilah & Sinurat 2021; Tian et al. 2019). *C. lentillifera* also consists of essential and non-essential amino acids, such as valine, lysine, leucine, aspartic acid, glutamic acid, arginine, and alanine (Syakilla et al. 2022). Glutamic and aspartic acids have been reported to contribute to umami flavor (Imchen 2021). Furthermore, various fatty acids are found in *C. lentillifera* including polyunsaturated fatty acids (PUFA) which are important to human and animal health (Nagappan & Vairappan 2014). This study also found that ash was the highest composition in A-CLE and M-CLE which indicated the presence of many kinds of mineral (Kumar et al. 2011; Matanjun et al. 2009; Nagappan & Vairappan 2014; Sompong et al. 2020). Previous studies showed that *C. lentillifera* contained essential minerals, such as Ca, Cu, Fe, Mg, Zn, K, Na, P, Mn and I, and the abundant mineral elements were Na, Mg, K, Ca, and Mn (Lozano Muñoz & Díaz 2020; Mann & Truswell 2017; Syakilla et al. 2022). Therefore, the concentration of *C. lentillifera* extracts for consumption should be further studied to prevent the excessive mineral consumption.

PHYTOCHEMICAL CONTENT AND DPPH RADICAL SCAVENGING ACTIVITY

The results of total phenolic, flavonoid, and triterpenoid determination showed that total flavonoid content was significantly higher in A-CLE (1.474 ± 0.046 mg QE g^{-1} extract) than M-CLE (1.179 ± 0.054 mg QE g^{-1} extract) (Figure 1, p<0.01) but there was no significant difference in total phenolic and triterpenoid contents between A-CLE and M-CLE (p>0.05). Total phenolic contents of A-CLE and M-CLE in this study were similar to the total phenolic contents produced by conventional methods and aqueous extraction (Nguyen, Ueng & Tsai 2011). Previous studies showed that phenolic compounds from marine algae had exhibited useful anti-oxidant, anti-cancer, anti-tumor, anti-inflammatory, anti-hyperglycemic, anti-microbial and antibiotic activities (Getachew, Jacobsen & Holdt 2020; Syakilla et al. 2022). In this study, both A-CLE and M-CLE exhibited antioxidant activity against DPPH radicals. The IC_{50} values for free radical inhibition of A-CLE and M-CLE were 43.94 ± 0.58 and 41.94 ± 0.92 mg mL^{-1}, respectively. Statistical analysis showed that there was no significant difference between two extraction methods (Figure 2, p>0.05). The results indicated that A-CLE and M-CLE had antioxidant properties which effectively inhibited free radicals.

Moreover, total flavonoid content was higher in A-CLE than M-CLE. Both autoclave- and microwave-assisted extraction methods are the environmentally-friendly extraction methods. Microwave heating caused dipole rotation of the polar solvent (water) and dissolved ions in the solvent, then the cells burst and cytosolic components were released (Getachew, Jacobsen & Holdt 2020). On the other hand, autoclave heating is a hydrothermal extraction combining high temperature and pressure. Heating and pressure ruptured the cells and organic compounds are released. Moreover, lignocellulose was hydrolyzed under the pressurized condition to produce saccharides and aromatic organic acids which are also useful compounds (Sereewatthanawut et al. 2008; Shiddiqi et al. 2014). Autoclave-assisted extraction more effectively broke the cell walls of *C. lentillifera* than microwave-assisted extraction due to the pressure and higher temperature inside the autoclave.

This study also investigated the total triterpenoid contents in both extracts, which were found to contain similar amounts of total triterpenoids. Triterpenoids have been found in marine algae including *C. lentillifera* (Nufus, Nurjanah & Abdullah 2017). Algae produce triterpenoids to discourage competitors and predators (Li, Himaya & Kim 2013). The biologically active triterpenoids of marine algae have anti-cancer and anti-tumor properties, therefore triterpenoids can cause cytotoxicity (Pacheco et al. 2011; Souto et al. 2003). So, the effective concentration of both extracts must be of concern.

CYTOTOXICITY IN HUMAN NORMAL COLON CELL AND ACUTE TOXICITY IN *Daphnia magna*

To establish the safety of both extracts, we determined the IC_{50} in human normal colon cells. The cytotoxicity assay showed that A-CLE and M-CLE exhibited very low toxicity toward human normal colon cells. The IC_{50} values of A-CLE and M-CLE were 12,260 ± 197 μg mL^{-1} and 10,950 ± 169 μg mL^{-1}, respectively (Figure 3(A)). Statistical analysis showed that the IC_{50} of M-CLE was significantly lower than that of A-CLE (p<0.01). M-CLE was therefore more toxic. Moreover, the cell viability in the control was not significantly different from the cell viability in the 1000 and 2000 μg mL^{-1} A-CLE treatments, and the 1000 μg mL^{-1} M-CLE treatment (p>0.05), but was significantly higher than the cell viability in the 4000-16000 μg/mL A-CLE and 2000-16000 μg mL^{-1} M-CLE treatments (p<0.05, Figure 3(B)). However, the IC_{50} of both A-CLE and M-CLE was very high (>10,000 μg mL^{-1}), indicating that both extracts of *C. lentillifera* were safe for consumption at the proper dose (<1,000 μg mL^{-1}). Previous

TABLE 1. Proximate composition of fresh and dried C. lentillifera, A-CLE and M-CLE

Proximate composition	Dried C. *lentillifera* (% DW)	A-CLE (% extract)	M-CLE (% extract)
Crude lipid	0.753± 0.041	0.030 ± 0.002 [b]	0.054 ± 0.001 [a]
Crude protein	4.923 ± 0.127	1.127 ± 0.010 [a]	0.645 ±0.007 [b]
Crude carbohydrate	30.900 ± 0.211	23.659 ± 0.281 [a]	22.344 ± 0.315 [b]
Ash	56.663 ± 0.652	73.870 ± 0.229 [b]	75.400 ± 0.337 [a]
Moisture	6.760 ± 0.420	1.319 ± 0.058 [b]	1.558 ± 0.024 [a]

*Different letters indicate significant differences between A-CLE and M-CLE (Student's t- test, $p<0.05$)

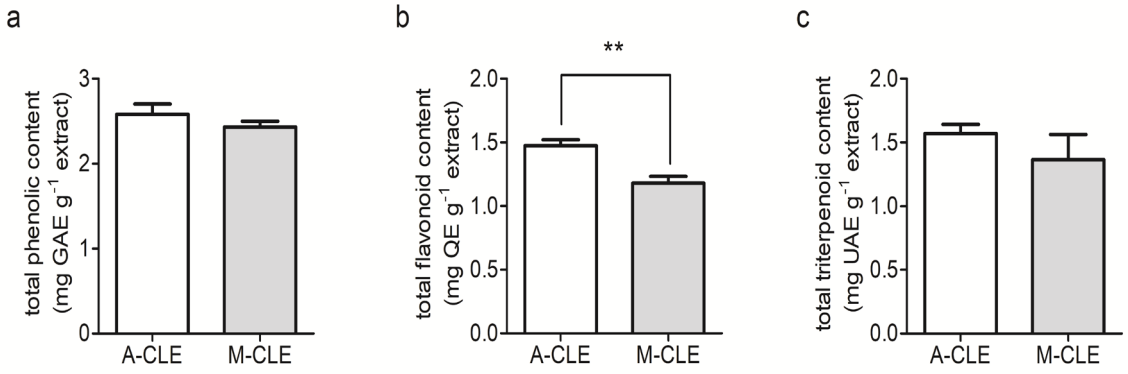

FIGURE 1. Total phenolic (A), flavonoid (B) and triterpenoid (C) contents of A-CLE and M-CLE. Asterisks above the bars indicate significant differences between A-CLE and M-CLE (Student's t- test, $p<0.01$)

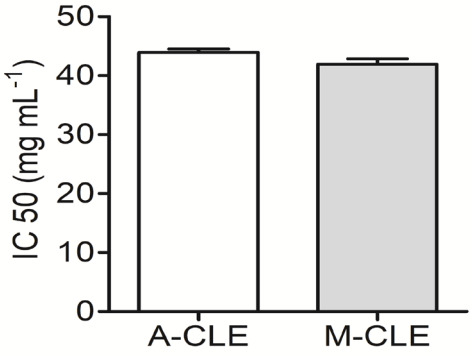

FIGURE 2. DPPH radical scavenging activity of A-CLE and M-CLE

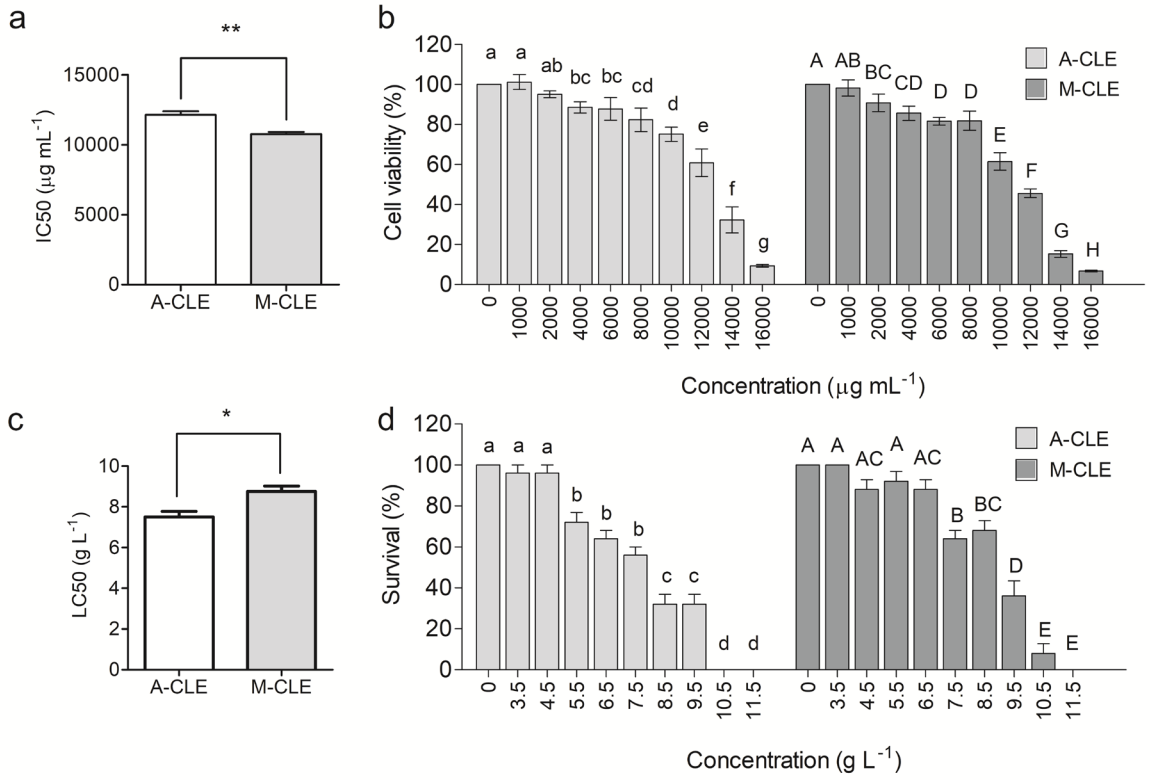

FIGURE 3. Toxicity test of A-CLE and M-CLE in human normal colon cells (A-B) and Daphnia magna (C-D). Asterisks above the bars indicate significant differences between IC50 and LC50 of A-CLE and M-CLE (Student's t- test, * and ** indicate $p<0.05$ and $p<0.01$, respectively). Different letters above the bars indicate significant differences between concentrations of A-CLE (lowercase letters) or M-CLE (uppercase letters) (Tukey's HSD, $p<0.05$)

study of *C. lentillifera* extract in aqueous fraction also showed the similar results that had no toxic effects in mouse fibroblast (L929), mouse macrophages (RAW 264.7), mouse hepatocyte (FL83B), keratinocytes (HaCaT), and human lymphoblast cells (TK6) at 1,000 µg mL^{-1} (Osotprasit et al. 2021). Moreover, IC_{50} of A-CLE and M-CLE were higher than the ethanolic extracts of *C. lentillifera* (Nurkolis et al. 2023; Osotprasit et al. 2021). Therefore, the use of aqueous extract of *C. lentillifera* was safer than ethanolic extracts of *C. lentillifera*.

Furthermore, we also concerned the safety of the extracts for use in aquaculture, so the LC_{50} was determined in neonate *D. magna*. The results showed that the LC_{50} of A-CLE (7.50 ± 0.28 g L^{-1}) was significantly lower than the LC_{50} of M-CLE (8.76 ± 0.26 g L^{-1}) (Figure 3(C)). Survival of *D. magna* was not different between the control and low concentrations of A-CLE (3.5 and 4.5 g L^{-1}) and M-CLE (3.5-6.5 g L^{-1}) ($p>0.05$), but was significantly higher in the control than in treatments at 5.5-11.5 g L^{-1} of A-CLE and 7.5-11.5 g L^{-1} of M-CLE ($p<0.05$, Figure 3(D)). These results indicated that A-CLE was more toxic to *D. magna* than M-CLE, however, the LC_{50} of both extracts was high (>7 g L^{-1}) when compared with purified phytochemicals of *Caulerpa* (Mert-Ozupek et al. 2022). Therefore, our results suggested that both A-CLE and M-CLE were safe for human and animal.

CONCLUSIONS

Both autoclave-assisted and microwave-assisted extraction methods were highly effective at extracting nutrients, minerals and bioactive compounds from the marine seaweed *C. lentillifera.* The autoclave-assisted extraction method extracted more crude carbohydrate, crude protein and flavonoids than the microwave-assisted extraction method. On the other hand, the microwave-assisted extraction has ability to extract more crude lipid than the autoclave-assisted extraction. Therefore, present findings will be useful for further studies related to the nutrient and mineral profiling, and utilization of *C. lentillifera* extract to improve human and animal health.

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REFERENCES

Abdelhamid, A., Lajili, S., Elkaibi, M.A., Ben Salem, Y., Abdelhamid, A., Muller, C.D., Majdoub, H., Kraiem, J. & Bouraoui, A. 2019. Optimized extraction, preliminary characterization and evaluation of the *in vitro* anticancer activity of phlorotannin-rich fraction from the brown seaweed, *Cystoseira sedoides*. *Journal of Aquatic Food Product Technology* 28: 892-909.

Akbary, P. & Aminikhoei, Z. 2018. Effect of polysaccharides extracts of algae *Ulva rigida* on growth, antioxidant, immune response and resistance of shrimp, *Litopenaeus vannamei* against *Photobacterium damselae*. *Aquacultue Research* 49(7): 2503-2510.

Association of Official Analytical Chemists (AOAC). 2000. *Official Methods of Analysis*. 17th ed. Washington: Association of Official Analytical Chemists.

Chaiklahan, R., Srinorasing, T., Chirasuwan, N., Tamtin, M. & Bunnag, B. 2020. The potential of polysaccharide extracts from *Caulerpa lentillifera* waste. *International Journal of Biological Macromolecules* 161: 1021-1028.

Chandra, S., Khan, S., Avula, B., Lata, H., Yang, M.H., Elsohly, M.A. & Khan, I.A. 2014. Assessment of total phenolic and flavonoid content, antioxidant properties, and yield of aeroponically and conventionally grown leafy vegetables and fruit crops: A comparative study. *Evidence-Based Complementary and Alternative Medicine* 2014: 253875.

Chang, C.L., Lin, C.S. & Lai, G.H. 2012. Phytochemical characteristics, free radical scavenging activities, and neuroprotection of five medicinal plant extracts. *Evidence-Based Complementary and Alternative Medicine* 2012: 984295.

Chemat, F., Vian, M.A. & Cravotto, G. 2012. Green extraction of natural products: Concept and principles. *International Journal of Molecular Sciences* 13(7): 8615-8627.

Chung, P.Y., Navaratnam, P. & Chung, L.Y. 2011. Synergistic antimicrobial activity between pentacyclic triterpenoids and antibiotics against *Staphylococcus aureus* strains. *Annals of Clinical Microbiology and Antimicrobials* 10: 25.

Dang, T.T., Bowyer, M.C., Van Altena, I.A. & Scarlett, C.J. 2018. Optimum conditions of microwave-assisted extraction for phenolic compounds and antioxidant capacity of the brown alga *Sargassum vestitum*. *Separation Science and Technology* 53: 1711-1723.

du Preez, R., Majzoub, M.E., Thomas, T., Panchal, S.K. & Brown, L. 2020. *Caulerpa lentillifera* (sea grapes) improves cardiovascular and metabolic health of rats with diet-induced metabolic syndrome. *Metabolites* 10(12): 500.

Fajriah, S., Fadhilah, I. & Sinurat, E. 2021. Characterization and analysis of the antidiabetic activities of sulphated polysaccharide extract from *Caulerpa lentillifera. Pharmacia* 68(4): 869-875.

Garcia-Vequero, M., Ravindran, R., Walsh, O., O'Doherty, J., Jaiswal, A.K., Tiwati, B.K. & Rajuaria, G. 2021. Evaluation of ultrasound, microwave,ultrasound–microwave, hydrothermal and high pressure assisted extraction technologies for the recovery of phytochemicals and antioxidants from brown macroalgae. *Marine Drugs* 19: 309.

Garcia-Vaquero, M., Ummat, V., Tiwari, B. & Rajauria, G. 2020. Exploring ultrasound, microwave and ultrasound– microwave assisted extraction technologies to increase the extraction of bioactive compounds and antioxidants from brown macroalgae. *Marine Drugs* 18: 172.

Getachew, A.T., Jacobsen, T. & Holdt, S.L. 2020. Emerging technologies for the extraction of marine phenolics: Opportunities and challenges. *Marine Drugs* 18(8): 389.

Herawati, V.E., Pinandoyo, Ariyati, R.W., Rismaningsih, N., Windarto, S., Prayitno, S.B., Darmanto, Y.S. & Radjasa, O.K. 2021. Effects of *Caulerpa lentillifera* added into culture media on the growth and nutritional values of *Phronima pacifica*, a natural fish-feed crustacean. *Biodiversitas* 22(1): 424-431.

Imchen, T. 2021. Nutritional value of seaweeds and their potential to serve as nutraceutical supplements. *Phycologia* 60(6): 534-546.

Khuituan, P., Huipao, N., Jeanmard, N., Thantongsakul, S., Promjun, W., Chuthong, S., Tipbunjong, C. & Peerakietkhajorn, S. 2022. *Sargassum plagiophyllum* extract enhances colonic functions and modulates gut microbiota in constipated mice. *Nutrients* 14(3): 496.

Koyande, A.K., Chew, K.W., Rambabu, K., Tao, Y., Chu, D.T. & Show, P.L. 2019. Microalgae: A potential alternative to health supplementation for humans. *Food Science and Human Wellness* 8(1): 16-24.

Kumar, M., Gupta, V., Kumari, P., Reddy, C.R.K. & Jha, B. 2011. Assessment of nutrient composition and antioxidant potential of Caulerpaceae seaweeds. *Journal of Food Composition and Analysis* 24(2): 270-278.

Lee, P.T., Lee, M.C., Ho, Y.T., Liao, Z.H., Huang, H.T., Ho, Y.S. & Nan, F.H. 2020. Polysaccharides from the green alga *Caulerpa racemosa* (Agardh, 1873) improve the immune response and antioxidant status in the white shrimp *Litopenaeus vannamei* (Boone, 1931) (Dendrobranchiata, Penaeidae). *Crustaceana* 93(6): 611-632.

Lee, S.H., Kang, S.M., Ko, S.C., Moon, S.H., Jeon, B.T., Lee, D.H. & Jeon, Y.J. 2014. Octaphlorethol a: A potent alpha-glucosidase inhibitor isolated from *Ishige foliacea* shows an anti-hyperglycemic effect in mice with streptozotocin-induced diabetes. *Food & Function* 5(10): 2602-2608.

Li, Y.X., Himaya, S.W.A. & Kim, S.K. 2013. Triterpenoids of marine origin as anti-cancer agents. *Molecules* 18(7): 7886-7909.

Li, Z., Wang, B., Zhang, Q., Qu, Y., Xu, H. & Li, G. 2012. Preparation and antioxidant property of extract and semipurified fractions of *Caulerpa racemosa*. *Journal of Applied Phycology* 24: 1527-1536.

Lourenço, S.O., Barbarino, E., De-Paula, J.C., Pereira, L.O.D.S. & Lanfer Marquez, U.M. 2002. Amino acid composition, protein content and calculation of nitrogen-to-protein conversion factors for 19 tropical seaweeds. *Phycological Research* 50(3): 233-241.

Lozano Muñoz, I. & Díaz, N.F. 2020. Minerals in edible seaweed: Health benefits and food safety issues. *Critical Reviews in Food Science and Nutrition* 62(6): 1592-1607.

Mann, J. & Truswell, A.S. 2017. *Essentials of Human Nutrition.* 5th ed. Oxford: Oxford University Press.

Mary, A., Mary, V., Lorella, A. & Matias, J.R. 2009. Rediscovery of naturally occurring seagrape *Caulerpa lentillifera* from the Gulf of Mannar and its mariculture. *Curr. Sci.* 97: 1418-1420.

Matanjun, P., Mohamed, S., Muhammed, K. & Mustapha, N.M. 2010. Comparison of cardiovascular protective effects of tropical seaweeds*, Kappaphycus alvarezii*, *Caulerpa lentillifera*, and *Sargassum polycystum*, on high-cholesterol/high-fat diet in rats. *Journal of Medicinal Food* 13(4): 792-800.

Matanjun, P., Mohamed, S., Mustapha, N.M. & Muhammad, K. 2009. Nutrient content of tropical edible seaweeds, *Eucheuma cottonii*, *Caulerpa lentillifera* and *Sargassum polycystum*. *Journal of Applied Phycology* 21: 75-80.

Mert-Ozupek, N., Calibasi-Kocal, G., Olgun, N., Basbinar, Y., Cavas, L. & Ellidokuz, H. 2022. *In-silico* molecular interactions among the secondary metabolites of *Caulerpa* spp. and colorectal cancer targets. *Frontiers in Chemistry* 10: 1046313.

Michalak, I. & Chojnacka, K. 2014. Algal extracts: Technology and advances. *Engineering in Life Sciences* 14(6): 581-591.

Mohaddes-Kamranshahi, M., Jafarizadeh-Malmiri, H., Simjoo, M. & Jafarizad, A. 2019. Evaluation of the saponin green extraction from *Ziziphus spina-christi* leaves using hydrothermal, microwave and Bain-Marie water bath heating methods. *Green Process and Synthesis* 8: 62-67.

Muller-Feuga, A. 2000. The role of microalgae in aquaculture: situation and trends. *Journal of Applied Phycology* 12: 527-534.

Nagappan, T. & Vairappan, C.S. 2014. Nutritional and bioactive properties of three edible species of green algae, genus *Caulerpa* (Caulerpaceae). *Journal of Applied Phycology* 26: 1019-1027.

Nagappan, S., Das, P., Quadir, M.A., Thaher, M., Khan, S., Mahata, C., Al-Jabri, H., Vatland, A.K. & Kumar, G. 2021. Potential of microalgae as a sustainable feed ingredient for aquaculture. *Journal of Biotechnology* 341: 1-20.

Nguyen, V.T., Ueng, J.P. & Tsai, G.J. 2011. Proximate composition, total phenolic content, and antioxidant activity of seagrape (*Caulerpa lentillifera*). *Journal of Food Science* 76(7): C950-C958.

Nufus, C., Nurjanah & Abdullah, A. 2017. Karakteristik rumput laut hijau dari perairan Kepulauan Seribu dan Sekotong Nusa Tenggara Barat sebagai antioksidan. *Jurnal Pengolahan Hasil Perikanan Indonesia* 20: 620-632.

Nurkolis, F., Taslim, N.A., Qhabibi, F.R., Kang, S., Moon, M., Choi, J., Choi, M., Park, M.N., Mayulu, N. & Kim, B. 2023. Ulvophyte green algae *Caulerpa lentillifera*: Metabolites profile and antioxidant, anticancer, anti-obesity, and *in vitro* cytotoxicity properties. *Molecules* 28(3): 1365.

Organisation for Economic Cooperation and Development (OECD). 2004. Test No. 202: *Daphnia* sp. acute immobilisation test. In *OECD Guidelines for the Testing of Chemicals*. Paris: OECD Publishing. pp. 1-12.

Osotprasit, S., Samrit, T., Chaiwichien, A., Changklungmoa, N., Meemon, K., Niamnont, N., Manohong, P., Noonong, K., Tamtin, M., Sobhon, P. & Kueakhai, P. 2021. Toxicity and anti-oxidation capacity of the extracts from *Caulerpa lentillifera. Chiang Mai University Journal of Natural Science* 20(3): e2021065.

Pacheco, F.C., Villa-Pulgarin, J.A., Mollinedo, F., Martín, M.N., Fernández, J.J. & Daranas, A.H. 2011. New polyether triterpenoids from *Laurencia viridis* and their biological evaluation. *Marine Drugs* 9(11): 2220-2235.

Pandey, K.B. & Rizvi, S.I. 2009. Plant polyphenols as dietary antioxidants in human health and disease. *Oxid. Med. Cell Longev.* 2(5): 270-278

Ratana-arporn, P. & Chirapart, A. 2006. Nutritional evaluation of tropical green seaweeds *Caulerpa lentillifera* and *Ulva reticulate. Kasetsart Journal (Natural Science)* 40(6): 75-83.

Roy, M.C., Anguenot, R., Fillion, C., Beaulieu, M., Bérubé, J. & Richard, D. 2011. Effect of a commercially-available algal phlorotannins extract on digestive enzymes and carbohydrate absorption *in vivo. Food Research International* 44(9): 3026-3029.

Sengkhim, R., Peerakietkhajorn, S., Jeanmard, N., Pongparadon, S., Khuituan, P., Thitiphatphuvanon, T., Surinlert, P. & Tipbunjong, C. 2021. Effects of *Sargassum plagiophyllum* extract pretreatment on tissue histology of constipated mice. *Tropical Journal of Pharmaceutical Research* 20(11): 2339-2346.

Sereewatthanawut, I., Prapintip, S., Watchiraruji, K., Goto, M., Sasaki, M. & Shotipruk, A. 2008. Extraction of protein and amino acids from deoiled rice bran by subcritical water hydrolysis. *Bioresource Technology* 99(3): 555-561.

Shiddiqi, Q.Y.A., Karisma, A.D., Machmudah, S., Widiyastuti, Nurtono, T., Winardi, S., Wahyudiono & Goto, M. 2014. Effect of hydrothermal extraction condition on the content of phenolic compound extracted from rind of mangosteen (*Garcinia mangostana*) and its antioxidant efficiency. *IPTEK The Journal for Technology and Science* 25(2): 54-59.

Sompong, U., Mingkaew, J., Amornlerdpison, D. & Mengumphan, K. 2020. Sea grape (*Caulerpa lentillifera*) cultivation in artificial seawater closed system. *Maejo International Journal of Energy Environmental Communication* 2(1): 17-22.

Souto, M.L., Manríquez, C.P., Norte, M., Leira, F. & Fernández, J. 2003. The inhibitory effects of squalene-derived triterpenes on protein phosphatase PP2A. *Bioorganic & Medicinal Chemistry Letters* 13(7): 1261-1264.

Sullivan, D. & Carpenter, D. 1993. *Method of Analysis for Nutrition Labeling*. Arlington: AOAC International.

Sun, K., Gao, M., Wang, Y.Z., Li, X.R., Wang, P. & Wang, B. 2022. Antioxidant peptides from protein hydrolysate of marine red algae *Eucheuma cottonii*: Preparation, identification and cytoprotective mechanisms on H_2O_2 oxidative damaged HUVECs. *Frontiers in Microbiology* 13: 791248.

Sun, Y., Liu, Y., Ai, C., Song, S. & Chen, X. 2019. *Caulerpa lentillifera* polysaccharides enhance the immunostimulatory activity in immunosuppressed mice in correlation with modulating gut microbiota. *Food & Function* 10(7): 4315-4329.

Suo, S.K., Zheng, S.L., Chi, C.F., Luo, H.Y. & Wang, B. 2022. Novel angiotensin-converting enzyme inhibitory peptides from tuna byproducts-milts: Preparation, characterization, molecular docking study, and antioxidant function on H_2O_2-damaged human umbilical vein endothelial cells. *Frontiers in Nutrition* 9: 957778.

Syakilla, N., George, R., Chye, F.Y., Pindi, W., Mantihal, S., Wahab, N.A., Fadzwi, F.M., Gu, P.H. & Matanjun, P. 2022. A review on nutrients, phytochemicals, and health benefits of green seaweed, *Caulerpa lentillifera*. *Foods* 11(18): 2832.

Tanawoot, V., Vivithanaporn, P., Siangcham, T., Meemon, K., Niamnont, N., Sobhon, P., Tamtin, M. & Sangpairoj, K. 2021. Hexane extract of seaweed *Caulerpa lentillifera* inhibits cell proliferation and induces apoptosis of human glioblastoma cells. *Science & Technology Asia* 26(2): 128-137.

Tian, H., Liu, H., Song, W., Zhu, L. & Yin, X. 2019. Polysaccharide from *Caulerpa lentillifera*: Extraction optimization with response surface methodology, structure and antioxidant activities. *Natural Product Research* 35(20): 1-9.

Wang, J., Hu, S., Nie, S., Yu, Q. & Xie, M. 2016. Reviews on Mechanisms of *in vitro* antioxidant activity of polysaccharides. *Oxidative Medicine and Cell Longevity* 2016: 5692852.

Wells, M.L., Potin, P., Craigie, J.S., Raven, J.A., Merchant, S.S., Helliwell, K.E., Smith, A., Camire, M.E. & Brawley, S.H. 2017. Algae as nutritional and functional food sources: Revisiting our understanding. *Journal of Applied Phycology* 29(2): 949-982.

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